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IMMUNOHISTOCHEMICAL EXPRESSION OF TLR4, MYD88 AND PCNA IN COLON POLYPS OF OVARIECTOMIZED FEMALE RAT INDUCTION BY AZOXY METHANE AND TREATED BY ESTROGEN HORMONE

Ali Yhea Salman[1]*, Huda Sadun[2] and Thaeer Jwad[3]

[1,2]Department of Microbiology, Collage of Veterinary Medicine, University of Baghdad, Iraq.

[3]Al-Yarmouk Teaching Hospital, Baghdad, Iraq.

Abstract

Worldwide, polyps and cancer of the colon is complex and involve genetic abnormalities and may be due to different environmental chemical carcinogens. This study was conducted at AL-Yarmouk Teaching Hospitals and collage of veterinary medicine, University of Baghdad from April 2018 to July 2019, of which colon polyps were taken from 32 colon sample of Ovariectomized rats and 16 controls to assess Toll like receptor 4 (TLR-4), Myeloid differentiation primary response 88 (MYD88) and Proliferating cell nuclear antigen (PCNA) level among them and to evaluate their roles in cancer development. Forty Eight Ovariectomized female rats were divided into three groups: Control Group: 16 ovariectomized female rats were administrated with vehicles once a weekly for 6 weeks. Azoxymethane Group: 16 ovariectomized female rats received intraperitoneal injections of Azoxymethane (10 mg/kg body weight) one dose weakly for two weeks without any treatment after 2nd weeks they received only 0.1 ml normal saline for 4 weeks. Azoxymethane + E2 group: 16 ovariectomized female rats administrated with both Azoxymethane and estrogen, estrogen (40 mg/kg body weight was given after dissolved in distilled water) once a week. The weekly drugs injection lasted for 6 weeks together with the weekly intraperitoneal injections of Azoxymethane (10 mg/kg body weight for 2 weeks). According to the histological and immunohistochemical examination of these groups the expression ratio of TLR4 was (6.25%, 93.75%,75.0%) respectively. The differences among groups were significant. The expression of myd88 for these groups (6.25%, 93.75%, 68.75%), respectively and the differences were significant. The PCNA expression for these groups was (6.25%, 87.50%, 62.50%) respectively and the differences were significant. All these results indicated the effect of estrogen hormone to prevent development of colon polyps.

Key words : Estrogen, colon polyps, TLR4, rats.

Introduction

Colon is the longest part of the large intestine a tube-like organ, it is located between the caecum and the rectum. The function of the colon removes water and some nutrients and electrolytes from partially digested food. The remaining material, solid waste called stool, moves through the colon to the rectum and leaves the body through the anus. the wall of colon consist from 4 layers mucosa, submucosa, muscular layer and serosa/ adventitia (Hounnou *et al*., 2002). Many diseases effected on colon such as Colitis, Inflammatory bowel disease, Crohn's disease, Ulcerative colitis, Colon polyps and colon cancer (Herrinton *et al*., 2012).

***Author for correspondence :** E-mail: dr.ali.yhea.salman@gmail.com

Colon polyps are uncontrolled growths on the lining of colon or large intestine, Polyp is a term derived from the Greek word polypous, which means 'morbid lump. Generally, this term describes any mass protruding into the lumen of a hollow vessel, anywhere in the gastro-intestinal, genito-urinary or respiratory tracts. Usually, polyps arise from the mucosal layer of these organs, although some submucosal pathologies may cause mucosal protrusion into the lumen and resemble mucosal polyps. Not all polyps necessarily exhibit neoplastic behavior (Williams *et al*., 2004). Most of them aren't harmful. But some can turn into colon cancer over time. The are four main types of polyps hyperplastic, neoplastic, hamartomatous and inflammatory (Mahasneh *et al*.,

2017). Colon polyps are not usually associated with symptoms. but When they occur, symptoms include rectal bleeding, bloody stools, abdominal pain and fatigue. And due to chronic blood loss from rectal bleeding and bloody stools, they sometimes present with iron deficiency anemia. A change in bowel habits may occur including constipation and diarrhea. Occasionally, if a polyp is big enough to cause a bowel obstruction, there may be nausea, vomiting and severe constipation (Grady *et al*., 2008). Colon polyps risk increased after menopause and decreased after hormone replacement treatment. Plenty of epidemiologic evidence demonstrated that estrogen might influence the incidence of colon polyps in women (Xu *et al*., 2010). Many hypotheses had been proposed and studied. Estrogen receptors were found in colon epithelium and the estrogen receptor beta was the dominant subtype. On cell models, many studies had found that estrogen could affect the growth of cells originated from colon mucosa (Wilkins *et al*., 2010), the colon cancer was created by treated with Azoxymethane.

Azoxymethane (AOM), a gene mutation agent, may be used with dextran sulfate sodium (DSS) to create cancer models in laboratory animals, which can be used to study mechanisms of cancer progression and chemoprevention. Also, it is a potent carcinogen used to induce colon cancer in rats and mice (Raju *et al*., 2009).

Estrogens are members of steroid hormone family and are traditionally associated with the female reproductive development. It is one of two main sex hormones that women have (Nelson *et al*., 2001). The other one is progesterone. The major sources of estrogens are the ovaries and the placenta additional small amounts are secreted by the adrenal glands and by the male testes. Three types of Estrogen hormone: Estrone (E1), Estradiol (E2), Estriol (E3) (Herynk *et al*., 2004). The different estrogen receptors (ER) have revealed their significant role in tissue types other than the female reproductive tract including the gastrointestinal system. Estrogens regulate these cellular effects through their intracellular receptors, ERα and ERβ. The two receptors are coded for by separate genes, ESR1 (ERα) and ESR2 (ERβ), with each gene producing different receptor isoforms from alternative splicing, resulting in three ERα and five ERβ variants. A role for estrogen has been demonstrated in multiple epidemiologic studies, which may benefit CRC prevention. A large body of evidence from preclinical studies indicates that expression of the estrogen receptor beta (ERβ/ESR2) demonstrates an inverse relationship with the presence of colorectal polyps and stage of tumors (Weyant *et al*., 2001).

The colon polyps can be detected by use biological marker or biomarkers. The biological marker is a characteristic that can be objectively measured and evaluated as an indicator of normal biological or pathogenic processes (a diagnostic biomarker), or pharmacological responses to a therapeutic intervention (a therapeutic biomarker).

TLR4 is expressed in various types of human cancer, including prostate, pancreatic, liver, colon, and breast cancer. TLR4, the receptor for lipopolysaccharide (LPS) (Kawai *et al*., 2010), primarily induces inflammatory cytokines in immune cells, but it is also involved in carcinogenesis and cancer cell survival. Furthermore, LPS can modify cytokine levels of the tumor microenvironment to promote tumor growth, invasion and metastasis (Davoodi *et al*., 2011).

The MYD88 is an adaptor protein in regulation of the innate immunity, functions to regulate immune responses against viral and bacterial infections in the human body. and altered MyD88 signaling also involved in cancer-associated cell intrinsic and extrinsic inflammation progression and carcinogenesis. Detection of MyD88 expression was to predict prognosis of various human cancers, *e.g.*, lymphoid, liver and colorectal cancers (Araki *et al*., 2005).

PCNA is an accessory protein of DNA polymerase and it is play an important role in the elongation or replication of the DNA chain. Its accumulation in the nucleus during the G-1 and S stages of the cell cycle, the percentage of PCNA-positive cells, has been reported to be correlated with the proliferative activity and the prognosis of various malignant tumors. Thus it is valuable to assess what role PCNA plays during the formation of the colonic polyps (Zhang *et al*., 2003).

The present study aimed to identify the effect of therapeutic agent Estrogen hormone in colon polyps formation, and expression of such immunological marker and distribution in the colon of rats.

Materials and Methods

Animals and housing condition

Forty healthy female rats were used in the present study. female rats (2 month of age and their weight range between 250-500 gm) were housed in clean and disinfectant plastic cages in the animal house of veterinary college at University of Baghdad; under the laboratory conditions, with controlled room temperature 25-28^{o}C, good ventilation and feed normal pellets and tap water. All these rats are Ovariectomized according to Parhizkar *et al*. (2008).

Preparation of azoxymethane injection

The Azoxymethane (AOM) (available as oily solution 25 mg in ampoule) was dissolved in 12.5 ml from sterile phosphate buffer saline, kept as a stock solution of 2 mg/ml in 4 Co, for induction colon polyps, the ideal dose is 10mg/Kg B.W (Penman *et al*., 2011).

Preparation of estrogen hormone dose

The Estrogen available as a tablet (2mg) the tablet dissolved in distal water complete volume to 50 ml. The concentration become 40 *M*g/ml. 2 /50 =0.04 mg/ml, mg = 1000 *Mg*

0.04X1000 = 40 *M*g/ml.

Study design

All female rats were Ovariectomized (OVX) at the age of 8 weeks. At the age of 9 weeks, these rats will be randomly divided into 3 groups:

Control Group : 16 ovariectomized female rats were administrated with vehicles once a weekly for 6 weeks. Azoxymethane Group: 16 ovariectomized female rats received intraperitoneal injections of Azoxymethane (10 mg/kg body weight) one dose weakly for two weeks (Papalois and Paidas, 2003) without any treatment ,after 2nd weeks they received only 0.1 ml normal saline for 4 weeks. Azoxymethane + E2 group: 16 ovariectomized female rats administrated with both Azoxymethane and estrogen, estrogen of a dose (40 µg/kg body weight was given orally after dissolved in distilled water) once a week, The weekly drugs injection lasted for 6 weeks together with the intraperitoneal injections of Azoxymethane (10 mg/kg body weight for 2 weeks).

All the animals were sacrificed. Colon tissue autopsy was taken and visual macroscopic examination carried out, the incidence, volumes and multiplicity of colon lesion in each group had been evaluated. Histopathological examination, the expressions of Toll-like receptors TLR, Myeloid differentiation primary response 88 (MYD88), and Proliferating cell nuclear antigen (PCNA) detected in each group by immunohistochemistry.

Harvesting of tissue specimen

After killing rats the entire colorectums will be collected and opened longitudinally and washed with PBS. Polyps will be identified through visual macroscopic examination and later verified with histopathological examination. The location and number of all the polyps were recorded and fixed in 4% paraformaldehyde and embedded in paraffin block.

Tissue preparation for histological and Immunohistochemical detection of TLR4, MYD88 and PCNA in paraffin-embedded section

The preparation of histological section depended on standard methods of Allen and Cameron (2004) Immunohistochemical according the manufacture company.

The slide was examined and stained cell was counted with the helping of an histopathologist by light microscope. the scoring system, corresponds to percentage of immunoreactive positive cells (nucleus and cytoplasmic Staining of polyps) score with evaluating by counting of number of cells staining. The immunostaining score of TLR4, MyD88 and PCNA was assessed by the percentage of positively stained cells as:

Score 0: No positive staining

Score1: 1-25% cell Positive.

Score 2: 26-50% cell Positive.

Score 3: 51-75% cell Positive.

Score 4: 76-100% cell Positive.

Specimens with scores ≥ 2 were labeled as 'positive (+) (Zhou *et al*., 2012; Lee *et al*., 2012).

Statistical analysis

The Statistical Analysis System- SAS (2012) program was used to detect the effect of difference factors in study parameters. Chi-square test was used to detect the significant differences between percentages. $P<0.05$ is considered significant.

Results

Effect of administration Azoxymethane and Estrogen hormone on the colon tissue of Ovariectomized rats

After administration of Azoxymethane (two dose in two weeks) the clinical singe was invisible (not viewed) and after sacrificed rats the macroscopic features was congestion on mesenteric and gastrointestinal tract and hepatomegaly was appeared ,after opening the large intestine and colon the features of irritation was visible and polyps was appeared (fig. 1). In the group treated with Estrogen Hormone the clinical signs also was not viewed except improved vitality of rats, and after sacrificed rats the previous macroscopically features are decreased or absent (fig. 3). All this feature appeared with was absent in control negative (normal rats) (fig. 2).

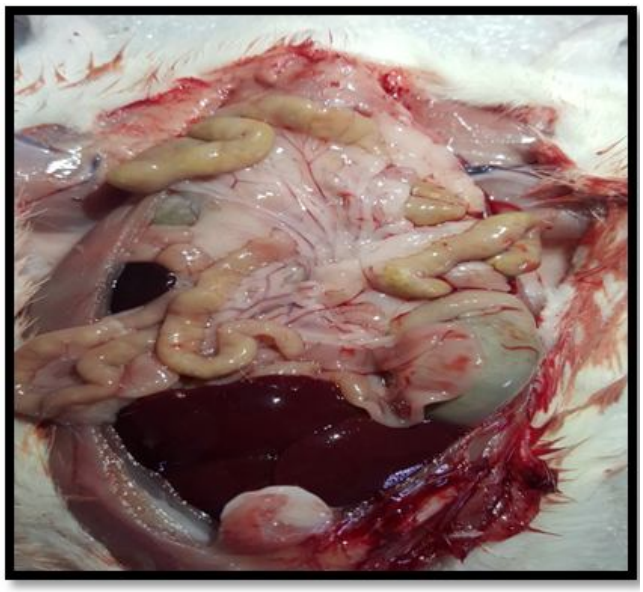

Fig. 1 : Rat administration with Azoxymethane (the sign of congestion of mesenteric and wall of intestine and hepatomegale).

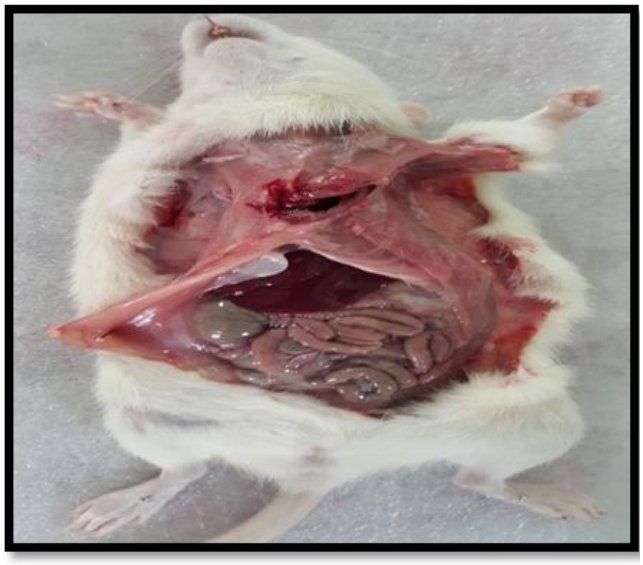

Fig. 2 : Control negative (normal rat).

Histopathological effect in the colon tissue of Ovariectomized rats administration with azoxymethane and treated with Estrogen hormone

Histopathological examination of the Ovariectomized rats administrated with two dose of Azoxymethane (10 mg/kg body weight of rats) showing focal hyperplastic polyps (fig. 5).

The group treated with Estrogen hormone (40 *M*g/ Kg B.W of rat) was reduce the polyps and showing reactive lymphoid follicular hyperplasia and mild regeneration of the hyperplastic polyps (fig. 6). All this feature compared with histopathological feature of control negative (normal rats) (fig. 4).

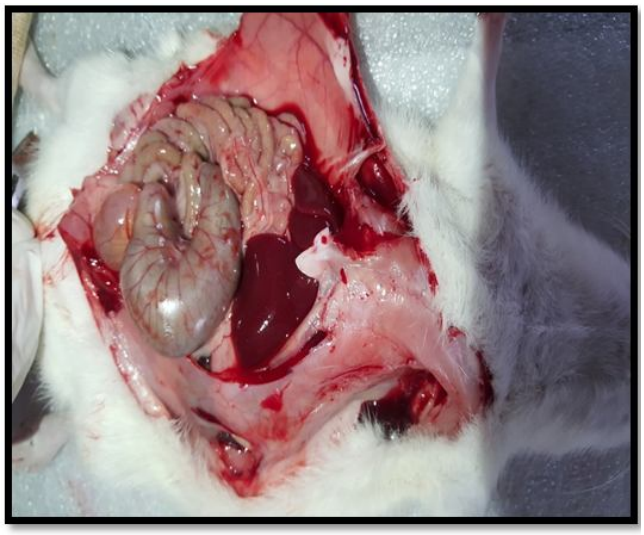

Fig. 3 : Rat infected with *E. coli* and treated with Estrogen Hormone.

Immunohistochemical expression TLR4, MYD88 and PCNA in the tissue of Ovariectomized rats administration with Azoxymethane and Estrogen hormone

Immunohistochemical expression of the (TLR4, Myd88, PCNA) markers in colon polyps of rats administration with Azoxymethane (10 mg/kg body weight of rats) showed significantly increased of the these markers when compared with Estrogen treated group. The expression was found in crypt foci and epithelial cells of colon of polyps group.

The cytoplasmic expression of toll like 4 in control group was negative, (the number of positive staining sample were 2 from 16 sample and the percentage was 12.5%) (fig. 7), while the group administrated with Azoxymethane (10 mg/kg body weight of rats) (2 dose in two weeks) showed significant increase in cytoplasmic expression of TLR4 (the number of positive staining samples were 15 from 16 samples and the percentage was 93.75%) (fig. 8), but in group of administration azoxymethane (10 mg/kg body weight of rats) and treated with Estrogen hormone (40 *M*g/Kg B.W of rat) showed a decreasing in the cytoplasmic expression of TLR4 (the number of positive staining samples were 12 from 16 samples and the percentage was 75.0%) (fig. 9). The statistical analysis showed a significant difference (table 1).

Positive cytoplasmic expression of MYD88 was seen in 93.75% of samples in rats administered with Azoxymethane. Regarding to Estrogen treatment group of colon polyps, MYD88 showed a significant reduction in the expression (68.75%), while the expression in control group was negative. The number of samples staining

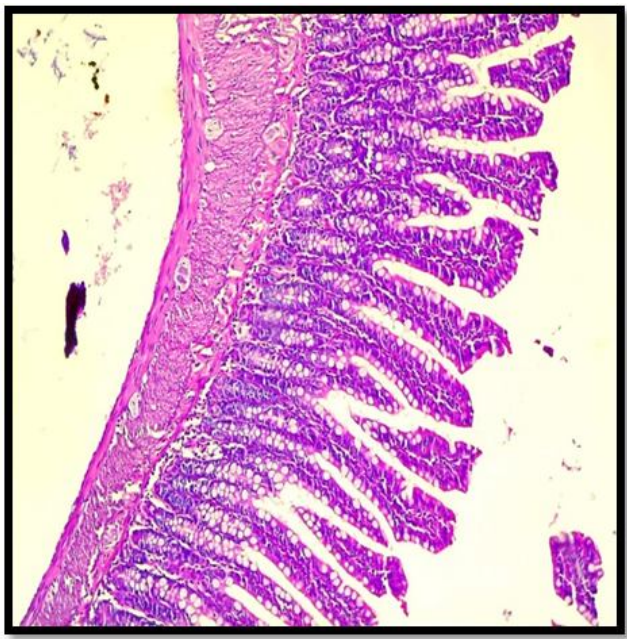

Fig. 4 : Histopathological section in the large intestine tissue of rat showing normal tissue (H&E stain 100 X).

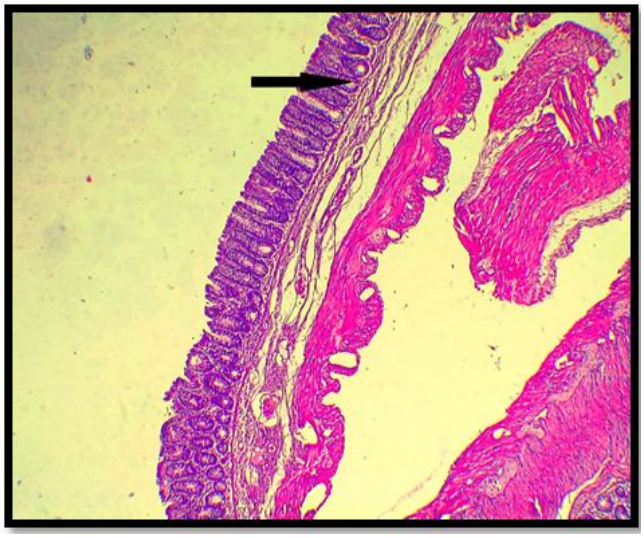

Fig. 5 : Histopathological section in the large intestine tissue of rat administrated with Azoxymethane showing focal hyperplastic polyps (H&E stain 100 X).

positive was 1 sample out of 16 samples and the percentage was 6.25% (fig. 10). After 2 weeks from administration of Azoxymethane, the cytoplasmic expression of MYD88 was found in the 15 samples out of 16 samples and the percentage was 93.75% (fig. 11). In group of administration of azoxymethane (2 dose in two weeks) (10 mg/kg body weight of rats) and treated with Estrogen hormone (40 *M*g/Kg B.W of rat) showing reduce cytoplasmic expression of MYD88, the number of positive staining samples were 11 samples from 16 samples and the percentage was 68.75% (fig. 12). The statistical analysis of MYD88 expression showed a

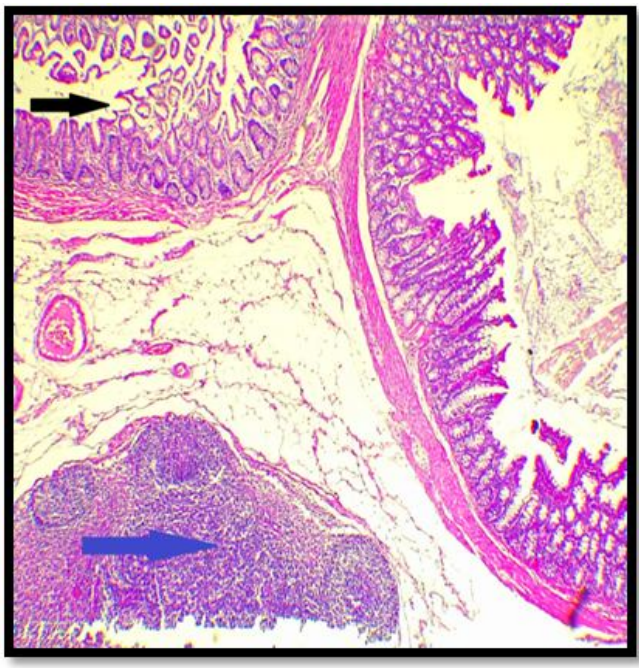

Fig. 6 : Histopathological section in the large intestine tissue of rat administrated with Azoxymethane and treated with Estrogen hormone showing (➡) reactive lymphoid follicular hyperplasia and (⟹) mild regeneration of the hyperplastic polyps (H&E stain 100 X).

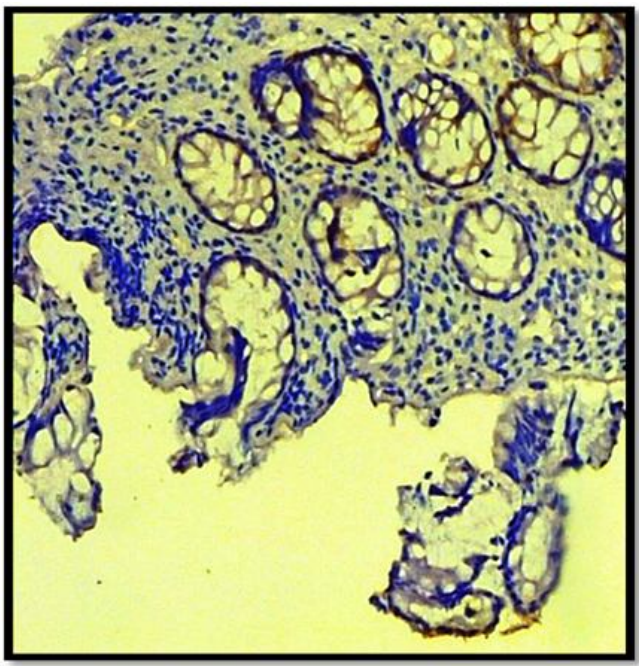

Fig. 7 : Immunohistochemical staining of cytoplasmic TLR4 for colon of control group, showed negative staining 100X).

significant decreasing in treated group (table 2).

The proliferation rates in different groups were assessed by PCNA. The PCNA of polyps from Azoxymethane group ranged 87.5%, when

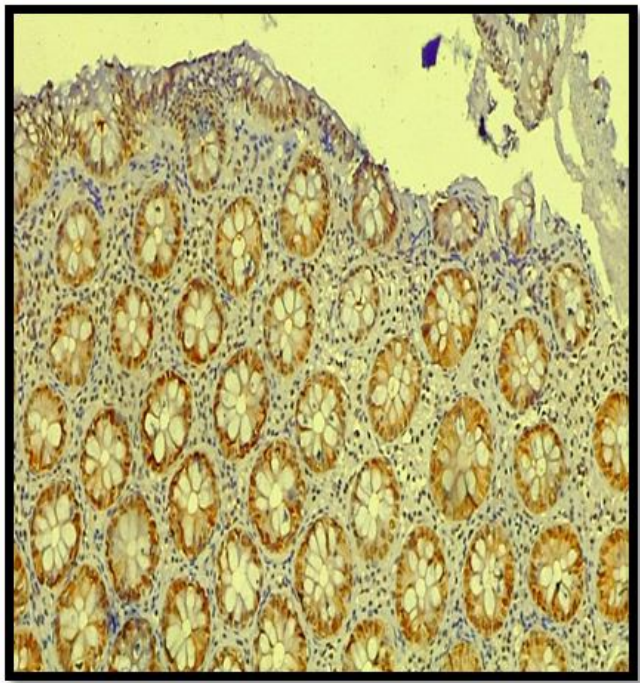

Fig. 8 : Immunohistochemical staining of cytoplasmic TLR4 in colon of rats group administrated with Azoxymethane group, showed strong positive staining (100X).

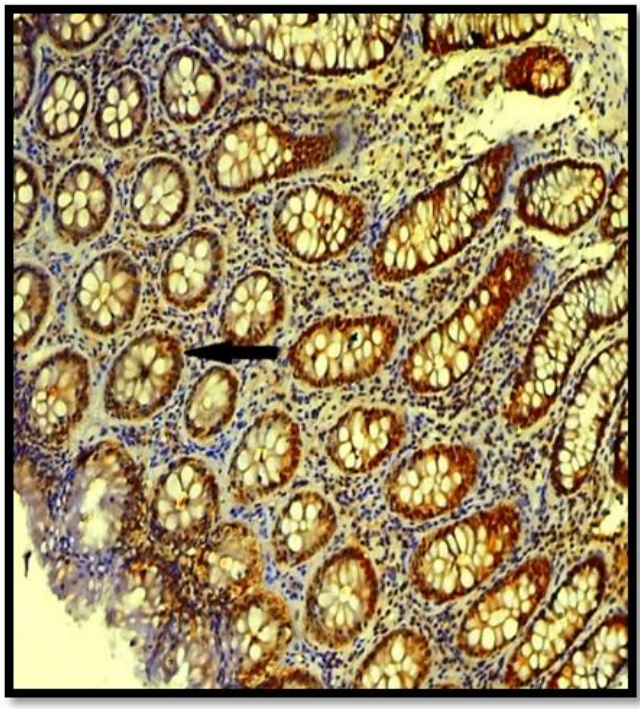

Fig. 9 : Immunohistochemical staining of cytoplasmic TLR4 in colon of rats group administrated with Azoxymethane and treated with Estrogen hormone, showed moderate positive staining, 100X).

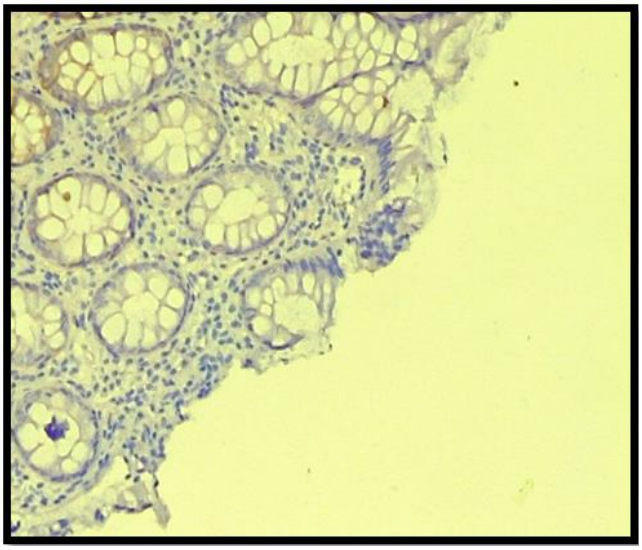

Fig. 10 : Immunohistochemical staining of cytoplasmic MYD88 for colon of control group, showed negative staining, 100X).

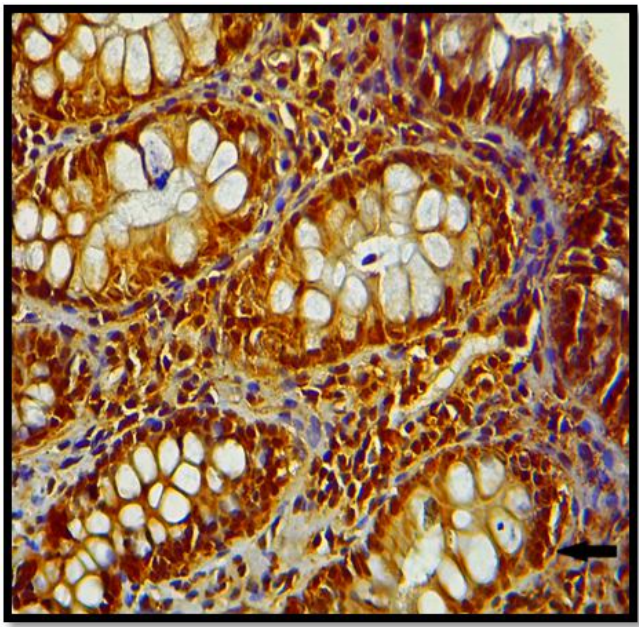

Fig. 11 : Immunohistochemical staining of cytoplasmic MYD88 in colon of rats group administrated with Azoxymethane group, showed strong positive staining, 400X).

Azoxymethane was administrated together with Estrogen the PCNA decreased significantly to 62.5%. The PCNA index in control group (6.25%) was significantly lower than that in other groups. The nuclear expression of PCNA in control group was negative staining, the number of positive staining samples were 1 from 16 sample and the percentage was 6.25% and showed complete lack of nuclear expression (fig. 13). And after 2 weeks from administration of Azoxymethane the nuclear expression of PCNA found in 14 from 16 samples and the percentage was 87.5%, the greater positive staining sample were in score 4) (fig. 14). In group of administration of Azoxymethane (2 dose in two weeks) (10 mg/kg body

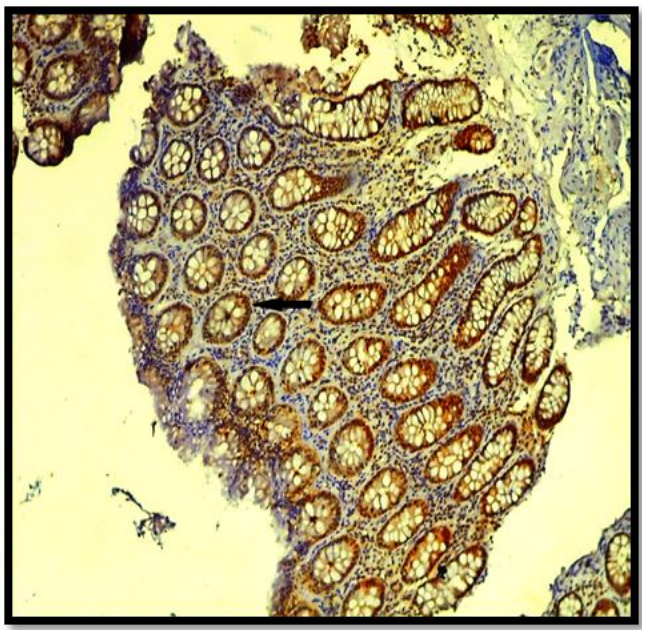

Fig. 12 : Immunohistochemical staining of cytoplasmic MYD88 in colon of rats group administrated with Azoxymethane and treated with Estrogen hormone, showed moderate positive staining,100X).

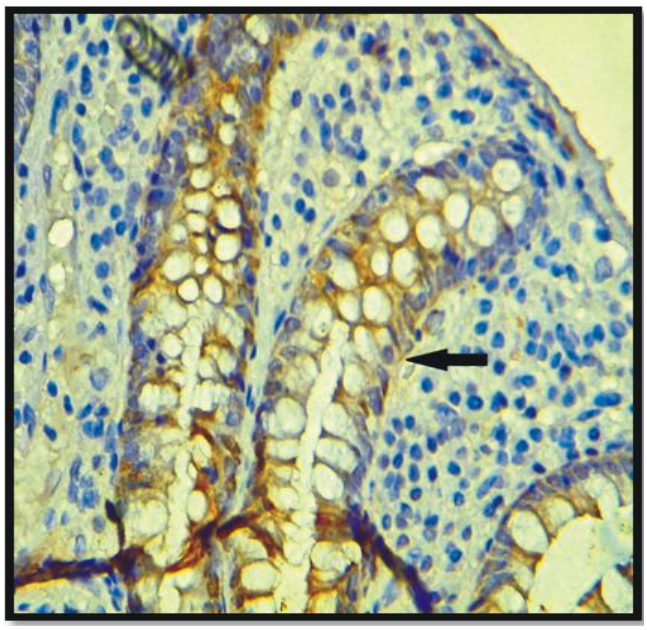

Fig. 13 : Immunohistochemical staining of nuclear PCNA for colon of control group, showed negative staining, 400X).

Table 1 : Immunohistochemical scoring of TLR4 in colon of ovariectomized rats Administration with Azoxymethane and treated with Estrogen hormone group.

Marker	Group	No. of rats	Score					Percentage of positive result
			Score 0	Score1	Score2	Score3	Score4	
TLR4	Control	16	13	1	2	0	0	12.5%
	Administration with Azoxymethane	16	0	1	1	2	12	93.75%
	Administration with Azoxymethane and treated with Estrogen hormone	16	0	4	8	3	1	75.0%
Chi-Square (χ^2)								14.528**

** (P<0.01). (positive score >2).

Table 2 : Immunohistochemical scoring of MYD88 in colon of ovariectomized rats administration with Azoxymethane and treated with Estrogen hormone group.

Marker	Group	No. of rats	Score					Percentage of positive result
			Score 0	Score1	Score2	Score3	Score4	
MYD88	Control	16	11	4	1	0	0	6.25 %
	Administration with Azoxymethane	16	0	1	2	4	9	93.75%
	Administration with Azoxymethane and treated with Estrogen hormone	16	0	5	8	2	1	68.75 %
Chi-Square (χ^2)		_	_	_	_	_	_	14.209**

** (P<0.01). (positive score >2).

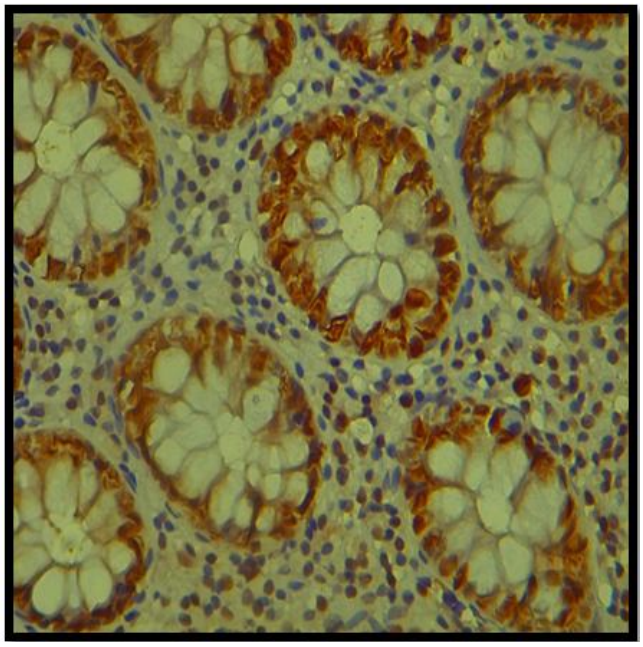

Fig. 14 : Immunohistochemical staining of nuclear PCNA in colon of rats group administrated with Azoxymethane group, showed strong positive staining, 400X).

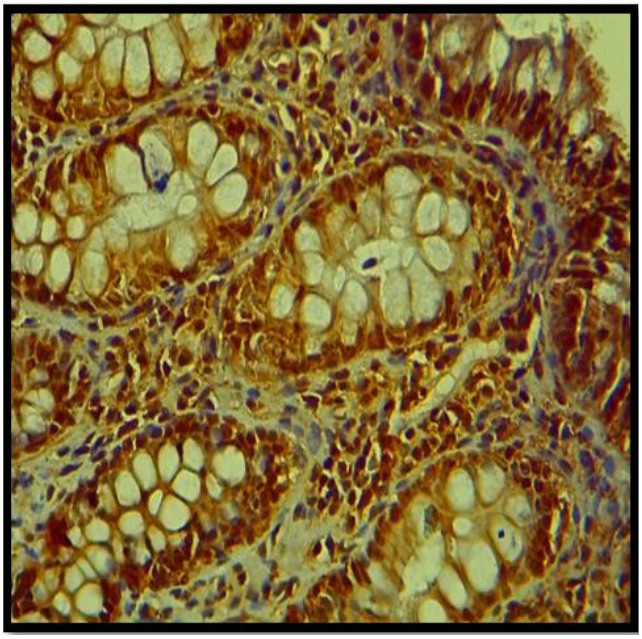

Fig. 15 : Immunohistochemical staining of nuclear PCNA colon of rats group administrated with Azoxymethane and treated with Estrogen hormone, showed moderate positive staining, 400X).

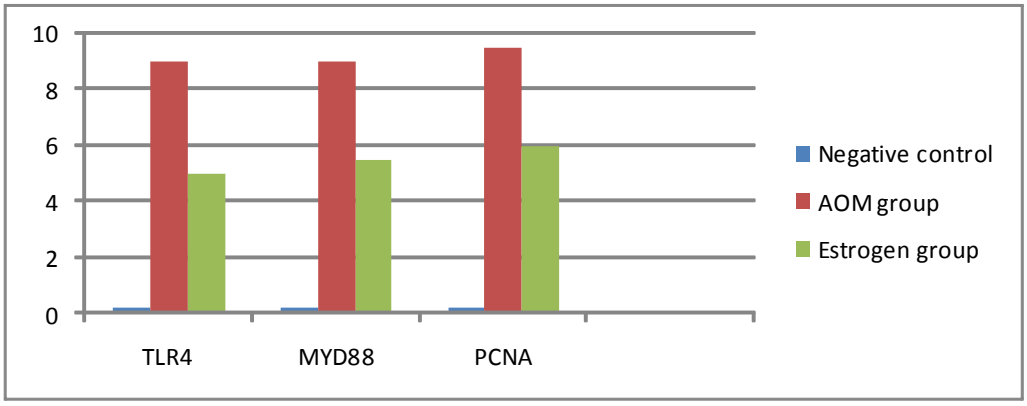

Fig. 16 : In this raw show increased in expression of three marker (TLR4, MYD88 and PCNA) when administration of Azoxymethane and decreased the expression when treated with Estrogen hormone.

Table 3 : Immunohistochemical scoring of PCNA in colon of ovariectomized rats administration with Azoxymethane and treated with Estrogen hormone group.

Marker	Group	No. of rats	Score					Percentage of positive result
			Score 0	Score1	Score2	Score3	Score4	
PCNA	Control	16	14	1	1	0	0	6.25%
	Administration with Azoxymethane	16	0	2	2	4	8	87.50%
	Administration with Azoxymethane and treated with Estrogen hormone	16	1	5	8	1	1	62.50 %
Chi-Square (χ^2)		_	_	_	_	_	_	13.477**

** (P<0.01). (positive score >2).

weight of rats) and treated with Estrogen hormone (40 *M*g/Kg B.W of rat). The nuclear expression of PCNA was reduced, the number of samples staining positive with PCNA were 10 samples from 16 samples and the percentage was 62.5%) (fig. 15) and the statistical analysis showed significantly reduce (table 3). All previous results are illustrated in the fig. 16.

Discussion

Colorectal polyps of its great health importance and it is one of the most common causes of cancer-related death in both men and women (McCashland *et al*., 2001). The risk of colon cancer is considerably higher in men than in women (Jung *et al*., 2014). The main cause of tumours of the colon are chemical carcinogens from the environment or produced within the body from foods are important candidates (Kim *et al*., 2007). Many chemicals cause colon cancer (Corpet *et al*., 2005). The sex-specific differences worldwide was reported by Kim *et al*. (2015). The colon cancer also related with female sex hormone, especially Estrogen (Parkin *et al*., 2005).

We used ovariectomized rats to get rid of and reduce Estrogen hormone as little as possible because this hormone have a protective role against the progression of colitis and colon cancer this agree with Naugler *et al*. (2007) and Kim *et al*. (2015). Based on this evidence, we expected that ovariectomy, which creates an animal model that mimics menopause through the elimination of endogenous female sex hormones and would increase colon cancer development.

Azoxymethane that used in this study as carcinogenic compound and this is one of the main causes of colon cancer (Suzuki *et al*., 2007; Wimberly *et al*., 2013).

Azoxymethane (AOM) is a common model for colon Polyps. It can specifically induce colon cancer similar to the pathogenesis of human sporadic colon cancer. Thus, it has been extensively used in the study of the molecular biology, prevention and treatment of colon cancer. After administration, AOM is metabolised into methylazoxymethanol by CYP2E1, which causes DNA mutations (Otori *et al*., 1999).

In the first group (Azoxymethane group), the histopathological result was crypt foci and Polyps was appeared focal hyperplastic polyps. Hyperplastic glands and proliferated surface epithelium without atypia and Mucin poor type demonstrates micropapillary architecture, mucin depletion and an absence of goblet cells, all these features refer to cancer began appeared because the non-controlling growth of the epithelial tissue this result from the mutation occur in the gene (Chen *et al*., 2003).

When rats treated with Estrogen hormone the histopathological signs of polyps are reduced also reduce the crypt foci and showed reactive lymphoid follicular hyperplasia and mild regeneration of the hyperplastic polyps all these signs referred to the enhance immunity of the rats and recover is began by reactive of lymphoid follicular (Taher *et al*., 2013).

In the first part (carcinogenic part) the three ovariectomized group (administrated with Azoxymethane group, administrated with Azoxymethane and treated with Estrogen Hormone group and control negative group) and used three immunological marker (TLR4,MYD88 and PCNA) to observation the changes in this marker in all group.

The result revealed increasing in expression of the these markers (toll like receptor 4 ,Myd88 and PCNA) as in the fig. 16.

The increase in expression of TLR4 in the Azoxymethane group (93.75%) when the Polyps appear because TLR4 act via MYD88-independent pathways with activation of NF-κB signalling. TLR4 play a vital role in activating immune responses. TLRs have been shown to mediate inflammatory responses (Francesco *et al*., 2015).

The Myd88 was (93.75%) increased in the group administered Azoxymethane when the Polyps was began appear because MyD88 mutation associated with lymphoma development and MyD88 signalling also involved in cancer-associated cell intrinsic and extrinsic inflammation progression and carcinogenesis.

The Detection of MyD88 expression was to predict prognosis of various cancers, *e.g.*, lymphoid, liver, and colorectal cancers. In polyps, MyD88 protein acts as a bridge between the inflammatory signalling from the TLR/IL-1R and R as oncogenic signalling pathway. However, the MyD88 signalling played dual functional roles in colorectal cancer, *i.e.* The tumour-promoting role that enhances cancer inflammation and intestinal flora imbalance to induce tumour invasion and tumour cell self-renewal, and the anti-tumour role that helps to maintain the host-microbiota homeostasis to induce tumour cell cycle arrest and immune responses against cancer cells. (Wang *et al*., 2018).

The PCNA also increased expression in its (87.5%) when administrated of Azoxymethane and the polyps begin appeared because PCNA is a 36-κDa 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 recognizes PCNA protein, which is at the maximum level in the late G1 and S phase of proliferating cells (Qasim *et al*., 2012).

The Estrogen hormone act to reducing the effect of carcinogenic material (Azoxymethane) suggesting a protective role for sex hormones in the development of the disease. Preclinical data supports a role for Estrogen and its receptors in the initiation and progression of CRC and establishes that protective effects of Estrogen are exerted through ERβ. Hormone replacement therapy (HRT) in postmenopausal women as well as consumption of soy reduces the incidence of CRC reduced the risk of colon cancer by 56% (95% CI, 0.38 to 0.81; P=0.003). A recent meta-analysis showed that in females, consumption of soy reduced the risk of colon cancer by 21% (95% CI, 0.03 to 0.35; P=0.026). This agrees with Barzi *et al*. (2013). Therefore, the expression of three markers was decreased (fig. 16).

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