

Histological evaluation of colonic anastomotic healing, during perioperative Capecitabine administration. Experimental study in rats

H.D. Konstantinidis¹, A.C. Sioga², L.D. Economou², K.D. Mpallas¹, C.I. Demertzidis¹ and T.T. Pissanidou¹

¹2nd Propedeutical Department of Surgery, Hippokration General Hospital, Thessaloniki, Greece and

²Laboratory of Histology and Embryology, Aristotelian University of Thessaloniki, Greece

Summary. Background: Neoadjuvant chemotherapy is a highly promising treatment modality for colorectal cancer. One of the basic side effects of this method is the possible impact on anastomotic healing. Capecitabine is a tumor selective pro-drug of 5-fluorouracil, indicated for the therapy of colorectal cancer. The aim of this study is to estimate the effect of perioperative Capecitabine administration on the colonic anastomotic healing process, by evaluating histopathological findings. Methods: We studied the effect of Capecitabine on hand sutured colonic anastomosis in rats. Sixty Wistar rats were randomized in two groups. In the study group (n=30) Capecitabine was given p.o. in therapeutic dose of 359 mg/kg, (2/3 of the mean toxic dose), 1 week prior the anastomosis and throughout the study. In the control group (n=30) placebo medication was administrated. Both groups were further subdivided into 3 groups, each consisting of 10 animals. Rats of each group were sacrificed on the 3rd, 7th and 14th postoperative day, in both study and control groups. Results: No negative impact on the healing process of colonic anastomosis was found. Histological findings indicated a more effective healing during the early postoperative days, with lesser necrosis effects on the anastomotic line for the study group, in comparison with the control group. The median bursting pressure was found to be significantly higher in the subdivision of the study group sacrificed on the 3rd day, in comparison to respective control subdivision. Conclusion: Perioperative administration of Capecitabine, as neoadjuvant chemotherapy, does not impair the healing

of colonic anastomosis in rats.

Key words: Colonic anastomosis, Neoadjuvant chemotherapy, Capecitabine, Healing, Histological findings

Introduction

Operation remains the main choice for a successful treatment in carcinomas of the colon and rectum. Despite curative resection, many patients develop local recurrence and incur the possibility of a distal metastasis during operative manipulation of the tumor (El-Malt et al., 2003). Perioperative administration of cytostatic agents promises to reduce these implications, but on the other hand, can cause a significant delay in wound healing and multiplies the risk of anastomotic failure, followed by increased morbidity and mortality rates (Bozdag et al., 2001).

Capecitabine is an oral administrated pro-drug of 5-fluorouracil (5-FU) with enhanced tumor selectivity, widely used in colorectal, breast and other malignancies (Cats, 2003, Schull et al., 2003). Capecitabine is non toxic in vitro and its activation follows a pathway with three enzymatic steps to form 5-FU (Malet-Martino et al., 2002). The final step requires thymidine phosphorylase, an enzyme that is significantly more active in tumor than in normal tissues (Gross et al., 2003). Our hypothesis is that this tumor selective attribute, of a generally cytotoxic agent, may hide a less negative impairment on the healing of colonic anastomosis.

This study investigated the histopathological aspects of colonic healing in experimental anastomoses

performed in rats, which were under Capecitabine administration throughout the perioperative period.

Materials and methods

Animals

Sixty Wistar rats, 10-12 weeks old, with mean body weight 224 gr (157-243) were used. The animals were randomly divided in two groups of 30. The study group received Capecitabine p.o. and the control group natural saline, also p.o. These two groups were further subdivided into three groups, each consisting of 10 animals. One subdivided group of 10 animals, from both study and control group, was sacrificed on the 3rd postoperative day and the same procedure was followed on the 7th and 14th postoperative day. The days of sacrifice were chosen based on the phases of the healing process (acute inflammatory stage, collagen proliferation and beginning of remodelling stage). The animals had free access to standard laboratory chow and water before and after the operation. They were kept in a stable environment of 20°C and natural lighting. The study was approved by the ethical committee of the Aristotelian University of Thessaloniki. All experiments were conducted in conformity with the European Community laws.

Chemicals

Capecitabine (Xeloda[®], F. Hoffman La -Roche, Basle, Switzerland) in form of white powder, was dissolved in 40 mM citrate buffer (PH 6,0) containing 5% gum Arabic as the vehicle and then administered p.o. to rats. We used a therapeutic dose of 359 mg/kg, 2/3 of the mean toxic dose (Del Vecchio et al., 1999), 1 week prior the anastomosis and throughout the study. In the control group natural saline was administered for the same period.

Operative procedures

Anesthesia was induced with a combination of light ether and intraperitoneal injection of 40 mg Ketamine Hydrochloride (Ketalar[®], Warner Lambert Co, USA) per kilogram of body weight. All rats were operated by the same surgeon, using the same technique. After a middle laparotomy was performed, a 2 cm segment of the colon was dissected, just above the peritoneum reflection. An one layer, end to end anastomosis was then performed with 10 interrupted sutures of 6.0 polypropylene (Prolene[®], Ethicon Ltd, Edinburgh, UK). Skin and fascia were closed in two layers with continuous sutures of 4.0 polypropylene (Prolene[®], Ethicon Ltd, Edinburgh, UK). Rats were re-operated in groups of 10 animals on the fixture postoperative day (3rd, 7th and 14th). The anastomosis site was gently dissected en block, as the mid-portion of a 5 cm colonic segment, and then rats were sacrificed with a lethal dose of pentobarbital.

Bursting pressure

Anastomotic segments were cleared of stools and washed with saline. The proximal end was ligated using a 3.0 silk suture and a plastic catheter was secured to the distal end. This catheter was connected to an infusion pump, while a manometer was interfaced by a side line. The intraluminal pressure was gradually raised by infusing air at a rate of 10 ml/min. The bursting pressure was defined as the maximal intraluminal pressure the segment resisted, expressed in mm of Hg column. The breaking site was noted and the anastomotic region was longitudinally divided in two pieces and collected for further examination. Pieces of normal intestinal wall, away from the anastomosis site, were also collected from both study and control group, in order to survey the effect of surgical stress and Capecitabine administration on large bowel tissue.

Histopathology

After measuring the bursting pressure, the anastomotic site was divided in two parts, which were prepared, one for light microscopy and the other for electron microscopy. For light microscopy histopathological studies the samples were fixed in 10% formaldehyde. The segments were embedded in paraffin following standard procedures. Serial longitudinal sections of 3-5 μ m paraffin blocks were stained with hematoxylin-eosin and evaluated at x20 to x200 magnifications.

For electron microscope histopathological assessment the samples were cut into small pieces and fixed in 3% glutaraldehyde in phosphate buffer PH 7.4 for 2 hours and post fixed in 2% osmium tetroxide for 1 hour. After staining with 1% aqueous solution of uranyl acetate for 14 hours, the tissue pieces were dehydrated in a series of alcohol solutions and then embedded in EPON 812. After semi-thin sections were observed, thin sections were stained with lead citrate and then observed in a Jeol TEM 2000 FX II at 80 KV. Findings from both electron and light microscopy are presented together.

Statistical analysis

All variables followed a skewed distribution and consequently all the results were expressed as median-interquartile range. Statistical analysis was performed using the nonparametric Mann-Whitney U test. P values of <0.05 were considered significant.

Results

Surgical morbidity and mortality

No deaths were reported during the experiment. No rats showed any macroscopic evidence of leakage or rupture from the anastomotic portions and septic complications were found to be absent in both groups.

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During sacrifice, inspection of the abdominal cavity did not reveal abnormalities which could cause late complications or death to any animal.

Bursting pressure

Bursting pressure measurement was used for the assessment of the mechanical strength of the anastomosis. The median bursting pressure was found to be significantly higher ($p=0.0015$) in the subdivision of the study group sacrificed on the 3rd postoperative day (68 mm/Hg), in comparison to the control group (46 mm/Hg). All ruptures took place on the anastomotic site. The median bursting pressure was not found to be significantly different ($p=1.17$), in the subdivisions

sacrificed on the 7th postoperative day (187.5 mm/Hg for the control group vs. 192.5 mm/Hg for the study group). Two ruptures took place on the anastomotic site at control group vs. none for study group. Likewise, no statistical significance ($p=0.486$), was reported between the bursting pressures of the subdivisions sacrificed on the 14th postoperative day (289.5 mm/Hg for the study group vs. 283.5 mm/Hg for the control group). No rupture took place on the anastomosis site for both control and study groups.

Histological findings

On the 3rd postoperative day in the control group, the mucosa was found well shaped, with multilayer epithelium and continuous basal lamina (Fig. 1).

Many fibroblasts with dilated RER were recognized, as well as regional assemblances of eosinophils and neutrophils, and some red blood cells. The vessels had normal structure. Many glands, with many goblet and

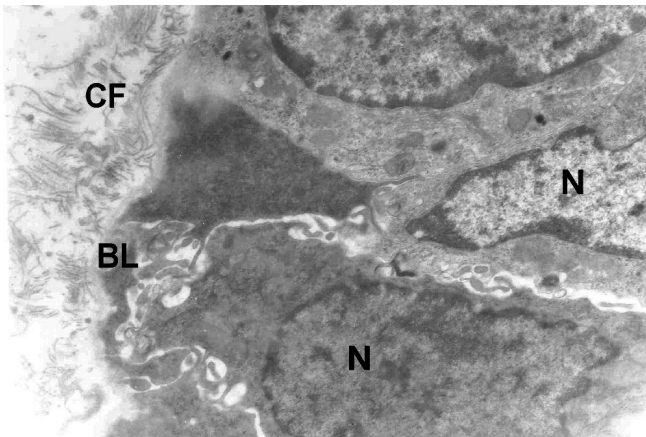


Fig. 1. Basic layers of the epithelium with columnar cells. Continuous basal lamina (BL). Epithelial cell nuclear (N), collagen fibres of the lamina propria (CF). x 20,000

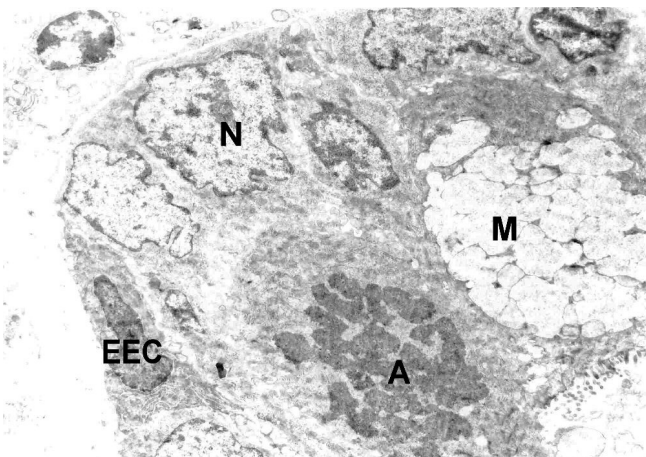


Fig. 2. Gland with mucosal (M) and absorptive (A) cells in scission phase. Basal cell nuclear (N), enteroendocrine cell (EEC). x 6,250

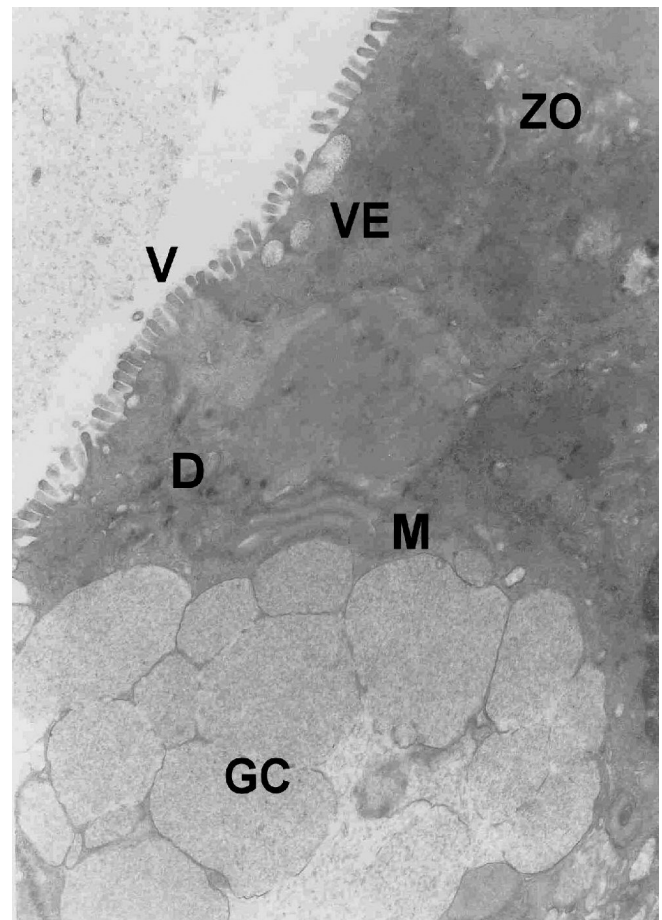


Fig. 3. Epithelium with short microvilli (V). Cells are bonded together with intercellular junctions. Zonula occludens (ZO), desmosomes (D), mitochondria (M), vesicles (VE). Goblet cell (GC). x 30,000

lesser enteroendocrine cells, were present (Fig. 2). The muscularis mucosa was found to be thin. Inside the submucosa many and dense collagen fibers were found, as well as some red blood cells.

On the 3rd postoperative day in the study group, total restitution of the bowel wall, well developed mucosa layer, continuous basal lamina, well developed muscularis mucosa and also submucosal layer were seen. The epithelium was stratified, with sparse and short microvilli and also many goblet cells among the absorptive cells were observed (Fig. 3).

The basal cells of epithelium were characterized by the presence of many polyribosomes. The largest part of lamina propria was filled with glands, most of which were goblet and secondary enteroendocrine cells. Many and dense collagen fibers were also seen in the lamina propria, as well as multiple fibroblasts with dilated RER, many cells with condensive nuclear, dispersive eosinophils, red blood cells, and neutrophils -especially close to the vessels (Fig. 4). Inside the submucosa,

dispersive collagen fibers, many destroyed cells, or cells with condensive nuclear were distinguished. Also, many capillary vessels were seen.

On the 7th postoperative day in the control group, the mucosa was found to be well developed. The epithelium was monolayer squamous, regionally multilayer, with or without microvilli, and firm cellular connections (Fig. 5).

The glands were dense, with many mucosal cells. At the lamina propria, many destroyed cells and cells with condensive nuclear (apoptosis) were recognized, as well as pervasive eosinophils, red blood cells and some neutrophils (Fig. 6).

On the 7th postoperative day in the study group, the mucosa was also found to be well developed. The epithelium was multilayer columnar, with firm cellular connections, cells with plenty of mitochondria and normal structure. The surface absorptive cells had sparse and short microvilli, locally absent. The glands had chiefly goblet cells, of which many had perinuclear

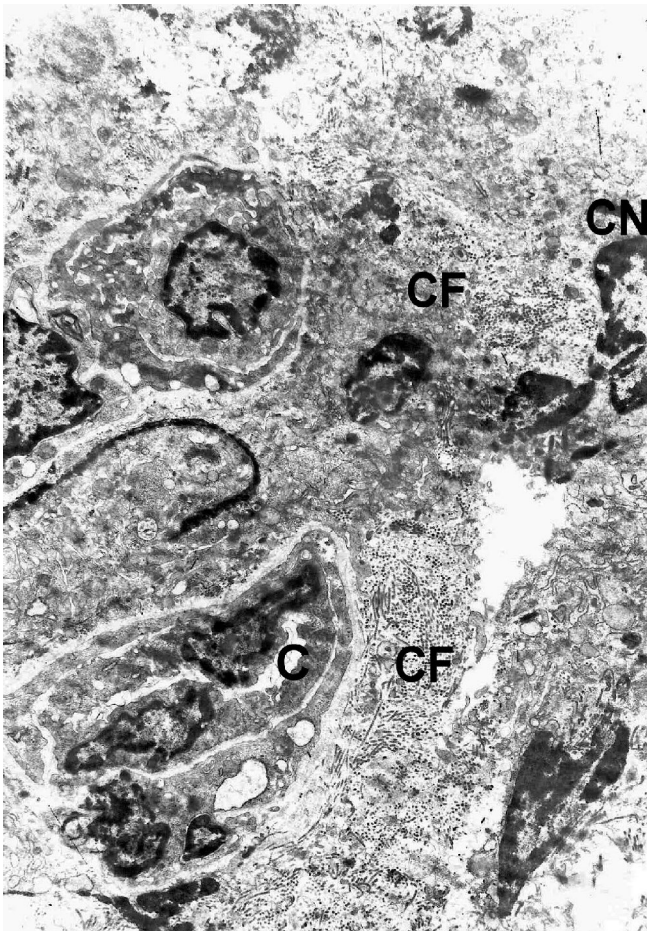


Fig. 4. Part of the lamina propria with normal capillary vessels (C) and dense collagen fibers (CF). Plenty of cells with condensive nucleus (CN) and cellular oddments are seen. x 7,500

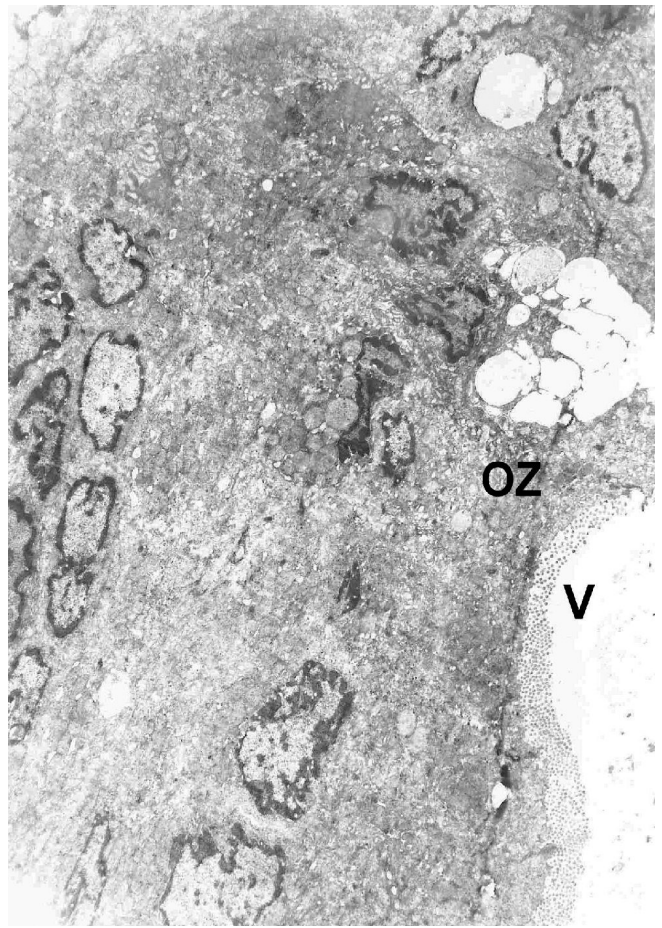


Fig. 5. The epithelium is locally multilayer squamous with plenty of microvilli (V) at the surface cells, which are bonded locally with occludeus zones (OZ). x 5,000

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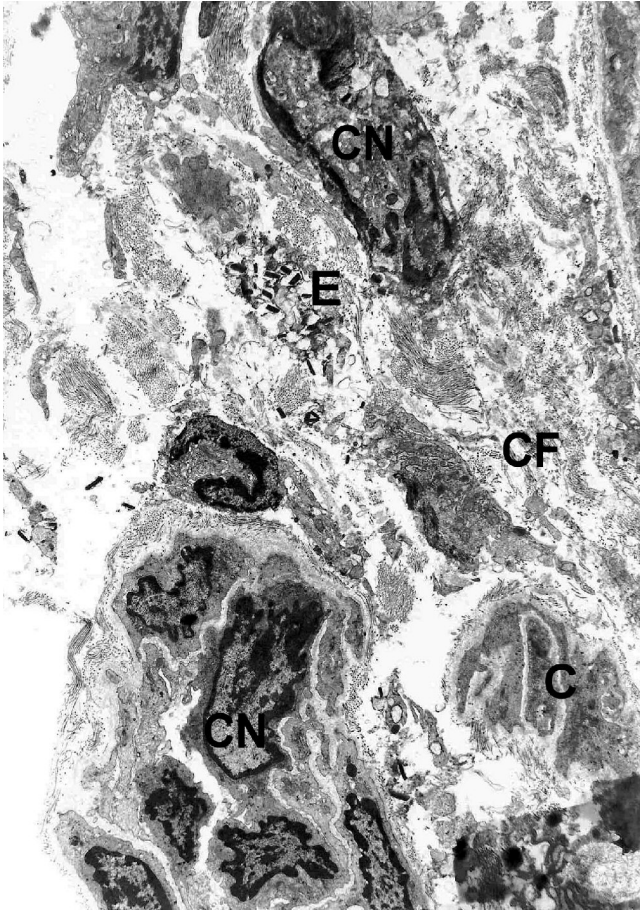


Fig. 6. Lamina propria with collagen fibers (CF), capillary vessels (C), cells with condense nucleus (CN), destroyed eosinophils (E). x 6,250

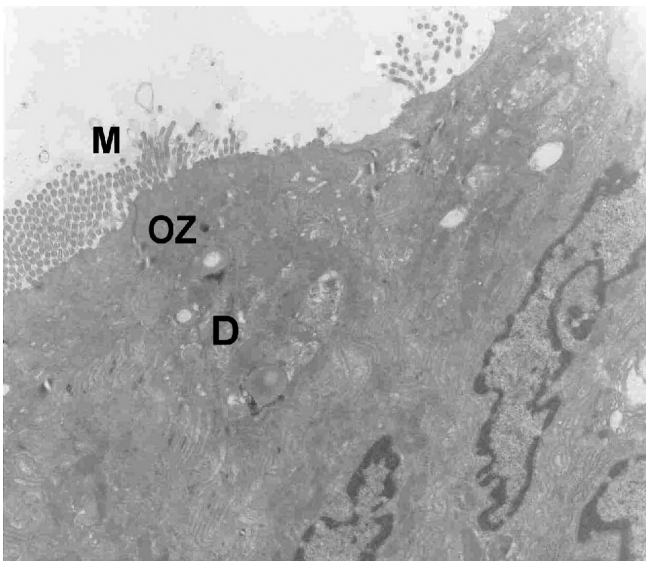


Fig. 7. Epithelium with short microvilli (M) which are locally absent. Cells are bonded together with intercellular junctions. Occludeus zones (OZ), desmosomes (D). x 10,000

dilatation and large dilatation of the RER (Fig. 7).

Some of the gland cells included large phagosomes. Inside the lamina propria many bands of collagen fibers and capillary vessels were distinguished (Fig. 8).

On the 14th postoperative day in the control group, the mucosa was to be found well developed, with multilayer epithelium and lamina propria full of collagen fibers, fibroblasts, eosinophils and vessels. Well developed muscularis mucosa, muscularis externa and submucosa were also seen (Fig. 9).

On the 14th postoperative day in the study group, all layers of the intestinal wall were also adequately developed, with normal morphology. The epithelium was multilayer. An incrassate lamina propria layer was

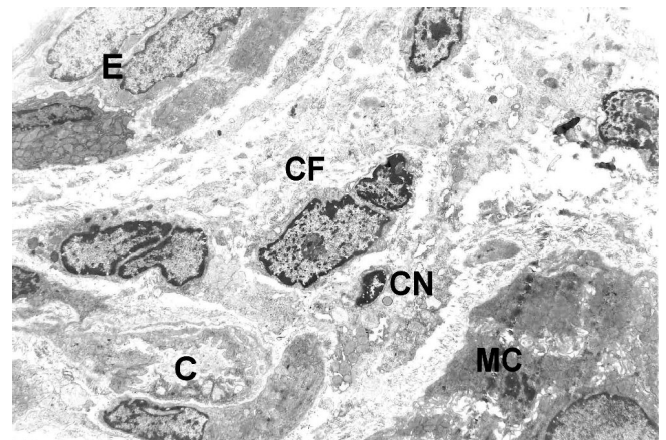


Fig. 8. Lamina propria with plenty of collagen fibers (CF), capillary vessel (C), fibroblasts, cells with condense nucleus (CN) and macrophages with lobulated nuclear (MC). Basal part of stratified epithelium (E). x 5,000

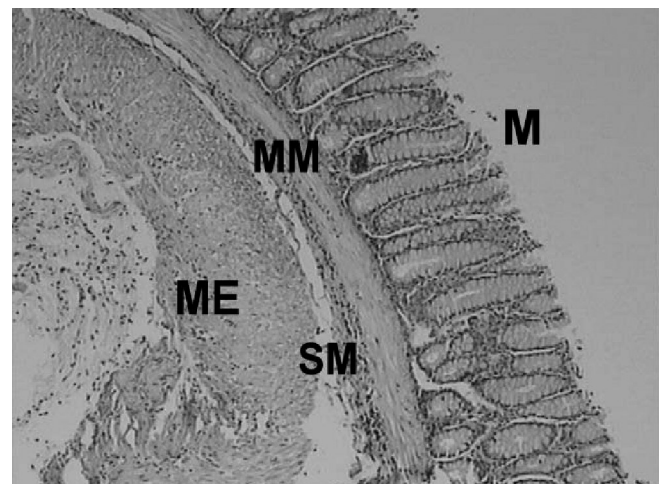


Fig. 9: Part of intestinal mucosa (M). Muscularis mucosa (MM), muscularis externa (ME) and submucosa (SM). x 50

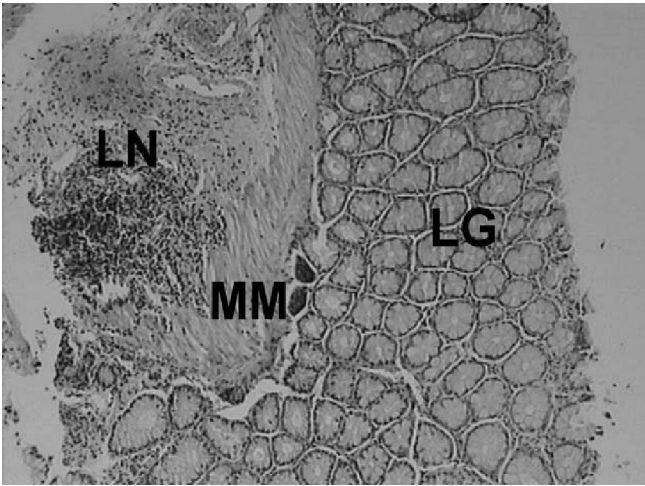


Fig. 10. Mucosa full of glands. Lieberkühn glands (LG), lymphonode (LN), muscularis mucosa (MM). x 50

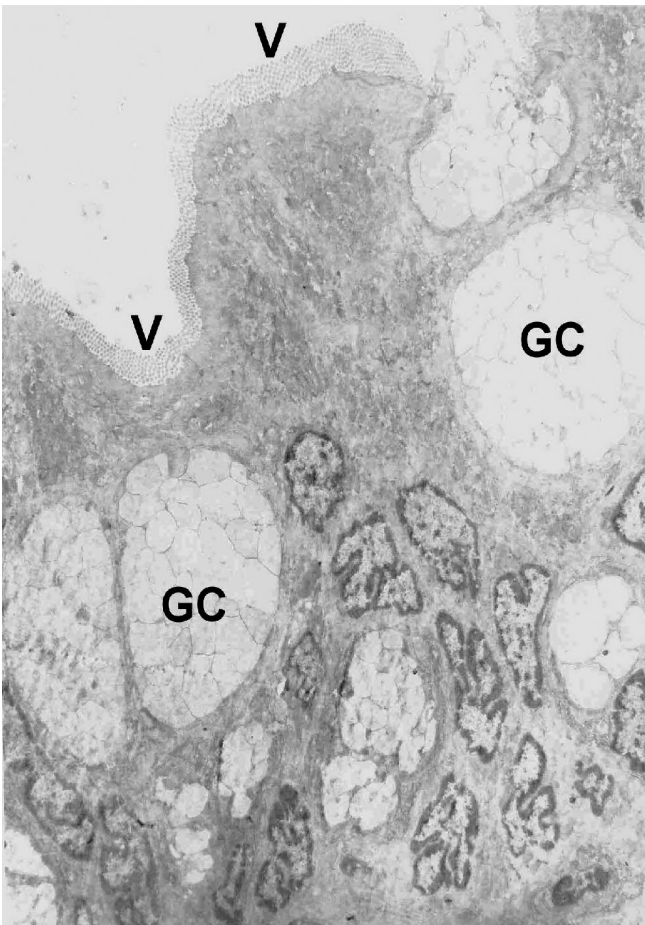


Fig. 11. Stratified epithelium with many microvilli (V) and plenty of goblet cells (GC). x 5,000

seen, as well as normal mucus layer and many eosinophils. The increased thickness of connective tissue, containing many collagen fibers and vessels, was also noticed (Fig. 10).

On the control group with 14 days placebo medication, from tissues away from the anastomosis, the mucus layer had normal morphology. The epithelium was found to be multilayer with short microvilli, locally absent. Many glands, full of columnar and lesser mucosal cells, were seen. The lamina propria had many collagen fibers, fibroblasts, red blood cells, neutrophils, capillary vessels and cells with pycnotic nucleus (Fig. 11). The muscularis mucosa was well developed and inside the submucosa many vessels were found.

On the study group with 14 days Capecitabine medication, from tissues away from the anastomosis, all intestinal wall layers were found to be well developed, with normal morphology. The epithelium was columnar multilayered. Inside the lamina propria many collagen fibers, eosinophil leucocytes, vessels and few cells with corrugated nucleus were seen (Fig. 12).

Discussion

Neoadjuvant chemotherapy has been proposed in order to increase respectability and to improve local control in advanced colorectal cancer. Nevertheless, it is

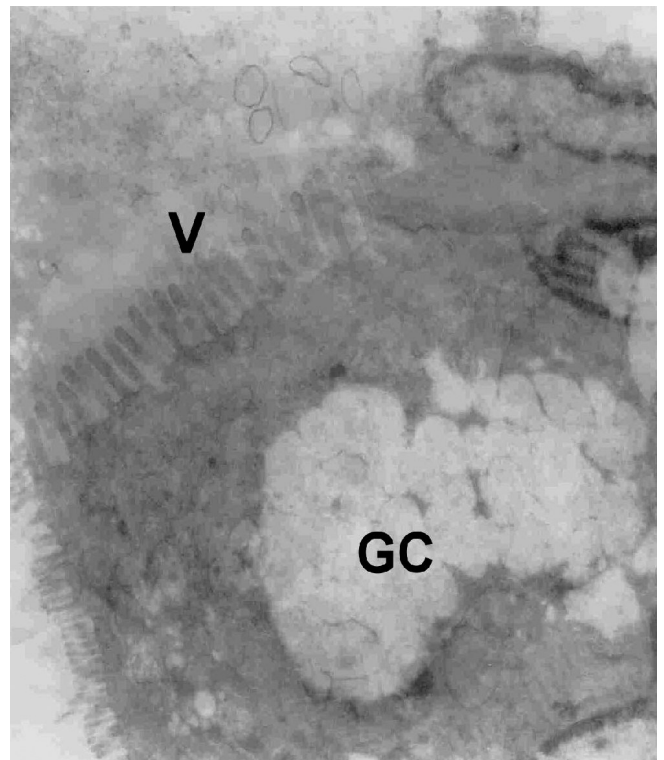


Fig. 12. Epithelium with goblet cells (GC) and microvilli (V). x 20,000

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well studied and known that the early phase of colonic healing sequence is characterized by a transient loss of strength in the anastomotic segment (Van der Kolk et al., 1999). Further reduction in wound healing at this period, such as perioperative chemotherapy, may compromise anastomotic integrity and increase the risk of anastomotic dehiscence.

Recently, a number of new agents, including 5-fluorouracil prodrugs have been studied with promising results. Capecitabine is the first in a new class of fluoropyrimidines. It is an oral, tumor-activated, anticancer prodrug of 5-fluorouracil (Miwa et al., 1998; Desmoulin et al., 2002). Capecitabine appears to be at least as active against metastatic colorectal cancer as conventionally administered intravenous 5-fluorouracil (Del Vecchio et al., 1999; Lewis and Meropol, 2000), with significantly less toxicity, improved quality of life, and lesser cost (Ishitsuka, 2000; De Bono and Twelves, 2001). Capecitabine may ultimately provide enhanced, more elective, antitumor activity to fluorouracil-containing regimes for advanced colorectal cancer (Ishikawa et al., 1998, Morita et al., 2001).

The effect of most antineoplastic factors on anastomoses healing, including 5-FU, is well studied and documented as strongly inhibitory (Van der Kolk et al., 1999; Nayci et al., 2003). In addition to this general rule, our study revealed that Capecitabine, not only does not impair mechanical strength of large bowel anastomoses, but it aggravates the healing process during the first postoperative days, as reflected by increased bursting pressure. The influence of Capecitabine on colonic anastomoses healing during the mid and late postoperative days was not found to be substantial, since no statistically significant differences were reported between the bursting pressure of control and study subdivisions sacrificed on the 7th and 14th postoperative day.

Capecitabine has the unique ability to be selectively metabolized to 5-FU from tumor cells. It is sequentially converted to 5'-Deoxy-fluoro-cytidine (5'-DFCR) by carboxylesterase in the liver, to 5'-Deoxy-fluoro-uridine (5'-DFUR) by cytidine deaminase (highly expressed in the liver and tumor tissues), and finally to 5-FU by thymidine phosphorylase, which is preferentially located in various types of cancer tissues (Verweij, 1999). As a result of the unique localization of these enzymes, Capecitabine is expected to deliver the active 5-FU selectively in tumor tissues and to have better safety profiles and fewer side effects (Tanaka et al., 1990). Our data supports the non toxic effect of Capecitabine on the healing process.

Even though wound healing is a complex and dynamic process, on the first three days the main pathophysiologic action is the acute inflammatory phenomenon, described as inflammatory stage (Portera et al., 1997; Steed, 1997). This is an essential phase of intestinal healing, in which macrophage and polymorphonuclears migrate from the circulation to the wound. Local ischemia and necrosis are present,

simultaneous with cell regeneration on the anastomotic line (Barbul et al., 1989; Martin and Muir, 1990). This phase is followed by fibroblastic proliferation, synthesis of collagen, remodeling of connective tissue and its parenchymal component, and the acquisition of wound strength (Goldman, 2004). The data obtained in this experiment shows that the animals of the study group sacrificed on the 3rd postoperative day, compared to those of the control group, had better restitution of the bowel wall layers, lesser necrosis effects, better regeneration of the epithelium, higher grade reformation of the glands and absorptive cells, as well as basic membrane cells with more profound polyribosomes, as a sign of more aggravated metabolism.

The submucosal layer of the animals sacrificed on the 3rd postoperative day, was almost presented with bundles of collagen fibers and many fibroblasts with dilated RER, especially in study group. The suture holding capacity and mechanical strength is strongly dependent on the structural collagen network in the submucosal layer (Kiyama et al., 2002), and this observation agreed with the elevated bursting pressure of the study group on the 3rd postoperative day. The same differences were not found between study and control groups sacrificed during later postoperative days.

Enhancing of bursting pressure during the early postoperative days has been reported in other studies, in which anti-inflammatory factors, vitamins, antibiotics and low doses of immunosuppressant drugs were used (Rostan et al., 2002; Siemonsma et al., 2003). The proposed mechanism of action for those factors, in order to explain the positive effect on wound healing, is the partial inhibition of the inflammatory phase, so that a positive balance is accomplished between regeneration and necrosis effects on the anastomosis line (Meddahi et al., 1996; Kiyama et al., 2002). Our experiments, in line with that interpretation, also revealed diminished cell destruction with Capecitabine treated animals, during the early and more vulnerable days of intestinal healing.

In conclusion, our results indicate that perioperative administration of Capecitabine, as an additional strategy to surgical excision, does not impair the healing of rat colonic anastomosis. Further experimental studies should be carried out to corroborate this outcome and make it standard practice cancer therapy in humans.

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