

Hepatoprotective Effect of Pioglitazone in Cases of Chemotherapy Induced Steatohepatitis

S. Celik¹, K. Kartal², H. Ozseker³, M. Hayran⁴, E. Hamaloglu⁵

¹Department of General Surgery, Van Training and Research Hospital, Van, Turkey

²Department of General Surgery Sisli Etfal Training and Research Hospital, Istanbul, Turkey

³Department of Pathology, Hacettepe University, Faculty of Medicine, Ankara, Turkey

⁴Department of Preventive Oncology, Hacettepe University, Faculty of Medicine, Ankara, Turkey

⁵Department of General Surgery, Hacettepe University, Faculty of Medicine, Ankara, Turkey

Rezumat

Efectul hepatoprotector al pioglitazonei în cazurile de steatohepatită indusă chimioterapic

Obiective: de a evalua efectele dăunătoare ale 5-fluoruracilului (5-FU) și Irinotecanului asupra ficatului și de a determina rolul Pioglitazonei în prevenirea afectării hepatice.

Metode: șaizeci de femele de șobolan de laborator Sprague-Dawley au fost împărțite în 4 grupuri. Primului grup (n=20) i s-au administrat 40 mg de 5-FU și 40 mg/kg de Irinotecan intraperitoneal timp de 4 cicluri, în timp ce cel de-al doilea grup (n=20) a primit 4 mg/kg de Pioglitazonă prin gavaj gastric la 5 zile timp de 20 de zile, adițional față de chimioterapie. Cel de-al treilea grup (n=10) a reprezentat grupul placebo; regimul de chimioterapie a fost administrat precum primului grup. În plus, ser fiziologic a fost administrat zilnic timp de 20 de zile prin gavaj gastric. Cel de-al patrulea grup (n=10) a primit ca grup de control doar un regim standard. Apoi, probele de sânge prelevate au fost evaluate în ceea ce privește nivelul de aspartataminotransferază AST și alaninaminotransferază ALT. Lobul stâng hepatic al șobolanilor a fost prelevat pentru analiză patologică.

Rezultate: Deși s-a administrat chimioterapie pe termen scurt, nivelurile de aspartataminotransferază și alaninaminotransferază au fost determinate a fi semnificativ crescute în primul și cel de-al treilea grup comparativ cu celelalte (p<0.0001). Nu s-au decelat diferențe semnificative între cel

de-al doilea grup și grupul de control. Pioglitazona reduce efectele adverse ale chimioterapiei asupra ficatului, dar nu are efect asupra modificărilor histopatologice.

Concluzii: Chimioterapia în cure scurte cauzează perturbări la nivelul hepatocitelor, dar fără conexiune cu steatohepatita indusă chimioterapic (CASH). Tratamentele preventive de tipul Pioglitazonei ar trebui utilizate cu mai multă atenție.

Cuvinte cheie: Pioglitazonă, chimioterapie, steatohepatită

Abstract

Background/Objectives: To evaluate the harmful effects of 5-fluoruracil (5-FU) and Irinotecan on the liver and to determine the role of Pioglitazone in averting liver damage.

Methods: Sixty Sprague-Dawley female rats were divided into 4 groups. The first group (n=20) was administered 40 mg of 5-FU and 40 mg/kg of Irinotecan intraperitoneally for 4 cycles, while the second group (n=20) received 4 mg/kg of Pioglitazone by gastric gavage at 5 days a week for 20 days in addition to chemotherapy. The third group (n=10) was the sham group; chemotherapy regimen was given as in the first group. In addition, normal saline was given daily for 20 days by gastric gavage. The fourth group (n=10) was only given a standard diet as a control group. Then, blood samples were studied for the evaluation of alanine aminotransferase (AST) and alanine aminotransferase (ALT) levels. And left liver lobes of rats were taken for pathological analysis.

Results: Although short-term chemotherapy was administered, aminotransferase (AST) and alanine aminotransferase (ALT) levels were found to be significantly higher in the first and third groups compared to the others (p<0.0001). No signifi-

Corresponding author:

Kinyas Kartal, MD
Sisli Etfal Training and Research Hospital
Sisli Istanbul 34371 Turkey
E-mail: drkinyaskartal@gmail.com

cant difference was determined between the second and the control group. Pioglitazone reduced the adverse metabolic effects of chemotherapy on the liver, but had no effect on the histopathological changes.

Conclusion: short-term CT causes metabolic disruption in hepatocytes, but not relevant with CASH. Preventive treatments like Pioglitazone should be used more carefully

Key words: Pioglitazone, chemotherapy, steatohepatitis

Introduction

Liver metastases of colorectal cancer are mostly unresectable. (1,2) Today, by the concurrent use of chemotherapeutic and biological agents, these patients can become candidates for resection surgery, which is the most effective treatment option for this patient group. Therefore, most of the patients with metastatic colorectal cancer are administered chemotherapy before and/or after surgery. (3) Additionally, patients with recurrences also require subsequent surgeries and chemotherapy.

Chemotherapy is used in combination with surgery in the treatment of most cases of metastatic cancer; however, pre-operative chemotherapy may increase the postoperative morbidity and mortality. (4-7)

Fatty liver disease is classically divided into alcoholic and non-alcoholic fatty liver disease. If steatosis is associated with inflammation, necrosis and fibrosis, this condition is termed as "steatohepatitis". Kleiner et al. (8) defined a new histopathological scoring system for steatohepatitis. According to this system, lobular inflammation, steatosis, and ballooning of hepatocytes are the basic properties of steatohepatitis and they are reversible. On the other hand, fibrosis is graded different from the grading of lobular inflammation, steatosis, and ballooning of hepatocytes and scored as a chronic and irreversible pathological change.

Non-alcoholic steatohepatitis (NASH) can be caused by chemotherapeutic agents, which is termed as chemotherapy associated steatohepatitis (CASH), and it is very important in the peri-operative morbidity and mortality of liver surgery. (4,5,9)

The development of histopathological changes like sinusoidal obstruction syndrome (SOS) and steatohepatitis vary according to the chemotherapy regimen (FOLFOX, FOLFIRI, FOLFOXIRI). (4,6,9,10) In this study, we used 5-fluorouracil (5-FU) and Irinotecan as chemotherapeutic agents, and we investigated the "steatohepatitis" associated with these agents.

There are many studies on the causes and prevention of NASH. (11) Pioglitazone, an agent used in type 2 Diabetes Mellitus, is one of the agents used for the prevention of steatohepatitis. (12,13) Pioglitazone is a member of the thiazolidinedione (TZD) family, a class of antidiabetic agents (troglitazone,

rosiglitazone, Pioglitazone) that regulate lipid metabolism and improve glycemic control in people with Type 2 diabetes. TZDs show their effects by activating ligand-dependent peroxisome proliferator-activated receptors (PPARs). (13,14)

There are many studies that show the protective effects of Pioglitazone in NASH. (12,14,15) In this study, we aimed to investigate the role of Pioglitazone in CASH.

Methods

Study protocol

The present study was carried out between December 2010 and February 2011 in the Experimental Animal Laboratory of Hacettepe University; sixty Sprague-Dawley female rats weighed between 150 to 250 g were included. Approval was obtained from the Ethics Committee of Hacettepe University. The study was conducted in accordance with European Commission Directive 86/609/EEC for animal experiments.

Rats were divided into 4 groups; Group 1 and 2, comprising 20 rats each, were the study groups, while group 3 was the sham group and group 4 served as control group.

The rats were housed in metal cages in a temperature-controlled environment ($24 \pm 1^\circ\text{C}$) under a 12-h dark/light cycle, ten each. All rats were fed with a standard diet and water.

Chemotherapeutic Agents and Pioglitazone

Chemotherapeutics were given at the highest non-lethal dose for rats. 16 40 mg/kg 5-FU and 40 mg/kg Irinotecan were given intraperitoneally (IP) weekly for a total of 4 weeks (Fig. 1A).

Group 1: Starting from the first day of the study, IP; 40 mg/kg 5-FU (5-fluorouracil EBEWE, 1000 mg/20 cc, IV, ia EBEWE Pharma Ges. Untrach-Austria) and 40 mg/kg Irinotecan (Campto. 100 mg/5 cc Aventis) were administered once a week (4 cycles).

Group 2: Chemotherapy regimen was given as in the first group. In addition, 4 mg/kg Pioglitazone (Glifix 30 mg.90 tablets, Bilim Pharmacy Turkey) were given daily for 20 days by gastric gavage.

Group 3: Served as sham group; chemotherapy regimen was given as in the first group. In addition, normal saline was given daily for 20 days by gastric gavage.

Group 4: Served as the control group, and rats were only given standard feeding.

Anesthesia

2 weeks after 4 cycles of chemotherapy and 20 days of Pioglitazone administration, all rats were sedated with 30 mg/kg of ketamine (Ketalar, Parke Davis, Turkey) and 5 mg/kg xylazine (Rompun, Bayer, Turkey). Under aseptic conditions, a 1 cc intracardiac blood sample was drawn. Immediately after taking the blood sample, a 2 cm laparotomy was performed (Fig. 1 B). The left liver lobe of the rats was removed according to the method described by Higgins and Anderson. (17) The tissue

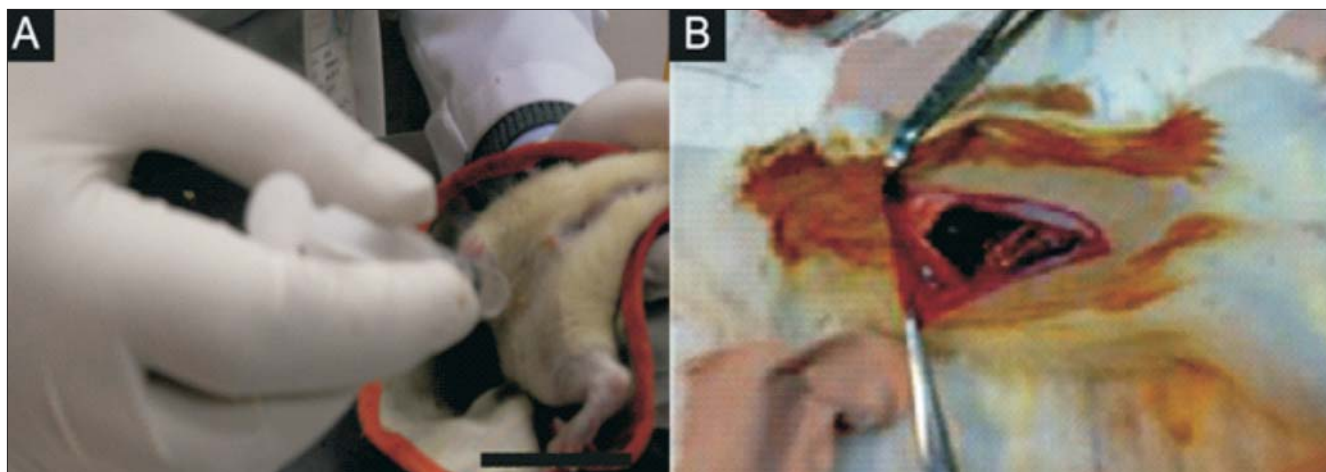


Figure 1. (A) Picture showing the intraperitoneal administration of chemotherapeutic agents. (B) Laparotomy in sterile conditions

samples were preserved in 10% formaldehyde solution. Then, the rats were sacrificed by a high dose of ketamine administered intra-abdominally.

Examination of blood samples

All blood samples were centrifuged (Eppendorf centrifuge 5810 R, Hamburg, Germany) at 4000 rpm for 3 minutes. Then, the serum samples were preserved at -22°C . After 1 month, these samples were taken out and left in room temperature to dissolve. Aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), gamma glutamyl-transferase (GGT), total bilirubin (TB), albumin (ALB) were studied for each sample by Hitachi-Modular analyser (Roche, Tokyo, Japan).

Pathological examination

All tissue samples were embedded in paraffin blocks, cross-sectional samples of $4\mu\text{m}$, were stained with hematoxylin-eosin (H&E) and were examined by a pathologist who was blinded to the groups.

Evaluation of steatohepatitis

Steatosis, lobular inflammation, ballooning and fibrosis were graded according to the scoring system proposed by Kleiner et al. (8)

Data collection and statistical analysis

Statistical analysis was performed by Statistical Package for the Social Sciences (SPSS) for Windows (version 20.0; SPSS Inc., Chicago, IL, USA). Biochemical and pathological variables were compared using non-parametric methods. Kruskal-Wallis test was used for comparing the groups. Mann-Whitney U test was used as a post hoc test, to determine the difference

between individual pairs of groups. Fischer Test was used to compare the histopathological scores between the groups. P values <0.05 were considered statistically significant.

Results

Liver function tests

No significant difference was found for GGT, TB, ALB and ALP levels between the groups.

However, serum AST and ALT were significantly different between the groups ($p<0.001$ for ALT and AST). In post-hoc analysis for ALT, there was no difference between group 1 and group 3 ($p=0.454$). Both group 1 and group 3 were significantly different from group 2 and group 4 in terms of ALT levels ($p=0.001$ for group 1 and group 2, $p<0.0001$ for group 1 and group 4, $p=0.002$ for group 3 and group 2 and $p<0.0001$ for group 3 and group 4). The mean ALT levels were: 132.3 (%95 CI: 86.27-178.33) in Group 1, 68.24 (%95 CI: 52.68-83.79) in Group 2, 135.10 (%95 CI: 86.05-185.15) in Group 3 and 63.3 (%95 CI: 55.54 – 71.06) in Group 4 (Fig. 2). No difference was seen between group 2 and group 4.

In post-hoc analysis for AST, as with ALT, group 1 and 3 were the same ($p=0.628$) and these two groups were significantly ($p<0.0001$ for group 1 and the other two groups, $p=0.014$ for group 3 and group 2 and $p=0.007$ for group 3 and group 4) different from the other two (group 2 and group 4) similar groups ($p=0.237$, Fig. 3). The mean AST levels were: 387.80 (%95 CI: 291.27-484.33) in Group 1, 182.29 (%95 CI: 144.70-219.89) in Group 2, 392.10 (%95 CI: 200.12-584.08) in Group 3 and 151.70 (%95 CI: 131.99-171.41) in group 4.

Histopathological results

Histopathological examination and grading was performed on the left lobes of the livers. Interestingly, steatosis and ballooning was not observed in any of the tissue samples.

Figure 2. Alanine aminotransferase (ALT) levels of the groups

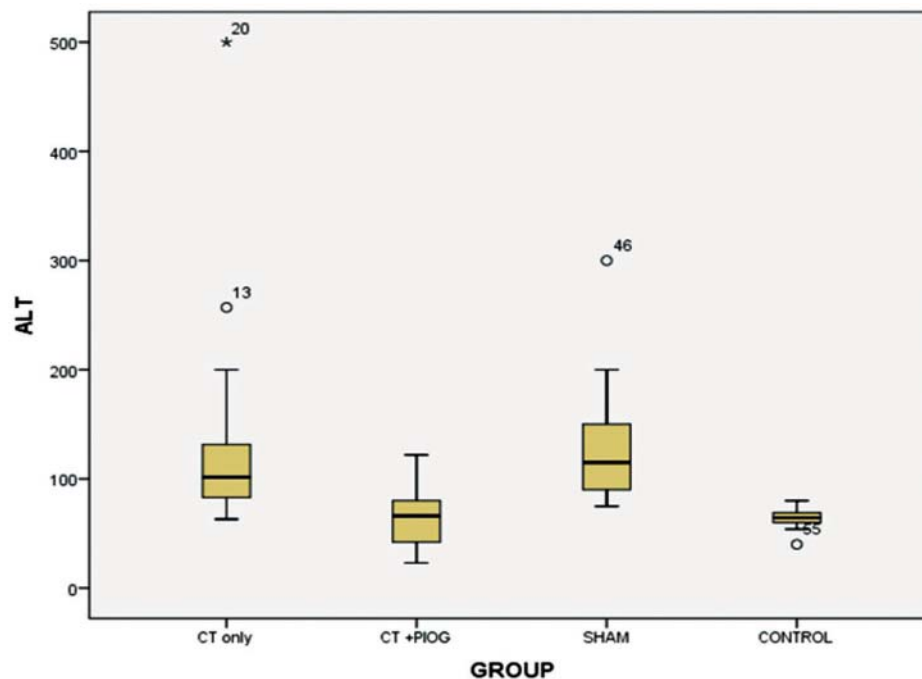
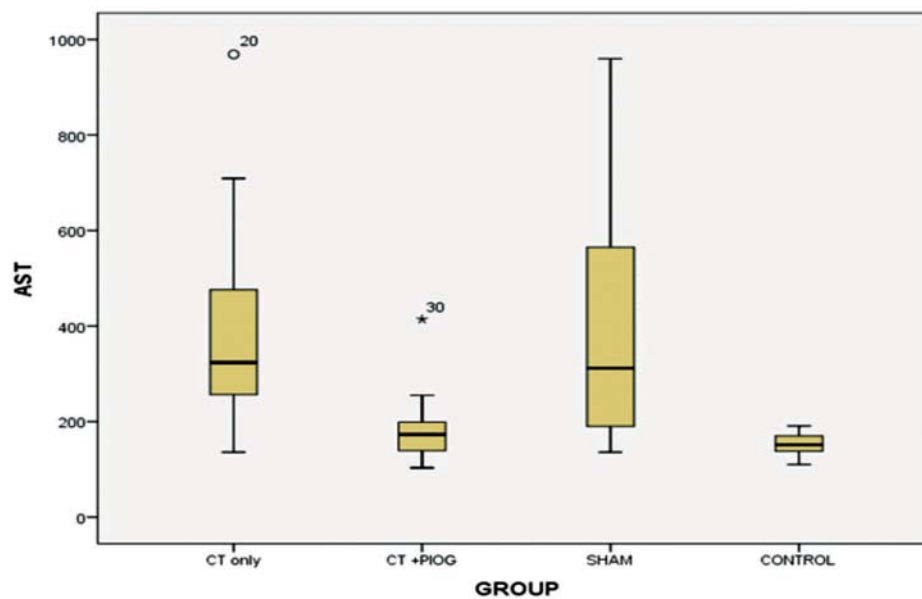


Figure 3. Aspartate aminotransferase (AST) levels of the groups



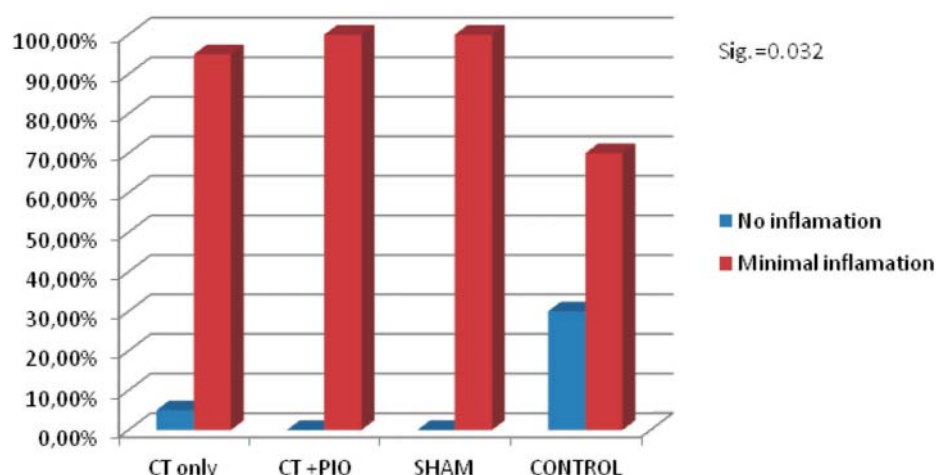
Fibrosis scores were similar among the groups ($p=0.123$). However, there was a significant difference in terms of lobular inflammation scores between the groups in post-hoc analysis ($p=0.032$). It was observed that 95% of the samples in group 1, 100% in group-2, 100% in group-3 and 70% in group-4 demonstrated minimal lobular inflammation (Fig. 4 and Fig. 5 A). Although no significant difference was identified between the groups in terms of fibrosis, the study groups (group 1 and group 2) had higher fibrosis scores than the control groups. Fibrosis

was periportal or perisinusoidal in all samples that fibrosis was observed in (Fig. 5B).

Discussion

Long term or high dose chemotherapy results in injury to the normal liver parenchyma. After neoadjuvant chemotherapy, 7 to 40% of unresectable liver metastases of colorectal cancer can become resectable (18). Additionally, 70% of the patients who

Figure 4. The rates of lobular inflammation in the groups



receive adjuvant chemotherapy, have recurrence in the liver, and have to be re-operated. Among these patients, those who receive long-term chemotherapy develop chemotherapy-associated liver injury, which increases the perioperative morbidity and mortality of these patients. Behrns et al claimed that the duration of surgery and the need for blood transfusion as well as postoperative complications increase in patients that have serious hepatic steatosis (19). Khan et al showed that it was particularly steatohepatitis, which increased the operative mortality by causing liver failure in the remnant liver (10).

The severity and type of chemotherapy associated liver injury varies according to chemotherapy protocols and number of cycles administered. It was pointed out in the EORTC 40983 study that post-operative complications were increased in patients who received more than 6 cycles of chemotherapy,

compared to chemotherapy naïve patients (25% vs. 16%). (20) In a study by Rubbia-Brandt L. et al it was reported that oxaliplatin caused a vascular injury called hepatic SOS; however, this condition did not affect the clinical condition of the patient (6). In other studies it was shown that oxaliplatin was related to vascular damage, while Irinotecan was associated with steatohepatitis (7,9). Vauthey et al claimed that Irinotecan was associated with steatohepatitis, and increased 90-day mortality by causing remnant liver failure (9). In another study, Fernandez et al reported that both Irinotecan and oxaliplatin could cause steatohepatitis (7). 5-FU was also reported to be associated with steatosis (7,21).

In light of these studies, biochemical and histopathological alterations in rat liver tissue in response to short-term (4 cycles) 5-FU and Irinotecan therapy, was investigated. To our

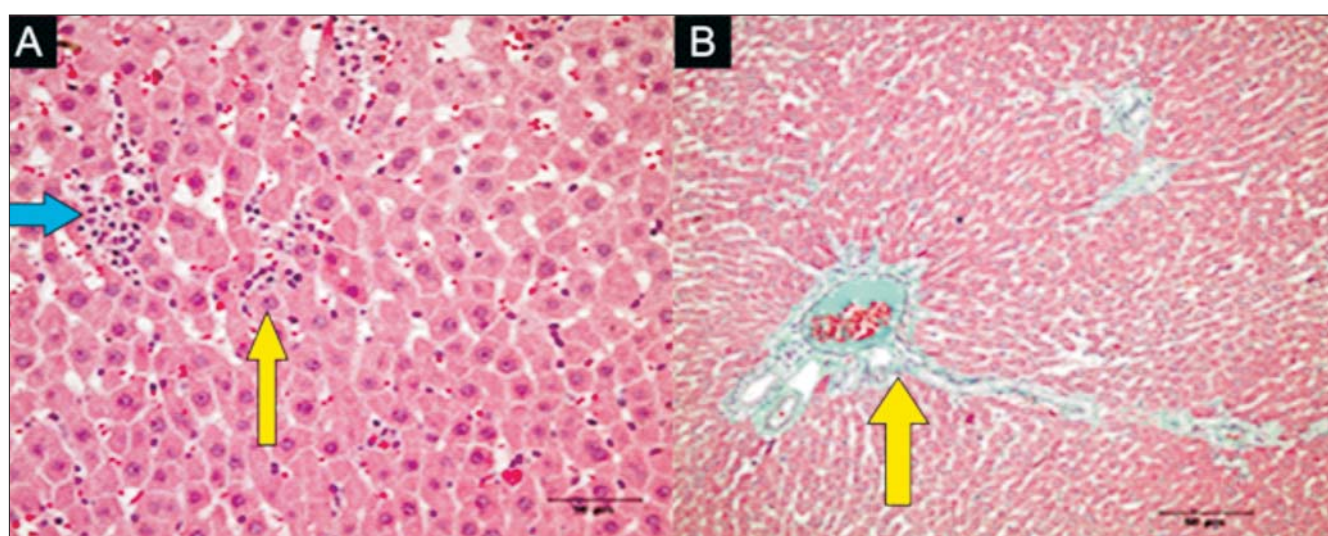


Figure 5. (A) A histopathological liver sample obtained from a rat from the chemotherapy group (Group-1). There are inflammatory cells (arrows) between the normal hepatocytes. (B) A histopathological sample from the chemotherapy group (group 1). Minimal fibrosis (arrow) is seen in the periportal region by trichrome staining

knowledge, the present study is the first study investigating the biochemical and histopathological changes in CASH, and also, the ways of prevention from CASH.

In our study, AST and ALT levels were significantly higher in the group that received chemotherapy compared to the control group ($p < 0.001$). In the Pioglitazone group, liver function tests were much the same as the control group, which shows that Pioglitazone prevents CASH, at least at cellular level.

Among the three fundamental features of steatohepatitis (steatosis, ballooning, lobular inflammation), only minimal lobular inflammation was observed in histopathological examination. So, we can say that we did not observe an accurate CASH in this study. This may be due to short-term chemotherapy, since the previous studies showed that steatohepatitis occurred after 6 or more cycles of chemotherapy (20,22).

In our study, 4 cycles of chemotherapy with 5-FU and Irinotecan caused only minimal lobular inflammation. On the other hand, the significant rise in the levels of AST and ALT may indicate that chemotherapy induces functional liver damage at cellular level.

Although the exact mechanism of Pioglitazone is not known, it has frequently been used in NASH and ultimately it is reported that Pioglitazone leads to mitochondrial modulation (12,13,15,23,24). Belfort et al claimed in their clinical study that in patients with biopsy proven NASH, Pioglitazone decreased AST and ALT levels by 40% and 58%, respectively (12). In the same study, it was reported that Pioglitazone caused a decrease in inflammation in 85% of the patients. However, in our study, lobular inflammation was more common in rats treated with Pioglitazone compared to that of controls (70% vs. 100% $p = 0.032$). It is interesting that although Pioglitazone decreases AST and ALT, it increases lobular inflammation. This situation may be explained in three ways. The first possible explanation may be the under or over-estimation of steatohepatitis, despite the fact that the samples were interpreted by an expert pathologist in liver histopathology. The other explanation is that Glitazones, the class of agents that Pioglitazone belongs to, may increase lobular inflammation in the liver (25). The last explanation is that the gavage procedure may cause stress which increases inflammation in the liver as seen in the sham group.

One of the limitations of this study was the low dose chemotherapy, as CASH did not develop with this dose, and the histopathological effects of Pioglitazone could not be demonstrated. In order to investigate the possible hepatic side effects of Pioglitazone, a sham group receiving only Pioglitazone may reveal significant results.

Conclusion

1. Four cycles of 5-FU and Irinotecan caused minimal lobular inflammation but not ballooning and steatosis, which may indicate that low dose chemotherapy, does not cause CASH.
2. 5-FU and Irinotecan caused abnormalities in liver function tests (especially AST and ALT), but when

Pioglitazone was added to the chemotherapy protocol, liver function tests were normalized.

3. Pioglitazone prevented chemotherapy associated metabolic liver injury, but not the histopathological changes.

Short term CT causes metabolic disruption in hepatocytes, but not relevant with CASH. Preventive treatments like Pioglitazone should be used more carefully.

Acknowledgments and Conflicts of Interest Statement

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None of the authors have any conflict of interests

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