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BRIEF ARTICLE

Hyperoxia accelerates progression of hepatic fibrosis by up-regulation of transforming growth factor-β expression

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Abstract

AIM: To investigate the effect of hypoxia or hyperoxia on the progression of hepatic fibrosis and to examine the role of transforming growth factor-β (TGF-β) in the livers of rats exposed to hypoxic or hyperoxic conditions.

METHODS: Male Sprague-Dawley rats were injected intraperitoneally with thioacetamide to induce hepatic fibrosis and were randomly divided into a hypoxia group, a hyperoxia group and an untreated control group. Ten rats in the hypoxia group were exposed to an altitude of 20000 ft for 1 h/d during 7 wk. Ten rats in the hyperoxia group were exposed to a water depth of 20 m with 100% oxygen supply for 1 h/d during 7 wk. We evaluated the degree of hepatic fibrosis using Masson trichrome stain and examined the expression level of hepatic TGF-β mRNA using quantitative real-time reverse transcriptase-polymerase chain reaction analysis.

RESULTS: Eight of 10 rats exposed to hypoxia showed diffuse and confluent fibrosis with the formation of structurally abnormal parenchymal nodules involving the entire liver, consistent with hepatic cirrhosis. Nine of 10 rats exposed to hyperoxia also demonstrated obvious histological findings of hepatic cirrhosis identical to those in hypoxic rat livers. In contrast, 8 of 10 untreated rats had periportal or septal fibrosis only. The frequency of hepatic cirrhosis in hypoxic rats (P = 0.009) and hyperoxic rats (P = 0.003) was significantly higher than that in untreated rats. In addition, hepatic TGF-β mRNA levels in hyperoxic rats were significantly higher than those in untreated rats. The mean value of the normalized TGF-β mRNA/β-actin expression ratio in the hyperoxic rats was 1.9-fold higher than that in the untreated rats (P = 0.027).

CONCLUSION: We demonstrated that both hypoxia and hyperoxia accelerated the progression of hepatic fibrosis in rats. Significant up-regulation of hepatic TGF-β in hyperoxic rats suggests that TGF-β is involved in the acceleration of hepatic fibrosis under hyperoxic conditions.

Key words: Hepatic fibrosis; Cirrhosis; Hypoxia; Hyperoxia; Transforming growth factor-β

Core tip: We observed that both hypoxic and hyperoxic rat livers exhibited significantly higher frequencies of hepatic cirrhosis than untreated rat livers. We also observed that hepatic transforming growth factor-β

(TGF-β) expression in hyperoxic rats was significant higher than that in untreated rats, suggesting that TGF-β is involved in the acceleration of hepatic fibrosis under hyperoxic conditions. To the best of our knowledge, up-regulated TGF-β expression in the livers of cirrhotic rats exposed to hyperoxia has not been reported.

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INTRODUCTION

Hepatic fibrosis is an essential pathophysiological consequence of various chronic liver injuries and a common underlying mechanism for hepatic insufficiency[1]. Hepatic fibrosis is currently known to be part of a dynamic process in the setting of liver injury that leads to an abnormal accumulation of extracellular matrix in the liver. The endpoint of hepatic fibrosis is cirrhosis, which is characterized by the formation of regenerative nodules of liver parenchyma separated by confluent fibrotic septa and is accompanied by significant morbidity and mortality[2]. Complex interaction between various liver cell types, such as hepatic stellate cells, hepatocytes, Kupffer cells, and liver sinusoidal endothelial cells, contributes to hepatic fibrosis. Each of these cell types releases a subset of mediators and cytokines that have diverse effects on the progression of hepatic fibrosis and the development of hepatic cirrhosis[1].

Extensive studies using animal models of hepatic fibrosis have revealed that several groups of key genes mediate liver fibrogenesis[3,4]. For example, genes regulating hepatocellular apoptosis and necrosis influence the extent of tissue damage and the subsequent profibrogenic response[5,6]. Genes regulating the generation of pro-inflammatory cytokines and reactive oxygen species (ROS) determine the profibrogenic response to injury and extracellular matrix deposition[7-10]. Among these, a particularly well-studied molecule is transforming growth factor-β (TGF-β), which is widely regarded as profibrogenic in liver injury[11]. A number of studies have identified TGF-β as the most important fibrogenesis-stimulating cytokine in the liver[1,2,12]. The findings of increased hepatic stellate cell activation and hepatic fibrosis in mice with elevated hepatic TGF-β level provide direct evidence for the critical role of TGF-β in hepatic fibrosis[13]. TGF-β is also considered profibrogenic because it inhibits hepatic stellate cell apoptosis, thereby contributing to the increased number of these cells in the setting of liver injury[14]. In addition, TGF-β stimulates extracellular matrix production by liver sinusoidal endothelial cells[15]. Strategies aimed at disrupting the TGF-β signaling pathway have markedly decreased hepatic fibrosis in experimental models[16,17].

Oxygen has important functions as a substrate for biochemical reactions and a modulator of gene expression. Impaired tissue oxygenation and cellular hypoxia are major components in the pathophysiology of a variety of clinical conditions, including infection, wounds, stroke, myocardial infarction, chronic lung disease, and hepatic fibrosis[18,19]. Hypoxia always occurs during liver injury or inflammation, in which swelling of hepatocytes, accumulation of extracellular matrix in the spaces of Disse, construction of regenerating parenchymal nodules and fibrotic septa, and the formation of abnormal vascular networks indicate that hepatocellular hypoxia is involved in the progression of hepatic fibrosis and tissue remodeling[20-25]. Conversely, exposure to hyperoxic conditions generally improves tissue oxygenation. A dramatic increase in oxygen content and arterial blood oxygen pressures accounts for the markedly facilitated diffusion of oxygen to tissues during hyperoxic exposure[26]. However, the advantage of hyperoxia in augmenting oxygen availability to tissues is challenged by the commonly accepted paradigm of cell injury, which emphasizes the role of ROS formation leading to the up-regulation of pro-inflammatory mediators and aggravation of tissue damage[27]. In particular, previous data have suggested that ROS generation is a fundamental mechanism of liver injury[28]. ROS can damage cellular macromolecules and therefore may participate in hepatocellular injury when produced in excess[29]. Moreover, increasing evidence implicates ROS in the pathogenesis of hepatic fibrosis and cirrhosis[29,30].

Although several studies have investigated the relationship between tissue fibrosis and hypoxia, they have focused on the effects of hypoxia in diseases of the heart, lung, and kidney[18,31,32]. Furthermore, the effect of hyperoxic exposure on hepatic fibrosis has not yet been reported. Therefore, the relationship between hepatic fibrosis and hypoxic or hyperoxic conditions *in vivo* remain to be elucidated. The aim of this study was to investigate the effects of hypoxia or hyperoxia on the progression of hepatic fibrosis and to examine the expression level of TGF-β in the livers of rats exposed to hypoxic or hyperoxic conditions.

MATERIALS AND METHODS

Experimental model

Throughout the experimental period (7 wk), 30 Sprague-Dawley adult male rats (Samtako Bio Korea Co., Ltd., Osan, Gyeonggi-do, South Korea) 6-7 wk of age and weighing between 200 and 230 g were fed standard laboratory rat chow, provided with free access to water, and maintained on a 12-h light-dark cycle under pathogen-free conditions. Temperature and moisture were controlled at 20-25 ℃ and 40%-45%, respectively. To induce hepatic fibrosis, the rats were injected intraperitoneally with 200 mg/kg of thioacetamide (dissolved in

Table 1 Degree of hepatic fibrosis

Descriptive diagnosis	Definition
No fibrosis	Normal connective tissue
Portal fibrosis	Fibrous portal expansion
Periportal fibrosis	Periportal fibrosis with short septa extending into lobules or rare porto-portal septa (intact architecture)
Septal fibrosis	Fibrous septa reaching adjacent portal tracts and terminal hepatic venules (architectural distortion, but no obvious cirrhosis)
Cirrhosis	Diffuse nodular formation

saline; Sigma-Aldrich Co., St. Louis, MO, United States) twice per week. The rats were randomly divided into 3 experimental groups. Ten rats in the hypoxia group were exposed to an altitude of 20000 ft for 1 h/d. Ten rats in the hyperoxia group were exposed to a water depth of 20 m with 100% oxygen supply for 1 h/d. The remaining 10 rats were used as an untreated control group. Animals were killed using diethyl ether 3 d after the last thioacetamide injection and laparotomized *via* a midline incision. Livers were removed from the animals and immediately preserved in a 10% formaldehyde (formalin) solution or frozen in liquid nitrogen and stored at -70 ℃ until quantitative real-time reverse-transcriptase polymerase chain reaction (RT-PCR) analysis was performed. The institutional animal ethics committee of the Republic of Korea Air Force Aerospace Medical Center approved all experimental procedures involving animals.

Histological evaluation

After 48 h of formalin fixation, the liver tissues were embedded in paraffin and processed for routine hematoxylin-eosin and for Masson trichrome stains. The degree of hepatic fibrosis was graded according to the standardized guideline proposed by the Korean Study Group for Pathology of Digestive Diseases (Table 1)[33].

Quantitative real-time RT-PCR analysis

Total RNA was extracted from the liver tissue samples using a NucleoSpin RNA Ⅱ extraction kit (Macherey-Nagel GmbH and Co. KG, Dueren, Germany). For complementary DNA (cDNA) synthesis, 1 μg of total RNA was reverse-transcribed using a ReverTra Ace-α-reverse transcriptase kit (Toyobo Co., Ltd., Osaka, Japan). The reverse-transcribed cDNA was used for real-time RT-PCR with SsoAdvanced SYBR Green Supermix (Bio-Rad Laboratories, Inc., Hercules, CA, United States). PCR was performed using a Bio-Rad CFX384 Real-Time PCR Detection System (Bio-Rad Laboratories, Inc.). The primer sequences used for TGF-β were as follows: forward 5'-AGGGCTACCATGCCAACTTC-3'; reverse 5'-CCACGTAGTAGACGATGGGC-3'. The primer sequences used for β-actin were as follows: forward 5'-TCTTCCAGCCTTCCTTCCTG-3'; reverse 5'-CACACAGAGTACTTGCGCTC-3'. PCR reactions for TGF-β and β-actin were initiated with an initial PCR activation step at 95 ℃ for 30 s, followed by 45 cycles at 95 ℃ for 2 s, 58 ℃ for 5 s, and 65 ℃ for 10 s. A melting curve, ramping from 65 ℃ to 95 ℃, was produced after each RT-PCR to test for the presence of primer dimers. When primer dimer formation was detected, the PCR was rerun using a separate aliquot of cDNA. Each measurement was repeated twice. The normalized expression ratio was calculated using the $2^{-\Delta\Delta Ct}$ method[34].

Statistical analysis

Fisher's exact test was performed to compare the degree of hepatic fibrosis between groups. The Kruskal-Wallis test was performed to examine whether the expression level of hepatic TGF-β messenger RNA (mRNA) differed significantly between the groups. Statistical analyses were performed using SPSS version 20.0 (IBM SPSS Inc., Chicago, IL, United States). A P value of less than 0.05 was deemed statistically significant.

RESULTS

Histologically, all specimens showed hepatic fibrosis, although the degree of fibrosis was variable. Eight of 10 rats exposed to hypoxia showed diffuse and confluent fibrosis with the formation of structurally abnormal parenchymal nodules involving the entire liver, consistent with hepatic cirrhosis (Figure 1A). Nine of 10 rats exposed to hyperoxia also demonstrated obvious histological findings of hepatic cirrhosis identical to those in hypoxic rat livers (Figure 1B). In contrast, only 2 of 10 untreated rats exhibited hepatic cirrhosis; the remaining 8 rats in the control group exhibited periportal (2 rats) or septal (6 rats) fibrosis only (Figure 1C). The frequency of hepatic cirrhosis in the hypoxic rats (P = 0.009) and hyperoxic rats (P = 0.003) was significantly higher than that in the untreated rats (Table 2).

Quantitative real-time RT-PCR revealed that the expression level of hepatic TGF-β mRNA in the hyperoxic rats was significantly higher than that in the untreated rats (P = 0.027; Figure 2). The mean value of the normalized TGF-β mRNA/β-actin expression ratio in the hyperoxic rats was 1.9-fold higher than that in the untreated rats (Table 3). In contrast, the TGF-β mRNA levels in the livers of hypoxic rats were not significantly different from those in the untreated rats (P = 1.000; Figure 2).

DISCUSSION

In this study, we investigated the effect of hypoxia or hyperoxia on hepatic fibrosis progression using an animal model of hepatic fibrosis. We observed that both hypoxic and hyperoxic rat livers exhibited frequencies of hepatic cirrhosis that were significantly higher than those in untreated, normoxic rat livers. Hypoxia is known to be a key factor in tissue damage and to play a crucial role in chronic liver injury. Exposure to hypoxic conditions stimulates the release of a variety of mediators from he-

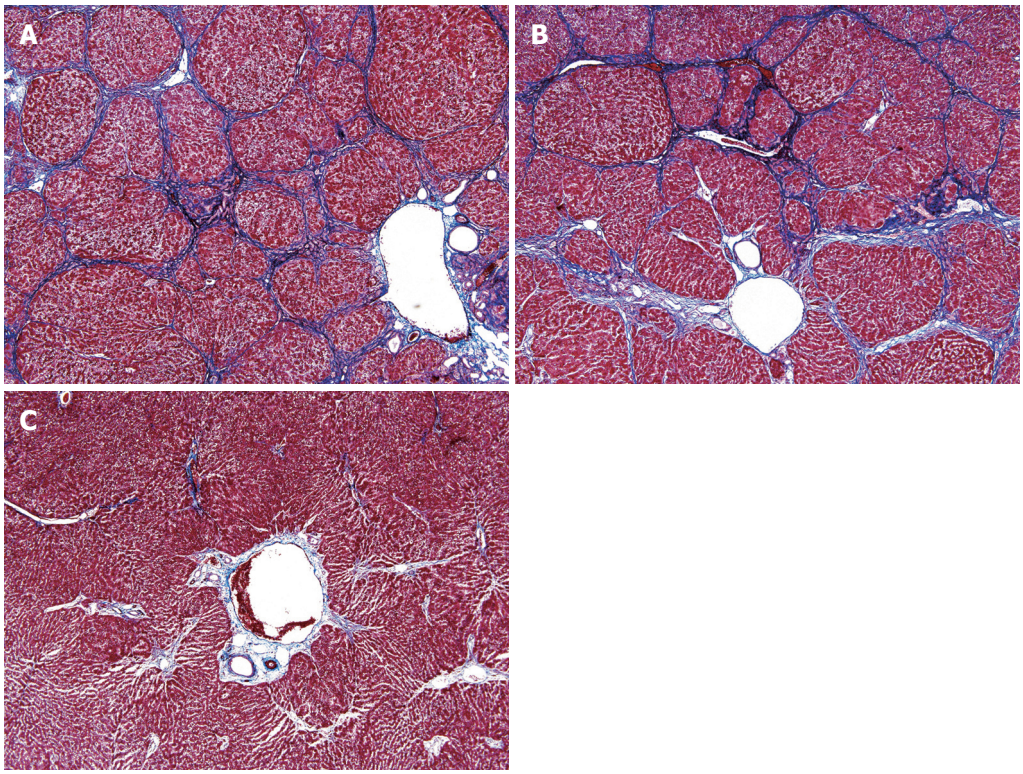

Figure 1 Histology of hepatic fibrosis and cirrhosis. A, B: The livers obtained from 8 of 10 hypoxic rats (A) and 9 of 10 hyperoxic rats (B) demonstrated obvious histological findings of hepatic cirrhosis, including the formation of structurally abnormal parenchymal nodules and confluent fibrotic septa; C: In contrast, 6 of 10 untreated rat livers showed periportal fibrosis only (Masson trichrome stain; original magnification, × 40).

Table 2 Difference in the degree of hepatic fibrosis among the three groups

Diagnosis	Number of rats		
	Control group (n = 10)	Hypoxia group (n = 10)	Hyperoxia group (n = 10)
Periportal fibrosis	2	0	0
Septal fibrosis	6	2	1
Cirrhosis	2	8[1]	9[2]

[1]P = 0.009 *vs* control group; [2]P = 0.003 *vs* control group.

patic stellate cells and affects the progression of hepatic fibrosis[21,35-37]. Hernandez-Guerra *et al*[38] revealed that hypoxia aggravates intrahepatic endothelial dysfunction in cirrhotic rat livers. Some *in vitro* studies have also reported that hypoxia activates hepatic stellate cells and accelerates hepatic fibrosis[22,23,25]. In this context, our finding supports the notion that exposure to hypoxia worsens hepatic fibrosis. In contrast, the effect of hyperoxia on the progression of hepatic fibrosis or the development of hepatic cirrhosis has very rarely been reported. Instead, some available data are sufficient to support the suggestion that hyperoxia exerts beneficial anti-inflammatory effects in models of tissue hypoxia and has protective effects on hemodynamic and metabolic parameters against splanchnic ischemia-reperfusion in rats[39,40]. However, hyperoxic exposure is generally accepted to often result in the formation of ROS in tissues directly implicated in the induction of cell injury *via* lipid peroxidation and up-regulation of pro-inflammatory cytokines[41-44]. In the liver, Kupffer cells and neutrophils can induce oxidative tissue damage both directly *via* the effect of ROS on cellular components and indirectly through the release of protease[45]. Increased oxidative stress and the formation of ROS cause extensive necrosis of the liver and ultimately contribute to the development of fibrosis and cirrhosis. Oxidative stress figures prominently in several scenarios of liver fibrogenesis[46,47]. Bhandari *et al*[48] demonstrated that patients with Child-Pugh class C cirrhosis have greater oxidative stress than those with class B cirrhosis, suggesting that the severity of hepatic cirrhosis is associated with the degree of oxidative stress. Given these data, our findings suggest that hyperoxic exposure accelerates the progression of hepatic fibrosis. In addition, this study can serve as a background for assessing the role of antioxidants in preventing the progression of hepatic cirrhosis.

To clarify the role of TGF-β on hepatic fibrosis under hypoxic or hyperoxic conditions, we investigated the expression of hepatic TGF-β mRNA in rats exposed to hypoxia or hyperoxia. Although a number of studies have examined the effect of TGF-β in experimental models of hepatic fibrosis, to the best of our knowledge, TGF-β expression in the livers of cirrhotic rats exposed to hypoxia or hyperoxia has not been reported. We observed that hepatic TGF-β mRNA level in hyperoxic rats was significantly higher than that in untreated rats. This finding is consistent with previous

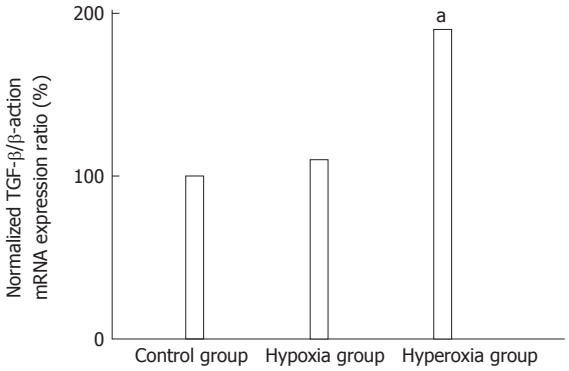

Figure 2 Quantitation of hepatic transformation growth factor-β mRNA expression level using real-time reverse-transcriptase polymerase chain reaction analysis. Results are mean values of 2 independent experiments with 10 rats in each group. The mean value of the normalized hepatic transformation growth factor-β (TGF-β)/β-actin mRNA expression ratio in the hyperoxic rats was significantly higher than that in the untreated rats. $^{a}P < 0.05$ *vs* the corresponding values in the control group.

data which showed that hyperoxic exposure elevated the expression level of TGF-β[49]. Similarly, Alejandre-Alcazar *et al*[50] demonstrated that hyperoxia up-regulates the expression level of key components of the TGF-β signaling pathway, including type Ⅱ TGF-β receptor, type I bone morphogenetic protein receptors, and Smad proteins. They further illustrated that TGF-β stimulation dramatically elevates the levels of tissue inhibitors of metalloproteinase-1 mRNA in fibroblasts, supporting the idea that dysregulation of TGF-β signaling impacts extracellular matrix deposition and tissue remodeling[50]. Together with these results, the observation in the present study that hyperoxic rats exhibited a significantly higher frequency of hepatic cirrhosis than untreated rats suggests that TGF-β is partially responsible for the acceleration of hepatic fibrosis progression in rats exposed to hyperoxia.

However, no significant difference between hepatic TGF-β mRNA levels in hypoxic rats and untreated rats was found. In fact, relevant experimental studies have provided conflicting results regarding the effect of hypoxia on TGF-β expression. An *in vitro* study demonstrated that hypoxic exposure induces an increase in the expression level of TGF-β mRNA[51]. A study using a model of carbon tetrachloride-induced hepatic fibrosis also showed that hypoxic rat livers release TGF-β protein[52]. Similarly, hypoxia has been shown to up-regulate TGF-β synthesis and enhance its effect in human skin and lung fibroblasts[53,54]. In contrast, a study using a cell culture method has shown that hypoxia increases the production of fibronectin, collagen I , and collagen Ⅳ, but not TGF-β, in placental fibroblasts, suggesting that increased extracellular matrix production under hypoxia is not mediated directly by increased TGF-β[55]. Our observation raises 2 possibilities. First, hepatic fibrosis might be accelerated independent of TGF-β under hypoxic conditions. Second, intermittent exposure to hypobaric hypoxia at the altitude of 20000 ft might be insufficient to affect TGF-β mRNA transcription in rat liver. Further investigations are necessary to clarify the relationship between TGF-β expression and the progression of hepatic fibrosis under hypoxic conditions.

Table 3 Quantitative real-time reverse-transcriptase polymerase chain reaction results

Measurement	Control group	Hypoxia group	Hyperoxia group
TGF-β mRNA average Ct	31.51 ± 1.49	30.84 ± 1.07	29.57 ± 0.96
β-actin mRNA average Ct	23.99 ± 1.18	23.35 ± 1.27	22.90 ± 0.72
$^{\Delta}$Ct	7.52 ± 0.63	7.49 ± 0.65	6.67 ± 0.67
$^{\Delta\Delta}$Ct	0 ± 0.63	-0.03 ± 0.65	-0.77 ± 0.74
Normalized expression ratio	1.0	1.1[1]	1.9[2]

[1]$P = 1.000$ *vs* control group, [2]$P = 0.027$ *vs* control group.

In conclusion, we demonstrated that both hypoxia and hyperoxia accelerated the progression of hepatic fibrosis in rats. In addition, a significant up-regulation of hepatic TGF-β in hyperoxic rats suggests that TGF-β is involved in the acceleration of hepatic fibrosis under hyperoxic conditions.

COMMENTS

Background

Hepatic fibrosis is a pathophysiological consequence of chronic liver injuries and a common underlying mechanism for hepatic insufficiency. Experimental models of hepatic fibrosis have provided a means to study the cell and molecular mediators of fibrosis and revealed that several groups of key genes mediate liver fibrogenesis. In particular, extensive studies have identified transforming growth factor (TGF)-β as the most important profibrogenic cytokine in the liver. Although a number of studies have investigated the relationship between tissue fibrosis and oxygen tension, they have focused on the effects of hypoxia in diseases of the heart, lung, and kidney. The effect of exposure to hyperoxic conditions on hepatic fibrosis and the role of TGF-β in the progression of fibrosis in hyperoxic livers have not yet been reported.

Research frontiers

Hyperoxic exposure is generally accepted to result in the formation of reactive oxygen species (ROS) in a variety of tissues directly implicated in the induction of cell injury *via* lipid peroxidation and up-regulation of pro-inflammatory cytokines. In the liver, Kupffer cells and neutrophils can induce oxidative tissue damage both directly *via* the effect of ROS on cellular components and indirectly through the release of proteolytic enzymes. Furthermore, the formation of ROS and increased oxidative stress cause extensive hepatocellular necrosis and ultimately contribute to the development of hepatic fibrosis and cirrhosis. Oxidative stress figures prominently in several scenarios of liver fibrogenesis.

Innovations and breakthroughs

The authors observed that hyperoxic livers exhibited frequencies of hepatic cirrhosis that were significantly higher than those in untreated, normoxic livers. In addition, they further observed that hepatic TGF-β mRNA level in hyperoxic rats was significant higher than that in untreated rats. To the best of our knowledge, no reports have been published demonstrating a significant relationship between exposure to hyperoxia and frequencies of hepatic cirrhosis and discussing the role of TGF-β in the progression of hepatic fibrosis in hyperoxic rat livers. The observations in the present study suggest that TGF-β is partially responsible for the acceleration of hepatic fibrosis progression in rats exposed to hyperoxia.

Applications

Based on the observation that hyperoxic exposure accelerates the progression of hepatic fibrosis, this study may serve as a background for assessing the role of antioxidants in preventing the progression of hepatic cirrhosis.

Terminology

Hepatic cirrhosis is the endpoint of hepatic fibrosis and various chronic liver diseases characterized by the formation of regenerative nodules of liver parenchyma separated by confluent fibrotic septa. It is typically accompanied by significant morbidity and mortality. TGF-β is a protein that controls the cell cycle, apoptosis, proliferation and differentiation in most cells. In the liver, it plays a crucial role in inhibiting hepatic stellate cell apoptosis, thereby contributing to the increased number of these cells in the setting of liver injury. In addition, TGF-β stimulates extracellular matrix production by liver sinusoidal endothelial cells.

Peer review

This is well performed study, in which authors analyzed the frequency of hepatic cirrhosis and TGF-β expression status in rats exposed to hyperoxia. The results are interesting and suggest that TGF-β accelerates the progression of hepatic fibrosis following hyperoxic exposure. Further investigations are recommended to elucidate the molecular biological functions of TGF-β and a potential role of ROS in association with hyperoxia in the liver.

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