American Journal of Biology and Life Sciences
2018; 6(2): 18-30
http://www.openscienceonline.com/journal/ajbls
ISSN: 2381-3784 (Print); ISSN: 2381-3792 (Online)

Open Science

The Effect of the Combination of Cetuximab (Erbitux®) and Sodium Butyrate on Mucous Secreting Cells during Rat Colon Carcinogenesis

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To cite this article

Farida Elmoursi Elbassiouni, Elsayed Ibrahim Salim, Doaa Hussein Zineldeen. The Effect of the Combination of Cetuximab (Erbitux®) and Sodium Butyrate on Mucous Secreting Cells during Rat Colon Carcinogenesis. *American Journal of Biology and Life Sciences.* Vol. 6, No. 2, 2018, pp. 18-30.

Received: August 30, 2017; **Accepted**: January 23, 2018; **Published**: July 23, 2018

Abstract

Colon carcinogenesis is a multistep process which originates from a series of histopathological and molecular alterations. To study the effect of the combination of Cetuximab (Erbitux™) and Sodium butyrate and the role of mucous secreting cells during short –term rat colon carcinogenesis, we used male Wistar rats divided into 5 groups. Group 1: Normal control rats, group 2: 1,2-dimethylhydrazine (DMH)- injected rats, group 3: Rats injected with DMH then received Cetuximab at a dose of 10 mg/kg body weight for two weeks in the 5th and 8th week of start, group 4: Rats injected with DMH then treated with Na-butyrate, i.p, 200mg/kg body weight from the 5th week till the end, and group 5: Rats injected with DMH then treated with both Cetuximab and Na-butyrate at the same doses and protocols as in groups 3 and 4. The rats from groups 3, 4 had significantly lower numbers of colonic cancer biomarkers; namely aberrant crypt foci (ACF) and mucin depleted foci (MDF) as compared with DMH group 2. Interestingly, the Cetuximab and Na-Butyrate-treated group 5 had significantly the lowest numbers of ACF and MDF as compared with DMH control, as well as retained the goblet cell numbers in the colonic crypts almost to control levels.

Keywords

Colon Carcinogenesis, Cetuximab, Erbitux, Sodium Butyrate, Aberrant Crypt Foci, Mucin Depleted Foci

1. Introduction

Colorectal cancer (CRC), also called colon cancer or bowel cancer includes cancerous growths in the colon, rectum and appendix (Zeeneldin, Saber et al. 2013). CRC is one of the leading causes of cancer death worldwide and is the third most common form of malignancy in both men and women after lung and breast cancers (Gunning, Kirby et al. 2013, Quintana-Hayashi, Mahu et al. 2015).

The gastrointestinal tract is lubricated by a continuously secreted mucus layer which can also act as a barrier against pathogens. (Quintana-Hayashi, Mahu et al. 2015). Mucins, high molecular weight glycoproteins predominantly expressed at the epithelial surface of tissues, provide protection under normal physiological conditions (McAuley, Linden et al. 2007); (Cone 2009).

DMH and its metabolite azoxymethane AOM are the agents widely used in experimental models of colorectal carcinogenesis in rodents. They are highly specific indirect colorectal carcinogens that induce the initiation and promotion steps of colorectal carcinogenesis yielding colorectal tumor lesions in a dose-dependent manner in rats,

mice and hamsters (Ansil, Prabha et al. 2013).

Aberrant crypt foci (ACF) can be easily induced in rodent colon after single or multiple administrations of DMH or its metabolite azoxymethane (Tanaka 2009). They are precancerous lesions of CRC which appear as larger crypts with a thicker layer of epithelial linings which slightly elevate and protrude toward the lumen (Bird and Good 2000) ACF show numerous similarities to those seen in human sporadic colon carcinoma. Thus, formation and growth of ACF caused by DMH induction in experimental models are being used as a potent marker to identify modulators of colon carcinogenesis (Tammasakchai, Chaiyasut et al. 2015). They are highly heterogeneous in number and crypt multiplicity, and moreover, the sequential changes in ACF during carcinogenesis are debated (Papanikolaou, Wang et al. 2000, Maurin, Forgue-Lafitte et al. 2007, Raju 2008).

Mucin depleted foci (MDF) are dysplastic colonic crypts characterized by the absence or low production of mucin and overexpression of β-catenin protein. They are precancerous lesions of the colon identified in carcinogen-treated rodents (Caderni, Femia et al. 2003). (Femia, Swidsinski et al. 2012).

Butyrate (BT) is the traditional name for the conjugate base of butyric acid. The formula of the butyrate ion is $C_4H_7O_2^-$. The name is used as part of the name of esters and salts of butyric acid, a short chain fatty acid. Examples include Sodium butyrate, Butyrates are important as food for cells lining the mammalian colon (colonocytes). Without butyrates for energy, colon cells undergo autophagy (self-digestion) and die (Donohoe, Garge et al. 2011). Short-chain fatty acids SCFA which include butyrate, are produced by beneficial colonic bacteria (probiotics) that feed on, or ferment prebiotics, which are plant products that contain adequate amounts of dietary fiber. These short-chain fatty acids benefit the colonocytes (cells of the colon) by increasing energy production and cell proliferation, and may protect against colon cancer (Lupton 2004).

Cetuximab is a monoclonal immunoglobulin G1 antibody that targets the epidermal growth factor receptor (EGFR) that plays important roles in the regulation of cell growth and development (Citri and Yarden 2006, Mitsudomi and Yatabe 2010, Wang 2016). The EGFR signalling pathway is involved in processes critical to tumor growth (Lenz, Van Cutsem et al. 2006). Cetuximab has a higher affinity than EGFR's natural ligands such as the epidermal growth factor (EGF) and transforming growth factor-α (TNF- α), thereby inhibiting the effects of EGFR activation. In July 2009, the Food and Drug Administration (FDA) approved Cetuximab (Erbitux) for treatment of colon cancer.

2. Materials and Methods

2.1. Animals

The experiments were carried out using male 6-weeks old Wister rats, obtained from the Central Animal House of Cairo University, Cairo, Egypt. After one week acclimation period to the animal facility conditions at Tanta University, 50 animals were divided according to body weights to minimizine group differences, into five groups of 10 animals each. The rats were kept in polypropylene cages with wood chips for padding and were kept in a room maintained at 25±2˚C with a 12 h light/dark cycle and a relative humidity of 50 ± 5. Drinking water and basal diet were offered to all rats. The department council of the Zoology Department, Faculty of science, Tanta University has approved the experimental design.

2.2. Chemicals

a. 1, 2- dimethylhydrazine (DMH) (purity 99.9%) was obtained from Sigma-Aldrich Co. (Saint Louis, MO, USA).
b. Sodium butyrate (Product No. 303410) was obtained from Sigma-Aldrich Co. (Saint Louis, MO, USA) (powder).
c. Cetuximab (Erbitux) was obtained from Merck (KGaA, Darmstadt, Germany) (5 mg\ml).

2.3. Experimental Groups

a. Group 1: Normal control rats injected with 0.9% saline.
b. Group 2: DMH- injected rats.
c. Group 3: Rats injected with DMH then received Cetuximab at a dose of 10 mg/kg body weight for two weeks (the 5th week and the 8th week) of start (Nautiyal et al., 2012).
d. Group 4: Rats injected with DMH then treated with Na-butyrate, i.p, 200mg/kg body weight from the 5th week till the end (Kumar et al., 2005).
e. Group 5: Rats injected with DMH then treated with both Cetuximab and Na-butyrate at the same doses and protocols in groups 3 and 4. Scarifying 12 weeks after start.

2.4. Whole Mount Preparation of Colon

The cecum and anus were removed and the colon was inflated with saline, longitudinally opened and then trisected starting at the distal end into three equal lengthwise segments that were operationally defined as representing proximal, middle and distal regions, respectively. The three areas were placed in filter paper. The entire colon was excised from cecum to anus in < 4 min from time of euthanasia till fixation in 10% buffered formalin (McGinley et al., 2011).

2.5. Methods

2.5.1. Identification of Aberrant Crypt Foci (ACF)

Colon whole mounts were removed from 10% phosphate buffered formalin and stained with 0.02% methylene blue in distilled water for 2-5 minute (Bird, 1995). The number of aberrant crypts in each focus was counted and divided by the total numbers of aberrant crypts to evaluate the crypt

multiplicity. ACF that showed variations in its multiplicity, such as foci containing 1 crypt (1AC), foci with 2 or 3 crypts (2ACs and 3ACs), or larger foci with- or more than 4 crypts (≥4ACs), were counted and separated in categories. Capture images were shot, and then the samples were stored again in buffered formalin.

2.5.2. Identification of Mucin Depleted Foci (MDF)

After ACF determination, whole colons were re-stained with alcian blue-neutral red (AB-NR) for MDF evaluation (Femia et al., 2007). The colonic epithelial normal mucosa appeared as a reddish background (NR staining) dotted with blue spots representing the opening of normal crypts full of mucus stained with AB. The MDF were distinguished from this blue-dotted background as a reddish spot in which the crypts do not produce mucin.

2.5.3. Mucin Staining

The periodic-acid-Schiff (PAS) staining according to Mc, M. J. (1946). (Mc, 1946). Glycogen, mucin and some basement membranes red/purple and Background is blue. Colloidal iron staining according to (Tickoo et al., 1998) The results are Acid mucopolysaccharides (blue) and Nuclei (red). Hematoxylin and eosin stains The histopathology was carried out according to Bancroft and Stevens (Bancroft and Gamble, 2008), using Harris hematoxylin and eosin staining technique (Bancroft and Layton, 2013).

2.6. Statistical Analysis

Results were expressed as means ± S.D. Multiple comparisons were performed by one-way analysis of variance ANOVA followed by Dunnet's multiple comparison test. Comparison between any two groups was analyzed by the unpaired Student's t-test using GraphPad Prism 7.00 software (GraphPad Software, San Diego, USA). Statistical significance was considered when the P 3.

3. Result

3.1. Data for Average Body, Absolute and Relative Liver and Kidneys Weights

The data for average body weight, absolute and relative liver and kidneys weights for rats in all groups are shown in Table 1. DMH treatment in group 2 caused a significant body weight reduction compared with untreated control. Rats treated with Na-Butyrate, Cetuximab or both after DMH in groups 3-5 showed a slight but non-significant body weight reduction as compared with the DMH-only treated group 2.

On the other hand, Table 1 shows that DMH treatment caused a significant decrease in absolute liver weights below normal control group 1. In groups 3 and 4 the treatment with Cetuximab and Na-Butyrate respectively caused a significant elevation in the absolute liver weights above DMH control group 2. However, combined treatment with both Na-Butyrate and Cetuximab has significantly decreased the absolute liver weight levels below the DMH control. Also DMH treatment showed a significant decrease in the relative liver weights below normal control. However, the relative liver weights levels detected in groups 3 and 4 treated with Na-Butyrate or Cetuximab, respectively were significantly elevated almost to control levels. Combined treatment with both Na-Butyrate and Cetuximab has also elevated the relative liver weights levels above the DMH control but without significant difference. The treatment with DMH in group 2 has significantly reduced absolute kidney weights as compared to normal control values. Treatment with Cetuximab, Na-Butyrate have not changed the absolute kidney weights as compared with group 2, however, the treatment with both drugs have elevated absolute kidney weights almost to control levels. Relative kidney weights were not significantly changed between groups. Figure 1 shows growth rates of rats in all experimental groups.

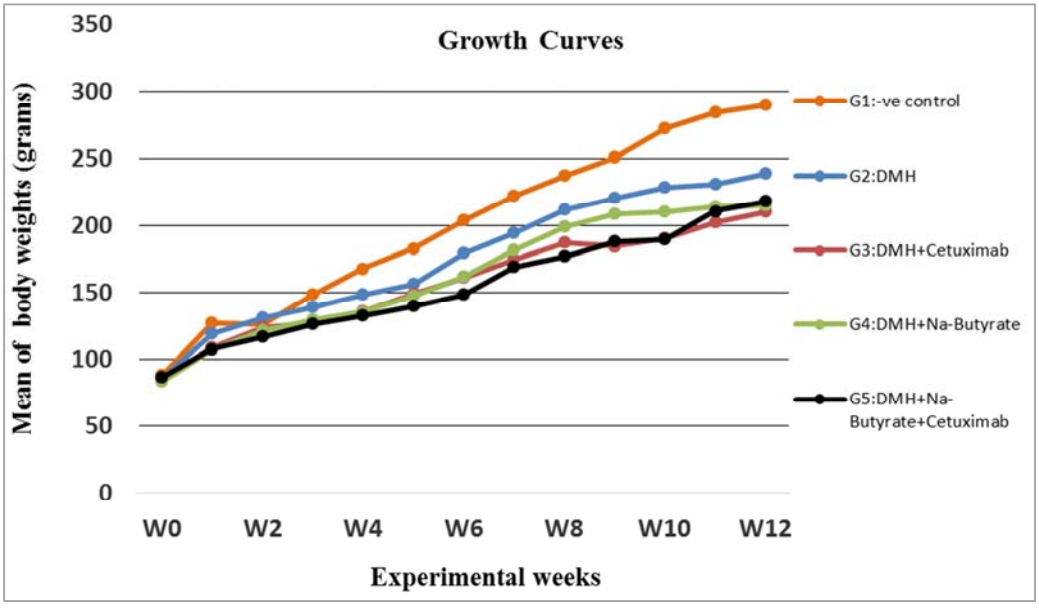


Figure 1. *Changes in body weights of different groups under study.*

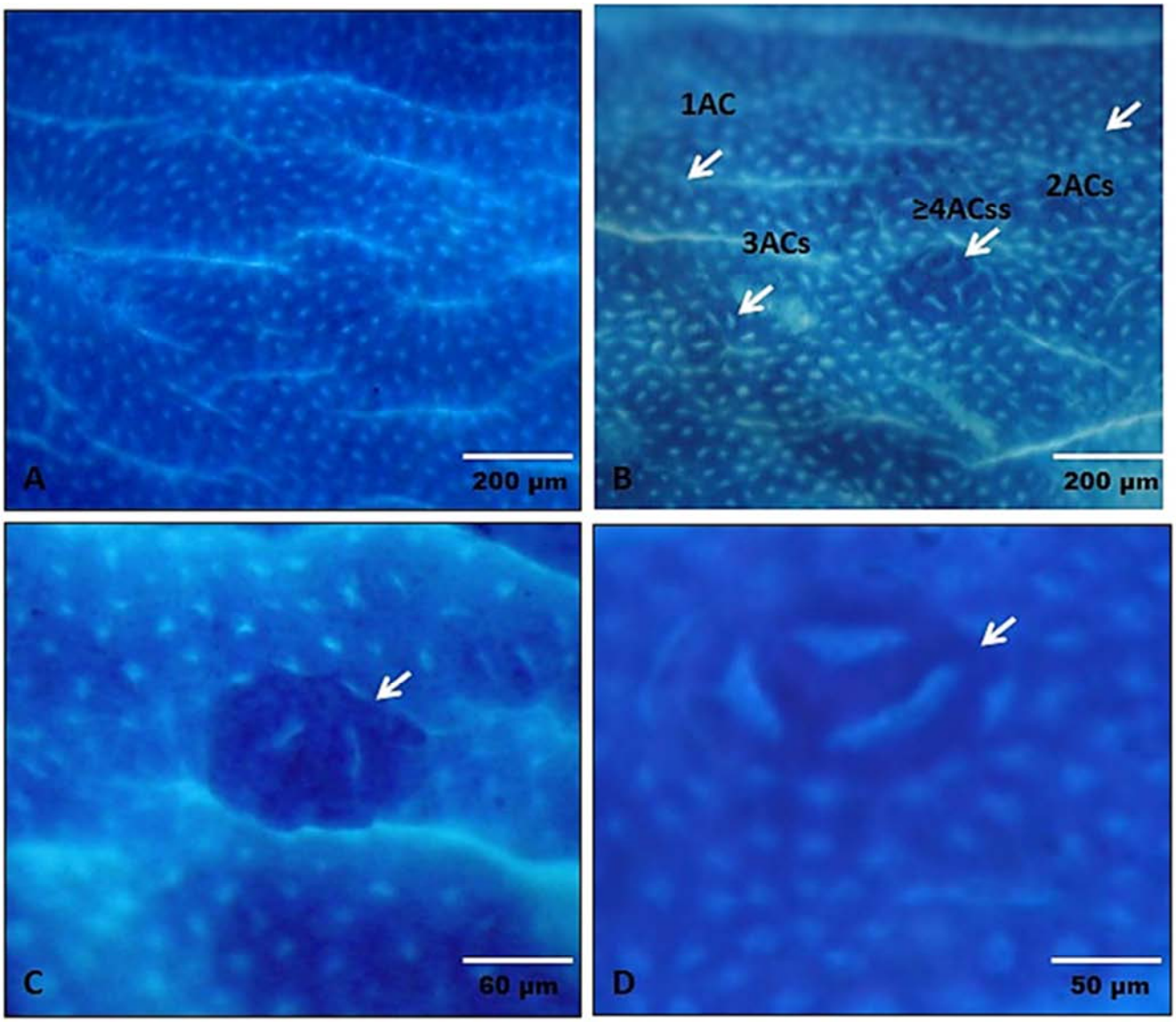
Table 1. *Data for Average Body weights, Absolute and Relative Liver and Kidneys Weights.*

Group	Treatment	Body Weight[a] (grams)		Organ Weight[b,c] (grams)	
		Initial	Final	Liver Weights	Both Kidneys Aver. Weights
1	0.09% Saline	87.9±6.2	290.0±23.4*	7.8±0.6[b]*(2.7)[c]*	1.7±0.18[b]*(0.5)
2	DMH	85.4±8.5	239.0 ±28.0	5.3±0.7 (2.2)	1.5±0.16 (0.6)
3	DMH+Cetuximab	85.3±7.6	210.4±12.8*	6.3±0.8*(3.0)*	1.5±0.17*(0.6)
4	DMH+Na-Butyrate	83.1±7.5	215.4±33.2	6.4±0.6*(2.9)*	1.3±0.17*(0.6)
5	DMH+Na-Butyrate+ Cetuximab	86.3±7.7	218.5±23.8	4.8±0.7*(2.3)	1.2±0.16*(0.5)

a: Values are means ± S.D; b: absolute organ weight; c: Numbers in parenthesis are relative organ weights (%) = Organ wt./body wt.×100; *: P<0.05 vs. group 2.

3.2. Morphology of ACF

ACF were observed in the whole colons of the DMH-treated rats after staining with methylene-blue, with the complete lack of such lesions in normal colons. The most distinguishable morphological features of ACF that allowed their detection and classification and distinguished them from the normal surrounding crypts were: that ACF were generally larger than normal (with reduced distance among crypts) with different crescent-shaped luminal openings. Figure 2 shows the normal shape of crypts in control group, as well as the different aberrant crypt foci with only 1 aberrant crypt (AC), the double (2ACs), triple (3ACs), four and more than four (≥ 4ACs) branching foci. Also, ACF were distinguished by their dimensions and wider luminal shapes. These features enabled detection of the focus and counting the number of crypts in the focus (crypt multiplicity). All ACF were surrounded by darkly stained pericryptal zones.



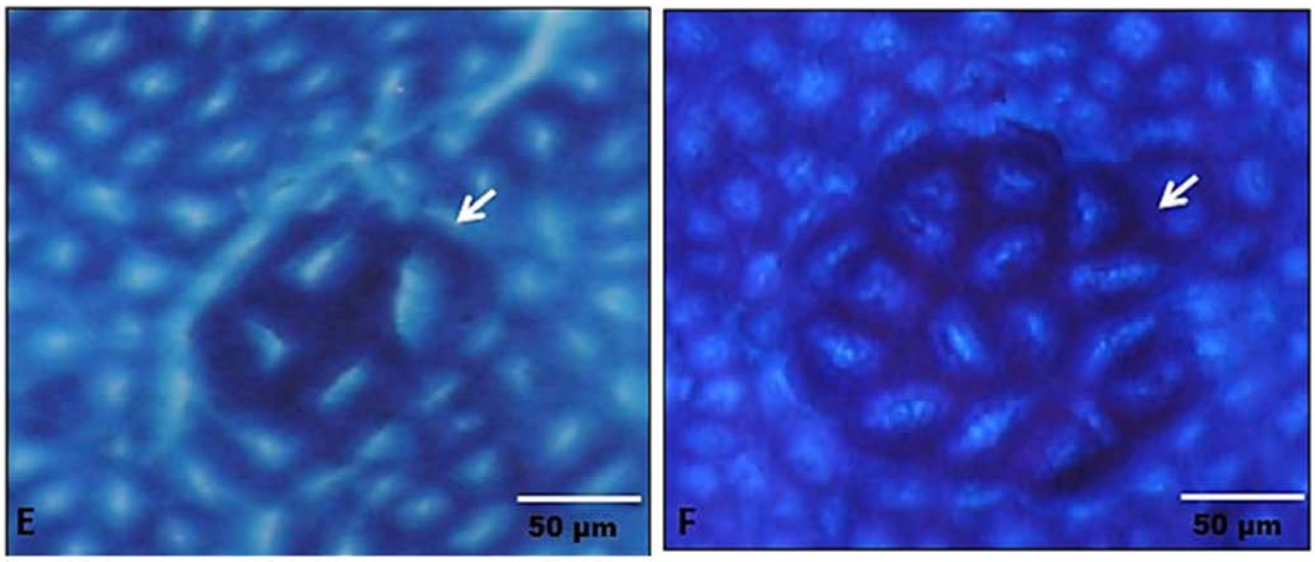


Figure 2. *Photomicrographs of rat whole colon stained with methylene blue. A): Normal colonic mucosa; B): DMH-administered rat colon showing ACF with different crypt multiplicities; C): Aberrant crypt with 2ACs; D): 3ACs; E): 4ACs; F):≥4ACs. Methylene Blue stain.*

3.3. Effect of Na-Butyrate and Cetuximab on DMH-Induced ACF Formation

Table 2 and Figure 3 show the total numbers of ACF as well as the numbers of different crypt criteria. It was found that the rats treated with Cetuximab, Na-Butyrate or both had significantly lower total numbers of ACF (P<0.05) as compared with the DMH control group. Treatment with both drugs in combination in group 5 showed the lowest total ACF numbers; which was significantly different (P<0.05) as compared with each drug alone. Interestingly, all treatments have significantly reduced the large ACF (≥4ACs) numbers, which are expected to develop into tumors, with the combination treatment in group 5 exerted the highest inhibition values (Table 2).

Table 2. *Effect of Na-Butyrate, Cetuximab or Their Combination on DMH-Induced ACF Formation in Rats.*

Group	Treatment	n	Total No. of ACFa	No. of Foci containing: a			
				1 AC	2 ACs	3 ACs	≥4 ACs
1	0.9% Saline	10	0	0	0	0	0
2	DMH	10	129.1±6.4	76.8±5.8	28.1±3.3	16.0±2.0	8.2±3.0
3	DMH + Cetuximab	10	86.8±6.1*	46.9±3.3*	24.0±3.4	11.0±2.0*	4.9±2.2*
4	DMH + Na-Butyrate	10	109.9±7.0	64.5±4.7	23.5±4.0	15.1±2.0	6.8±1.3
5	DMH + Na-Butyrate + Cetuximab	10	51.6±7.7*	27.1±5.0*	13.5±3.0*	6.4±2.4*	4.6±1.2*

n: Number of rats tested per group; a: Values are means ± S.D.; AC: Aberrant crypts; *: P<0.05 vs. group 2.

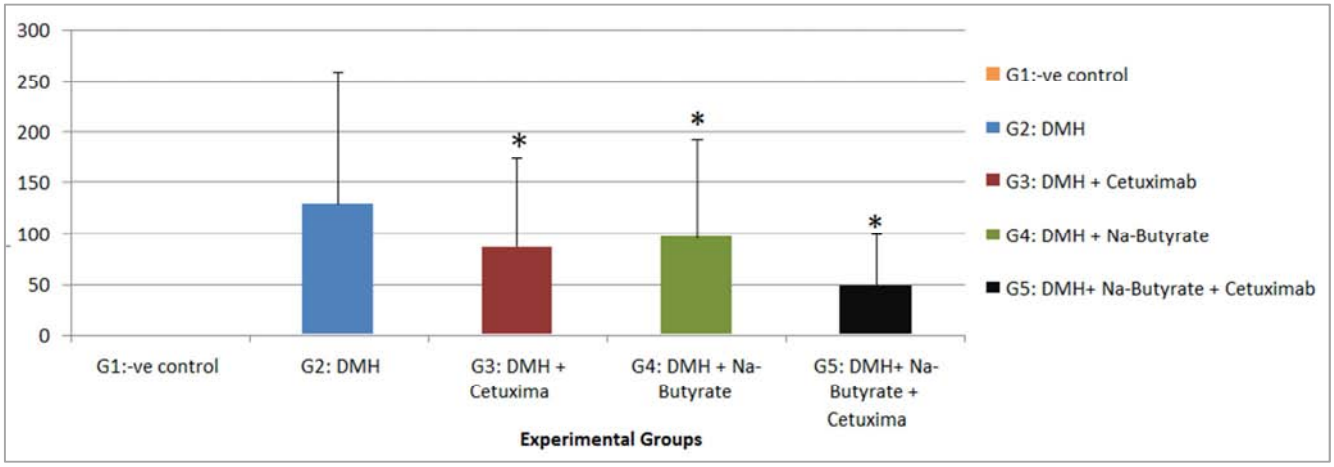


Figure 3. *Average total numbers of DMH-induced ACF in all groups under study ;(*): P<0.05 vs. group 2.*

3.4. Incidence and Numbers of Mucin Depleted Foci (MDF)

MDF were identified as focal lesions by the following criteria: (a) absence or very small production of mucins; (b) distortion of the opening of the lumen compared with normal surrounding crypts; (c) elevation of the lesion above the surface of the colon; and (d) multiplicity (i.e., the number of crypts forming each focus) higher than 3 crypts. To be defined as MDF, a focus had to fulfill the first criterion (absence or very low production of mucins) and at least two of the other criteria listed above. Figure 4 shows the gross appearance of the MDF within colonic mucosa.

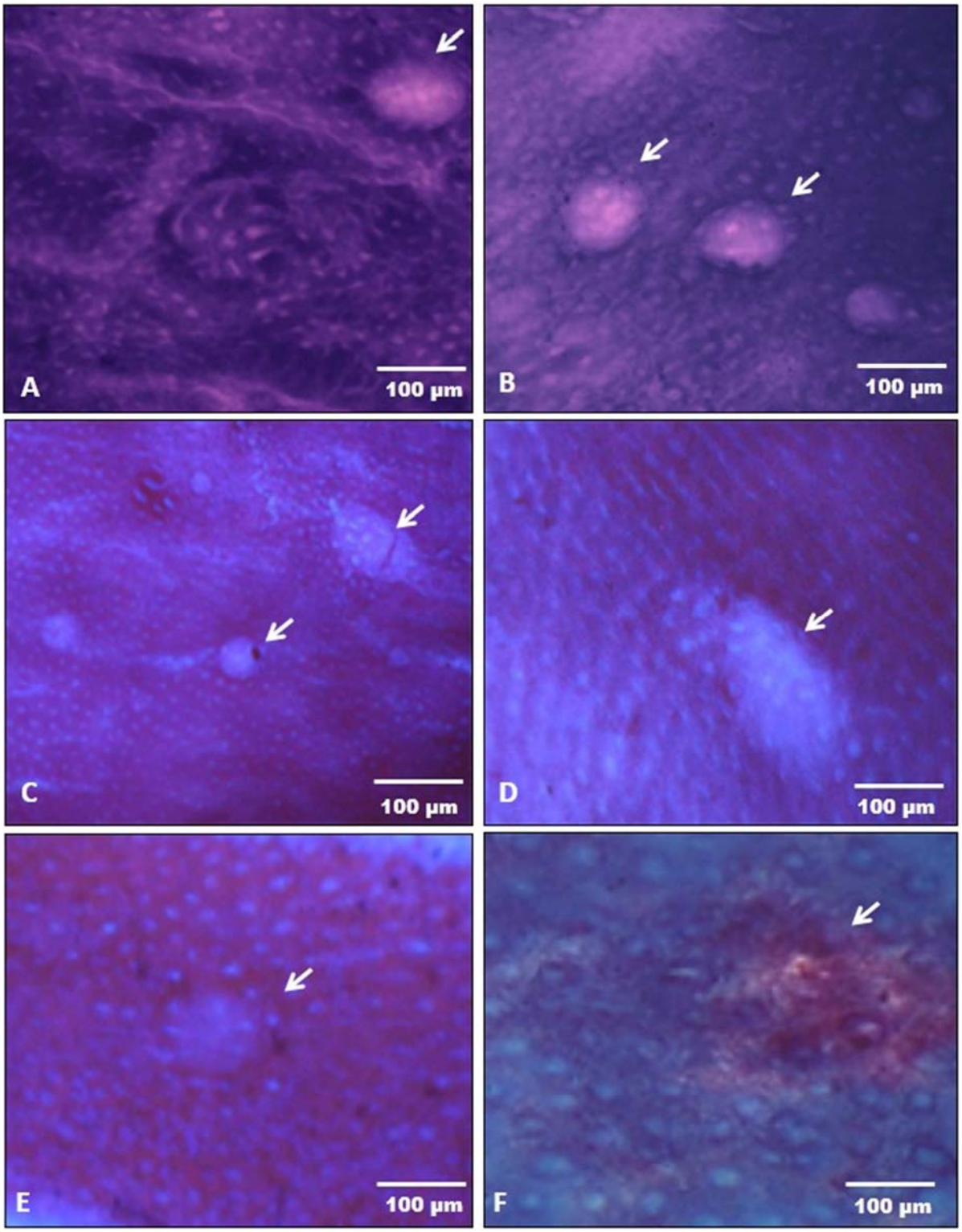


Figure 4. *Photomicrographs of rat whole colon stained with Alcian blue- neutral red (AB-NR) showing different shapes and size of MDF distinguished from the blue-dotted background as a reddish spot in which the crypts do not produce mucin. AB-NR stain.*

3.5. Effect of DMH on the Development of MDF in Colonic Epithelium

MDF were identified in Alcian blue-neutral red (AB-NR) stained whole colons (Figure 4) as focal lesions by the absence or very scant production of mucin, and a distortion of the opening of the lumen compared with normal surrounding crypts (Figure 4). Table 3 and Figure 5 show the data of MDF calculations. The incidence of MDF in the colonic epithelia was 100% in groups 2-4 and 90% in group 5. The rats of groups 3-5 had significantly lower total numbers of MDF (P<0.05) as compared with the DMH-only treated group 1, with group 5 exhibited the lowest total MDF numbers among the DMH-induced groups.

Table 3. *Incidence and Average Total Numbers of Mucin Depleted Foci (MDF) in Control and Treated Groups.*

Group	Treatment	*n*	MDF Incidence[a]	Average Total No. of MDF/Colon/Rat[b]
1	0.09% Saline	10	0%	0
2	DMH	10	100%	18.1±8.6*
3	DMH+Cetuximab	10	100%	6.5±11.7*
4	DMH+Na-Butyrate	10	100%	11.0±6.8*
5	DMH+Na-Butyrate+Cetuximab	10	90%	8.1±7.6*

n: Number of rats tested per group; a: Incidence= No of rats bearing MDF/group; b: Values are Means ± S.D; *: P<0.05 vs. group 2.

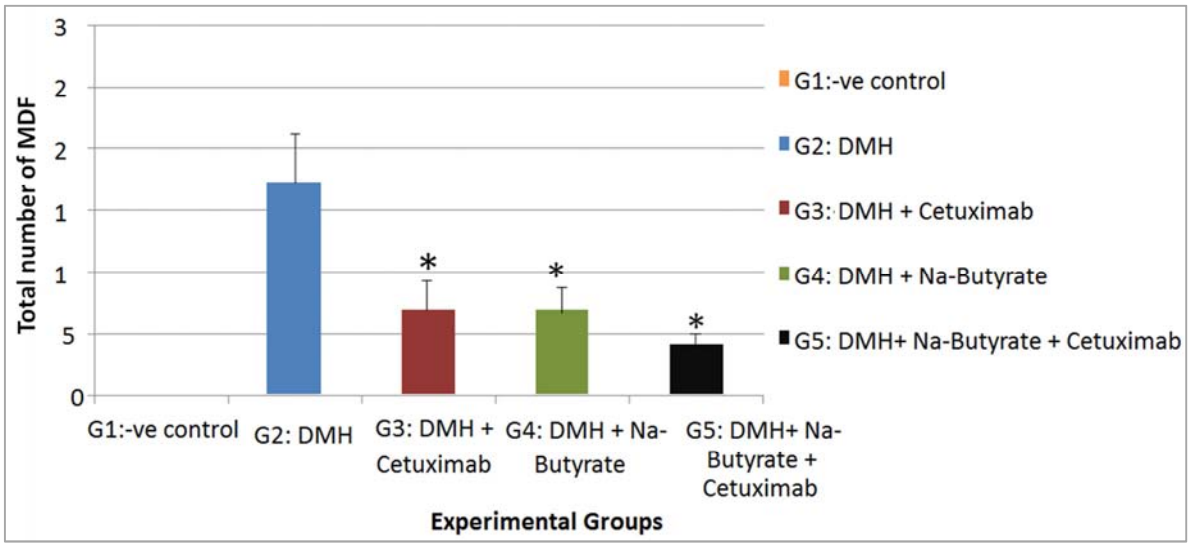


Figure 5. *Effect of Na-Butyrate and Cetuximab or their combination on the average total numbers of Mucin Depleted Foci (MDF) of control and treated groups; (*): P<0.05 vs. group 2.*

3.6. Histology of the Colon

Figure 6 shows the histological structure of the rat colon from control and DMH treated groups. The normal colon was found to be compromised of three distinct histologically different regions; the proximal, the middle, and the distal colons. The colon wall is composed of an external serosal area of squamous simple epithelium followed by muscularis area, then with a lamina propria of connective tissues. The inner most layer, the mucosa, is formed of long mucosal crypts which are branched tubular glands for mucin secretion. Proximal colonic areas were distinguished histologically by the presence of mucosal folds. The areas of mid colons are distinguished by longer and higher numbers of mucosal crypts, while distal colonic areas are distinguished by shorter mucosal crypts, thicker muscular layer and less density of mucosal crypts. The mucosal crypts from rats administered by DMH are histologically distinguished by paler eosinophilia, slight dysplastic colonic crypts with wider openings and obvious fewer numbers of mucous secreting cells. Parts of hyperplasia could be distinguished occasionally and higher numbers of lymphocytic cells are frequently observed among the crypts (Figure 6).

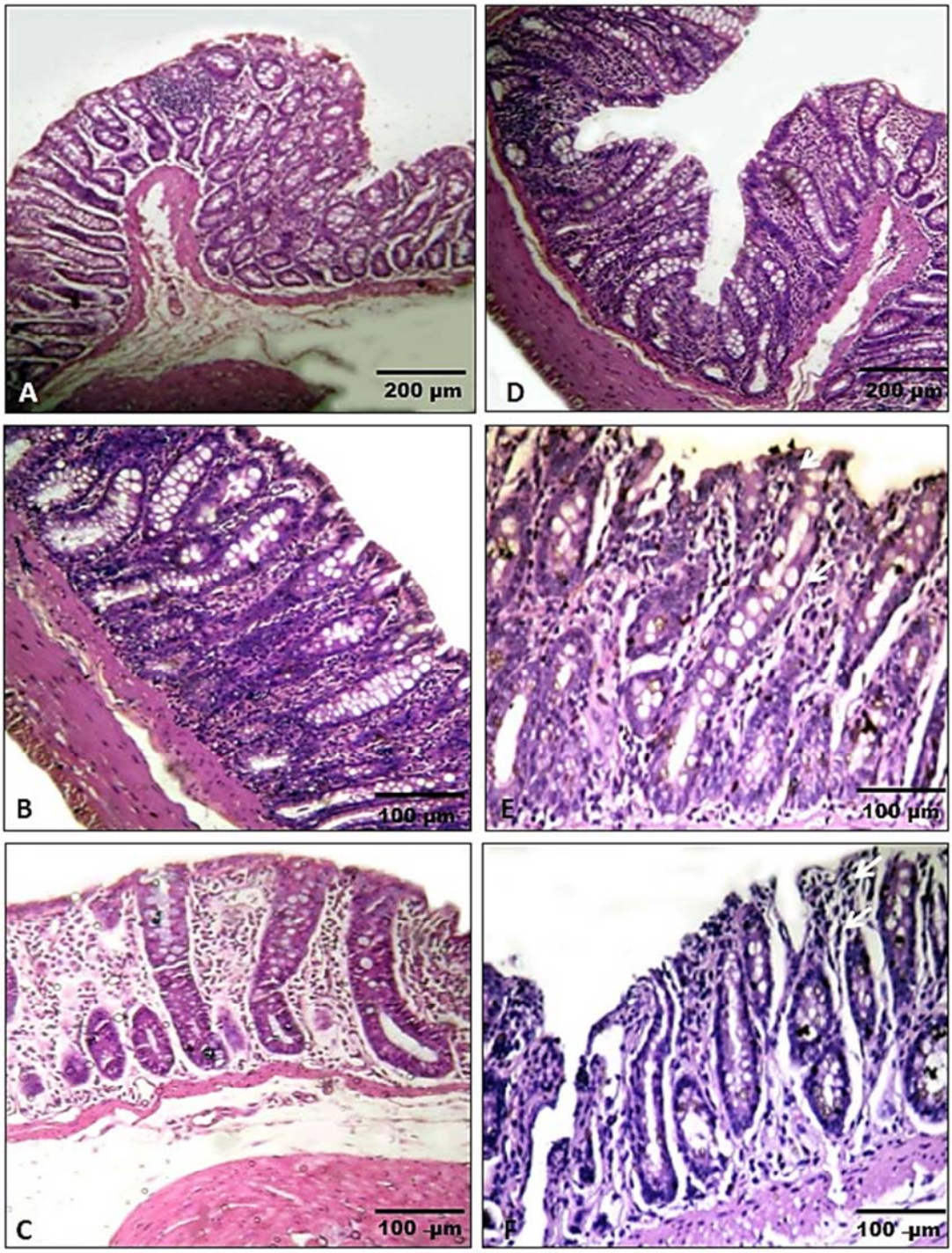


Figure 6. *Photomicrographs of rat colon histology. A-C: Normal colonic epithelium of A): proximal colon; B): Mid colon; C): Distal colon. D-F: Colonic epithelia from rat treated with DMH. D): Proximal colon E): Mid colon; F): Distal colon showing mild dysplastic colonic crypts. H&E stain.*

3.7. Mucin Histochemistry

3.7.1. PAS Histochemistry for Neutral Mucins

Figure 7 shows the PAS stained glycoproteins (neutral mucins) in the mucous secreting cells in normally appearing mucosa of control, DMH-only-treated rats, Cetuximab-treated rats, Na- Butyrate treated rats or rats received both Cetuximab and Na- Butyrate. In non-treated control rats, the PAS staining was strong and intense in all colonic areas (proximal, middle and distal colons). On the other hand, the mucosa of DMH-treated rats showed faint and weak PAS staining with obvious less numbers of mucous secreting cells. The mucosa of DMH-treated group rats that received Cetuximab and the group that received Na- butyrate showed a significant increase in the goblet cell sizes and numbers, while the group that received both Cetuximab and Na-butyrate had higher sizes and numbers of mucous secreting sells comparing with the other groups and almost similar to normal control.

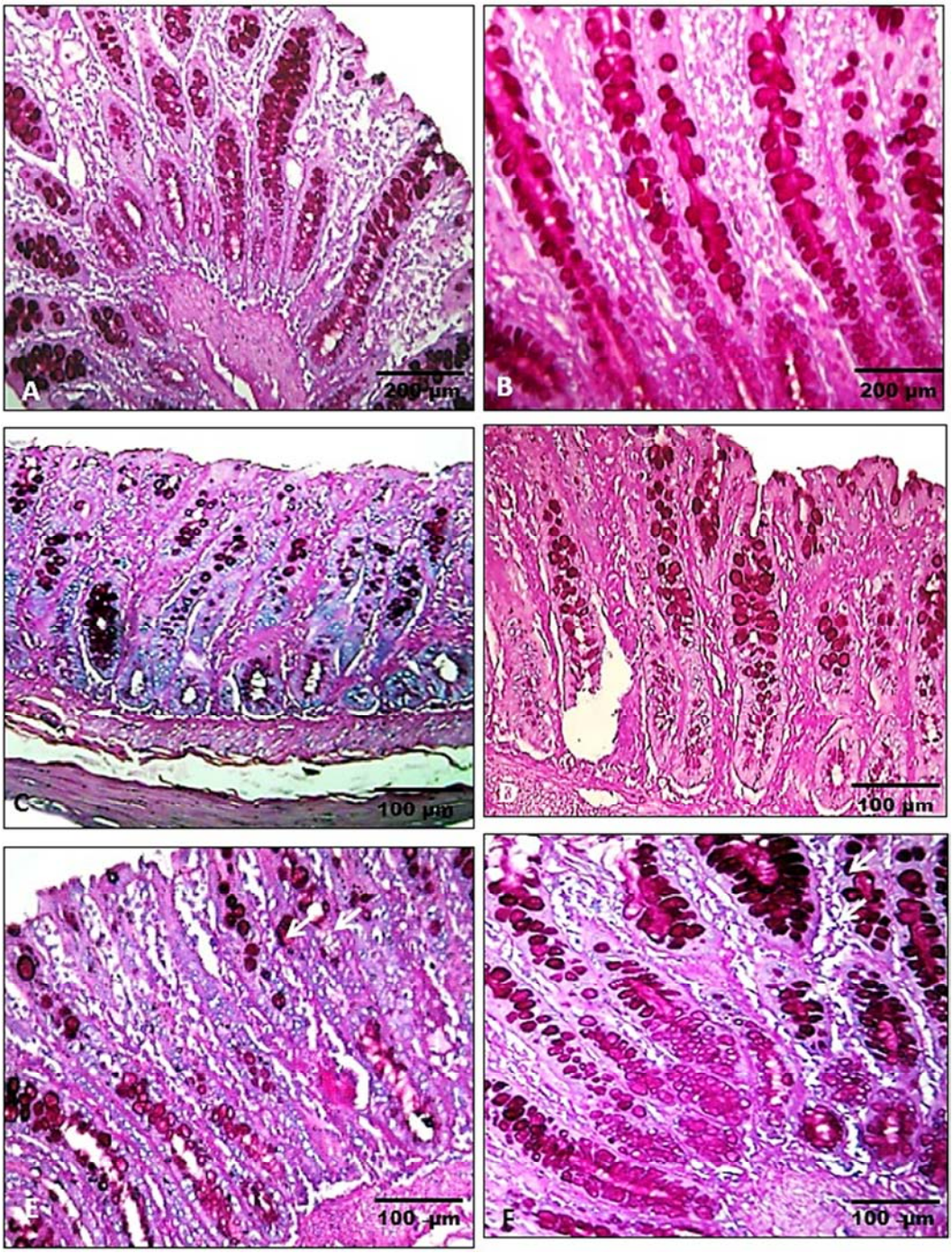


Figure 7. *Photomicrographs of rat colonic epithelia stained with PAS showing goblet cells. A.B: Normal colonic epithelium with high numbers of goblet cell; C): DMH-treated colon epithelium showing mucin depletion; D): Increased goblet cell numbers after cetuximab treatment; E): Increased goblet cell numbers after Na-Butyrate treatment; F): Higher increase in goblet cell.*

3.7.2. Calculations of Mucous Cell Numbers

Table 4 show the average numbers of PAS-positive goblet cells as average of the three colonic areas of each experimental group. The epithelia of DMH-treated rats that received Cetuximab, Na- butyrate or combination treatment showed a significant increase in the average total goblet cell numbers (P<0.05), particularly those received the combination treatment comparing to normal control (Table 4).

Table 4. *Mucous Cell Counts in Colonic Epithelium of Rats.*

Group	Treatment	*n*	Goblet cells No.[a]
1	0.09% Saline	10	511/1000*
2	DMH	10	198/1000*
3	DMH+Cetuximab	10	334/1000*
4	DMH+Na-Butyrate	10	318/1000*
5	DMH+Na-Butyrate+Cetuximab	10	484/1000*

n: Number of rats evaluated per group; a: No. of PAS-positive cells per 1000 epithelial cells; *: P<0.05 vs. group 2.

3.7.3. Hale's Colloidal Iron Staining for Acid Mucins

Figure 8 shows Hale's Colloidal Iron staining for acidic mucins in different groups. The trend for acidic mucin staining in all groups was a little different than that of neutral mucin in goblet cells. The acidic mucin was stained light green in different locations within the colonic crypts. However the quantity of acidic mucin appeared less than that of neutral mucins. Normal controls showed higher histochemical reactivity for acidic mucins, while DMH treated sections were evaluated to be markedly with lower expression. Treatment with Cetuximab or Na-butyrate showed a little increase in neutral mucins. In contrast, treatment with both Cetuximab and Na-butyrate has markedly elevated expression levels of neutral mucins in all colonic areas of treated rats.

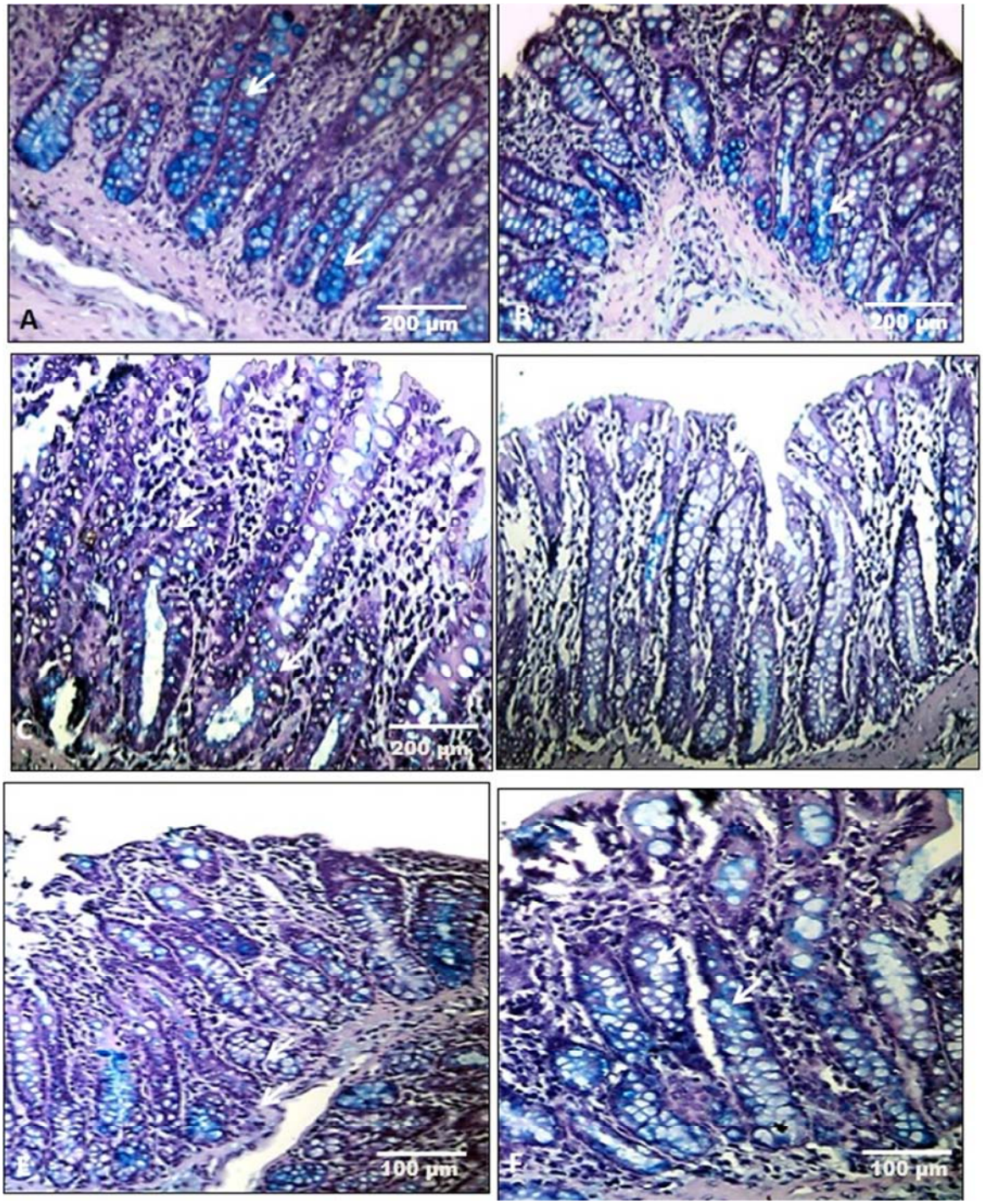


Figure 8. *Photomicrographs of rat colonic epithelia stained with Hale's Colloidal Iron staining showing acid mucins. A, B: Normal colonic epithelium with high levels of acidic mucins (arrows); C): DMH-treated colon epithelium showing acid mucin depletion; D): Relative increase in acid mucin after cetuximab treatment; E): Relative increase in acid mucin after Na-Butyrate treatment; F): Higher increase in acid mucin after cetuximab+Na-butyrate treatment. Acid mucin stained with Hematoxylin.*

4. Discussion

Colorectal cancer comprises histopathological alterations in the colon, rectum and appendix due to accumulating molecular changes in the epithelia (Jänne and Mayer 2000). It has been recently documented that mucin depletion is a hallmark for colon malignant transformation in both human and rodents. MUC-2 is particularly the main and most predominantly secreted mucin glycoprotein, and is confined to goblet cells as well as other colonic epithelial cells. (Sylvester, Myerscough et al. 2001).

Na-butyrate is the name used as part of esters and salts of butyrate, a short chain fatty acid (these include Cellulose acetate butyrate, Methyl butyrate, Ethyl butyrate, Sodium butyrate and others) *(Donohoe, Garge et al. 2011).* One mechanism underlying butyrate protective effect is partly through acting as a histone deacetylase (HDAC) inhibitor, that is bound to the Fas gene promoter resulting in hyperacetylation of the Fas promoter and up regulation of Fas receptor on the immune cell surface (Zimmerman, Singh et al. 2012).

Even though that butyrate modulates cell cycle kinetics (Hong, Turner et al. 2015). The effect of butyrate on colon cancer development is still debated. Results from some studies suggest that butyrate is chemopreventive by decreasing tumor growth via a reduction in cell proliferation and an increase in differentiation and apoptosis (Kelman, Zuo et al. 1999) (Hong, Turner et al. 2015). Also, it was shown that butyrate in combination with fish oil decreased ACF formation compared to corn oil/butyrate diet (Crim, Sanders et al. 2008). These results recommended that whether or not butyrate defense against colon carcinogenesis is reliant on nutritional fat and other mechanisms.

The present study aimed to test targeting EGFR with the presence of Na-butyrate, a histone deacetylase inhibitor, in a combined treatment. Cetuximab blocks EGFR which is known to obstruct cellular signals for cell growth, angiogenesis and proliferation (Herbst, Kim et al. 2005). Since butyrate is known to nourish cells and a key factor for cellular proliferation, we sought that targeting EGFR will overcome the increasing proliferation and differentiation effects of butyrate on the cancerous colonocytes, thus, this may give better chance to enhance butyrate's antitumor capacity exerted by histone deacetylation. Recently, HDAC inhibitors are recommended for cancer therapy or adjunct (Marks and Dokmanovic 2005). The meticulous mechanisms by which HDAC inhibitors may work are yet indistinct, but epigenetic pathways are projected *(Monneret 2007).* HDAC inhibitors could increase p21WAF1 mRNA expression. HDACs were previously found tangled with the retinoblastoma protein (pRb) that may suppress cellular proliferation. pRb protein is a fragment of a composite that combines HDACs to the nuclear chromatin to deacetylate histones *(Brehm, Miska et al. 1998).* Previous data specified that chromatin silencing mediated by HDAC and DNA methylation is a critical part of ERα inhibition in human breast cancer cells (Donohoe, Garge et al. 2011).

The histochemical examination in the present study revealed that a recovery has occurred to the mucous secreting cells as indicated by the PAS staining for neutral mucins or Hale's Colloidal Iron staining for acidic mucins. It is clear from this study that the best results were obtained after combination treatment of Cetuximab and Na-butyrate as compared with each treatment when used alone. The four-carbon fatty acid n-butyrate were shown to have miscellaneous effects on cellular morphology and metabolism in vitro, while it caused differentiation followed by apoptosis in the same cell lines (Dyson, Daniel et al. 1992). Also found that Na-butyrate could significantly constrain cellular proliferation in vitro. Butyrate made in the colonic lumen significantly preserves the homeostasis of the colonic epithelium, and it is known as a favored energy source for colonocytes (Xiao, Liu et al. 2014). These short-chain fatty acids benefit the colonocytes by increasing energy production (Lupton 2004). Butyrate has also been a supporter for the role of histone acetylation in chromatin integrity and function. It was postulated previously that inhibition of histone acetylation interrupts the expression of approximately 2% of mammalian genes (Davie 2003).

ACF and MDF are recognized precursors for colon cancer. Interestingly, their numbers were significantly reduced with all treatments used here with the combination treatment in group 5 resulting in best results. Thus, the results of the current study suggest that the combination of Cetuximab and Na-butyrate rendered better resistance against DMH-induced colon carcinogenesis. The present treatments did not show signs of serious toxicity or side effects on blood parameters as indicated by serum levels of the liver function, kidney functions, lipid profile or electrolytes. Previous studies also support our findings (Hong, Turner et al. 2015). and suggested that treatment with Na-butyrate alone although was less effective than administered to rats combined with other treatments such as fish oil, had no certain side effects.

Cetuximab shows many-sided advantages in treatment of certain epithelial tumors by blocking proliferation and metastasis while increasing tumor cell apoptosis. (Son, Hong et al. 2015). Cetuximab inhibits the proliferation of colon cancer cells in vitro (Sridhar, Seymour et al. 2003) Some cancer cells have epidermal growth factor (EGF) receptors on their surface. EGF is a protein that been produced naturally in the body attaches to the receptors. Cetuximab works by attaching itself to the EGF receptors This blocks the EGF protein from reaching the cancer cells and stops them from growing. (Raben, Helfrich et al. 2003). Investigated the antitumor activity of Cetuximab on four non-small cell lung cancer (NSCLC) cell lines Cetuximab reduced the rate of cellular proliferation along with downregulation of EGFR mRNA expression (Seshacharyulu, Ponnusamy et al. 2012) This is in line with our results about the ability of Cetuximab to decrease cellular proliferation.

Mucins are high molecular weight, heavily glycosylated proteins secreted by epithelial cells of the colon, which form a protective mucous layer in the form of gel in intestinal lumen (Robbe, Capon et al. 2004). In support to the present

results, the data of (Chen, Lin et al. 2013) confirmed the chemopreventive potential of butyrate in DMH-induced colon carcinogenesis in mice. They have shown that most of the tumor-related signaling pathways in mice treated with DMH and butyrate were MAPK pathway, Wnt pathway, insulin growth factor (IGF) pathway, and VEGF pathway which were downregulated by butyrate. These clear effects related to cell differentiation, cell cycle, cell proliferation, apoptosis, cell adhesion and cell migration, were significantly modulated by butyrate without certain side effects.

Acknowledgements

This work was carried out in cooperation with Central laboratory of zoology department, Faculty of science, University of Tanta. The authors thank Ms. Eman Zakaria and Mr. Amr Salah for their help and supports. This research received no specific grant from any funding agency in the public, commercial, or not for-profit sectors.

Abbreviations Used

Colorectal cancer (CRC); 1,2-dimethylhydrazine (DMH); Aberrant crypt foci (ACF); azoxymethane (AOM); Mucin depleted foci (MDF); Butyrate (BT); epidermal growth factor receptor (EGFR); Transforming growth factor-α (TNF- α); Food and Drug Administration (FDA); Alcian blue-neutral red (AB-NR); The periodic-acid-Schiff stain (PAS); Stander deviation (S.D).

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