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RESEARCH ARTICLE

COMPARISON OF EFFICACY OF HYPERTHERMIC INTRAPERITONEAL CHEMOTHERAPEUTICS MITOMYCIN C VERSUS OXALIPLATIN IN EXPERIMENTAL COLORECTAL PERITONEAL METASTASIS MODEL

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ABSTRACT

Aim: Our study aimed to establish a mouse model with colorectal cancer-induced peritoneal metastasis(PM) and to compare the efficacy of hyperthermic intraperitoneal chemotherapeutic agents, mitomycin C and oxaliplatin.
Materials and Methods: The peritoneal metastasis model was established in nude mice using the CC531 colon carcinoma cell line. Models with PM were randomized into four groups of seven animals each: Group-1, control group; Group-2, hyperthermic intraperitoneal chemotherapy(HIPEC) with mitomycin C(MMC), and Group-3, HIPEC with Oxaliplatin(OXA).
Results: Tumor development was achieved in all animals. While the tumor burden decreased significantly in the treatment Group-2(p=.013). In the PM mouse model, hyperthermic intraperitoneal administration of MMC had a higher tumoricidal effect than hyperthermic intraperitoneal administration of OXA.
Conclusions: Our PM model provided a good opportunity to examine the efficacy of HIPEC and IPIP. Hyperethermic intraperitoneal mitomycin applied in the colorectal PM animal model was found to have higher tumoricidal activity than oxaliplatin. In future studies, we plan to evaluate efficacies of different drugs in the PM models we have created.

Keywords: peritoneal metastasis model, colorectal cancer, HIPEC

INTRODUCTION

According to the data of the World Health Organization, colorectal cancer is the third most common cancer in both genders in Turkey [1]. 25 % of recurrences in colorectal cancer(CRC) patients are located in the peritoneum [2-4]. Approximately 5% of CRC patients are simultaneously diagnosed with peritoneal metastases (PM) at the time of initial diagnosis. During the course of their disease, PM develop in 2-19% of them [5-8]. While an average of

one year survival can be achieved with systemic chemotherapy in patients with peritoneal metastases of CRC, 5-year survival rates can reach 40-58% with cytoreductive surgery (CRS) and hyperthermic intraperitoneal chemotherapy (HIPEC) [9]. Therefore, it is recommended that CRS&HIPEC be performed as standard treatment in selected patients [10]. Although they are more successful when compared with systemic chemotherapy, there is not enough scientific evidence about CRS&HIPEC. Although hyperthermia per se has a cytotoxic effect on cancer cells, CRS&HIPEC potentiate each other's effects with chemotherapy[11]. When compared with systemic chemotherapy, intraperitoneal administration of chemotherapy provides a more intense concentration of chemotherapeutic agents on tumor cells with lower systemic toxicity[12]. Because of all these effects, when HIPEC is applied, 20-50 times more tumoricidal effect occurs compared to systemic chemotherapy [13].

In our study, we planned to create a PM model in athymic mice by using CC-531 (colon adenocarcinoma cell line) and to administer intraperitoneal chemotherapy using the infusion pump we developed in this model. We compared the efficacy of mitomycin C versus oxaliplatin, which we use as hyperthermic intraperitoneal chemotherapeutic agents in patients with colorectal PM.

MATERIAL AND METHODS

Our study was conducted at Dokuz Eylül University Faculty of Medicine Experimental Animals Laboratory (DEUFMEAL) between January and July 2022, with the approval of the Dokuz Eylul University Multidisciplinary Laboratory Animal Experiments Local Ethics Committee (Date: 23.01.2018, Decision No: 03/2018). In the process of establishing the peritoneal metastasis model, 7-8 week- old 21 male athymic nude mice bred by Experimental Animals Laboratory were used. Nude mice being caged in groups of seven under laboratory conditions in air-filtered laminar flow cabinets were monitored. Mice were fed with irradiated food and autoclaved reverse osmosis treated water, and all treatments were carried out under sterile conditions in a laminar flow hood.

İntraperitoneal Tumor cell inoculation: Cancer cells from the CC531 colon adenocarcinoma cell line were harvested during the logarithmic growth stage by incubating them at 37 °C under a humidified 5% CO_2 atmosphere. Cells were then resuspended in phosphate- buffered saline (PBS) for intraperitoneal injection. By providing the necessary sterilization in the laminar flow hood, suspended cells were given by intraperitoneal (IP) injection using a 16 mm long and 0.45 mm diameter needle.The amount to be injected into all groups was determined as $5x10^6$ cells, 0.3cc, in 200µl PBS, taking the previous studies as an example [11]. We detected development of distension and palpable nodular lesions due to the formation of intraabdominal ascites between the 7th and 10th days in the subjects who were checked daily by inspection and palpation starting from the 5th day. After the presence of tumor was detected (day 10), the subjects were divided into three groups [Group-1(G-1), Control Group, (0.9% NaCl), Group-2(G-2), hyperthermic chemotherapy with mitomycin C(MMC); Group-3(G-3), hyperthermic chemotherapy with Oxaliplatin(OXA) groups (Table 1).

Surgical Intervention and HIPEC Procedure: Athymic nude mice were weighed before administration of anesthesia.Their mean weight was 34 ± 2g . Diethyl ether inhalation anesthesia was applied. After anesthesia; the abdominal skin was cleaned with povidone-iodine. Necessary sterilization conditions were provided by covering the mouse with sterile covers. A midline abdominal incision of approximately 1 cm was made and the abdomen was entered. Peritoneal metastases were found. After the inlet and outlet catheters of the intraperitoneal infusion pump (IPIP) were placed lateral to the abdomen, the midline incision was closed primarily with 4/0 prolene sutures (Figure-1). Then, different chemotherapeutic agents were infused under hyperthermic (41ºC) (mitomycin C 20mg/m², oxaliplatin 100mg/m²) conditions for 45 minutes to

Table 1. Groups and intraperitoneal treatment procedures.

GROUPS	*Intraperitoneal Treatment Procedure*
Group I *(Control)*	Normothermic (37º) 0.9%NaCl
Group II	Hyperthermic (41º) MMC
Group III	Hyperthermic (41º) OXA

MMC: Mitomycin C, OXA: Oxaliplatin

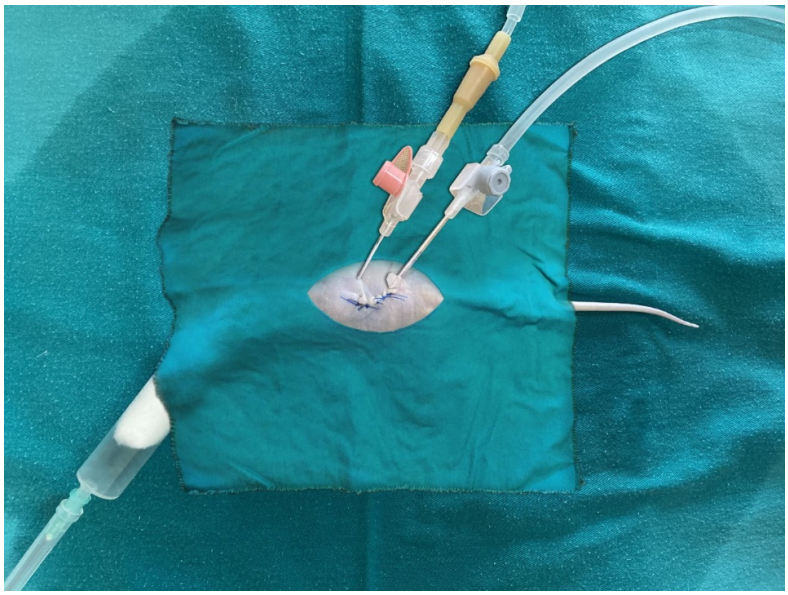

Figure 1. Preparation of model before the application of intraperitoneal chemotherapy, placement of the catheters.

the previously determined groups. This process was developed by us as a prototype and was carried out with IPIP (Figure-2). Intraoperatively, the required tissue temperature was reached within 4-6 minutes in the HIPEC group. Steady temperatures were then maintained for an additional 45 minutes with an average of 40.5 ± 0.5°C, and approximately 5 ml of the solution was required to fill the abdomen. Using the thermostat of the device, the temperature of the fluid given and instilled into the abdomen was controlled and the temperature was kept constant. Chemotherapeutic agents were given as MMC (20 mg/m²), and OXA (100mg/m²) prepared in 30cc 0.9% NaCl and 5% glucose solution served as carrier. This process was done in a laboratory environment, taking safety precautions. Body surfaces area was estimated usin Meeh's with an empirical Meeh constant of *k=9.6*[14]. formula of mice were calculated in square meters (m^2) using the formula: ($A(m^2) = k \times W^{2/3}/100$). Intraperitoneal perfusion was maintained for 45 minutes. After the procedure was completed, the perfusion cannulas were taken out of the abdomen.The liquid boiler of the system was sterilized after each operation.All infusion tubing and cannulas were changed after each procedure. Sterilization was provided under optimal conditions for each mouse.

Intraperitoneal Infusion Pump (IPIP): It consists of intraperitoneal infusion pump (IPIP), 1000 milliliter

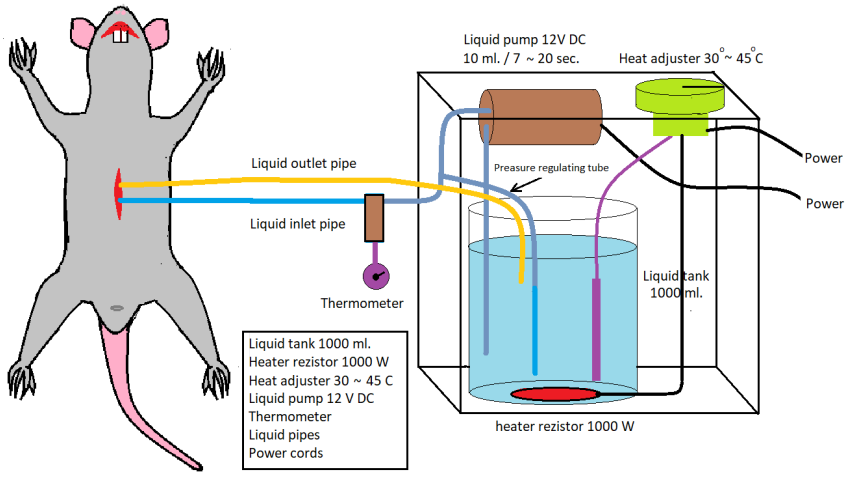

Figure 2. Intraperitoneal infusion pump system working diagram.

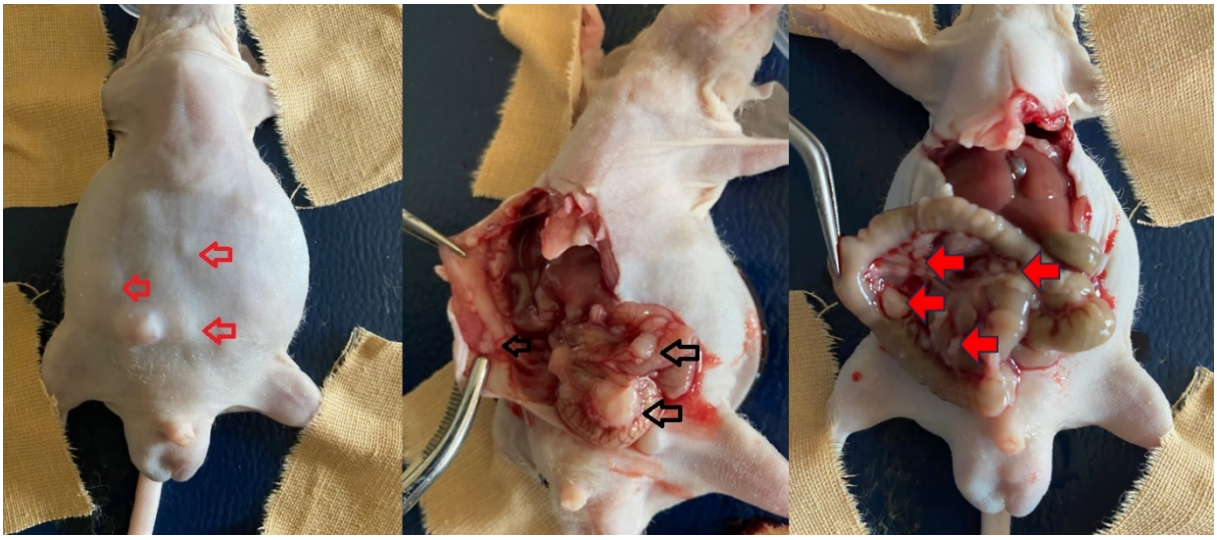

Figure 3. Determination of peritoneal carcinomatosis index in animals, acid amount measurement.

liquid tank, liquid pump, thermostat that can control the range of temperature between 30-45°C, heater resistance (1000W), liquid temperature control probe, liquid flow and collection cannulas, liquid pressure adjustment cannula designed and developed by us. The temperature of the fluid coming from the fluid outlet cannula and the inside of the abdomen passes through the second control cannula and a constant temperature is provided. The flow rate of the liquid pump can be adjusted. Intraperitoneal chemotherapy can be applied to CPC models created in different types with the IPIP system we have developed.

Follow-up, Sacrification and Evaluation of Subjects: Subjects undergoing daily follow-ups were evaluated by performing laparotomy 5 days after intraperitoneal infusion of chemotherapeutic agents. Peritoneal cancer index (PCI) was determined and scoring was done. Scoring was done considering the involved organ and tumor diameter, and evaluated out of 8 points as follows: small bowel and/or mesenteric involvement: 1 point; peritoneal involvement: 1 point; diaphragmatic involvement: 1 point, ascites (+): 1 point; involvement of other organs: 1 point. Tumor diameters were measured and scored as follows: 0 : no tumor growth; 1 point:nodule diameter ≤ 2 mm; 2 points: nodule diameter 2-5mm or > 5 tumor nodules; 3 points: nodule diameter ≥ 5mm or >10 tumor nodules (Figure-3). Ascitic fluid was aspirated, and its quantification was carried out. Small intestine, peritoneum, intraabdominal fluid and blood samples were taken and the subjects were sacrificed.Tissue and intraabdominal fluid samples were then evaluated histopathologically and biochemically. Tissue samples were fixed in 10% formaldehyde, cassetted, and embedded in a paraffin block after tissue follow-up. Frozen sections of 5 µm thickness were obtained from the optimum section surface. Sections were then stained with hematoxylin and eosin (H&E) and examined under Olympus X50 light microscope.Tissues were evaluated for the presence of tumor, tumoral pattern, differentiation, apoptosis, mitosis and necrosis.Evaluation was made by calculating the total number of mitoses in 10 different tumor areas by magnifying the field of vision 400 times under a 40X objective of a light microscope.The number of apoptosis was calculated by evaluating 5000 cells and determining its percentage in 1000 cells. Tissue samples were evaluated for tumor necrosis. The intensity and expression levels of lymphatic vessel endothelial hyaluronan receptor-1(LYVE-1), Angiopoietin-1(Ang-1) and Angiopoietin-2(Ang-2) in cancerous tissue were examined by immunohistochemical methods. Supernatants remaining after centrifugation of intraabdominal fluid samples of the mice were studied using Lysyl Oxidase-like protein 1(LOXL1) and TWIST Transcription factor (TWIST) mouse compatible ELISA kits.Vascular endothelial growth factor(VEGF) levels were studied by diluting the samples in fluids, taking into account the mouse-compatible ELISA kit application steps. According to the absorbance values obtained from the standards, standard graphs of each test were created. Concentrations were expressed by calculating the absorbances obtained from the samples. The

Table 2. Group II mean PCI, tumor diameter and amount of ascites were significantly less than the other groups (Kruskal-Wallis test, T-test)

Mean(std±) *Median(25-75th)*	**G-I**	**G-II**	**G-III**	
PCI	6.71(±1.38) 7(6-8)	4.42(±0.53) 4(4-5)	6.28(±1.60) 7(6-7)	*.013*
Tumor diameter(mm)	5.85(±3.28) 5(4-8)	2.42(±0.53) 2(2-3)	5.71(±1.79) 5(4-7)	*.001*
Ascites(ml)	4(±1.93) 4.5(3-5)	1.28(±1.77) 0(0-2.5)	3.57(±1.74) 4(3-5)	*.032*

PCI: peritoneal cancer index, std: standard deviation.

Table 3. The mean of tumor tissues in the groups; mitosis counts, apoptosis counts and tumor necrosis rates(Kruskal-Wallis Test, T-Test, p=.003, .008, .015).

Mean(std±) *Median(25-75th)*	**Mitosis count(40X)**	**Apoptosis count/1000cell**	**Tumor Necrosis (+) Subject Ratio in Groups (%)**
G I *(Control)*	12.714(std 1.79) 12(12-15)	5.42(std 2.29) 5(4-6)	14.2
GII *(Hyperthermic MMC)*	5.857(std 1.34) 6(5-7)	131.42(std 48.79) 130(80-180)	100
GIII *(Hyperthermic OXA)*	9.000(std1.82) 8(8-11)	65.71(std 23.70) 60(50-80)	42.8
	.003	***.008***	***.015***

MMC: Mitomycin C, OXA: Oxaliplatin, std: standard deviation.

measuring range of the LOXL1 ELISA kit was 78-5000 pg/mL and the measurement sensitivity of the test kit was 29 pg/ml.The measuring range of the kit for the TWIST test was 0.156-10 ng/mL, and the measurement sensitivity of the test kit was 0.056 ng/mL.The measuring range of the kit for the VEGF test was 15-1000 pg/mL, and the measurement sensitivity of the test kit was 9.375 pg/mL.

Statistical analysis: Before the study, the number of subjects was determined by power analysis.The maximum number of animals allowed by the animal experimentation ethics committee was used to obtain statistically significant results. Statistical analysis was performed using IBM SPSS 24.0 statistics. The significance of differences was assessed by the Kruskal-Wallis-test. Continuous variables were compared by independent samples t-test. Descriptive statistics were presented in median (25-75th percentile) format.Fisher exact chi-square test and T-test were used in the analysis of qualitative data, and descriptive statistics were shown in the form of frequency. P values <0.05 were defined as statistically significant.

RESULTS

There was no mortality in the postoperative period in the mice that received intraperitoneal chemotherapy**.** Mild side effects (anorexia and lethargy) were observed in five animals in the group given hyperthermic chemotherapy which disappeared within two days (G-2 n=2, G-3 n=3). Although minimal dehiscence was observed in the incision line in seven animals, no infection or wound dehiscence, which would cause mortality, was observed until the sacrification process.

Macroscopic findings: When the peritoneal cancer index (PCI) was compared between the groups, G-2 had the lowest mean and statistically significant PCI (p=.013) value [4.42±0.53)]. When tumor diameters and amount of ascites were compared between groups, G-2 again had the lowest, and statistically significant values [2.42±0.53 mm vs 1.28±1.77 ml, and p=.001 vs p=.032, respectively]. However, in our

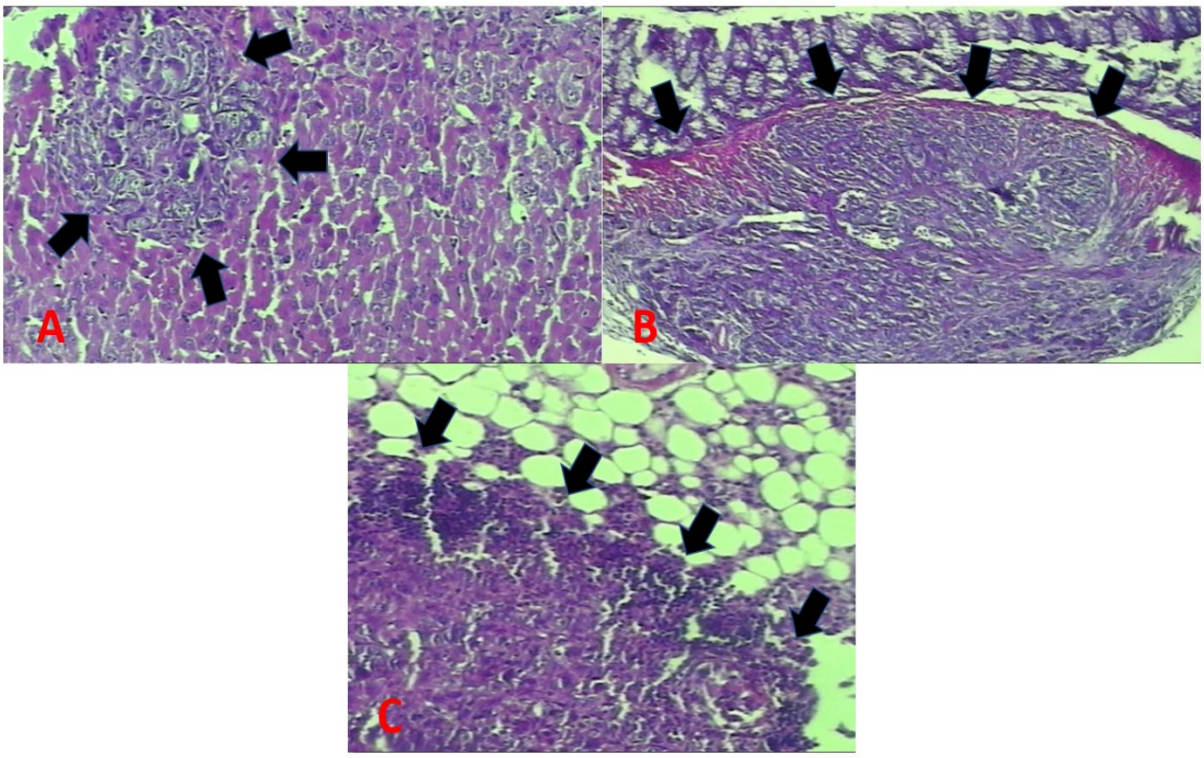

Figure 4. Group 1 (control) tumor images; A: Nodular tumor implanted in the liver, B: Tumor implantation in the intestinal wall, C: Tumor infiltrating the peritoneal adipose tissue.

study, no significant difference was found between the groups in terms of these parameters, except for G-2(Table 2).

Microscopic findings: When the tissue samples obtained from the intestinal system, peritoneum, liver after sacrification were evaluated under microscope, tumor cell infiltration was observed in all tissues.The tumor was found to be nodular and undifferentiated.When the groups were compared in terms of number of mitotic, and apoptotic cells and tumor necrosis, statistically significant intergroup differences were found ($p<0.05$). In G-2, the number of apoptotic cells and areas of tumor necrosis were found to be statistically significantly higher than the other groups ($p=.008$, $p=.015$). The number of mitosis was found to be significantly lower than the other groups. ($p=.003$) (Table 3) (Figures 4 and 5).

Biochemical findings:The mean values of VEGF, LOX1 and TWIST in the intraabdominal ascites fluid

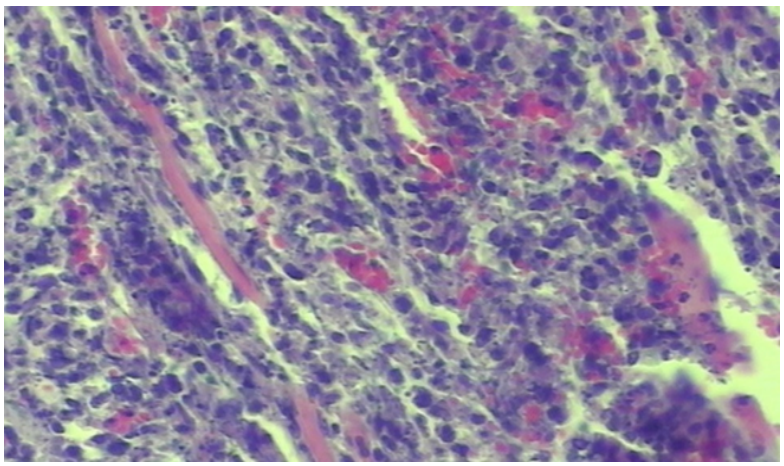

Figure 5. Group II (Hyperthermic MMC) tumor images; areas of increased apoptosis and tumor necrosis in tumor cells.

Table 4. VEGF, LOX1 ve TWIST values in intra-abdominal fluid (Kruskal-Wallis Test, T-Test, p=0.004, 0.037, 0.012).

	VEGF Mean(std±) Median(25-75th)	LOX1 Mean(std±) Median(25-75th)	TWIST Mean(std±) Median(25-75th)
GI	274.625 (std±192.498) 267 (165-314)	552.142 (std±326.622) 525 (415-745)	1.416 (std±1.164) 1.13 (1.05-1.49)
GII	17.960 (std±24.728) 0(0-31)	225.000 (std±308.801) 0 (0-430)	0.254(std±0.407) 0 (0-0.83)
GIII	150.782 (std±97.396) 149 (107-235)	553.571 (std±204.587) 540 (320-800)	1.799 (std±1.328) 2.18 (0-2.96)
	.004	**.037**	**.012**

VEGF:Vascular Endothelial cell Growth Factor, LOX1: Lysyl Oxidase Like Protein-1, TWIST: Twist Transcription Factor, std: standard deviation

were found to be statistically significantly lower in G-2 compared to the other groups [VEGF; 17.960±24.728 pg/mL, LOX1; 225.000 ±308.801 pg/mL, TWIST; 0.254 ±0.407 ng/mL (p=.004, .037, and .012, respectively)]. No significant difference was found between the other groups in terms of these parameters (Table 4).

Immunohistochemical Findings: Tumors from all groups had LYVE-1 positivity. The strong positivity rate in the control group and group III were found to be statistically significantly higher than G-2(p<.001, p=.031). There was no significant difference between the groups in terms of Ang-1 staining intensities. Ang-2 was found to be significantly stronger positive in the control group compared to the groups that received intraperitoneal chemotherapy (p=0.002).

DISCUSSION

Hyperthermic intraperitoneal chemotherapy (HIPEC) with mitomycin C has been applied following cytoreductive surgery for various peritoneal surface malignancies. Spratt et al. first performed HIPEC in a patient with pseudomyxoma peritonei [15]. A significant survival benefit has been shown for HIPEC when compared to systemic chemotherapy alone [16,17]. The complete cytoreductive surgery is the most important prognostic factor. Incomplete cytoreduction results in limited survival [18,19].

The drugs used in the HIPEC procedure have a limited depth of penetration. For this reason, HIPEC is applied in patients whose macroscopic tumor burden was eliminated or minimal residual tumor remained following radical cytoreductive surgery [20]. Therefore, tumor cells can be implanted into an intraperitoneal fat pad to simulate cytoreductive surgery as described by Veenhuizen et al.[21]. Using this technique, the spread of tumor implants is limited. This simulates an abdomen that has undergone cytoreductive surgery and has a reduced tumor burden. In our study, we provided widespread implantation of tumor cells by injecting tumor cells into the intraperitoneal cavity. Diffuse peritoneal implants formed in all subjects within 7-10 days. In many studies, and tumors are produced by intraperitoneal injection [22,23]. The widespread creation of peritoneal implants made it easier for us to determine the macroscopic PCI score. Apart from this, we think that this approach enables us to better detect the differences in efficacies of different drugs administered to the groups. In some studies, the tumor formation rate after intraperitoneal tumor transplantation was reported as 80% [24,25], while tumor formation rate of 100% was reported in a study where tumor cells were implanted in an intraperitoneal fat pad [21].In our study, tumor formation was observed at a rate of 100% after intraperitoneal inoculation.

We have seen that with the IPIP system we developed, HIPEC can be performed effectively in the athymic mouse PM model. There was no loss of subjects during and after perfusion. Animals were observed for 5 days after administration of intraperitoneal chemotherapy. No serious complications were observed. Mild side effects (anorexia and lethargy) were observed in four animals in the group given only hyperthermic chemotherapy. All these side effects disappeared within two days. Late-term effects, morbidities, and effects of sacrification on the 5th day could not be fully evaluated. Basically, HIPEC is a proven procedure with cytoreductive surgery. However, in our study, only HIPEC was applied since it was not appropriate

to perform cytoreductive surgery on the model. In this case, the administered chemotherapeutic drugs demonstrated limited effectiveness.

It is difficult to achieve homogeneous distribution of temperature, and cytotoxic drugs, but it is crucial for ensuring the tumoricidal efficacy of this procedure. For this reason, an open abdomen approach can be chosen to ensure homogeneous temperature and drug distribution [11]. The biggest disadvantage of this method is exposure to cytotoxic drugs. In our study, after perfusion catheters were placed, the abdominal wall was closed and then peritoneal infusion was started. Optimal intraperitoneal circulation was ensured by continuous temperature control and adjustment of the infusion rate in the fluid outflow and inflow catheters, and the desired temperature and homogeneous drug distribution were maintained.The closed abdomen facilitated the control and maintenance of the same drug temperature. After the treatment period was completed, the cannulas were withdrawn and the procedure was terminated.

In the study of Liesenfeld et al.[26] on dose-related side effects and mortality in the mouse model, the doses of mitomycin and oxaliplatin with the lowest loss of subjects were determined. In our study, we applied the same doses in this study Liesenfeld et al.[26] by calculating MMC as 20mg/m² and OXA as 100mg/m². In the postoperative follow-up, mild side effects (anorexia and lethargy) were observed in five mice in the group that hyperthermic chemotherapy was given, which disappeared within two days. Mortality was not observed in the early period.

Oxaliplatin and Mitomycin C are the most commonly used chemotherapeutics as intraperitoneal agents in HIPEC[27,28]. The reason why oxaliplatin and MMC are suitable for intraperitoneal use is that due to the large molecular weight of the chemotherapeutics, they may undergo limited systemic absorption and reach high intraperitoneal concentrations. In this case, it increases their intraperitoneal activity[29]. In recent years, the use of oxaliplatin as a HIPEC regimen in patients with colorectal PM has become increasingly popular[30]. Studies have been conducted comparing MMC, which is commonly used in PM patients with colorectal cancer, and oxaliplatin [31-34]. In the study by Zhang X.[31] et al., no significant difference was found in terms of survival between patients who underwent cytoreductive surgery and hyperthermic oxaliplatin and those who underwent MMC in colorectal PM patients. However, the rate of major complications (bleeding, renal toxicity, hepatic toxicity, neurotoxicity) was higher in the group receiving Oxaliplatin. In our study, no significant difference was found in terms of complications in the two groups until the sacrification process, which is the 5th day. In general, the perfusion time is about 30 minutes for oxaliplatin, which is significantly shorter than that of MMC (60-90 minutes) [32]. This is one of the reasons why it has been preferred in recent years. In our study, however, we applied HIPEC for equal duration (45 minutes) to both groups in order to avoid inconsistency in the results, since their efficacy may vary depending on the duration. Leung V.[33], et al. reported that oxaliplatin provides a better overall survival advantage than MMC in colorectal PM patients. In the study by Eden W. J. V.[34] et al., it was determined that the survival times were significantly longer in the patient group given oxaliplatin compared to the patient group given MMC. When the two groups were compared in terms of postoperative complications, no significant difference was found. When these studies are examined, it is seen that oxaliplatin given for a shorter time comes to the fore. However, the cytoreductive surgery technique applied to the patients, the different drug doses and durations applied in the HIPEC procedure may affect these results. In the study of Delhorme J P[35] et al., it was determined that disease-free survival in patients with colorectal peritoneal metastasis was significantly higher in the group that underwent MMC. In the study of Villaverde A P[36] et al., it was shown that the median overall survival was significantly longer in the MMC group. In the study of Woeste M R. [37] et al. it is reported that hyperthermic intraperitoneal administration of MMC or Oxaliplatin together with an effective cytoreductive surgery is a safe and effective treatment in colorectal PM patients. They argued that both perfusion treatments should be considered in all patients receiving modern induction chemotherapy. In the PM animal model study conducted by Raue W.[38] and his colleagues, MMC was applied hypertemic intraperitoneally and it was shown to have high tumoricidal activity.

In our study, when we evaluate all the findings, macroscopic, microscopic, biochemical and immunohistochemical examination results jointly have shown that the strongest tumoricidal activity was achieved in the hyperthermic MMC group. However, no significant difference was found between the early complication rates. Long-term survival could not be

evaluated because it was an experimental study on an animal model.

Limitations of this study can be stated as small number of mice included in the nude mouse peritoneal carcinomatosis model, and very dificult application of chemotherapy procedure. The mice were followed up and sacrificed until the 5th postoperative day. Therefore, the long-term efficacy of the drugs and the late-term postoperative complications could not be evaluated. Due to the limited number of studies in this area, it was not possible to foresee the difficulties that may be encountered. Before this experiment, preliminary experimental studies were carried out in order to establish peritoneal carcinomatosis and to gain experience in IPIP procedure.

CONCLUSION

Hyperthermic intraperitoneal chemotherapy procedure can be applied in the created peritoneal carcinomatosis model and the results can be evaluated qualitatively and quantitatively. We compared the two most effective chemotherapeutic agents used in cytoreductive surgery and the HIPEC procedure in an animal model of colorectal peritoneal metastasis and found that tumoricidal activity was statistically significantly higher in the MMC group. In addition, we observed that there were tolerable side effects in two different groups that underwent hyperthermic intraperitoneal chemotherapy, and there was no difference between two groups in terms of postoperative complications. We also aim to further increase the efficiency of the HIPEC procedure and further reduce its side effects by conducting experimental in vitro and in vivo studies in the future.

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Author contribution: The concept, design, control of the study, data collection and/or processing was done by B.M. and A.E.C. B.M. and O.Y. created an animal model of peritoneal carcinomatosis and carried out the experimental study. S.A. performed histopathological examinations. Z. A. performed biochemical analyzes of intra-abdominal fluid samples. B.M. and T.Y. performed the statistical analysis with the data obtained. B.M. T.B. and A.E.C. wrote the main manuscript text and prepared figures and tables. Literature search was done by all authors. All authors reviewed the manuscript.

Conflict of interest: Authors declare that they have no conflict of interest.

Ethical Approval: The study was approved by Dokuz Eylul University Multidisciplinary Laboratory Animal Experiments Local Ethics Committee (Date: 23.01.2018, Decision No: 03/2018).

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