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AVALIAÇÃO DA FRUTOSE-1,6-BISFOSFATO SOBRE O ESTADO DE ATIVAÇÃO EM LINHAGEM CELULAR GRX

FERNANDA CRISTINA DE MESQUITA

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Dissertação apresentada como requisito para a obtenção do grau de Mestre pelo Programa de Pós-graduação em Biologia Celular e Molecular da Pontifícia Universidade Católica do Rio Grande do Sul.

Orientador: Prof. Dr. Jarbas Rodrigues de Oliveira

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Dedico à minha mãe, que dividiu comigo
as vitórias e obstáculos desta jornada.

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Á minha mãe, que sempre me incentivou e me apoiou em tudo. Além de ter proporcionado mais esta etapa da minha formação.

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RESUMO

A fibrose hepática é a resposta cicatricial do fígado a lesões continuadas, caracterizada pelo rompimento da arquitetura hepática associada ao aumento da expressão dos componentes da matriz extracelular. As células estreladas hepáticas (HSC) desempenham um papel fundamental no processo de fibrogênese. No fígado normal, as HSC encontram-se em sua forma quiescente de depósito de vitamina A. Durante a lesão hepática, essas células passam por uma ativação fenotípica, tornam-se miofibroblastos e adquirem propriedades fibrogênicas. O processo de fibrose hepática envolve vários mediadores inflamatórios e, portanto, substâncias anti-inflamatórias tem sido empregadas na tentativa de reverter a fibrose e bloquear a ativação e diferenciação das HSC. A ativação de PPARγ (receptor ativado por proliferador de peroxissomo Gama) e a inibição de moléculas fibrogênicas são possíveis estratégias para estes fins. O objetivo deste estudo foi investigar os efeitos *in vitro* da frutose-1,6-bisfosfato (FBP) sobre o fenótipo das HSC. Os resultados demonstraram que a FBP é capaz de induzir o fenótipo quiescente das HSC via ativação de PPARγ. Foi observado nas primeiras 24h de tratamento uma diminuição significativa da expressão de mRNA de colágeno tipo I. Posteriormente, houve uma redução do colágeno total e de TGF-β1 (fator de transformação do crescimento beta). Assim, a FBP diminui o estado de ativação das HSC por suas ações antifibróticas e anti-inflamatórias. Estas descobertas demonstram que a FBP pode ser um potencial novo agente terapêutico para o tratamento de fibrose hepática.

Palavras-chave: Frutose-1,6-bisfofato, células estreladas hepáticas, fibrose hepática, receptor ativado por proliferador de peroxissomo gama, fator de transformação do crescimento beta.

ABSTRACT

Liver fibrosis is the wound healing response to repeated injury of the liver. It is characterized by disruption of the liver architecture associated with increased expression of extracellular matrix components. Hepatic stellate cells (HSC) play a key role in liver fibrogenesis. In normal liver, HSC are quiescent and its main function is to store vitamin A. During liver injury, these cells undergo activation, become myofibroblasts and acquire fibrogenic properties. Activation of PPARγ (peroxisome proliferator-activated receptor gamma) and inhibition of fibrogenic molecules are potential strategies to block HSC activation and differentiation. Aware that the process of hepatic fibrosis involves inflammatory mediators, various anti-inflammatory substances have been studied in an attempt to revert fibrosis. The purpose of this study was to investigate the *in vitro* effects of fructose-1,6-bisphospahte (FBP) on HSC phenotype. The results demonstrated that FBP induced quiescent phenotype in HSC via PPARγ activation. Significant decrease in type I collagen mRNA expression was observed in the first 24h of treatment. These events preceded the reduction of TGF-β1 (transforming growth factor-beta) and total collagen secretion. Thus, FBP promoted downregulation of HSC activation by its antifibrotic and anti-inflammatory actions. These findings demonstrate that FBP may have potential as a novel therapeutic agent for the treatment of liver fibrosis.

Key-words: Fructose-1,6-bisphosphate, hepatic stellate cell, hepatic fibrosis, peroxisome proliferator-activated receptor gamma, transforming growth factor-beta.

LISTA DE ABREVIATURAS

ECM – Matriz extracelular

FBP – Frutose-1,6-bisfosfato

FBS – Soro fetal bovino

HCV – Vírus da hepatite C

HSC – Célula estrelada hepática

PPARγ – Receptor ativado por proliferador de peroxissomo gama

TGF-β1 – Fator de transformação do crescimento beta 1

TNF-α – Fator de necrose tumoral alfa

SUMÁRIO

1. INTRODUÇÃO

1.1 Fibrose hepática

A fibrose hepática é uma resposta cicatricial que ocorre em quase todos os pacientes com lesão crônica do fígado (Friedman 2008). Ocasionalmente, a fibrose hepática pode progredir rapidamente como resultado de uma lesão aguda causada por drogas, pelo vírus da hepatite C (HCV), ou por co-infecção pelo vírus da imunodeficiência humana adquirida (HIV) e o HCV. Porém, na maioria dos casos, a fibrose hepática acumula-se durante décadas, proveniente de uma lesão crônica (Jang and Chung 2011). O prolongamento natural dessa resposta, em contraste com a progressão mais rápida da fibrose nos rins ou pulmões, é creditado à capacidade excepcional do fígado de regeneração, pois se sabe que deve haver uma redução de 80 a 90% da capacidade funcional do fígado para que ocorra falência hepática, tamanha a reserva funcional desse órgão (Friedman 2008; Krizhanovsky, Yon et al. 2008).

A cirrose hepática é considerada um estágio avançado de fibrose, constituindo-se em um dos maiores problemas de saúde mundial (Friedman 2008) e compreende todas as complicações da doença hepática no estágio final, que inclui hipertensão portal, ascite, encefalopatia e alterações no metabolismo, principalmente de lipídeos e da glicose (Friedman 2008). O maior determinante da cirrose hepática é a dificuldade em degradar a matriz fibrótica, o que provoca destruição da arquitetura do fígado, interrupção das funções hepáticas, acarretando em disfunção (Sarem, Znaidak et al. 2006; Friedman 2008; Friedman 2008; Iredale 2008; Fallowfield and Hayes 2011).

1.2 Células estreladas hepáticas

As Células Hepáticas Estreladas (HSC, do inglês, *Hepatic Stellate Cells*) são também chamadas de lipócitos, células armazenadoras de gordura, células armazenadoras de vitamina A, células intersticiais ou células de Ito (Senoo 2004; Sarem, Znaidak et al. 2006). Foram descobertas por Kupffer em 1876 e caracterizadas quase um século depois por Ito e Nemoto como células armazenadoras de gordura (Winau, Quack et al. 2008).

Estas células estão localizadas no espaço perissinusoidal de Disse. Classicamente este espaço define-se como uma zona limitada pelos hepatócitos e pela parede sinusoidal formada pelas células endoteliais sinusoidais (Senoo 2004; Friedman 2008; Winau, Quack et al. 2008). O espaço perisinusoidal de Disse contém fibras nervosas e componentes da matriz extracelular (ECM) como fibras de colágeno dos tipos I e III e componentes da membrana basal (Vicente, Fortuna et al. 1998).

As HSC constituem 5-8% do número total de células hepáticas considerando as parenquimatosas (hepatócitos), endoteliais (sinusoidais e vasculares), células de Kupffer (macrófagos hepáticos) e as células epiteliais da via biliar. As HSC manifestam dois fenótipos distintos: miofibroblástico (quando estão ativadas) e lipocítico (quando estão quiescentes), como demonstrado na figura 1. Quando expressam o fenótipo miofibroblástico estão essencialmente envolvidas com a produção da ECM e controle da homeostasia do tecido conectivo hepático. O aumento do número e ativação dos miofibroblastos hepáticos está associado com fibrose e cirrose (Vicente, Fortuna et al. 1998).

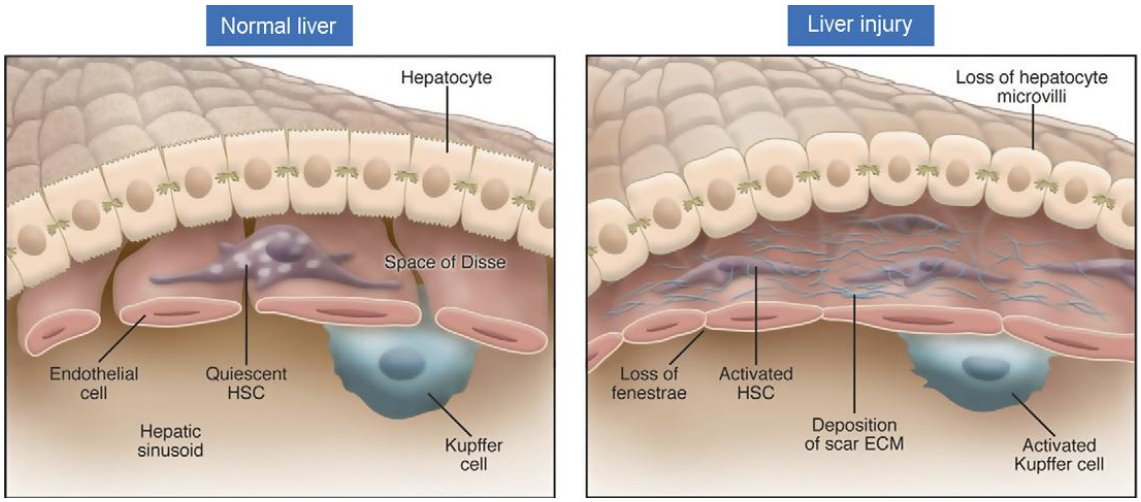

Figura 1 – Papel das células residentes do fígado na lesão hepática. As mudanças no espaço perisinusoidal de Disse durante o desenvolvimento da fibrose em resposta a alguma lesão no fígado incluem alterações tanto no comportamento celular quanto na composição da ECM. A ativação das HSC leva a síntese de colágeno e consequentemente a deposição de matriz fibrótica precedendo a falência hepática. A ativação das células de Kupffer tem ação parácrina sobre as HSC (Iredale 2008).

Expressando o fenótipo lipocítico, seu citoplasma se caracteriza por conter gotículas de gordura onde está a vitamina A (retinol) (Senoo 2004; Sarem, Znaidak et al. 2006; Friedman 2008). Mais de 90% da vitamina A hepática (aproximadamente 80% do total do organismo) é captada, armazenada e metabolizada nas HSC, que podem ser identificadas pela autofluorescência desta vitamina (Friedman 2008; Winau, Quack et al. 2008).

Em condições normais as HSC cumprem várias funções no fígado, entre elas as mais importantes são: armazenamento da vitamina A, controle da comunicação intercelular através da liberação de mediadores e remodelamento da ECM (Winau, Quack et al. 2008).

Além disso, alguns estudos relatam que as HSC têm um papel crucial no sistema imune hepático. Elas expressam receptores para os lipopolissacarídeos bacterianos (Toll-like receptor 4 – TLR4), moléculas co-estimulatórias e citocinas necessárias para a modulação da resposta imune além de atuarem como células apresentadoras de antígenos (APCs) (Winau, Quack et al. 2008).

O desenvolvimento de fibrose hepática é baseado na ativação das HSC que sofrem mudanças fenotípicas características. Essa ativação é um processo patológico que se caracteriza pela perda das gotículas de gordura onde a vitamina A é armazenada, pelo aumento do número e tamanho celulares e pela diferenciação das HSC em células proliferativas, fibrogênicas, contráteis muito similares aos miofibroblastos (Sarem, Znaidak et al. 2006; Friedman 2008).

A ativação das HSC consiste em duas subfases: iniciação e perpetuação, seguidas pela fase final chamada resolução. A primeira fase está associada ao estímulo parácrino de células inflamatórias e hepatócitos lesados. As células de Kupffer podem estimular a proliferação, síntese de matriz, e perda dos retinóides pelas HSCs através da ação de citocinas como fator de transformação do crescimento β (TGF-β) e fator de necrose tumoral α (TNF-α) (Li, Liao et al. 2008). A perpetuação resulta dos efeitos desses estímulos que mantêm o fenótipo ativado principalmente através do aumento da expressão de fatores de crescimento. Essa fase de ativação envolve no mínimo sete mudanças discretas no comportamento celular: proliferação, quimiotaxia, fibrogênese, contratilidade, degradação da matriz, perda de lipídios e liberação de citocinas. Durante essa fase há uma liberação de estimulos pró-inflamatórios, pró-fibrogênicos e pró-mitogênicos que agem de forma autócrina e parácrina, além do remodelamento acelerado da ECM (Lee and Friedman 2011).

Na resolução, as HSC podem sofrer apoptose ou voltarem a exibir um fenótipo quiescente, o que seria benéfico para o tecido hepático (Winau, Quack et al. 2008).

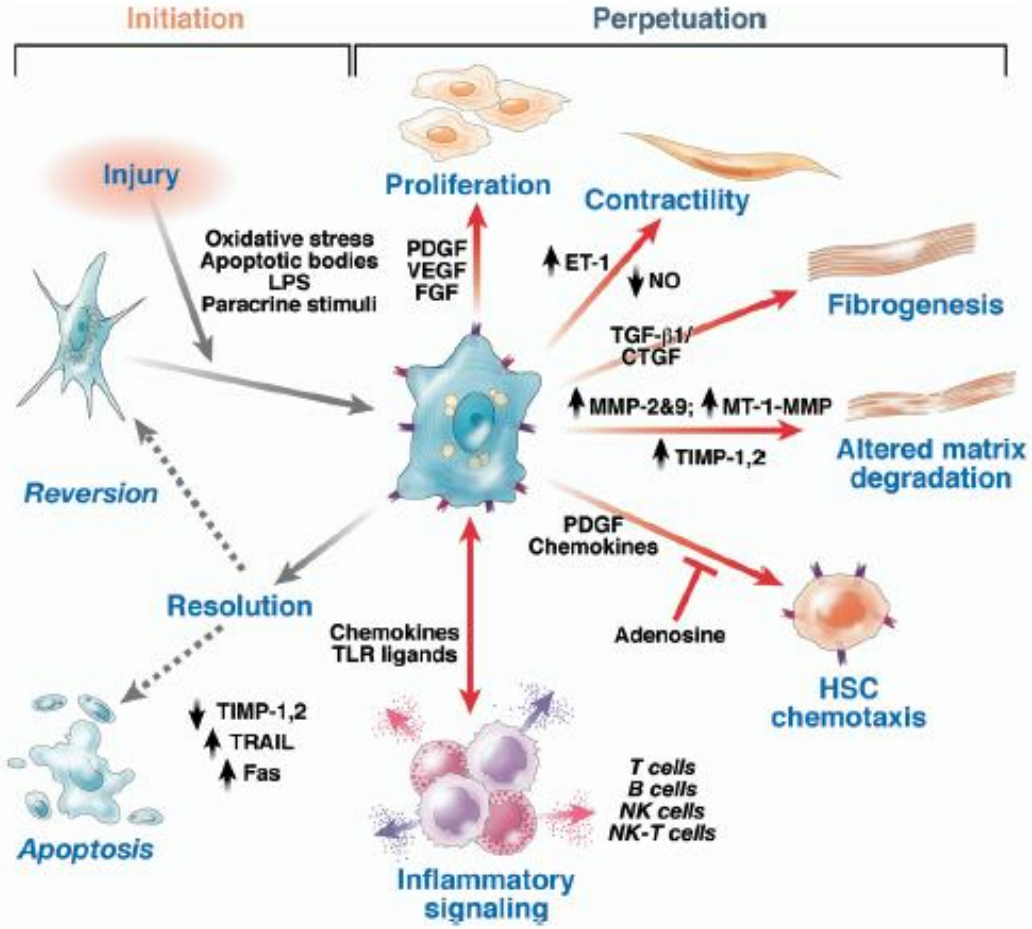

Figura 2 – Vias de ativação das HSC. As **c**aracterísticas da ativação de células estreladas podem ser distinguidas entre aquelas que estimulam a iniciação e aquelas que contribuem para a perpetuação. A iniciação é provocada por estímulos, que incluem espécies reativas de oxigenio, substâncias apoptóticas, lipopolissacarídeos (LPS) e os estímulos parácrinos de células vizinhas, que incluem macrófagos e hepatócitos. A perpetuação que se segue é caracterizada por várias mudanças fenotípicas como proliferação, contratilidade, fibrogênese, alteração na degradação da matriz, quimiotaxia e sinalização inflamatória (Friedman 2008).

1.2.1 A linhagem celular GRX

A linhagem celular GRX é representativa de HSC em camundongos e foi obtida de uma reação fibrogranulomatosa hepática de camundongo infectado com *Schistosoma mansoni* (Vicente, Fortuna et al. 1998; Guimaraes, Franceschi et al. 2006). Sob condições padrão de cultivo essas células expressam fenótipo de miofibroblastos e estão no estado transicional entre o lipócito quiescente e o estado ativado (miofibroblasto ativado) (Guimaraes, Franceschi et al. 2006; Souza, Martins et al. 2008). Elas podem expressar o fenótipo de miofibroblastos ativados em resposta ao tratamento *in vitro* com citocinas profibrogênicas ou expressar o fenótipo lipocítico quiescente se forem tratadas com retinol, ácido retinóico ou drogas que modifiquem o metabolismo lipídico como a indometacina (Guimaraes, Franceschi et al. 2007; Souza, Martins et al. 2008). Além disso, foi demonstrado que esta linhagem se comporta muito similarmente às HSC no que diz respeito à captação, armazenamento e liberação de retinóides. Sendo assim, a linhagem celular GRX é considerada um modelo *in vitro* das HSC para avaliar o destino das HSC (Vicente, Fortuna et al. 1998).

1.2.2 PPARγ

O receptor ativado por proliferador de peroxissomo gama (PPARγ) é um fator de transcrição associado à membrana nuclear que regula muitos processos biológicos, incluindo o metabolismo lipídico, homeostase da glicose, inflamação e aterogênese (Queiroz, Alonso-Vale et al. 2009). O PPARy tem sido descrito como o regulador central da adipogênese, é altamente expresso em HSCs quiescentes e opera na via de diferenciação celular, promovendo o armazenamento intracelular de lipídios e suprimindo o gene do colágeno tipo I (Guimarães, Franceschi et al. 2007).

Além disso, a ativação de PPARy é necessária para inibir a proliferação celular, induzir a apoptose e a expressão do gene supressor de ECM (Wang, Xu et al. 2011; Zhang, Lu et al. 2012). Ligantes PPARy mostram a grande promessa na moderação inflamação em vários tecidos (Annese, Rogai et al. 2012; Kulkarni, Woeller et al. 2012).

O mecanismo molecular preciso pelo qual PPARγ atua ainda é desconhecido, porém é provável que ele interaja com vários processos de sinalização que regulam a expressão de moléculas fibrogênicas pelas HSCs (Zhang, Lu et al. 2012).

1.3 Frutose-1,6-bisfosfato

A frutose-1,6-bisfosfato (FBP) é um dos metabólitos encontrados na rota glicolítica, apresentando estruturas estáveis anoméricas: α e β furanose. Este açúcar bisfosforilado, além de ser um subproduto da via glicolítica também exerce papel importante junto a diversas rotas metabólicas do organismo (Alva, Carbonell et al. 2011). Entre as suas ações, aparece a sua capacidade de alterar o metabolismo de carboidratos estimulando a glicólise e inibindo a gliconeogênese (Kirtley and McKay 1977).

A FBP tem demonstrado efeitos terapêuticos em várias situações patológicas como: isquemia, choque e lesões tóxicas (Fortes Aiub, Bortolini et al. 2003). Também foram documentados os efeitos benéficos de FBP em deficiências orgânicas cardíacas, renais, cerebrais, intestinais e hepáticas (De Oliveira, Rosa et al. 1992; Nunes, Graziottin et al. 2003; Calafell, Boada et al. 2009).

Os mecanismos pelos quais FBP protege células e tecidos ainda não são claros. Um possível mecanismo de proteção inclui o metabolismo anaeróbio da FBP para gerar adenosina trifosfato (ATP) (Gobbel, Chan et al. 1994) ou reduzir a sua perda (Gregory, Welsh et al. 1990), e/ou pela sua propriedade quelante de cálcio (Hassinen, Nuutinen et al. 1991). Esta capacidade da FBP em diminuir a quantidade de cálcio extracelular, melhora o rendimento mecânico e respiratório do coração isquêmico (De Oliveira, Rosa et al. 1992), este mediado pela ativação de fosfoquinase-C que modula a atividade intracelular do cálcio (Donohoe, Fahlman et al. 2001). A FBP também aumenta a captação celular de potássio que resulta em uma diminuição intracelular da concentração de sódio, reduzindo assim, o edema celular citotóxico (Cattani, Costrini et al. 1980).

O mecanismo pelo qual a FBP reduz a formação de O_2^- pode ser decorrente do aumento nos níveis de ATP, tendo em vista que este pode ser o regulador fisiológico da atividade catalítica da enzima nicotinamida adenina dinucleotídeo fosfato (NADP) oxidase, uma das enzimas responsáveis pela produção destes radicais (Babior and Peters 1981). A FBP inibe a formação de

espécies reativas de oxigênio e a ativação de neutrófilos (Sola, Panes et al. 2003), reduz a proliferação e a viabilidade de linfócitos T (Nunes, Graziottin et al. 2003) e também inibe a apoptose em hepatócitos (Calafell, Boada et al. 2009).

Ano	Revista	Autores	Ações da FBP
1980	Agressologie	Cattani, Costrini et al. 1980	Aumenta a captação celular de potássio e diminui a concentração de sódio, reduzindo o edema celular citotóxico
2003	Journal of Leukocyte Biology	Sola, Panes et al.	Inibe ERO e a ativação de neutrófilos.
2003	International Immunopharmacology	Nunes, Graziottin et al.	Reduz proliferação e viabilidade de linfócitos.
2009	European Journal of Pharmacology	Calafell, Boada et al.	Inibe a apoptose em hepatócitos, aumenta a produção de NO e reduz o estresse oxidativo.
2011	European Journal of Pharmacology	Alva, Carbonell et al.	Previne acidose metabólica e estresse oxidativo.

Tabela 1. Ações da frutose-1,6-bisfosfato classificadas por ano, revista e autores.

2. JUSTIFICATIVA

Estudos prévios relataram as propriedades terapêuticas antioxidantes e antiinflamatórias da frutose-1,6-bisfosfato em modelos animais (Nunes, Graziottin et al. 2003; Alva, Carbonell et al. 2011). Sabendo que o processo de fibrose hepática envolve mediadores inflamatórios como citocinas, quimiocinas, espécies reativas de oxigênio, é importante considerar que a FBP possa ser útil na tentativa de reversão da fibrose. Por essa razão, esse estudo tem como finalidade avaliar a ação antifibrótica da FBP.

3. OBJETIVOS

3.1 Objetivo geral

Avaliar o efeito da frutose-1,6-bisfosfato sobre a reversão fenotípica em linhagem celular GRX.

3.2 Objetivos específicos

- Avaliar alterações no fenótipo das células GRX tratadas com FBP.

- Determinar a concentração de lipídios, TGF-β1 e colágeno total.

- Avaliar a expressão do mRNA de PPAR e colágeno tipo I das células tratadas com FBP.

4. ARTIGO CIENTÍFICO

Os resultados do presente trabalho foram submetidos ao periódico *Journal of Cellular Biochemistry.*

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Fructose-1,6-bisphosphate induces phenotypic reversion of activated hepatic stellate cell

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Key-words: fructose-1,6-bisphosphate, hepatic stellate cell, fibrosis, peroxisome proliferator-activated receptor gamma, transforming growth factor-beta.

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ABSTRACT

Hepatic stellate cells (HSC) play a key role in liver fibrogenesis. Activation of PPARγ and inhibition of fibrogenic molecules are potential strategies to block HSC activation and differentiation. Aware that the process of hepatic fibrosis involves inflammatory mediators, various anti-inflammatory substances have been studied in an attempt to revert fibrosis. The purpose of this study was to investigate the *in vitro* effects of fructose-1,6-bisphospahte (FBP) on HSC phenotype. The results demonstrated that FBP induced quiescent phenotype in GRX via PPARγ activation. Significant decrease in type I collagen mRNA expression was observed in the first 24h of treatment. These events preceded the reduction of TGF-ß1 and total collagen secretion. Thus, FBP promoted downregulation of HSC activation by its antifibrotic and anti-inflammatory actions. These findings demonstrate that FBP may have potential as a novel therapeutic agent for the treatment of liver fibrosis.

Fibrosis is a reversible scarring response that occurs in almost all patients with chronic liver injury [Friedman, 2008a]. Although the mechanisms of acute injury activate fibrogenesis, the signs associated with chronic lesions caused by infections, drugs, metabolic disorders, alcohol abuse, non-alcoholic hepatitis, or immune attack are necessary to accumulate fibrosis [Friedman, 2008a; Iredale, 2008; Jang and Chung, 2011; Krizhanovsky et al., 2008].

Hepatic stellate cells (HSC) are a major regulator in liver homeostasis and play a central role in the development and maintenance of liver fibrosis [Friedman, 2008b]. Under normal conditions, the HSC reside in the space of Disse in a quiescent phenotype storing vitamin A in cytoplasmic lipid droplets, showing a low proliferate rate and expressing markers that are characteristic of adipocytes (*e.g.* PPARγ) [Chakraborty et al., 2012; Friedman, 2008b]. Upon liver injury, HSC transform to an active phenotype and produce excessive fibrillar collagens, inhibitors of matrix proteases and proinflamatory cytokines [Chakraborty et al., 2012]. These cytokines include profibrotic transforming growth factor (TGF-β), a central mediator of fibrotic response. Fibrosis is potentially a reversible process in early stages [Friedman, 2008b].

The GRX cell line was established from hepatic inflammatory fibrogranulomatous reactions and became an important tool for the study of HSC physiology [Borojevic et al., 1985; Guimarães et al., 2006]. Under standard conditions, these cells express a transitional myofibroblast phenotype and secretes collagen type I and III [Guimarães et al., 2007]. These cells have pro-inflammatory and pro-fibrogenic properties but it has been previously described the GRX induction to quiescent phenotype by several agents that modify lipid metabolism, decrease collagen production and store lipid droplets [Bitencourt et al., 2012; Borojevic et al., 1990; Bragança de Moraes et al., 2012; Cardoso et al., 2003; Martucci et al., 2004].

Fructose-1,6-bisphosphate (FBP) is a metabolite found in cells and has been reported as showing therapeutic effects in a number of pathological situations such as isquemia, shock[Markov, 1986], and toxic lesions [Markov, 1986]. The beneficial effects of FBP have been documented in cardiac [Hardin et al., 2001; Wheeler and Chien, 2012], renal [Azambuja et al., 2011], cerebral [Gobbel et al., 1994; Liu et al., 2008], intestinal (small bowel) [Sola et al., 2004] and hepatic dysfunctions [Cuesta et al., 2006; De Oliveira et al., 1992]. The protective action increases the interest of FBP as a therapeutic agent [Calafell et al., 2009]. Empirical evidence indicates that the protective action of FBP in stress situations is explained by its incorporation as an energy substrate and by the prevention of critical alterations in membrane function. The relative contribution of each factor depends on the cell type [Calafell et al., 2009].

The protective action of FBP against hepatic injury involves both energy metabolism and cell-membrane function of liver parenchyma cells [De Oliveira et al., 1992], as well as of other cells involved in liver inflammation [Cuesta et al., 2006; Hirokawa et al., 2002; Nunes et al., 2003].

Aware that the process of hepatic fibrosis involves inflammatory mediators such as cytokines, chemokines and reactive oxygen species, it is important to investigate if FBP can be useful in reversing fibrosis. Therefore, the aim of this study was to assess the FBP effect on the phenotype of GRX liver cells.

MATERIALS & METHODS

CELL CULTURE: The murine HSC cell line, GRX [Borojevic et al., 1985], were obtained from Rio de Janeiro Cell Bank (Federal University, Rio de Janeiro, Brazil). Cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 5% fetal bovine serum (FBS) (Invitrogen, USA), 2 g/L HEPES buffer, 3.7g/L NaHCO3 and 1% penicillin and streptomycin (Invitrogen) and incubated at 37°C in a humidified atmosphere with 5% CO_2.

FRUCTOSE-1,6-BISPHOSPHATE TREATMENT: Fructose-1,6-bisphosphate (Sigma Chemical Co., USA) was dissolved in DMEM at concentration of 0.6 mM and 1.25 mM [Bordignon Nunes et al., 2003]. GRX cells were incubated and the analyses were performed 24h or seven days after treatment. Based on previous studies [Bitencourt et al., 2012], all experiments were performed thrice and in triplicates.

DETECTION OF LIPID DROPLETS BY OIL RED O STAINING: To visualize cell morphology and lipid accumulation, on day 7, cells were stained with Oil Red-O (ORO) (Sigma Chemical Co., USA) [Ramírez-Zacarías et al., 1992]. Briefly, after fixing cells with 10% formaldehyde, ORO (0.35g, 60% isopropanol) was added for 15min. Intracellular lipid droplets were examined using an inverted light microscope.

QUANTIFICATION OF LIPID ACCUMULATION: Lipid was measured on day 7. The procedure is based on ORO staining of intracellular lipid droplets and Coomassie brilliant blue staining (Sigma) of cellular proteins [Bouraoui et al., 2008]. Briefly, cells were fixed with perchloric acid and incubated with ORO dissolved in propylene glycol (2 mg/mL) for 2h. The ORO within the lipid droplets was extracted using isopropanol. The absorbance was read at 492 nm using an ELISA plate reader. Next, the wells were washed with water thrice for protein determination. Cells were incubated with Coomassie brilliant blue staining for 1h. After washing, the cells were incubated with propylene glycol for 3h at 60°C. The absorbance was read at 620nm. The specific lipid content was calculated as the ratio of absorbance value obtained for ORO and Coomassie brilliant blue staining.

TGF-β1 QUANTITATION: TGF-β1 concentration was measured, on day 7, in cell supernatant using commercially available ELISA kit (R&D Systems, USA). The kit contained a specific monoclonal antibody immobilized on a 96-well microtiter plate that bound TGF-β1 in the aliquot and a second enzyme-conjugated specific polyclonal antibody. Following several washings in order to remove unbound substances and antibodies, a substrate solution was added to the wells. Color development was stopped by sulfuric acid and optical density

was determined at 540nm with the correction wavelength set at 570nm in an ELISA plate reader. Results were calculated on a standard curve concentration and multiplied for the dilution factor. TGF-β1 levels were expressed as picograms per milliliter.

MEASUREMENT OF COLLAGEN CONTENT: Collagen content in GRX cells was measured on day 7, using picro-sirius red. Briefly, picro-sirius red was added to cell supernatant to form a collagen-dye complex. After centrifugation, unbound dye was removed and collagen-dye complex dissolved in NaOH. The absorbance was measured at 540nm in an ELISA plate reader. Each sample was normalized to the relative amount of total protein measured by the Bradford method [Bradford, 1976]. Results were calculated on a standard curve concentration. Collagen levels were expressed as the ratio of milligrams of collagen and milligrams of proteins.

RNA EXTRACTION AND RT-PCR: Total RNA was extracted from cells using TRIzol reagent (Invitrogen, USA) on 24h and day 7. RNA was reverse transcribed into cDNA, using Superscript III First-Strand Synthesis SuperMix (Invitrogen, USA) according to the manufacturer's instructions. Table 1 shows the primers sets used. Polymerase chain reaction products were electrophoresed using 1.5% agarose gel containing ethidium bromide 5 μg/ml. The gel was visualized using ultraviolet light and photographed. The band intensities were measured using the public domain National Institutes of Health Image program (Image J) and the signals were expressed relatively to the intensity of the b-actin amplicon in each coamplified sample.

STATISTICS: Data are reported as mean ± SD. Each experiment was performed at least three independent times and in triplicate. Statistical test was performed with SPSS software (version 13.0, SPSS Inc., USA). Results were analyzed by one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test. The level of significance was set at $P < 0.05$.

RESULTS

To study the effect of FBP on the GRX cell, concentrations of 0.6mM and 1.25mM were used after 24 h and 7 days of treatment.

FBP led to phenotype conversion of myofibroblast-like (activated) phenotype into fat storing (quiescent) phenotype. Typical changes in cell morphology during de-differentiation were observed. Cells lost their elongated and parallel strand appearance and acquired a larger and polygonal shape. Intracellular lipid droplets were mostly visible in GRX cells treated with all FBP concentrations. After 7 days most cells exhibited the fat storing phenotype as detected by phase contrast microscopy after staining with Oil- Red-O (ORO) (Fig. 1). In contrast, control cells preserved their myofibroblast- like morphology, devoid of large lipid droplets (Fig. 1). These findings were confirmed by colorimetric quantification of intracellular lipids concentration (Fig. 2). The amount of ORO detected at day 7 was up to 2-fold higher compared with that of control cells staining. Aware that PPARγ regulates lipid metabolism, to confirm cell phenotypic reversion, the mRNA expression of PPARγ was measured after 24h of treatment. The expression of PPARγ was significantly increased in groups treated with FBP compared to the control group (Fig. 3).

To measure the antifibrotic effects of FBP, the amount of secreted TGF-β1 present in media from cultured GRX was quantified by ELISA. Thus, as the cells began to differentiate and change in morphology, a significantly decreasing of TGF-β1 levels was detected. After 7 days, GRX cells treated with FBP exhibited a reduction of TGF-β1 secretion when compared to the control group (Fig. 4). As the activated HSC are the predominant hepatic cell type in the liver and responsible for the increased synthesis and deposition of type I collagen during fibrosis, we decided to quantify the amount of total collagen secreted by GRX cells and to measure the mRNA expression of type I collagen. The total collagen secretion decreased after 7 days (Fig. 5) and the mRNA expression of type I collagen was downregulated at 24h (Fig. 6).

DISCUSSION

The permanent cell line GRX experimental model has allowed the analysis and comparison of several parameters of hepatic stellate cell metabolism in the myofibroblast versus lipocyte-like phenotype, such as: neutral lipid and phospholipid synthesis [Guaragna et al., 1992], activity of enzymes involved in lipid and retinol metabolism [Fortuna et al., 2001], extracellular matrix and collagen synthesis [Margis et al., 1992; Pinheiro-Margis et al., 1992], intermediate filament expression [Guma et al., 2001], and actin organization [Mermelstein et al., 2001].

In this study, we investigated the activity of FBP on the phenotypic reversion of GRX cells. We demonstrated that FBP is capable of inducing de-differentiation of myofibroblast to lipocyte phenotype in GRX. The culture of GRX in the presence of FBP resulted in an increased lipid accumulation, as evidenced by ORO staining and confirmed by lipid quantitation. Therefore, FBP acts as a potent inducer of the quiescent phenotype.

PPARγ regulates many biological processes, including lipid metabolism, glucose homeostasis, inflammation and atherogenesis. [Queiroz et al., 2009]. The PPARγ has been described as the central regulator of adipogenesis, it is highly expressed in quiescent HSCs and operates in cellular differentiation pathway through a variety of mechanisms [Guimarães et al., 2007; Tsukamoto et al., 2006]. Furthermore, PPARγ activation is necessary for inhibiting cell proliferation, inducing apoptosis, suppressing ECM gene expression, and restoring lipid storage capacity [Wang et al., 2011; Zhang et al., 2012]. PPARγ ligands show great promise in moderating inflammation in various tissues [Annese et al., 2012; Kulkarni et al., 2012]. For this reason, knowing that the FBP also acts as anti inflammatory agent, we investigated if the FBP would be changing the GRX cell phenotype through the PPARγ pathway. We thought that FBP-induced de-differentiation may be associated with the PPARγ pathway. The PPARγ mRNA expression in GRX cells treated with FBP for 24 h increased 2.0-fold compared to the group of untreated cell. These results suggest that PPARγ participates as a transcriptional factor of adipogenesis during GRX de-differentiation induced by FBP. The experiment was conducted only in 24 hours with the aim of discovering if activation of PPAR is the trigger for cell differentiation.

Inhibition of matrix production has been the primary target of most antifibrotic therapies. This has been attempted directly by blocking matrix synthesis and processing or indirectly by inhibiting the activity of TGFb1.

This is the most powerful mediator of HSC activation and plays a central role in liver fibrosis [Qian et al., 2012]. It not only activates HSC, but also stimulates ECM synthesis, and regulates cell proliferation and differentiation [Bataller and Brenner, 2005]. The inhibition of extracellular matrix production is crucial for the prevention and resolution of fibrosis [Hernández-Ortega et al., 2012]. TGF-b1 is the major isoform implicated in hepatic fibrosis. During fibrogenesis, tissue and blood levels of active TGF-b1 are elevated. Thereby, its concentrations are correlated with the severity of liver fibrosis [Friedman, 2008a; Gressner et al., 2002]. As TGF-b is the central regulator cytokine of tissue fibrosis, we analyzed TGF-b1 concentrations in GRX cells supernatant. A significant decrease of TGF-b1 protein levels was detected after 7 days treatment with FBP. The results indicate a possible antifibrotic effect of FBP in HSC via reduced generation of active TGF-b1. The decrease in levels of TGFβ corroborate with the increased PPARγ once many studies have shown that activation of PPARγ interrupts the signaling pathway of TGFβ and inhibits its effects pro-fibrotic [Gong et al., 2011; Sakurai et al., 2011; Zhang et al., 2012]. Accordingly, we suggest that FBP mediates the blockage of the TGF-β signaling pathway via PPARγ activation.

Stellate cells generate fibrosis not only by increased cell number, but also by increasing matrix production. The best-studied component of hepatic scar is collagen type I, the expression of it is regulated both transcriptionally and postranscriptionally in hepatic stellate cells by a growing number of stimuli and pathways [Friedman, 2008a]. GRX cells, as many activated HSCs, secrete high levels of collagen type I and III [Borojevic et al., 1985; Herrmann et al., 2007]. Therefore, type I collagen is a critical parameter reflecting the metabolism of collagen in the process of liver fibrosis [Gressner et al., 2002]. The results presented in this report demonstrate not only that the FBP significantly reduced the amount of total collagen in the culture media, but also downregulated expression of type I collagen mRNA within the first 24 h of incubation. Resulted by the activation of PPARγ that was already operational in 24 h.

In conclusion, the FBP induced phenotype reversion of GRX cell by its anti-inflammatory and antifibrotic actions. The primary mechanism, that appears to be involved is the up-regulation of PPARγ mRNA expression, ends inducing the decrease of TGFβ and consequently decrease profibrogenics mediators. Knowing that cirrhosis is a worldwide problem of high incidence and high impact, whose treatment for reversal or prevention of disease are still lacking, these data may be important for the development of novel anti-inflammatory and antifibrotic agents in fibrosis.

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FIGURE LEGENDS

Fig. 1. Oil Red-O (ORO) staining and lipid quantitation of GRX cells at day 7. (A) Control cells; (B), (C) cells treated with FBP at 0.6 mM and 1.25, respectively. Bar length = 20 µm.

Fig. 2. Specific lipid content expressed spectrophotometrically as the ratio of absorbance value obtained for ORO and Coomassie brilliant blue staining. Results are expressed as mean ± SD. **P < 0.01: control vs. treated cells.

Fig. 3. Effects of Fructose-1,6-bisphosphate (FBP) on PPARγ mRNA expression of GRX cells treated for 24 hours. β-actin was an internal control for equal loading. Data are expressed as mean ± SD (n = 3). Results are presented as relative optical density PPARγ/β-actin. ** P < 0.01 compared with control.

Fig. 4. ELISA assay of TGF-β1 in cell supernatant of 7 days treatment. Data represent the mean ± SD (n = 3). TGF-β1 levels were expressed as picograms per milliliter. * P < 0.05 and ** P< 0.01 compared with control.

Fig. 5. Total collagen content in cell supernatant of 7 days treatment. Data represent the mean ± SD (n = 3). Results were expressed as percentage of control. ** P < 0.01 and *** P < 0.001 compared with control.

Fig. 6. Effects of fructose-1,6-bisphosphate (FBP) on type I collagen mRNA expression of GRX cells treated for 24 hours. Data represent the mean ± SD (n = 3). b-actin was an internal control for equal loading. Results are presented as relative optical density type I collagen/actin. *** P < 0.001 compared with control.

Figure 1.

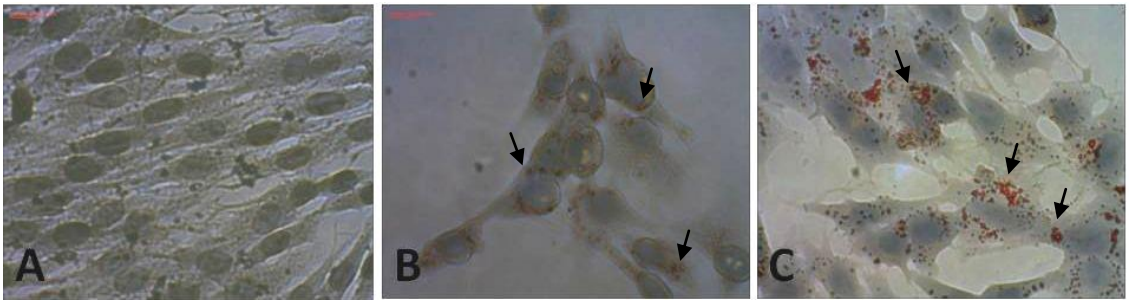

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Figure 2.

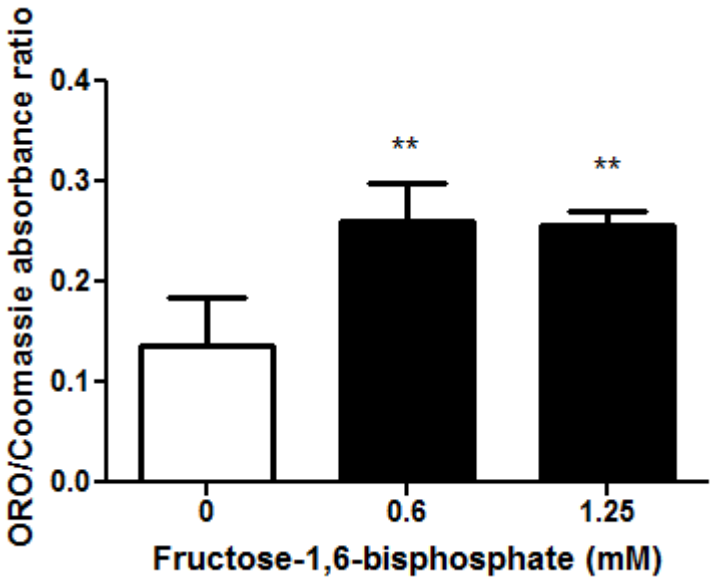

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Figure 3.

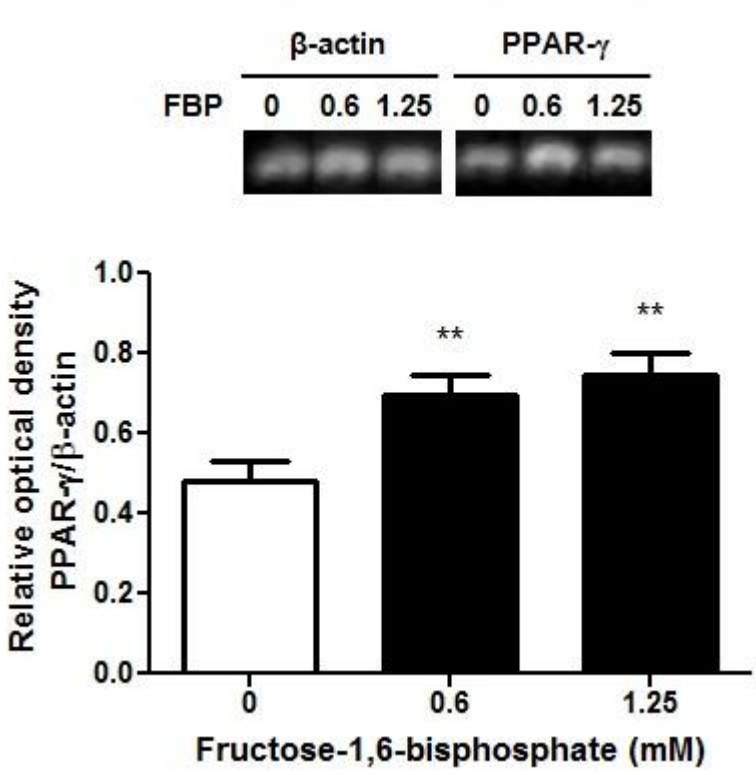

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Figure 4.

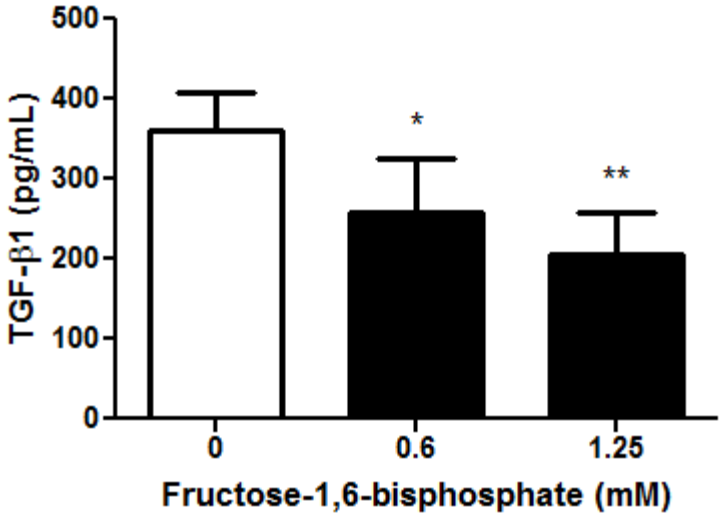

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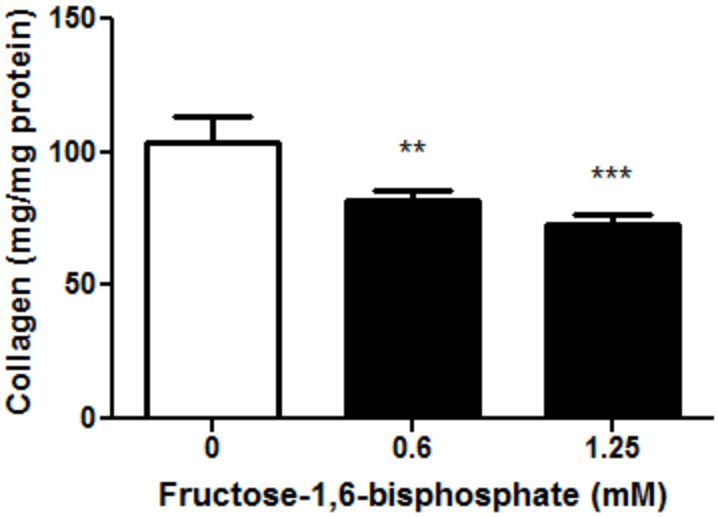
Figure 5.



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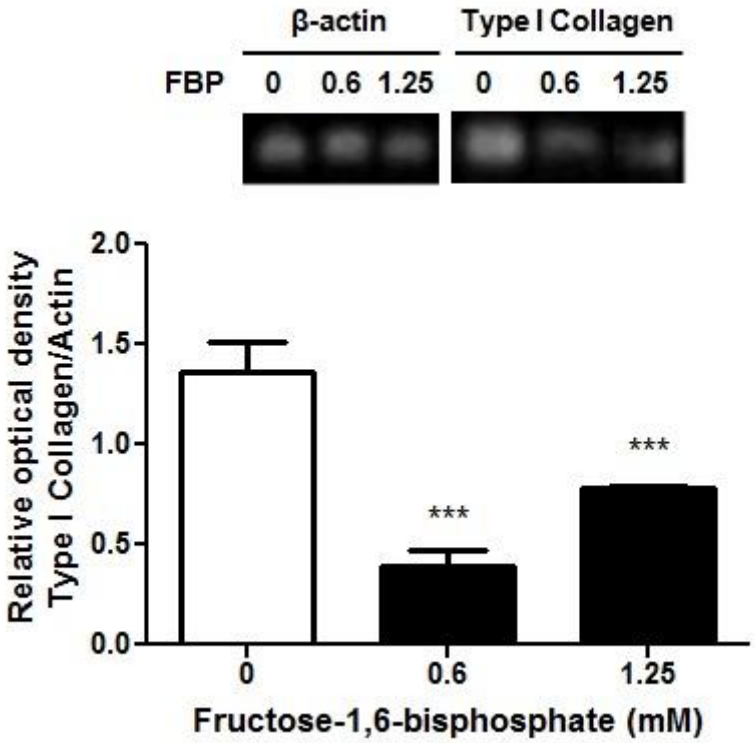

Fig. 6. Effects of fructose-1,6-bisphosphate (FBP) on type I collagen mRNA expression of GRX cells treated for 24 hours. Data represent the mean ± SD (n = 3). b-actin was an internal control for equal loading. Results are presented as relative optical density type I collagen/actin. *** P < 0.001 compared with control.

Table

Table 1 – Sequence of primers used for RT-PCR.

Primers	Forward primer (5'– 3')	Reverse primer (5'– 3')	Reference
PPAR-γ	TGGAATTAGATGACAGTGACTTGG	CTCTGTGACGATCTGCCTGAG	(Guimaraes et al. 2007)
Type I Collagen	AGAACATCACCTATCACTGCAAGA	GTGGTTTTGTATTCGATGACTGTCT	(Brun et al. 2005)
β-actin	TATGCCAACACAGTGCTGTCTGG	TACTCCTGCTTGCTGATCCACAT	(Guimaraes et al. 2007)

5. CONSIDERAÇÕES FINAIS

O difícil diagnóstico e a falta de tratamento realmente eficaz tem acelerado o interesse em descobrir novos agentes capazes de reduzir a progressão da fibrose hepática (Friedman 2008). Substâncias antiinflamatórias que inibam a ativação e proliferação das HSC têm sido muito estudadas como estratégias terapêuticas, já que estas células são consideradas a chave da fisiopatologia da fibrogênese e o estado inflamatório das mesmas é um fator determinante para o início e manutenção da fibrose (Chakraborty 2012).

Considerando a ação antiinflamátoria da FBP, este trabalho teve como objetivo verificar os efeitos deste metabólito sobre a linhagem GRX, modelo murino de HSC, visando estudar seus efeitos antifibróticos, através da reversão fenotípica das células.

Nosso trabalho mostrou que a FBP é capaz mudar o estado de ativação das células, fazendo com que as células deixem de estar ativadas e voltem ao seu estado quiescente. A reversão pôde ser visualizada no microscópio invertido através da coloração de Oil-Red, que cora as gotículas de gorduras, estas visíveis somente no estado lipocítico (quiescente). Para confirmação foi realizada a dosagem de lipídios, onde foi possível perceber que a concentração de lipídios era duas vezes maior nas células que foram tratadas com FBP comparadas ao grupo que não recebeu nenhum tratamento.

Corroborando com os dados já apresentados, a expressão de mRNA de PPARγ apresentou um aumento considerável nas células tratadas, mostrando que a FBP ativa a rota lipogênica. O PPARγ quando ligante-ativado possui um papel fundamental na reversão da fibrose hepática e na regulação da inflamação (Elias-Miro 2012).

O efeito antifibrótico da FBP pode ser evidenciado também pela inibição de TGF-β e a consequente diminuição da expressão do colágeno tipo I e da secreção de colágeno total. O TGF-β não só ativa as HSC, mas estimula a síntese de ECM e regula a proliferação e diferenciação celular (Bataller e Brenner 2005). Durante a fibrogênese, os níveis de TGF-β1 são elevados, desse modo, as suas concentrações estão correlacionados com a gravidade da fibrose hepática (Friedman 2008; Gressner et al 2002).

Como conclusão deste estudo, podemos dizer que a FBP induz a reversão do fenótipo das células GRX por suas ações antiinflamatórias e antifibróticas. O primeiro mecanismo que parece estar envolvido é a super-expressão de PPARγ, que acaba induzindo um decréscimo de TGFβ e consequente redução de mediadores pró-fibrogênicos.

Ainda são necessários mais estudos para aprofundar o conhecimento da atividade antifibrótica da FBP, porém, esta tem se mostrada não só benéfica nos casos de inflamação, mas também para as doenças crônicas do fígado.

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7. ANEXO

Carta de Submissão *Journal of Cellular Biochemistry.*

11-Jan-2013

Manuscript number: JCB-13-0026

Dear Miss de Mesquita:

We are pleased to receive your manuscript entitled "Fructose-1,6-bisphosphate induces phenotypic reversion of activated hepatic stellate cells" by de Mesquita, Fernanda; Bitencourt, Shanna; Caberlon, Eduardo; Silva, Gabriela; Basso, Bruno; Schmid, Julia; Ferreira, Gabriela; Oliveira, Jarbas.

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