

Original Article

Niacin regresses collagen content in human hepatic stellate cells from liver transplant donors with fibrotic non-alcoholic steatohepatitis (NASH)

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Abstract: In patients with non-alcoholic steatohepatitis (NASH), the onset of fibrosis is a major predictor of cirrhosis and its deadly complications. There is no approved effective pharmacologic therapy for liver fibrosis. Niacin (in pharmacologic concentrations or dose) reverses hepatic steatosis and steatohepatitis. Niacin's efficacy on human hepatic fibrosis is unknown. We investigated the effect of niacin on reversal of preexisting collagen content, in cultured primary human hepatic stellate cells (HSC) obtained from 7 donor livers (processed for transplantation) selected from 5 deceased patients having histologically diagnosed NASH with fibrosis (F1-F3) and 2 non-NASH-fibrosis subjects (Samsara Sciences, Inc., now LifeNet Health). Pharmacologically relevant concentrations of niacin produced a robust and significant dose and time-dependent regression of pre-existing fibrosis by an average of 47.6% and 60.1% (0.25 and 0.5 mM niacin at 48 h incubation) and 53.5% and 65.0% (0.25 and 0.5 mM niacin at 96 h incubation), respectively. In stellate cells from non-NASH-fibrosis subjects, niacin prevented, and regressed fibrosis induced by liver fibrosis stimulators, transforming growth factor- β (TGF- β) and hydrogen peroxide. Niacin significantly inhibited oxidative stress induced by stressors, palmitic acid, or hydrogen peroxide by 52% and 50%, respectively. Translationally, these human HSC data, coupled with emerging *in vivo* animal data and *in vitro* human hepatocyte data, suggest that niacin (used clinically for dyslipidemia) could be repurposed as an effective drug for the clinical treatment of patients with NASH-fibrosis or liver cirrhosis. This is in addition to its known efficacy for reversing steatohepatitis and steatosis which can also result in liver cirrhosis.

Keywords: Nicotinic acid, collagen, hepatic fibrosis, liver cirrhosis, oxidative stress, non-alcoholic steatohepatitis (NASH)

Introduction

Non-alcoholic steatohepatitis (NASH), a severe sub-type of non-alcoholic fatty liver disease (NAFLD), is characterized by steatosis (fatty liver), inflammation, and hepatocellular injury and/or liver fibrosis and it affects about 20 million people in the United States alone [1, 2]. The severity of liver fibrosis in patients with NASH is the strongest predictor of liver-related mortality [3]. Hepatic fibrosis (excessive deposition of Collagen type 1 produced by activated stellate cells) is a wound healing response of the liver, and is caused by chronic liver injuries with diverse etiologies including NASH, viral hepatitis, alcoholic and autoimmune liver diseases [4]. Increased hepatic oxidative stress plays a crucial role in stellate cell activation and

hepatic fibrosis [5]. Hepatic fibrosis with persistent deposition of collagen results in distortion of hepatic parenchyma and vascular structure, clinically manifesting as liver cirrhosis, with fatal complications. The unmet need is a therapeutic agent that will reverse liver fibrosis in a patient with cirrhosis, thus reducing portal hypertension and its fatal sequelae including esophageal varices and hemorrhage.

Although several drugs and targets have been under investigation, currently there are no FDA-approved pharmacological agents (s) for treating liver fibrosis or cirrhosis [6, 7]. Several pre-clinical models to test antifibrotic drugs have been used for drug development. These include *in vitro* methods including HSC lines, precision-cut tissue culture slices, and artificial organ tis-

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Table 1. Human donor subjects demographics, medical history, and liver histology

Donor Subjects	Gender, Race, Age (years)	Weight (kg), BMI	Past Medical History	Cause of Death	Liver Pathology**			
					NAS*	Steatosis	Inflammation	Fibrosis
1	Female, Caucasian, 59	63.6, 27.4	Hyperlipidemia	ICH/Stroke	0 of 8	0 of 3	0 of 4	0 of 4
2	Male, African American, 24	72.7, 22.9	None	Trauma	0 of 8	0 of 3	0 of 4	0 of 4
3	Female, Hispanic, 35	87.6, 34.2	Diabetes type II, Pancreatitis, high cholesterol, fatty liver, history of Thyroid cancer	CVA/Stroke	2 of 8	1 of 3	1 of 4	2-3 of 4
4	Male, Hispanic, 45	89.5, 35	Diabetes Type II, Hypertension, Schizophrenia	Anoxia/CVD	3 of 8	1 of 3	2 of 4	2 of 4
5	Female, Caucasian, 53	127, 48.06	Hypertension, Diabetes Type II, Depression	Cardiac arrest/ Anoxia	3 of 8	2 of 3	1 of 4	1-2 of 4
6	Female, Asian, 44	100, 34.5	Diabetes Type II, Kidney disease, Hyperlipidemia, Asthma, Arthritis	Anoxia/Diabetic Ketoacidosis	4 of 8	3 of 3	1 of 4	1-2 of 4
7	Male, African American, 66	113, 37.0	CAD/CABG, Hypertension, Hypercholesterolemia	Cardiac arrest/ Anoxia	5 of 8	2 of 3	2 of 4	1 of 4

*NAS = NAFLD Activity Score; **NASH CRN Scoring System (Hepatology 41: 1313-1321, 2005).

sue culture engineering. Various animal models in rats and mice using hepatotoxins or transgenic gene approaches or NASH models have also been used [8]. As pointed out by Schuppan and associates, there are pros and cons to all these methods, and none are ideal for human fibrosis/cirrhosis. The use of primary HSC for transplant livers from patients with NASH-fibrosis has not been researched much because of the difficulty in coordinating the collection of these samples. In this research, we have uniquely obtained primary human HSC from livers that were professionally processed for transplantation. We believe that the data obtained from our initial research reported herein have important translational implications as discussed below.

Based on our research on niacin, we proposed the concept that niacin, a currently used drug for dyslipidemia and cardiovascular disease, can be repurposed for the treatment of liver fibrosis [9]. Research from our group showed that niacin prevented and regressed steatosis in an animal model of NAFLD [10]. Additionally, we showed that niacin significantly decreased the production of reactive oxygen species (ROS, an index of oxidative stress) and inflammation in human hepatocytes [11]. In a small clinical trial in 39 hypertriglyceridemic patients with steatosis, treatment with niacin extended-release for 6 months showed a reduction of liver fat by 47% and reductions in liver enzymes and C-reactive protein from baseline [12].

In this communication, we present data indicating that niacin significantly regresses preexisting elevated collagen content in hepatic stel-

late cells selected from donor livers (in process for transplantation) from recently deceased patients and with histologically diagnosed NASH with fibrosis (F1-F3), and from non-NASH-Fibrosis donors.

Materials and methods

Human hepatic stellate cells

Cryopreserved primary cultures of human hepatic stellate cells (HSC, passage 0) isolated from 7 cadaveric livers that were processed for transplantation, were commercially purchased from Samsara Sciences, Inc., San Diego, CA (now LifeNet Health). Clinical data on these deceased individuals were obtained from the same company. All 7 subjects had sudden cardiorespiratory arrest from a variety of causes including intracerebral hemorrhage, vehicular accident, cardiac arrests, or diabetic ketoacidosis. The clinical course was mainly in resuscitation attempts including intensive care monitoring of vital signs and other relevant treatments related to survival. After death pronouncement, their liver was harvested as soon as possible by a team specializing in liver transplantation. Liver biopsies were performed by the team as part of their assessment. For this investigation reported herein, stellate cells were harvested from patients 3-7 (as detailed in **Table 1**) who had NASH with Fibrosis. Patients 1 and 2 were controls who had no evidence of NASH. None of the patients had liver CT scanning done as part of their management prior to final death. After reviewing all the available data, relevant demographic details and known medical history are given in **Table 1**.

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Detailed donor subjects' history reports including donor demographic characteristics, cause of death, medical history, and liver pathology provided by Samsara Sciences are summarized in **Table 1**. None of these human subjects were executed prisoners or institutionalized individuals. Detailed product information on these individual human hepatic cells including isolation of stellate cells from the liver, their morphology, and phenotypic characteristics was established by the vendor and included in the product sheets.

Liver pathology assessment by a certified pathologist revealed a varying degree of liver histology in patients with fibrosis including NAFLD activity scores 2-5, steatosis grades 1-3, inflammation scores 1-2, and fibrosis scores 1-3 (**Table 1**). Histological assessment performed on liver samples in non-NASH-Fibrosis donor subjects showed a NAFLD activity score of 0, steatosis grade 0, inflammation score 0, and fibrosis score 0.

Cryopreserved HSCs were grown in DMEM + 10% FBS media containing 1% antibiotic/antimycotic according to the recommended media and procedures provided by Samsara Sciences. HSCs in passage 2 were used for all *in vitro* studies described below. During experimental incubations with niacin, TGF- β , or H₂O₂, cells were incubated in DMEM + 0.5% FBS.

Histochemical and Enzyme-linked Immunosorbent Assay (ELISA) for hepatic stellate cell total collagen and collagen type 1 quantification: Histochemical visualization of total collagen was performed using Sirius Red staining procedure. Sirius Red is a unique dye that specifically binds to all collagen types. In brief, stellate cells grown in glass slides were stained with Sirius Red (0.1% dissolved in aqueous saturated picric acid) for 1 hour followed by thorough washing in 1% acetic acid (three times), dehydrated, and mounted with Clarion mounting medium and coverslips. Slides were visualized and imaged using a 10X objective on a microscope. Sirius Red stained cells were used for photographic images (magnification 10x) for a qualitative visual representation of total collagen content showing red in color in various treatment groups.

Additionally, quantitative total collagen content in stellate cell lysates was also measured using the commercially available Sirius Red Total

Collagen Detection Assay kit, Catalog # 9062 (Chondrex, Inc., Redmond, WA) as described in the kit procedure instructions. The Sirius Red stained total collagen content in stellate cell lysate was quantitated by measuring the optical density at 510-550 nm using a Spectrophotometer. Total Collagen contents were calculated based on the standard curve.

Type 1 Collagen content in stellate cells were measured by the commercially available human Type 1 Collagen Detection ELISA kit, Catalog # 6021 (Chondrex, Inc., Redmond, WA) using a human monoclonal antibody specific for Type I Collagen. Cellular digestion of collagen with pepsin, solubilization, and assay procedures for measuring type 1 collagen by ELISA kit was performed according to the assay protocols provided in the assay kit by Chondrex, Inc. Cellular Collagen Type I contents were measured based on the standard curve.

Fluorescence Spectrophotometry and Fluorescent Microscopy measurement of Reactive Oxygen Species (ROS): ROS production, as an index of oxidative stress, in stellate cells were measured using a cell-permeable fluorescent probe 2'-7'-dichlorodihydrofluorescein diacetate (DCFDA) as described previously [11]. In brief, cells were incubated with DCFDA (10 μ mol) for 30 min. After thorough washing, cells were collected and lysed in PBS containing Triton X-100 (0.5%). The fluorescent intensity in the cell lysate was measured at the excitation and emission wavelength of 480 nm and 520 nm respectively using a Fluorescence Spectrometer.

Additionally, Fluorescent microscopy was used to visualize cellular ROS content. For these measurements, after thoroughly washing the DCFDA stained cells were visualized (magnification 10x) using a fluorescent microscope for photographic images and for representation of cellular ROS content.

Assessment of cell viability: Human hepatic cell viability was assessed by using a commercially available PrestoBlue Cell Viability Reagent kit by Invitrogen, Carlsbad, CA.

Statistical analysis

Data presented are mean \pm SE of 3 separate experiments for each subject's stellate cells. Statistical analysis for two-group comparisons

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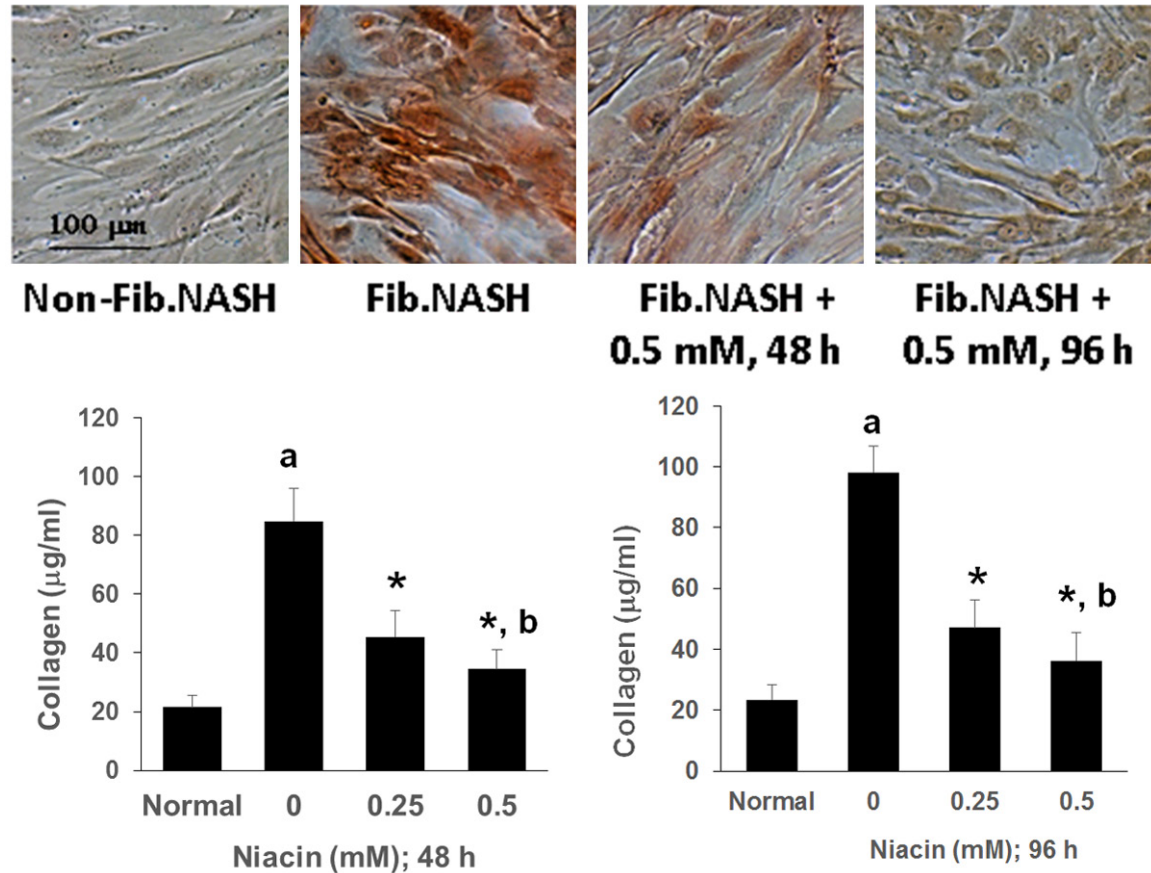


Figure 1. Niacin significantly decreases fibrosis in hepatic stellate cells from human donor subjects with fibrotic NASH (Donors 3-7). Human hepatic stellate cells from donor subjects with varying degrees of fibrosis and NASH (NAFLD activity scores 2-5, and fibrosis scores 1-3) were incubated with pharmacologically relevant concentrations of niacin (0.25 mM and 0.5 mM) for 48 or 96 hours. Cells were stained with Sirius Red for total collagen content, cellular photographic images, and quantitation. Top Panel: Representative photographic images of stellate cells (left to right): “Non-NASH” from donor 1, “NASH” from donor 6 without niacin, with 0.5 mM niacin at 48, and 96 h incubation. Microscopy images were captured at 10X magnification. Scale bar: 100 μm. Bottom Panel: Quantitative composite Mean ± SE total collagen content data from all 5 NASH-Fibrosis patients (donors 3-7) showing the efficacy of niacin to regress pre-existing fibrosis in stellate cells from patients with NASH and fibrosis. The left bar marked “Normal” refers to the mean collagen content in stellate cells from non-NASH subjects (donors 1, 2). Right 3 bars refer to stellate cells from NASH patients (donors 3-7) with fibrosis treated with niacin at 0, 0.25, 0.5 mM. Statistical analysis for two-group comparisons was performed by using an unpaired Student’s t-test (two-tailed). *P<0.001 vs 0 mM Niacin, ^aP<0.0001 vs Normal (Non-NASH subject), ^bP<0.03 vs 0.25 mM Niacin.

was performed by using an unpaired Student’s t-test (two-tailed), and a value of $P<0.05$ was considered significant.

Results

Niacin reverses collagen content in hepatic stellate cells from human subjects with NASH-fibrosis

For these studies, human hepatic stellate cells from donor patients with varying degrees of fibrosis (F1-3) and NASH (NAFLD activity scores 2-5) and stellate cells from normal non-fibro-

sis subjects (NAFLD activity score of 0) were incubated with pharmacologically relevant concentrations of niacin (0.25 mM and 0.5 mM) in DMEM + 0.5% serum media for 48 or 96 hours. Cells were stained with Sirius Red and collagen content was quantitated according to the procedure noted in Sirius Red Collagen Detection Kit.

In the representative photographic image of collagen content in stellate cells from non-fibrosis and from fibrotic NASH subjects (**Figure 1**, top panel), collagen content in stellate cells from NASH patients (donor 6) had the expected

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Table 2. Effect of Niacin on collagen content in hepatic stellate cells from non-NASH normal Subjects and NASH patients with fibrosis

Donor Subject/Patient	Incubation time		48 h			96 h		
	Niacin (mM)		0	0.25	0.5	0	0.25	0.5
			Collagen ($\mu\text{g/mL}$)			Collagen ($\mu\text{g/mL}$)		
Normal Non-Nash								
1			21.5 \pm 2.4	20.0 \pm 0.2	20.1 \pm 0.1	23.4 \pm 2.8	21.9 \pm 1.5	ND
2			20.4 \pm 0.2	20.6 \pm 0.1	24.0 \pm 1.1	25.2 \pm 0.6	23.9 \pm 0.1	ND
Mean \pm SE (Patients 1-2)			21.0 \pm 1.1	20.3 \pm 0.2	22.1 \pm 1.0	24.3 \pm 1.4	22.9 \pm 0.8	
NASH with Fibrosis								
3			60.1 \pm 1.7	32.7 \pm 2.7	24.6 \pm 2.4	94.6 \pm 5	46.4 \pm 5.1	29.7 \pm 5.1
4			73.3 \pm 5.3	42.3 \pm 2.2	28.6 \pm 3.2	88.4 \pm 3.1	31.4 \pm 2.7	21.5 \pm 3.2
5			70.4 \pm 5.0	24.9 \pm 4.5	24.3 \pm 2.0	78.4 \pm 7.0	22.7 \pm 3.6	18.8 \pm 4.0
6			96.5 \pm 5.6	49.2 \pm 2.0	34.7 \pm 2.0	98.9 \pm 7.6	60.7 \pm 2.4	40.4 \pm 4.6
7			123.1 \pm 7.6	77.9 \pm 6.4	60.2 \pm 6.8	130.3 \pm 7.0	74.3 \pm 7.2	70.8 \pm 8.8
Mean \pm SE (Patients 3-7)			84.7 \pm 11.3	45.4 \pm 9.1 ^a	34.5 \pm 6.7 ^a	98.1 \pm 8.7	47.1 \pm 9.3 ^a	36.2 \pm 9.4 ^a
Mean % Regression (Patients 3-7)				47.6 \pm 4.6	60.1 \pm 2.5 ^a		53.5 \pm 6.2	65.0 \pm 5.7 ^a

Statistical analysis for two-group comparisons was performed by using an unpaired Student's t-test (two-tailed). *, P<0.001 vs 0 mM Niacin for respective incubation period. a, P<0.03 vs 0.25 mM Niacin for respective incubation period; ND, Not determined.

strikingly higher content than in stellate cells from normal non-fibrosis subject (donor 1) which showed very minimal Sirius Red stainable collagen content. **Table 2** displays quantitative changes in collagen content at baseline, 48 and 96 hours of incubation with 0.25 mM and 0.50 mM niacin in stellate cells from individual normal non-fibrosis subjects (donors 1, 2) and from patients with fibrosis (donors 3-7). **Figure 1**, bottom panel displays the composite Mean \pm SE collagen content data from all 5 Fibrosis patients (donors 3-7) showing the niacin's efficacy to regress pre-existing fibrogenic collagen content in stellate cells from patients with fibrosis.

Collagen content in stellate cells from fibrotic patients was strikingly higher (4 fold) than in stellate cells from control non-fibrosis subjects (**Table 2; Figure 1**). Treatment of hepatic stellate cells from patients with fibrosis (donor patients 3-7) with niacin (0.25 mM and 0.5 mM) for 48 h or 96 h, respectively, produced a robust and significant regression of pre-existing fibrosis by an average of 47.6 and 60.1 percent (at 48 h incubation) and 53.5 and 65.0% (at 96 h incubation, P<0.001), respectively (**Table 2; Figure 1**, bottom panel).

It is striking to note that niacin caused significant regression of pre-existing fibrosis in stellate cells from all 5 donor subjects.

In the next set of experiments, using human hepatic stellate cells from normal non-fibrosis

human donor subjects we examined whether niacin prevents and reverses stellate cell fibrosis induced by major physiological stimulators of liver fibrosis such as TGF- β or oxidative stress mediator hydrogen peroxide (H_2O_2).

Niacin prevents TGF- β or H_2O_2 -induced stellate cell fibrogenic collagen

For these studies, human hepatic stellate cells from non-fibrosis subjects were incubated with TGF- β (20 ng/ml) or H_2O_2 (25 μM) in the absence or presence of niacin (0.5 mM) for 24 h. Hepatic stellate cell fibrosis was assessed by measuring total collagen content by a Sirius Red staining kit. As shown in the representative photographic images of stellate cells after staining with Sirius Red (**Figure 2**, Top histogram panel), incubation of cells with either TGF- β or H_2O_2 , known inducers of stellate cell fibrosis, markedly increased collagen content as compared to vehicle treatment (VEH). Co-incubation of these cells with either TGF- β or H_2O_2 and niacin for 24 h noticeably prevented collagen production as shown in cellular photographic images after Sirius Red staining (**Figure 2**, Top histogram panel).

Quantitative data in **Figure 2**, top bar diagram showed that both H_2O_2 and TGF- β robustly and significantly increased total collagen content by 266% and 233% respectively in human stellate cells from non-fibrosis patients. Treatment of these cells with niacin in the presence of either H_2O_2 or TGF- β markedly and significantly

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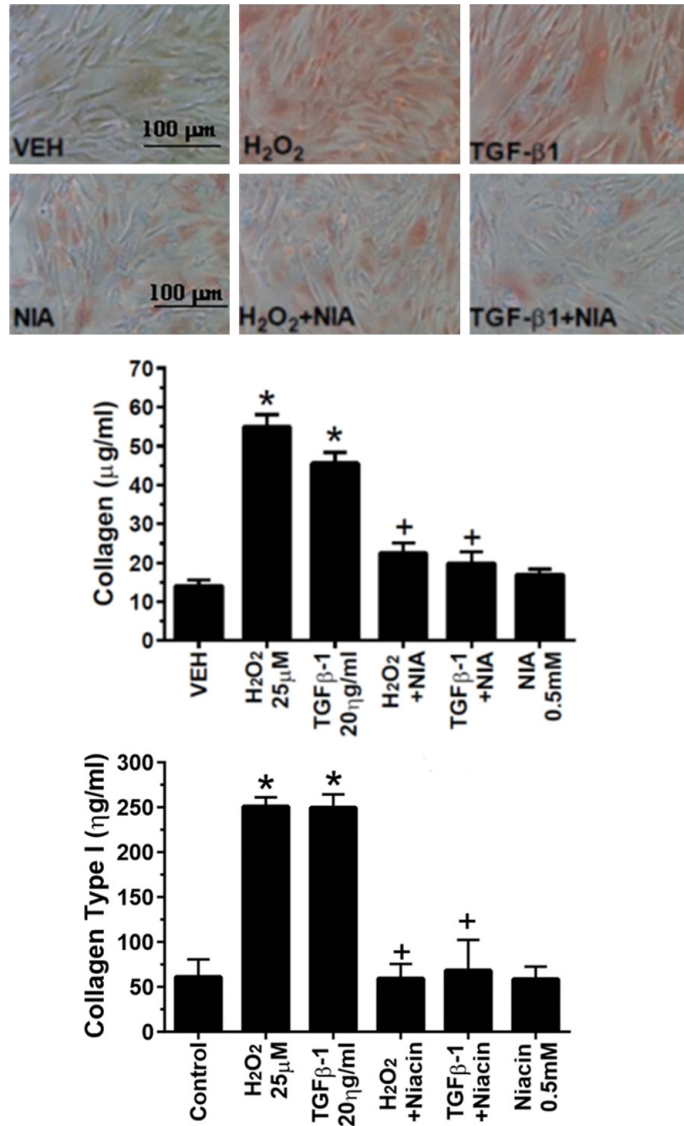


Figure 2. Niacin Prevents and Reverses TGF- β or H₂O₂-induced collagen production in human hepatic stellate cells from human subjects without liver fibrosis. Top Panel: Cells were incubated with TGF- β (20 ng/ml) or H₂O₂ (25 μ M) in the absence or presence of niacin (0.5 mM) for 24 h. Cells were stained with Sirius Red for assessment of total collagen content. Top Panel, histogram: Representative cellular photographic images of total collagen deposition. Microscopy images were captured at 10X magnification. Scale bar: 100 μ m. Top Panel, bar diagram: Quantitation of total collagen content in stellate cells. VEH, PBS vehicle, NIA, treatment with niacin (0.5 mM) alone without H₂O₂ or TGF- β . Statistical analysis for two-group comparisons was performed by using an unpaired Student's t-test (two-tailed). *, P<0.05 vs VEH; +, P<0.05 vs respective H₂O₂ or TGF- β . Bottom Panel: Human hepatic stellate cells from control subjects without liver fibrosis were first stimulated with TGF- β (20 ng/ml) or H₂O₂ (25 μ M) for 24 h to induce collagen deposition. These cells were then continued to incubate for an additional 24 h in the absence or presence of niacin (0.5 mM). Cellular content of Type 1 Collagen was assessed. Control, no prior treatment with H₂O₂ or TGF- β ; Niacin 0.5 mM, treatment with only niacin without prior treatment with H₂O₂ or TGF- β . Statistical analysis for two-group comparisons was performed by using an unpaired Student's t-test (two-tailed). *, P<0.05 vs Control; +, P<0.05 vs respective H₂O₂ or TGF- β .

prevented total collagen production by 59% and 56% respectively when compared to cells treated with H₂O₂ or TGF- β alone (Figure 2, top bar diagram).

Niacin reverses TGF- β or H₂O₂-induced hepatic stellate cell fibrosis

In these studies, human hepatic stellate cells from non-fibrosis subjects were first stimulated with TGF- β (20 ng/ml) or H₂O₂ (25 μ M) for 24 h to induce fibrosis. These cells were then continued to incubate for an additional 24 h in the absence or presence of niacin (0.5 mM). Cellular content of Type 1 Collagen was measured by ELISA as noted in Methods. As shown in Figure 2 bottom panel, both H₂O₂ and TGF- β robustly increased by 4-5 fold cellular collagen type I content as compared to vehicle (control). Treatment of these cells with pre-existing fibrosis (induced by H₂O₂ or TGF- β) with niacin (0.5 mM) almost completely reversed cellular fibrosis, and collagen type I content was like the controls (Figure 2 bottom panel). Treatment of cells with niacin (labeled as Niacin 0.5 mM) without previous stimulation with H₂O₂ or TGF- β had no effect on collagen type I content (Figure 2 bottom panel, last column).

Niacin decreases human hepatic stellate cell ROS production induced by palmitic acid (PA) or H₂O₂

Human hepatic stellate cells were first stimulated with either palmitic acid (0.5 mM) or H₂O₂ (25 μ M) for 24 h to induce oxidative stress and then continued to incubate an additional 24 h in the absence or presence of niacin (0.5 mM). Niacin markedly reduced ROS induced by PA and H₂O₂ by 52% and 50% respectively (Figure 3).

Niacin did not affect human hepatic stellate cell viability

For these studies, human hepatic stellate cells were incubated with

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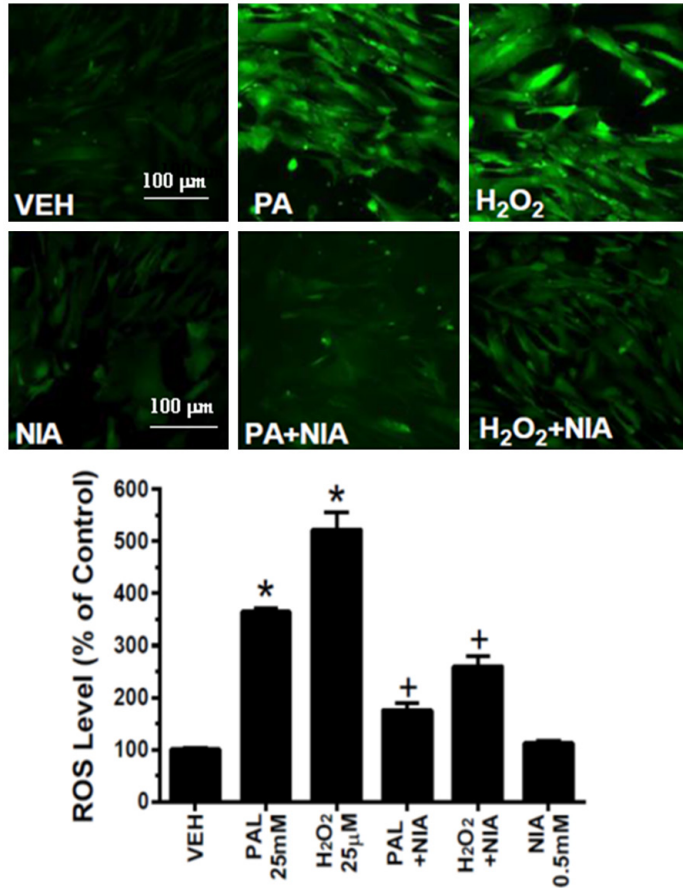


Figure 3. Niacin prevents human hepatic stellate cell oxidative stress induced by palmitic acid or H₂O₂. Stellate cells were first stimulated with either palmitic acid (0.5 mM) or H₂O₂ (25 μM) for 24 h to induce oxidative stress. These cells were then continued to incubate for an additional 24 h in the absence or presence of niacin (0.5 mM) for measurement of ROS levels. Top Panel, Histogram: Representative cellular photographic images after staining with DCFDA. Fluorescent Microscopy images were captured at 10X magnification. Scale bar: 100 μm. Bottom Panel, bar diagram: Quantitative levels of ROS presented as percent control. VEH, PBS vehicle alone, NIA, treatment with niacin (0.5 mM) alone without H₂O₂ or palmitic acid. PA or PAL, palmitic acid. Statistical analysis for two-group comparisons was performed by using an unpaired Student's t-test (two-tailed). *, P<0.05 vs VEH; +, P<0.05 vs Palmitic acid or H₂O₂, respectively.

niacin (0-0.5 mM) for 48 or 96 h. Cellular viability was measured by cell viability assay kit as noted in Methods. Treatment of cells with niacin (0.25 mM or 0.5 mM) for 48 h and 96 h had no significant effect on cell viability as compared to stellate cells without incubation with niacin (data not shown).

Discussion

To the best of our knowledge, this is the first study to evaluate niacin's efficacy on human stellate cell fibrosis reversal and prevention in a

preclinical *in vitro* human model of liver fibrosis. What makes this study unique and significant from preclinical animal models or immortalized cell lines is that primary stellate cells used for these studies were isolated and cultured *in vitro*, from donor livers (processed for transplantation) of patients who had died of various causes (Table 1) and patients who did not have fibrotic NASH. As shown, these patients included males and females, wide age range, had comorbidities of varying etiologies including risk factors for NASH, and racial backgrounds giving a diverse background.

Pharmacological concentrations of niacin (0.25-0.5 mM) used in our *in vitro* studies in hepatic stellate cells are clinically relevant and comparable to the niacin concentrations observed in human plasma after oral administration of commonly used niacin doses of 1-3 g/daily [13]. Because of the first pass effect via the portal vein, niacin concentration in liver tissue will be much higher than in plasma levels after oral administration of 1-3 g of niacin suggesting that lower doses of niacin may be clinically effective for potential fibrosis treatment or liver cirrhosis.

Of pathophysiologic significance, in addition to the direct effects of niacin on hepatic fibrosis reversal (and prevention) demonstrated herein, there is also an indirect effect via the mitigating effects of niacin on steatosis and inflammation [9]. By reducing the front end of this progressive disease, niacin reduces the sequential cascade effect on inflammation and fibrosis. Thus, niacin attacks fibrosis in 3 ways: (a) by reversing preexisting fibrosis, (b) by suppressing fibrosis production, and, (c) by mitigating steatosis and steatohepatitis which can lead to fibrosis.

Oxidative stress plays a key role in the pathogenesis of hepatic fibrosis via lipotoxicity, tissue inhibitors of metalloproteinases (TIMP), and other mediators [14]. Our data shows that niacin significantly inhibited the production of

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reactive oxygen species induced by oxidative stressors, palmitic acid, or hydrogen peroxide by half. This data is consistent with our previous reports that niacin reduces oxidative stress in several cells including human aortic endothelial cells, neutrophils, and hepatocytes [11, 15, 16]. We suggest that the reduction of stellate cell oxidative stress is an important mechanism for fibrosis reversal observed in this investigation. Complementary *in vivo* data has been reported earlier in which niacin prevented fibrosis induced in rats by thioacetamide (TAA) administration [17]. In this model, niacin significantly decreased hydroxyproline (a measure of liver fibrosis), oxidative stress and lipid peroxidation, α -SMA (a marker for activated HSC), TGF- β , and CTGF (amplifier of TGF- β actions). Additionally, niacin decreased MMP-2 and MMP-9 in this TAA-induced animal model of fibrosis. Of note, this study did not assess fibrosis regression and demonstrated that niacin prevented fibrosis in a rat model induced by the administration of a toxic chemical.

TGF- β induces collagen production and fibrosis mainly through SMAD-mediated intracellular signaling pathways. However, a growing body of evidence indicates that oxidative stress and reactive oxygen species (ROS) regulate TGF- β fibrogenic effects through different pathways including a) ROS induces TGF- β production, and b) it activates TGF- β -mediated SMAD signaling pathways leading to increased collagen production and fibrosis [18]. Additionally, TGF- β increases ROS production and suppresses antioxidant enzymes resulting in an imbalance in the cellular redox state [18]. Thus, current literature suggests a reciprocal regulative relationship between TGF- β and ROS for fibrosis [18]. It is likely that niacin-mediated rescuing effects of TGF- β on fibrosis may be due to its ability to inhibit ROS production and in turn, its effect on decreasing TGF- β -mediated SMAD signaling for fibrosis.

The limitations of this study are that these *in vitro* preclinical studies are performed in isolated primary human hepatic stellate cells and do not necessarily reflect cellular interactions that may be present in the whole liver. Because stellate cells interact with other hepatic cells in the liver (e.g., hepatocytes, Kupfer cells, etc.) interpretation of observations in isolated stellate cells places a limit on the full understanding of the mechanisms of action of niacin on

fibrosis. Although the findings presented in this report are crucial in their clinical implications, the data do not delineate detailed mechanisms of action of niacin on oxidative stress reduction and steps involved in the reversal of liver fibrosis.

However, these results in patients with histologic fibrosis provide unique proof of concept that pharmacologic (high-dose) niacin offers a potential treatment for liver cirrhosis, the clinical manifestation of liver fibrosis. This study offers a clue that niacin may reverse fibrosis resulting from other causes of cirrhosis including viral, alcohol, chemicals, etc. Further research including a clinical trial is needed to assess the efficacy of niacin for fibrosis and cirrhosis resulting from NASH and other etiologies.

In conclusion, this study shows for the first time that niacin reverses preexisting collagen deposition in human stellate cells isolated from patients with liver fibrosis associated with NASH. Niacin also prevents and reverses collagen deposition induced by oxidative stress in non-fibrotic stellate cells. Translationally, the totality of this human data, coupled with emerging *in vivo* animal and *in vitro* human hepatocyte published data on steatosis and steatohepatitis, suggest that niacin (currently used clinically for dyslipidemia) could be repurposed as an effective drug for clinical treatment of patients with NASH-fibrosis or liver cirrhosis. This is in addition to its known efficacy for reversing steatohepatitis and steatosis which can also result in liver cirrhosis. Further research including more detailed elucidation of mechanisms and a clinical trial in patients with fibrotic NASH or liver cirrhosis is suggested as a cost-effective therapy for a major unmet need in clinical medicine.

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Disclosure of conflict of interest

Neil Hoa and Jayant Kamanna: None. Dr. Kashyap has received research grants from AbbVie, Amgen, Amylin, Arisaph, AstraZeneca,

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Bristol Meyers Squibb, Eli-Lilly, Kos, Sanofi-Aventis, Merck and has been an advisor and/or speaker for AbbVie, Amarin, Kos, Bristol Meyers Squibb, and Merck. Drs. Moti Kashyap, Shobha Kamanna, and Vajjinath Kamanna are inventors of the patent issued by the US Patent Office titled "Indication for use of niacin (nicotinic acid) for treatment and reversal of fatty liver disease (Patent no.: US 9,072,732 B2) and a pending patent related to this communication. Drs. Kamanna and Kashyap are principals in Aasta Pharmaceuticals, LLC.

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