

The Effects of Halofuginone and Ursodeoxycholic Acid in Prevention of Sclerosing Cholangitis Caused by Scolocidal Agents

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Öz

Skolosidal Ajanlardan Kaynaklanan Sklerozan Kolanjitin Önlenmesinde Halofuginon ve Ursodeoksikolik Asidin Etkileri

Amaç: Biliyer skleroz, otoimmünite, operatif travma, toksik ajanlar, kanser ve kronik inflamatuvar koşulların neden olduğu yaşamı tehdit eden durumdur. Karaciğer dokusu, fibrotik skar dokusunun ilerlemesi ile belirgin siroza doğru ilerleyebilir. Alkaloid febrifugine bitkisinin aktif bileşeni olan halofuginonun, fibrozisi inhibe ettiği gösterilmiştir.

Gereç ve Yöntem: Elli sıçan, bir tanesi salin infüzyonu içeren bir kontrol grubu da içinde olmak üzere beş gruba randomize edilerek, koledoka skolosidal bir ajan olan povidon iyot (PI) enjeksiyonu ile bir biliyer skleroz modeli oluşturuldu. PI grupları (kontrol hariç tüm gruplar) daha sonra ya halofuginon, ya ursodeoksikolik asit (UDCA), ya ikisi beraber, ya da hiçbir madde verilmeyerek 90 gün takip edildi. Takip sonunda sıçanlar, intrahepatik ve hiler fibrozis tespiti için sakrifiye edildi. Karaciğer hasarının laboratuvar parametreleri ve kollajen degradasyonunu göstermek üzere hidroksiprolin düzeyleri serumda çalışıldı. Fibrotik değişikliklerin analizi için karaciğer ve koledokun histolojik incelemesi yapıldı.

Bulgular: Ursodeoksikolik asit kullanılmış olsun veya olmasın, halofuginon kullanılan grupların histolojik analizinde diğer gruplara göre istatistiksel açıdan anlamlı derecede az skleroz mevcut idi. SGOT, SGPT ve ALP'nin serum analizleri incelendiğinde; "sadece PI kullanılan grup" ve halofuginon grupları arasında anlamlı farklılıklar vardı. GGT sadece PI kullanılan grup için anlamlı derecede yüksekti. Bilirubin düzeyleri açısından gruplar arasında anlamlı fark yoktu. Hidroksiprolin serum düzeyleri "sadece PI kullanılan grup" için en yüksek olup, bunu sadece UDCA kullanılan grup ve ardından halofuginon grupları izledi.

Sonuç: Halofuginon, sıçanlarda indüklenen bir sklerozan kolanjit modelinde UDCA'ya ek bir tıbbi tedavi olarak karaciğer ve safra yollarında fibrozun önlenmesinde etkili olmuştur.

Anahtar Kelimeler: Halofuginon, siroz, ursodeoksikolik asit, sklerozan kolanjit, fibrozis, skolosidal

Abstract

The Effects of Halofuginone and Ursodeoxycholic Acid in Prevention of Sclerosing Cholangitis Caused by Scolocidal Agents

Aim: Biliary sclerosis is a life threatening condition caused by auto-immunity, operative trauma, toxic agents, cancer and chronic inflammatory conditions. The liver tissue may progress into overt cirrhosis by the progression of fibrotic scar tissue. Halofuginone, which is the active component of the plant alkaloid febrifugine, has been shown to inhibit fibrosis.

Material and Methods: Fifty rats were randomized on 5 groups to form a biliary sclerosis model by a scolocidal agent povidone iodine (PI) injection into the common bile duct with a control group of saline infusion included. The four PI groups were later treated by halofuginone, ursodeoxycholic acid (UDCA), with both and none. The rats were sacrificed 90 days later for intrahepatic and hilar fibrosis. Laboratory parameters of liver damage and levels of hydroxyproline to show collagen degradation were obtained and histological examination of the liver and the common bile duct for fibrotic changes were carried out.

Results: With or without application of ursodeoxycholic acid, the halofuginone groups showed significantly less sclerosis according to histological analysis. In regard to serum analysis of SGOT, SGPT and ALP; there were significant differences between "PI only" group and halofuginone groups. GGT was significantly high in "PI only" group. There was not any significant difference between the groups in regard to bilirubin levels. Hydroxyproline serum levels were highest in "PI only" group, followed by "UDCA only" group and then halofuginone groups.

Conclusion: Halofuginone was effective in preventing fibrosis as an additional medical therapy to UDCA in an induced sclerosing cholangitis model in rats.

Keywords: Halofuginone, cirrhosis, ursodeoxycholic acid, sclerosing cholangitis, fibrosis, scolocidal

1. INTRODUCTION

Biliary sclerosis is a chronic inflammatory disease of the biliary epithelium, in which the bile ducts are distorted by obliterative concentric fibrosis and thus eventually causing biliary strictures (1). The most common biochemical marker pertaining is the elevated serum alkaline phosphatase (2). Cholangiography displays the diagnostic and characteristic multifocal strictures within the intra and extra-hepatic bile ducts (3). Continuous injury leads to chronic inflammation, cell proliferation and fibrosis. The hepatic stellate cells in the Disse space are the main contributors to liver fibrosis, which is the key factor in the whole process that ends up with the loss of hepatic parenchyma (4,5). The main aspects of secondary sclerosing cholangitis is inflammation, biliary cholestasis, obliterative fibrosis, stricture formation and progressive destruction of the biliary tree. The main reasons are longlasting biliary obstruction, surgical trauma to the bile duct, ischemic injury and intra-arterial chemotherapy (6). Halofuginone (HF) (Collgard Biopharmaceuticals) is a less toxic form of the plant derivative of the febrifugine which is found in the roots of Blue Evergreen Hydrangea, Dichroa Febrifuga Lour. Halofuginone is an orally-active quinazolinone alkaloid with potential antineoplastic activity. Halofuginone interferes with the signaling pathway of transforming growth factor beta (TGF beta) and inhibits expression of matrix metalloproteinase 2, thereby inhibiting collagen type I synthesis and inducing extracellular matrix degradation, resulting in inhibition of angiogenesis, tumor growth, or metastasis (7). It has been shown that HF selectively inhibits the differentiation of pro-inflammatory T-helper17 cells (8). Also that, febrifugine derivatives are found to compete with proline for the tRNA synthetase active site, causing the accumulation of uncharged tRNA, and mimicking reduced cellular proline availability. HF is able to inhibit tissue fibrosis and tumor progression as well as overproduction and deposition of extracellular matrix components without global effect on protein synthesis (9).

Ursodeoxycholic acid (UDCA) is an endogenous biliary acid (10). When ingested, it is absorbed 30-60 % in the terminal ileum and distal jejunum whence it enters the enterohepatic cycle (11). Ursodeoxycholic acid is used for the treatment of cholestatic liver diseases in three mechanisms: protection of cholangiocytes against toxic hydrophobic bile acids by reduction of the cytotoxicity via decreasing the concentration of hydrophobic bile acids, stimulation of membrane molecules and by inhibiting bile acid induced apoptosis (12). UDCA is the main medical therapy for sclerosing cholangitis. However, it is far from being perfect, unfortunately. We aimed to show the antifibrotic effects of halofuginone on a chemically induced biliary sclerosis model on rats in comparison with UDCA.

2. MATERIALS AND METHODS

Fifty female Sprague-Dawley rats weighing between 250-300 grams were involved in this study at the Animal Study Laboratory of the Cerrahpasa Medical Faculty of Istanbul University. Five groups were provided each with 10 rats: the control group (saline group) (group-1), the povidone iodine group (group-2), halofuginone group (group-3), ursodeoxycholic acid group (group-4), halofuginone plus ursodeoxycholic acid group (group-5). The rats were fed regularly with the same standard chow. The rats were operated on under general anesthesia to isolate the common bile duct to create a biliary sclerosis model through common bile duct infusion. The distal part of the common bile duct was clamped with a bulldog clamp and was catheterized with a no:33 angiocatheter. The control group was given 0,2 cc saline and the other four groups were injected with 0.2 cc povidone iodine (PI), a sclerocidal agent that causes fibrosis in the biliary system. Five minutes later, the bulldog clamps were removed. The 3rd and 5th groups were administered 10 mcg of HF via transperitoneal route for the next six days. For 90 days the groups 4 and 5 were given 20 mg/kg/day ursodeoxycholic acid via oral feeding tube. Ninety days later the rats were sacrificed. The common bile duct and the hepatic hilum were found to be densely sclerotic in the second (polyvinylpyrrolidone only) group and fourth (povidone iodine plus oral ursodeoxycholic acid) groups.

For biochemical studies; 5 cc blood from the right ventricle were obtained by a transcardiac catheter. The whole common bile duct, in addition to specimens from right and left liver were obtained. Biochemical tests of alkaline phosphatase (ALP), serum glutamic oxaloacetic transaminase (SGOT), serum glutamic pyruvic transaminase (SGPT), gamma glutamyl transferase (GGT), total and direct bilirubin tests were carried out immediately. The serum hydroxyproline levels were detected by hidroxyprognosticon kit (Organon, Netherlands). All the subjects were tested for biochemical tests of ALP, SGOT, SGPT, GGT, total and direct bilirubin before the initial operation and before the re-operation at 90th day to predict any defect in the liver functions. The groups were compared with Tukey Multiple Comparison test for pairwise comparisons against $p < 0,05$. All the pathological specimen were stained by hematoxyline-eosin and parenchymal necrosis, hyperemia, portal inflammation, portal fibrosis, proliferation of the ductal epithelium of the biliary tract were studied in the liver tissues. Increased vascularity, mononuclear cell infiltration indicating active inflammation in the common bile duct, and fibroblastic proliferation leading to fibrosis were studied in common bile duct specimens.

Table-1. The severity of inflammation for the liver lobes and the common bile ducts

| | Liver parenchymal necrosis | Liver hyperemia | Portal inflammation within liver | Portal fibrosis in within liver | Proliferation in biliary within tract | Active inflammation in common bile duct | Increased vascularity in common bile duct | Fibrosis in common bile duct |
|--|----------------------------|--------------------|----------------------------------|---------------------------------|---------------------------------------|---|---|------------------------------|
| Saline (Group-1) | 0 | 1±0,66 0,7±0,82 | 0,8±0,69 1,1±0,87 | 0,6±0,5 0,8±0,63 | 0 | 0 | 0 | 0 |
| Povidone iodine only (Group-2) | 2,0±0,9 2,5±0,7 | 2,0±0,8 2,3±0,6 | 1,2±1,21 2,1±0,87 | 1,9±1,1 1,2±1,1 | 2,,7±0,4 2,1±0,31 | 1,7±0,82 | 1,5±0,84 | 2,1±0 |
| Halofuginone (HF) (Group-3) | 1,0±0,81 1,0±0,6 | 1,7±0,5 1,7±0,9 | 0,2±0,4 0,2±0,4 | 0,3±0,6 0 | 0 0,4±0,84 | 1,2±0,63 | 0,2±0,42 | 0,2±0,42 |
| Ursodeoxycholic acid (UDCA) (Group-4) | 0 0 | 1,4±0,9 1,9±0,8 | 1,9±0,9 2,0±0,5 | 1,5±0,7 1,3±0,8 | 1,3±1,1 1,9±0,56 | 1,9±0,7 | 1,0±0,6 | 0,8±0,6 |
| UDCA+HF (Group-5) | 1,±0,7 0,7±0,5 | 1,4±0,7 1,5±0,7 | 0,7±0,5 1,1±0,7 | 0,4±0,5 0,5±0,8 | 1,1±1,3 0,8±1,13 | 1,5±0,5 | 1,0±0,7 | 0,3±0,5 |

Ethical Declaration

This study was composed by reorganization of speciality thesis of the first author dated 2002. There is no ethical approval, but Helsinki Declaration and other bioethical guidelines have been followed during this study.

3. RESULTS

Laboratory examination: The groups were tested for alkaline phosphatase (ALP), serum glutamic oxaloacetic transaminase (SGOT), serum glutamic pyruvic transaminase (SGPT), gamma glutamyl transferase (GGT), total and direct bilirubin and hydroxyproline at the initial operation day and at the 90th day, the day of re-operation. There was no significant difference among the groups preoperatively before the initial operation in terms of SGOT levels. When 5 groups were evaluated preoperatively at the reoperation day, there was no significant difference between control group (saline group) (no 1), UDCA (no 4) and HF plus UDCA (no 5) groups. However, there was significant difference between group 2 (povidone iodine) and HF groups (3 and 5). When postoperative levels were considered, group 1 had the lowest mean value of SGOT, while the group 2 had the highest. When considering the SGPT, there was no significant difference among the groups preoperatively before the initial operation. When 5 groups were evaluated preoperatively at the reoperation, there was significant difference between group 1 and others. When we investigate the ALP levels, there was no significant difference among the groups preoperatively before the initial operation. When 5 groups were evaluated preoperatively at the reoperation, there was no significant difference between control group (no 1), UDCA (4) and HF plus UDCA (no 5) groups. How-

ever, there was significant difference between group 2 (PI) and HF groups (3 and 5). When postoperative levels were considered, group 1 had the lowest mean ALP levels, while the group 2 had the highest. For GGT, there was significant difference between the PI group and others. For bilirubin levels, there were no significant differences between preoperative and pre-reoperation levels among the groups. Hydroxyproline levels were highest in group 2, followed by group 4 and the HF groups (groups 3 and 5).

Pathologic examination: With or without application of ursodeoxycholic acid, the halofuginone groups showed significantly less sclerosis according to histological analysis (Table-1).

The control group (saline group) had no parenchymal necrosis, and no proliferation in the biliary tract, but minimal portal fibrosis, portal inflammation and hyperemia. There were also no active inflammation, no increased vascularity and no fibrosis in the common bile duct (CBD), either.

The second group (povidone iodine only group) showed medium increase in vascularization, high level of fibrosis, active inflammatory infiltration in CBD, and advanced hyperemia, paranchymal necrosis, portal inflammation, portal fibrosis and proliferation of the biliary tract epithelium in liver.

The third group (halofuginone plus povidone iodine) showed no increase in vascularity and fibrosis but mild active inflammation in CBD. There was mild pranchymal necrosis, medium hyperemia, mild portal inflammation, mild portal fibrosis and mild proliferation in the biliary tract epithelium.

The fourth group (ursodeoxycholic acid plus povidone iodine) displayed mild vascularization, medium active inflammation and mild fibrosis in CBD. There was no parenchymal necrosis, but mild hyperemia, medium portal inflammation, portal fibrosis, and biliary tract epithelium proliferation in the liver.

The fifth group (halofuginone plus ursodeoxycholic acid plus povidone iodine) group showed mild fibrosis and mild vascularization and medium inflammation in the CBD. There was mild parenchymal necrosis, mild hyperemia, mild portal inflammation and fibrosis, and mild biliary tract epithelium proliferation in the liver.

Overall, in histological examination, the best results were detected in the saline group (Group-1) followed by HF (Group-3) and HF plus UDCA (Group-5) groups. They were followed by the UDCA (Group-4) group. The worst results are seen in the povidone iodine (Group-2) group, as expected.

4. DISCUSSION

Primary biliary cirrhosis (PBC) is a progressive inflammatory liver disease that leads to the destruction of small interlobular bile ducts, progressive cholestasis, and, eventually, fibrosis and cirrhosis of the liver (13). Cirrhosis, which is the end stage of progressive fibrosis, is characterized by the accumulation of extracellular matrix proteins and the distortion of the hepatic architecture (14). Especially the fibrogenic growth TGF- β is said to play an important role in fibrosis due to its stimulating effect on matrix protein generation and it also inhibits the matrix protein removal (15). Elevation of serum markers of cholestasis, i.e., alkaline phosphatase and gamma-glutamyl transferase play an important role in diagnosis. The only contemporary established treatment for PBC is UDCA 13-15 mg/day, which improves the serum biomarkers such as bilirubin, ALP, GGT. UDCA may slow down histologic progression to liver cirrhosis, improve quality of life, disease-free survival of transplant patients, and overall survival. Survival of PBC patients has been largely improved with the widespread use of ursodeoxycholic acid (UDCA), however, one third of patients still do not respond to the treatment and proceed to liver cirrhosis, requiring liver transplantation as a last chance for cure (13). There is a clear need for additional therapy which could slow down the fibrotic process for any sclerosing cholangitis to prolong survival and to improve the quality of life. Our study proposes that the addition of HF to UDCA therapy can aid in slowing down the fibrotic process in the biliary sclerosing cholangitis. HF has been shown to be effective in situations, which progressive sclerosis is the key pathology. Sclerosis, with progressive replacement of muscle tissue, is a prominent feature in some muscular dystrophy (MD) diseases. TGF- β

is the leading candidate for activating fibroblasts and reducing overproduction of extracellular matrix (ECM) protein in such disorders. Pines et al, found that HF improved muscle histopathology and muscle functions in various MDs, by inhibition of muscle fibrosis, and increased myotube fusion (16). This may justify the idea of using HF in a chronic progressive fibrotic disease like the sclerosing cholangitis. Halofuginone is not only found to be effective in reducing the amount of type-1 collagen, it has also been found to be effective in reducing the cartilage degeneration and subchondral bone deterioration in a similar pathway. Cui et al found that HF was effective in inhibition of subchondral bone TGF- β activity and aberrant angiogenesis (17). HF seems to be effective in various conditions by a similar mechanism which can be of use in many progressive fibrotic situations that may have the same underlying pathological processes. Pines et al, studied an experimental model of chronic hepatitis-associated fibrosis induced by intraperitoneal administration of dimethylnitrosamine (DMN) in mice. Hepatic cirrhosis is characterized by excessive deposition of collagen, resulting from an increase in type I collagen gene transcription. HF treatment, before the onset of fibrosis, inhibited the rise in the collagen type I gene expression, and resulted in reduced liver collagen. HF reduced plasma alkaline phosphatase activity as well as mortality. This study, which was similar to our study, confirmed our results as well (18). There are various other agents used to create a fibrotic process in the liver. In a similar model, Bruck et al (19), investigated the effect of halofuginone on a model of thioacetamide (TAA) induced liver fibrosis in rats. Halofuginone given orally before fibrosis induction prevented the activation of most of the stellate cells and the remaining cells produced low levels of collagen. When given to rats with established fibrosis, they found out that HF caused almost complete resolution of the fibrotic condition. By investigating the effect of HF on a TAA induced hepatic fibrosis model, they administered HF orally 2-4 weeks. HF inhibited the collagen synthesis by the stellate cells. These results were similar with our pathological findings. Liang et al (20), investigated concavaline A (Con A) to induce liver fibrosis. Con A infusion significantly elevated the ALT, AST levels, while the oral administration of HF significantly reduced the level of the transaminases. The histologic examination showed a significant reduction in the severity of liver fibrosis. These two findings were similar to our results. There are also other studies to justify the use of HF in any condition with excess collagen accumulation. Yavas et al, investigated the use of HF in a lung model. Lung is very sensitive to ionizing radiation leading to fibrosis and this may limit the therapeutic effects of radiation on thorax. HF has been shown to protect the lung tissue against radiation caused fibrosis by suppressing the TGF- β signaling pathway(21). Choi et al (22), evaluated the effect of HF on a rabbit model for intimal hyperplasia in an

end to end anastomosis of the right common carotid artery in a double blind study. Intimal hyperplasia is caused by smooth muscle proliferation and extracellular matrix accumulation. Oral HF was found to inhibit muscle cell proliferation and reduced the anastomotic intimal thickness. HF can be of use to prevent arterial stenosis in vascular surgery. HF has been studied on rabbits about prevention of arterial stenosis and atherosclerosis. Arteriosclerosis and restenosis after angioplasty are caused by the proliferation of vascular smooth muscle cells and collection of extracellular matrix components within the arterial wall as a response to local injury. HF inhibits the extracellular matrix deposition and collagen type I synthesis and this ability to reduce injury-induced intimal hyperplasia may place halofuginone alone or in combination with other antiproliferative compounds as a potential candidate for prevention of arterial stenosis and atherosclerosis (23). The rationale behind the use of HF as an adjunct to UDCA is that, like our study and other similar studies have shown, HF inhibits the overproduction and deposition of extracellular matrix components without changing the overall protein synthesis. HF has been shown to be effective in reducing the fibrosis in different parts of the body such as liver, cartilages and lungs. Our results indicate that the combined use of HF with UDCA provided better results than only UDCA in a rat model. By far, this is the only study we know that compares the therapeutic effect of UDCA against HF, and combination of the two, as well.

In conclusion; halofuginone may be effective in any chronic fibrotic disease which is mediated by TGF- β and TH17 activation that causes accumulation of extracellular matrix protein and overproduction of type 1 collagen. HF was effective in preventing fibrosis as an additional medical therapy to UDCA in an induced sclerosing cholangitis model in rats, which would be interesting for both surgical and medical investigators and their branches.

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