

# EFFECTS OF HYPERTHERMIC INTRAPERITONEAL MITOMYCIN-C TREATMENT AUGMENTED WITH RADIOTHERAPY ON THE ANASTOMOTIC HEALING OF THE RAT COLON

## *Radyoterapi ve İntraperitoneal Mitomisin-C Kemohipertermisinin Sıçanlarda Kolon Anastomoz İyileşmesine Etkileri*

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### ARAŞTIRMA

#### ÖZET

**Amaç:** Preoperative radyoterapi ile intraperitoneal mitomisin-C kemohipertermisinin (HIMMC) kolon anastomoz iyileşmesine olan etkilerini araştırmak

**Materyal ve Metod:** Wistar-Albino ratlar beş gruba ayrıldı. Radyoterapi (RT) ve RT+ HIMMC gruplarına tek doz (20 Gy) preoperative eksternal radyoterapi uygulandı. RT ve RT+HIMMC gruplarında radyoterapiden beş gün sonra ve bütün gruplarda peritoneal refleksiyonun 1.5 cm proksimalinde kolon transvers planda kesildi ve anastomoz uygulandı. Batın kapatıldıktan sonra, HIMMC ve RT+HIMMC gruplarında 0.4 mg/kg dozunda MMC-saline solüsyonu 50 ml/dk ve 30 dakikada zerkedildi. İntraperitoneal sıcaklık 41-42°C olarak ayarlandı. HT grubunda ısıtılmış saline solüsyonu perfüzyonu yapıldı. Postop 7. günde sıçanlar katledildi ve anastomozların patlama basınçları ölçüldü ve hidrokspirolin düzeyleri belirlendi. Segmentin mikroskopik incelemesine ilaveten anastomoz kaçığı ve mortalite oranları belirlendi.

**Bulgular:** HIMMC ve RT+ HIMMC gruplarında anastomoz sızıntısı ve mortalite oranları diğer gruplardan anlamlı olarak yüksek çıktı (P<0.05). RT, HIMMC ve bu tedavi modalitelerinin kombine olarak uygulanmasının patlama basınçlarını kötü etkilediği görüldü. En düşük hidrokspirolin seviyeleri RT+HIMMC grubunda bulundu. Doku iyileşme indeksi RT grubunda en düşük olarak saptandı, RT+HIMMC, HIMMC ve HT gruplarında ise artan oranlarda bulundu.

**Sonuç:** Bu veriler bize HIMMC tedavisi ve onun preop radyoterapi ile kombinasyonunun kolon anastomozu üzerinde istenmeyen yan etkilerinin olabileceğini gösterdi.

**Anahtar kelimeler:** Preoperatif radyoterapi, hipertermik intraperitoneal perfüzyon, mitomisin C, kolon, yara iyileşmesi, sıçan.

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### RESEARCH

#### ABSTRACT

**Aim:** To investigate the effects of hyperthermic intraperitoneal mitomycin-C (HIMMC) treatment plus preoperative radiotherapy on colonic anastomotic healing.

**Materials and Methods:** Wistar-Albino rats were divided into five groups. The rats in the radiotherapy (RT) group and RT+HIMMC group were received single dose (20 Gy) external preoperative radiotherapy. Cutting of the colon in transverse plan 1.5 cm proximal from the peritoneal reflection and anastomosis were performed in all but five days after radiotherapy in RT and RT+HIMMC groups. After closure, MMC in saline solution with a dose of 0.4mg/kg was infused 50ml/min for 30 minutes in HIMMC and RT+HIMMC groups. The intraperitoneal temperature was maintained between 41 and 42°C. Heated saline solution was perfused in the HT group. At the 7th postoperative day, the rats were sacrificed and bursting pressures and hydroxyproline levels of anastomoses were measured. Mortality and anastomotic leak rates were also determined in addition to microscopic examination of the segment.

**Results:** Anastomotic leak and mortality rates were significantly higher in HIMMC and RT+ HIMMC groups compared to others (P<0.05). RT, HIMMC and combination of these treatment modalities were affected bursting pressures adversely. The lowest hydroxyproline levels were found in RT+HIMMC group. Tissue healing index was found to be lowest in RT group, RT+HIMMC, HIMMC and HT groups were followed in increasing proportion.

**Conclusion:** These data suggest that HIMMC treatment and its combination with preoperative radiotherapy have unwanted side effects on colonic anastomosis.

**Key words:** Preoperative radiotherapy, hyperthermic intraperitoneal perfusion, mitomycin C, colon, wound healing, rat.

## INTRODUCTION

Peritoneal carcinomatosis and local recurrence are common problems in the eradication of malignant tumors derived from gastrointestinal tract. In the absence of detectable metastatic focus in the intra or extra-peritoneal area, microscopic disease left after resection or peri-operative tumor-cell spillage are accepted causes of the intra-peritoneal recurrence. In the cases after curative resection of the gastric carcinoma, local or intra-abdominal recurrence is reported up to 50% (1,2). Like the cases with gastric carcinoma, intra-abdominal recurrence of the tumor after curative resection is a major problem in the cases with colon and rectal carcinoma (3). The median survival in the patients with peritoneal carcinomatosis was reported between 5.2 to 6 months in the colorectal carcinoma (4-6).

In general, peritoneal carcinomatosis is considered a terminal condition. However, cytoreductive surgery and intra-peritoneal chemotherapy allows long-term disease free survival in this group (7-11). Recently, studies aimed to prevent peritoneal carcinomatosis at the time of curative surgery for gastric carcinoma are published in the literature (12-14). Intra-operative or early post-operative hyperthermic intra-peritoneal chemotherapy is used for the prevention in these studies. In contrast to preventive approaches in the cases with gastric carcinoma, pre-operative radiotherapy is generally accepted method for the prevention from peritoneal or local recurrences in the colorectal carcinoma (15). Also, post-operative chemotherapy decreases local recurrences, increases disease free interval and increases survival in colorectal carcinoma on both clinical and experimental settings (16-18). 5-Fluorouracil is considered to be the most effective single agent using in the colorectal carcinoma treatment (19). Mitomycin C is another chemotherapeutic agent gaining popularity against digestive tract cancers in the recent studies (8-13,20,21). Hyperthermic intra-peritoneal infusion is considered to be enhancing mitomycin's clinical efficiency compared to 5-fluorouracil (11,22).

Well prepared experimental studies for the colonic anastomotic healing after pre-operative radiotherapy are published in the literature to date (23-25). Intra-peritoneal administration of the 5-fluorouracil is showed encouraged results on the colonic anastomotic healing in the rat model when combining with pre-operative radiotherapy (26). In clinical settings, possible additive adverse affects of pre-operative radiotherapy and intra-peritoneal chemotherapy threaten the clinicians for preventive usage of this modality. However, the effects of hyperthermic intra-peritoneal administration of mitomycin C, the most popularized technique against peritoneal seeding of the digestive tract carcinomas, on the early anastomotic healing have not been investigated. Therefore, we examined the effect of combined pre-operative irradiation with a dose of 25 Gy and

intra-operative hyperthermic intra-peritoneal mitomycin C chemotherapy on anastomotic healing in the rat colon. Also, the effects of combined treatment were compared with either single treatment modality or hyperthermic intra-abdominal perfusion.

## MATERIALS AND METHODS

A hundred adult male Wistar-Albino rats weighing from 250 to 280 g were used. All studies were carried out under guidelines of the Ankara University, institutional animal ethics committee. The rats were randomly divided into five groups [control group, preoperative radiotherapy group (RT), preoperative radiotherapy plus hyperthermic intraperitoneal mitomycin C perfusion group (RT+ HIMMC), hyperthermic intraperitoneal mitomycin C perfusion group (HIMMC), hyperthermic saline perfusion group (HT)] of twenty animals each. The rats were kept at room temperature and provided with free access to standard chow and tap water. The rats in the RT group and RT+ HIMMC group were received external preoperative radiotherapy. An area 3X2 cm<sup>2</sup> in width on the abdomino-pelvic wall where cover the left colonic segments was irradiated by ATC Cobalt 60 machine pre-operatively with a single dose of 20 Gy. The source-skin distance was restricted to 80cm. All animals were fasted for 12 h except for water before the operation. Median laparotomy and cutting of the colon in transverse plan 1.5 cm proximal from the peritoneal reflection was performed under ketamine (40mg/kg, Ketalar, Parke-Davis Inc., USA) anesthesia. Operation was performed five days after irradiation in the RT and RT+HIMMC groups. End-to-end anastomosis was performed in a single-layer, interrupted and inverted manner with 5/0 polypropylene sutures in every animals. After anastomosis was completed, a silicone inflow catheter (Dura-Sil; Biometrix, Jerusalem, Israel) was introduced into the left subdiaphragmatic space and another silicone catheter was inserted into the left paracolic gutter for outflow drainage in the HT, HIMMC group and RT+HIMMC groups. Temperature sensors (Mon-a-therm™; Mallinckrodt Medical, St Louis, Missouri, USA) were attached to the catheters. Laparotomy was closed with interrupted 3/0 polypropylene sutures. After closure of the laparotomy, MMC in saline solution with a dose of 0.4mg/kg was infused 50ml/min for 30 minutes in HIMMC and RT+HIMMC groups. The intraperitoneal temperature was maintained between 41 and 42°C with a heat exchanger (Baxter, Uden, The Netherlands) and a roller pump (Polystan, Copenhagen, Denmark) was provided solution's back to the abdomen. Only heated saline solution (41-42°C) was perfused after anastomosis in the HT group. Drains were taken out when drainage was ceased and drain holes were closed. Rats in the control group were received laparotomy, cutting of the colon and anastomosis. The animals were returned their cages after experiment and they were kept in an

ambient temperature of 22°C. They were fed with a standard rat diet for a period of 6 days. At the 7<sup>th</sup> postoperative day, the rats were sacrificed in ether tank. Anastomotic segment resected with 1.5 cm proximal and the distal margins of the colon. Bursting pressures of the anastomotic segments were measured with a stable flow-pump (Perfusor E Braun, Melsunger AG, Germany) and a mercury manometer (27). Hydroxyproline measurement in the anastomotic line was done with the method first described by Bergman and Loxley (28). Tissue samples obtained from anastomotic line were fixed with 10% formaldehyde and embedded in paraffin. Sections processed with hematoxylin-eosin dye for light microscopy were examined. Fibroblastic activity, inflammatory cell infiltration, collagen quantity, neo-vascularization and necrosis/ulceration were determined and graded between scores 0 to 4 in the histopathologic examination (29). Also tissue healing index of the groups was determined (29).

Animals died within 7 day after experiments were rated to initially operated twenty rats in each group for mortality rate estimation. Animals with macroscopic anastomotic leak or gas leak determined during the bursting pressure measurement were excluded from bursting pressure and tissue hydroxyproline data. Instead of the excluded animals, same procedures were repeated to new animals for completion of twenty rats in each group. Animals showed upper and lower bursting pressure and tissue hydroxyproline values were excluded from the statistical analysis. Thus, values obtained from eighteen rats in each group were used in statistical analyses for bursting pressure and tissue hydroxyproline levels.

### Statistical analysis

Histological values are expressed as median (minimum-maximum) and values obtained from bursting pressure measurements and tissue hydroxyproline measurements are expressed as mean  $\pm$  SEM. Values were analyzed using Kruskal-Wallis test. If differences were found to be significant, Mann-Whitney-U test is used to compare the groups. Mortality rates of each group were compared with others using a Fisher's exact test. P values less than 0.05 were considered significant.

## RESULTS

Mortality rates for each group were given in Table 1. Anastomotic leak and secondary abdominal sepsis was found to be a leading cause of mortality. Only two animals (one in control group, one in HIMMC group) were died during the induction of anesthesia. Mortality rates in HIMMC group and RT+ HIMMC group were significantly different compared to other groups ( $p<0.05$ ). Anastomotic leak determined in the initial twenty animals per group were shown in Table 2. Except died animals before bursting pressure me-

**Table 1—** Mortality Rates Within The Initial Twenty Animals in Each Group

Group	Number of Dead Animals	%
Control	3	15
HT	1	5
HIMMC*	12	60
RT	3	15
RT+HIMMC*	9	45

\*Statistically significant difference compared to control, HT and RT groups ( $P<0.05$ ). HT; hyperthermic perfusion, HIMMC; hyperthermic intraperitoneal mitomycin C perfusion, RT; preoperative radiotherapy.

**Table 2—** Anastomotic Leak Rates Within the Initial Twenty Animals in Each Group

Group	Number of Anastomotic Leaks	%
Control	2	10
HT	1	5
HIMMC*	12	60
RT	3	15
RT+ HIMMC*	10	50

\*Statistically significant difference compared to control, HT and RT groups ( $P<0.05$ ). HT; hyperthermic perfusion, HIMMC; hyperthermic intraperitoneal mitomycin C perfusion, RT; preoperative radiotherapy.

**Table 3—** Mean Levels of Bursting Pressures (Mean  $\pm$  Standard Deviation)

Groups	Bursting Pressures (mmHg)
Control	219.44 $\pm$ 35.00
HT NS	200.22 $\pm$ 20.33
HIMMC*	186.33 $\pm$ 37.18
RT	178.33 $\pm$ 22.62
RT+HIMMC	149.00. $\pm$ 37.80

NS, no significant difference between control group.

Significant difference between control group,  $P=0.02$ .

Significant difference between control group,  $P=0.01$ .

Significant difference between control group,  $P=0.001$ .

HT;-hyperthermic perfusion, HIMMC; hyperthermic intraperitoneal mitomycin C perfusion, RT; preoperative radiotherapy.

asurement, anastomotic leak was determined one in HIMMC group, one in RT+HIMMC group during the measurement. Anastomotic leak rate was increased significantly in HIMMC and RT+ HIMMC groups compared to others ( $p<0.05$ ).

### Bursting pressures

Bursting pressure values for each group (mean  $\pm$  standard deviation) were given in Table 3. Bursts were occurred in the anastomotic lines in every animal. Both radiotherapy and HIMMC treatment were decreased bursting pressures of anastomoses significantly compared to control group ( $p=0.01$ ,  $p=0.02$ ). Combination of these treatment modalities (RT+HIMMC group) was affected bursting pressures

**Table 4—** Tissue Hydroxyproline Levels from The Anastomosis Site (Mean  $\pm$  Standard Deviation)

Group	Mean Tissue Hydroxyproline Levels (mcg/mg)
Control	2.27 $\pm$ 0.59
HT*	3.38 $\pm$ 0.67
HIMMC	2.48 $\pm$ 0.66
RT†	2.74 $\pm$ 0.65
RT+HIMMC	2.17 $\pm$ 0.63

\*Significant differences were found compared to others ( $P<0.05$ ).

†Significant differences were found with control, HT and RT+HIMMC comparisons ( $P<0.05$ ).

HT; hyperthermic perfusion, HIMMC; hyperthermic intraperitoneal mitomycin C perfusion, RT; preoperative radiotherapy.

adversely ( $p=0.001$ ). Hyperthermic saline perfusion was not influence significantly to the anastomotic line pressure measurement ( $p=0.1$ ).

#### Hydroxyproline levels in the anastomotic line

Hydroxyproline values expressed as mean  $\pm$  SEM for groups were given in Table 4. The lowest hydroxyproline levels were found in RT+HIMMC group and the highest values were determined in the HT group. Significant differences could be observed between control and HT groups ( $p=0.0001$ ), control and RT groups ( $p=0.003$ ).

#### Histopathology

Values obtained from microscopic examination (median, minimum-maximum) were given in Table 5. Animals in the control group had best values of tissue healing except low collagen levels. Also, tissue healing index (fibroblastic activity + inflammatory cell infiltration + tissue collagen level + neo-vascularization/necrosis-ulcer) was determined highest in the control group. Despite highest collagen levels determined in RT group, tissue healing index was found lower than the others. HT, HIMMC and RT+HIMMC groups were followed to control group decreasing proportion for tissue healing index.

## DISCUSSION

Heating of cells to 42 to 45°C leads to inhibition of DNA, RNA and protein synthesis (30). In addition, hyperthermia improves lysosomal activity with cytoplasmic and nuclear destruction which is shown exaggerated in malignant cells (31). Later, hyperthermia is found to be effective for the treatment of carcinoma when applied concomitantly with radiotherapy and chemotherapy (32). In the earliest studies, hyperthermia applied externally or intraluminally in combination with radiotherapy and/or chemotherapy in colorectal cancer treatment (33, 34). With the excellent works of Fujimoto and his colleagues, hyperthermic intraperitoneal perfusion is placed in the oncology nomenclature (35-37). Mitomycin C, a well known alkylating agent, is extensively studied in hyperthermic chemotherapy trials (8-13,20-22,35-37). The cytotoxic interaction of the MMC is appeared significantly greater in hypoxic cells under hyperthermic conditions (38). Reported rates of anastomotic complications after hyperthermic MMC treatment are varied in clinical studies. The characteristics of patients and complexity of the treatment modalities are affected healthy determinations negatively. Dependent to our results, hyperthermic (41-42°) saline perfusion did not affect tissue healing capacity negatively. In addition, highest level of tissue hydroxyproline content was determined in hyperthermic perfusion group. When the MMC was administered intraperitoneally in the hyperthermic conditions, highest anastomotic leak rate and mortality were observed. It was suggested that traumatized and hypoxic cells located close to the anastomotic line were seemed to be affected negatively from hyperthermic MMC treatment.

With the preoperative external beam radiation therapy, tumor shrinkage or ablation, decrease in metastatic lymph node numbers, decrease in recurrence rates and, in many studies, survival advantages are well demonstrated in patients with colorectal carcinoma (15, 39). However, negative effects of radiation on healing process of the anastomosis are reported in the literature (23-25,40,41). Cumulative radiation dosage, administration period and radiotherapy-operation interval are prominent factors on the anastomotic healing. In this study, single dose, preoperati-

**Table 5—** Microscopic Examination of The Anastomotic Lines (Median, Minimum-Maximum)

Group	Fibroblast Activity	Inflammatory Cell Infiltration	Collagen Levels	Neovascularization	Necrosis/Ulcer
Control	3.15 (2.81-3.32)	2.70 (2.53-3.1)	1.30 (0.73-1.57)	3.40 (2.95-3.51)	1.20 (0.92-1.39)
HT	2.70 (2.45-2.92)	2.40 (1.9-2.63)	1.90 (1.52-2.26)	2.60 (2.33-2.78)	1.60 (1.35-1.88)
HIMMC	2.60 (2.27-2.79)	1.65 (1.37-1.78)	0.90 (0.28-1.21)	2.70 (2.41-2.9)	1.90 (1.67-2.03)
RT	2.45 (2.15-2.65)	1.30 (0.71-1.62)	2.30 (1.82-2.51)	2.50 (2.22-2.68)	2.50 (2.23-2.68)
RT+ HIMMC	2.80 (2.63-3.07)	1.65 (1.42-1.76)	1.10 (0.69-1.33)	2.85 (2.6-2.95)	1.95 (1.63-2.18)

HT; hyperthermic perfusion, HIMMC; hyperthermic intraperitoneal mitomycin C perfusion, RT; preoperative radiotherapy.

ve radiotherapy to the both ends of anastomosis was showed unfavorable anastomotic strength. Milsom and his colleagues are demonstrated reduction in colorectal anastomotic blood flow using with laser doppler velocimetry after preoperative radiation (23). We also noted a significant reduction in neo-vascularization in the radiotherapy group. In contrast to previous reports about increased inflammatory reaction in the perianastomotic area after radiotherapy, we found reduction in the inflammatory reaction close to the anastomotic area (23, 42). Despite, determined highest collagen levels in RT group, tissue healing index found to be lowest in animals undergone preoperative radiotherapy. Additive effects of preoperative irradiation and postoperative chemotherapy on the colorectal carcinoma treatment are well established in the literature (43-47). However, anastomotic healing processes under chemo-radiotherapy regimen are under investigated. 5-Fluorouracil is considered one of the most effective single agent using in the chemotherapeutic regimen. In the study performed by Biert and his colleagues, colonic anastomotic healing was investigated in the rats exposed to postoperative intraperitoneal 5-FU and preoperative radiotherapy (26). Combination therapy found to be unaffected anastomotic healing process. However, authors were concluded increasing frequency of rectal stenosis with high dose intraperitoneal 5-FU plus preoperative radiotherapy group. In our study, combination of preoperative radiotherapy and hyperthermic MMC treatment was impaired anastomotic strength significantly. Lowest values of bursting pressure measurement and tissue hydroxyproline levels were suggested that additive negative effects of both therapies. But, additive toxicity did not detect in tissue healing parameters. These effects explained with increased MMC activity in the hypoxic conditions triggered by radiotherapy.

In conclusion, our data show that intraperitoneal MMC treatment in a hyperthermic condition and its combination with preoperative external radiotherapy lead to detrimental effects on anastomotic strength. The possible late side effects of HIMMC with or without radiotherapy treatment is not examined in this study, it should be subject of further investigation.

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