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RESEARCH ARTICLE

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Hydroxyurea attenuates hepatic stellate cell proliferation in vitro and liver fibrogenesis in vivo

Floris Haijer[1] | **Shiva Koets-Shajari**[1] | **Janette Heegsma**[2] | **Sandra Serna-Salas**[1] | **Tjasso Blokzijl**[2] | **Manon Buist-Homan**[2] | **Han Moshage**[1,2] | **Klaas Nico Faber**[1,2]

[1]Department of Gastroenterology and Hepatology, University Medical Center Groningen, University of Groningen, Groningen, The Netherlands

[2]Department Laboratory Medicine, Center for Liver, Digestive, and Metabolic Diseases, University Medical Center Groningen, University of Groningen, Groningen, The Netherlands

Correspondence
Floris Haijer, Department of Gastroenterology and Hepatology, University Medical Center Groningen, University of Groningen, Hanzeplein 1, 9713GZ, Groningen, The Netherlands.
Email: f.haijer@ozg.nl

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Abstract

Liver fibrosis results from excessive proliferation of, and collagen production by hepatic stellate cells (HSCs) that is caused by chronic liver injury. No drugs are available to cure liver fibrosis. Hydroxyurea is an anti-proliferative drug that is used in benign and malignant disorders. Here, we studied the effect of hydroxyurea on primary HSCs and its anti-fibrotic effect in the CCl_4 mouse model of liver fibrosis. Primary rat HSCs were cultured in the absence or presence of hydroxyurea (0.1–1.0 mmol/L). CCl_4 or vehicle was administered to C57BL/6/J mice for 4 weeks, with or without hydroxyurea (100 mg/kg/day) co-treatment. We used real-time cell proliferation analysis, Oil Red O (lipid droplet) staining, immunohistochemistry, Acridine Orange staining (apoptosis), Sytox green staining (necrosis), RT-qPCR, ELISA, and Western Blotting for analysis. Hydroxyurea dose-dependently suppressed lipid droplet-loss and mRNA levels of *Col1α1* and *Acta2* in transdifferentiating HSCs. In fully-activated HSCs, hydroxyurea dose-dependently attenuated PCNA protein levels and BrdU incorporation, but did not reverse *Col1α1* and *Acta2* mRNA expression. Hydroxyurea did not induce apoptosis or necrosis in HSCs or hepatocytes. Hydroxyurea suppressed accumulation of desmin-positive HSCs and hepatic collagen deposition after CCl_4 treatment. CCl_4-induced regenerative hepatocyte proliferation, *Col1α1* and *Acta2* mRNA expression and α-SMA protein levels were not affected. This study demonstrates that hydroxyurea inhibits HSC proliferation in vitro and attenuates early development of liver fibrosis in vivo, while preserving hepatocyte regeneration after

Abbreviations: Acta2, actin alpha 2; aHSC, activated hepatic stellate cell; a-sma, alpha smooth muscle actin; BrdU, bromodeoxyuridine; CCl4, carbon tetrachloride; Col1a1, collagen type 1 alpha 1; DMOG, dimethyloxalylglycine; ECM, extracellular matrix; ELISA, enzyme-linked immuno sorbent assay; FCS, fetal calf serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HSC, hepatic stellate cell; HU, hydroxyurea; IL-1b, interleukin-1 beta; IL-6, interleukin 6; IMDM, Iscov's Modified Dulbecco's Medium; ip, intraperitoneal; MLV, Molony murine leukemia virus; MOM, mouse on mouse; P/S/F, penicillin/Streptomycin/Amphotericin B; PCNA, proliferating cell nuclear antigen; PDGF-b, platelet-derived growth factor beta; PDGFR-b, platelet-derived growth factor receptor beta; PMF, portal myofibroblast; qHSC, quiescent hepatic stellate cell; RNR, ribonucleotide reductase; RTCA, real-time cell analyzer; RT-qPCR, real time-quantitative polymerase chain reaction; SEM, standard error of the mean; TGF-a, transforming growth factor beta; TNF-a, tumor necrosis factor alpha.

toxic insults by CCl_4. Thus, hydroxyurea may have therapeutic value against liver fibrosis.

KEYWORDS
anti-fibrotic, cirrhosis, fibrosis, hydroxycarbamide, hydroxyurea, stellate cell

1 | INTRODUCTION

Liver fibrosis is a prevalent consequence of chronic liver disease that affects millions of people worldwide.[1] It is characterized by the accumulation of scar/connective tissue in the liver that is produced by activated hepatic stellate cells (HSCs). In addition, portal myofibroblasts (PMFs) may contribute to liver fibrogenesis, particularly in cholestatic liver disease.[2] In a healthy liver, HSCs are quiescent, meaning that they show low proliferation rates and are non-fibrogenic. Quiescent HSCs (qHSCs) produce extracellular matrix proteins that are required for the liver architecture and are involved in vitamin A storage, immunity and inflammation.[3,4]

Ongoing liver damage triggers HSC activation. During HSC activation, qHSCs lose their characteristic lipid-rich and vitamin A-rich phenotype and transdifferentiate to alpha-smooth muscle actin (α-SMA)-expressing myofibroblast-like cells. Activated HSCs (aHSCs) become proliferative and produce excessive amounts of extracellular matrix proteins (ECM), particularly collagen type 1 and 3, that are major components of scar tissue in the liver.[5] Liver fibrosis may progress to cirrhosis and predisposes for hepatocellular carcinoma (HCC). There is no anti-fibrotic therapy available for clinical use and liver transplantation is currently the only curative treatment option for end-stage cirrhosis.[6]

However, liver fibrosis may largely be reversible, as evidenced in cases where the disease-causing trigger can be eradicated or treated, such as in viral and auto-immune hepatitis.[7–9]

Still, treatment options fail or are not available at all for many chronic liver diseases, including non-alcoholic steatohepatitis (NASH).[10] Thus, there is great need for drugs that halt and/or regress liver fibrosis. HSCs are prime target cells to develop such drugs, and drug targets include HSC proliferation, activation, ECM production, and/or HSC viability. The importance of cell proliferation for the development of liver fibrosis is evidently demonstrated by cyclin E1 whole body knock-out mice being less susceptible to liver fibrogenesis.[11] The most potent mitogen for HSCs is platelet-derived growth factor beta (PDGF-β). In HSCs, PDGFR-β is strongly induced during activation[12] and overexpression of PDGF-β induces liver fibrosis in mice, whereas blocking PDGF-β attenuates liver fibrogenesis.[13,14] However, PDGF-β receptors are expressed in both aHSCs and hepatocytes, and blocking hepatocyte proliferation would inhibit liver regeneration.[15,16] A lack of specificity combined with toxicity limits the use of many anti-proliferative drugs in general.[16]

Hydroxyurea is and inhibitor of ribonucleotide reductase (RNR), a key enzyme involved in the synthesis of deoxyribonucleotides.[17,18] It is a naturally occurring inhibitor of cell proliferation in many animal species, which may reach tissue concentrations of up to 250 μmol/L.[19] Hydroxyurea is in part metabolized in the liver though metabolic pathways that are not fully characterized. In addition, it is excreted unchanged via the kidneys.[20]

Despite the general effect on DNA synthesis and cell proliferation, hydroxyurea treatment is considered safe with very little reported adverse effects.[21–23] In the present study, we studied the effect of hydroxyurea on HSC proliferation in vitro, as well as liver fibrogenesis in vivo in order to evaluate its anti-fibrotic potential.

2 | MATERIALS AND METHODS

2.1 | Animals

Specified pathogen-free male Wistar rats obtained from Charles River Laboratories Inc. (Wilmington, MA, USA) were used for liver cell (HSC, PMF and hepatocyte) isolations (see below). Twelve week-old male C57BL/6/J mice were obtained from Charles River Laboratories and used for CCl_4-induced liver fibrosis in vivo.

Animals were housed under standard laboratory conditions with free access to water and chow. Animal experiments were approved by the local ethical committee and experiments were performed following the Dutch guidelines for care and use of laboratory animals.

2.2 | Experimental design

Mice were divided into five experimental groups, each group n = 8. One group was used as untreated control group. Mice were treated with CCl_4 (Sigma Aldrich, Zwijndrecht, The

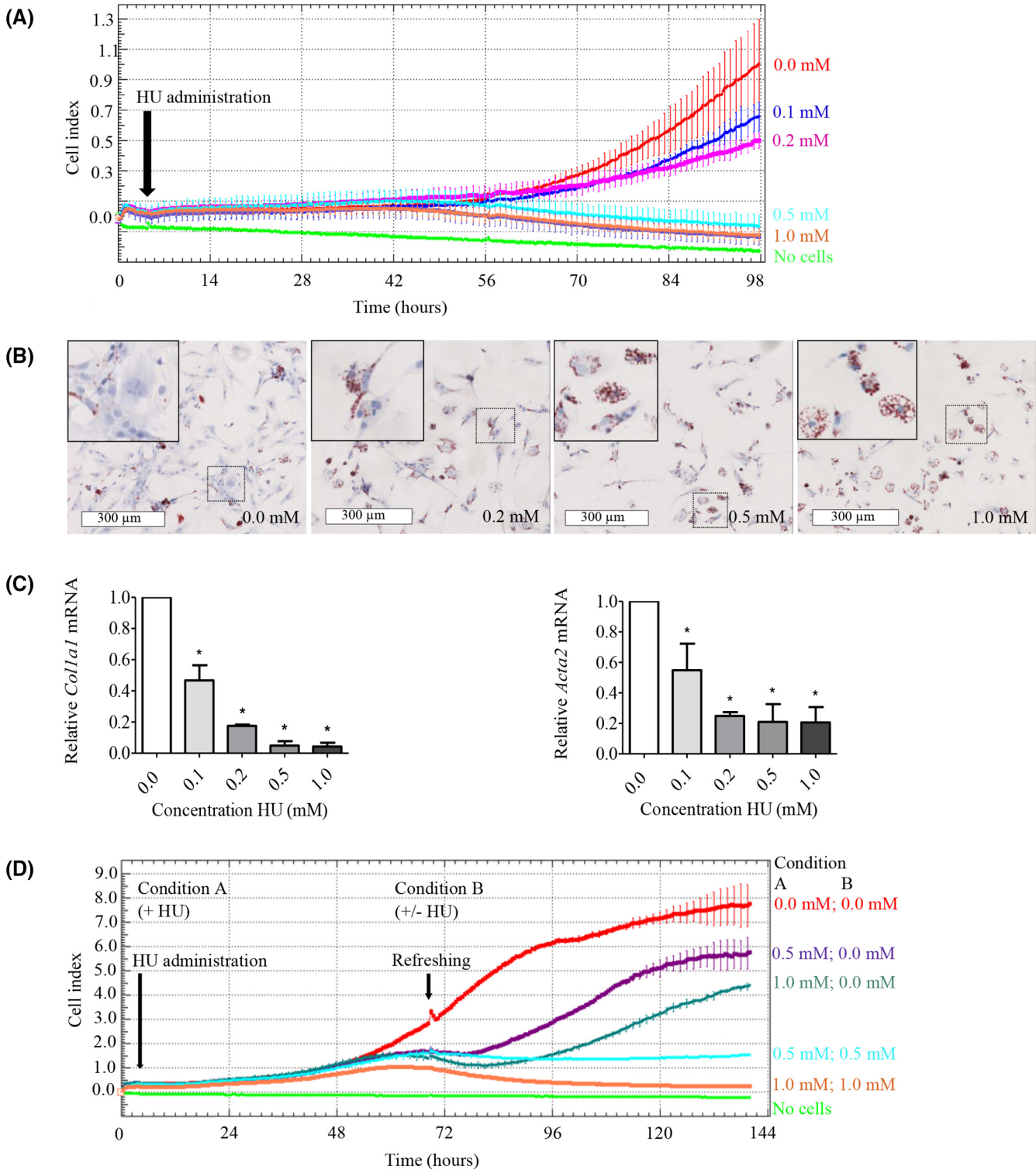


FIGURE 1 Hydroxyurea inhibits HSC activation and proliferation without inducing cell death. (A) Freshly-isolated rat HSCs were culture-activated in the absence and presence of 0, 0.1, 0.2, 0.5, or 1.0 mmol/L hydroxyurea and analyzed by real-time cell proliferation/stretching monitoring (xCELLigence) during 98 h. Hydroxyurea dose-dependently reduced HSC proliferation and/or activation/stretching (cell index). Error bars present the standard deviation between two different measurements. (B and C) Quiescent HSCs were exposed to hydroxyurea for 4 days and analyzed using Oil-red O staining and RT-qPCR. Hydroxyurea suppressed lipid droplet-loss from cultured HSCs (B) and dose-dependently suppressed the expression of HSC activation markers *Col1α1* and *Acta2* (C). (D) To investigate whether hydroxyurea induced HSC death, hydroxyurea-treated (0.5 or 1.0 mmol/L for 72 h) HSCs were further cultured in fresh medium without hydroxyurea for an additional 72 h. Removing hydroxyurea led to a rapid increase in cell index in the following 72 h (purple and dark green lines), indicating that HSC did not die under hydroxyurea treatment.

Netherlands), or corn oil (Sigma Aldrich) with hydroxyurea (Sigma Aldrich) or 0.9% NaCl (Sigma Aldrich) co-treatment. Hydroxyurea (100 mg/kg) or 0.9% NaCl was administered by daily intraperitoneal (*ip*) injections for 4 weeks. CCl_4 (Sigma Aldrich) was administered twice a week for 4 weeks. The first week, mice were treated with CCl_4 at a dose of 0.5 mL/kg twice a week. The second week, mice were treated with 0.8 mL/kg CCl_4 twice a week. From the third week mice were treated with 1.0 mL/kg twice a week. Mice were sacrificed 1 day after the final CCl_4 injection in week 4. CCl_4 was dissolved 1:10 (v/v) in corn oil, and kept in glass bottles.

2.3 | Cell isolation and culture

HSCs were isolated using pronase (Merck, Amsterdam, The Netherlands) and collagenase-P (Roche, Almere, The Netherlands) perfusion after cannulating the portal vein, and dissecting the inferior caval vein, as described before.[24] HSCs were further purified using Nycodenz (Axis-Shield POC, Oslo, Norway) gradient centrifugation.[25] Hepatocytes were isolated using collagenase perfusion as described before.[25] Portal myofibroblasts (PMFs) were isolated from the portal tree as described before.[25]

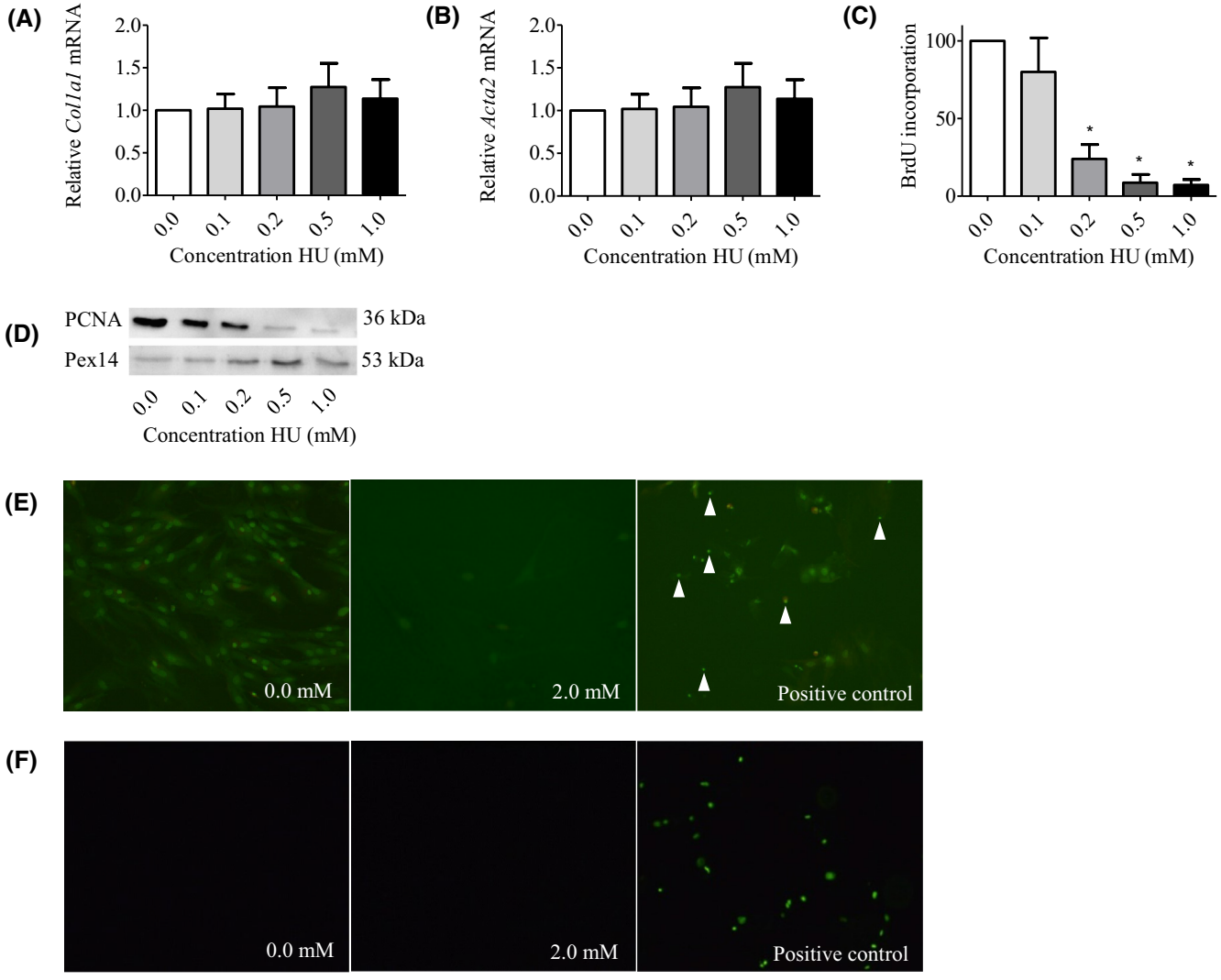


FIGURE 2 Hydroxyurea inhibits proliferation, but not the fibrogenic expression profile of activated HSCs. Fully-activated HSCs were exposed to 0, 0.1, 0.2, 0.5, or 1.0 mmol/L hydroxyurea for 7 days and analyzed by RT-qPCR (A,B), BrdU incorporation (C) and Western blotting (D). (A), (B) Hydroxyurea did not change mRNA levels of *Col1α1* and *Acta2*. (C and D) In contrast, hydroxyurea dose-dependently reduced BrdU incorporation and PCNA protein levels, indicative of reduced cell proliferation. Peroxin 14 (Pex14) was used as loading control. (E and F) Fully-activated HSCs were exposed to 0 and 2.0 mmol/L hydroxyurea and did not reveal significant signs of apoptosis (E, Acridine Orange staining) or necrosis (F, Sytox green staining). HSCs were treated for 5 h with 25 μmol/L menadione (E, right panel, arrowheads indicate apoptotic bodies) or for 24 h with 5 mmol/L H_2O_2 (F, right panel) as positive controls for apoptosis and necrosis, respectively.

Cells were cultured at 37°C and 5% CO_2 in a humidified incubator (ThermoFisher Scientific, Waltham, Massachusetts, USA). HSCs and PMFs were cultured using Iscov's Modified Dulbecco's Medium (IMDM) with Glutamax (Invitrogen, Breda, The Netherlands) supplemented with 20% FCS (Sigma Aldrich), 1% P/S/F (Lonza, Basel, Switzerland), 1% Non-essential amino acids (Sigma Aldrich), and 1% sodium pyruvate (Sigma Aldrich). HSCs and PMFs were cultured for at least 7 days on culture plastic in order to activate, or grow out of the portal tracts respectively. Hepatocytes were cultured using supplemented William's E (Invitrogen) medium as described before.[26]

2.4 | Real time cell analysis (RTCA)

Cell proliferation and activation were assessed by changes in the electrical impedance in the xCELLigence Real-time Cell Analyzer DP system (RTCA Biosciences, San Diego, USA).[27]

Per well 7500 quiescent HSCs were seeded, and cells were stimulated after attachment. Results were recorded and analyzed by RTCA software (RTCA Biosciences).

2.5 | BrdU incorporation assay

BrdU incorporation ELISA assay (Roche Diagnostics, Almere, The Netherlands) was performed according to the manufacturer's instructions using spectrophotometry (BioTek Instruments, Inc., Bad Friedrichshall, Germany).

2.6 | Cell death analysis

Cell apoptosis and necrosis were analyzed by Acridine Orange (Sigma-Aldrich) nuclear condensation staining and Sytox green (Invitrogen) staining, respectively, as described before.[28]

2.7 | RNA isolation, cDNA synthesis, and quantitative polymerase chain reaction

RNA was isolated using TRI reagent according to the manufacturer's instruction (Sigma Aldrich). RNA quality and quantity were assessed using Nanodrop spectrophotometry (Thermo Scientific, Wilmington, USA). Reverse

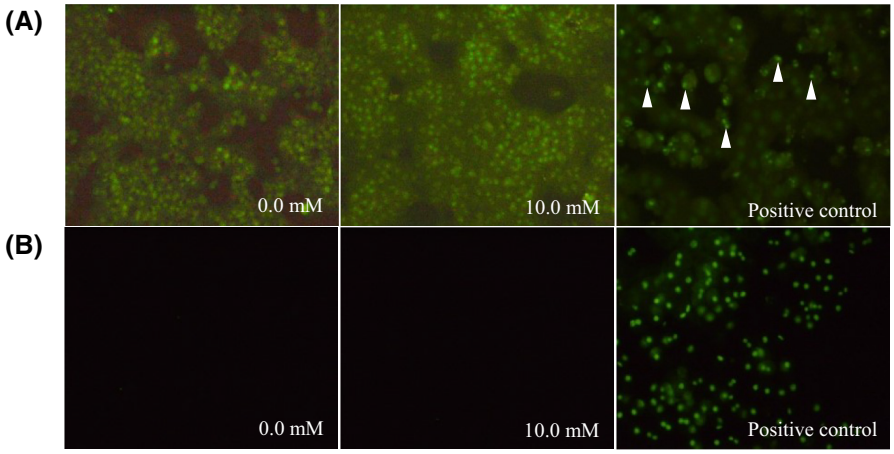


FIGURE 3 Hydroxyurea does not induce cell death in primary rat hepatocytes. (A), (B) Freshly-isolated rat hepatocytes were exposed to 0 or 10 mmol/L hydroxyurea for 2 days. Acridine Orange staining and Sytox green staining were performed to evaluate apoptotic and necrotic cell death, respectively. Hydroxyurea did not induce apoptotic (A) nor necrotic (B) cell death in hepatocytes. Rat hepatocytes were treated for 9 h with 50 μmol/L menadione (A, right panel; arrowheads indicate apoptotic bodies) or for 5 h with 5 mmol/L H_2O_2 (B, right panel) as positive controls for apoptosis and necrosis, respectively.

TABLE 1 Blood counts and transaminases.

	Untreated	Corn oil + NaCl	CCl_4 + NaCl	Corn oil + HU	CCl_4 + HU
Hemoglobin (mmol//L)	8.1 (0.3)	8.3 (0.1)	8.8 (0.1)	6.2 (0.1)	5.4 (0.5)
Leukocytes (10^9/L)	4.8 (0.3)	4.6 (0.3)	5.3 (0.7)	3.0 (0.4)	1.5 (0.2)
Thrombocytes (10^9/L)	441 (54)	530 (27)	613 (28)	354 (16)	351 (55)
AST (U/L)	76.0 (7.6)	80.2 (10.1)	2263.0 (566.5)	71.8 (6.0)	2114.6 (440.4)
ALT (U/L)	20.9 (1.1)	18.0 (1.6)	4595.1 (940.0)	23.25 (1.6)	4010.1 (816.1)

transcription was performed using 2.5 μg RNA, random nanomers (Life technologies, Breda, The Netherlands), and Molony murine leukemia virus (M-MLV) reverse transcriptase (Sigma-Aldrich). RT-qPCR was performed using the TaqMan protocol in a 7900HT Fast Real-Time PCR system (Applied Biosystems, Nieuwekerk a/d IJssel, The Netherlands), as described before.[29] Primer and probe sequences are given in Table S1. 18S was used as a house-keeping gene. Data are presented as relative expression using the ΔΔCT method.

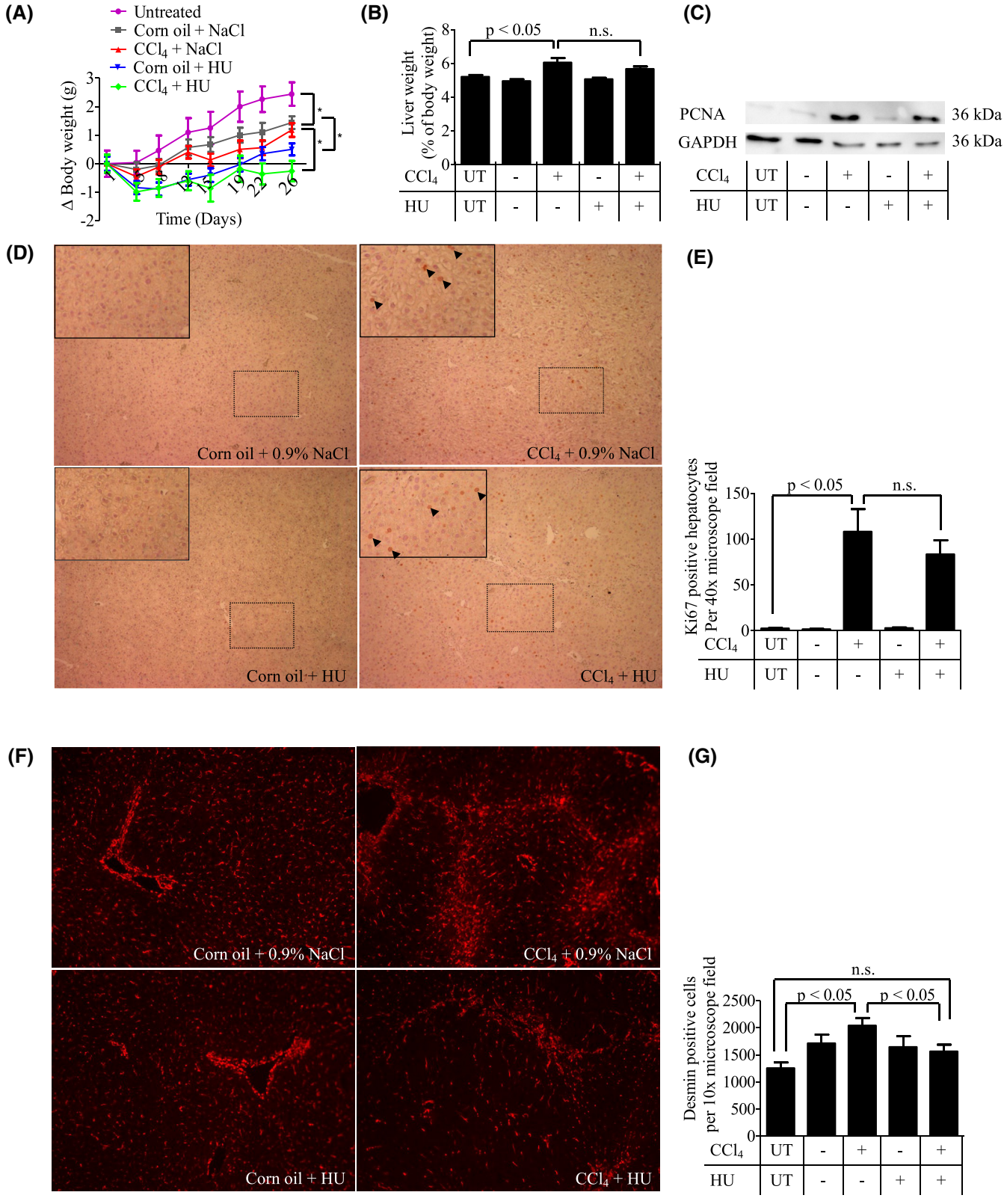


FIGURE 4 Hydroxyurea prevents stellate cell proliferation, but does not affect regenerative hepatocyte proliferation in CCl_4-treated mice. (A) Change in bodyweight and (B) relative liver weight as a percentage of body weight of mice that were treated for 4 weeks with or without CCl_4 and co-treated with or without hydroxyurea. Corn oil and 0.9% NaCl served as vehicle for CCl_4 and hydroxyurea, respectively. (C) Western blot analysis for hepatic PCNA (cell proliferation marker) reveals CCl_4-induced expression, which is maintain under co-treatment with hydroxyurea. Samples contain an equal amount of protein of all eight animals per experimental group and GAPDH was used as loading control. (D and E) Ki67 (cell proliferation marker) staining of liver tissue reveals abundant Ki67-positive hepatocytes in CCl_4 treated animals with and without hydroxyurea co-treatment (D). The quantification is given in (E). (F) Quantification in (G) Desmin (HSC marker) staining of liver tissue reveals a significant accumulation in CCl_4-treated mice, which is suppressed by hydroxyurea co-treatment. No significant difference was observed between untreated mice and CCl_4 + hydroxyurea co-treated mice.

2.8 | Protein quantification and western blotting

Protein concentrations were quantified using the BioRad DC protein assay (BioRad, Veenendaal, The Netherlands) according to the manufacturer's instructions. Western blot analysis of cell lysates and liver extracts was performed as described previously.[30] Protein expression was assessed using primary antibodies listed in Table S2.

2.9 | Chemical and immunohistochemical staining of cultured cells and liver tissue

Intracellular lipids in HSCs were stained with Oil Red O solution (Sigma-Aldrich) as described before.[26] Mouse liver tissue was stained using Sirius Red (Sigma-Aldrich) on paraffin sections according to standard protocols.[30] For α-SMA staining we used a MOM-kit, peroxidase PK-2200 (Vector, Brunschwig, Germany) according to the manufacturer's instructions.

Desmin was stained on cryosections. Primary antibodies that were used are listed in Table S2. CD68 was stained using paraffin sections, microwave cooking in citated buffer (Sigma Aldrich) for antigen retrieval.

Liver tissue was studied using a Leica DMI6000B microscope (Wetzlar, Germany) and the Hamamatsu NanoZoomer 2.0-HT digital slide scanner (Hamamatsu city, Japan). Sirius red surface area was determined and cell numbers were counted using ImageJ software (ImageJ; National Institutes of Health, Bethesda, MD, USA, http://rsbweb.nih.ogv/ij/).

2.10 | Blood parameters

Routine blood cell counts and transaminases were analyzed in the clinical laboratory of the University Medical Center Groningen (Groningen, The Netherlands).

2.11 | Statistics

Results of in vitro experiments are presented as the mean of three independent experiments ±SEM, unless specified otherwise. Mann–Whitney *U* test was used to determine statistical significance. For statistical analysis of the in vivo experiments, it was assumed treatment groups were normally distributed. A two way T-test was used to compare groups. A p value of less than .05 was considered to be statistically significant.

3 | RESULTS

3.1 | Hydroxyurea prevents HSC activation without inducing cell death

In order to determine whether hydroxyurea affects HSC activation, freshly-isolated primary quiescent rat HSCs (qHSCs) were allowed to attach for 4 h to plastic culture wells or xCELLigence® (real time cell analyzer; RTCA) plates and were subsequently culture-activated in the presence and absence of hydroxyurea (concentrations ranging from 0.0 to 1.0 mmol/L). The xCELLigence® works by measuring electron flow that is transmitted between gold microelectrodes. Adhering cells disrupt the interaction between the electrodes and culture medium and thus impede electron flow. The electrical impedance (resistance to alternating current) is expressed as arbitrary units called cell index. Cell index is dependent on cell number, cell morphology, cell size, and on the strength of cell attachment to the culture plate.[31] After a lag phase of approximately 48–60 h, the cell index steadily increased when HSCs are cultured in the absence of hydroxyurea (red line in Figure 1A), indicative of HSC activation and/or proliferation. Hydroxyurea dose-dependently inhibited the culture-induced increase in cell index, which was completely blocked at concentrations of 0.5 mmol/L and higher. As a difference in cell index was observed after 4 days of culture, HSC activation was assessed at this time point in subsequent analyses.

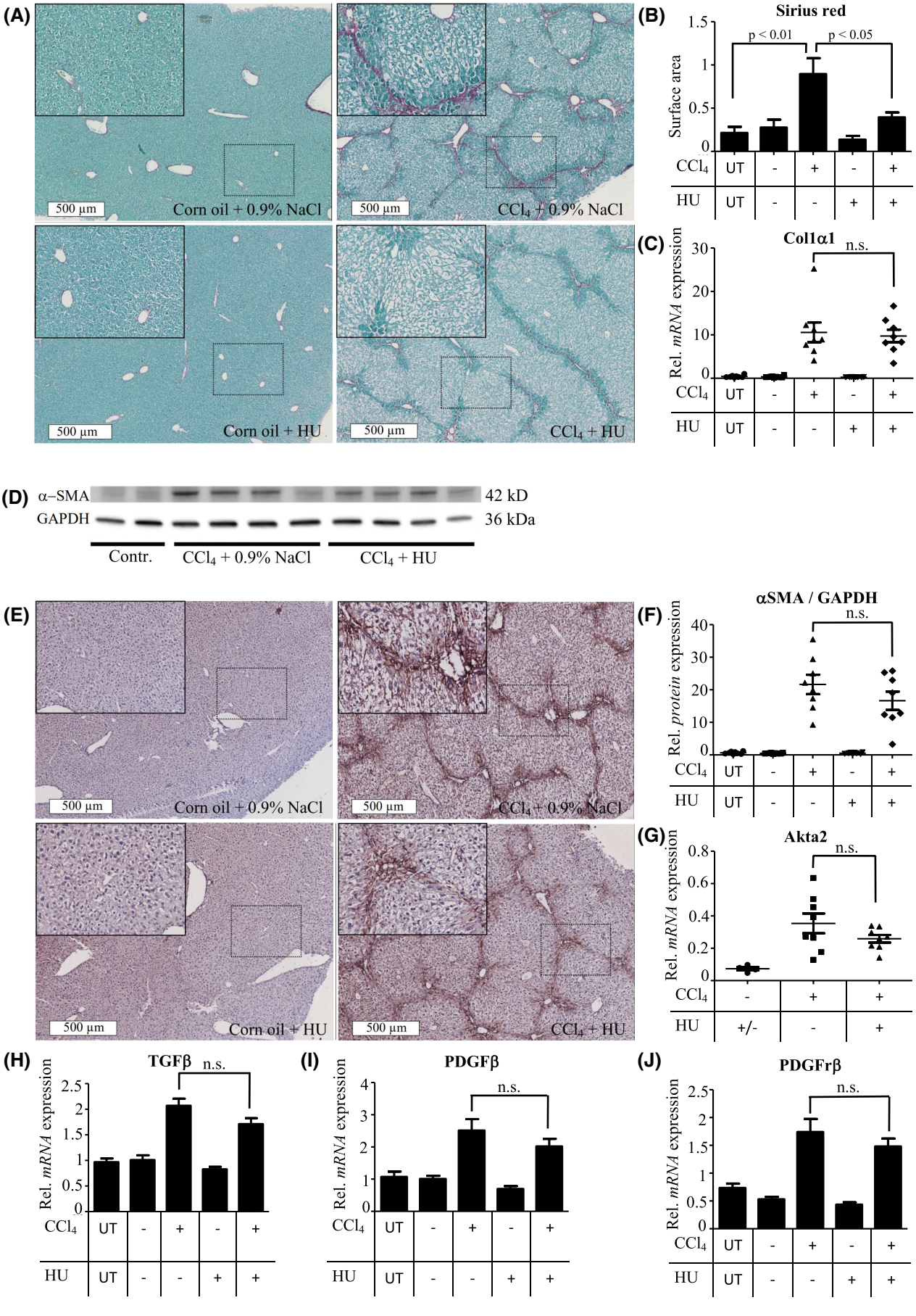


FIGURE 5 Hydroxyurea ameliorates collagen deposition without affecting *Col1α1* or *Acta2* mRNA expression. (A), quantification in (B) Hepatic collagen deposition was analyzed by Sirius Red staining. CCl_4 treatment significantly enhanced collagen deposition compared to sham-treated animals, which was suppressed by hydroxyurea co-treatment. (C) CCl_4 treatment significantly enhanced hepatic *Coll1α1* mRNA levels, which was not significantly reduced by hydroxyurea co-treatment. Western Blotting (D, quantification in F), immunohistochemistry and RT-qPCR (G) show that α-SMA expression was slightly but not significantly lower in hydroxyurea + CCl_4 treated mice compared to 0.9% NaCl + CCl_4 treated controls. RT-qPCR (H-J) analyses shows the CCl_4-induced expression of TGF-β, PDGF-β and PDGFR-β was not significantly lower in hydroxyurea treated mice compared to their controls.

Quiescent HSCs contain large lipid droplets, which are quickly lost during culture activation (Figure 1B, left panel). Lipid droplets were, however, still readily detectable by Oil red O staining in HSCs cultured for 4 days in the presence of increasing concentrations hydroxyurea (Figure 1B). Moreover, hydroxyurea-treated HSCs were much smaller and showed a more rounded phenotype characteristic of qHSCs when compared to control-grown HSCs. In line, hydroxyurea dose-dependently suppressed mRNA levels of the HSC activation markers *Col1α1* and *Acta2*, which was already significantly reduced by approximately 50% at the lowest concentration (0.1 mmol/L) hydroxyurea (Figure 1C) and further decreased by ~80%–95% at higher hydroxyurea concentrations. The effect of hydroxyurea on HSC cell index appeared reversible, as the cell index increased again when HSCs that were treated for 3 days with high concentrations hydroxyurea (0.5 and 1.0 mmol/L; purple and dark green lines, respectively) were refreshed with medium without hydroxyurea (Figure 1D). Still, after an additional 72 h culture in the absence of hydroxyurea, the cell index did not reach the same level as untreated HSCs (red line). These data show that hydroxyurea prevents HSC activation without inducing overt cell death.

3.2 | Hydroxyurea inhibits proliferation of activated HSCs, but does not reverse the fibrotic phenotype

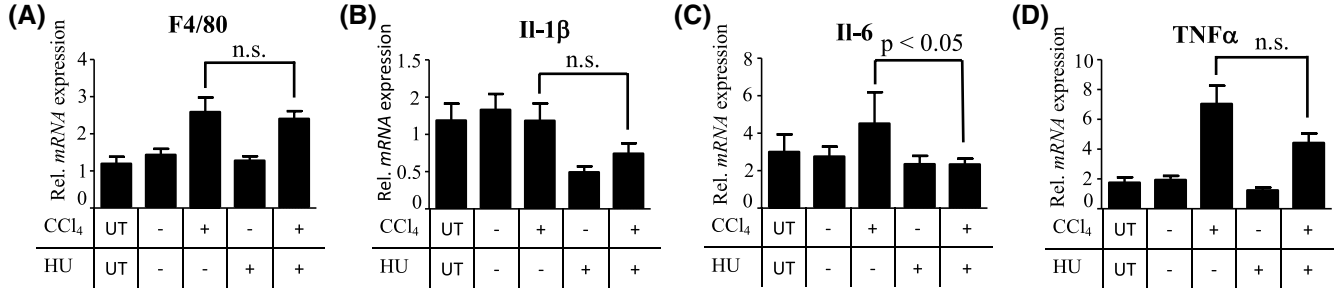
Next, we analyzed the effect of hydroxyurea on HSC proliferation and reversal of HSC activation in fully-activated HSCs (aHSCs). Primary rat HSCs were culture-activated for at least 7 days and were subsequently treated with hydroxyurea 0.0–1.0 mmol/L for an additional 7 days. In sharp contrast to the effect of hydroxyurea on transdifferentiating HSCs, hydroxyurea did not suppress the mRNA levels of *Acta2* and *Col1α1* in fully-activated HSCs at any of the tested concentrations after 7 days of treatment (Figure 2A,B). However, BrdU incorporation, as a measure of cell proliferation, was dose-dependently reduced at concentrations from 0.2 mmol/L hydroxyurea and higher (Figure 2C). PDGF-β is the most potent mitogenic cytokine for hepatic stellate cells. In vitro, PDGF-β promotes HSC proliferation in serum starved conditions. Figure S1A shows that PDGF-β at a dose of 10 ng/mL or 100 ng/mL clearly promotes HSC proliferation to a similar level compared to 20% FCS treatment that was used in all other HSC in vitro experiments that are described in this study. In addition, the antiproliferative effect of hydroxyurea treatment was similar in PDGF-β treated cells, compared to 20% FCS treated HSCs (Figure S1B). A significant reduction in BrdU incorporation was observed in cells that were treated with hydroxyurea 0.1 to 2.0 mM.

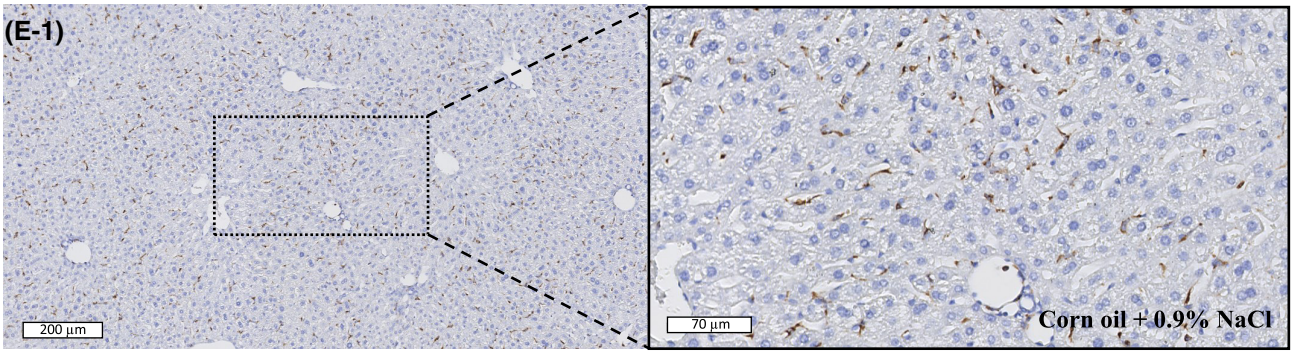
In line, Western blot analysis showed that 0.5–1.0 mmol/L hydroxyurea reduced the protein levels of the proliferating cell nuclear antigen (PCNA) in aHSCs (Figure 2D). Similar results were obtained for portal myofibroblasts (Figure S2).

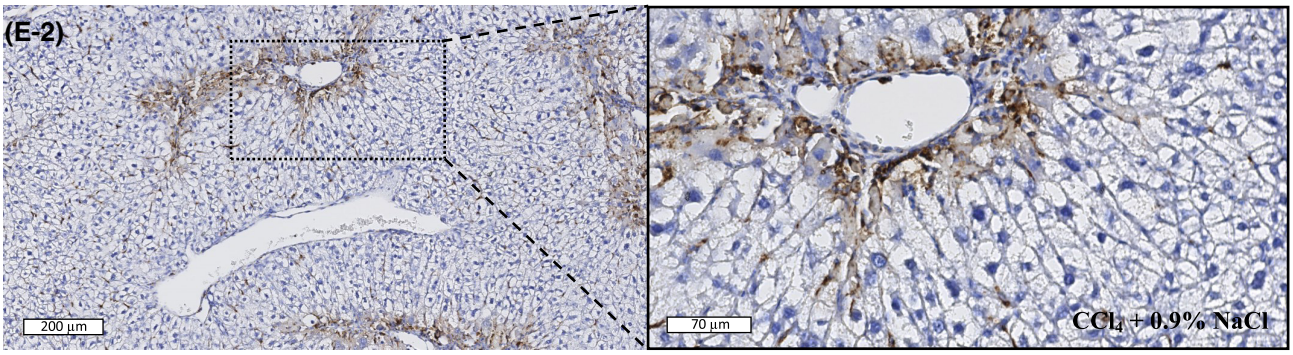
No signs of apoptotic (analyzed by Acridine Orange staining, Figure 2E) or necrotic (analyzed by Sytox green stain, Figure 2F) were observed in hydroxyurea-treated aHSCs. Menadione (25 μmol/L for 5 h)- and H_2O_2 (5 mmol/L for 24 h)-treated aHSCs were included as positive controls for apoptotic and necrotic stimuli, respectively (Figure 2E,F; right panels). Bright field-immunofluorescence overlay images are shown in Figure S3.

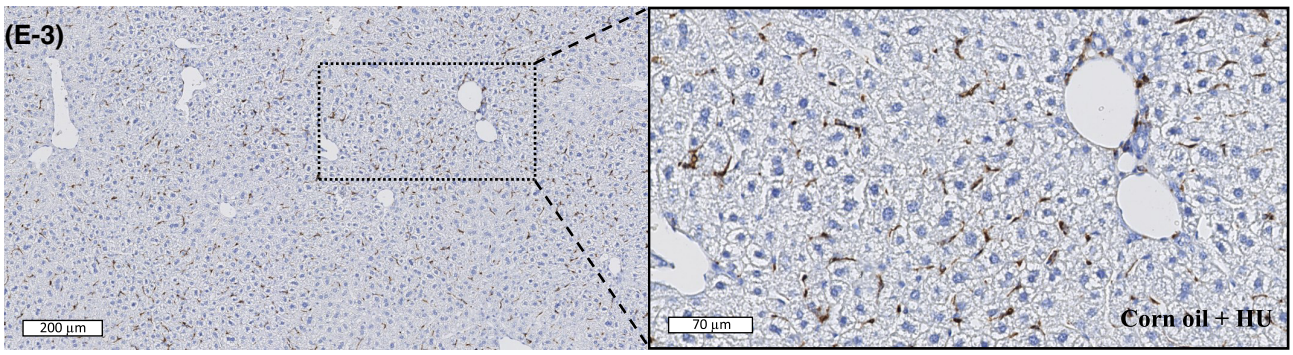
3.3 | Hydroxyurea does not induce cell death in primary rat hepatocytes

Pharmacological inhibition of HSC proliferation may aid in antifibrotic therapies, but should not be cytotoxic to other hepatic cell types, especially hepatocytes. To examine this, primary rat hepatocytes were exposed for up to 48 h to hydroxyurea concentrations up to 10 mmol/L, which did not give rise to evident cell morphological changes or induction of apoptotic (Acridine Orange staining, Figure 3A) or necrotic (Sytox green staining, Figure 3B) cell death. Menadione (50 μmol/L for 9 h)- and H_2O_2 (5 mmol/L for 5 h)-treated hepatocytes were included as positive controls for apoptotic and necrotic stimuli, respectively (Figure 3, right panels). Bright field-immunofluorescence overlay images are given in Figure S4.









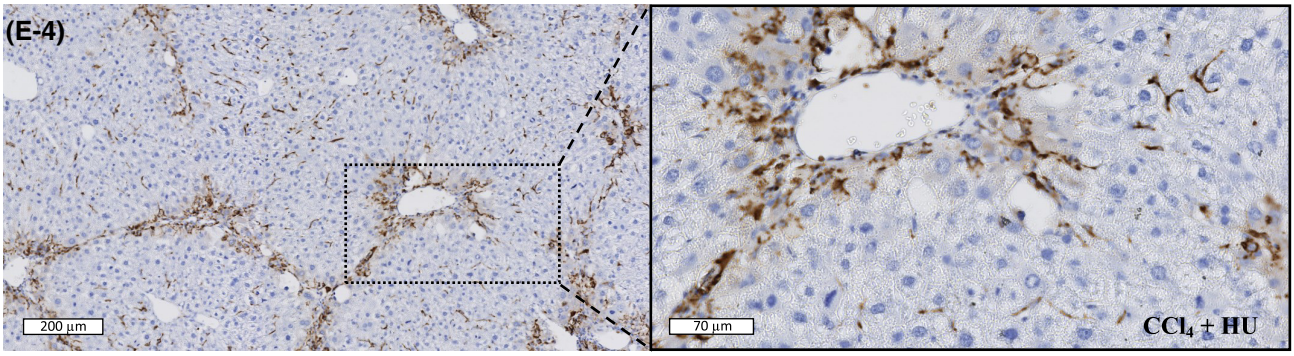


FIGURE 6 Hydroxyurea attenuates liver inflammation. RT-qPCR (A–D) shows that F4/80, Il-1β, and TNF-α were not significantly different between hydroxyurea + CCl_4 treated mice compared to 0.9% NaCl + CCl_4 treated control mice. Il-6 expression was significantly lower in hydroxyurea + CCl_4 treated mice compared to the CCl_4 treated control group. Liver macrophages were stained using the CD68 antibody (E1-E4). E1 presents corn oil + 0.9% NaCl treated mice. E2 presents CCl_4 + 0.9% NaCl treated mice. E3 presents corn oil + hydroxyurea treated mice. E4 presents CCl_4 + hydroxyurea treated mice. CD68 was present in the liver parenchyma of corn oil + 0.9% NaCl treated mice and corn oil + hydroxyurea treated mice. CCl_4 treatment led to a decrease in CD68 parenchymal staining and an increase of CD68 in portal/fibrotic areas. No clear difference was observed between CCl_4 + 0.9% NaCl treated mice compared with CCl_4 + hydroxyurea treated mice.

3.4 | Hydroxyurea suppresses CCl_4-induced accumulation of desmin positive HSCs in mice, without affecting regenerative hepatocyte proliferation

To establish potential antifibrotic effects of hydroxyurea in vivo, mice were treated 2 times per week with CCl_4 (*ip*) for 4 weeks, with or without daily *ip* co-treatment with hydroxyurea.

Forty mice were randomly divided into five groups ($n = 8$ per group): (1) untreated control mice; (2) vehicles (corn oil + 0.9% NaCl)-treated mice; (3) CCl_4 + 0.9% NaCl treated mice; (4) corn oil + hydroxyurea treated mice and (5) CCl_4 + hydroxyurea treated mice. No animals died during the experiment. Animals were sacrificed 1 day after the 8th CCl_4 injection. Table 1 shows blood cell counts and serum levels of liver damage markers AST and ALT. AST and ALT levels were sharply increased in CCl_4-treated mice, which was not changed by hydroxyurea treatment. Hemoglobin, leukocytes, and thrombocytes were reduced as an expected effect of the hydroxyurea treatment. Body weight gain of the mice over the 4-week period was significantly reduced in mice that were treated with corn oil + 0.9% NaCl (both vehicles) when compared to untreated mice. No significant difference in body weight development was observed between mice treated with CCl_4 + 0.9% NaCl and the vehicle-treated mice. Co-treatment with hydroxyurea lead to a significant decrease in body weight gain compared to both vehicle-treated and CCl_4 + 0.9% NaCl-treated animals. However, none of the experimental groups (#1–5) showed body weight loss (Figure 4A). The relative liver weight (as ratio to body weight) was significantly enhanced after CCl_4 treatment, which was not different after co-treatment with hydroxyurea (Figure 4B). CCl_4 induced protein levels of PCNA, indicating regenerative liver cell proliferation in these mice (Figure 4C). PCNA levels remained enhanced after co-treatment with hydroxyurea, though the absolute levels appear slightly reduced. Ki67 staining of liver tissue revealed that CCl_4 induced massive regenerative hepatocyte proliferation, which was not significantly suppressed by hydroxyurea co-treatment (Figure 4D, and quantification in Figure 4E). As expected, CCl_4 also induced the number of desmin-positive HSCs in the liver (Figure 4F and quantification in Figure 4G), in particular accumulating in the lining between portal tracts. In contrast to the lack of effect of hydroxyurea on hepatocyte proliferation, hydroxyurea did significantly suppress the CCl_4-induced accumulation of desmin-positive HSCs in mouse livers.

3.5 | Hydroxyurea suppresses CCl_4-induced collagen deposition

Sirius Red staining of liver tissue revealed that CCl_4 treatment significantly induced hepatic collagen deposition, especially around the portal tracks with very minor portal bridging (Figure 5A top right panel, with quantification in Figure 5B), which is an early stage of liver fibrosis. Hydroxyurea reduced the Sirius Red-positive surface area in livers from CCl_4-treated mice (Figure 5A bottom right panel, with quantification in Figure 5B). However, the CCl_4-induced *Col1α1* mRNA levels were not significantly reduced by co-treatment with hydroxyurea (Figure 5C). Hydroxyurea tended to also suppress the CCl_4-induced α-SMA protein levels (Figure 5D and quantification in Figure 5F), α-SMA staining pattern (Figure 5E) and corresponding *Acta2* mRNA levels (Figure 5G), but none of these effects reached statistical significance (Figure 5D–G).

Other hallmarks of liver fibrogenesis are induced expression of TGF-β as well as PDGF-β and the PDGF-β receptor. Figure 5H–J shows no significant reduction of these markers after hydroxyurea treatment.

3.6 | Hydroxyurea does not significantly inhibit liver inflammation

Besides a direct effect of hydroxyurea on HSC proliferation, hydroxyurea might inhibit hepatic inflammation as it inhibits hematopoiesis.

RT-qPCR was performed on the liver inflammation markers F4/80, IL1-β, IL-6, and TNF-α (Figure 6A–D). No significant difference in F4/80, IL1-β, and TNF-α expression was found between hydroxyurea + CCl_4 treated mice compared to vehicle + CCl_4 treated mice. Il-6 expression

was significantly lower in hydroxyurea + CCl_4 treated mice compared to vehicle + CCl_4 treated mice.

Next we performed a CD68 staining, which stains hepatic macrophages. In corn oil treated control mice, CD68 staining showed a homogenous infiltration pattern of the liver parenchyma (Figure 6E1,E3). No clear difference was observed between corn oil + 0.9% NaCl treated mice compared to corn oil + hydroxyurea treated mice. CCl_4 treatment led to a decrease in CD68 positive cells in the liver parenchyma and an increase in the portal (fibrotic) areas in both groups of CCl_4 treated mice (Figure 6E2,E4). No clear qualitative differences were observed between CCl_4 + 0.9% NaCl treated mice compared to CCl_4 + hydroxyurea treated mice.

Taken together, these results show that hydroxyurea did not deplete the liver of macrophages and hydroxyurea treatment did not result in a significant overall decrease of liver inflammation.

4 | DISCUSSION

In this study, we show that hydroxyurea inhibits HSC proliferation in vitro, and suppresses collagen deposition in vivo without blocking regenerative hepatocyte proliferation. Thus, hydroxyurea may be a relevant drug to inhibit early fibrogenesis or it may be used in a future combination therapy against liver fibrosis.

Hydroxyurea suppressed HSC proliferation in vitro at similar or even lower drug concentrations than those reported to inhibit proliferation of hematological cell lines (0.2 mmol/L vs. 0.2–1.1 mmol/L).[32] Although hydroxyurea was not cytotoxicity to hepatocytes in vitro, even at very high (10 mM) concentrations, the effect of hydroxyurea on proliferation of hepatocytes is difficult to test in vitro because primary hepatocytes have a low proliferative capacity in vitro and spontaneously lose many of their metabolic functions soon after cell isolation.[33,34] In this study, hydroxyurea did not significantly suppress regenerative hepatocyte proliferation. Our results are in line with an earlier report that showed that thymidine incorporation was normal in regenerating rat liver, 3 hours after injecting 100 mg/kg hydroxyurea in rats.[35] However, it has been described that, at very high concentrations (10 mmol/L in vitro or 500 mg/kg in vivo), hydroxyurea inhibited hepatocyte proliferation.[35,36] We did not study why hepatocyte proliferation was not significantly affected while HSC proliferation was. A possible explanation is the hepatic metabolism of hydroxyurea that most likely occurs in hepatocytes. Efficient hepatic metabolism (in addition to renal excretion) is illustrated by an experiment of Van den berg, et al. in a pharmacokinetic study, injecting mice with hydroxyurea (100 mg/kg *ip*). In this study, hydroxyurea was clearly detectable in lung, kidney and brain tissue (100–300 μM), but not in the liver.[37] It has been speculated that urease, catalase, and the monooxygenase cytochrome P-450 metabolize hydroxyurea.[20] Catalase and cytochrome P-450 enzymes are expressed in hepatocytes, and therefore hepatocytes might be less susceptible to hydroxyurea compared to activated hepatic stellate cells.[38,39]

Collagen deposition was reduced in CCl_4-exposed mice that were treated with hydroxyurea. However, hydroxyurea did not significantly reduce mRNA levels of the fibrosis markers *Col1α1* and *Acta2* in vitro nor in vivo. It is well known that collagen synthesis is more rapid in proliferating compared to non-proliferating fibroblasts and stellate cells.[40–43] However, the precise mechanism that explains why collagen synthesis is higher in proliferating cells is still unclear.

Besides the beneficial effect of inhibiting HSC proliferation, hydroxyurea may have an additional protective effect against fibrogenesis in humans as it theoretically could ameliorate fibrosis-associated hypoxia and thereby prevent fibrogenesis. Hypoxia is considered to be a key factor in promoting the progression of liver fibrosis.[44,45] In humans, hydroxyurea induces the production of fetal hemoglobin (HbF) to approximately 20% of the total hemoglobin pool,[46,47] while normal levels are below 1% soon after birth.

Fetal hemoglobin differs from adult hemoglobin (HbA) because it has a higher affinity for oxygen. Therefore, more oxygen can be carried through the circulation at a lower oxygen tension. Blood supply to the liver occurs predominantly via poorly-oxygenated portal venous blood, which results in a partial oxygen pressure of periportal and pericentral blood of approximately 60–65 and 30–35 mmHg,[48] respectively, compared to 75–100 mmHg in arterial blood. Hydroxyurea-induced fetal hemoglobin could theoretically carry more oxygen to the liver via the portal circulation. As only humans and primates can synthesize HbF after birth, this effect did not contribute to the observed anti-fibrotic effect in CCl_4-treated mice, but could synergize with the hydroxyurea-mediated reduction in HSC proliferation in humans.

An important concern associated with hydroxyurea treatment is the reduction in blood-levels of hemoglobin, thrombocytes, and leukocytes, as also observed in the hydroxyurea-treated mice. In particular leukopenia increases susceptibility for infections that can be a threat for patients with cirrhosis. Thus, in future application of hydroxyurea for the treatment of liver fibrosis, it will be of utmost importance to closely monitor blood counts and adjust the hydroxyurea dose to individual patients.

Despite a clear effect on hematopoiesis, hydroxyurea did not significantly suppress expression of liver inflammation markers. This is in line with the finding that transaminases were similar between hydroxyurea treated mice and controls. However, a slight inhibitory effect of hydroxyurea on liver inflammation cannot completely be excluded as Il-6 expression was significantly lower

in hydroxyurea + CCl_4 treated mice compared to CCl_4 treated controls.

While our study reveals potential anti-fibrotic properties of hydroxyurea, our study also has various limitations. First of all, we used cellular and animal models of liver fibrosis. It will be very interesting to analyze patient cohorts that are treated with hydroxyurea for possible effects on early liver fibrosis (F1-F2). Nowadays, this can easily be performed with the use of transient elastography.[49,50] Another limitation of our study is that we only show that hydroxyurea suppresses de novo-fibrogenesis and we did not analyze its capacity to reverse established liver fibrosis. As HSC proliferation is an early event in fibrogenesis, its potential to reverse advanced fibrosis is likely limited. We therefore hypothesize that application of hydroxyurea would be best in combination with drugs that inhibit ECM production and/or promote ECM degradation. Our group recently showed that the pan-hydroxylase inhibitor Dimethyloxalylglycine (DMOG) inhibits collagen type 1 release and suppressed *Col1α1* and *Acta2* mRNA expression by hepatic stellate cells.[51] In theory, combining for DMOG and hydroxyurea could act synergistically against liver fibrosis.

In conclusion, our study reveals that hydroxyurea inhibits stellate cell proliferation and liver fibrogenesis without significantly affecting regenerative hepatocyte proliferation. Since hydroxyurea is an FDA- and EMA-approved drug that can be prescribed safely for longer times, it may hold promise as part of an anti-fibrotic therapy.

AUTHOR CONTRIBUTIONS

Study concept and design: Floris Haijer, Klaas Nico Faber. Acquisition of data: Floris Haijer, Shiva Koets-Shajari, Sandra Serna-Salas, Janette Heegsma, Tjasso Blokzijl, Manon Buist-Homan. Analysis and interpretation of data: Floris Haijer, Shiva Koets-Shajari, Sandra Serna-Salas, Han Moshage, Klaas Nico Faber. Drafting of the manuscript: Floris Haijer, Shiva Koets-Shajari, Han Moshage, Klaas Nico Faber. Statistical analysis: Floris Haijer. Obtained funding: Floris Haijer, Klaas Nico Faber. Technical assistance: Janette Heegsma, Tjasso Blokzijl, Manon Buist-Homan. Study supervision: Klaas Nico Faber.

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DISCLOSURES

The authors certify that they have no affiliations with or involvement in any organization or entity with any financial or non-financial interest in the subject matter or materials described in this manuscript.

DATA AVAILABILITY STATEMENT

All methods that were used are described in the methods section. Data of this manuscript are available upon reasonable request to the corresponding author.

ORCID

Floris Haijer https://orcid.org/0000-0002-2641-5170

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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