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Could be Reversed the Detrimental Effect of 5-Fluorouracil on Healing of Colonic Anastomosis by Oral Glutamine Supplementation?

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Abstract

Background/Aim: The anti-neoplastic agents are known to impair tissue healing which may lead to significant post-operative complications, like anastomotic leaks. There has been a number of studies that have shown the protective affects of glutamine on the enteric mucosa. Our study aimed to test whether the addition of enteral glutamine to 5-fluorouracil (5FU) used as immediate post-colectomy chemotherapy caused less anastomotic complications compared to 5-FU alone.

Materials and methods: Thirty-six female Wistar-Albino rats were initially divided into three groups of twelve rats. The first and the second twelve formed the control (CG) and the 5-FU groups respectively, and were fed on standard laboratory diet and water for seven days. The third group was the Glutamine group (GG) which had oral glutamine supplements in addition to the standard diet. All animals had a laparotomy on day 7. The left colon was transected and a hand sewn colocolic anastomosis was undertaken (hangi teknik, hand-sewn, single layer, sero-submucosal). All groups were further divided into two subgroups (a total of six groups). The first subgroup in each main group was sacrificed on post-operative day 3, the remainder were killed on day 7. Bursting pressures, tissue-hydroxyproline and histopathology were compared by Anova test.

Results: Bursting pressure values were significantly reduced by 5FU treatment, both at day 3 and day 7 postoperatively. Glutamine treatment prevented the reduction of bursting pressure in 5FU treated animals, which was not significantly different from animals not treated with 5FU. The lowest mean tissue hydroxyproline levels were found in the 5FU-day 3 & day 7 groups, histopathology was superior in 5FU-glutamine-day 7 group.

Conclusion: Glutamine neutralised the detrimental affects of 5-FU on tissue healing. This may enable the early inititiation of adjuvant chemotherapy.

Keywords: Glutamine; 5-Fluorouracil; Wound healing; Colonic anastomosis

Introduction

Colorectal cancer is a common malignancy in most developed countries. In the UK, colorectal cancer is the third most common cause of cancer deaths after lung and prostate cancer in the male, and following breast and lung cancer in the female [1]. In Turkey, colorectal cancer is the third leading cause of cancer death [2]. Metastasis frequently occurs before clinical detection of the primary tumour. Despite the advances in surgical techniques, this characteristic of the malignancy prevents a significant improvement in cure rates for colorectal cancers and almost half of all patients with colorectal cancer will eventually die of recurrent disease. In selected patients, 5-FU based adjuvant chemotherapy has improved survival rates after curative resection [3]. Adjuvant chemotherapy is commonly used in patients with Astler Coller stage B2, C and resectable stage D colorectal cancer. A number of studies showed that the proliferation of cells in the metastatic foci increase after the primary tumor resection. This makes the interval between surgery and the administration of adjuvant chemotherapy critical. According to animal experiments, the most effective reduction of malignant proliferation occurs when the chemotherapeutic agent is administered immediately after tumor removal [4,5].

One of the most important criteria that affects post-operative mortality and morbidity is the integrity of the colonic anastomosis. 5-FU can inhibit the collagen synthesis and when used in the immediate post-operative setting may lead to wound and anastomotic dehiscence [6-11]. This risk often delays the inititiation of the adjuvant chemotherapy until the surgical wounds are healed.

Glutamine is traditionally considered a nonessential amino acid, but appears to be conditionally essential nutrient during injury. It is produced in the body from glutamate and ammonia by the enzyme glutamine synthetase. The process takes place mainly in the skeletal muscle. Glutamine is the most abundant amino acid in plasma and skeletal muscle. The circulating and tissue concentrations of glutamine decrease after injury or surgery [12,13]. Glutamine is also the preferred fuel for the intestine, and clinical studies have revealed that both parenteral and enteral glutamine supplementation is beneficial in patients after multiple trauma and surgery [14,15]. Utilization of glutamine by the intestine increases after surgery and appears to play a

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vital role in mucosal healing [16,17]. In this view, perioperative enteral glutamine supplementation results in improved wound healing and reduces anastomotic complications [18-23].

Our study aimed to find out whether addition of enteral Glutamine supplements made the 5-FU treatment safer in the immediate post-operative period.

Materials and Methods

Thirty-six female Wistar-Albino rats weighing between 170 and 220 g were used in this study. All rats were clinically healthy and were fed with standard laboratory food and water. The animals were numbered at the beginning of the study and weighed daily. The study was approved by the local Ethics Committee of Gazi University.

Thirty-six rats were equally divided into six groups. The daily dosage of 5-FU was set at 20 mg/kg which is the maximum non-lethal dose for rats [24-26]. The glutamine (Resource Glutamine, Nestle) was given as a bolus via an orogastric tube in a dose of 50 mg/kg/day [18,19,27].

The details of the six groups were as follows:

1) Control-day 3 (C-day 3): This group had free access to standard laboratory diet and water in the preoperative 7 days. After the operation, free access to standard laboratory diet and water were continued till the postoperative day 3. All rats in this group were killed on post-operative day 3.

2) Control 2 (C-day 7): Identical preoperative feeding regime to C-day 3. The rats were fed for 7 days after the laparotomy and all were sacrificed on day 7.

3) 5FU-day 3: Preoperative 7 days had free access to a standard laboratory diet and water. 5-FU was administered 20 mg/kg/day intraperitoneally at the time of the operation and it was repeated intraperitoneally once daily on the first and second postoperative days. Free access to standard laboratory diet and water were continued for the postoperative 3 days. Animals were killed on day 3.

4) 5FU-day 7: Identical to 5FU1 except animals were killed on day 7.

5) 5FU-gutamine-day 3 (5FUG-day 3): Preoperative 7 days 50 mg/kg Glutamine (Resource Glutamine, Nestle) feeding via orogastric tube as well as free access to standard laboratory diet and water. 5-FU was administered 20 mg/kg/day intraperitoneally at the time of operation and it was repeated intraperitoneally once daily for the first and second postoperative days. Glutamine feeding (50 mg/kg) via orogastric tube and free access to a standard laboratory diet and tap water were continued for the postoperative 3 days. Animals were killed on day 3.

6) 5FU-glutamine-day 7 (5FUG-day 7): Identical to 5FUG1 except animals were killed on day 7.

Operative Procedure

All rats were anesthetized with an intramuscular injection of 50 mg/kg ketamine hydrochloride and 10 mg/kg xylazine hydrochloride. Surgical procedures were performed under semi sterile conditions. Laparotomies were performed through midline incisions of approximately 5 cm. The left colon was completely transected approximately 1 cm proximal to the peritoneal reflection and an end-to-end anastomosis was performed consisting of eight interrupted 6-0 polipropylene sutures (Prolene, Ethicon®) (Figure 1). The abdominal layers and skin incision were closed en bloc with a running 3-0 monocryl (Ethicon®) suture. Postoperatively, rats in the group of C-day 3, C-day 7, 5FU-day 3 and 5FU-day 7 were allowed free access to tap water and food and libitum and in the group of 5FUG-day 3 and 5FUG-day 7 rats were allowed same diet plus 50 mg/kg bolus Glutamine feeding via orogastric tube immediately.

End of study procedures

Groups C-day 3, 5FU-day 3 and 5FUG-day 3 were killed on postoperative day 3 and groups C-day 7, 5FU-day 7 and 5FUG-day 7 were killed on postoperative day 7. All animals were anesthetized in the same manner and laparotomy was performed via the previous incision scar. All anastomoses and intra-abdominal adhesions were examined *in vivo* before the resection. Then a 10 cm segment of the left colon which included the anastomosis was resected. The bursting pressure measurements were performed *ex vivo* as follows:

Measurement of bursting pressures

On the back table a 10 Fr catheter was inserted to the proximal part of the colon and fixed with 4/0 silk sutures then connected to the perfusor (B.Braun Perfusor®Space). The distal part of the colon was connected to the arterial line transducer set with a 10 Fr connector. The strength of each anastomosis was assessed by measuring its burst pressure using the perfusor operating at 150 ml/h with an invasive arterial blood pressure transducer (Datex-Ohmeda Cardiocap/5®) and measured systolic pressures were accepted as the bursting pressure (mmHg). The maximum pressure immediately preceding the sudden fall as a result of explosion was recorded as the bursting pressure. Results were recorded with Statistical Package for the Social Sciences version 13.0 software (SPSS, Inc., Chicago, IL, USA).

After measurement of bursting pressures, anastomotic segments of 2 cm were separated from the specimen, wrapped in aluminium foil, and frozen in liquid nitrogen. Samples were then stored (-20°C) until the end of the experiment for hydroxyproline measurement. Animals were killed by overdose injection of ketamine hydrochloride (100 mg/kg) and xylazine hydrochlo ride (20 mg/kg).

Measurement of tissue hydroxyproline content

Briefly, the tissue specimen was homogenized to a fine solution in cold saline, hydrolyzed in alkali and oxidized with chloramine T. The chromophore was developed with the addition of Ehrlich's aldehyde and the absorbance of the chromophore was measured at 550 nm.

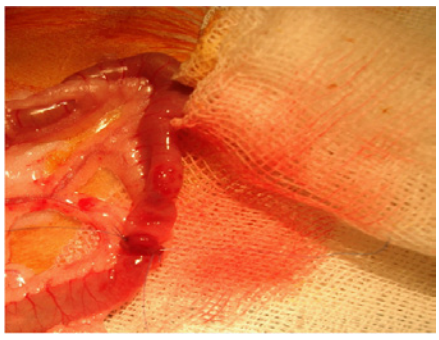

Figure 1: End-to-end left colon anastomosis with eight interrupted 6-0 propylene sutures.

The concentration of hydroxyproline in each tissue specimen was deduced from a standard calibration curve. Hydroxyproline levels were expressed as micrograms per milligram of tissue [28,29].

Anastomotic Complications and Intra-Abdominal Adhesions

All anastomoses were examined during the second laparotomy before the bursting pressure measurements. Intra-abdominal adhesions were classified according to the Blauer scoring system (0 = no adhesions; 1 = thin or narrow, easily separable adhesions; 2 = thick adhesions, limited to one area; 3 = thick and widespread adhesions; 4 = thick and widespread adhesions, plus adhesions of viscera to anterior or posterior abdominal wall) [30,31].

Microscopic Examination

For histological comparisons, one rat from each group was not subjected to measurement of bursting pressure and hydroxyproline. All tissue specimens were obtained from the anastomosis area and fixed in 10% buffered formalin, processed by routine protocols for embedding in paraffin wax, and cut into serial 5 µ thick sections by microtome. The sections were stained with haematoxylin and eosin and examined using a photomicroscope (BX50; Olympus, Tokyo, Japan). The accumulation of Polymorphonuclear Cells (PMNs), lymphocytes and macrophages (inflammation); thickness of the wall at the anastomosis relative to the thickness of the normal intestinal wall; submucosal-muscular layer repair; and amounts of necrosis and vascularisation on histopathological examination under light microscopy were scored from 0 to 3 [19].

Statistical Analysis

Data are expressed as mean ± SD for continuous outcomes. Statistical tests were chosen according to the distribution of data. Differences of the mean bursting pressures between the control and the treatment groups were assessed by one-way analysis of variance (ANOVA). The mean hydroxyproline content differences among all six groups were analyzed by one-way ANOVA. When the p value from ANOVA was significant, post-hoc Tukey HSD multiple comparison tests were performed. Intra-abdominal adhesions were analyzed by Kruskal–Wallis nonparametric test. The value of $p < 0.05$ was accepted as significant.

Results

Complications and weight loss

No wound complications were observed in the study or control groups and none of the rats died. There were no significant weight loss in the C-day 3 & day 7 and 5FUG-day 3 & day 7 groups, but there were significant weight loss in the 5FU-day 3 and day 7 groups when compared with the respective Control and Glutamine groups. Mean differences of the weight(MDW) until killing day during the study are shown in Table 1.

Anastomotic complications and intra-abdominal adhesions

No perforation, intra-abdominal abscess, or anastomotic dehiscence was observed except the groups of C-day 3, 5FU-day 3 and 5FU-day 7. Adhesions were scored on a scale of 0–4 according to Blauer's scoring system and the evaluation of the differences of adhesions between the groups were shown in Table 2.

Bursting site and pressure

In most animals the bursting site was along the anastomosis line.

Groups (n=6)	MDW ± SD (g)	MDW of C-day3 group ± SD (g)	p
5FU-day3	-7.6 ± 5.04	14.3 ± 4.08	0.001
5FUG-day3	6.0 ± 4.04	14.3 ± 4.08	0.014
p	0.001		
Groups (n=6)	MDW ± SD (g)	MDWof C-day7 group ± SD (g)	p
5FU-day7	-10.5 ± 9.09	34.3 ± 6.80	0.001
5FUG-day7	15.8 ± 3.76	34.3 ± 6.80	0.001
p	0.001		

Table 1: Mean differences of the weight among the groups until sacrifice day.

Groups (n=6)	Mean rank of the intra-abdominal adhesions	p
C-day3	4.00	
5FU-day3	15.50	0.001
5FUG-day3	9.00	
C-day7	3.67	
5FU-day7	15.50	0.001
5FUG-day7	9.33	

Table 2: Mean rank of the intra-abdominal adhesions and the p value among the study groups according to the Kruskal Wallis test on sacrifice day 3 and 7.

Anastomotic ruptures were more common in the C-day 3, 5FU-day 3 and 5FU-day 7 groups than in the C-day 7, 5FUG-day 3 and 5FUG-day 7 groups ($p < 0.01$). The bursting pressure values were lower in the 5FU-day 3 and 5FU-day 7 groups than in the respective control and glutamine groups. The differences of the Mean Bursting Pressures (MBP) between and within the study groups on postoperative day 3 and 7 were significant (p=0.022 and 0.001 respectively).

According to this result, the corelation between the study groups were calculated with Multiple Comparisons of Tukey test, the difference between the C1 vs 5FU-1 (p=0.017) groups was found significant.

On the other hand, the differences of the MBP between and within the study groups on postoperative day 7 were also found significant (p=0.001). According to this result, the corelation between the study groups were also calculated with Multiple Comparisons of Tukey test, and the differences between the, 5FU-2 vs C2 (p=0.007) and 5FU-2 vs 5FU-G2 (p=0.001) groups were found significant (Table 3).

Tissue hydroxyproline content

The Mean Tissue Hydroxyproline Content (MTHC) of the colonic anastomosis in the study groups were compared. The lowest MTHC of the colon anastomosis were found in the 5FU-1 and 5FU-2 groups than the respective control and glutamine groups. However, the differences of MTHC of the colon anastomoses between and within the study groups of postoperative day 3 were not found significant (p=0.334). On the other hand, the differences were found significant in the study groups of postoperative day 7 (p=0.001).

According to this result, the corelation between the study groups of postoperative day 3 and 7 were calculated with Multiple Comparisons of Tukey test. The differences of the MTHC between the groups of 5FU-G1 vs 5FU-1 (p=0.004) and C1 vs 5FU-1 (p=0.042) were found significant, but 5FU-1 vs C1 were insignificant. On the other hand, the differences of the MTHC between the groups of 5FU-G2 vs both C2 (p=0.002) and 5FU-2 (p=0.001) were found significant (Table 4).

Histopathological evaluation

The mucosal epithelium integrity was poor in the 5FU-1 group (Figure 2), but the mucosal epithelium integrity was better in the 5FU-G2 group illustrated in (Figure 4). According to the colon anastomosis

	MBP ± SD(mmHg)	C-day3	5FU-day3	5FUG-day3	C-day7	5FU-day7	5FUG-day7
C-day3	41.50 ± 6.31		p=0.017				
5FU-day3	20.33 ± 14.05			p=0.282			
5FUG-day3	31.00 ± 13.05	p=0.293					
C-day7	179.33 ± 77.41					p=0.007	
5FU-day7	47.00 ± 37.40						p=0.001
5FUG-day7	215.50 ± 68.00				p=0.594		

Table 3: The differences of mean bursting pressures (MBP) between the groups according to the Multiple Comparisons of Tukey test.

	MTHC±SD(µmol/g)	C-day3	5FU-day3	5FUG-day3	C-day7	5FU-day7	5FUG-day7
C-day3	0.375 ± 0.08		p=0.042				
5FU-day3	0.207 ± 0.07			p=0.004			
5FUG-day3	0.448 ± 0.14	p=0.488					
C-day7	0.574 ± 0.07					p=0.002	
5FU-day7	0.353 ± 0.06						p=0.001
5FUG-day7	0.859 ± 0.12				p=0.001		

Table 4: The differences of mean tissue hydroxyproline content (MTHC) between the groups according to the Multiple Comparisons of Tukey test.

healing scores, the inflammation, type of the mucosal epithelium, submucosal-muscular healing and necrosis scores of the 5FU-G2 group were found better than 5FU-2 group (Figure 3) significantly (p=0.01).

Discussion

Adjuvant chemotherapy after curative resection of colorectal cancer may be beneficial in some patients to reduce the risk of metastasis. However the timing of adjuvant chemotherapy is one of the most important problems after resection. Perioperative use of chemotherapeutics causes poor wound healing and increases the risk of wound dehiscence because of inhibition of collagen synthesis. Tissue hydroxyproline level is an important parameter in the tissue repair process [32,33]. Some studies indicated that 5-FU decreases the hydroxyproline content in the wounds. In the study of van der Kolk et al. [34], after the 5-FU treatment the anastomotic hydroxyproline content was found lower than the respective controls. In another study, Kuzu et al. [35] demonstrated that, although no significant differences were found in anastomotic burst pressures between the groups, hydroxyproline content were found significantly lower following 5-FU chemotherapy. As a result, adjuvant treatment is usually withheld, until the wound is healed.

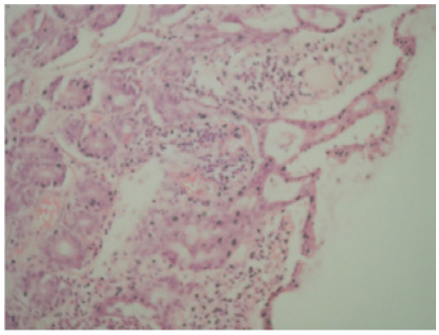

Figure 2: Ch1 Intestinal anastomosis: Mucosal desquamation-mucositis and submucosal lymphocytic infiltration (Haematoxylin and eosin dyes, x20), mucosal regularity was not observed.

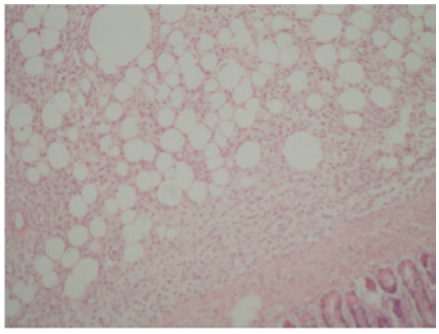

Figure 3: Ch2 Intestinal anastomosis. Submucosal lymphocytic infiltration , fat necrosis and fibroblastic proliferation in the periserosal fatty tissue and serositis (Haematoxylin and eosin dyes, x20), poor mucosal healing was observed.

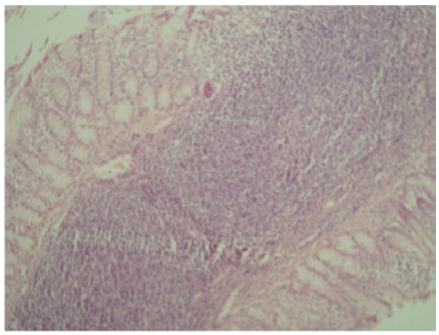

Figure 4: G2 Intestinal Anastomosis: Mucosal lymphocytic infiltration and fibroblastic proliferation in the periserosal fatty tissue (Haematoxylin and eosin dyes, x10). Fat necrosis, mucositis and serositis are absent, perfect mucosal healing was observed.

Glutamine is the main energy substrate for the enterocytes. In the study of Rouse et al. [36], glutamine was a primary respiratory fuel and also as a necessary substrate for nucleotide synthesis in most dividing cells such as enterocytes. In this study, protecting effect of oral glutamine was also demonstrated in normal tissues and possibly sensitizing tumor cells from chemotherapy-related injury via the effects of oral glutamine on tumor and host glutathione metabolism. In another study Sukhotnik et al. [37] researched the effects of oral glutamine in preventing intestinal mucosal damage caused by Methotrexate (MTX)

in rats. According to this study, MTX-glutamine in rats indicated better mucosal healing compared to MTX animals. On this basis, we can say perioperative glutamine intake causes improved wound healing and may reduce the risk of wound dehiscence. Gut's glutamine requirement is increased in catabolic states like surgery and immunosuppression. Although, glutamine is not classified as an essential amino acid, some studies emphasized that glutamine was an essential dietary element for the intestinal mucosal proliferation and growth [20]. As shown in our study, glutamine supplementation enhanced the healing of the colonic anastomosis and significantly reduced the negative affects of 5-FU on the healing process. We believe that this improvement can lead to early initiation of adjuvant chemotherapy which will give our patients the best chance in disease free survival.

In view of the administration route of Glutamine, our findings are very much in line with the previous publications that enteric route is both practical and efficient. Demetriades et al. [21], showed the positive affects of early postoperative glutamine enriched enteral feeding. Gokpinar et al. [22] also reported improved anastomotic healing with early enteral nutrition and glutamine enrichment in the postoperative period. In another study, da Costa et al. [23] reported that, total rupture strength and the percent area of mature collagen at the anastomoses sites on postoperative days 3 and 8 were increased by perioperative oral glutamine supplementation. On the contrary, Cihan et al. [38] demonstrated that, under normal conditions there was no beneficial affect on anastomotic bursting pressures with early enteral feeding with glutamine enriched diet.

Through a literature search, we could not identify any other study that that looked into the affects of glutamine supplementation on anastomotic healing in the presence of chemotherapy and this present study is the first one to show that glutamine strongly protects the integrity of anastomoses and levels of tissue hydroxyproline content in the presence of 5-FU .

Postoperative weight loss is an important indicator of nutritional status. Weight loss is also an important side effect of antineoplastic agents. In this study, weight loss was significant in 5-FU treated rats. This weight loss was substantially reduced by oral glutamine therapy. From this point of view, it is assumed that glutamine supported the nutritional status of the animals both in the early phase (postoperative day 3) and the late phase of the anastomotic healing (postoperative day 7).

Intra-abdominal adhesions were significantly more common in 5FU-treated animals that were on a normal diet compared to the ones that received glutamine supplementation. This suggested that, glutamine may have some protective effect on adhesion formation if given with 5-FU.

The most important observation in our study was the significant reduction in anastomotic leaks. The leaks only occurred in 5FU treated animals that did not receive any glutamine. In fact, negative effects of 5-FU on anastomotic healing were shown before. Kanellos et al. [39] demonstrated that, the perioperative intraperitoneal administration of 5-FU inhibits the healing of colonic anastomoses in rats.

In another study, Ersoy et al. [11] showed that bursting pressures of colonic anastomosis in rats that received 5-FU treatment were significantly lower than those of the control animals. Anastomotic leakage was a predicted complication in 5-FU treated animals. Our data suggests that glutamine has a protective effect on tissue healing which almost neutralises the negative affects of 5-FU.

In conclusion, higher bursting pressures in the glutamine groups indicate that glutamine prevents the negative effects of antineoplastic agents on wound healing. Higher tissue hydroxyproline levels and lower inflammation and necrosis scores in the glutamine groups also support this conclusion. Starting from the preoperative and in the early enteral nutrition period, addition of the glutamine to the nutritional intake could be beneficial for the healing of the colonic anastomosis. Perioperative glutamine supplementation can enable the immediate post-operative use of 5-FU in treatment of colorectal cancer.

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Burak Kavlakoglu and other co-authors have no conflict of interest.

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