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Effects of a long-acting somatostatin analogue, lanreotide, on bile duct ligation-induced liver fibrosis in rats

Uzun etkili somatostatin analoğu, lanreotidin sıçanlarda safra yolu bağlanarak oluşturulan karaciğer fibrozu üzerine etkisi

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Background/aims: *Somatostatin receptors have been shown on hepatic stellate cells, and somatostatin infusion has been shown to inhibit hepatic stellate cells activation. We aimed to test the effects of a long-acting somatostatin analogue, lanreotide, on bile duct ligation- induced liver fibrosis in rats.* ***Methods:*** *Thirty-seven Wistar rats were divided into 5 groups as follows: Group 1, bile duct ligation+lanreotide; Group 2, bile duct ligation; Group 3, sham+lanreotide; Group 4, sham; and Group 5, control group. Lanreotide-autogel (20 mg/kg/month) or saline in intraperitoneal doses was administered. Serum biochemical parameters, liver collagen level, and oxidative stress and histological parameters were determined after 28 days.* ***Results:*** *The tissue collagen level, biochemical parameters (AST, ALT, bilirubins, alkaline phosphatase, γ-glutamyl transpeptidase) and oxidative stress parameters (malondialdehyde, luminal, lucigenin) in the bile duct ligation groups were higher than in the sham-operated and control groups (p<0.001 for all). Lanreotide improved malondialdehyde and glutathione levels in the bile duct ligation+lanreotide group. In histopathological examination, bile duct ligation groups showed stage-3 liver fibrosis, while all the controls were normal. Lanreotide did not improve the liver fibrosis histologically or biochemically.* ***Conclusions:*** *A monthly active somatostatin analogue, lanreotide, improved malondialdehyde and glutathione; however, it was not able to improve bile duct ligation-induced liver fibrosis in rats. Although lanreotide is a long-acting medication, it did not show anti-fibrotic effects in the model.*

Key words: Anti-fibrotic, somatostatin analogue, lanreotide, bile duct ligation, hepatic fibrosis, liver

Amaç: *Somatostatin reseptörlerinin hepatik stellat hücreler üzerinde varlığı gösterilmiş ve somatostatin infüzyonunun hepatik stellat hücreler aktivasyonunu inhibe ettiği bildirilmiştir. Uzun etkili somatostatin analoğu, lanreotidin sıçanlarda safra yolu bağlanarak oluşturulan karaciğer fibrozu üzerine etkisinin değerlendirilmesi amaçlandı.* ***Yöntem:*** *Otuz yedi Wistar soyu sıçan 5 gruba ayrıldı. Grup 1: safra yolu bağlı+lanreotid, grup 2: safra yolu bağlı, grup 3: sham opere+lanreotid, grup 4: sham opere ve grup 5: kontrol grup olarak çalışıldı. Lanreotid-otojel (20 mg/kg/ay) veya serum fizyolojik intra-peritoneal yolla verildi. Serum biyokimyasal testleri, karaciğer kollajeni, oksidatif stres ve histolojik parametreler 28 gün sonra çalışıldı.* ***Bulgular:*** *Safra yolu bağlı grupların doku kollajen düzeyi, biyokimyasal parametreleri (AST, ALT, bilirubinler, alkalen fosfataz, γ-glutamil transferaz) ve oksidatif stres parametreleri (malondialdehid, luminal, lusigenin) sham opere edilen gruplardan ve kontrol grubundan yüksek bulundu (tüm p<0.001). Lanreotid malondialdehid ve glutatyon düzeylerini safra yolu bağlı+lanreotid grubunda düzeltti. Histopatolojik incelemede, safra yolu bağlı gruplarda stage-3 karaciğer fibrozu izlenirken, kontrol grupları bulundu. Lanreotid histolojik veya biyokimyasal olarak karaciğer fibrozunu iyileştiremedi.* ***Sonuç:*** *Aylık etkili somatostatin analoğu lanreotid tedavisi malondialdehid ve glutatyon düzeylerini iyileştirdi. Bununla beraber, safra yolu bağlanarak indüklenen karaciğer fibrozunda gerilemeye neden olmadı. Uzun aktiviteli bir ajan olduğu halde lanreotid tedavisinde fibroz modelde antifibrotik etkinlik görülmedi.*

Anahtar kelimeler: Antifibrotik, somatostatin analoğu, lanreotid, safra yolu bağlanma, hepatik fibroz, karaciğer

INTRODUCTION

Liver fibrosis is one of the main components of cirrhosis and is characterized by the accumulation of collagen and extracellular matrix proteins in the space of Disse. Hepatic stellate cells (HSCs)

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are the main component in producing these extracellular matrix products that lead to fibrosis. There is still no effective treatment available for liver fibrosis. Recent experimental studies have reported only partial success against liver fibrosis (1, 2).

Somatostatin and its analogue, octreotide, are known to inhibit DNA synthesis and proliferation in hepatocytes. Somatostatin receptors have been suggested to be involved in mediating the effect of somatostatin on HSCs in an activation-dependent way (3). Song et al. (4) reported that the somatostatin analogue octreotide exerted an inhibitory effect on HSCs by down-regulating the expression of the connective tissue growth factor gene and transforming growth factor (TGF)-beta. Octreotide can perfectly combine with HSCs in rats, and thereby can exert its biological activity on HSCs.

Bogazzi et al. (5) showed the anti-fibrotic effect of somatostatin analogues on the cardiac fibrosis degree in acromegalic patients. Recently, a somatostatin analogue (SOM230) has also been shown to attenuate bleomycin-induced pulmonary fibrosis in mice and human lung fibroblast activation (6).

Chatterjee et al. (7) explored the beneficial anti-fibrotic therapeutic potential of somatostatin in *Schistosoma mansoni*-mediated liver pathology. Tracy et al. (8) investigated the effect of octreotide on the biliary epithelial cell proliferative responses to biliary obstruction in rats. Octreotide decreased bile duct proliferation and periportal extracellular matrix deposition in response to biliary obstruction in a seven-day period. Turkcapar et al. (9) showed that nine days of octreotide therapy was effective in preventing bile duct proliferation and hepatic fibrosis in obstructive jaundice. However, these studies were restricted to short-term results only. These findings are in favor of octreotide as a potential agent for the prevention and management of hepatic fibrosis. We hypothesized that lanreotide could be a potential therapeutic candidate for fibrotic liver diseases with its long-acting feature, leading to much easier administration than conventional somatostatin or its analogues. To the best of our knowledge, this is the first study to investigate the possible anti-fibrotic effect of lanreotide in bile duct ligation (BDL)-induced liver fibrosis in rats.

MATERIALS AND METHODS

Procedures Related to Experimental Animals

This experimental protocol had the full approval of the Ethical Committee on Animal Research, Marmara University School of Medicine, Turkey, and was performed according to the criteria of the International Guidelines for Animal Research. All animals received humane care.

Forty-seven male Wistar rats, between 3.5-4 months old, weighing 190-220 g, were obtained from Marmara University Animal Research Laboratory. Animals were kept at a constant temperature (22 ± 1°C) with 12-hour light and dark cycles, in the same unit and allowed to acclimatize to their new conditions for one week before beginning the study. All animals received humane care in compliance with the National Institutes of Health Criteria for Laboratory Animals. Rats had free access to standard rat chow and water.

Under general pentobarbital anesthesia, 37 male Wistar rats underwent BDL or sham operation. Briefly, the common bile duct was exposed after laparotomy. Subsequently, 2 double knots were placed proximally and distally and the part of the bile duct between the 2 double knots was excised. In the sham-operated rats, the abdomen was closed without BDL. Sixteen of the rats underwent BDL and 14 were sham operated. BDL rats were divided into 2 groups. The first group (BDL + Lanreotide; n = 8) received 20 mg/kg lanreotide autogel intraperitoneally (i.p.). Sterile saline (1 ml/kg/i.p.) was administered initially to the second group (BDL group; n = 8). The sham-operated rats were also divided into 2 groups. After the sham operation, lanreotide was given to the sham + lanreotide group (n = 7) and sterile saline was administered to the sham group (n = 7). During this administration, the same dose of lanreotide was given to the BDL + lanreotide group and saline was administered to the BDL group immediately after BDL. An additional group of 7 healthy control rats were studied as the control group. After the 28 days required fibrosis induction time in the model (1, 2), the rats were weighed and decapitated; their trunk blood was collected and centrifuged (3000 rpm, 10 min, 4°C) and serum samples were obtained for biochemical analyses of aspartate aminotransferase (AST), alkaline aminotransferase (ALT), alkaline phosphatase (ALP), γ-glutamyl transpeptidase (GGT), and total and direct bilirubin. The serum samples were stored at –80°C and measured with automated standardized procedures (Roche Hitachi 917/747, Mannheim, Germany).

Liver Tissue Sampling

The left, middle and right lobes of each liver were explored. Six different 5 x 5 x 5 mm slices were fi-

xed in 10% buffered formalin, routinely processed, and blocked into paraffin for detecting collagen content by image analysis (2).

Biochemical Collagen Content Determination

The collagen content of the liver was assayed by the colorimetric method described by Lopez de Leon and Rojkind (10). The principle is the coloring of collagenous protein by Sirius red (36554–8, 2610-10-8; Aldrich Chemical, Deisenhofen, Germany) and noncollagenous proteins by fast green (14280; MERCK, Darmstadt, Germany). Fifteen micrometer-thick liver slices taken from each paraffin block were layered on glass slides. Slices were deparaffinized and assayed as originally described. Collagen content was calculated using the formula described by the authors as microgram collagen per milligram protein (10).

Histopathologic Investigations

Five-micrometer liver sections were stained by hematoxylin and eosin and Masson trichrome/Gomori reticulum staining. The necroinflammatory activity grading and fibrosis staging were set by Knodell's criteria (11).

Tissue Homogenization

Liver samples were weighed and homogenized in 0.15 mol NaCl to determine reactive oxygen species. Homogenates were diluted with 0.15 mol NaCl up to 20%. Tissue homogenates were sonicated twice at 30-second intervals at 4°C. After sonication, homogenates were centrifuged at 3000 rpm for 10 min and at 15,000 rpm for 15 min. Aliquots of the supernatants were used for studies.

Oxidative Stress Parameters

Malondialdehyde measurements

Measurements of thiobarbituric acid reactive species (TBARS) were done according to Yagi (12). Liver tissues were homogenized in icy trichloroacetic acid (TCA) (10%) solution and then centrifuged. The superficial liquid portion was mixed with equal volume of TBARS (0.67%) and heated at 90°C for 15 min. TBARS were measured in nmol/g tissue according to absorbance at 532 nm.

Chemiluminescence measurements

Reactive oxygen metabolites (ROM) were measured at room temperature via chemiluminescence technique using Mini Lumat LB 9506 Luminometer (EG&G, Berthold, Germany). Samples were placed into a 2-ml 0.02 mol HEPES buffer (pH 7.4) containing 0.5 mol phosphate-buffered saline. For measurement of ROM, 0.2 nmol concentrated lucigenin (specific for superoxide radicals) or luminal ($HOCl^-$, H_2O_2, OH^-) was used. Serial measurements at 15-second intervals for 5 min were done and results were calculated as area under the curve and relative light unit (RLU); correction for fresh tissue weight was done (RLU per milligram of tissue area under the curve) (13, 14).

Glutathione level measurement

Glutathione (GSH) levels were measured spectrophotometrically using Ellman's reagent and method (15). Results were calculated as Ìmol GSH/g tissue.

Statistical Evaluation

Data were expressed as mean values ± standard deviation or, in case of non-normal distribution, as median and range, and were compared using the Kruskal–Wallis test. When significant, subsequent multiple comparison test was performed. Values of p<p0.05 were considered significant. Comparisons between the groups were tested for significance by Mann–Whitney U and χ^2 tests.

RESULTS

Biochemical Findings

All biochemical parameter levels, including AST, ALT, ALP, GGT, and total and direct bilirubin were significantly increased in both of the BDL groups in contrast to the control groups ($p<0.001$ for all). No biochemical parameter difference was observed between the BDL subgroups. There was no significant difference with respect to these biochemical parameters between the sham and the healthy control groups (Table 1).

Oxidative Stress Parameters

The mean liver malondialdehyde levels of the BDL groups were significantly higher than in the control groups ($p<0.001$ for all). Lanreotide therapy improved the MDA level between the BDL subgroups ($p<0.01$; Figure 1).

The tissue luminal and lucigenin levels in the BDL groups were higher than in the control groups ($p<0.001$ for all). The tissue luminal and lucigenin levels were not statistically different between the BDL groups (Figure 2).

The tissue GSH levels in the BDL groups were significantly lower than in the control groups ($p<0.001$ for all). The tissue GSH level in the BDL + lanreotide group was higher than in the BDL group ($p<0.001$; Figure 3). No difference in the oxi-

Table 1. Biochemical parameters measured in the current study

Mean ± Standard deviation	BDL + Lanreotide	BDL	Sham + Lanreotide	Sham	Normal
AST (U/L)	461 ± 127*	431 ± 156*	270 ± 54	258 ± 34	233 ± 71
ALT (U/L)	94 ± 21*	97 ± 18*	59 ± 6	76 ± 10	75 ± 14
Alkaline phosphatase (U/L)	765 ± 460*	747 ± 99*	352 ± 237	487 ± 104	379 ± 120
GGT (U/L)	43 ± 27*	51 ± 21*	10 ± 5	8 ± 2	7 ± 2
Total bilirubin (mg/dl)	10.7 ± 2.3*	11.6 ± 2.1*	0.2 ± 0.03	0.2 ± 0.04	0.2 ± 0.03
Direct bilirubin (mg/dl)	6.3 ± 1.7*	7.0 ± 2.2*	0.02 ± 0.01	0.02 ± 0.01	0.02 ± 0.01

AST: Aspartate aminotransferase. ALT: Alkaline aminotransferase. GGT: Gamma-glutamyl transpeptidase. BDL: Bile duct ligation.
* $P<0.001$ versus the sham + lanreotide, sham, and normal control groups.

dative stress parameters was observed among the control groups.

Biochemical Collagen Content Measurement

Hepatic collagen contents in both BDL rat groups were significantly higher than in the control groups ($p<0.001$). However, the hepatic collagen levels were not found to be significantly affected by lanreotide administration. Similarly, no significant statistical difference was found between the control groups ($p>0.05$; Figure 4).

Histopathologic Findings

In the histopathologic examination of the liver sections, prominent stage 3 fibrosis was demonstrated in all BDL groups. The histologic activity and fibrosis observed in the BDL groups by Knodell scoring did not change with lanreotide therapy (Figure 5).

DISCUSSION

At present, no effective treatment for liver fibrosis is available for clinical use. Some experimental studies reported partial success with some substances such as melatonin (1), pegylated interferon (16) and N-acetylcysteine (17) in this setting. A valuable therapeutic approach against the development of hepatic fibrosis is still needed.

Anti-fibrotic effects of somatostatin analogues in different diseases such as cardiac fibrosis in acromegalic patients (5) and bleomycin-induced pulmonary fibrosis in mice (6) were reported. Chatterjee et al. (7) explored beneficial anti-fibrotic therapeutic potential of somatostatin in *Schistosoma mansoni*-mediated liver pathology.

Somatostatin receptors have been investigated on HSCs (3), and octreotide had an inhibitory effect on the expression of connective tissue growth factors in HSCs (4). Valatas et al. (18) studied chemokine secretion by activated rat Kupffer cells cultured with lipopolysaccharide and the possible effect of the somatostatin analogue octreotide on the hepatic inflammation and fibrosis in many types of liver injury. In their study, octreotide had an inhibitory effect on lipopolysaccharide-induced chemokine secretion, indicating a possible involvement of the PI3-kinase pathway. They demonstrated that chemokine secretion by Kupffer cells can be differentially regulated by octreotide, and suggested that somatostatin analogue may have immunoregulatory effects on resident liver macrophages.

Tracy et al. (8) investigated the effect of octreotide (6 μg/kg) subcutaneous injections, twice daily, on a BDL model of fibrosis in rats for seven days. The

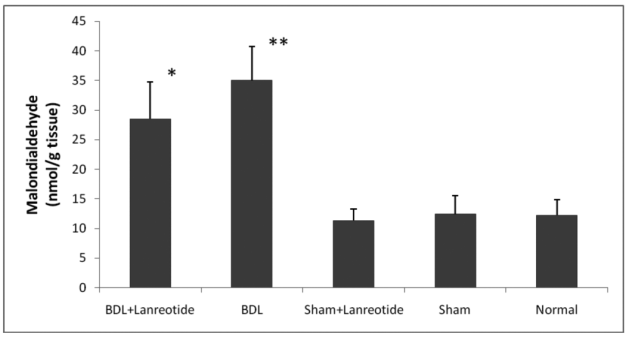

Figure 1. Tissue malondialdehyde levels.
* p<0.01 versus the bile duct ligation (BDL) group.
** p<0.001 versus the sham + lanreotide, sham, and normal control groups.

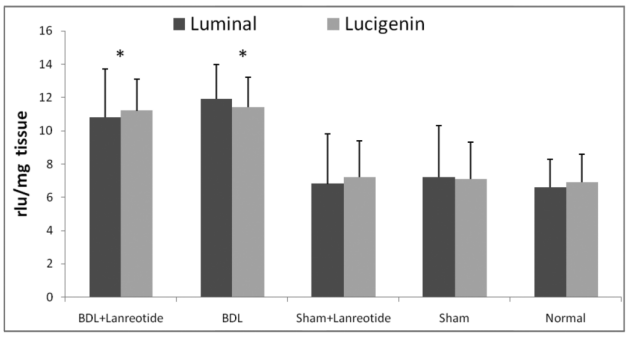

Figure 2. Tissue luminal and lucigenin levels.
* p<0.001 versus the sham + lanreotide, sham, and normal control groups. Tissue luminal and lucigenin levels were not statistically different between the BDL groups.

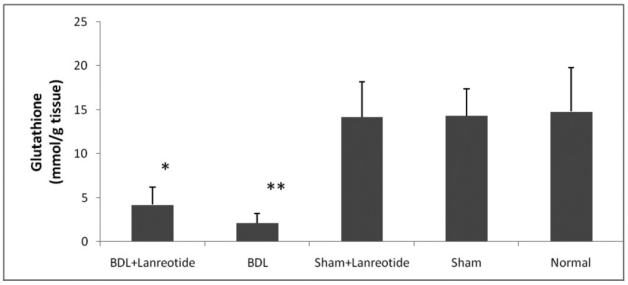

Figure 3. Tissue glutathione levels.
* p<0.001 versus the bile duct ligation (BDL) group. ** p<0.001 versus the sham + lanreotide, sham, and normal control groups.

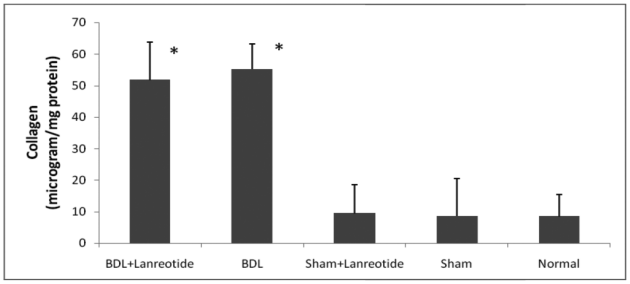

Figure 4. Tissue collagen levels.
* p<0.001 versus the sham + lanreotide, sham, and normal control groups. BDL: Bile duct ligation.

therapy decreased bile duct proliferation and periportal extracellular matrix deposition. Although extensions of the ductal epithelial cells into the lobule were prominent in the BDL group, these lesions were almost totally limited to the portal area in the BDL+ octreotide group.

Turkcapar et al. (9) studied the effects of octreotide (20 μg/kg) subcutaneous injections, thrice daily, on a BDL model of fibrosis in rats for nine days. The therapy significantly decreased bile duct proliferation and periportal fibrosis in the BDL + octreotide group compared to the BDL group. Additionally, they showed that octreotide was efficient in preventing bacterial translocation. However, these studies had short-term results with a short-acting conventional octreotide. We purposed that lanreotide treatment was a promising anti-fibrotic therapy having a long-acting effect with infrequent application.

Oxidative stress is related to the activation of HSCs, which are the central mediators in the pathogenesis of liver fibrosis (19, 20). Current data for the effects of somatostatin on oxidative stress are very limited. Chao et al. (21-23) demonstrated the modulating roles of somatostatin and octreotide on release of nitric oxide, tumor necrosis factor and hydrogen peroxide from macrophage and Kupffer cells. These data supported the hypothesis of anti-proliferative and anti-fibrotic effects of lanreotide. We planned to study some basic oxidative stress markers and antioxidant GSH to determine if lanreotide with its sustained high dosage (20 mg/kg) has any effect on this pathway during 28 days of BDL-induced liver fibrosis. This study showed for the first time that lanreotide improved the oxidative stress by decreasing malondialdehyde and increasing GSH in liver tissue levels. In the absence of literature data on the topic, we may speculate that this free radical scavenging activity of lanreotide may be related to its hepatoprotective actions by modulating Kupffer cell functions. However, these effects were insufficient in inhibiting HSC activation, associated with advanced fibrosis.

In conclusion, intraperitoneal administration of a long-acting somatostatin analogue, lanreotide, in the BDL model was found to have beneficial effects on oxidative stress parameters (malondial-

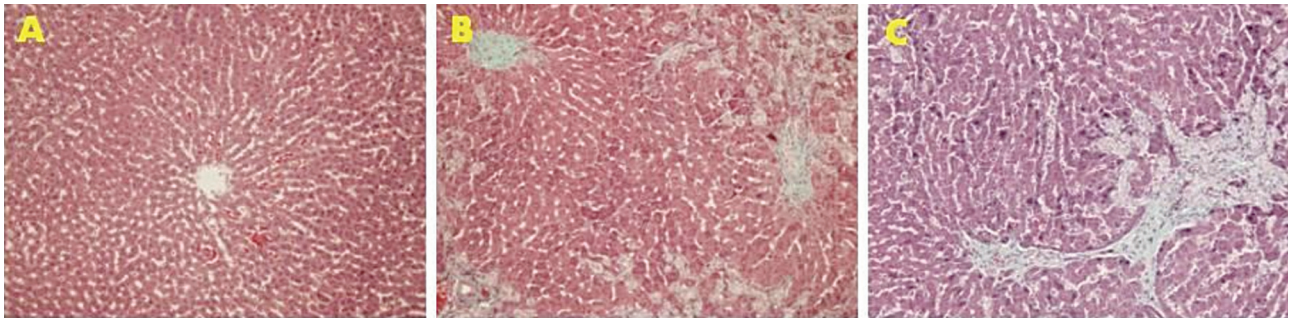

Figure 5. (A) sham-operated group rat liver section with normal finding (HE, original magnification x20). **(B)** A bile duct ligation (BDL) + lanreotide group liver slide with cellular loss and portal fibrous septa (Trichrome-stained original magnification x20). **(C)** A BDL group liver slide with cellular loss and bridging fibrous septa, connecting portal areas and lobule centers (Trichrome-stained original magnification x20).

dehyde and GSH). However, it was not able to improve BDL-induced liver fibrosis in rats. Lanreotide did not show an anti-fibrotic effect in the model; however, it is a long-acting therapeutic choice.

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