Effects of intraperitoneal administration of gemcitabine and paclitaxel on hepatic regeneration in rats

Gemsitabin ve paklitakselin intraperitoneal uygulamasının farelerdeki hepatik rejenerasyona etkileri

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Background/aims: *We aimed to test clinical implications of intra-peritoneally administered gemcitabine and paclitaxel on hepatic regeneration after hepatic resection in rats.* ***Methods:*** *Fifty male, Swiss albino rats weighing between 200 and 240 g were used. After a 30% partial hepatectomy was performed (except Sham group), animals were divided into five groups as: high-dose gemcitabine, low-dose gemcitabine, paclitaxel, control, and Sham operation groups. In the high-dose and low-dose gemcitabine groups, animals received 200 and 12.5 mg/kg intraperitoneal gemcitabine for five days after partial hepatectomy respectively. In the paclitaxel group, animals were administered 6mg/kg paclitaxel in the same fashion. Control and Sham groups received intraperitoneal 0.9% NaCl. On the sixth postoperative day, the animals were killed liver tissues were resected, proliferating cell nuclear antigen immunopositivity was determined and weight loss and diarrhea were assessed.* ***Results:*** *Gemcitabine and paclitaxel treated animals lost weight and had more severe diarrhea than control and Sham group animals. No significant difference was observed between treatment groups in terms of weight loss, diarrhea, and proliferating cell nuclear antigen. When treatment groups were compared to the control group in terms of proliferating cell nuclear antigen immunopositivity, no significant differences were detected.* ***Conclusions:*** *It can be concluded that adjuvant chemotherapy with gemcitabine and paclitaxel is a safe option in terms of liver regeneration and side effects such as diarrhea and weight loss.*

Key words: : Proliferating cell nuclear antigen, regeneration, chemotherapy, liver cancer.

Amaç: *Bu çalışmada hepatik rezeksiyon yapılan farelerde intraperitoneal olarak uygulanan gemsitabin ve paklitakselin hepatik rejenerasyona olan etkileri ve bunun klinik uygulanabilirliği araştırıldı.* ***Metod:*** *Ağırlıkları 200-240 arasında değişen erkek, Swiss albino fareleri kullanıldı. Denekler %30 parsiyel hepatektomi yapıldıktan sonra (sham grubu hariç) yüksek doz gemsitabin, düşük doz gemsitabin, paklitaksel, kontrol ve sham grubu olmak üzere beş gruba bölündü. Parsiyel hepatektomi yapıldıktan sonra, yüksek doz gemsitabin grubundaki farelere 200 mg/kg, düşük doz gemsitabin grubundaki farelere ise 12.5 mgl'kg gemsitabin beş gün boyunca intraperitoneal olarak uygulandı. Paklitaksel grubunda farelerede 6 mg l kg paklitaksel intraperitoneal olarak uygulandı. Kontrol ve Sham gruplarına intraperitoneal olarak %0.09 NaCl verildi. Fareler postoperatif 6. gün sakrifiye edildi. Karaciğer dokularında PCNA (proliferating cell nuclear antigen) ya bakıldı ve ayrıca kilo kaybı ve diyare gibi semptomlar da kaydedildi.* ***Sonuçlar:*** *Gemsitabin ve paklitaksel verilmiş farelerde, kontrol ve sham gruplarındakilere göre belirgin kilo kaybı ve şiddetli diyare gözlendi. Tedavi grupları arasında kilo kaybı, diyare ve PCNA düzeyleri arasında belirgin farklılık görülmedi. PCNA immunpozitifliği açısından da tedavi grupları ile kontrol grupları karşılaştırıldığında belirgin istatistiksel farklılık saptanmadı.* ***Tartışma:*** *Gemsitabin ve paklitaksel ile yapılan adjuvant kemoterapi karaciğer rejenerasyonu açısından güvenli bir seçenektir fakat diyare ve kilo kaybı gibi yan etkileri vardır.*

Anahtar kelimeler: PCNA, rejenerasyon, kemoterapi, karaciğer kanseri.

INTRODUCTION

Liver tumors are one of the leading causes of death in the western world, ranking seventh among tumors in males and ninth in females according to UICC statistics (1). Since hepatic resection is the only potential curative treatment modality in liver tumors, understanding the mechanism of proliferation of the liver cells following hepatectomy is crucial in treatment. It is not clear which factors are involved in cell proliferation during nodule formation and regeneration in liver parenchyma (2-5). Furthermore, several newer therapeutic agents, proposed to be helpful in adjuvant therapy of liver resections following hepatectomy, have appeared in recent years (6-14).

In third world countries, most liver tumors develop following hepatitis B and/or C infection and even a regular resection of the tumor may be a life-threatening intervention because of deteriorated liver regeneration and functional capacity. In case of resection, efforts to predict functional capacity and monitor regeneration of the remnant

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liver have revealed that a gold standard has yet to be determined (15).

Some authors reported better survival data using intraperitoneal normo-or hyper-thermic chemotherapy as an adjuvant treatment modality in abdominal tumors (16,21). Sugarbaker et al.(22,23) reported that while the intraperitoneal defense mechanism against tumor cells being released during resection was depressed in the early postoperative period, the adhesive capacity of tumor cells was increased. These two factors yield implantation of potential tumor cells on the peritoneal surface and peritoneal or local recurrences, prevention of which is the major objective of intraperitoneal administration of anti-tumoral agents as an adjuvant therapy. It also has the advantages of high drug concentrations locally and lack of adverse effects.

Gemcitabine and paclitaxel are two newer chemotherapeutic agents. It is expected that they will become major components of multi-modal therapy of abdominal tumors in the future.

The aim of the present study was to test clinical implications of these two intra-peritoneally administered anti-tumoral agents on hepatic regeneration in a rat model. The immunoreactivity patterns of proliferating cell nuclear antigen (PCNA) were examined to determine whether hepatic proliferation after partial hepatectomy was altered by intraperitoneally administered anti-cancer drugs. The PCNA is essential for cellular DNA synthesis and was originally defined as an intranuclear polymerase synthesized maximally during the S-phase of the cell cycle (1,4,20,24). This model mimics the early postoperative period of human hepatectomized patients and it would be helpful to assess the possible role of these agents in adjuvant chemotherapy following partial hepatectomy.

MATERIALS AND METHODS

Animals: Fifty male Swiss albino rats (Ege University Animal Research Laboratories, Izmir, Turkey) weighing between 200 and 240 g were maintained in standard cages in Dokuz Eylül University Animal Research Laboratories and had access to standard rat chow and water ad libitum. The room temperature and humidity were maintained at 25°C and 40%, respectively. All animals received human care in compliance with the Guide for the Care and Use of Laboratory Animals prepared by The National Academy of Sciences and published by the National Institute for Health (NIH publication No. 85-23 revised 1985). Under general ether anesthesia, proper antiseptic techniques were applied and 30% partial hepatectomy (PH) was performed in all animals except for the Sham group. Animals were divided into five groups as high-dose gemcitabine (HDG), low-dose gemcitabine (LOG), paclitaxel group (PG), control group (CG) and Sham operation group (SG). Each group consisted of 10 rats. While HDG animals received 200 mg/kg intraperitoneal gemcitabine (Gemzar®, Lilly Pharmaceuticals, Fegersheim, France) for five days starting just after PH, LOG group animals were administered 12.5 mg/kg gemcitabine in the same fashion. PG animals were administered 6 mg/kg intraperitoneal paclitaxel (Taxol®, Bristol-Myers-Squibb Company, Princeton, NJ, USA) for five days, while CG and SG received only 0.9% NaCl instead of antitumoral agent. The SG animals had undergone only a laparatomy and manipulation of liver. During these five days all animals were maintained in the same conditions as in the preoperative period and clinical conditions were recorded. The severity of diarrhea, the most common change in clinical condition was scored and scores were compared using non-parametric statistical test, Kruskal-Wallis one-way ANOVA on ranks test. All pairwise multiple comparisons were performed by Student-Newman-Keuls method. Body weights of all animals were also recorded at the end of the study and compared using one-way ANOVA test.

On the sixth postoperative day, all animals were killed and whole liver tissues were resected. Thin liver slices (5 mm) were put into a 10% buffered neutral formalin solution 24 hours before processing and embedding in paraffin wax.

Light Microscopy: The paraffin blocks were cut into 5m sections which were stained with hematoxyline-eosin. Hepatocellular proliferation was signified by mitoses, thickening of the hepatocyte cords, or some disorganization of the paranchymal structure. Hot spot areas with the highest hepatocellular regeneration were selected and marked for immunohistochemical evaluation.

Immunohistochemistry: The blocks were sectioned on poly-lysin-coated slides. The avidin-biotin-peroxidase method was performed using the primary monoclonal antibody against PCNA protein (1:100, DAKO Corp, Carpinteria, CA, USA).

Briefly, the sections were deparaffinized in xylene, rehydrated and immersed in distilled water; endogenous peroxidase activity was blocked using a 0.3% solution of hydrogen peroxidase in phosphate-buffered saline (PBS), 0.01 mol/L, ph 7.5. After antigen retrieval by heating in 10 mmol/L citrate buffer (pH 6.0), the primary antibodies were applied for 30 minutes at room temperature and washed in PBS. Biotinylated secondary antibodies and sterptavidin-peroxidase complex (DAKO Corp, Copenhagen, Denmark) were added consecutively for 10 minutes at room temperature and washed in PBS. The peroxidase activity was observed with 0.03% 3-3'diaminobenzidine tetrahydrochloride (Sigma Chemical, St. Louis, MO, USA) applied for five minutes. After rinsing in de-ionized water and counterstaining in hematoxylin, the slides were dehydrated and mounted. Appropriate tissue sections as positive and negative controls for primary antibody were also labelled.

Proliferating cell nuclear antigen (PCNA) immunopositivity was determined by counting the number of immunopositive nuclei among 100 tumor cells in at least five representative high power fields across the slide. Mean counts of each case were calculated and statistical analysis was performed with one-way ANOVA using statistical software (SPSS, Chicago, IL, USA).

RESULTS

All anti-cancer drug administered animals demonstrated skin changes such as hair loss and desquamation. The HDG, LDG and PG animals lost weight. The differences between treatment and control and Sham groups separately were statistically significant (one-way ANOVA, p<0,001). Mean weights of animals (±SEM) at the end of the study were 173.32±4.12, 176.10±3.94, 178.92±3.23, 224.84±4.99 and 230.68+4.54 in HDG, LDG, PG, CG and SG, respectively. Table 1 shows average weights of animals at the end of the experimental period.

In experiment groups watery diarrhea was detected in all animals. Severity of diarrhea was scored between + and ++++ according to the frequency as follows:

\+ no watery defecation, frequency between 1 and 3
++ watery defecation, frequency between 1 and 3
+++ watery defecation, frequency between 3 and 6
++++ watery defecation, frequency >6

Table 1. Weights (g) of animals in study groups at the end of study.

	HDG	LDG	PG	CG	SG
1	165.8	170.0	172.0	220.6	240.0
2	168.0	169.2	170.4	224.0	234.4
3	170.2	172.6	174.2	210.8	220.0
4	180.1	184.4	184.0	240.0	254.4
5	194.8	200.0	198.6	212.2	232.0
6	165.0	168.8	174.8	200.0	200.0
7	157.0	160.0	168.0	254.8	240.6
8	180/6	180.0	186.4	222.0	224.8
9	160.0	165.2	170.8	234.0	230.6
10	192.2	190.8	190.0	230.0	230.0
Mean ± SEM	173.37 ± 4.12	176.10 ± 3.94	178.92 ± 3.23	224.84 ± 4.99	230.68 ± 4.54

Values were defined as mean ± standard error of mean (SEM). The differences between treatment and control and Sham groups separately were statistically significant (one-way ANOVA, p<0,001)

HDG : High-dose gemcitabine group, LDG: Low-dose gemcitabine group, PG: Paclitaxel group, CG: Control group

While all treatment groups had significantly more severe diarrhea than control and Sham group animals (p<0.001), there were no significant differences between treatment groups (Kruskal-Wallis one-way ANOVA on ranks test. All pair-wise multiple comparisons were performed by Student-Newman-Keuls method). Table 2 indicates findings related to diarrhea.

Table 2. Severity of diarrhea in rats.

	HDG	LDG	PG	CG	SG
1	+++	+++	+++	+	+
2	++++	++++	++	+	+
3	++	+++	+++	+	+
4	++++	++++	++++	+	+
5	++++	++++	++++	+	+
6	+++	+++	+++	+	+
7	++++	++	+++	+	+
8	++++	++++	++	+	+
9	+++	+++	+++	+	+
10	++++	+++	++++	+	+

While all treatment groups revealed significantly more severe diarrhea than control and Sham group animals (p<0.001), there were no significant differences between treatment groups (Kruskal-Wallis one way ANOVA on ranks test. All pair-wise multiple comparisons were performed by Student-Newman-Keuls method).

Scores: + no watery defecation, frequency between 1 and 3
++ watery defecation, frequency between 1 and 3
+++ watery defecation, frequency between 3 and 6
++++ watery defecation, frequency >6

HDG: High-dose gemcitabine group, LDG: Low-dose gemcitabine group, PG: Paclitaxel group, CG: Control group

Table 3. Nuclear PCNA immunopositivity in each group.

	HDG	LDG	PG	CG
1	68.10	88.20	64.00	97.20
2	89.30	71.60	97.80	91.90
3	87.80	93.30	72.20	95.20
4	88.70	76.20	95.80	95.70
5	71.00	65.70	85.20	80.00
6	34.70	84.50	81.20	61.10
7	65.90	62.50	98.20	99.00
8	64.10	86.00	88.50	73.80
9	73.90	90.50	97.90	95.10
10	83.50	70.80	77.20	67.10
Mean±	72.70 ±	78.93±	±3.81	±4.41
SEM	5.23	3.46	85.80	85.61

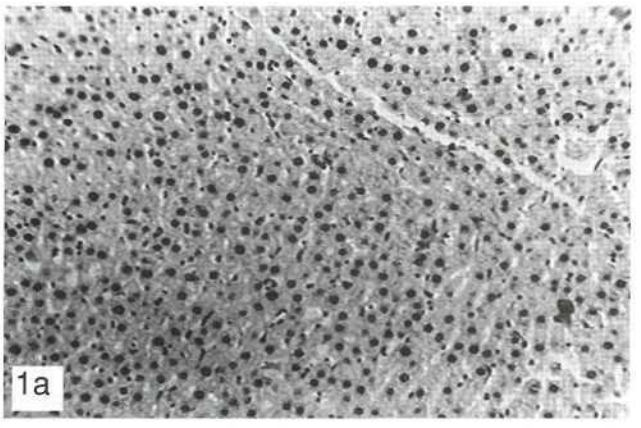
While all treatment groups revealed significantly more severe diarrhea than control and Sham group animals ($p<0.001$), there were no significant differences between treatment groups (Kruskal-Wallis one way ANOVA on ranks test. All pair-wise multiple comparisons were performed by Student-Newman-Keuls method).

Scores: + no watery defecation, frequency between 1 and 3
++ watery defecation, frequency between 1 and 3
+++ watery defecation, frequency between 3 and 6
++++ watery defecation, frequency >6

HDG: High-dose gemcitabine group, LDG: Low-dose gemcitabine group, PG: Paclitaxel group, CG: Control group

Observation of motor activity revealed fatigue in all anti-tumor agent groups,but it was not scored.

The percentage of nuclear PCNA immunopositivity for each case is summarized in Table 3. In the Sham group, PCNA immunostaining was either completely negative or minimally positive (Figure 1). When all treatment groups were compared to the control group and each other, no statistically significant differences were detected(one-way ANOVAtest).



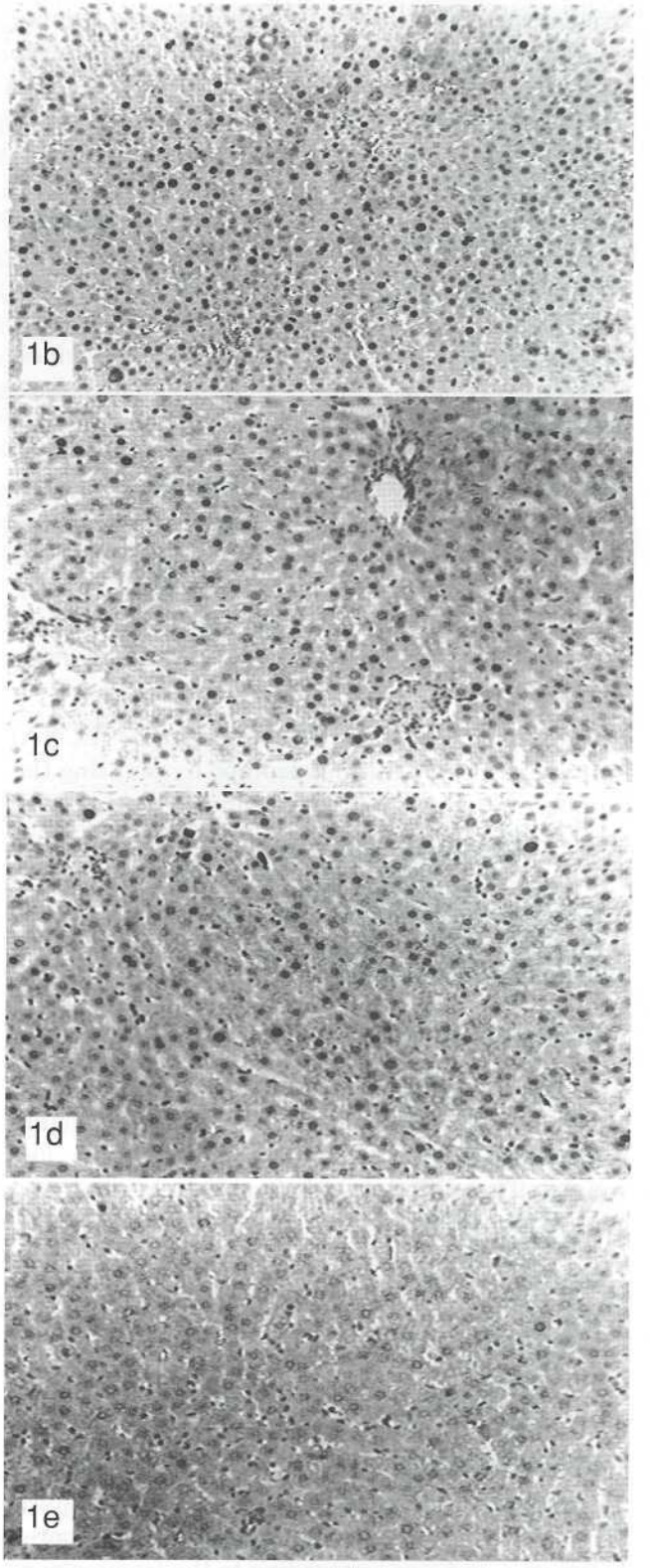


Figure 1. Immunostaining of PCNA in the CG (la), PG (Ib), HDG (Ic), LDG (Id) and SG (le) groups. Note relatively higher percentage of nuclear immunopositivity in the CG and PG groups (Immunoperoxidase staining xlOO). PCNA: Proliferating cell nuclear antigen; CG: control group; HDG: high-dose gemcitabine; LDG: low-dose gemcitabine; SG: Sham group; PG: paclitaxel group.

DISCUSSION

Gemcitabine (difluorodeoxycytidine; dFdC) is one the newer nucleoside analogs that has a broad spectrum of anti-tumoral activitiy in various solid tumor models (6,14,25,26). This agent not only inhibits DNA synthesis but also reduces intracellular deoxynucleoside triphosphate pools. In clinical trials gemcitabine has been found to be effective against some solid tumors like non small cell lung, ovary and pancreatic cancers.

Paclitaxel has also been demonstrated as a good choice for intraperitoneal chemotherapy in treatment of some solid tumors (10,11,13,14,24). Paclitaxel is a member of taxans and has previously been shown to be effective against primarily ovarian cancer along with other solid tumors like breast and hepato-pancreato-biliary cancers. Gemcitabine and paclitaxel used intraperitoneally have demonstrated anticancer effects and both have had effective target tissue concentrations (26,27) similer to previously well-defined intraperitoneal chemotherapeutic agents (16-21).

As can be seen in the literature, liver regeneration following resection is an ideal model for rapid cell division or proliferation such as in carcinogenesis. Liver resection is also one of the factors in hepatic carcinogenesis (1,3,4,22,28,29). Using this rationale we considered liver regeneration following a hepatectomy to be an appropriate model for testing antiproliferative agents like gemcitabine and paclitaxel. We also hypothesized that, because these anticancer drugs inhibit cell proliferation, they may be dangerous in liver cancer treatment as an adjuvant therapy following resection in terms of remnant liver tissue regeneration. Since adjuvant therapies based on these two agents may blockade liver regeneration , this should be take into account in liver reserve prediction in high risk patients such as hepatocellular carcinoma in cirrhosis.

Liver regeneration is an essential component of the reparative process following hepatic resections. It has been monitored by DNA synthesis rates (29), mitotic indices (1), immunohistochemical stainings of nuclear antigenic bodies (22,30) and enzyme (15) and protein marker levels like fibronectin (29). Many tissue and serum based methods have been employed in clinical and experimental studies to evaluate liver regeneration, but a gold standard has yet to be identified. In view of the pros and cons of each method, researchers should combine a minimum of two of these methods (22,28-30) or choose the most well-known, cost-effective and least invasive. Since our laboratory co-workers had a wide experience with PCNA, which has become a reliable immunohistochemical marker during the last decade (22,28-30), we preferred PCNA assessment as a marker of liver regeneration. Further more, crossreactivity has been observed in tissues from cat, cattle, dog, horse, mouse, rat and swine using Ki-67 (DAKO Anti-human Ki-67 antigen [Code No: N1574] datasheet).

Several authors (3,22,30) have shown that this proliferation-associated antigen is expressed during all active parts of the cell division cycle (Gl, S, G2 and M), but is absent in resting cells (GO). Thus, the assessment of proliferation in human tissues with PCNA has the additional advantage of detecting all cells in the cell cycle and not only those in S phase or mitosis.

In this study, there was no statistical difference between any study groups in terms of PCNA. It has been very well demonstrated previously that liver regeneration could be observed from 0-120 hours following partial hepatectomy in rats (31-34). We thus chose the 5th postoperative day to determine PCNA expression rate. In the Sham group, PCNA immunostaining was either completely negative or minimally positive (Figure 1). Study of two dosages of gemcitabine was preferred since both dosage regimens have been demonstrated as possible (8,25,26). Paclitaxel and gemcitabine introduction thus had no effect on liver regeneration independent of dosage.

As expected, both gemcitabine and paclitaxel had some adverse effects in animals. The leading symptom was diarrhea and hair loss in rats in the present study, but hair loss was so minimal that we chose not to take this parameter into account as a toxic side effect. When diarrhea was scored according to frequency and watery ingredient of feces, we defined four degrees of severity. All treatment groups had more severe diarrhea than the control and Sham groups, as was expected. In humans, as it is routine to administer anti emetic agents prior to chemotherapy it was felt that this could not be an important factor in deciding anticancer agent treatment.

It can be concluded that adjuvant chemotherapy is a safe choice in terms of liver regeneration, and that agents such as gemcitabine and paclitaxel

may be preferable for treatment of liver tumors following resection. These two agents revealed no negative effect on liver regeneration as determined by PCNA expression.

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