

**EFFECT OF ASPIRIN ON THE PROLIFERATION PARAMETERS  
OF RAT COLONIC CRYPTS : A HISTOLOGICAL AND  
IMMUNOHISTOCHEMICAL STUDY**

**Dina Mohamed Radwan, Hanan Ali Amin and Ashraf Mahmoud Kamel**  
Histology Department, Faculty of Medicine, Cairo University

**INTRODUCTION**

Tumorigenesis has been recognized for years as a multistep process, accompanied by accumulation of mutations and changes in oncogenes and tumor suppressor genes leading to changes in behaviour of the affected cell and transformation into malignant cell (Shpitz et al., 1995).

One of the mechanisms that contributes to malignant transformation is increased cell proliferation (Hall & Coates, 1995). Gastrointestinal tract epithelium is characterized by active and rapid cell turnover. During their growth, proliferating cells divide by the cell cycle which is controlled by numerous regulatory proteins such as oncoproteins and cyclins including the proliferating cell nuclear antigen (PCNA) (Murakami et al., 1995).

Despite the most recent technologic advances, cancer of the large bowel is still a major cause of neoplastic morbidity and mortality. The first step in multistage colonic carcinogenesis is increased cell proliferation and an upward shift of the proliferating zone in colonic crypts (Shpitz et al., 1997).

Aspirin (acetylsalicylic acid) has been well known for its anti-pyretic and anti-inflammatory action over the past century. However, in recent years there has been an evidence suggesting that aspirin could reduce the risk of colorectal cancer but the exact mechanism by which aspirin exerts this chemopreventive effect is unclear and complex (Law et al., 2000).

Therefore, this work was designed to assess the effect of aspirin intake on epithelial cell proliferation in the colonic crypts of adult rats aiming at clarifying how aspirin protects against development of cancer colon.

## MATERIAL AND METHODS

Thirty adult male albino rats weighing 150 - 170 grams were included. Divided into two groups :

### **Group I :**

Consisted of 10 rats. They were given saline daily orally for 30 days.

### **Group II :**

Consisted of 20 rats. They were given aspirin (acetylsalicylic acid) at a dose 50 mg / kg daily orally for 30 days.

Aspirin was given in the dosage that had significant anti-inflammatory action and would significantly inhibit prostaglandin (PG) biosynthesis (Laneville et al., 1994).

All animals were fed ad libitum and allowed for free water supply. One day after the last dose, they were sacrificed by decapitation. Each colon was immediately dissected, rinsed with ice-cold phosphate buffered saline (PBS, pH 7.4) and processed for paraffin blocks. They were sectioned at 5  $\mu$ m and stained with hematoxylin and eosin, and periodic acid Schiff reaction.

### **Immunohistochemical study :**

To study the effect of aspirin on cell proliferative activity of rat colonic crypts, immunostaining for PCNA (proliferating cell nuclear antigen) was performed on paraffin sections. This was done using a primary antiserum to PCNA (Clone P<sub>c10</sub>, DAKO corp.) followed by biotinylated horse antimouse serum, avidin-biotin complex, and DAB as chromogen. Tonsil and small intestine were used as positive control specimens. Negative colonic tissue control sections were prepared by omitting the application of the primary antibody. A positive reaction was expressed as a relatively dark brown nuclear color (Bancroft & Cook, 1994).

### **Morphometric study :**

This was done using "Leica Quin 500" Image analyzer computer system (England) to assess :

### **A) The proliferation parameters :**

They were scored in PCNA-immunostained sections. Crypts were included in the assessment if they spanned from immediately adjacent to the muscularis mucosa to the surface. A minimum of 12 crypts per section per animal was analyzed. A single column of cells was counted in each complete midaxially sectioned crypt (Fig. 1 - a) and the number of darkly stained PCNA-positive cells was counted (Fig. 1 - b). The labeling index (LI) was calculated as follows : (Hammann et al., 1992).

$$LI = \frac{\text{Number of labeled cells in crypt column} \times 100}{\text{Total number of cells in crypt column}}$$

This was calculated for crypts both adjacent to and away from lymphoid patches.

### **B) The amount of mucosecretion :**

In serially cut PAS- Stained sections, the area and area percent of mucus were measured in five randomly chosen fields per section using the binary image (Figs. 2 - a, b) and their mean values were obtained (Hammann et al., 1992).

The mean values and standard deviations of the labeling index of proliferation and of the area percent of mucosecretion in control and experimental sections were calculated. Comparison of significance between the control and experimental groups was done using Student's "t" test (Mould, 1989).

## **RESULTS**

### **I. Histological results :**

#### **A) Control group :**

Examination of Hx & E - stained sections revealed that the colonic mucosa consisted of two types of cells : columnar absorptive cells and mucus secreting cells. They were arranged in closely packed tubular glands (colonic crypts). The muscularis mucosa was a prominent feature of the mucosa (Figs. 3 - a, b). The wall contained numerous leucocytes including lymphocytes which formed large aggregations in the lamina propria and submucosa (Fig. 3 - c). Dividing epithelial cells at different stages of mitosis were seen (Figs. 4 - a, b) while few apoptotic cells were detected within the crypts (Fig. 5).

Examination of PAS-stained sections revealed the presence of mucus-secreting cells within the crypts whereas the luminal surface was almost entirely lined with

columnar absorptive cells. It was noted that crypts adjacent to lymphoid patches had less mucus-secreting cells than those away from lymphoid aggregations (Figs. 6- a, b).

#### **B) Experimental group :**

On examining Hx & E-stained colonic sections after regular aspirin intake, it was noted that the number of apoptotic cells seen within the colonic crypts were increased. They were detected along the whole length of the crypts (Fig. 7). The apoptotic cells had different morphological features as nuclear marginalization of chromatin, condensation of cytoplasm, and cell shrinkage (Figs. 8 - a, b, c, d).

It was also noted that mucus-secreting cells seen in PAS-stained sections were greater than those seen in the control group both in crypts away from lymphoid patches (Fig. 9 - a) and in crypts adjacent to lymphoid patches (Fig. 9 - b).

### **II. Immunohistochemical results :**

#### **A) Control group :**

In PCNA-immunostained sections of control rats, positive proliferating cells were located in the lower one-third to two-thirds of the crypts (Figs. 10 - a, b). However, in crypts adjacent to lymphoid tissue, the proliferating cells were seen along the whole length of the crypt (Figs. 11 - a, b).

#### **B) Experimental group :**

Aspirin intake resulted in an overall decrease in the proliferating epithelial cells in colonic crypts compared with the control sections (Figs. 12 - a, b, c). It had also been found that in crypts adjacent to lymphoid aggregates, there was a downward shift of the proliferating positive cells to the lower one-third (Fig. 13).

### **III Morphometric and statistical results :**

#### **A) The proliferation parameters :**

The mean labeling index of proliferation in control rats was found to be  $48.73 \pm 2.34$  in crypts away from lymphoid aggregates and  $54.73 \pm 4.43$  in crypts bordering lymphoid follicles. Aspirin intake resulted in a significant decrease of labeling index in crypts both away from and adjacent to lymphoid tissue where it reached  $35.73 \pm 2.08$  and  $39.26 \pm 4.11$  respectively.

The mean values of labeling index in the studied groups are shown in Table (1) and Fig. (14).

**B) The amount of mucosecretion :**

In PAS-stained colonic sections of control rats, the mean area percent of PAS-positive mucus was  $24.32 \pm 3.14$ . It had been noted that regular aspirin intake caused a significant increase in the area percent of mucus secretion to  $43.90 \pm 5.04$ .

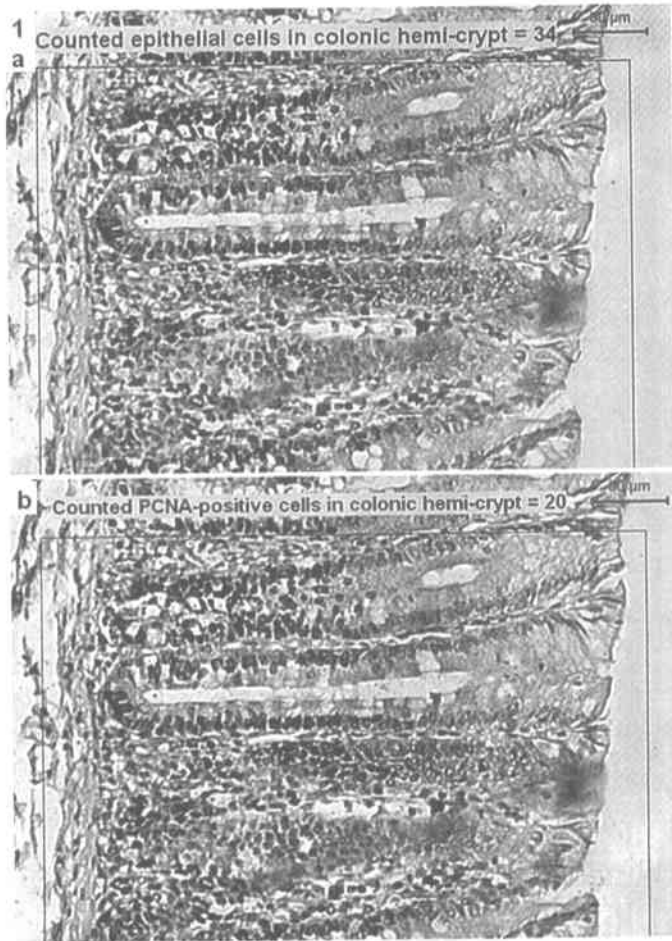
The area percent of PAS positive area in both control and experimental groups is shown in Fig. (15).

**Table 1 : The mean labeling index in the studied groups.**

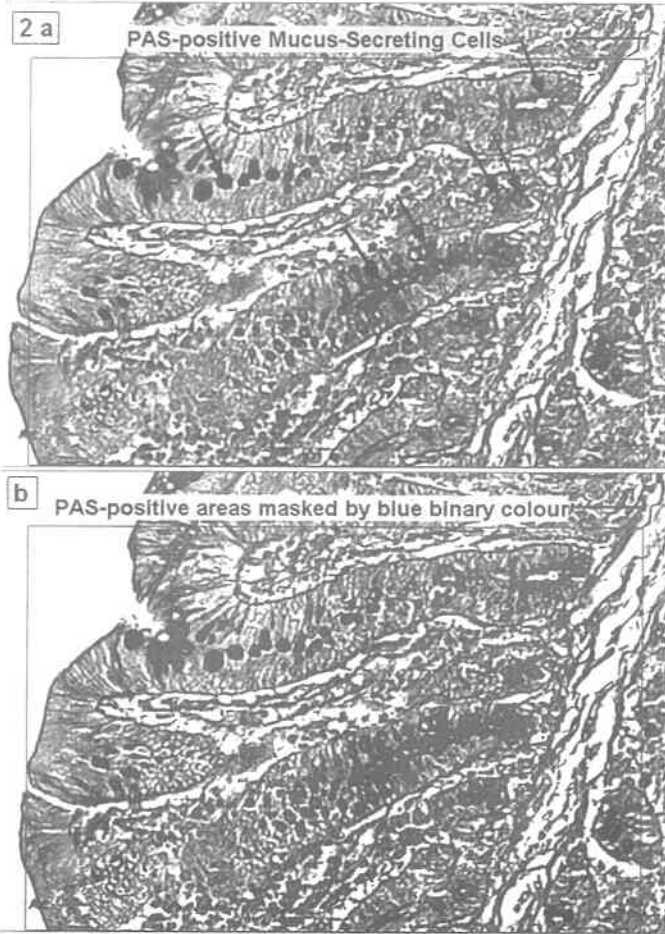
	The mean labeling index $\pm$ SD	
	Control	Experimental
Crypts away from LP	$48.73 \pm 2.34$	$35.73 \pm 2.08^*$
Crypts adjacent to LP	$54.73 \pm 4.43$	$39.26 \pm 4.11^*$

SD = standard deviation

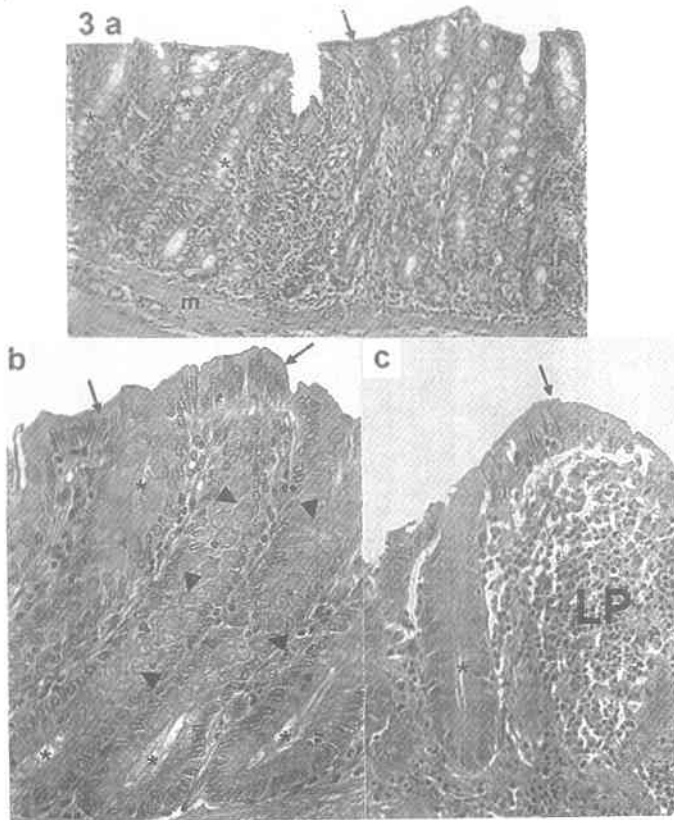
\* = significant ( $P \leq 0.05$ )



**Fig. (1)** :Photomicrographs of display seen on the monitor's screen of the image analyzer showing :  
a) Counted epithelial cells in colonic hemicrypt.  
b) Counted PCNA-positive cells in colonic hemicrypt.



**Fig. (2)** :Photomicrographs of display seen on the monitor's screen of the image analyzer showing :  
a) PAS-positive Mucus-Secreting cells.  
b) PAS-positive areas masked by blue binary colour.



**Fig. (3) :**Photomicrographs of colonic sections of control rats showing :

a) The mucosa consisting of tubular glands (\*) extending from the luminal surface (arrow) to the muscularis mucosa (m).

(Hx. & E.; x 200)

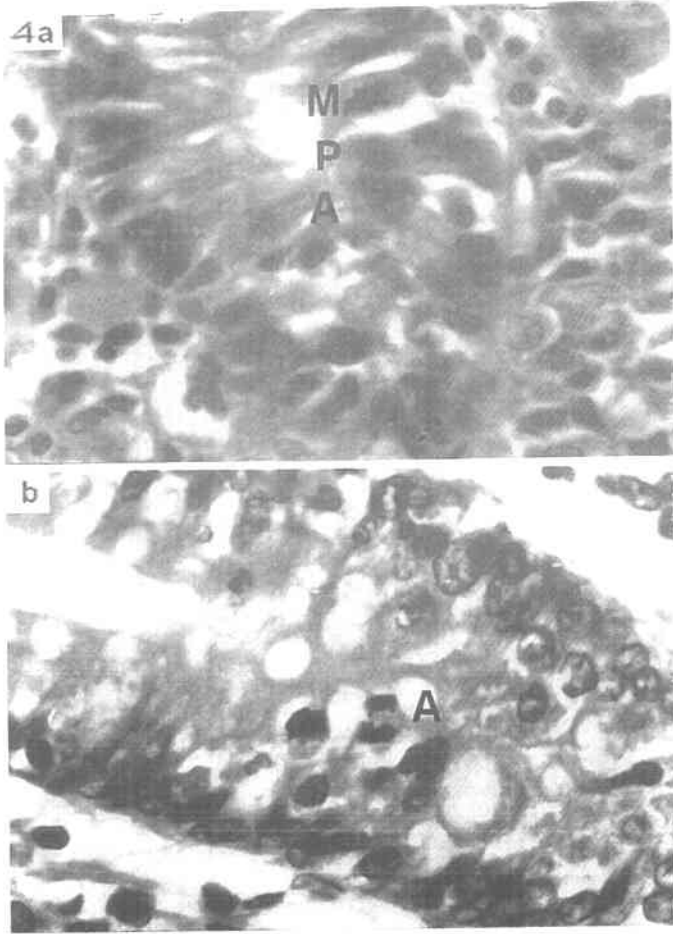
b) Two cell types comprising the colonic glands (\*) columnar absorptive cells (arrows) and mucus-secreting cells (arrow heads).

(Hx. & E.; x 400)

c) Colonic crypt (\*) extending from the luminal surface (arrow). It is adjacent to a lymphoid patch (LP) lying within the lamina propria.

(Hx. & E.; x 400)



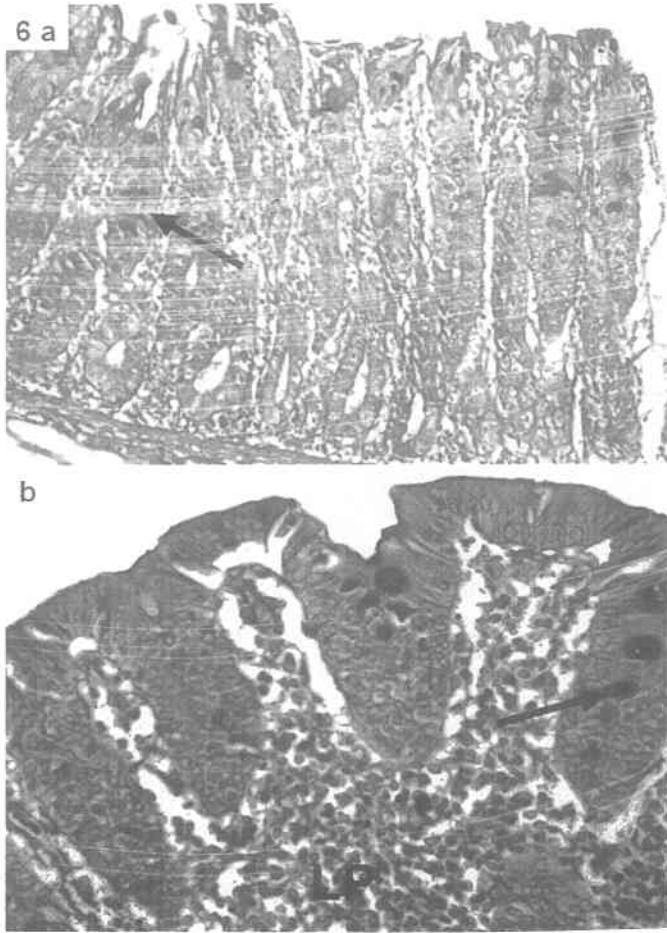


**Fig. (4) :**Photomicrographs of colonic epithelial cells of control rats showing different stages of mitosis :  
a) Prophase (P), metaphase (M) and anaphase (A).  
(Hx. & E.; x 1000)  
b) Dividing epithelial cell at the anaphase stage (A).  
(Hx. & E.; x 1000)



**Fig. (5)** :Photomicrograph of colonic section of a control rat revealing few apoptotic cells (arrows) detected within colonic crypts.

(Hx. & E.; x 400)



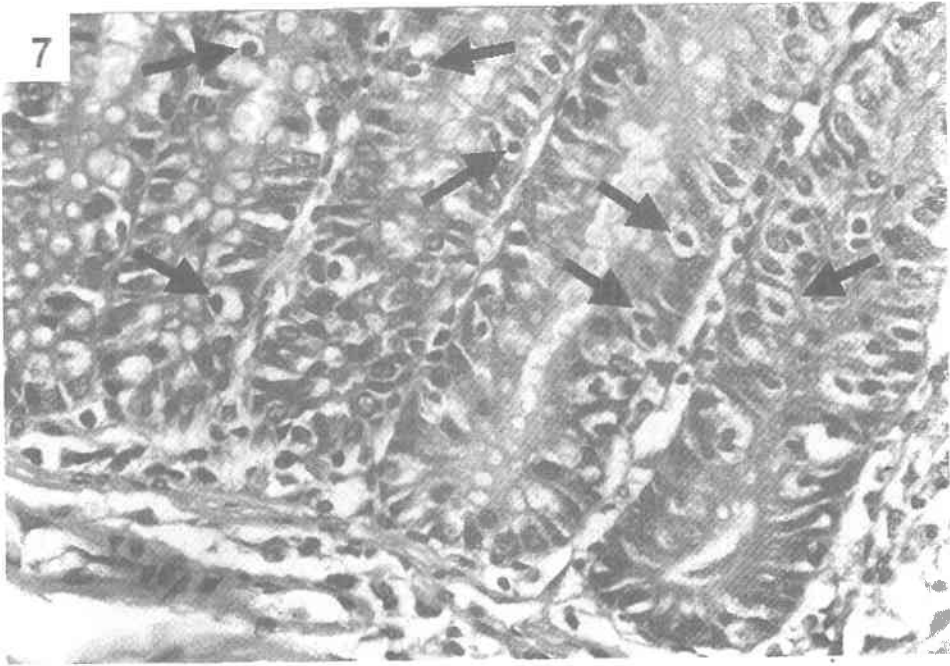
**Fig. (6)** :Photomicrographs focusing on colonic mucosa of control rats showing :

a) Mucus stained with strong magenta color in crypts away from lymphoid follicles (arrow).

(PAS & Hx.; x 400)

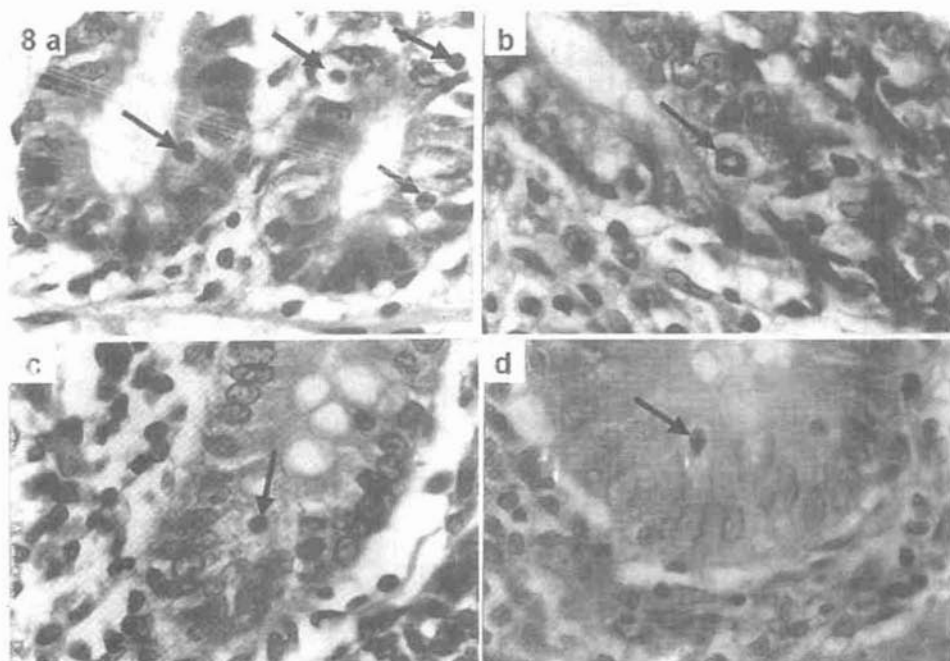
b) Crypts adjacent to lymphoid patches (LP) having less mucus content (arrow).

(PAS & Hx.; x 400)



**Fig. (7) :**Photomicrograph of colonic section after aspirin intake showing increased apoptotic cells within the crypts (arrows).

(Hx. & E.; x 400)



**Fig. (8) :**Photomicrographs of representative apoptotic figures within colonic crypts after regular aspirin intake showing :

a) Cell shrinkage (arrow).

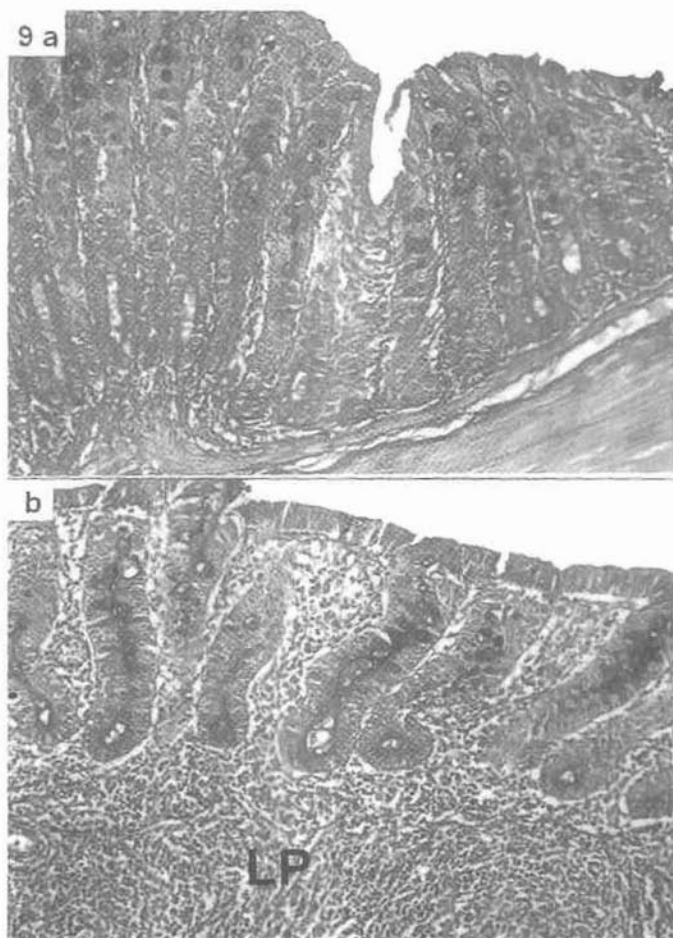
(Hx. & E.; x 1000)

b) Chromatin marginalization (arrow).

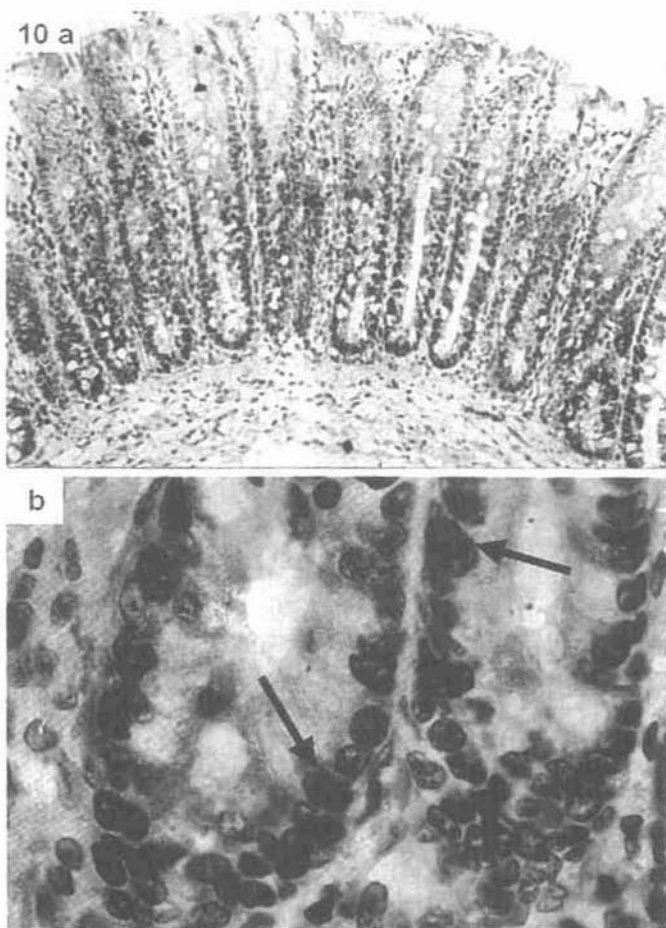
(Hx. & E.; x 1000)

c & d) Cytoplasmic condensation (arrows).

(Hx. & E.; x 1000)



**Fig. (9) :**Photomicrographs of colonic sections of experimental rats showing increased mucus secretion in :  
a) Crypts away from lymphoid patches. (PAS & Hx.; x 400)  
b) Crypts adjacent to lymphoid patches (LP). (PAS & Hx.; x 400)



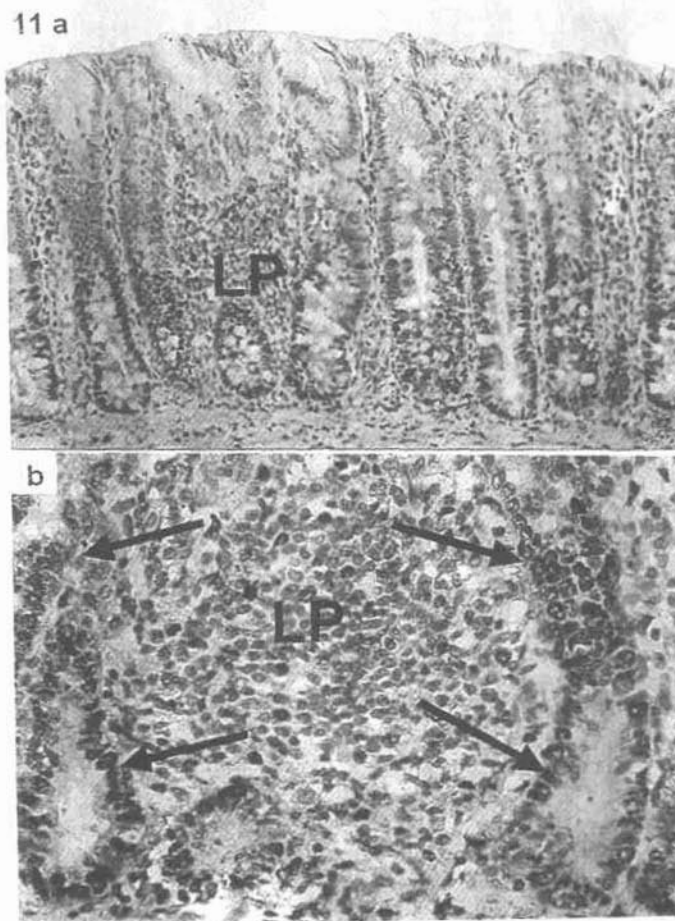
**Fig. (10)** :Photomicrographs of PCNA-immunostained colonic section of control rat showing :

a) Positive cells located in lower two-thirds of colonic crypts away from lymphoid patches.

(x 200)

b) Higher magnification revealing positively stained nuclei at the base of crypts (arrows).

(x 1000)



**Fig. (11) :**Photomicrographs of PCNA-immunostained sections of control rats showing :

a) Positive cells located in crypts bordering lymphoid patch (LP).

(x 200)

b) Positive cells (arrows) along the whole length of crypt.

(x 400)



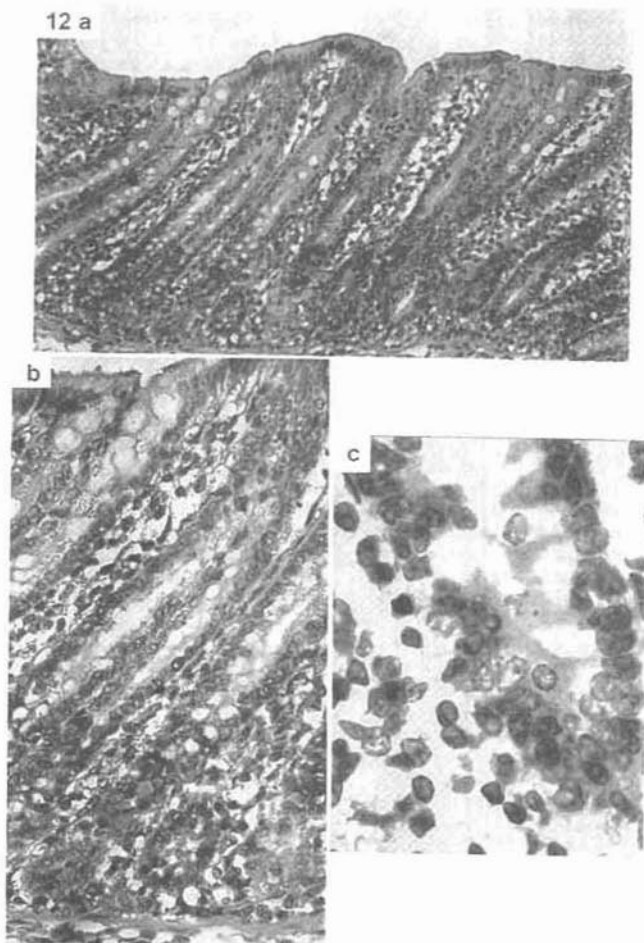


Fig. (12) :Photomicrographs of PCNA-immunostained sections after aspirin intake showing :

a) Decreased proliferative activity within crypts away from lymphoid aggregates.

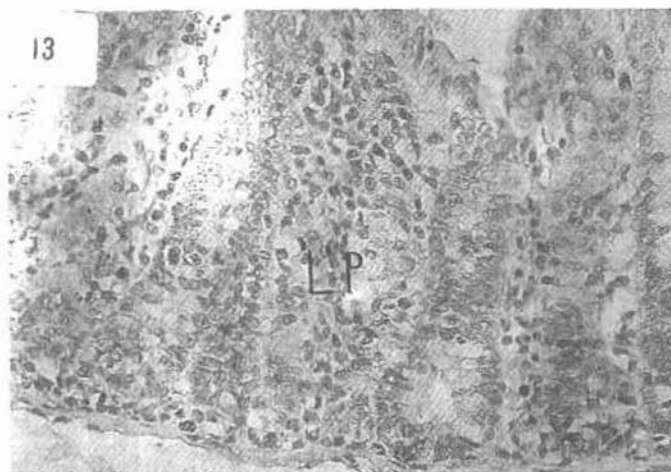
(x 200)

b) Higher magnification with downward shift of proliferating cells.

(x 400)

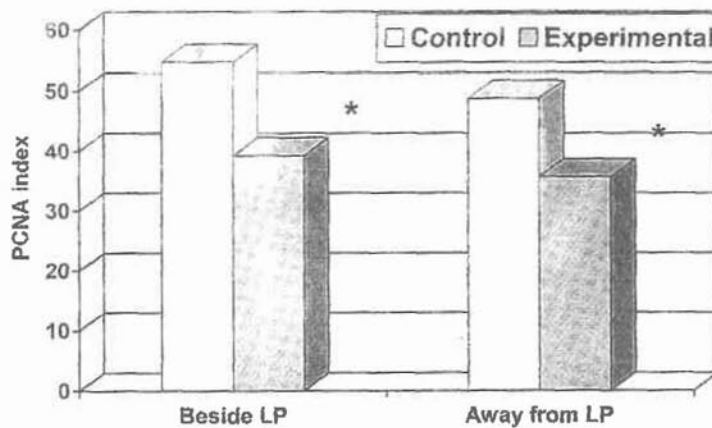
c) Decreased nuclear reaction in cells at the crypt base.

(x 1000)



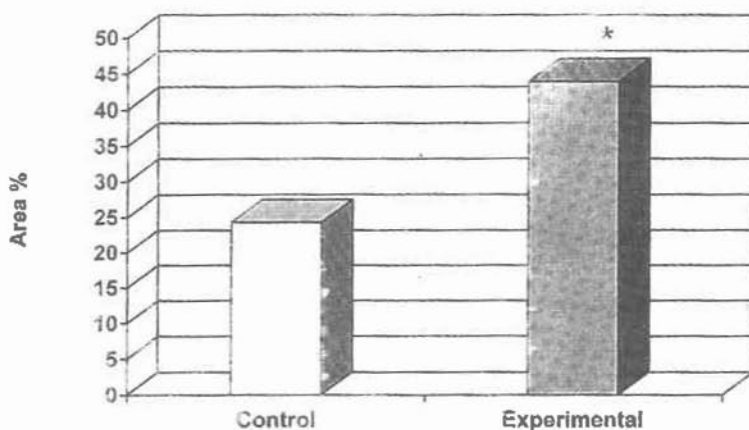
**Fig. (13)** :Photomicrograph of PCNA-immunostained colonic section after aspirin intake. Note the decreased proliferating cells in crypts adjacent to lymphoid patches.

(x 400)



\* significant difference from control ( $p < 0.05$ ).

**Fig. (14)** :Mean PCNA index in the studied groups.



\* significant difference from control ( $p < 0.05$ ).

Fig. (15) :Mean area % of PAS positivity in the studied groups.

## DISCUSSION

This study evaluated the effect of aspirin (acetylsalicylic acid) in its anti-inflammatory dose on the proliferative activity of rat colonic epithelial cells after daily oral administration for 30 days.

Aspirin like other non steroidal, anti-inflammatory drugs (NSAIDs) inhibits the binding of the prostaglandin substrate (arachidonic acid) to the active site of the cyclo-oxygenase enzyme. Two isoforms of the enzyme were recognized : Cox-1 and Cox-2. The unwanted side effects of NSAIDS, such as damage to the gastric mucosa and kidneys are due to the ability to inhibit Cox-1 while their anti-inflammatory (therapeutic effects) are due to inhibition of Cox-2 which is potently expressed in human colon cancer cells (Vane and Botting, 1998).

In control animals, the calculated labeling index was higher in crypts adjacent to lymphoid follicles compared with crypts located away from lymphoid follicles. This finding was expected based on the fact that, there is a strong correlation between sites of lymphatic nodules and sites of high incidence of colon and rectal tumors (Cameron et al., 1996).

Regular aspirin intake resulted in decreased proliferation and significantly lower labeling index in crypts both away from and adjacent to lymphoid patches compared with the control. In Support of our finding, Shpitz et al. (1997) found a step wise increase in PCNA-labeling index during neoplastic progression of colonic

lesions starting from aberrant foci, hyperplastic polyps, adenomas and adenocarcinomas of human colon.

According to **koike (1996)** the transformation of colorectal epithelium to carcinoma has been associated with progressive inhibition of apoptosis and an increase in the proliferative - apoptotic cell ratio.

It is reported that, the incidence of cancer colon increases with advancing age, that may lie in the fact that aging enhances proliferation and attenuates apoptosis in colonic mucosa (**Xiao et al., 2001**)

In this study, aspirin intake resulted also in increased apoptotic figures detected along the whole length of crypts. Similarly **Castano et al. (1999)** analyzed the induction of cell death by aspirin but in HT-29 colon carcinoma cells and found that aspirin induced two hallmarks of cell death; nuclear chromatin condensation and an increase in phosphatidylserine externalization. **Law et al. (2000)** stated that salicylate induced growth arrest is associated with down regulation of cyclin D1, cyclin A and proliferating cell nuclear antigen. According to **Reuter et al. (2002)** the onset of mitochondrial permeability transition (MPT) causes both necrotic and apoptotic death in cultured hepatocytes. Salicylate potentiates both necrotic and apoptotic cell killing by promoting the onset of MPT. They also concluded that aspirin's ability to promote apoptosis may render it suitable for use in combinatorial chemotherapy but not a stand-alone treatment for established Gastrointestinal cancer.

On the contrary to our results **Friis et al. (2003)** did not support a major protective effect of low dose aspirin on the development of colorectal cancer or other cancers.

In PAS-stained control sections, less mucosecreting cells were found in crypts adjacent to or bordering lymphoid patches. This is supported by the fact that, atypical crypts with high proliferative activity and decreased number of mucosecreting cells may be preferential targets for preneoplastic changes (**Cameron et al., 1996**). Aspirin treatment significantly increased the area of mucus in all crypts protecting against malignant transformation.

So, from the above findings it could be concluded that chemopreventive mechanism of aspirin may lie in decreased proliferation, increased cell deaths and increased mucus secretion especially in crypts adjacent to lymphoid patches.

## SUMMARY

Aspirin (acetylsalicylic acid), the most common non steroidal anti-inflammatory drug has been shown to have a protective effect against the incidence and mortality of colorectal cancer. However, the mechanism of its anticancer function remains unclear.

The aim of the study was to determine the effects of aspirin on the proliferation parameters of rat colonic crypts. Thirty adult male albino rats divided into control group (10 rats) and Experimental group (20 rats) were used. They received saline and aspirin (50 mg / kg) daily orally respectively for 30 days. Colonic sections were stained with Hx / E, periodic acid Schiff and subjected to proliferating cell nuclear antigen (PCNA) immunostaining. PCNA- labeling index (PCNA-LI) and area of mucus secreting cells were determined using Leica Quin (500) image analysis computer system. Results indicated that, compared to control, regular aspirin intake led to significant decrease of cell proliferative activity associated with increased apoptotic figures in crypts both adjacent to and away from lymphoid patches as well as significantly increased area of mucus secreting cells.

It could be concluded that attenuation of proliferation and enhancement of apoptosis and mucus secretion may account for aspirin chemopreventive effect against cancer colon.

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**تأثير الأسبرين على معاملات التكاثر فى ارداب القولون للفار :  
دراسة هستولوجية وهستوكيميائية مناعية**

**دينا محمد رضوان - حنان على امين - اشرف محمود كامل**

قسم الهستولوجيا - كلية الطب - جامعة القاهرة

يبدو أن لتعاطى الأسبرين وهو أكثر مضادات الالتهاب غير الستيرويدية شيوعاً ، دوراً فى الحماية ضد حدوث وتطور سرطان القولون والمستقيم ، إلا أن آلية هذا الأثر المضاد للسرطنة مازالت غير معلومة .

يهدف هذا البحث إلى دراسة تأثير الأسبرين على معاملات التكاثر فى خبايا القولون لذكور الفار الأبيض . تم استخدام ثلاثين فأراً ، تم تقسيمهم إلى مجموعة ضابطة ضمت عشرة فئران تم إعطاؤها محلولاً ملحياً بالفم ، فى حين أعطيت المجموعة الأخرى والمكونة من عشرون فأراً جرعة يومية من الأسبرين ( ٥٠ ملجم / كجم ) عن طريق الفم لمدة ثلاثون يوماً . فى نهاية التجربة تم تحضير مقاطعات القولون وصبغت بالهيماتوكسيلين والأيوسين والبيروديك أسيد شيف ، كما أجريت دراسة هستوكيميائية مناعية للكشف عن مستحضرات النواه للخلايا المتكاثرة PCNA .

تم قياس معامل التكاثر ومساحة الخلايا المفرزة للمخاط بواسطة جهاز تحليل الصور ، وقد أظهرت النتائج أن التعاطى المنتظم للأسبرين قد أدى إلى انخفاض فى معاملات تكاثر خلايا القولون مصحوباً بتزايد فى الموت المبرمج للخلايا فى الخبايا المجاورة والبعيدة عن تجمعات الخلايا اللمفاوية ، كما أدى أيضاً إلى زيادة الخلايا المفرزة للمخاط .

وقد خلصت هذه الدراسة إلى أن إضعاف التكاثر والزيادة في موت الخلايا وإفراز المخاط قد يكون وراء دور الاسبرين في الوقاية من سرطان القولون .

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مجلة التشريح المصرية، ٢٦ (١)، يناير ٢٠٠٣