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The Efficacy of 5-Fluorouracil Therapy on Liver Fibrosis in Rats: Experimental Study

Sıçanlarda Karaciğer Fibrozu Üzerine 5-Florourasil Tedavisinin Etkinliği: Deneysel Çalışma

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ABSTRACT Objective: Liver fibrosis is a common pathological condition that can lead to liver failure. Previous studies demonstrated that 5-fluorouracil (5-FU) is an effective agent in preventing fibrosis. This experimental study aimed to evaluate the efficacy of 5-FU therapy on liver fibrosis in rats. **Material and Methods:** Thirty-eight rats were divided into 3 groups: Group 1 (sham-operated, n=10), the bile duct (BD) was exposed but not ligated; Group 2 [bile duct ligation (BDL)-saline, n=14], the BD was ligated and normal saline solution was given; Group 3 (BDL-5-FU, n=14), the BD was ligated and treated with 5-FU. The liver and blood samples were harvested at postoperative day 21. Liver function analyses, tissue hydroxyproline (HP) content, and serum and tissue transforming growth factor beta 1 (TGF-β1) levels were determined. The liver sections were analyzed and percentage of tissue collagen content was measured. **Results:** There were no significant differences between the groups in terms of tissue (p=0.748) and serum (p=0.123) levels of TGF-β1. Mean HP level in the sham-operated, BDL-saline, and BDL-5-FU groups were 253±42 ng/L, 360±32 ng/L and 305±41 ng/L, respectively. 5-FU significantly decreased HP level (p=0.036). Mean percentage of collagen content in the sham-operated, BDL-saline, and BDL-5-FU groups were 0.99±0.39%, 4.92±1.03%, and 2.62±0.64%, respectively. 5-FU significantly reduced collagen accumulation (p<0.001). **Conclusion:** 5-FU has a preventive effect on liver fibrosis in rats. This result suggests that 5-FU can be a potential agent for prevention of liver fibrosis in clinical practice.

Keywords: 5-fluorouracil; liver; fibrosis

ÖZET Amaç: Karaciğer fibrozu, karaciğer yetersizliğine yol açabilen yaygın bir patolojik durumdur. Önceki çalışmalar, 5-florourasilin (5-FU) fibrozu önlemede etkili bir ajan olduğunu göstermiştir. Bu deneysel çalışmada, sıçanlarda 5-FU tedavisinin karaciğer fibrozu üzerindeki etkinliğinin araştırılması amaçlandı. **Gereç ve Yöntemler:** Otuz sekiz sıçan 3 gruba ayrıldı: Grup 1 (sham ameliyatı, n=10), safra kanalı (SK) açığa çıkarıldı ancak bağlanmadı; Grup 2 [safra kanalı ligasyonu (SKL)-salin, n=14], SK bağlandı ve normal salin solüsyonu verildi; Grup 3 (SKL-5-FU, n=14), SK bağlandı ve 5-FU ile tedavi edildi. Karaciğer ve kan örnekleri postoperatif 21. günde alındı. Karaciğer fonksiyon analizleri, doku hidroksiprolin (HP) içeriği, serum ve doku dönüştürücü büyüme faktörü beta 1 [transforming growth factor beta 1 (TGF-β1)] düzeyleri biyokimyasal olarak belirlendi. Karaciğer kesitleri analiz edildi ve doku kollajen içeriği yüzdesi ölçüldü. **Bulgular:** Doku (p=0,748) ve serum (p=0,123) TGF-β1 düzeyleri açısından gruplar arasında anlamlı fark yoktu. Sham ameliyatı, SKL-salin ve SKL-5-FU gruplarında ortalama HP seviyeleri sırasıyla 253±42 ng/L, 360±32 ng/L ve 305±41 ng/L idi. 5-FU tedavisi HP düzeyini anlamlı olarak düşürdü (p=0,036). Sham ameliyatı, SKL-salin ve SKL-5-FU gruplarında ortalama kollajen içeriği yüzdeleri sırasıyla %0,99±0,39, %4,92±1,03 ve %2,62±0,64 idi. 5-FU tedavisi kollajen birikimini önemli ölçüde azalttı (p<0,001). **Sonuç:** 5-FU, sıçanlarda karaciğer fibrozu üzerinde önleyici bir etkiye sahiptir. Bu sonuç, 5-FU'nun klinik uygulamada karaciğer fibrozunun önlenmesi için potansiyel bir ajan olabileceğini düşündürmektedir.

Anahtar Kelimeler: 5-florourasil; karaciğer; fibrozis

Liver fibrosis is a complex process caused by an imbalance between collagen synthesis to extracellular matrix (ECM) and its degradation.[1] It is a common pathological condition which gradually disrupts the normal architecture of the liver. If the fibrotic process continues without treatment, advanced stage of fibrosis results in cirrhosis, and eventually leads to liver failure and even death.[2] Unfortunately, this long-term disease remains a major concern for clinicians due to the lack of effective therapy till now.[3,4]

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Liver fibrosis is the result of the chronic diseases including viral, cholestatic, autoimmune, metabolic, alcoholic or drug-induced diseases.[5] The ongoing damage to the hepatocytes activates hepatic stellate cells (HSCs), which normally exhibit a quiescent phenotype in the healthy liver.[6,7] When activated, they transdifferentiate into myofibroblasts (MFs), which are special type of fibroblasts that contained microfilaments in their cytoplasm similar to those of smooth muscle cells.[1,7,8] HSCs are the major source of MFs in the fibrotic liver except for cholestatic liver diseases, in which portal fibroblasts (PFs) predominate in fibrogenesis. However, recent data indicate that bone marrow derived mesenchymal cells and fibrocytes also contribute to the fibrotic process in the liver due to their ability to transdifferentiate into MFs, but these cells are not a significant source of ECM.[9-12] After transdifferentiation, MFs migrate and accumulate at sites of tissue repair, and secrete large amounts of ECM, in particular collagen Type I.[13,14] MFs are currently considered to be the main source of collagen in the fibrotic liver.[15] Transdifferentiation of activated HSCs and PFs to MFs are controlled by a number of cytokines. Among them, transforming growth factor beta 1 (TGF-β1) is a key mediator in the pathogenesis of liver fibrosis.[16,17] Some studies suggested a correlation between increased levels of TGF-β1 and the severity of hepatic fibrosis.[14,18] In addition, tissue hydroxyproline level is the best indicator of collagen synthesis. It is an amino acid and a subproduct of collagen synthesis, which shows a parallel increase with collagen synthesis.[19]

While cirrhosis has been traditionally regarded as an irreversible condition, more recent understanding of liver fibrosis has indicated that even advanced fibrosis is reversible.[20-22] Theoretically, any medication that prevents collagen synthesis could be used in the treatment of liver fibrosis. Therefore, several antifibrotic agents have been evaluated in recent studies. Although numerous agents have been shown to alleviate fibrotic process in animal models, the efficacy of most of the suggested agents has not been tested in humans.[8] 5-fluorouracil (5-FU) is a commonly used anticancer drug. Previous studies showed that 5-FU is an effective agent in preventing fibrosis.[23,24] To our knowledge, the role of 5-FU in the treatment of liver fibrosis is still obscure. Thus, we hypothesized that 5-FU could be a potential therapeutic agent in preventing or reducing liver fibrosis. This experimental study aimed to evaluate the efficacy of 5-FU therapy on liver fibrosis in rats. For this purpose, bile duct ligation (BDL) was used as an experimental model to induce liver fibrosis.

MATERIAL AND METHODS

The experiment was approved by the Animal Experiments Local Ethics Committee of Süleyman Demirel University, Isparta, Turkey (approval date and number: 2019-02, February 14, 2019). The study was performed in accordance with the principles of the Guide for the Care and Use of the Laboratory Animals, and the principles of the Declaration of Helsinki.

EXPERIMENTAL GROUPS

Thirty-eight adult male Wistar-Albino rats weighing 415±48 g were used for the study. The animals were kept under standardized conditions, and caged separately. They were randomly divided into 3 groups: Group 1 (sham-operated group, n=10), the bile duct (BD) was exposed but not ligated; Group 2 (BDL-saline, n=14), the BD was ligated, and normal saline solution was given; Group 3 (BDL-5-FU, n=14), the BD was ligated, and treated with 5-FU.

SURGICAL PROCEDURE AND HARVESTING OF SAMPLES

After 12 hours of fasting, rats were weighed and anesthetized intraperitoneally with ketamine hydrochloride (90 mg/kg) and xylazine hydrochloride (10 mg/kg). A midline laparotomy was performed using sterile surgical techniques, and the BD was isolated. The BD was exposed, doubly ligated with 4/0 silk suture and transected between 2 ligatures in the BDL-saline and BDL-5-FU groups. After this process, 1 mL normal saline solution was given intraperitoneally to the BDL-saline group, whereas 20 mg/kg 5-FU (1 mL of solution contains 50 mg of 5-FU) was given intraperitoneally to the BDL-5-FU group. The incision was closed, and 10 mL normal saline solution was administered subcutaneously to all animals. The rats were fed with standard diet and

water during the experimental period. On the 10th day of the experiment, 1 cc normal saline solution and 20 mg/kg 5-FU were given intraperitoneally again to the BDL-saline and BDL-5-FU groups, respectively. On the 21th day, they were sacrificed, and the abdominal cavity was opened. The blood samples were collected from the vena cava inferior, centrifuged at 3,000 rpm for 10 min, and then obtained serums were stored in the aliquots at -80 °C until assayed for biochemical evaluation. Then, the liver of each animal was harvested. The right lobes stored at -80 °C for biochemical evaluation, and the left lobes were stored in 10% neutral formaldehyde solution until used for the histopathologic evaluation.

BIOCHEMICAL ASSAYS

Biochemical assays were carried out blindly by the same observers. Briefly, the frozen sample of rat liver was weighed and homogenized (Janke & Kunkel Ultraturrax T25, Germany) (1:10, w/v) in 100 mmol/L phosphate buffer (pH 7.4) containing 0.05% sodium azide in an ice bath. The homogenate was sonicated (UW-2070 Bandeun, Germany) for 30 s, and centrifuged at 5000 rpm for 15 min. The supernatant was frozen at -80 °C in the aliquots until used for the biochemical assays.

LIVER FUNCTION ANALYSIS

Serum alanine transaminase, aspartate transaminase, gamma-glutamyl transpeptidase, total bilirubin and direct bilirubin levels were analyzed by spectrophotometric method using automatic analyzer (Beckman Coulter AU 5800, CA, USA).

TISSUE HYDROXYPROLINE CONTENT

Quantitative measurement of hepatic hydroxyproline (HP) level was determined using the commercial enzyme linked immunosorbent assay (ELISA) kit (BT Lab, Shanghai, China) according to the manufacturer's instructions. Results were expressed as nanograms per liter (ng/L).

DETERMINATION OF SERUM AND TISSUE TGF-B1 LEVELS

Quantitative measurement of serum and tissue TGF-β1 levels was done using the commercial ELISA kit (Elabscience, Wuhan, China). The kit contained 7 spot calibrators (0.16 ng/mL, 0.32 ng/mL, 0.63 ng/mL, 1.25 ng/mL, 2.5 ng/mL, 5 ng/mL, 10 ng/mL) and all standards were studied in duplicate. A calibration curve was plotted and TGF-β1 concentration of each sample was determined by interpolation from the calibration curve. Results were expressed as nanograms per milliliter (ng/mL).

HISTOPATHOLOGIC EVALUATION AND CALCULATION OF TISSUE COLLAGEN CONTENT

Histopathologic analysis was carried out blindly by the same observer. Liver sections from routinely embedded paraffin blocks were stained with Masson's trichrome for the quantification of liver collagen content. Ten high resolution images of each sample from random fields were taken at 100x magnification using a light microscope (Eclipse Ni-U, Nikon Instruments Inc., Japan). Images were analyzed using Adobe Photoshop CC Software (Adobe Inc., San Jose, CA, USA). Briefly, blue-stained areas which are marker of collagen content were selected using color filtering, and then total pixels of the selected areas were calculated. Then, the ratio of these calculated pixels to the total texture pixels gave the percentage of tissue collagen content. The data were pooled to determine the mean.

STATISTICAL ANALYSIS

The statistical analyses of the study were performed by SPSS 20.0 (IBM Inc., Chicago, IL, USA). One-way ANOVA or Kruskal-Wallis test were used to compare the measurements between the study groups with Tukey HSD or Kruskal-Wallis post-hoc tests. The normality test was performed by Kolmogorov-Smirnov analysis. The correlation between continuous variables were found by Pearson correlation coefficient. Continuous variables with normal distribution were presented as mean±standard deviation (SD); non-normal variables were reported as median (minimum-maximum). In all analyses, statistical significance level was taken as $\alpha=0.05$.

RESULTS

All animals survived in the sham-operated group, but 6 rats died in both BDL-saline and BDL-5-FU groups during the study.

LIVER FUNCTION ANALYSIS

The results of the liver function tests of the groups are summarized in Table 1. The liver function tests of BDL-saline and BDL-5-FU groups were significantly increased when compared to sham-operated group. There was no significant difference between the BDL-saline and BDL-5-FU groups.

SERUM AND TISSUE TGF-B1 LEVELS

There were no significant differences between the groups in terms of tissue and serum levels of TGF-β1 (Table 2).

TISSUE HP LEVEL

HP level was significantly increased in both BDL-saline and BDL-5-FU groups than sham-operated group. 5-FU treatment significantly decreased HP level (Table 3).

HISTOPATHOLOGIC EVALUATION AND TISSUE COLLAGEN CONTENT

Histopathological evaluation showing the collagen content of the groups is presented in Figure 1. Collagen content was significantly increased in both BDL-saline and BDL-5-FU groups. 5-FU treatment significantly reduced collagen accumulation (Table 4).

No correlation was observed between tissue collagen content and tissue (r=-0.088, p=0.683) or serum (r=0.005, p=0.983) TGF-β1 levels.

DISCUSSION

This study found that 5-FU has a preventive effect on liver fibrosis. This antimetabolite is commonly used to treat many types of cancer. Some studies demonstrated that 5-FU inhibits effectively fibroblast proliferation and differentiation to MFs for prolonged periods by impairing DNA functions.[23,24] Another proposed mechanism of its antifibrotic activity include inhibition of TGF-β1 expression, which is also an important target for antifibrotic strategies.[25] Moreover, it has also been shown that 5-FU selectively reduce collagen Type I synthesis which is the main component of fibrosis.[26] Thus, 5-FU can reduce excessive collagen Type I accumulation without affecting normal ECM synthesis. These evidences have made this antimetabolite as a promising agent in the treatment of fibrosis-related disorders. Although the exact antifibrotic mechanism of 5-FU still remains unknown, it has so far been tried in the treatment of various fibrosis-related disorders. Prevention of scar tissue formation after glaucoma filtering surgery was the first successful use of 5-FU in clinical setting.[27] Furthermore, 5-FU has shown some success in the treatment and prevention of fibrosis in different pathologies, such as tendon adhesions after tendon repairs, scar formation after skin incisions, capsule formation around breast implants and stricture formation after corrosive esophageal damages.[28-31] Based on all this, we assumed that 5-FU can also prevent fibrotic process in the liver by inhibiting the functions of MFs or by suppressing portal fibroblasts, which are an important source of MFs in cholestatic liver injury. To prove this, we used biochemical and histopathologic

TABLE 1: Liver function analysis of the groups. Results are expressed as median (minimum-maximum).

Groups	AST (U/L)	ALT (U/L)	GGT (U/L)	TB (mg/dL)	DB (mg/dL)
Sham-operated (G1)	111 (101-204)	59 (47-90)	3.4 (1.5-4.9)	0.13 (0.09-0.17)	0.01 (0.01-0.02)
BDL-saline (G2)	455 (256-1,173)	248 (71-301)	28.8 (18.8-172.4)	5.41 (2.11-9.40)	3.24 (1.19-6.08)
BDL-5-FU (G3)	662 (419-1,458)	129 (103-616)	83.7 (15.4-165.4)	8.94 (1.80-12.30)	5.74 (1.04-7.84)
p value	<0.001	0.008	<0.001	<0.001	<0.001
	0.010 G1-G2	<0.001 G1-G2	0.004 G1-G2	0.003 G1-G2	0.003 G1-G2
	<0.001 G1-G3	0.004 G1-G3	<0.001 G1-G3	<0.001 G1-G3	<0.001 G1-G3
	0.643 G2-G3	0.988 G2-G3	0.728 G2-G3	0.856 G2-G3	0.764 G2-G3

AST: Aspartate transaminase; ALT: Alanine transaminase; GGT: Gamma-glutamyl transpeptidase; TB: Total bilirubin; DB: Direct bilirubin; BDL: Bile duct ligation, 5-FU: 5-fluorouracil.

TABLE 2: Comparison of serum and tissue TGF-β1 levels of the groups. Results are expressed as mean±SD.

Groups	TGF-β1 tissue (ng/mL)	TGF-β1 serum (ng/mL)
Sham-operated	3.11±1.93	44.16±10.40
BDL-saline	2.49±1.23	45.50±9.00
BDL-5-FU	2.66±1.89	34.03±15.51
p value	0.748	0.123

TGF-β1: Transforming growth factor beta 1; SD: Standard deviation; BDL: Bile duct ligation; 5-FU: 5-fluorouracil.

TABLE 3: Tissue hydroxyproline levels of the groups. Results are expressed as mean±SD.

Groups	HP (ng/L)
Sham-operated (G1)	253.64±42.02
BDL-saline (G2)	359.89±32.42
BDL-5-FU (G3)	305.50±40.98
p value	<0.001
	<0.001 G1-G2
	0.047 G1-G3
	0.036 G2-G3

SD: Standard deviation; HP: Hydroxyproline; BDL: Bile duct ligation; 5-FU: 5-fluorouracil.

parameters to determine the degree of fibrosis. Of these, HP level was significantly decreased in the BDL-5-FU group when compared to the BDL-saline group. This finding also correlated with the histopathologic evaluation of tissue collagen content.

Although TGF-β1 levels are expected to increase during fibrogenesis, neither tissue nor serum levels of TGF-β1 differed significantly between the groups, and no correlation was observed between the severity of hepatic fibrosis and tissue or serum TGF-β1 levels. These conflicting results have been explained by a previous study investigating time-related changes after experimental BDL as follows. BDL in mice activates the fibrotic process, and leads to an increase in transcript level of TGF-β1 for 2 weeks. Thereafter, transcript level for TGF-β1 decreases.[32] Since TGF-β1 levels were determined after 3 weeks of BDL in rats, we could not achieve a significant difference in TGF-β1 levels between the groups. Clarification of this requires further studies, where tissue and blood samples will be obtained earlier.

Any therapy aimed at preventing or ameliorating fibrosis formation should not cause significant side effects when administered. In this perspective, 5-FU may have a disadvantage in the clinical practice. Because it can cause some significant side effects such as nausea, vomiting, diarrhea, hair loss, myelosuppression, leukopenia and allergic reactions.[33] But 5-FU causes DNA damage and cell death only in high doses.[24] At much lower doses, it has no cytotoxic effects but only decreases cellular metabolism and acts as a negative regulator of collagen synthesis, causing a significant reduction in collagen synthesis by fibroblasts.[26] In this respect, we used intraperitoneal 5-FU at a dose of 20 mg/kg, which is the highest non-lethal dose of this drug for rats.[34] Although 6 rats died in the BDL-5-FU group, these deaths cannot be said to be caused by its detrimental effects. Because the same number of rats died in the BDL-saline group. In our study, the main reasons behind this increased mortality should be considered as complications from BDL and progressive cholestatic

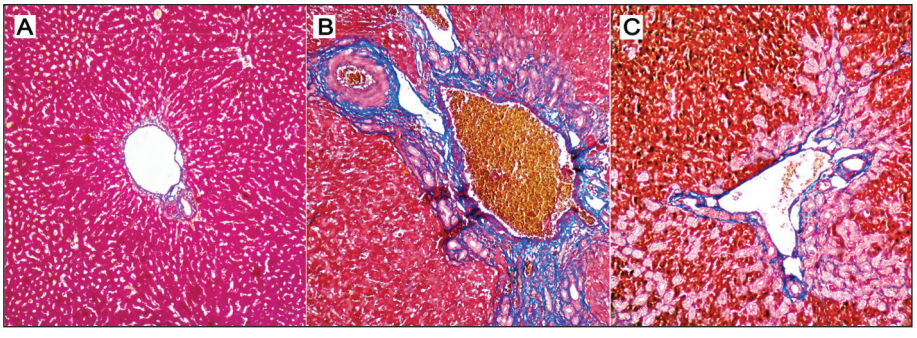

FIGURE 1: Histopathologic evaluation showing the collagen content of the groups. **A)** Sham-operated group, **B)** BDL-saline group, **C)** BDL-5-FU group. The blue-stained areas show the tissue collagen content (Masson trichrome staining, 100x). 5-FU treatment significantly reduced collagen accumulation. **BDL:** Bile duct ligation; **5-FU:** 5-fluorouracil.

TABLE 4: Mean percentage of liver collagen contents of the groups. Results are expressed as mean±SD.

Groups	Tissue collagen content (%)
Sham-operated (G1)	0.99±0.39
BDL-saline (G2)	4.29±1.03
BDL-5-FU (G3)	2.61±0.68
p value	<0.001
	<0.001 G1-G2
	<0.001 G1-G3
	<0.001 G2-G3

SD: Standard deviation; BDL: Bile duct ligation; 5-FU: 5-fluorouracil.

damage. Previous studies suggested that a single-dose of 5-FU results in inhibition of fibroblasts at prolonged periods.[35] Our study supports this data by showing that fibrosis was reduced by giving 5-FU therapy at 10-day intervals. All these data suggest that prolonged inhibition of fibroblasts by 5-FU reduces the frequency of use of the drug. In this way, ease of use, better cost-effectiveness and less side effects can be achieved in the clinical practice.

Our study has some limitations that should be considered. First, we could not show a relationship between TGF-β1 levels and hepatic fibrosis in the samples taken at the end of the 3rd week. This result requires new studies in which TGF-β1 could be examined earlier than 2 weeks. Second, we do not know exactly the dose with the least side effects and the highest antifibrotic effect of 5-FU. In this regard, experimental studies using lower doses of 5-FU can be done. Third, we were unsure how long a single dose of antifibrotic activity lasted in rat liver, so we gave two doses of 5-FU. New studies can be conducted to investigate the efficacy of a single dose of 5-FU on liver fibrosis.

CONCLUSION

To our knowledge, this is the first study showing 5-FU has a preventive effect on liver fibrosis. This result suggests that 5-FU may be useful in preventing or slowing the progression of liver fibrosis in clinical setting. Clinical trials are now required to verify potential benefits of this antimetabolite on diseases that cause liver fibrosis.

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Conflict of Interest

No conflicts of interest between the authors and / or family members of the scientific and medical committee members or members of the potential conflicts of interest, counseling, expertise, working conditions, share holding and similar situations in any firm.

Authorship Contributions

Idea/Concept: *Levent Duman, Gökhan Okay;* ***Design:*** *Levent Duman, İbrahim Metin Çiriş, İlter İlhan;* ***Control/Supervision:*** *Levent Duman;* ***Data Collection and/or Processing:*** *Gökhan Okay, Adnan Karaibrahimoğlu;* ***Analysis and/or Interpretation:*** *İbrahim Metin Çiriş, İlter İlhan, Hülya Mete Arıcan, Adnan Karaibrahimoğlu;* ***Literature Review:*** *Gökhan Okay;* ***Writing the Article:*** *Levent Duman, Gökhan Okay;* ***Critical Review:*** *Levent Duman;* ***References and Fundings:*** *Levent Duman;* ***Materials:*** *Gökhan Okay.*

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