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Protective Effect of Omega-3 Fatty Acids on 5-fluorouracil-induced Small Intestinal Damage in Rats: Histological and Histomorphometric Study

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ABSTRACT

Omega-3 fatty acids have a therapeutic role in many inflammatory conditions. The aim of the study, to investigate the protective effect of omega-3 fatty acids on 5-fluorouracil-induced small intestinal cytotoxicity. Fluorouracil (FU) and Omega group rats were given a single intraperitoneal injection of 5-fluorouracil (150 mg kg^{-1}). Rats of the second group also received oral omega-3 (1 mL $animal^{-1}$ day^{-1}). Four rats from both FU subgroups (FU1, FU3, FU6 and FU9) and Omega subgroups (Omega1, Omega3, Omega6, Omega9) were sacrificed 1, 3, 6, 9 days after 5-fluorouracil injection. Histological, histomorphometric and statistical studies for small intestine were done. FU1 and FU3 subgroups demonstrated signs of mucositis such as separation of the epithelium and denudation of the villi. Histological signs of initial recovery appeared in FU6 and was followed by full recovery in FU9. Preserved structural integrity and faster recovery were observed in the Omega group. The conclusion of this study, omega-3 fatty acids ameliorate 5-fluorouracil-induced small intestinal damage.

Key words: 5-fluorouracil, omega-3, small intestine, mucositis

INTRODUCTION

The chemotherapeutic agent 5-Fluorouracil (5-FU) is a widely used antimetabolite drug which acts by blocking DNA synthesis via inhibition of thymidylate synthase enzyme (Benson *et al*., 2004). However, its indiscriminate mechanism of action targets not only cancer cells, but all rapidly dividing cells within the body. Chemotherapy affects the gastrointestinal tract resulting in mucositis which occurs in approximately 40% of cancer patients (Naidu *et al*., 2004; Wright *et al*., 2009). The symptoms of mucositis are debilitating and include severe inflammation and ulceration of the gastrointestinal tract, resulting in abdominal pain, nausea, vomiting, diarrhea and weight loss (Gibson and Keefe, 2006).

Omega-3 fatty acids (omega-3 F.A.) are polyunsaturated essential fatty acids. The major omega-3 fatty acid, the Alpha-linolenic Acid (ALA) is metabolized in the body to Eicosapentaenoic Acid (EPA) and Docosahexaenoic Acid (DHA). Both are found in large quantities in fish oils (Plourde and Cunnane, 2007).

Omega-3 fatty acids are strong antioxidants (Pauwels and Kostkiewicz, 2008) and have beneficial effects in the treatment or prevention of many acute and chronic inflammatory conditions such as cardiovascular diseases (Vrablik *et al*., 2009), arthritis (Goldberg and Katz, 2007) and inflammatory bowel disease (Ross, 1993).

Aim of the work: The aim of this study was to assess the histological and histomorphometric aspects of 5-FU-induced cytotoxicity on the different segments of small intestine and to investigate the possible protective effect of omega-3 fatty acids.

MATERIALS AND METHODS

Animals: Forty eight adult male albino rats weighing (200-250) grams were used in this study. The animals were housed in separate cages under constant environmental conditions and were allowed free access to food and water.

Drugs:

- The drug, 5-Fluorouracil is a product of Biosyn, Arzneimittel, Gmbh, Germany with a trade name; 5-fluorouracil biosyn (1000 mg/20 mL of 5-fluorouracil per ampoule)
- Omega-3 is a product of Puritan's pride, INC, USA with a trade name; natural omega-3 (300 mg of omega-3 per capsule)

Experimental design: In a fourteen-day-long experiment, the rats were randomly assigned into three groups (16 rats each):

Control group: Rats were given water daily by orogastric tube from the first day of the experiment till the time of sacrifice. On the fifth day of the experiment, they received saline (a single intraperitoneal injection), equivalent to the 5-fluorouracil dose.

FU group: Rats were given water daily as the control group. In addition; they received a single intraperitoneal dose of 5-fluorouracil (5-FU) (150 mg kg^{-1}) at the fifth day (Torres *et al.*, 2008). This group was further subdivided into four equal subgroups; FU1, FU3, FU6, FU9 sacrificed 1, 3, 6, 9 days after 5-FU injection, respectively.

Omega group: Rats were given omega-3 treatment (1 mL $animal^{-1}$) daily by orogastric tube from the first day of the experiment till the time of sacrifice. On the fifth day, the animals were injected with 5-Fluorouracil as the FU group. This group was further subdivided into four equal subgroups; Omega1, Omega3, Omega6 and Omega9 sacrificed 1, 3, 6, 9 days after 5-FU injection, respectively.

Methods: The animals were weighed on the first day, fifth day and at time of sacrifice. Four animals from each group were anesthetized with intraperitoneal thiopental sodium (10-15 mg kg^{-1}) and sacrificed 1, 3, 6, 9 days after 5-FU. Samples from the duodenum, jejunum and ileum were obtained and flushed with isotonic saline.

Histological study: Paraffin sections (5um thick) from the duodenum, jejunum and ileum were prepared and stained with:

- Haematoxylin and Eosin (H and E) stain (Gamble and Wilson, 2002)
- Periodic acid-Schiff's (PAS) reaction (Totty, 2002)

Histomorphometric study

Computer-aided image analysis

Slide imaging and digitizing: The slides were photographed using Olympus® digital camera installed on Olympus® microscope with 0.5 X photo adaptor, using 10 or 40 X objective lenses depending on the required analysis and saved in Tagged Image Format File (TIFF). Then, the images were analyzed on Intel® Core I3® based computer using videotest Morphology® software (Russia) with a specific built-in routine for calibrated distance measurement and automated object counting.

The following histomorphometric parameters were assessed:

- **Villus height and crypt depth:** Ten measurements of villus heights and crypt depths were taken from the H and E stained sections of each intestinal segment from each animal in all groups
- **Thickness of submucosa and muscularis externa:** Ten measurements for the thickness of the submucosa and ten measurements for the thickness of muscularis externa were taken at different sites in the H and E stained sections of each intestinal segment from each animal in all groups

Cell counting study

- **Goblet cell count:** Goblet cells were counted in 5 fields at a magnification (X 400) from PAS stained sections of each intestinal segment from each animal in all groups
- **Apoptotic cell count:** This was done by manual counting of the apoptotic bodies in H and E stained sections under light microscopy at high magnification (X1000) in 20 randomly chosen crypts for each intestinal segment from each animal in all groups
- **Mitotic cell count:** This was done by manual counting of well-defined mitotic figures at crypt bases in H and E stained sections under light microscopy at X400 magnification in 20 randomly chosen longitudinal crypts for each intestinal segment from each animal in all groups

Statistical analysis: Statistical analysis of the data was done by using Statistical Package for Social Science (SPSS) version 15.0. The data were parametric by using Kolmogrov-Smirnov test and were expressed as Mean±SD. Comparisons were carried out by Analysis of Variance (ANOVA) with the Least Significance (LSD) Post hoc analysis for inter group comparison. Significance was considered when p value<0.05.

The most affected intestinal segment was determined by comparing the percentages of affection which was calculated by dividing each of the studied parameters (except the apoptotic count) by its control in the most affected duration; then subtracting the result from 100. The most affected segment was that with the highest percentage of affection.

RESULTS

Histological results

Control group: The wall of the control rat small intestine (duodenum, jejunum and ileum) was formed of mucosa, submucosa, muscularis externa and serosa. The mucosa consisted of villi, crypts, lamina propria and muscularis mucosa (Fig. 1). The intestinal villi were covered by columnar absorbing cells and goblet cells and had connective tissue cores containing mononuclear cells, smooth muscle fibers, central lacteals and blood capillaries. Intraepithelial lymphocytes were seen

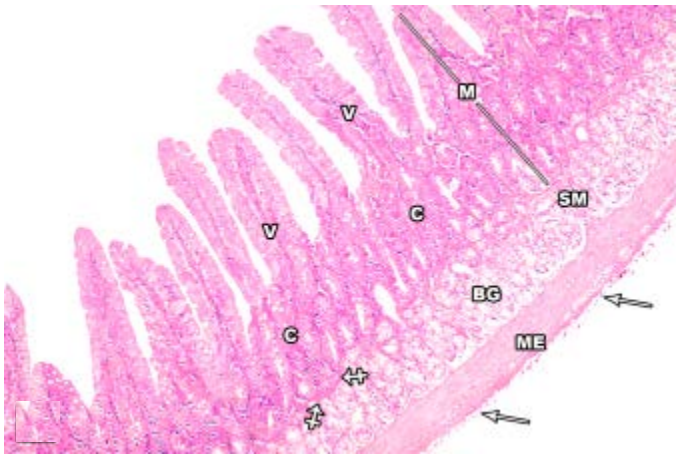

Fig. 1: A photomicrograph of the control rat duodenum. The wall is formed of M: mucosa, SM: Submucosa, ME: Muscularis externa and Arrows: Serosa. The mucosa consists of V: Villi, C: Crypts and Crossed arrows: Muscularis mucosa. The submucosa is occupied by BG: Brunner's glands (H and EX100)

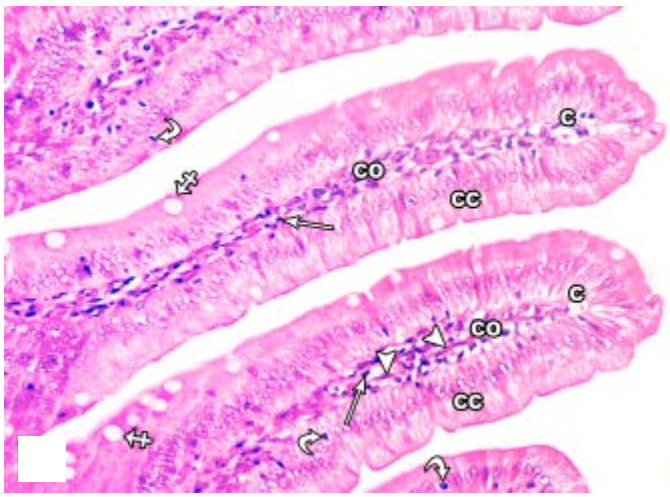

Fig. 2: A photomicrograph of the control rat duodenum showing the intestinal villi covered by CC: Columnar absorbing cells and Crossed arrows: Goblet cells. The villi have a connective tissue core (CO) containing mononuclear cells (arrows), smooth muscle fibers (arrowheads) and blood capillaries (C). Note the intraepithelial lymphocytes (curved arrows) between the epithelial cells (H and EX400)

migrating between the epithelial cells (Fig. 2). The lamina propria surrounded the crypts and extended upward to form the core of the vilh. Many mitotic figures were observed at the base of the crypts (Fig. 3). A strong PAS positive reaction was observed in the brush border and goblet cells (Fig. 4).

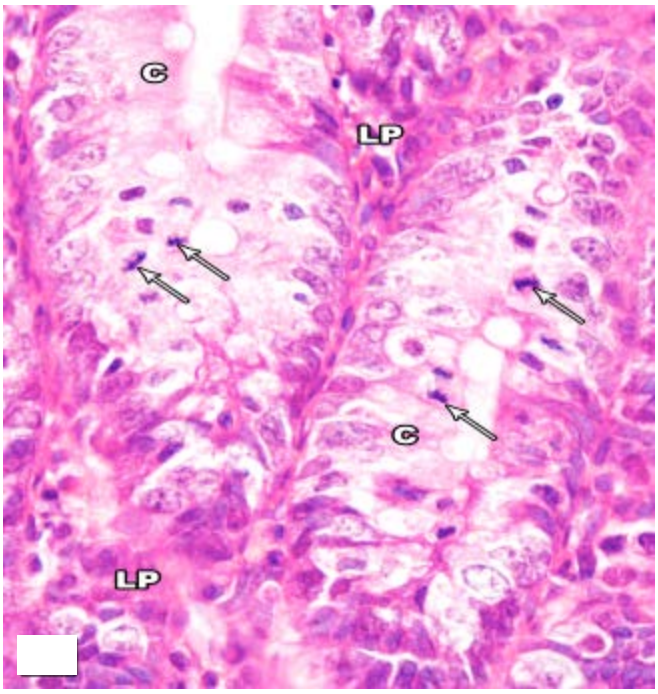

Fig. 3: A photomicrograph of control rat ileum showing the lamina propria (LP) surrounding the intestinal crypts (C). Numerous mitotic figures (arrows) are seen at the base of the crypts (H and EX1000)

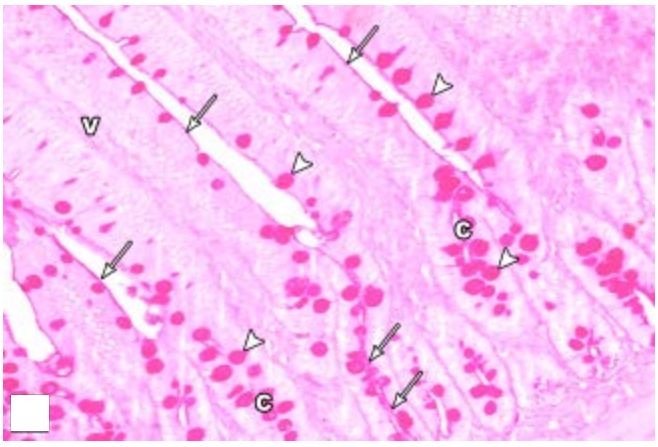

Fig. 4: A Photomicrograph of the control rat duodenum showing a strong PAS positive reaction in the brush border (arrows) and goblet cells (arrowheads) of the villi (V) and crypts (C) (PASX400)

FU group: Haematoxylin and Eosin-stained sections of the FU1 small intestine revealed areas of markedly reduced mucosal thickness with apparent reduction in the number and depth of the

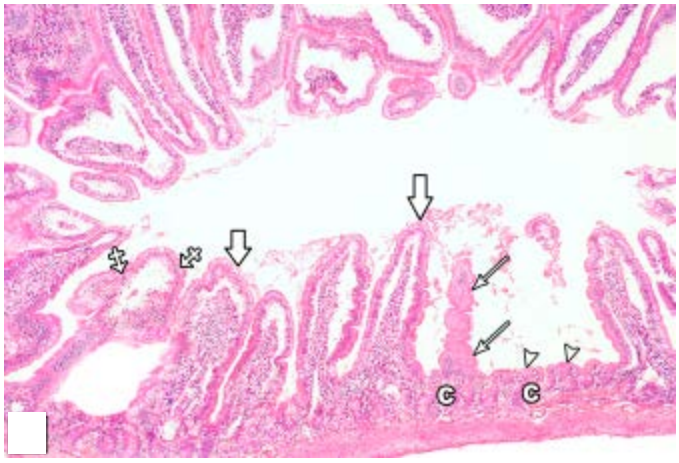

Fig. 5: A Photomicrograph of the FU1 duodenum showing areas of markedly reduced mucosal thickness (arrowheads) with an apparent reduction in the number and depth of the crypts (C). Some of the villi are distorted (arrows) or club-shaped (crossed arrows). Separation of the villus epithelium is also seen (thick arrows) (H and EX100)

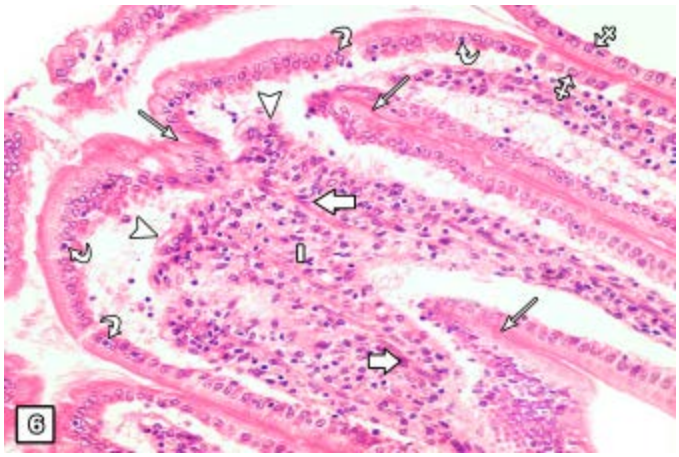

Fig. 6: A photomicrograph of the FU1 duodenum showing fused villi (arrows) and detached epithelium (arrowheads). The lamina propria shows inflammatory cell infiltration (I) and myofibroblasts (thick arrows). The columnar absorbing cells are apparently decreased in height (crossed arrows). Many intraepithelial lymphocytes (curved arrows) are seen (H and EX400)

crypts. The surface epithelium of the villi was markedly detached and separated from the underlying lamina propria (Fig. 5). The intestinal villi were variable in size and shape. Some were distorted or had club-shaped appearance (Fig. 5) and others were fused and covered with detached low columnar epithelium with many intraepithelial lymphocytes (Fig. 6). Short, broad and blunt villi with disrupted or low columnar surface epithelium were also observed (Fig. 7). The lamina

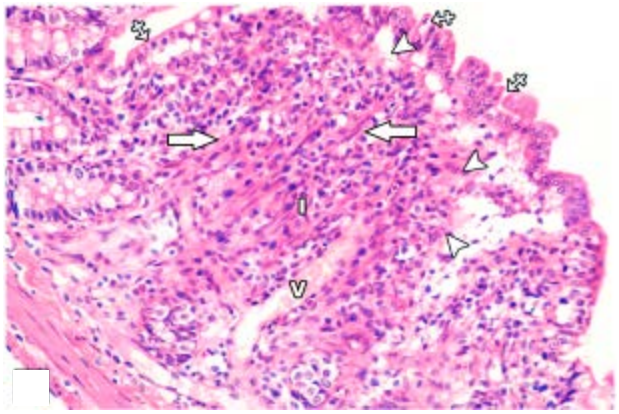

Fig. 7: A photomicrograph of the FU1 jejunum showing a broad and blunt villus with heavy inflammatory cell infiltrate (I) in its core. The epithelium appears detached in some areas (arrowheads). The absorbing cells are reduced in height and some are disrupted (crossed arrows). Note the congested blood vessel (V) and myofibroblasts (thick arrows) in the lamina propria (H and EX400)

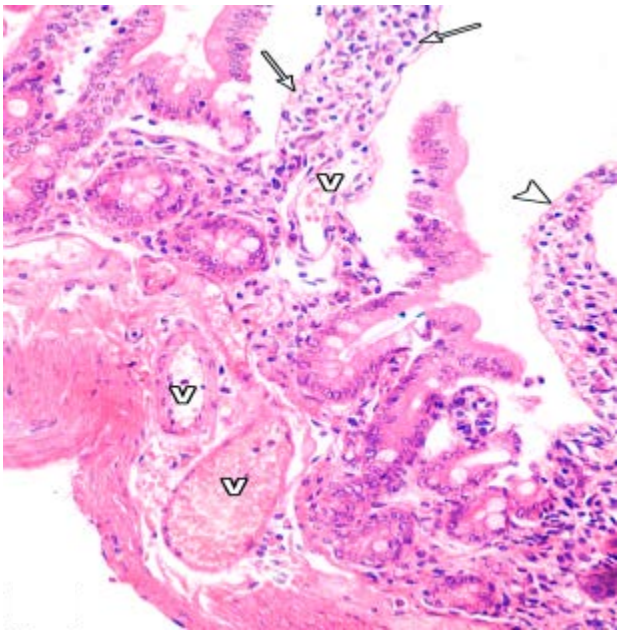

Fig. 8: A Photomicrograph of the FU1 jejunum. The epithelium is completely separated from the lamina propria (arrows) and is even lost in some areas (arrowhead). Congested dilated blood vessels (V) are seen in the mucosa and in the thickened submucosa (H and EX400)

propria showed cellular infiltration and myofibroblasts (Fig. 6, 7). Complete separation and loss of surface epithelium were seen in some areas with apparent submucosal thickening (Fig. 8).

Congested dilated blood vessels were observed in the mucosa and submucosa (Fig. 7, 8). Some vacuolated cells and many acidophilic apopotic bodies were observed in the lower parts of the intestinal crypts (Fig. 9).

The small intestine of FU3 showed reduced mucosal thickness with shortening, fusion and complete loss of intestinal villi (Fig. 10). Sloughing of the epithelium into the lumen was evident

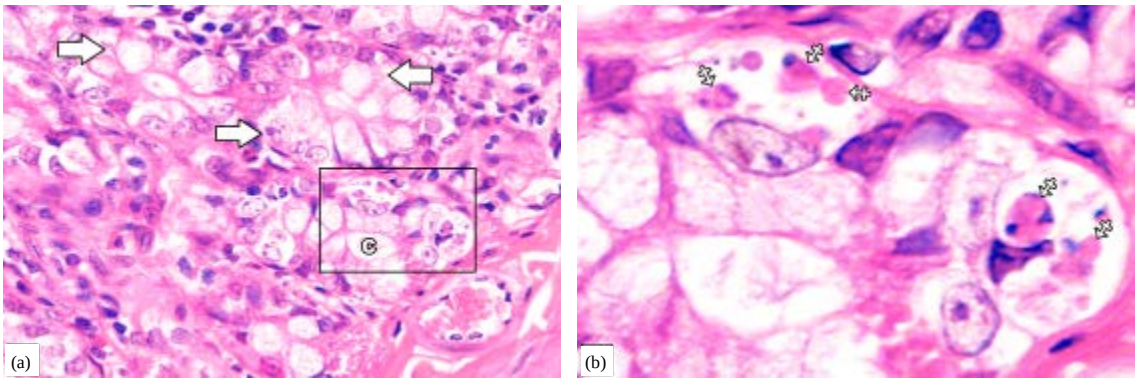

Fig. 9(a-b): Photomicrographs of the FU1 duodenum. (a) Pale vacuolated cells (thick arrows) and apoptotic bodies (inset) in the lower parts of the intestinal crypts (C). (b) Zoomed-in part of (a) showing the acidophilic apoptotic bodies (crossed arrows) (H and E X1000)

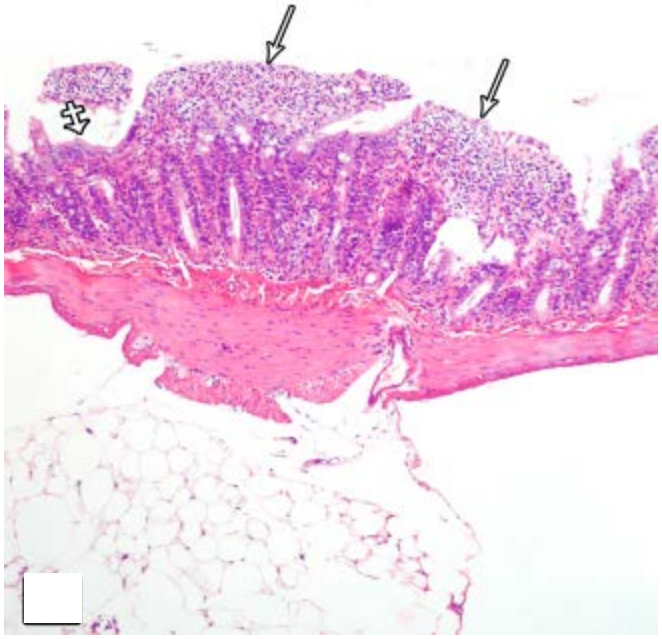

Fig. 10: A Photomicrograph of the FU3 ileum showing reduced mucosal thickness with shortening, fusion (arrows) and complete loss of intestinal villi (crossed arrow) (H and EX100)

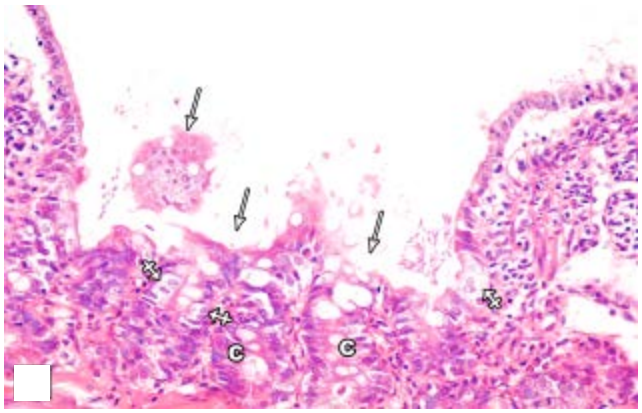

Fig. 11: A photomicrograph of the FU3 jejunum showing an area with complete loss of villi and sloughing of the epithelium into the lumen (arrows). The crypts (C) are short, distorted and lined with enlarged cells (crossed arrows) with pale vacuolated cytoplasm (H and EX400)

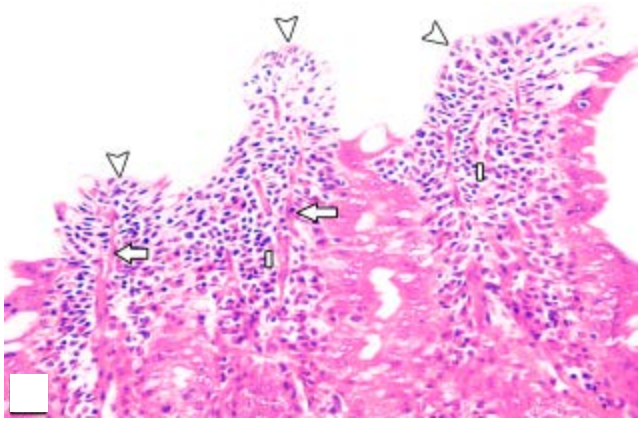

Fig. 12: A photomicrograph of the FU3 duodenum showing fused villi with raw surfaces (arrowheads). Note the heavy cellular infiltration (I) and myofibroblasts (thick arrows) in the lamina propria (H and EX400)

(Fig. 11) and extensive villus denudation leaving completely raw surfaces was frequently seen (Fig. 12). Some villi appeared atrophic and were covered with flattened cells or enlarged pale vacuolated cells with pale nuclei (Fig. 13). Short broad blunt villi covered with disrupted or flattened epithelium were also observed (Fig. 14). The core of the intestinal villi contained heavy cellular infiltration, myofibroblasts and extensively dilated lymphatics (Fig. 12-14). The intestinal crypts appeared few, short and distorted and were mostly lined by enlarged pale vacuolated cells (Fig. 11, 14). Dilated blood vessels were found in the submucosa (Fig. 14). In PAS stained sections, the brush border was interrupted and exhibited weak to moderate PAS reaction or was even lost in some areas. Goblet cells were markedly reduced in number over the vilh and crypts and either contained few mucous granules or were completely depleted and appeared PAS negative (Fig. 15).

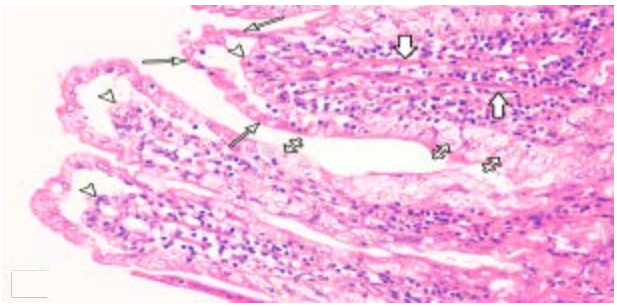

Fig. 13: A photomicrograph of the FU3 duodenum showing atrophic villi with detached epithelium (arrowheads). The columnar absorbing cells are reduced in height (arrows) and some appear enlarged (crossed arrows) with pale vacuolated cytoplasm and pale nuclei. Note the myofibroblasts (thick arrows) in the core of the villi (H and EX400)

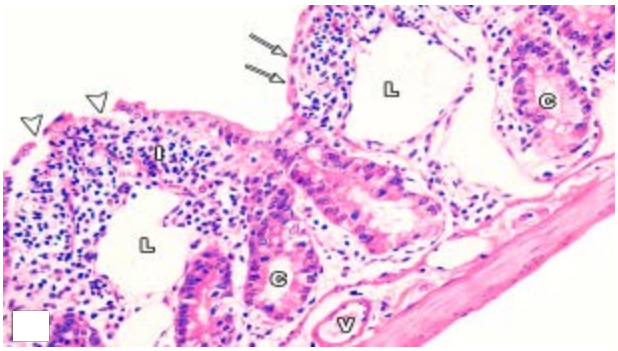

Fig. 14: A photomicrograph of the FU3 ileum showing short and broad villi with marked flattening (arrows) and loss of the surface epithelium (arrowheads). The crypts (C) are few and short. A dilated blood vessel (V) is observed in the submucosa. Note the extensive dilated lymphatics (L) and the cellular infiltration (I) in the lamina propria (H and EX400)

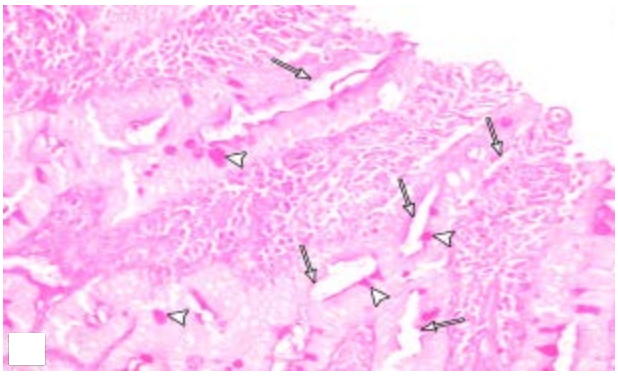

Fig. 15: A photomicrograph of the FU3 duodenum. The brush border is interrupted over the villi and crypts (arrows) and demonstrates weak or moderate PAS reaction. The moderately PAS positive goblet cells (arrowheads) are obviously reduced in number over the villi and crypts and contain few mucous granules (PASX400)

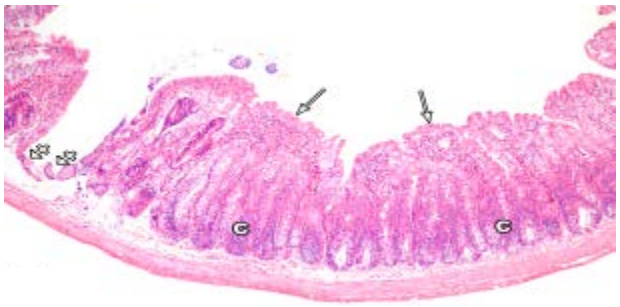

Fig. 16: A photomicrograph of the FU6 duodenum. The surface of the mucosa is completely covered with low columnar epithelium (arrows); however, no well-formed villi are seen. An area of complete mucosal loss is observed (crossed arrows). The crypts (C) are apparently increased in both number and depth (H and EX100)

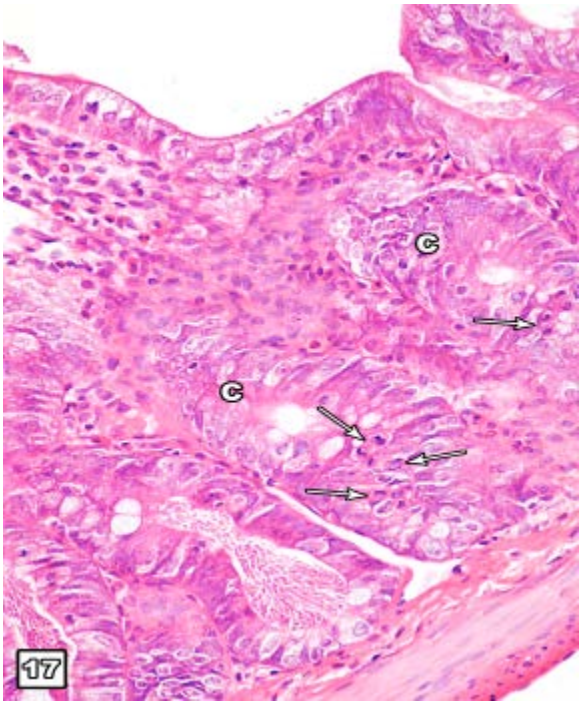

Fig. 17: A photomicrograph of the FU6 jejunum. The lining epithelium of the crypts (C) appears more or less normal. Numerous mitotic figures (arrows) are frequently seen at the base of the crypts (H and EX400)

Histological examination of FU6 small intestine revealed signs of initial recovery. The surface of the mucosa and the villi was completely covered with low continuous epithelium. However, in some areas no well-formed villi were seen and a complete loss of mucosa and vilh was still observed (Fig. 16). The intestinal crypts apparently increased in number and depth, compared to those of FU1 and FU3 and the lining cells were more or less normal. Numerous mitotic figures were observed at the crypt bases (Fig. 16, 17).

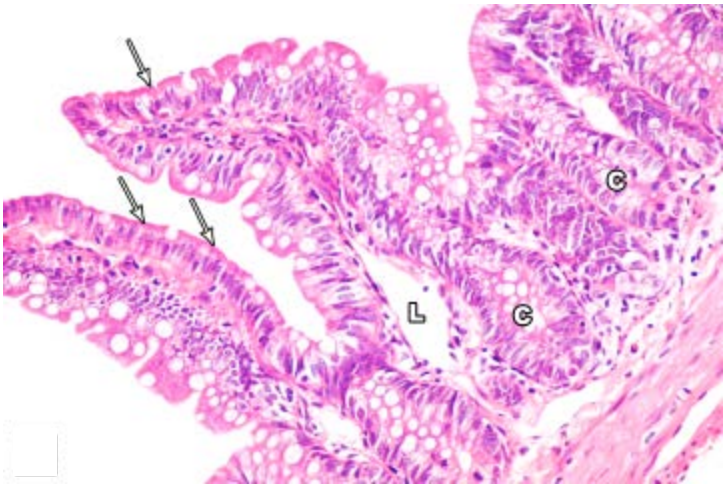

Fig. 18: A Photomicrograph of the FU9 ileum showing intact mucosa, villi (V), crypts (C) and epithelium (arrows). Some dilated lymphatics (L) are seen in the lamina propria (H and EX400)

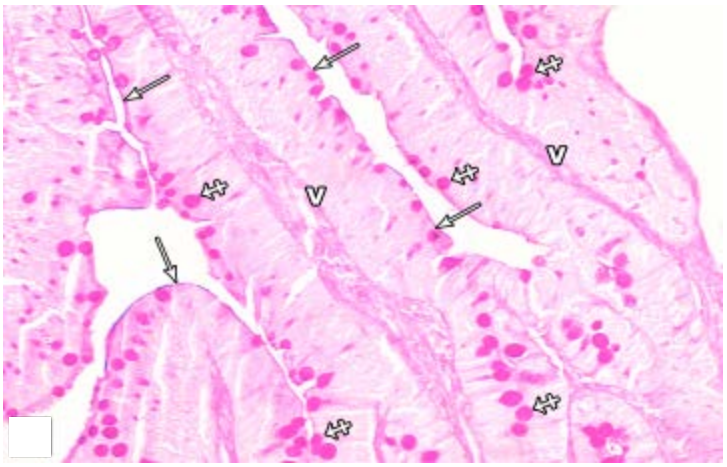

Fig. 19: A Photomicrograph of the FU9 duodenum showing a continuous brush border (arrows) with moderate to strong PAS positive reaction. Goblet cells are strongly PAS positive (crossed arrows) and more numerous over the villi (V). Most of the cells are distended with mucous granules (PASX400)

In FU9, the small intestine almost showed histological signs of full recovery. All the intestinal villi and crypts appeared intact and displayed a normal appearance similar to that of the control. Dilated lymphatics were still seen in the core of some villi (Fig. 18). A moderate to strong PAS positive reaction was demonstrated in the continuous, well-defined brush border. Goblet cells apparently increased in number, especially over the villi and displayed a strong PAS positive reaction. Most of the cells were distended with mucous granules (Fig. 19).

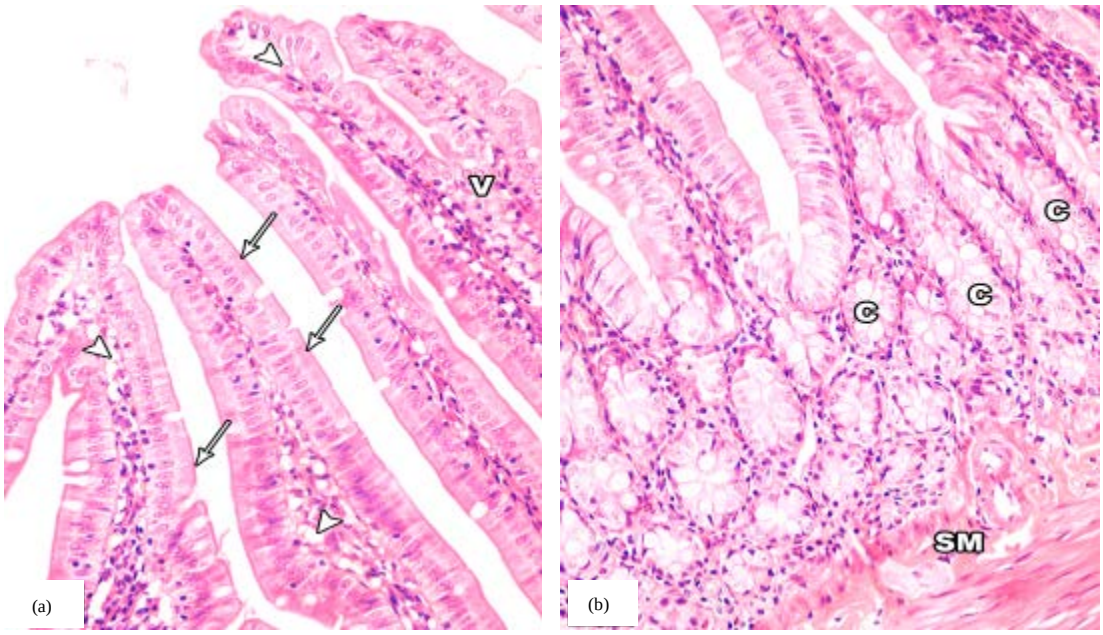

Fig. 20(a-b): Photomicrographs of the Omega1 duodenum, (a) Some fused villi (V) are seen. The epithelium (arrows) covering the villi is almost normal and shows slight detachment (arrowheads) and (b) The crypts (C) and the submucosa (SM) exhibit intact structure (H and EX400)

Omega group: The histological structure of the small intestine of Omega1 was preserved and showed more or less normal appearance, compared to FU1. The villi and crypts appeared intact; however, some fused villi and slight separation of the villus epithelium from the lamina propria were observed (Fig. 20).

In Omega3 rats, the appearance and structure of the small intestine were mostly preserved. The surface epithelium covering the villi was intact and continuous with a slight reduction in the height of some cells. Areas of slight epithelial detachment and some cellular infiltration were seen in the core of some villi. Prominent myofibroblasts were also observed (Fig. 21). PAS stained sections revealed a well-defined, strong PAS positive brush border. The goblet cells exhibited moderate to strong PAS positive reaction and were relatively reduced in number compared to control (Fig. 22).

The small intestine of Omega6 exhibited preserved integrity with intact villi and crypts. The villus epithelium appeared almost intact and of normal height. Some fused villi were detected and many mitotic figures were observed at the base of the crypts (Fig. 23).

In Omega9, the structure of the small intestine appeared normal and similar to that of the control (Fig. 24). A strong PAS positive reaction was seen in the brush border and in the numerous goblet cells which were distended with mucous granules (Fig. 25).

Statistical results

The mean body weight: (Fig. 26): In the control group, a progressive significant increase in the mean body weight was detected. The difference in the mean body weight between the FU and

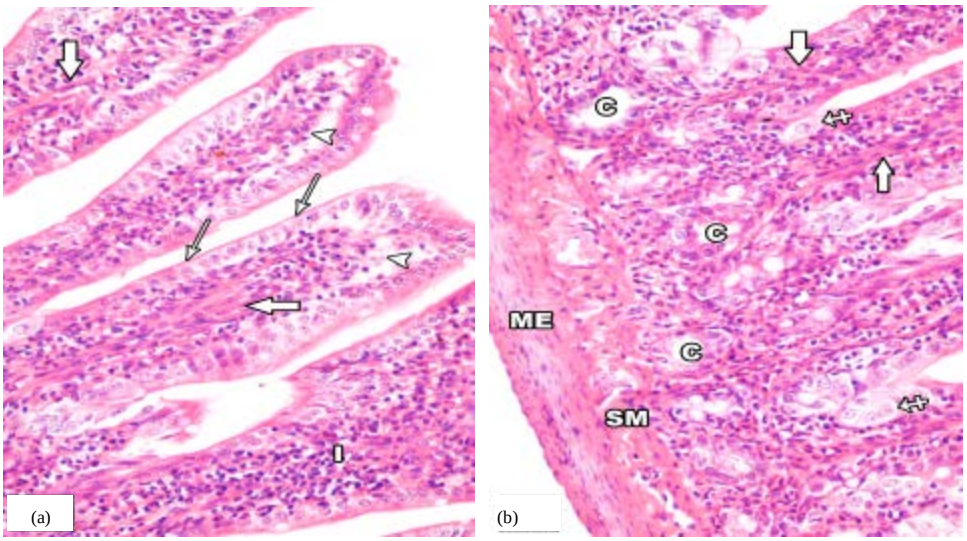

Fig. 21(a-b): Photomicrographs of the Omega3 jejunum, (a) shows intact villus epithelium with slight reduction in the height of some cells (arrows). Areas of slight epithelial detachment (arrowheads) are seen. The core of the villi shows some cellular infiltration (I) and prominent myofibroblasts (thick arrows) and (b) Most of the cells lining the crypts (C) are more or less intact. Few large pale vacuolated cells (crossed arrows) and prominent myofibroblasts (thick arrows) are seen. The submucosa (SM) and the muscularis externa (ME) appear almost intact (H and EX400)

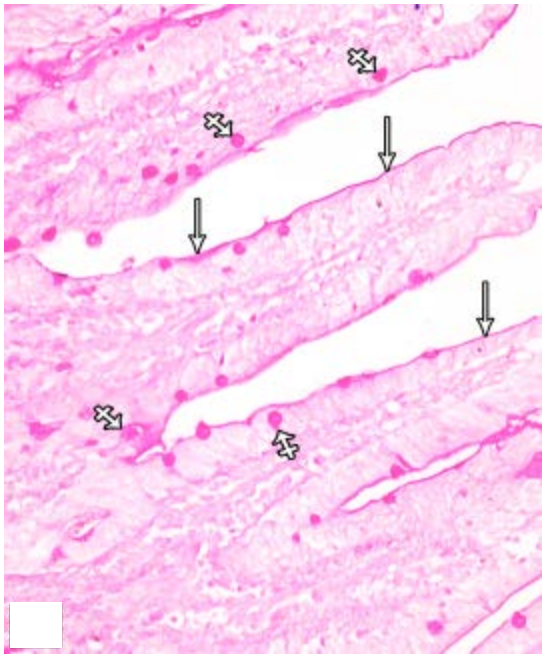

Fig. 22: A Photomicrograph of the Omega3 duodenum showing a strong PAS positive brush border (arrows). The goblet cells (crossed arrows) are relatively reduced in number and show moderate to strong PAS positive reaction (PASX400)

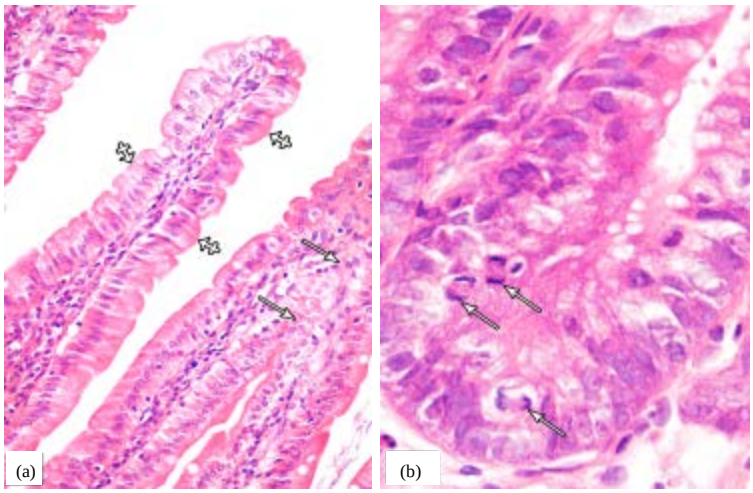

Fig. 23: Photomicrographs of the Omega6 jejunum. 23a: Fused villi (arrows) are seen. The villus epithelium appears almost intact and of normal height (crossed arrows). 23b: Many mitotic figures (arrows) are observed at the crypt base (H and E, a X 400, b X 1000)

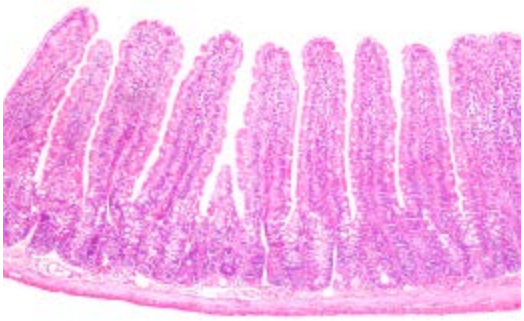

Fig. 24: A Photomicrograph of the Omega9 jejunum showing normal appearance and structure of the small intestine (H and EX100)

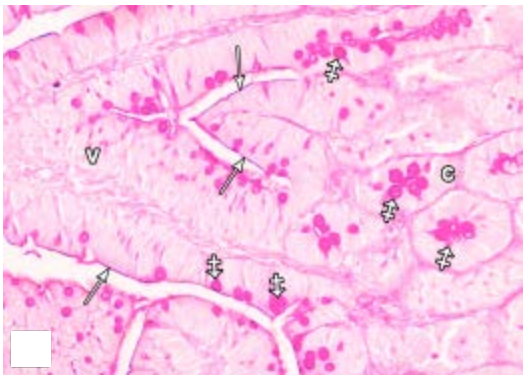

Fig. 25: A Photomicrograph of the Omega9 duodenum. A strong PAS positive reaction is shown in the brush border (arrows) and in the numerous goblet cells (crossed arrows) of the villi (V) and crypts (C). The Goblet cells appear distended with mucus (PASX400)

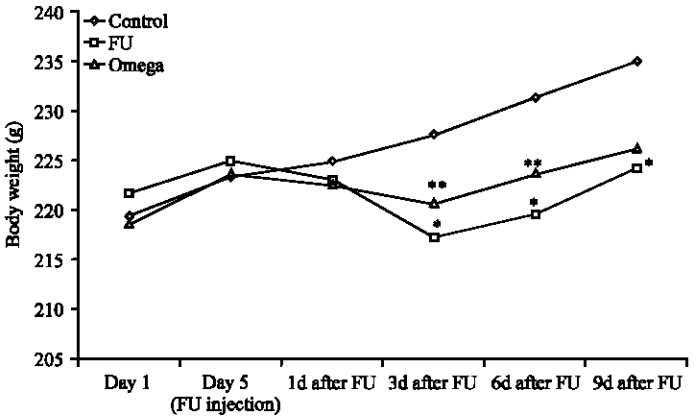

Fig. 26: Mean body weight of rats in different groups, *Significant difference between FU and control groups, **Significant difference between Omega and FU groups

control groups was non-significant both at the first and fifth days (day of FU injection) of the experiment. However, one day after FU injection (FU1), a non-significant decrease in the mean body weight was detected, in comparison to the day of FU injection and to the control. Three days after FU (FU3), the mean body weight significantly decreased, compared to that recorded one day after FU and to the control, reaching the maximum detected weight reduction. After that, there was a non-significant increase in body weight 6 days after FU injection (compared to FU3), followed by a significant increase 9 days after FU (compared to FU6); however, the mean body weight remained significantly lower than that of the control.

As regards the Omega group, there was an initial weight gain similar to FU group, with no significant differences between the 2 groups in mean body weight at both the first and fifth days (day of FU injection). One day after FU injection there was a non-significant difference in the body weight between FU and Omega groups. The mean body weight significantly increased in both Omega3 and Omega6, compared to the corresponding FU subgroups. However, this change was non-significant between Omega9 and FU9.

Histomorphometric study

The mean villus height: The mean villus height in the duodenum, jejunum and ileum of FU1 (658.24±18.16, 374.1±9.32, 302.7±5.4 μm); FU3 (400.5±8.6, 190±7.3, 222.7±8.3 μm) and FU6 (420.18±8.9, 227.8±16.3, 246.4±11.01 μm) decreased significantly, compared to Control 1 (690.92±25.84, 395.7±9.9, 320.9±8.9 μm); Control 3 (693.5±24.84, 398.9±10.6, 323.1±10.9 μm) and Control 6 (694.3±24.8, 402.3±11.2, 324.6±6.5 μm), respectively with the maximum reduction recorded in FU3. This change was non-significant in FU9, compared to the control. On the other hand, the mean villus height of the duodenum, jejunum and ileum significantly increased in Omega1 (683.9±13.03, 390.2±8.7, 314.6±9.08 μm); Omega3 (480.5±23.5, 249±7.48, 266.3±8.1 μm) and Omega6 (578.6±8.5, 373.3±5.6, 299.02±8.3 μm) compared to the corresponding FU subgroups. However, this change was non-significant in Omega9, compared to FU9 (Table 1-3).

The mean crypt depth: The mean crypt depth in the duodenum, jejunum and ileum for FU1 (179.9±6.7, 205.4±11.2, 192.2±8.3 μm) and FU3 (190.20±10.04, 210±6.5, 203±4.9 μm) decreased

Table 1: The villus height, crypt depth, thickness of submucosa and muscularis externa (in micrometer) of the duodenum in different groups

	Control 1	FU1	Omega1	Control3	FU3	Omega3	Control 6	FU6	Omega6	Control9	FU9	Omega9
Villus height												
Mean±SD	690.92±25.84	658.24±18.16	683.9±13.03	693.5±24.84	400.5±8.6	480.5±23.5	694.3±24.8	420.18±8.9	578.6±8.5	700.7±17.2	690.8±12.9	699.6±8.9
P1		<0.05*			<0.05*			<0.05*			>0.05	
P2			<0.05*			<0.05*			<0.05*			>0.05
Crypt depth												
Mean±SD	203.6±7.6	179.9±6.7	190.5±5.7	210.2±7.9	190.20±10.04	200.6±6.04	212.5±5.2	221±4.9	230.5±9.5	213.3±9.3	211.5±9.2	215.5±11.3
P1		<0.05*			<0.05*			<0.05*			>0.05	
P2			<0.05*			<0.05*			<0.05*			>0.05
Thickness of submucosa												
Mean±SD	73.7±7.3	75.2±5.2	72.9±6.61	74.3±6.8	100.3±6.4	87.9±6.3	74.4±10.9	79.8±5.7	76.08±4.83	77.7±10.7	74.3±7.06	75.4±11.6
P1		>0.05			<0.05*			>0.05			>0.05	
P2			>0.05			<0.05*			>0.05			>0.05
Thickness of muscularis externa												
Mean±SD	150.6±13.8	154.1±5.1	148.3±4.03	150.9±14.02	183.7±3.3	167.1±1.9	151.7±13.5	157.3±4.4	153.5±2.2	151.5±13.2	150.2±2.4	149.3±4.1
P1		>0.05			<0.05*			>0.05			>0.05	
P2			>0.05			<0.05*			>0.05			>0.05

All results are expressed as Mean±standard deviation (SD), Non significant: p>0.05, Significant (*): p<0.05, P1 significance between control group and FU group, P2 significance between FU group and Omega group

Table 2: The villus height, crypt depth, thickness of submucosa and muscularis externa (in micrometer) of the jejunum in different groups

	Control 1	FU1	Omega1	Control 3	FU3	Omega3	Control 6	FU6	Omega6	Control 9	FU9	Omega9
Villus height												
Mean±SD	395.7±9.9	374.1±9.32	390.2±8.7	398.9±10.6	190±7.3	249±7.48	402.3±11.2	227.8±16.3	373.3±5.6	402.4±11.8	398.8±15.3	401.2±16.06
P1		<0.05*			<0.05*			<0.05*			>0.05	
P2			<0.05*			<0.05*			<0.05*			>0.05
Crypt depth												
Mean±SD	235.6±13.5	205.4±11.2	219.19±11.9	237.2±12.3	210±6.5	225±5.1	239.4±6.08	250±5.6	262±9.05	237.5±12.2	236±13.2	239.9±6.6
P1		<0.05*			<0.05*			<0.05*			>0.05	
P2			<0.05*			<0.05*			<0.05*			>0.05
Thickness of submucosa												
Mean±SD	33.2±3.2	34.1±3.8	31.0±3.02	34.5±4.4	62.8±2.2	45.08±1.3	35.08±3.3	39.4±2.6	37.2±1.7	35.2±4.3	34.2±4.2	33.8±3.8
P1		>0.05			<0.05*			>0.05			>0.05	
P2			>0.05			<0.05*			>0.05			>0.05
Thickness of muscularis externa												
Mean±SD	127.1±8.3	129.7±5.2	126.3±3.3	128.2±8.1	157±4.5	144±4.8	128.5±6.8	134.3±3.6	131.03±2.9	128.7±8.2	127.6±3.7	124.3±2.7
P1		>0.05			<0.05*			>0.05			>0.05	
P2			>0.05			<0.05*			>0.05			>0.05

All results are expressed as Mean±standard deviation (SD), Non significant: p>0.05, Significant*p<0.05, P1 significance between control group and FU group, P2 significance between FU group and Omega group

Table 3: The villus height, crypt depth, thickness of submucosa and muscularis externa (in micrometer) of the ileum in different groups

	Control 1	FU1	Omega 1	Control 3	FU3	Omega3	Control6	FU6	Omega6	Control 9	FU9	Omega9
Villus height												
Mean±SD	320.9±8.9	302.7±5.4	314.6±9.08	323.1±10.9	222.7±8.3	266.3±8.1	324.6±6.5	246.4±11.01	299.02±8.3	324.1±8.2	320.5±10.04	323.6±8.04
P1		<0.05*			<0.05*			<0.05*			>0.05	
P2			<0.05*			<0.05*			<0.05*			>0.05
Crypt depth												
Mean±SD	220.1±10.1	192.2±8.3	205.6±5.6	221.6±8.6	203±4.9	216±9.03	221.1±8.9	233.6±5.5	245.4±8.6	222.6±9.27	220±18.5	223.8±22.01
P1		<0.05*			<0.05*			<0.05*			>0.05	
P2			<0.05*			<0.05*			<0.05*			>0.05
Thickness of submucosa												
Mean±SD	47.2±14.3	49.2±2.7	44.5±4.3	48.8±14.5	71.4±2.1	58±5.2	53.4±18.2	58.7±4.03	54.6±4.6	52.5±16.02	50.3±2.8	49.3±5.7
P1		>0.05			<0.05*			>0.05			>0.05	
P2			>0.05			<0.05*			>0.05			>0.05
Thickness of muscularis exterua												
Mean±SD	96.2±7.6	99.5±4.1	94.9±6.02	96.6±7.3	111.5±2.7	104.7±3.5	97.2±7.08	102.6±3.6	99.8±4.4	97.7±7.02	95.8±3.5	96.09±7
P1		>0.05			<0.05*			>0.05			>0.05	
P2			>0.05			<0.05*			>0.05			>0.05

All results are expressed as mean±standard deviation (SD), Non significant: p>0.05, Significant*p<0.05, P1 significance between control group and FU group, P2 significance between FU group and Omega group

significantly, while that of FU6 (221±4.9, 250±5.6, 233.6±5.5 µm) increased significantly, compared to Control 1 (203.6±7.6, 235.6±13.5, 220.1±10.1 µm); Control 3 (210.2±7.9, 237.2±12.3, 221.6±8.6 µm) and Control 6 (212.5±5.2, 239.4±6.08, 221.1±8.9 µm), respectively. A non-significant decrease in the mean crypt depth was recorded in FU9, compared to the control. The mean crypt depth in Omega1 (190.5±5.7, 219.19±11.9, 205.6±5.6 µm); Omega3 (200.6±6.04, 225±5.1, 216±9.03 µm) and Omega6 (230.5±9.5, 262±9.05, 245.4±8.6 µm) increased significantly compared to the corresponding FU subgroups, while that of Omega9 showed a non-significant increase compared to FU9 (Table 1-3).

The mean thickness of submucosa and muscularis externa: The mean thickness of submucosa and muscularis externa increased in each of the three intestinal segments of FU group compared to the control. This increase was significant in FU3 and non-significant in FU1 and FU6. A non-significant decrease was recorded in FU9, compared to the control. On the other hand, there was a decrease in the mean thickness of the submucosa and muscularis externa in Omega group, compared to FU group. This decrease was significant in Omega3, compared to FU3 and non-significant in Omega1, Omega6 and Omega9, compared to the corresponding FU subgroups (Table 1-3).

Cell counting study

Apoptotic count: The mean number of apoptotic bodies in the duodenum, jejunum and ileum of FU1 (24±5.2, 29±6.3, 26±5.4) increased significantly, compared to the Control 1 (1, 1, 1), respectively. However, this change was non-significant in FU3. A non-significant difference was also recorded in both FU6 and FU9, compared to the control. On the other hand, the mean number of apoptotic bodies in Omega1 (16±3.1, 17±3.3, 12±2.1) decreased significantly, compared to FU1 (24±5.2, 29±6.3, 26±5.4), while in Omega3 there was a non-significant decrease, compared to FU3. A non-significant difference was also observed in Omega6 and Omega9, compared to the corresponding FU subgroups (Table 4).

Mitotic count: A significant decrease in the mean number of mitotic cells was detected in the duodenum, jejunum and ileum of FU1 (2±0.3, 1±0.5, 3±1.1) and FU3 (13±3.9, 11±2.7, 14±2.5) compared to Control 1 (28±3.2, 25±3.7, 21±4.5) and Control 3 (29±5.4, 26±3.4, 22±2.5), respectively with the maximum reduction recorded in FU1. The mean number of mitotic cells in FU6 (38±3.4, 35±3.9, 33±2.2) increased significantly, compared to Control 6 (29±6.6, 27±4.3, 23±5.2); however, a non-significant decrease was recorded in FU9. On the other hand, a significant increase was observed in the mean number of mitotic cells in Omega1 (7±0.6, 5±1.2, 8±2.1), Omega3 (21±2.3, 20±2.9, 20±3.6) and Omega6 (47±4.1, 46±2.4, 43±3.1) compared to FU1 (2±0.3, 1±0.5, 3±1.1); FU3 (13±3.9, 11±2.7, 14±2.5) and FU6 (38±3.4, 35±3.9, 33±2.2), respectively. However, the increase was non-significant in Omega9 compared to FU9 (Table 4).

Goblet cell count: The mean goblet cell number in the duodenum, jejunum and ileum of FU1 (176±19.1, 190±9.7, 225±20.5); FU3 (116±15.1, 102±13.23, 121±17.45) and FU6 (195±30.44, 190±21.8, 205±18.5) decreased significantly, compared to Control 1 (233±23.2, 257±25.3, 281±28.5); Control 3 (236±35.4, 255±28.44, 283±31.21) and Control 6 (235±27.9, 257±23.33,

Table 4: Apoptotic count, mitotic count and goblet cell number of the duodenum, jejunum and ileum in different groups

	Control 1	FU1	Omega1	Control 3	FU3	Omega3	Control 6	FU6	Omega6	Control 9	FU9	Omega9
Apoptotic count												
Duodenum	1	24±5.2	16±3.1	1	3±0.3	2±0.5	1	1	1	1	1	1
P1		<0.05*			<0.05			>0.05			>0.05	
P2			<0.05*			<0.05			>0.05			>0.05
Jejunum	1	29±6.3	17±3.3	1	3±0.4	2±0.3	1	1	1	1	1	1
P1		<0.05*			<0.05			>0.05			>0.05	
P2			<0.05*			<0.05			>0.05			>0.05
Ileum	1	26±5.4	12±2.1	1	2±0.3	1±0.5	1	1	1	1	1	1
P1		<0.05*			<0.05			>0.05			>0.05	
P2			<0.05*			<0.05			>0.05			>0.05
Mitotic count												
Duodenum	28±3.2	2±0.3	7±0.6	29±5.4	13±3.9	21±2.3	29±6.6	38±3.4	47±4.1	30±4.7	28±5.1	30±3.2
P1		<0.05*			<0.05*			<0.05*			>0.05	
P2			<0.05*			<0.05*			<0.05*			>0.05
Jejunum	25±3.7	1±0.5	5±1.2	26±3.4	11±2.7	20±2.9	27±4.3	35±3.9	46±2.4	27±3.6	26±4.4	24±5.3
P1		<0.05*			<0.05*			<0.05*			>0.05	
P2			<0.05*			<0.05*			<0.05*			>0.05
Ileum	21±4.5	3±1.1	8±2.1	22±2.5	14±2.5	20±3.6	23±5.2	33±2.2	43±3.1	24±2.3	23±3.4	21±3.7
P1		<0.05*			<0.05*			<0.05*			>0.05	
P2			<0.05*			<0.05*			<0.05*			>0.05
Goblet cell number												
Duodenum	233±23.2	176±19.1	198±29.6	236±35.4	116±15.1	132±20.7	235±27.9	195±30.44	229.1±29.3	237±25.7	232±22.5	236±26.64
P1		<0.05*			<0.05*			<0.05*			>0.05	
P2			<0.05*			<0.05*			<0.05*			>0.05
Jejunum	257±25.3	190±9.7	211±11.22	255±28.44	102±13.23	130±15.34	257±23.33	190±21.8	241±19.25	256±24.36	254±21.43	257±18.32
P1		<0.05*			<0.05*			<0.05*			>0.05	
P2			<0.05*			0.05*			0.05*			>0.05
Ileum	281±28.5	225±20.5	267±24.34	283±31.21	121±17.45	191±15.89	278±21.35	205±18.5	268±15.8	280±24.78	277±27.7	281±24.45
P1		<0.05*			<0.05*			<0.05*			>0.05	
P2			<0.05*			0.05*			0.05*			>0.05

All results are expressed as Mean±standard deviation (SD). Non significant: at p>0.05, Significant *p<0.05, P1 significance between control group and FU group, P2 significance between FU group and Omega group

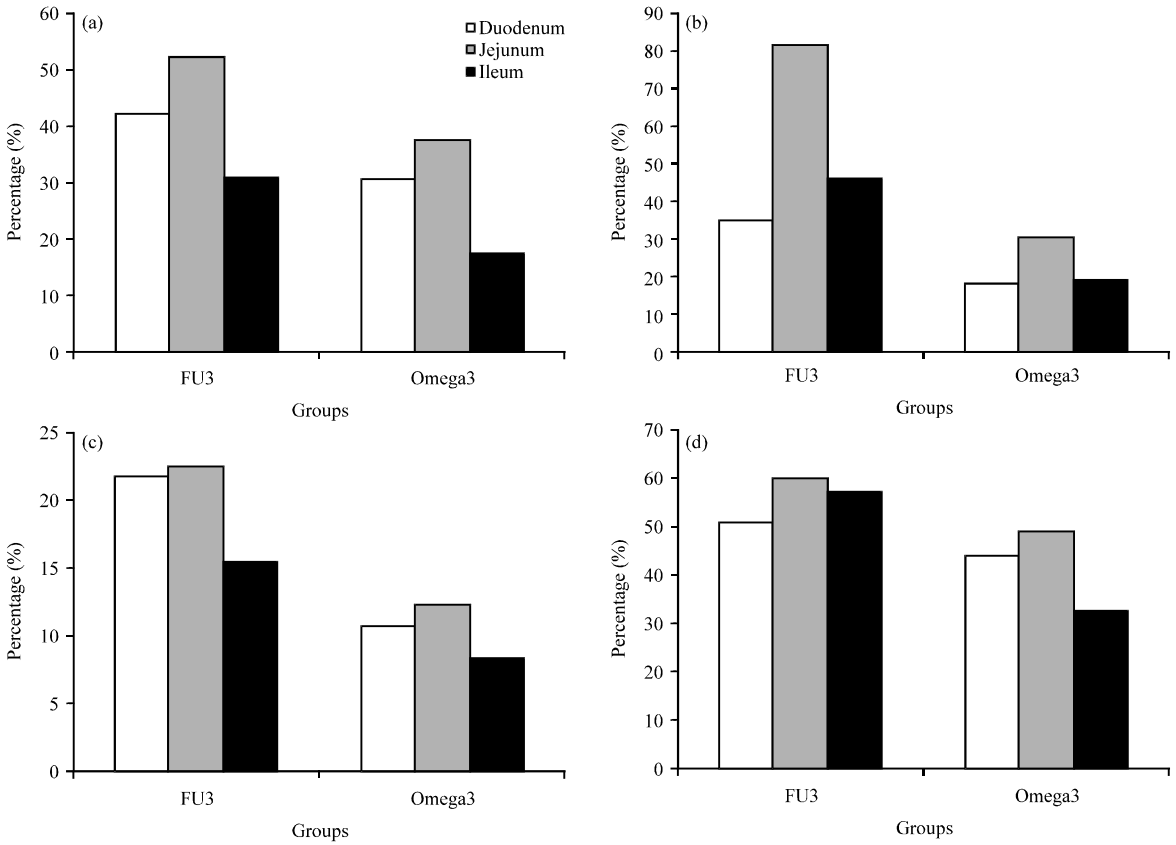

Fig. 27(a-d): The most affected intestinal segment according to percentage of affection in (a) Villus height, (b) Thickness of submucosa, (c) Thickness of muscularis externa and (d) Goblet cell number in FU3 and Omega3 groups

278±21.35), respectively with the maximum reduction recorded in FU3. However, the decrease was non-significant in FU9, compared to the control. The mean goblet cell number in Omega1 (198±29.6, 211±11.22, 267±24.34); Omega3 (132±20.7, 130±15.34, 191±15.89) and Omega6 (229.1±29.3, 241±19.25, 268±15.8) increased significantly compared to the corresponding FU subgroups. However, this change was non-significant in Omega9 compared to FU9 (Table 4).

The most affected intestinal segment: The jejunum was the most affected intestinal segment according to the percentage of affection in villus height, thickness of submucosa, thickness of muscularis externa and goblet cell number. The comparison was done between FU3 and Omega3 since these parameters were mostly affected in these subgroups (Fig. 27).

The jejunum was also the most affected intestinal segment according to the percentage of affection in apoptotic count, crypt depth and mitotic count. The comparison was done between FU1 and Omega1 since these parameters were mostly affected in these subgroups (Fig. 28).

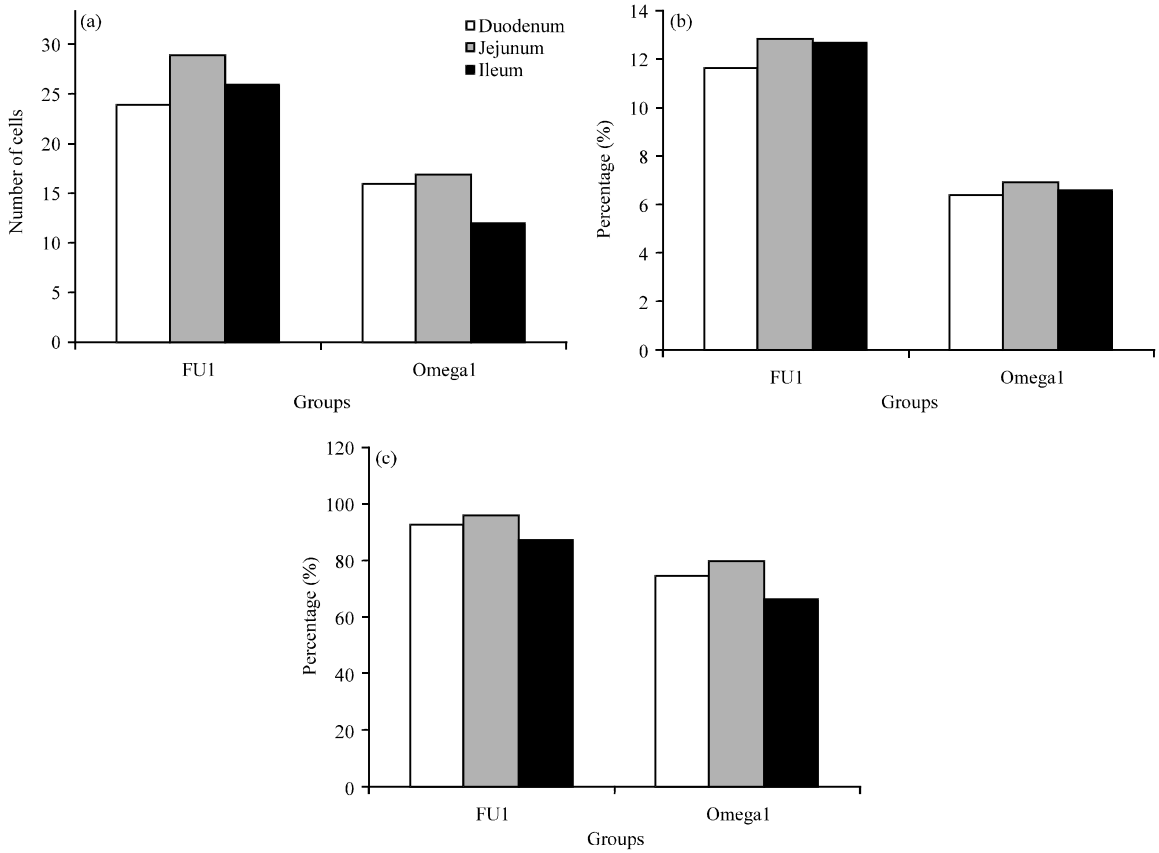

Fig. 28(a-c): The most affected intestinal segment according to, (a) Apoptotic count, (b) Percentage of affection in crypt depth and (c) Percentage of affection in mitotic count, in FU1 and Omega1 groups

DISCUSSION

The chemotherapeutic agent 5-Fluorouracil (5-FU) is a widely used antimetabolite drug which acts by blocking DNA synthesis (Benson *et al*., 2004). However, its mechanism of action targets not only cancer cells, but all rapidly dividing cells such as cells of the gastrointestinal tract (Wright *et al*., 2009). Fatty acids from fish oil (omega-3 PUFAs) are powerful safe disease-modifying nutrients which improve the immune system and protect the body against the systemic inflammatory response syndrome (Sukhotnik *et al*., 2011). The current study has been performed to assess the histological and histomorphometric aspects of 5-FU-induced cytotoxicity on the different segments of the rat small intestine and to investigate the possible protective effect of omega-3 fatty acids.

Fluorouracil administration was accompanied by weight reduction starting with a non-significant decrease in FU1, followed by a significant decrease in FU3. A similar finding was previously reported in 5-FU and methotrexate-treated rats (Soares *et al*., 2008; Fijlstra *et al*., 2011) and was attributed to diarrhea and altered intestinal absorptive capacity.

Microscopic signs of intestinal mucositis were observed in FU group. Marked detachment of surface epithelium was observed in FU1 and is suggested to be due to stromal edema. Different signs of mucosal damage such as distortion, fusion, shortening and blunting of villi were observed in FU1 and FU3. This was associated with a significant decrease in the villus height and crypt

depth. These changes are consistent with those reported by Soares *et al.* (2008) and Stringer *et al.* (2009). Loss of surface epithelium and marked exfoliation of the vilh were also noticed in FU1 and were more evident in FU3. Similar findings were reported in methotrexate treated rats (Kolli *et al.*, 2008). Intestinal cells with vacuolated cytoplasm and pale nuclei were seen in FU3. Such pale vacuolated cells were described by Soares *et al.*, 2008) and thought to have undergone hydropic degeneration as a consequence of 5-FU injury.

A significant decrease in goblet cell number was observed in both FU1 and FU3 with maximum reduction in FU3. This reduction may be a consequence of early stem cell death which has been reflected on the renewal of all cell lineages including goblet cells. Furthermore, it was reported that the intestinal absorption of 5-FU may cause an up-regulation of neurotransmitter release from enteric neurons, resulting in increased mucin secretion and depletion of goblet cells mucus (Stringer *et al.*, 2009). The heavy cellular infiltration, dilatation of lymphatics and congestion of blood vessels seen in the mucosa and submucosa of FU1 and FU3 are similar to those reported in the 5-FU-induced oral mucositis (Sonis, 1998) and in small intestinal irradiation mucositis (Cameron *et al.*, 2012). It was stated that chemotherapy increases the release of proinflammatory cytokines which cause tissue damage and inflammatory response resulting in increased subepithelial vascularity (Sonis, 1998).

The appearance of intestinal myofibroblasts in the lamina propria of FU1 and FU3 indicates repair trials. Contraction of subepithelial myofibroblasts may play a role in closing of gaps in the epithelial layer (Wilson and Gibson, 1997). In addition, myofibroblasts produce bioactive and growth factors and regulates adhesion, movement, proliferation and differentiation of epithelial cells (Fritsch *et al.*, 1997; Powell *et al.*, 1999).

Numerous apoptotic bodies were detected in FU1 and this was supported by a significant increase in the apoptotic count. Meanwhile, a significant decrease in the mitotic count has been recorded in FU1 and FU3. The increased apoptotic count and reduced mitotic count with the consequent loss of cells from the villi and crypts may explain mucosal damage observed in the present study. Apoptotic bodies were exclusively located in the lower parts of the crypts which correspond to the location of stem cells. Duncan and Grant (2003) stated that, following chemotherapy, stem cells in the crypts are damaged and prevented from subsequent replication and differentiation. This early apoptotic response to 5-FU may be p53 mediated (Pritchard *et al.*, 1998). It seems that inhibition of DNA synthesis, DNA damage and the production of reactive oxygen species by chemotherapy impair the metabolism in progenitor cells and cause inhibition of mitosis and increase of apoptosis (Sonis, 2004).

A significant increase in the mean thickness of submucosa was recorded in FU3 and may be due to increased collagen deposition (Dadhani *et al.*, 2010).The associated increase in the thickness of the muscle layer could be due to 5-FU-induced edema (Carr, 2001). The concomitant increase in the thickness of both submucosa and muscularis externa observed in FU1 and FU3 may also occur in response to growth factors regulating the repair process of the intestinal mucosa (Wilson and Gibson, 1997).

The jejunum appeared to be the most affected intestinal segment, according to all statistically studied parameters in FU1 and FU3. It has been reported that 5-FU preferentially damages the upper small intestine due to the higher cell turnover rate (Lindsay *et al.*, 2010).

Signs of initial recovery with significant increase in crypt depth and mitotic count were detected in FU6 group. The elongation of the crypts seems to be a sign of crypt regeneration via

hyperproliferation after the initial crypts damage. Similarly, crypt hyperplasia, greater crypts and a burst in the crypt mitotic activity were previously observed in mice, 6 days after 5-FU administration (Carneiro-Filho *et al.*, 2004).

Signs of full intestinal recovery were demonstrated in FU9 group with non-significant decrease in all studied histomorphometric parameters, compared to the control. This recovery was accompanied by an increase in goblet cell number, indicating a complete cell renewal and migration of goblet cells to the vilh. Carneiro-Filho *et al.* (2004) observed similar findings by day 8 after 5-FU injection in mice. Duncan and Grant (2003) stated that the structure and functionality of the villi and absorptive surfaces of human gut can return to normal around 1 week after onset of chemotherapy.

In the Omega group, the mean rat body weight was significantly higher than that of FU group, 3 and 6 days after 5-FU injection. This could be attributed to the improved intestinal mucositis and subsequent improvement of intestinal absorptive function. A similar effect of omega-3 F.A. was reported in patients with colorectal carcinoma (Read *et al.*, 2007).

The current microscopic study demonstrated preserved structural integrity with minimal mucosal damage in the three intestinal segments of the Omega group. The presence of prominent myofibroblasts in the lamina propria of the Omega group may suggest an accelerated repair process. Similarly, Torres *et al.* (2008) observed that Lyprinol (marine oil rich in omega-3 F.A.) provided some protective effect on 5-FU-induced mucositis. Camuesco *et al.* (2006) also found that omega-3 F.A. administration improves experimental colitis in rats. Moreover, the number of apoptotic bodies in Omega1 was significantly decreased, compared to FU1, suggesting a protective effect of omega-3 F.A. against apoptosis. A similar effect of omega-3 F.A. on the apoptotic index was previously reported in intestinal ischemia reperfusion injury (Sukhotnik *et al.*, 2011) and 5-FU-induced intestinal mucositis (De Segura *et al.*, 2004). It was reported that omega-3 F.A. have a role in the protection of the intestine from the genomic damage which results in programmed cell death (Hardman *et al.*, 2002).

The intestinal brush border was preserved with strong PAS positive reaction in Omega3, compared to the weak or lost reaction in the interrupted brush border of FU3. Subsequently, the PAS reaction appeared stronger in the Omega group than in the FU group. It was reported that the biosynthesis of glycoprotein layer associated with the microvillar membranes is highly sensitive to nutritional variations (Teitelbaum and Walker, 2001). Omega-3 deficiency decreases the intestinal brush border glycosylation whereas omega-3 supplementation increases this process (Alessandri *et al.*, 1995).

Significant increases in the mean villus height, crypt depth and mitotic count were observed in Omega1, Omega3 and Omega6, compared to the corresponding FU subgroups, indicating less intestinal damage and a faster recovery in omega-treated rats. The non-significant difference between Omega9 and FU9 groups as regards to these parameters suggests that the beneficial effect of omega-3 treatment is specially exerted early during damage and the initial recovery phase.

The mean goblet cell number was significantly higher in Omega1, 3 and 6, compared to the corresponding FU subgroups. This increase may be explained by decreased apoptosis and increased stem cell proliferation. This change indicates another protective mechanism of omega-3 F.A. against the 5-FU-induced damage through the preservation of the intestinal mucous barrier integrity. This observation is consistent with that reported in rats with experimental ulcerative colitis (Nieto *et al.*, 2002; Camuesco *et al.*, 2006).

There is good evidence that omega-3 F.A. exert their protective effect by many different mechanisms. Omega-3 F.A. competes with arachidonic acid and inhibits the pro-inflammatory eicosanoids (PGE2 and LTB4) production. The eicosanoids alter the absorptive and secretory functions of the intestine, cellular immunology and act as chemotactic factors to leucocytes and neutrophils into the mucosa resulting in tissue damage. These fatty acids also produce eicosanoids with anti-inflammatory actions and inhibit the production of inflammatory cytokines (Camuesco *et al.*, 2006; Calder, 2009). Omega-3 F.A. is also the origin of other anti-inflammatory lipid mediators known as resolvins and protectins that antagonize the pro-inflammatory mediators and actively promote the return to health (Weylandt *et al.*, 2012). Moreover, omega-3 F.A. have antioxidant activity by scavenging free radicals and inhibiting lipid peroxidation (Pauwels and Kostkiewicz, 2008). In addition, incorporation of omega-3 F.A. into the membrane phospholipids may alter intestinal mucosal function and promote mucosal integrity (Teitelbaum and Walker, 2001).

CONCLUSION

Fluorouracil chemotherapy has a deleterious effect on the small intestine leading to marked morphometric and microscopic changes, with the jejunum being the most affected segment. Omega-3 F.A. can protect the small intestine from fluorouracil-induced cytotoxicity, ameliorate the associated injury and fasten the recovery.

RECOMMENDATIONS

It is recommended for patients receiving chemotherapy to take omega-3 F.A. either in diet or in the form of drug supplementation (omega-3 capsules) to improve their quality of life during treatment. Cancer patients are advised to receive omega-3 F.A. prior to, concurrently with and just after chemotherapy as omega-3 F.A. are mostly effective in the early days post-chemotherapy.

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