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AVALIAÇÃO DA ATIVIDADE DA FRUTOSE-1,6-BISFOSFATO EM CÉLULAS GRX EXPOSTAS A FERRO LIVRE

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

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Para sua Mãe e seu Pai:

Sinta-se livre para culpá-los por tudo o que há de errado com você, mas não se esqueça:

Dê a eles o crédito por tudo que está certo.

Neil Strauss

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À minha esposa, pelo apoio, carinho e, principalmente, paciência.

Aos meus pais e irmão, por sempre me estimularem a seguir adiante e nunca desistir dos meus sonhos.

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RESUMO

Hemocromatose hereditária (HH) é uma doença genética onde o balanço do ferro está desregulado e esse metal se acumula no fígado, causando efeitos tóxicos e, principalmente, fibrose. Fibrose é uma resposta de cicatrização exacerbada com depósito de matriz extracelular (ECM). Células estreladas hepáticas (HSC) quando ativadas são as maiores responsáveis pela produção de ECM. Frutose-1,6-bisfosfato (FBP) é um açúcar e possui inúmeros efeitos benéficos, como melhorar o potencial antioxidante da célula, proteger o fígado de lesão e reverter o fenótipo de HSC ativadas. Por causa disso, nosso objetivo foi testar os efeitos da FBP em uma linhagem imortalizada de HSC (GRX) expostas a ferro livre (Fe), na tentativa de simular o que ocorre em pacientes com HH.O tratamento com Fe (1mg/L) por 8 dias aumentou a proliferação celular enquanto o tratamento com Fe + FBP (1mg/L + 0.6mM) a diminuiu para níveis menores que os do controle. A atividade da LDH, taxa de apoptose e ciclo celular não foi alterada em nenhum grupo. A coloração com OilRed-O (ORO) mostrou uma diminuição na quantidade de lipídio intracelular quando as células foram tratadas com Fe por 8 dias. No grupo Fe + FBP, houve um aumento do conteúdo lipídico e as células apresentaram características morfológicas de células quiescentes. A expressão de PPAR-γ foi diminuída no grupo Fe e igual ao controle no grupo Fe + FBP. Ao contrário, o Fe aumentou os níveis de expressão de Colágeno tipo I (Col-1) e o tratamento concomitante com FBP reverteu esse efeito, ficando igual ao controle. A produção de TGF-β1 se manteve inalterada no grupo Fe e foi menor que o controle no tratamento com Fe + FBP, mostrando uma atividade antifibrótica da FBP. O teste de DPPH mostrou que a FBP não possui atividade antioxidante em nenhuma dose testada. O teste de Ferrozine mostrou uma diminuição da absorbância depois de 120 minutos de incubação de FBP + Fe em todas as doses testadas, mostrando que a FBP é um quelante de ferro.Esses dados demonstram que FBP reverte o fenótipo das células GRX mesmo quando em presença do Fe e que isso pode ser causado pela regulação da expressão do PPAR-γ e COL-1.Em conclusão, a FBP diminuiu o crescimentoe reverteu o fenótipo de células GRX, mostrando um possível efeito antifibrótico.

Palavras-chave: Ferro, Hemocromatose, Fibrose, Células Estreladas Hepáticas, Frutose-1,6-bisfosfato.

ABSTRACT

Hereditary hemochromatosis (HH) is a genetic disease where iron balance is deregulated and this metal accumulates in the liver, causing toxic effects and fibrosis. Fibrosis is an exacerbated wound-healing response with extracellular matrix (ECM) deposition. Hepatic stellate cells (HSC), when activated, are the main responsible for ECM production. Fructose-1,6-bisphosphate (FBP) is a sugar and possess innumerous beneficial effects, like enhance cell antioxidant potential, protects liver from damage and reverts the phenotype of activated HSC. Because of this, we aimed to test the effects of FBP in immortalized HSC line (GRX) exposed to free iron (Fe) tempting to simulate what occurs in patients with HH.Fe (1mg/L) treatment for 8 days increased cell growth, whereas Fe + FBP (1mg/L + 0.6mM) decreased cell proliferation to levels below of control. LDH activity, apoptosis rate and cell cycle were not altered in any group. Oil Red-O (ORO) staining showed a decrease in lipid content when GRX cells were Fe-treated (1mg/L) for 8 days. In Fe + FBP (1mg/L + 0.6mM), GRX cells showed increased lipid content and characteristics of quiescent HSC. PPAR-γ expression was diminished on Fe group and same as control on Fe + FBP group. On the contrary, Fe treatment rose Col-1 expression and Fe + FBP reversed it to control levels. TGF-β1 was unaltered in Fe group. However, on Fe + FBP group, TGF-β1 levelswas far bellow of control and Fe-treated group, showing an antifibrotic activity. FBP didn't present antioxidant activity by DPPH assay. Ferrozine assay showed a decreased absorbance after 120 min in all FBP-tested doses, demonstrating that FBP is an iron chelator. These data demonstrate that FBP reverse the phenotype of GRX cells even when in Fe-presence and that this could be caused by regulation of PPAR-γ and COL-1. In conclusion, FBP diminished the growth rate and reversed the phenotype of GRX cell, showing a possible antifibrotic effect.

Key-words: Iron, Hemochromatosis, Fibrosis, Hepatic Stellate Cell, Fructose-1,6-bisphosphate

LISTA DE SIGLAS

BMP - Proteína Morfogênica De Osso

Col-1 – Colágeno Tipo 1

DMT1 – Transportador de Metal Divalente 1

ECM – Matriz Extracelular

FBP – Frutose-1,6-Bisfosfato

Fe – Ferro Livre

FPN - Ferroportina

GPI –Glicofosfatidilinositol

HH – Hemocromatose Hereditária

HSC – Células Estreladas Hepáticas

IRPs – Proteínas de Resposta ao Ferro

NO – Óxido Nítrico

NTBI – Ferro não Ligado à Transferrina

PPARγ – Receptor Ativado por Proliferadores do Peroxisoma γ

ORO – Coloração por OilRed-O

ROS – Espécies Reativas de Oxigênio

Tf - Transferrina

TfR1 – Receptor de Transferrina 1

TfR2 – Receptor de Transferrina 2

TGF-β1 – Fator de Crescimento Transformante β1

SUMÁRIO

1 INTRODUÇÃO

1.1 Fibrose e Células Estreladas Hepáticas

Fibrose é uma resposta cicatricial reversível que ocorre em quase todos pacientes com doença hepática crônica e é caracterizada por um aumento do depósito de componentes de matriz extracelular (ECM), como colágeno e fibronectina. Esse depósito ocorre preferencialmente ao redor da camada sinusoidal no espaço de Disse e há uma reorganização molecular da matriz, resultando em uma matriz fibrótica de composição alterada. O dano crônico causado por drogas, desordens metabólicas, ataque imune ou infecção é requerido para o acúmulo da fibrose. A cirrose é o estágio mais avançado da fibrose e se caracteriza pela distorção do parênquima hepático com formação de nódulos e septos, fluxo sanguíneo alterado e, principalmente, o risco de insuficiência hepática (Friedman 2008b; Gressner 1998; Wynn and Ramalingam 2012).

As células estreladas hepáticas (HSC), quando ativadas, são as maiores produtoras de componentes de ECM e, por isso, acredita-se que sejam as principais células envolvidas no processo fibrótico. Estão localizadas no espaço perisinusoidal de Disse entre o endotélio fenestrado hepático e os hepatócitos epiteliais (Winau et al. 2008). Essas células podem ser encontradas em dois estágios fenotípicos diferentes: quiescente e ativadas. As HSC apresentam características bem diferentes quando ativam seu fenótipo e isso está relacionado a uma série de alterações de metabolismo de lipídeos, composição de matriz extracelular, contratilidade e citocinas produzidas. As HSC quiescentes acumulam retinol (vitamina A) em seu citoplasma. Também, contribuem para a arquitetura tridimensional do fígado e para a síntese de proteínas que vão formar e degradar a matriz extracelular (metaloproteases). Além disso, podem ajudar a controlar o tônus microvascular respondendo a vários agentes vasoativos, como endotelina-1 e óxido nítrico (NO). Finalmente, exercem controle sobre a regeneração hepática, expressando fator de crescimento de hepatócito (Burt 1999; Tsukamoto 2005; Winau et al. 2008). Quando ativadas, essas células perdem a capacidade de armazenar retinol, alteram a composição e aumentam a síntese dos componentes de ECM, como relina(Kobold et al. 2002), actina de músculo liso alfa e colágeno tipo I (Col-1). A ingesta de debrisapoptótico, derivado dos hepatócitos, pelas HSC ativadas aumenta a expressão e secreção do fator de crescimento transformante- β1 (TGF-β1). Ainda, expressam altos níveis de inibidores de metaloprotease tecidual, o que impede a

degradação da nova matriz formada, e de matriz extracelular, especialmente Col-1 (Iredale 2008).

O receptor ativado por proliferadores do peroxisoma γ (PPARγ) é um receptor nuclear que está relacionado à regulação do metabolismo de lipídeos (Corzo and Griffin 2013). A ativação desse receptor por seus ligantes inibe a produção de Col-1 e TGF-β1, a proliferação celular e induz células estreladas a realizarem uma reversão fenotípica e retornarem ao seu estado quiescente (Wang et al. 2011; Zhang et al. 2013). OCol-1 é o principal componente de matriz extracelular liberado quando HSC estão ativadas e sua produção é característica desse fenótipo celular. O TGF- β1 é a maior citocina profibrogênica no fígado (Friedman 2008b), induz a produção de ECM e a ativação de HSC. Além disso, é um potente estímulo para a proliferação dessas células. O tratamento de HSC ativadas com agonistas PPAR-γ é capaz de reverter mudanças bioquímicas características de células ativadas, aumentar expressão de catalase, inibir a atividade do gene promotor do colágeno e aumentar degradação de ECM(Guimaraes et al. 2007; Miyahara et al. 2000). Porém, a expressão de PPAR-γ é suprimida quando a célula se ativa (Tsukamoto 2005).

Estudos prévios tem demonstrado que a sobrecarga de ferro é capaz de gerar fibrose em vários tecidos (Friedman 2008b). Em 1997, foi demonstrada pela primeira vez a correlação entre a concentração de ferro hepático e a ativação de HSC na hemocromatose em humanos (Ramm et al. 1997). O ferro pode estimular a ativação de HSC quiescentes quando diretamente expostas a esse metal (Gardi et al. 2002) ou pode se acumular no hepatócito, causando danos e estimulando essas células a liberarem citocinas que irão ativar as HSC (Parkes and Templeton 2003). O tratamento com antioxidantes e quelantes de ferro é capaz de inibir a ativação, diminuir a proliferação e impedir a síntese de proteínas antiapoptóticas em HSC (Brittenham 2003; Jin et al. 2007). Também são capazes de impedir a formação de espécies reativas de oxigênio (ROS) e a ativação do TGF-β1 (Pociask et al. 2004).

1.2 Ferro e Hemocromatose

Ferro é um micronutriente essencial para quase todos os organismos. É o segundo elemento mais abundante na crosta terrestre,pode existir em múltiplos estados

de oxidação, o que é essencial para a transferência de elétrons, tem um grande potencial redox e sistemas biológicos podem ajustar sua reatividade química de acordo com as necessidades fisiológicas. Participa como cofator de diversas proteínas que atuam no armazenamento, transporte (hemoglobina) e metabolismo de oxigênio (catalases e peroxidases), respiração celular, transporte de elétrons, síntese de DNA, proliferação e diferenciação celular, regulação gênica, metabolismo e síntese de esteroides(Mitchell and Mendes 2013; Pantopoulos et al. 2012; Ponka 1999). Por outro lado, o ferropode apresentar efeitos extremamente tóxicos, gerar radicais livres e ROS. Esses radicais causam danos à estruturas celulares vitais, como proteínas, lipídios e ácidos nucleicos, alterando suas estruturas e funções.

O ferro pode existir em dois estados oxidativos, Fe^{+2} e Fe^{+3}, ferroso e férrico respectivamente.Na reação de Fenton, a transição dos íons ferrosos para íons férricos na presença de peróxido de hidrogênio leva à produção de radical hidroxil (OH°) que é altamente reativo (Droge 2002; Ponka 1999).Uma maneira de isso não ocorrer é diminuir a quantidade de ferro livre disponível. Proteínas específicas que se ligam ao ferro são responsáveis pelo transporte, armazenamento e liberação desses íons. Assim, a quantidade de ferro livre circulante é muito baixa e apenas o necessário para as necessidades da célula. Já que não existem mecanismos de excreção do ferro absorvido, seu principal modo de regulação é através do controle de sua absorção no intestino delgado pelos enterócitos.

O Ferro pode ser absorvido de duas formas: ferro não heme, derivado de vegetais e grãos, e o ferro heme, derivado de carnes vermelhas (Chen and Chloupkova 2009; Wessling-Resnick 2000). A ferroportina (FPN) é responsável pela exportação do ferro absorvidonosenterócitose reciclado pela eritrofagocitose. A hefaestina auxilia a etapa de exportação convertendo o Fe^{+2} a Fe^{+3} que, então, se liga a transferrina (Tf). A Tfé a responsável pelo transporte desses íons pela circulação e distribuição pelos tecidos. Quase todas as células adquirem ferro através da ligação da Tf no seu receptor, denominado receptor de Tf-1 (TfR1). Após essa ligação, o TfR1 é internalizado e levado ao endossomo, onde o ferro é reduzido de Fe^{+3} a Fe^{+2}pela ferroredutase STEAP3 e encaminhado ao citoplasma pelo transportador de metal divalente 1 (DMT1). Então, pode ser usado em outros compartimentos celulares, ser exportado novamente pela FPN ou estocado ligado àferritina. Oferro intracelular é controlado por proteínas de resposta ao ferro (IRPs) que atuam junto da ferritina e receptores de transferrinapara regular a

transcrição de mRNA de outras proteínas envolvidas no metabolismo desse metal (Byrne et al. 2013; Hentze and Kuhn 1996; Mitchell and Mendes 2013). A Figura 1 mostra a regulação do metabolismo do ferrosistêmico.

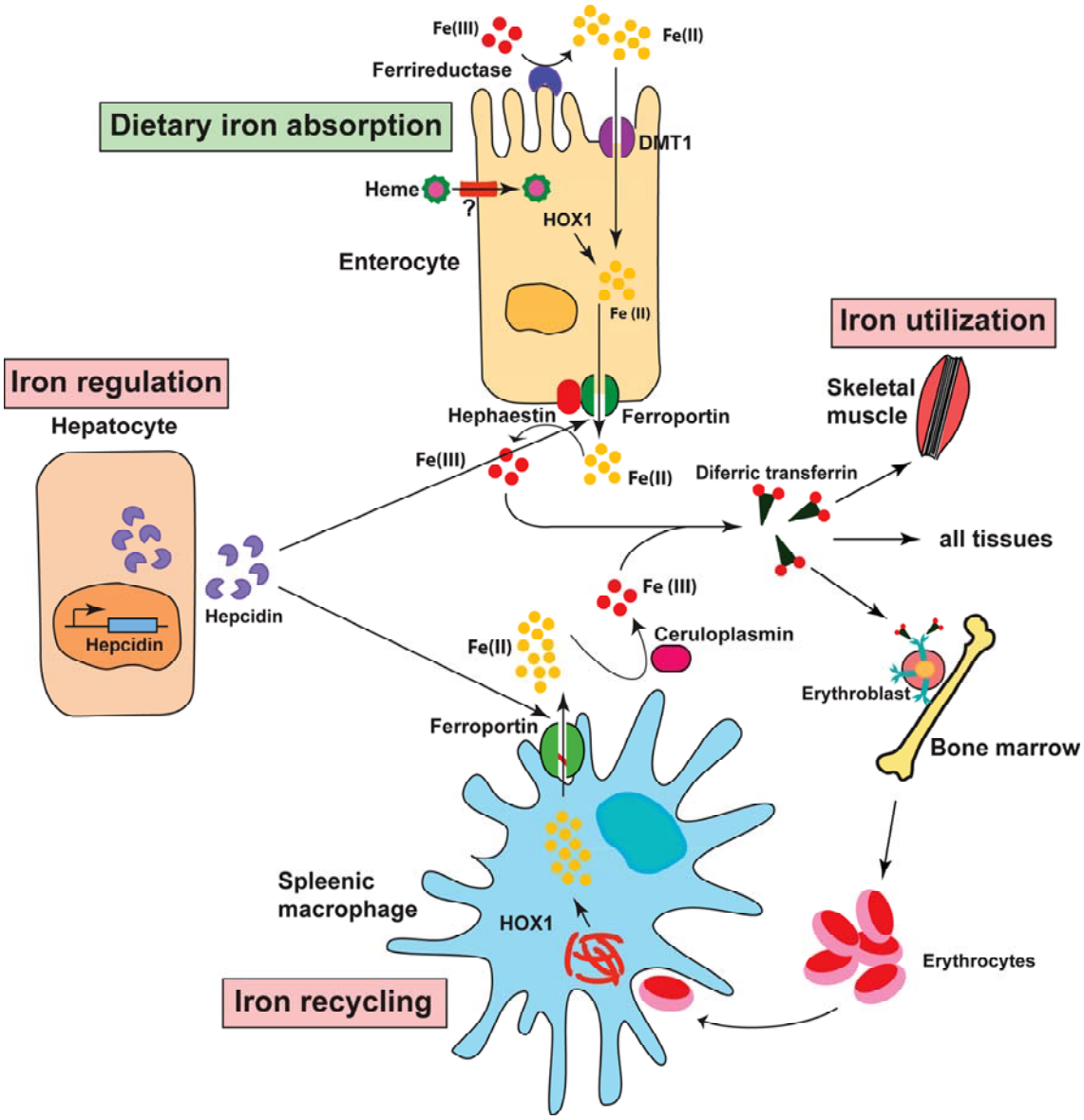

Figura 1. Regulação do metabolismo do ferro sistêmico. Órgãos e células envolvidas no equilíbrio do ferros são mostrados. Enterócitos duodenais absorvem o ferro proveniente da dieta via DMT1 após a redução de Fe^{+2} para Fe^{+3}. Macrófagos reciclam o ferro de eritrócitos senescentes. Ambos os tipos celulares liberam ferro através da ferroportina com auxílio da hefaestina, que oxida Fe^{+3} paraFe^{+2}. O Ferro também é oxidado pela ceruloplasmina na circulação. A transferrina plasmática captura e promove a circulação do ferro pelo organismo. A hepcidina regula o efluxo do ferro dessas células através da estabilização da ferroportina (Pantopoulos*et al.* 2012).

O ferro circulante que não está ligado à Tf, ao grupo heme ou à ferritina, é denominado ferro não ligado à trasferrina (NTBI).Essa forma de ferro torna-se importante em desordens de sobrecarga, pois o ferro presente no plasma está acima da capacidade de ligação da Tf (Breuer et al. 2000). O fígado remove o NTBI do plasma de maneira eficiente (Brissot et al. 1985) e isso é um mecanismo de se remover o ferro circulante potencialmente tóxico e que poderia causar dano celular. (Breuer et al. 2000). Contudo, o hepatócito tem uma taxa limite onde o acúmulo de ferro se torna tóxico e começa a causar dano, levando a patologias como fibrose, cirrose e hepatocarcinoma. Essas condições são características de desordens que envolvem a sobrecarga de ferro, como a hemocromatose hereditária.

A hemocromatose hereditária (HH) é uma doença de caráter genético, autossômica recessiva que está associada com a mutação de 5 genes: da proteína da hemocromatose HFE, da hemojuvelina, da hepcidina, do receptor de transferrina 2 (TfR2) e da ferroportina(Chen and Chloupkova 2009). Essas mutaçõescausam uma absorção aumentada de ferro no lúmen intestinal, o que gera uma sobrecarga sistêmica de ferro e olento acúmulo desse metal nos tecidos podendolevar a fibrose e cirrose hepáticas, cardiopatias, diabetes artrite, pigmentação cutânea e câncer (Means 2013; Zarrilli et al. 2013). A HH é resultado de uma resposta inadequada à hepcidina. Hepcidina é um peptídeo sintetizado pelo fígado e é o principal modulador do metabolismo do ferro devido a sua capacidade de se ligar à FPN e induzir sua degradação, inibindo sua atividade com consequente diminuição da absorção intestinal de ferro. Assim, quando os níveis de ferro se encontram altos, a hepcidina inibe a exportação de ferro para a circulação através do bloqueio da FPN deenterócitos, hepatócitos e a absorção intestinal(Canavesi et al. 2012; De Falco et al. 2013; Knutson et al. 2005).

O tipo mais comum de HH é a associada ao gene HFE e pode ser chamada, também, HH clássica. O produto desse geneHFE é uma proteína que está complexada com o TfR1.Estudos mostramque HFE, TfR2 e hemojuvelina interagem entre si para formar um complexo sensível às concentrações de ferro (D'Alessio et al. 2012). Se os níveis de Fe^{+3} aumentam, HFE é deslocada do TfR1 para permitir sua interação com o TfR2, o que ativa a transcrição do gene da hepcidina(De Falco et al. 2013). Hemojuvelina é uma proteína ancorada a um glicofosfatidilinositol (GPI) que age como um co-repector da proteína morfogênica de osso (BMP), o que leva à transcrição de

hepcidina via cascata de sinalização BMP-SMAD (Babitt et al. 2006).A patogênese da lesão hepática na hemocromatose se dá pelo dano oxidativo induzido pelo ferro seguido de peroxidação lipídica da mitocôndria, membrana de lisossomos e outras estruturas celulares. As células de Kupffer, macrófagos residentes do fígado, reagem a esse dano e liberam citocinas que estimulam células estreladas a produzirem colágeno, levando à fibrose (Zarrilli et al. 2013).Na HH clássica, o acúmulo de ferro pode ocorrer nos primeiros 20 anos de vida. A expressão dessa doença é menos comum em pessoas do sexo feminino devido às perdas menstruais e a apresentação dos primeiros sintomas ocorrem normalmente nas terceira e quarta décadas de vida. Como em estágios tardios da doença ocorrem alterações irreversíveis, é importante que o diagnóstico precoce ocorra. É importante que, uma vez identificada essa doença em um indivíduo, se faça uma análise de todos outros membros da família (Fletcher and Halliday 2002). O diagnóstico é realizado através de marcadores laboratoriais tradicionais, como ferritina sérica, que avalia estoques de ferro sistêmicos, índice de saturação da transferrina, enzimas hepáticas, como alanina amino transferase e aspartato amino transferase(Fletcher and Halliday 2002)e análises de mutações nos genes relacionados à hemocromatose.Surpreendentemente, uma incidência maior é encontrada quando o diagnóstico é baseado em marcadores laboratoriais tradicionais devido ao fato de uma série de doenças causarem hemocromatose secundária (Whitlock et al. 2006).

Muitas das alterações encontradas na HH não são características específicas dessa doença. Por esse motivo, o diagnóstico diferencial torna-se bastante difícil, sendo que frequentemente ele não ocorre. Por essas razões, sua incidência é subestimada e normalmente não é tratada,fazendo dessa doença um problema relevante. Uma incidência de 1:250 indivíduos é comumente aceitável, o que faz da HH uma das doenças de causa genética mais comum em caucasianos (Zarrilli et al. 2013).Pacientes com HH tem um maior risco de morte quando comparados à população geral (Fletcher and Halliday 2002; Yang et al. 1998). A sobrecarga de ferro tem sido associada a uma variedade de tipos de câncer. Sua diminuição pode ser útil em casos de pacientes com cirrose, o que diminuiria o risco de hepatocarcinoma(Kanwar and Kowdley 2014).Substâncias quelantes, como a desferroxamina, também podem ser utilizados. Porém sua absorção oral é muito baixa e a meia vida curta, o que faz com que se necessite a administração subcutânea ou endovenosa diária para uma terapia eficaz (Brittenham 2003).Não existe cura e a flebotomia terapêutica permanece sendo o

principal tratamento de pacientes com hemocromatose. Dependendo do estágio de progressão da fibrose ou cirrose hepática, o transplante de fígado também é praticado (Kanwar and Kowdley 2014).

1.3 GRX como modelo experimental

A linhagem celular GRX é a linhagem de HSC mais antiga que existe (Herrmann et al. 2007). Foi obtida de granulomas hepáticos de camundongos da linhagem C3h/HeN que foram infectados com cercarias de *Scistosoma mansoni*. As GRX se encontram em progressão fenotípica intermediária entre os estágios quiescente e ativado e por isso, apresentam algumas características de ambos os fenótipos, como a morfologia de miofibroblastos. Além disso, secretam colágeno do tipo I, III e IV, fibronectina, possuem baixas concentrações de lipídio intracelular, sintetizam TGF- β1, expressam PPAR-γ e possuem a capacidade de se proliferar indefinidamente se mantidos meios adequados. Outra característica importante é que agentes antioxidantes, como retinol, são capazes de reverter o fenótipo ativado dessas células e reorganizar seu citoesqueleto. Assim, apresentam algumas vantagens em relação às culturas primárias, por serem uma fonte ilimitada de células, exibirem alto grau de homogeneidade, poderem ser congeladas por longos períodos e restringirem o uso de animais (Herrmann et al. 2007; Means 2013)

1.4 Frutose-1,6-bisfosfato

A Frutose 1,6-Bisfosfato (FBP) é um intermediário do metabolismo da glicose e diversos efeitos protetores tem sido atribuídos a essa molécula em diferentes situações. A FBP é capaz de aumentar a sobrevida de animais submetidos à sepse experimental, diminuir a liberação enzimas hepáticas que são usadas como marcadores de dano, como as transaminases e LDH (de Mello et al. 2011). Ainda, FBP protege contra dano hepático (De Oliveira et al. 1992) e aumenta as taxas de apoptose, enquanto diminui taxas de necrose em animais tratados com galactosamina (Fortes Aiub et al. 2003).

Além disso, estudos mostram que a FBP possui ação antioxidante, sendo capaz de eliminar radicais livres (Bajic et al. 2011; Bochi et al. 2012; Spasojevic et al. 2009).

Outros trabalhos mostram que a FBP é capaz de aumentar o potencial antioxidante das células por aumentar a glutationa e acatalase(de Mello et al. 2011; Guimaraes et al. 2007) e isso impede a ativação de HSC (Dong et al. 2014). Entretanto, um estudo recente mostrou resultados conflitantes, sugerindo que a eficácia da FBP contra hepatocarcinoma foi causada por uma atividade oxidante, aumentando a produção de peróxido de hidrogênio (Lu et al. 2013).

Em outro trabalho, a FBPfoi capazde sequestrar íons ferrosos (Bajic et al. 2011) e, recentemente, nosso grupo demonstrou que a FBP é capaz de reverter o fenótipo de células estreladas hepáticas ativadas da linhagem GRX por atuar na regulação das expressões de PPAR-γ e Col-1, além de diminuir a quantidade de TGF- β1 secretada (de Mesquita et al. 2013).

2. JUSTIFICATIVA

Devido à alta incidência e prevalência de hemocromatose na população, às complicações provenientes dessa doença, à falta de diagnóstico ou ao diagnóstico comumente tardio que leva à perpetuação da fibrose,novos estudos tem sido realizados com o intuito de diminuir a sobrecarga de ferro no organismo e de reverter o processo fibrótico. Entretanto, até o momento, nenhum tratamento é efetivo para conter a progressão do processo fibrótico.

A Frutose-1,6-bisfosfatoapresenta a capacidade de quelar ferro, sequestrar radicais livres, promover a reversão fenotípica em células GRX e regularcitocinasfibrogênicas que são fatores chave no metabolismo de lipídios e produção de matriz extracelular.Por essas razões, a FBP pode apresentar um grande potencial para a diminuição e reversão da fibrose em pacientes com hemocromatose.

3. OBJETIVOS

3.1 Objetivo Geral

Avaliar os efeitos da frutose-1,6-bisfosfato sobre a proliferação e fenótipo de células da linhagem GRX expostas a ferro livre.

3.2 Objetivos Específicos

Avaliar a proliferação de células GRX expostas a ferro e tratadas com FBP;

Avaliar os efeitos da FBP sobre marcadores de apoptose e ciclo celular em células GRX expostas a ferro;

Avaliar a morfologia celular e quantificar lipídios em células GRX expostas a ferro e tratadas com FBP;

Avaliar o efeito da FBP sobre a expressão de PPAR-γ e Col-1 em células GRX expostas a ferro;

Avaliar o efeito da FBP sobre a concentração de TGF- β1 em sobrenadante de culturas de células GRX expostas a ferro;

Avaliar o potencial antioxidante da FBP;

Avaliar a capacidade quelante da FBP.

4 ARTIGO CIENTÍFICO

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Fructose-1,6-Bisphosphate Decreases Proliferation Rate and Reverts Iron-Induced Phenotype of Activated Hepatic Stellate Cells

Running Head: Action of FBP on Iron-induced GRX cells

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ABSTRACT

Hereditary hemochromatosis (HH) is a genetic disease where iron balance is deregulated and this metal accumulates in the liver, causing toxic effects and fibrosis. Fibrosis is an exacerbated wound-healing response with extracellular matrix (ECM) deposition. Hepatic stellate cells (HSC), when activated, are the main responsible for ECM production. Fructose-1,6-bisphosphate (FBP) is a sugar and possess innumerous beneficial effects, like enhance cell antioxidant potential, protects liver from damage and reverts the phenotype of activated HSC. Because of this, we aimed to test the effects of FBP in immortalized HSC line (GRX) exposed to free iron (Fe) tempting to simulate what occurs in patients with HH. Fe (1mg/L) treatment for 8 days increased cell growth, whereas Fe + FBP (1mg/L + 0.6mM) decreased cell proliferation to levels below of control. LDH activity, apoptosis rate and cell cycle were not altered in any group. Oil Red-O (ORO) staining showed a decrease in lipid content when GRX cells were Fe-treated (1mg/L) for 8 days. In Fe + FBP (1mg/L + 0.6mM), GRX cells showed increased lipid content and characteristics of quiescent HSC. PPAR-γ expression was diminished on Fe group and same as control on Fe + FBP group. The contrary, Fe treatment rose Col-1 expression and Fe + FBP reversed it to control levels. TGF-β1 was unaltered in Fe group. However, on Fe + FBP group, TGF-β1 was far bellow of control and Fe levels, showing an antifibrotic activity. FBP didn't present antioxidant activity by DPPH assay. Ferrozine assay showed a decreased absorbance after 120 min in all FBP-tested doses, demonstrating that FBP is an iron chelator. These data demonstrate that FBP revert the phenotype of GRX cells even when in Fe-presence and that this could be caused by regulation of PPAR-γ and COL-1. In conclusion, FBP diminished the growth rate and reversed the phenotype of GRX cell, showing a possible antifibrotic effect.

Key-words: Iron, Hemochromatosis, Fibrosis, Hepatic Stellate Cell, Fructose-1,6-bisphosphate

1. INTRODUCTION

Hereditary Hemochromatosis (HH) is a genetic disease that results in toxic iron overload and its deposit in the tissues, specialty in the liver (Means 2013). This is a late onset disease commonly undiagnosticated and untreated (Fletcher and Halliday 2002). Besides, HH is the most prevalent genetic disease in caucasians, with an incidence of 1:250 individuals (Zarrilli et al. 2013). Subjects with HH have a higher mortality risk when compared to general population (Fletcher and Halliday 2002; Yang et al. 1998) and manual phlebotomy remains the principal treatment for this genetic disorder. Moreover, iron overload can result in hepatic fibrosis, cirrhosis and has been associated to many cancer types, (Kanwar and Kowdley 2014; Zarrilli et al. 2013).

Fibrosis is an exacerbated wound-healing response with excessive extracellular matrix (ECM) produced and accumulated in the liver (Friedman 2008b). These ECM components are synthesized specially by hepatic stellate cells (HSC). In normal liver, these cells are in a quiescent state and maintain a lipocyte phenotype, accumulating retinol droplets in cytoplasm. In injured liver, HSC become activated by cytokines and reactive oxygen species (ROS), change to a myofibroblast-like phenotype, lose their retinol droplets and begin to synthesize and secrete large amounts of ECM proteins, like type I collagen (Col-1), and transforming growth factor-β1 (TGF- β1). For this reason, HSC are believed the principal responsible for the fibrotic process and maintenance of fibrotic state (Friedman 2000; Gressner 1998). TGF- β1 is the major fibrogenic cytokine in the liver (Friedman 2008b). Is responsible for proliferation and activation of HSC besides the stimulus for ECM production (Casini et al. 1993).

Peroxisome proliferator-activated receptor γ (PPAR-γ) is a nuclear receptor involved in lipid metabolism and exerts antifibrotic effects. PPAR-γ is downregulated in activated HSC (Corzo and Griffin 2013; Guimaraes et al. 2007) and its ligands are shown to inhibit liver fibrosis in animal models (Galli et al. 2002).

Iron deposition is seen in fibrosis (Guo et al. 2006). Iron is an essential micronutrient for almost all organisms. Iron is a required cofactor for protein synthesis, oxygen transport and metabolism, DNA synthesis and others (Mitchell and Mendes 2013; Pantopoulos et al. 2012). However, the redox cycling of ferrous (Fe^{+2}) and ferric (Fe^{+3}) iron in H_2O_2 presence leads to hydroxyl radicals production (Fenton chemistry)

that can attack and damage lipids, proteins, DNA and other cellular components (Droge 2002; Ponka 1999).

The correlation between iron overload and activated HSC had been made in subjects with HH since 1997 (Ramm et al. 1997). Iron overload can stimulate quiescent HSC to become activated by directly exposed (Gardi et al. 2002) or can generate hepatocyte damage, resulting in cytokine production that will activate HSC (Parkes and Templeton 2003). Iron chelators inhibit the activation and proliferation of HSC, upregulateproapoptotic proteins (Jin et al. 2007) and reduces fibrosis (Brittenham 2003). Activated HSC have low levels of natural antioxidants (Jameel et al. 2009). Thereby, the administration of exogenous antioxidants or the enhancement of antioxidant capacity of HSC is an effective manner of revert the phenotype of stellate cells or prevent its activation (Dong et al. 2014).

Fructose-1,6-bisphosphate (FBP) is a sugar that participates as a glycolytic intermediate in the pentose-phosphate pathway that has been shown to exert a protective effect in a wild type of harmful conditions by our group (de Mello et al. 2011; De Oliveira et al. 1992; Fortes Aiub et al. 2003; Santos et al. 2012) and others (Alva et al. 2011; Bochi et al. 2012; Gawarammana et al. 2010; Lu et al. 2013). Furthermore, antioxidant effects (Bochi et al. 2012; Spasojevic et al. 2009) and the ability of sequester ferrous ions (Bajic et al. 2011) had been attributed to this sugar. These effects could have a potential role in HH by diminish the damage conditions generated by iron and free radicals. FBP has a protector effect in the liver injury (De Oliveira et al. 1992). Moreover, de Mesquita et al. 2013, showed a decreased proliferation rate and phenotypic reversion FBP-induced in GRX cell line. These effects were caused by changes on Col-1 and PPAR-γ expression and TGF- β1 release modulation. GRX is an immortalized cell line of HSC and became an important tool for the study of HSC physiology (Guimaraes et al. 2006). These cells express a transitional myofibroblast-like phenotype and produces Col-1 and TGF- β1 (Herrmann et al. 2007). Since hereditary hemochromatosis don't present an effective treatment and it can result in hepatic fibrosis, in this study, we aimed to evaluate if FBP could maintain this protective effects in GRX cell line when exposed to free iron.

2. MATERIALS AND METHODS

2.1 Cell culture

The murine GRX cell line (Borojevic et al., 1985) was obtained from the 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, Carlsbad, CA), 2g/L HEPES buffer, 3.7g/L NaHCO3 and 1% penicillin and streptomycin (Invitrogen) and incubated at 37ºC in a humidified atmosphere with 5% CO2.

2.2 Free iron and fructose-1,6-bisphosphate treatment

$FeSO_4.7H_2O$ (*Ferrous Sulfate*Heptahydrate) (Sigma Chemical Co., St. Louis, Mo) was dissolved in DMEM at final concentrations of 0.01 mg/L, 0.1 mg/L, 1.0 mg/L and 15.0 mg/L of free iron (Fe). GRX cells were incubated with Fe and cell number was assessed by direct counting in Neubauer´s chamber in 1, 5 and 8 days after treatment.

Fructose-1,6-bisphosphate (Sigma Chemical Co., St. Louis, Mo) was dissolved in DMEM at final concentrations of 0.6 mM and 1.25 mM(de Mesquita et al. 2013). GRX cells were, then, incubated with Fe (1.0 mg/L) and with Fe + FBP (1.0 mg/L + 0.6mM). Analysis were performed in 1 or 8 days after treatment. All experiments were performed in triplicate.

2.3 Cell growth

Cell growth was assessed by direct counting on Neubauer´s Chamber. GRX cells were seeded into 24-well plates ($1x10^3$ cells/well) and treated with Fe and Fe + FBP (as described above) for 8 days. All groups were performed in triplicate. The control group received only DMEM and 5% SFB for 8 days. The results are presented as total number of cells/well.

2.4 Cell viability

Cell viability of Fe and Fe + FBP treatment was evaluated by lactate dehydrogenase (LDH) activity. LDH activity was assessed in culture supernatants, using a commercial kit (Labtest, MG, Brazil), after treatment with Fe or Fe + FBP for 1 and 8 days and compared with control group.

2.5 Analysis of apoptosis and cell cycle by flow cytometry

Apoptosis was assessed using the PE Annexin V Apoptosis Detection Kit I (BD Bioscience, San Jose, CA). Briefly, after treatment with Fe and Fe + FBP for 1 day, cells were washed twice with PBS and ressuspended in binding buffer before the addition of Annexin V, 7-AAD and Phycoerythrin (PE). For the apoptosis positive control, cells were treated with 20 mMcisplatin and washed with PBS following the same protocol. Cells were vortexed and incubated for 15 min in the dark at room temperature. A total of 20.000 events were acquired with FACSCanto II flow cytometer (BD Bioscience). The data were analyzed using FlowJo 10.0.7 software (Tree Star Inc., Ashland, OR) to detect the percentage of apoptotic cells (Annexin V - PE -stained cells).

To determine the effect of Fe and Fe + FBP on the cell cycleGRX cells were first synchronized by serum starvation for 1 day and then, exposed to Fe and Fe + FBP for 24 h. Cell cycle phase analysis was performed using the FICT BrdU Flow Kit (BD Bioscience). After treatment and BrdU labeling, cells were harvested by trypsinization and adjusted to $1x10^6$ cells/mL. Samples were fixed with BD Citofix/Cytoperm Buffer (BD Bioscience). Following this, cells were treated with DNase for 20 min to expose BrdU epitopes. DNA was stained using 7-AAD dye. The DNA profiles were determinate by FACSCanto II flow cytometer (BD Bioscience) and analyzed using the FlowJo 10.0.7 software (Tree Star Inc., Ashland, OR).

2.6 Detection of lipid droplets by Oil Red-O staining

To visualize cell morphology and lipid accumulation cells were stained with Oil Red-O (ORO) (Sigma) (Ramirez-Zacarias et al. 1992) on day 8. Briefly, after fixing cells with 10% formaldehyde, ORO (0.35g in 60% isopropanol) was added for 15 min.

Intracellular lipid droplets were examined using an inverted light microscope (BestScope, China).

2.7 Quantification of lipid accumulation

The procedure is also based on ORO staining of intracellular lipid droplets and Coomassie brilliant blue staining (Sigma Chemical Co., St. Louis, Mo) of cellular proteins (Bouraoui et al. 2008). Briefly, cells were fixed with perchloric acid and incubated with ORO dissolved in propylene glycol (2mg/mL) for 2h. The ORO within the lipid droplets was extracted using isopropanol. The absorbance was read at 492nm using an ELISA plate reader. Next, the wells were washed with water thrice for protein determination. Cells were incubated with Coomassie brilliant blue for 1h. After washing, the cells were incubated with propylene glycol for 3h at 60ºC. 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.

2.8 RNA extraction and RT-PCR

To analyze the PPAR-γ and Col-1 expression total RNA was extracted from cells using TRIzol reagent (Invitrogen, CA, USA). RNA was reverse transcribed into cDNA, using Super-script III First-Strand Synthesis SuperMix (Invitrogen,CA, 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 5µg/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 (ImageJ v. 1.47 for Windows) and the signals were expressed relatively to the intensity of the β-actin amplicon in each co-amplified sample.

2.9 TGF- β1 Quantification

TGF-β1 concentration was measured, on day 8, in cell supernatant using commercially available ELISA kit (R&D Systems, Minneapolis, MN). Results were calculated on a standard curve concentration and multiplied for the dilution factor. TGF-β1 levels were expressed as picograms per milliliter/mg protein.

2.10 Antioxidant potential by DPPH

To evaluate antioxidant potential of FBP we used DPPH (2,2-diphenyl-1-picrylhydrazyl) (Sigma Chemical Co., St. Louis, Mo) assay, which is based on measurement of scavenging ability of antioxidants toward the stable radical DPPH. The FBP concentrations tested were of 0.6 mM, 1.2 mM, 2.5 mM, 5 mM, 10 mM and 20 mM. Briefly, a 0.2 ml aliquot of a 0.0062 mM of DPPH solution, in 20 ml methanol (95%) was added to 0.04 ml of 1 mg/mL ascorbic acid, as positive control, and to each concentration of FBP and shaken vigorously. Absorbance changes in samples were measured at 517 nm after 1 h. Methanol (95%) was used as a blank.

2.11 Analysis of FBP as a potential iron chelator

To evaluate the potential capacity of FBP as an iron chelator, we performed the Ferrozine assay. Ferrozine is acknowledged as an effective chelator of ferrous iron and has been used for the determination of iron in biological samples. Briefly, Fe 1mg/L solution (1.0 mL) was mixed with FBP dissolved in PSB (phosphate saline buffer) at final concentrations of 5 mM, 2.5 mM, 1.25 mM and 0.6 mM. The mixture was incubated for 120min (120') at 37ºC. Past incubation time, 25 µL of Ferrozine was added at the samples and the same were incubated more 15 min at 37ºC. The final color was read in 560nm. To Fe control, the same procedure was realized but no FBP was added.

2.12 Statistical analysis

Data were analyzed by one-way ANOVA followed by Tukey's test using SPSS for windows (V. 17.0, Chicago, USA). Results were presented as mean ± S.D. *p*-values $< 0,05$ were considered statistically significant.

3. RESULTS

3.1 Effects of free iron and FBP in GRX cell growth

In order to evaluate the effects of free iron on GRX cell proliferation rate we analyzed cell number by direct count in Neubauer's chamber. We observed an increase in the number of cells after treatment by 8 days only in 1 mg/L dose when compared to control (Figure 1) and all experiments were performed with this concentration. The 15 mg/L dose was toxic (data not shown). The 0.01 mg/L and 0.1 mg/L doses showed no significant results (data not shown) and were discarded from the following experiments. On days 1 and 5, difference was not significant in any dose (data not shown). Next, we evaluated the effects of Fe + FBP treatment in GRX cell growth. A decrease in cell number was noted at concentrations of 1 mg/L + 0.6 mM of Fe and FBP respectively (Figure 2). These doses were used to perform the following experiments. The doses of 1mg/L + 1.2mM of Fe and FBP were toxic (data not shown). To verify if the lower number of cells presented in the Fe + FBP group was entailed by citotoxicity or necrotic cell death, we measured the activity of the LDH released in the cell supernatant. There was no significant difference amongst the groups (Figure 3), demonstrating no toxic effect neither in the Fe treatment nor on the associated doses of Fe and FBP.

3.2 Effects of free iron and fructose-1,6-bisphosphate in apoptosis and cell cycle

To evaluate if the decrease proliferation was caused by apoptotic death we stained the cells with Annexin-V. There was no significant difference between groups. However, the group Fe + FBP presented a slightly tendency to increase the apoptotic rate (Figure 4). To analyze if Fe or Fe + FBP were capable of influence the cell cycle, we measured BrdU incorporation. We found no significant difference between Fe and Fe + FBP groups when compared to control (Figure 5A e 5B).

3.3 Effects of free iron and fructose-1,6-bisphosphate in cell phenotype

At microscopic single glance, we noticed modifications in cell shape and increased refringency, which is phenotype-conversion characteristic, when these cells were treated with Fe + FBP. To test the hypothesis that FBP could alter GRX cells

phenotype even in the presence of Fe, we stained these cells with ORO to evaluate morphology and lipid content. We observed that Fe + FBP treatment led to phenotypic conversion from myofibroblast-like (activated) phenotype to a fat storing (quiescent) phenotype (Figure 6C). Typical changes were observed. Cells lost their elongated and parallel strand appearance and acquired a larger and polygonal shape. Intracellular lipid droplets were most visible in GRX cells treated with Fe + FBP (arrows). After 8 days most cells exhibited the fat storing phenotype, as detected by phase contrast microscopy after staining with ORO. Control cells preserved their myofibroblast-like morphology (Figure 6A). In contrast, Fe treated cells preserved their activated state and lose their lipid droplets almost totally (Figure 6B). These findings were confirmed by colorimetric quantification of intracellular lipid content (Figure 6D). It demonstrates that FBP can revert the phenotype of GRX cells even in the presence of free iron.

To confirm cell-differentiation, PPAR-γ and Col-1 mRNA expression were measured after 1 day of treatment. The expression of PPAR-γ was significant decreased in cells Fe treated (Figure 7A). However, Fe + FBP treatment recovered this effect and PPAR-γ mRNA expression in this group was similar to control. Col-1 mRNA significant increased in Fe treatment (Figure 7B) when compared with control. Fe + FBP treatment also recovered this effect with same Col-1 mRNA expression of control. These expression profiles presented on Fe treatment are characteristic of activated HSC. The Fe + FBP group had its expression of PPAR-γ significant increased and Col-1 significant decreased (Figures 7A and 7B) when compared with Fe group.

3.4 Effects of free iron and fructose-1,6-bisphosphate in TGF- β1

To evaluate the antifibrotic and antiproliferative effects of FBP we analyzed the amount of TGF- β1 fibrogeniccytokinethis cytokine released in cell supernatant. Fe didn't increase the TGF- β1 levels. However, when FBP was administered, the cells showed a significant decrease in the amount of TGF- β1 released (Figure 8).

3.5 Effects of fructose-1,6-bisphosphate on free radical DPPH assay

Previous studies demonstrated an antioxidant and free radical scavenger effect from FBP (Bajic et al. 2011; Bochi et al. 2012; Spasojevic et al. 2009). Therefore, we performed DPPH assay to verify the potential antioxidant of FBP. We found that FBP did not present antioxidant effects in any tested concentration (0.6 mM, 1.2 mM, 2.5 mM, 5.0 mM, 10 mM and 20 mM) (Figure 9).

3.6 Effects of fructose-1,6-bisphosphate on free iron sequestering

To test the hypothesis that FBP can act as an iron chelator, we performed Ferrozine assay. FBP did chelates iron after 120 minutes even in the lower dose tested (0.6mM) and this effect was time and dose-dependent (Figure. 10).

4. DISCUSSION

Iron overload is responsible for liver fibrosis in hemochromatosis. Amongst liver cells, there is hepatic stellate cells that play a major role in liver fibrogenesis(Friedman 2008b). Iron can activate these cells by accumulating in other hepatic cells, i.g. hepatocytes (Parkes and Templeton 2003) and Kupffer cells, which release cytokines and profibrotic factors, and can act directly on HSC and cause its activation, increase of Col-1 and proliferation (Gardi et al. 2002). In our study, GRX cells directly exposed to free iron with a 1mg/L dose increased proliferation rate of GRX cells (Fig. 1). Accordingly with de Mesquita et al. 2013, FBP decreased the growth rate of GRX cells for phenotypic reverse. So, in this study, we aimed to evaluate if FBP could maintain this protective effects in GRX cell line when exposed to free iron. We showed that FBP can diminish the proliferation rate even when in presence of Fe (Fig. 2) and that this effect was not caused by cytotoxicity, as proved by LDH assay (fig 3).

As the aggressor agent disappears on liver injury, the activated HSC begin to be cleaned up by apoptosis, thus the increase of apoptotic process is a key factor to reverse the fibrotic state (Elsharkawy et al. 2005; Friedman 2008a). It was demonstrated that FBP protects the liver preventing depletion of intracellular ATP, decreasing necrosis and increasing apoptotic rate (Fortes Aiub et al. 2003). For this reason, we assessed the possible effect of FBP on apoptosis induction and observed that there was no difference between groups treated with Fe and with Fe + FBP when compared to control on Annexin V test (Fig. 4). Yet, trying to understand the lower proliferation rate, we analyzed cell cycle. We found neither Fe nor Fe + FBP alter the cell cycle. Until now, the lower growth presented by Fe + FBP group was not caused by death (necrosis or apoptosis) or cell cycle regulation.

PPAR-γ, a nuclear receptor, is the main regulator of adipogenesis and possesses antifibrotic effects. It is expressed in quiescent HSC but is downregulated in activated HSC (Guimaraes et al. 2007). We showed PPAR-γ expression downregulated on Fe treatment and its upregulation by FBP to similar control levels. PPAR-γ upregulation inhibits the production of Col-1, cell proliferation and leads HSC to undergo to lypocite phenotype (Wang et al. 2011; Zhang et al. 2013). As HSC are responsible for the increased synthesis and deposition of Col-1 through perpetuation of fibrosis and as Col-1 expression is a characteristic of activated HSC, we decided to measure its mRNA

expression. Our Fe treatment increased Col-1 expression, proliferation and caused a decreased on intracellular lipid content. When cells concomitantly received FBP, there was a decreased Col-1 expression to control levels, a lower proliferation rate and much more intracellular lipid droplets. This indicates that FBP maintained the ability of induce quiescent phenotype even in iron presence. Besides, looks like Col-1 serves as a survival signal for activated HSC. During resolution of fibrosis, activated HSC are sources of tissue inhibitor of metalloprotease, which prevent ECM decomposition. This is associated to fibrosis persistency and lower apoptosis rate (Iredale 2008). PPAR-γ activation interrupts the signaling pathway of TGF- β1 and inhibits its profibrogenic effects (Zhang et al. 2013)

TGF- β1 is the major profibrogenic cytokine in the liver (Friedman 2008b). It induces ECM production and its activation is a necessary wound-repair mechanism in response to cellular damage (Leask and Abraham 2004). Moreover, it's a potent stimulus for HSC proliferation. Our results show that low levels of TGF- β1 on Fe + FBP group (Fig. 8) could explain the lower proliferation rate in comparison with Fe group and control. TGF- β1 is synthesized as a proproteincomplexed to a latency associated peptide (LAP) and HSC release latent TGF- β1 (Kmiec 2001). LAP disassembly allows TGF- β1 to be biologically active and this is the major point of control in its activity (Zi et al. 2012). ROS can oxidize specific LAP amino acids, which results in a structure change of the complex and the release of active TGF- β1 (Barcellos-Hoff and Dix 1996; Pociask et al. 2004). By its turn, the main mechanism of iron-associated cell injury is through ROS production by Fenton reaction. By this mean iron can activate latent TGF- β1. GRX cells are in a transitional state between lipocyte/quiescent and myofibroblast/activated phenotype. For this, presents fibrogenic characteristics and produces high amounts of TGF- β1. Our Fe treatment didn't increased TGF- β1 levels even increasing growth (Fig. 8). This find is in agreement with prior results showing that hepatic iron-deposit was not relationed with increase of TGF- β1 expression (Guo et al. 2006).

Antioxidants can prevent the ROS production and, as consequence, inhibit TGF-β1 activation (Pociask et al. 2004). Previous studies report FBP as an antioxidant (Bajic et al. 2011; Bochi et al. 2012; Spasojevic et al. 2009). To test this potential of FBP we performed DPPH assay. DPPH is free radical and the potential scavenging of FBP was measured spectrophotometrically. We did not find difference in any of tested doses,

even highest ones (Fig. 9). Inclusive, recent work pointed out the pro-oxidant effects of FBP in cancer cells and the raise of H_2O_2 levels (Lu et al. 2013).

PPAR-γ activation raises catalase expression (Guimaraes et al. 2007) in HSC. In the same way, many effects FBP-related are based on enhancement of natural antioxidants proteins like glutathione (Alva et al. 2013; Calafell et al. 2009) and catalase (Guimaraes et al. 2007). By these reports and our results, we can conclude that FBP don't act as an antioxidant agent but improve the cell antioxidant natural ability. On a yet to be published work of our group, FBP was capable of diminish the levels of Interleukin 8 in HepG2 cells. IL-8 is a proinflammatory cytokine with crucial role in neutrophils recruitment and regulated by nuclear factor- κB (NF- κB). IL-8 induces ROS generation (Miyoshi et al. 2010) and antioxidants downregulate is expression (Shimada et al. 1999). This work point out that the immunosuppressant activity of FBP through inhibition of NF- κB could induce cytokine suppression, improve antioxidant state and, perhaps, posses anti-proliferative effects. Besides, iron has been implicated on activation of the ERK pathway. ERK-induced activation of IL-8 needs a strictly iron balance (Kina et al. 2009).

Iron chelators inhibit activation, proliferation and antiapoptotic proteins in HSC (Jin et al. 2007). Ferrozine binds ferrous iron, but not ferric iron, in a complex that absorbs strongly in 550nm (Fish 1988). We found that, in agreement with previous reports (Bajic et al. 2011), FBP chelates ferrous iron and that this effect was time and dose-dependent. In this assay, Fe and FBP were added at same time of Ferrozine or Fe and FBP was allowed to react during 120 min and, only after this time, Ferrozine was added to the samples. We found that only after 120 min. of reaction FBP was capable of chelate iron, indicating that this action is time-dependent. As we had a progressive decline on levels of iron detected by this method as FBP concentration increase, we showed a time and dose-dependent effect of FBP on chelating iron. This discovery is extremely important because highlights the principal mechanism of action of FBP in this study and can explain many of the encountered results. Looks like FBP not only sequester the free iron presented in the culture medium, but can exerts its beneficial effects even when bound to Fe. FBP, also possesses the ability to chelate extracellular calcium (Hassinen et al. 1991). Both ions have +2 charge. Looks like the ions charge are fundamental to the chelating property of FBP, since it does not bind in ferric iron, which charge is +3 (Bajic et al. 2011).

5. Conclusion

We demonstrate that FBP lead to a decreased proliferation rate of GRX cells that was occasioned by reverse of phenotype even in Fe presence. Moreover, it shows that FBP posses multiple useful properties to counteract HH. First, FBP, by activating PPAR-γ and increasing lipid droplets, leads cells to become quiescent and decrease proliferation rate, Col-1 expression and TGF- β1 release. Second, FBP chelates iron in a dose-dependent way. FBP could lower the overload state on HH and promote a relief of saturated transferrin and ferritin. Thereby, it also could avoid Fenton reaction and inhibits free radical production. Third, by enhancing antioxidant natural-proteins activity, FBP could diminish cell damage by ROS, revert activation of hepatic stellate cells and block TGF- β1 synthesis and release. Because all of it, we suggest that fructose-1,6-bisphosphate protects the liver against the fibrotic effects of Hemochromatosis. However, studies must to be made to evaluate mechanisms of FBP and the potential application on *in vivo* models.

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

	Forward primer (5' - 3')	**Reverse primer (3' - 5')**
PPAR-γ	TGGAATTAGATGACAGTGACTTGG	CTCTGTGACGATCTGCCTGAG
Type I Collagen	AGAACATCACCTATCACTGCAAGA	GTGGTTTTGTATTCGATGACTGTCT
β-actin	TATGCCAACACAGTGCTGTCTGG	TACTCCTGCTTGCTGATCCACAT

Figure legends

Figure 1.

Effect of free iron (Fe) in GRX cells growth. Cells were treated with Fe for 8 days. Data represent the mean ± SD (*n*=3). Results were expressed as cell number per well. (*) $p < 0.05$ vs. Control.

Figure 2.

Effect of Fe and Fe + FBP in GRX cells growth. Cells were treated with Fe and Fe + FBP for 8 days. Data represent the mean ± SD (*n*=3). Results were expressed as cell number per well. (**) $p < 0.01$ vs. Control. (###) $p< 0.001$ vs. Fe.

Figure 3.

Effect of Fe and Fe + FBP in LDH release on cell supernatant. Data represent the mean ± SD (*n*=3). Results were expressed as percent of LDH.

Figure 4.

Effects of Fe and Fe + FBP on apoptosis. Annexine-V staining. Cells were treated with Fe and FE + FBP for 24h. Data represent the mean ± SD (*n*=3). Results were expressed as percent of positive PE cells.

Figure 5.

Effects of Fe and Fe + FBP on BrdU fluorescence. Cells were treated with Fe and FE + FBP for 24h. (A) Represents a single experiment which illustrates a general profile of cell distribution. (B) Data represent the mean ± SD (*n*=3). Results were expressed as percentage of cell number for each cell cycle phase.

Figure 6.

Effects of Fe and Fe + FBP in Oil Red-O (ORO) staining and lipid quantification of GRX cells at day 8. (A) Control cells (400x); (B) cells treated with Fe (1mg/L) (400x); (C) cells treated with Fe + FBP (1mg/L + 0.6mM) (400x). Typical changes were observed in Fe + FBP group when compared to control and Fe group. Cells lost their elongated and parallel strand appearance and acquired a larger and polygonal shape. Intracellular lipid droplets were most visible in this group. (Arrows) indicate intracellular lipid droplets. (D) Lipid content of GRX cells at day 8. Data represent the mean ± SD (*n*=3). Results were expressed spectrophotometrically as the ratio absorbance value obtained for ORO and Coomassie brilliant blue staining (Specific Lipi Content). (*) $p < 0.05$ vs. Control. (***) $p< 0.001$ vs. Control. (###) $p< 0.001$ vs. Fe.

Figure 7.

Effects of Fe and Fe + FBP on mRNA expression of GRX cells treated for 24h. β-actin was used as an internal control for equal loading. Data represent the mean ± SD (*n*=3). (A) PPAR-γ. Results were expressed as relative optical density of PPAR-γ/β-actin. (*) $p< 0.05$ vs. Control. (##) $p< 0.01$ vs. Fe. (B) Col-1. Results were expressed as relative optical density of Col-1/β-actin. (*) $p< 0.05$ vs. Control. (##) $p< 0.01$ vs. Fe.

Figure 8

Effects of Fe and Fe + FBP on TGF- β1. Cells were treated with Fe and Fe + FBP for 8 days. Data represent the mean ± SD (*n*=3). Results were expressed as pg/mL. (*) $p< 0.05$ vs Control. (#) $p < 0.05$ vs Fe.

Figure 9

Effects of FBP on DPPH assay. FBP was added to a DPPH solution for 1h. Data represent a single analysis for each dose. Results were expressed as absorbance in 517 nm. C+ represent the positive control with ascorbic acid 1 mg/mL.

Figure 10

Effects of FBP on Ferrozine assay. FBP was incubated at different concentrations with a 1mg/L iron solution. Data represent the mean ± SD (n=3). Results were expressed spectrophotometrically as the absorbance value obtained with Ferrozine reaction after 120 min of incubation of Fe and FBP. (*) $p< 0.05$ vs. Fe. (***) $p< 0.001$ vs. Fe.

Figure 1

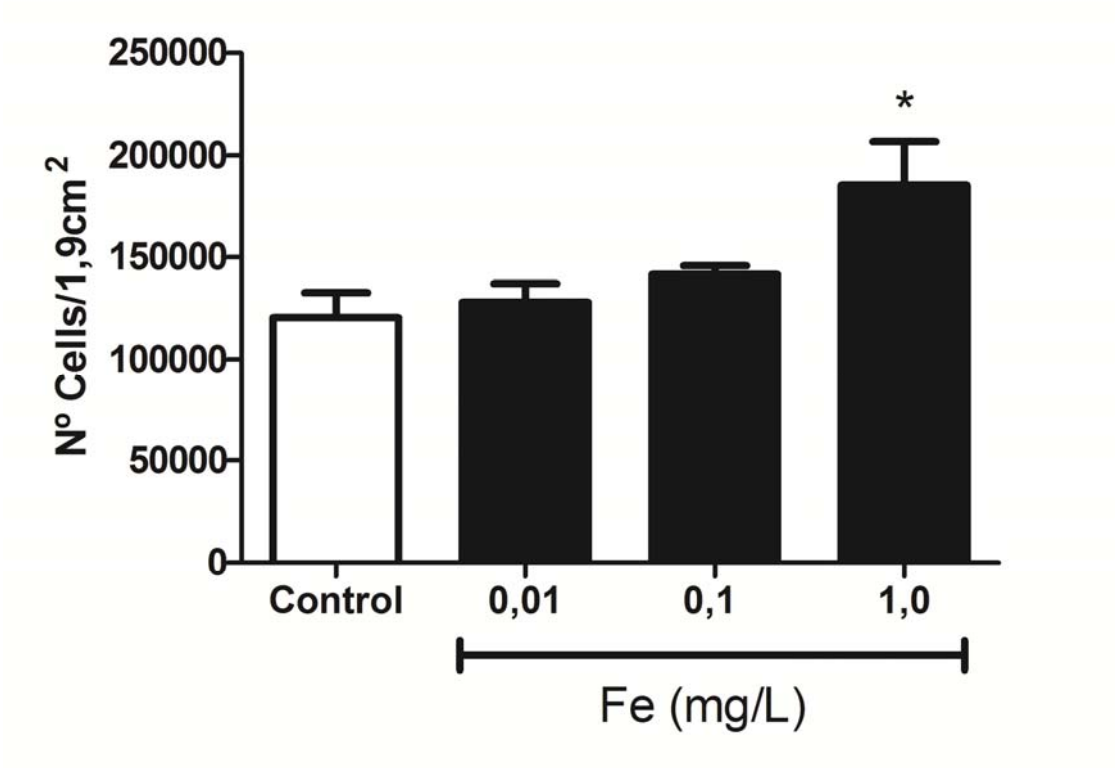


Figure 2

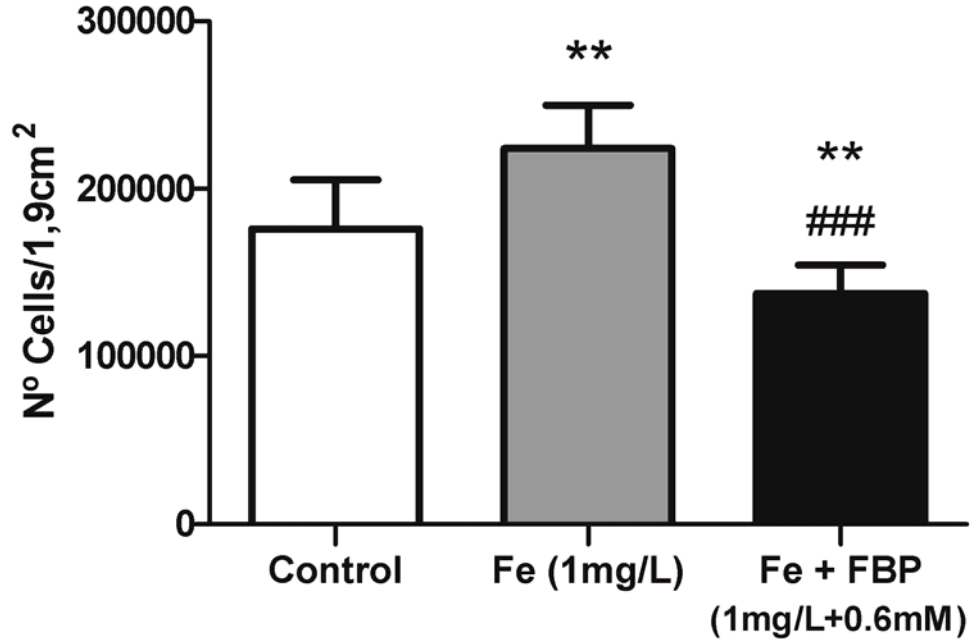


Figure 3

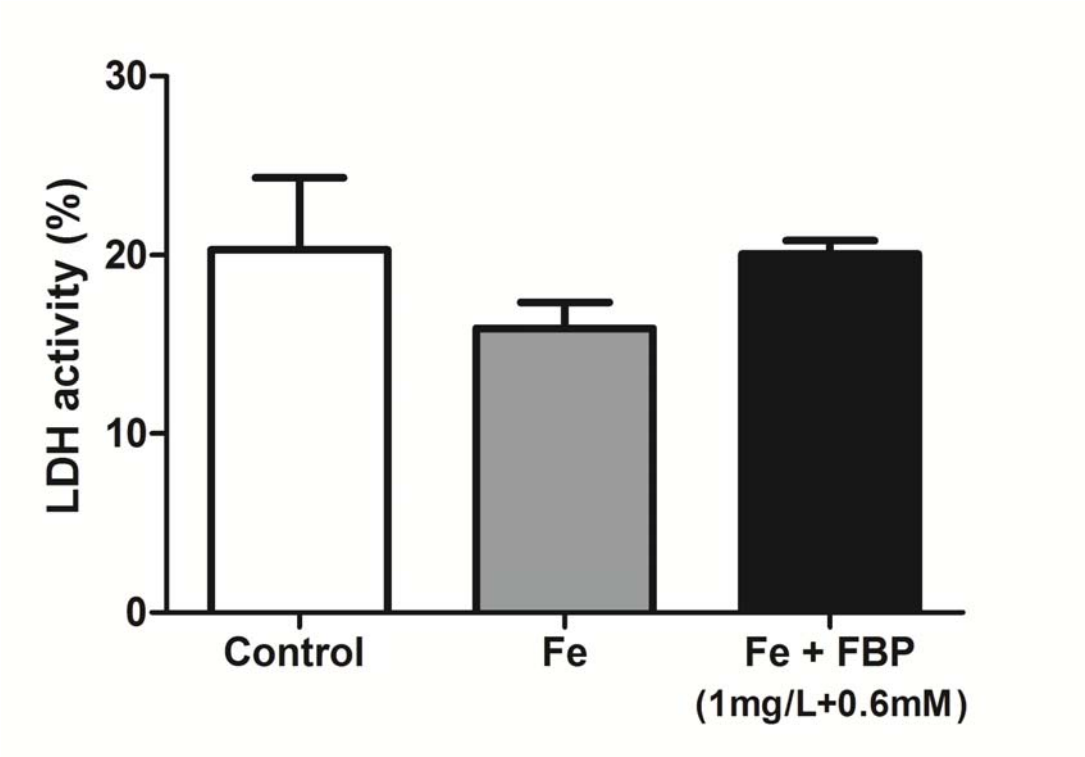


Figure 4

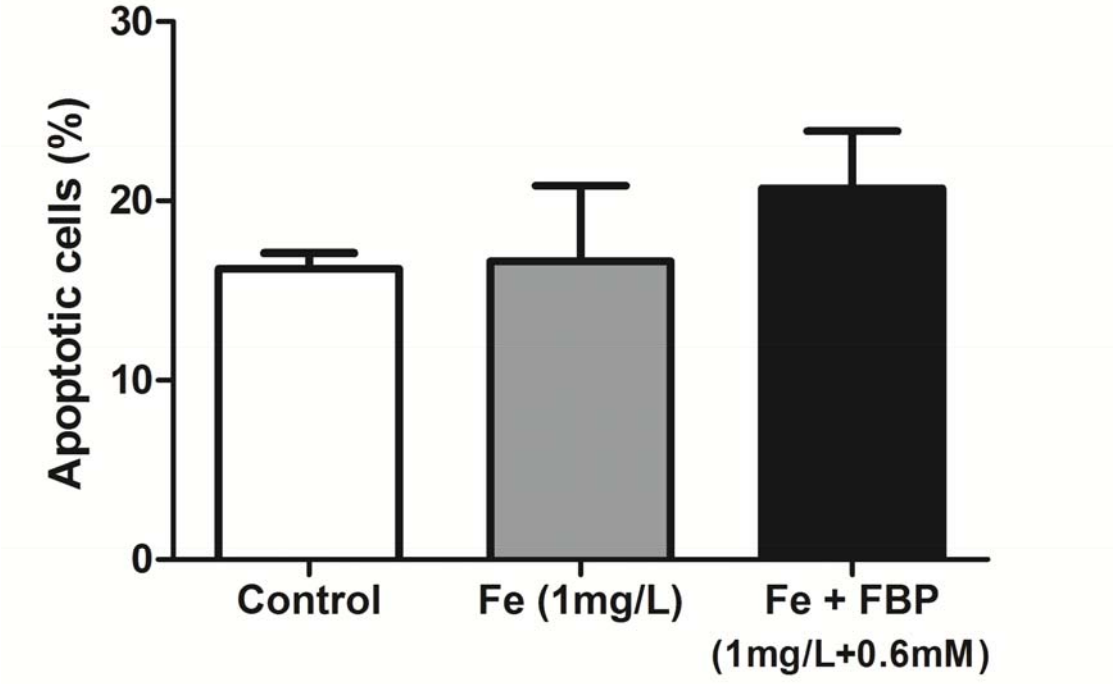


Figure 5A

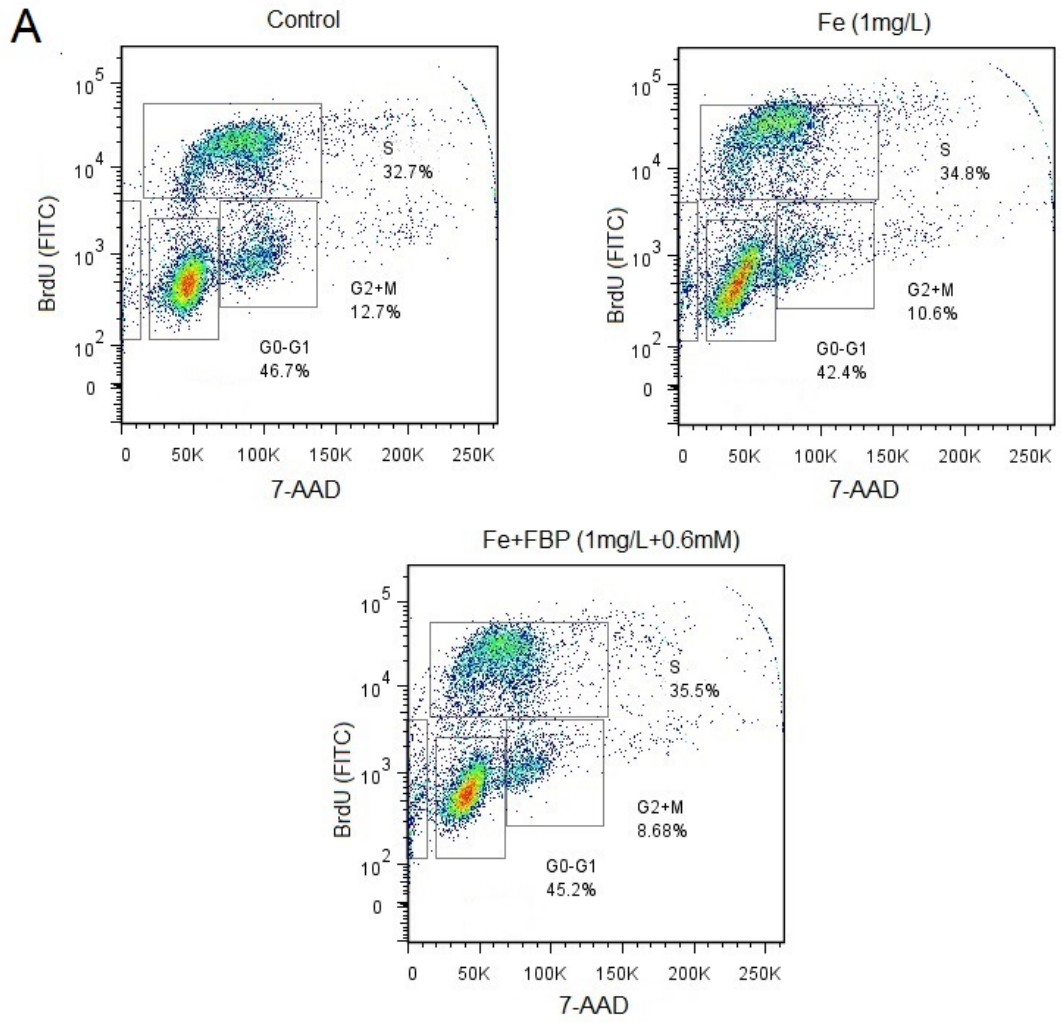

A
Control
S
32.7%
G2+M
12.7%
G0-G1
46.7%
BrdU (FITC)
7-AAD
Fe (1mg/L)
S
34.8%
G2+M
10.6%
G0-G1
42.4%
BrdU (FITC)
7-AAD
Fe+FBP (1mg/L+0.6mM)
S
35.5%
G2+M
8.68%
G0-G1
45.2%
BrdU (FITC)
7-AAD

Figure 5B

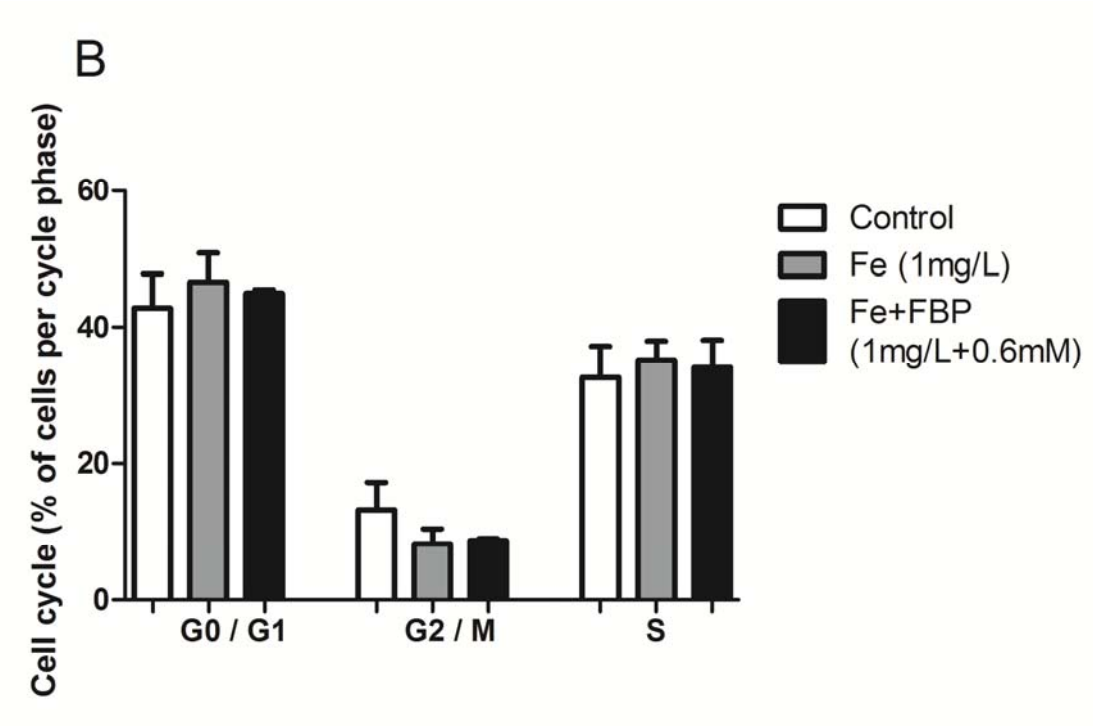

B
Cell cycle (% of cells per cycle phase)
60
40
20
0
G0 / G1
G2 / M
S
Control
Fe (1mg/L)
Fe+FBP
(1mg/L+0.6mM)

Figure 6A

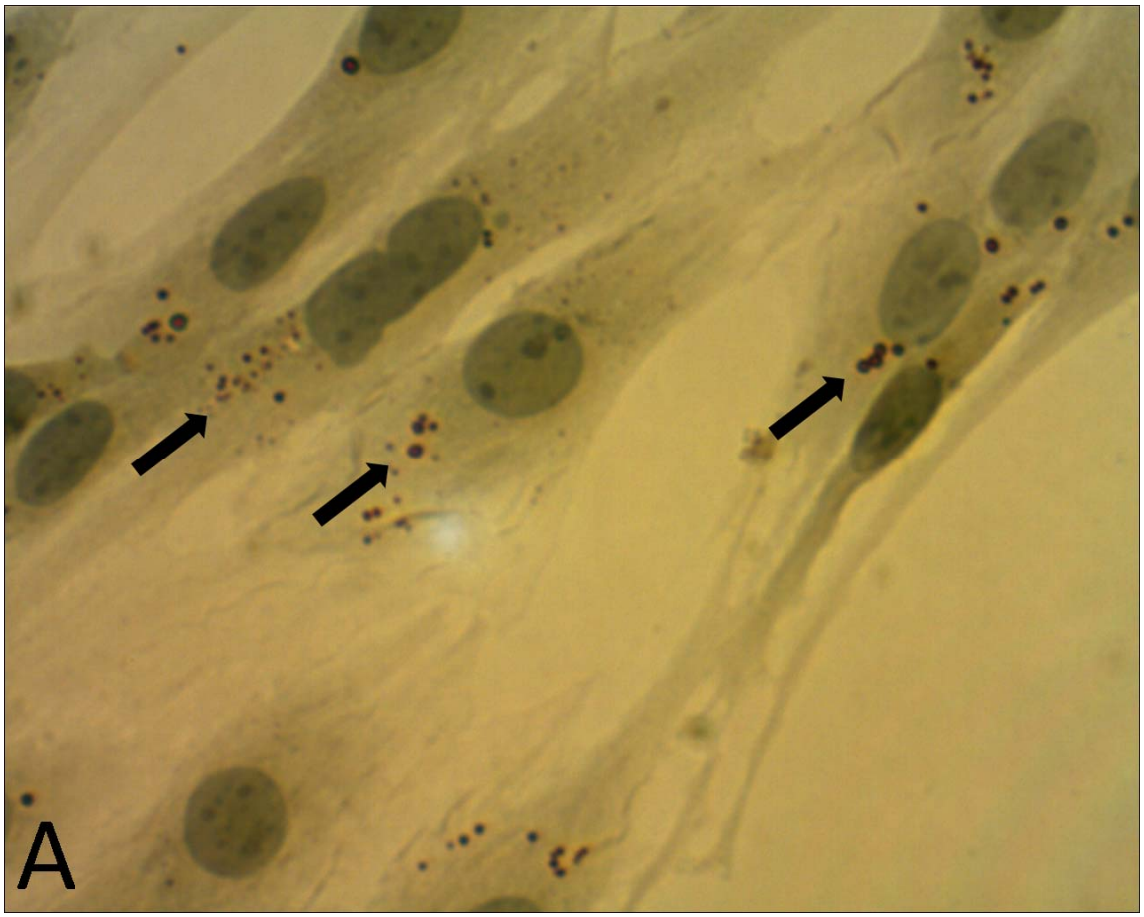


Figure 6B

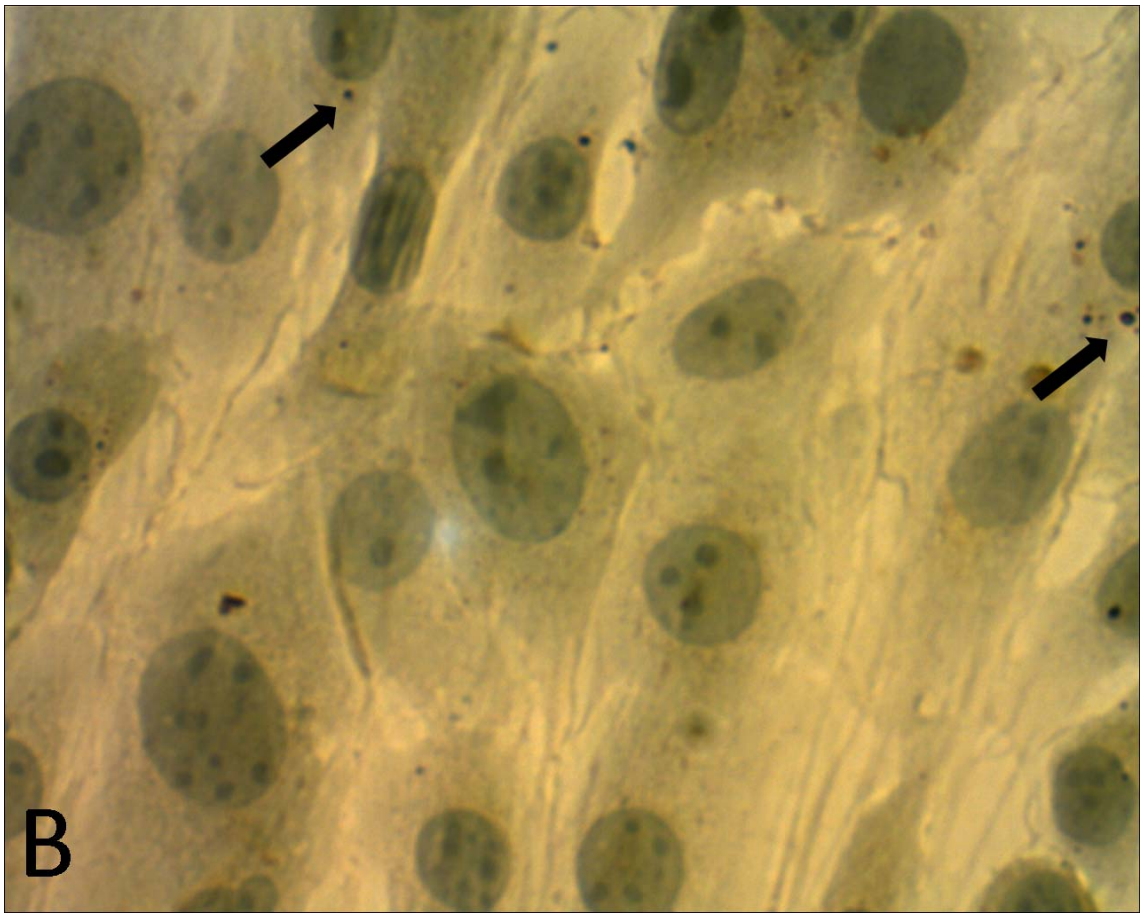


Figure 6C

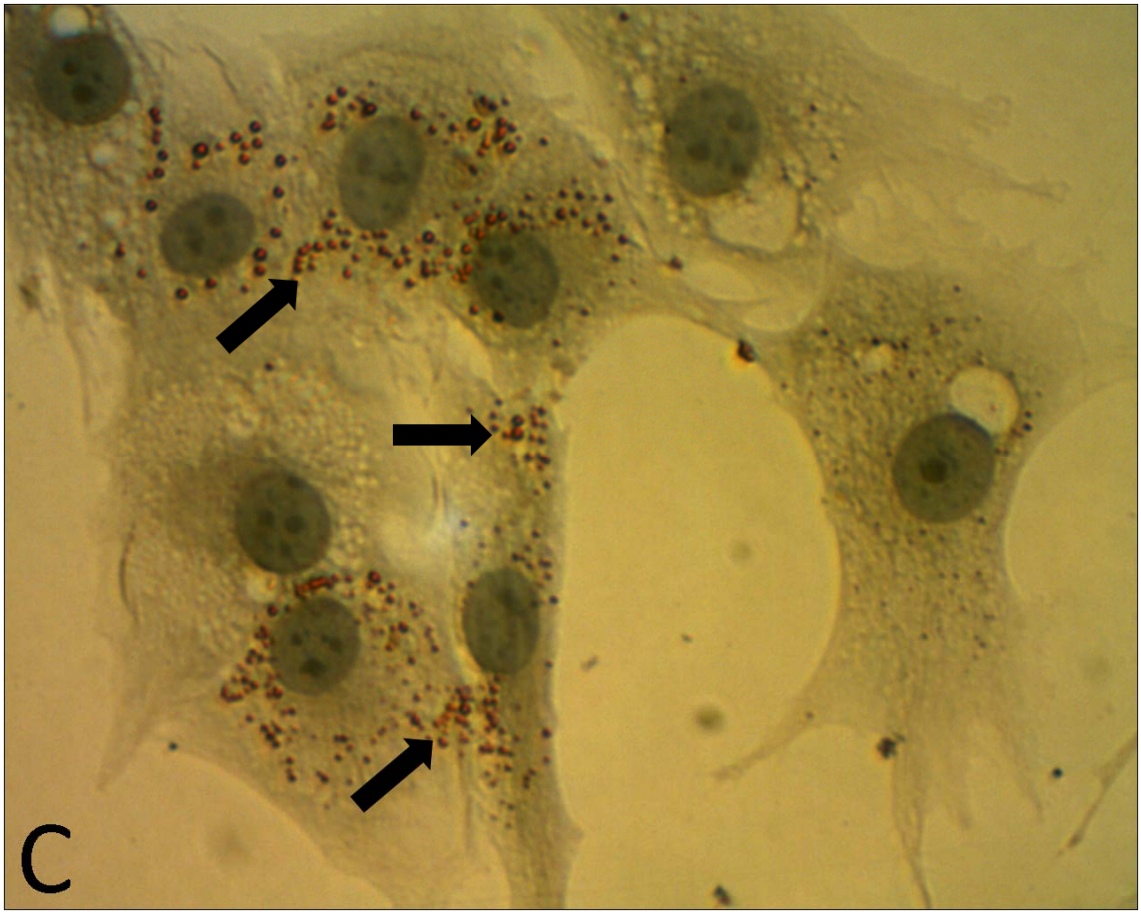


Figure 6D

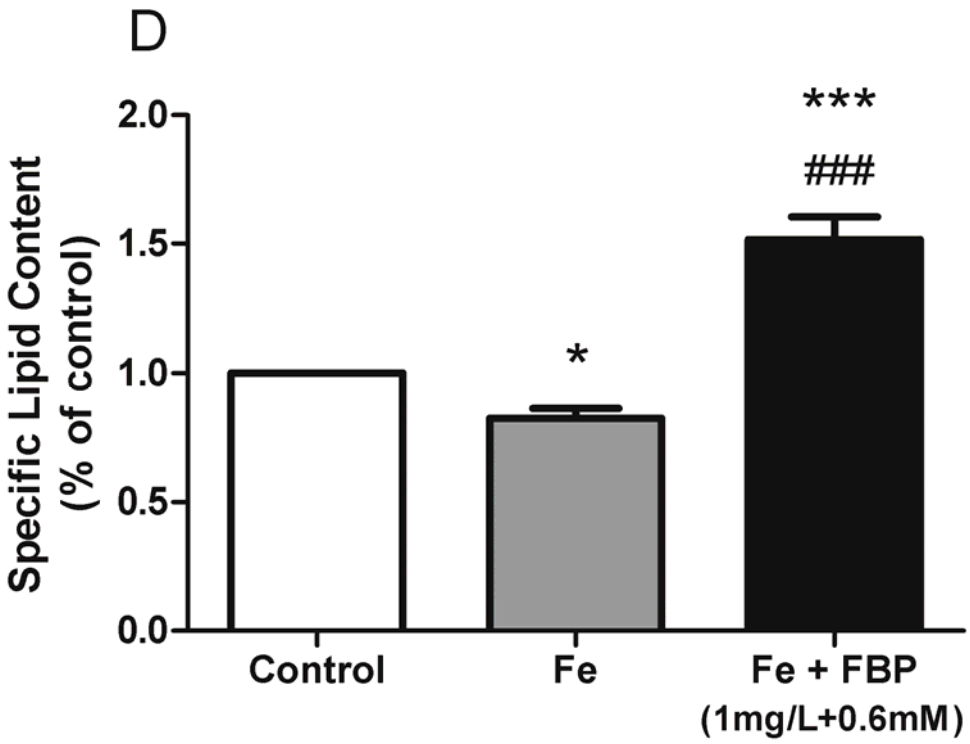

D
Specific Lipid Content
(% of control)
2.0
1.5
1.0
0.5
0.0
*
***
###
Control
Fe
Fe + FBP
(1mg/L+0.6mM)

Figure 7A

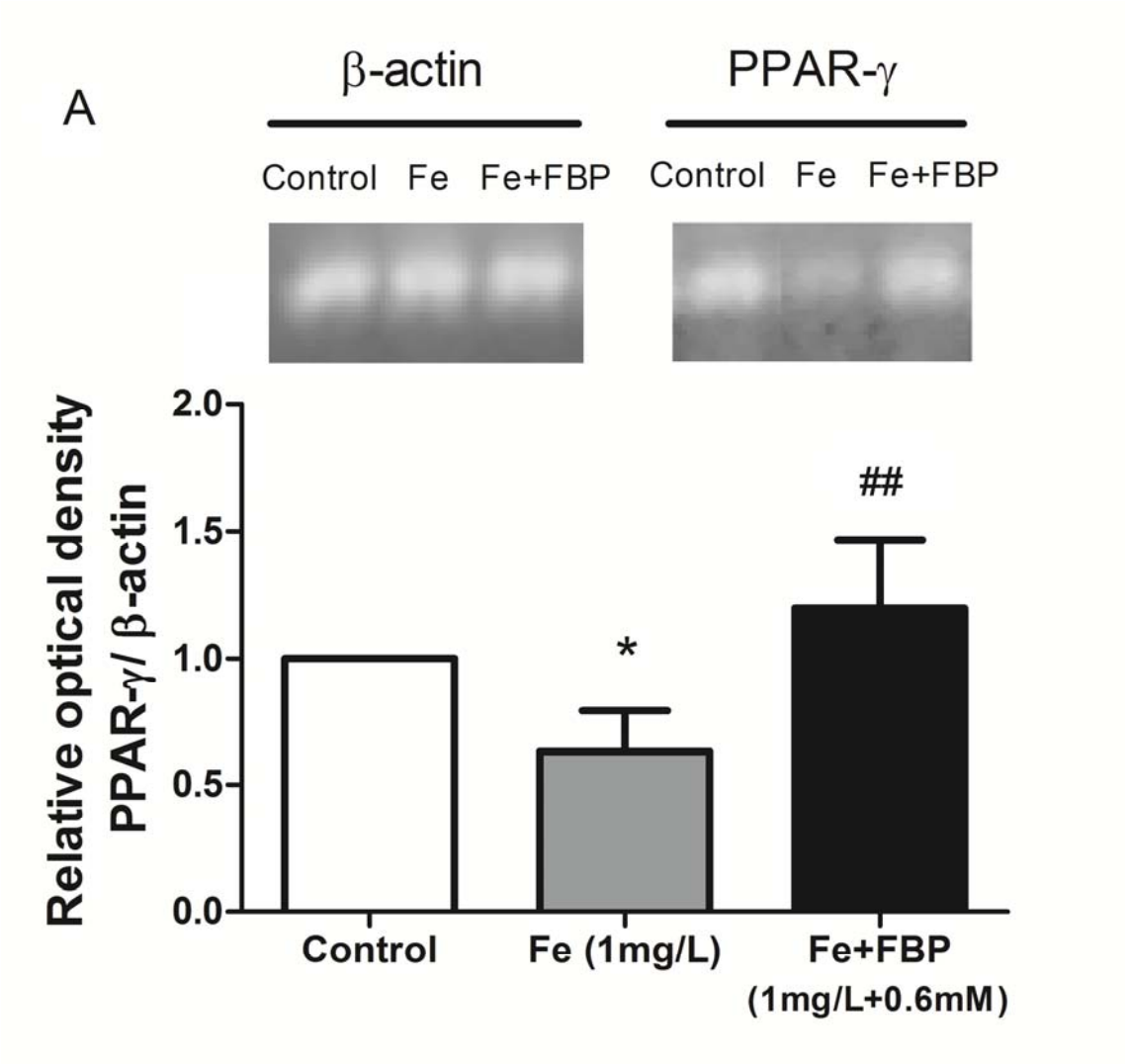

A
β-actin
PPAR-γ
Control Fe Fe+FBP
Control Fe Fe+FBP
Relative optical density
PPAR-γ/ β-actin
2.0
1.5
1.0
0.5
0.0
*
##
Control
Fe (1mg/L)
Fe+FBP
(1mg/L+0.6mM)

Figure 7B

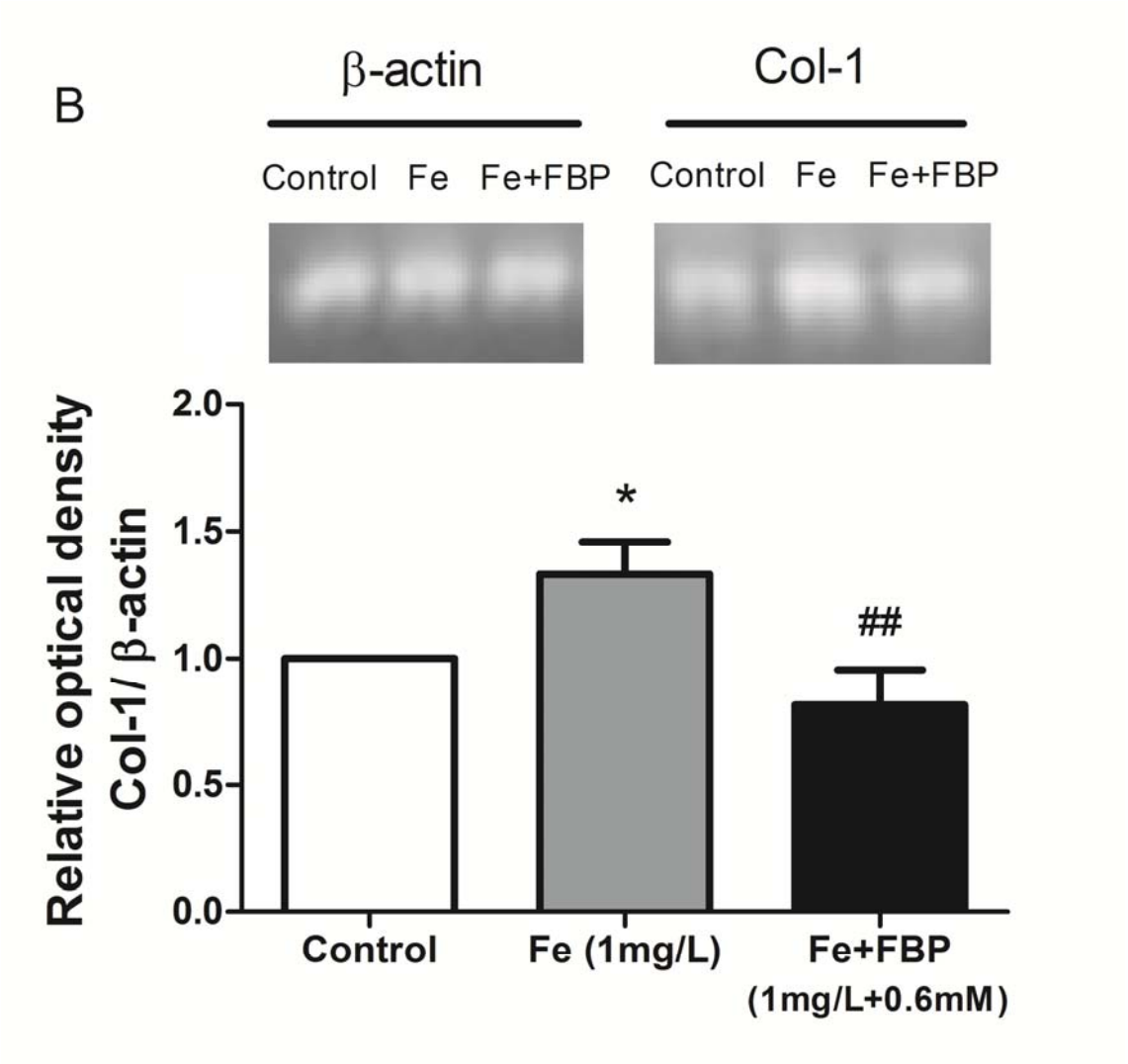

B
β-actin
Col-1
Control
Fe
Fe+FBP
Control
Fe
Fe+FBP
Relative optical density
Col-1/ β-actin
2.0
1.5
1.0
0.5
0.0
*
##
Control
Fe (1mg/L)
Fe+FBP
(1mg/L+0.6mM)

Figure 8

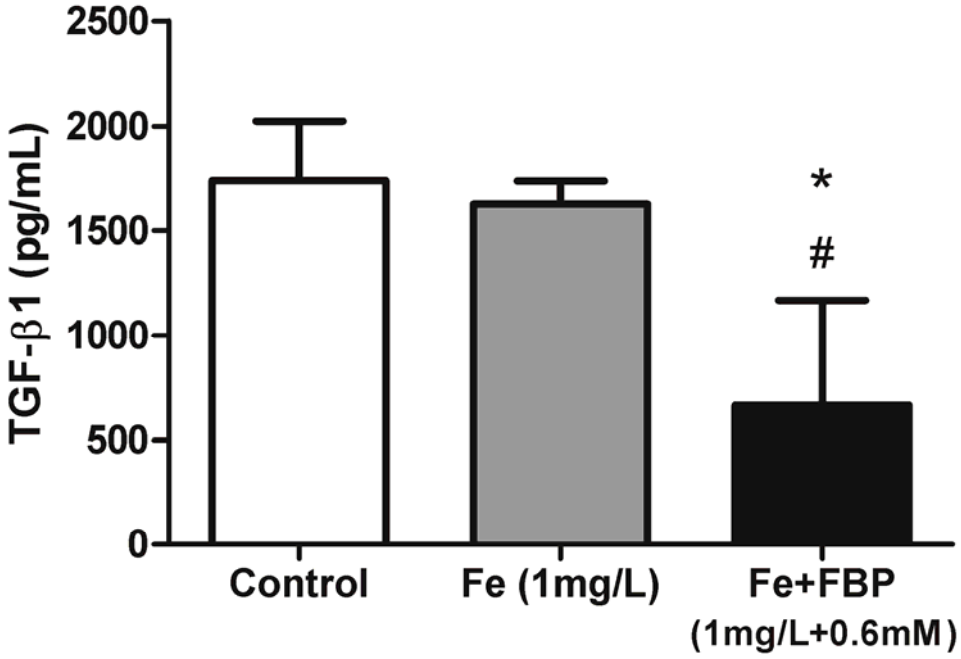


Figure 9

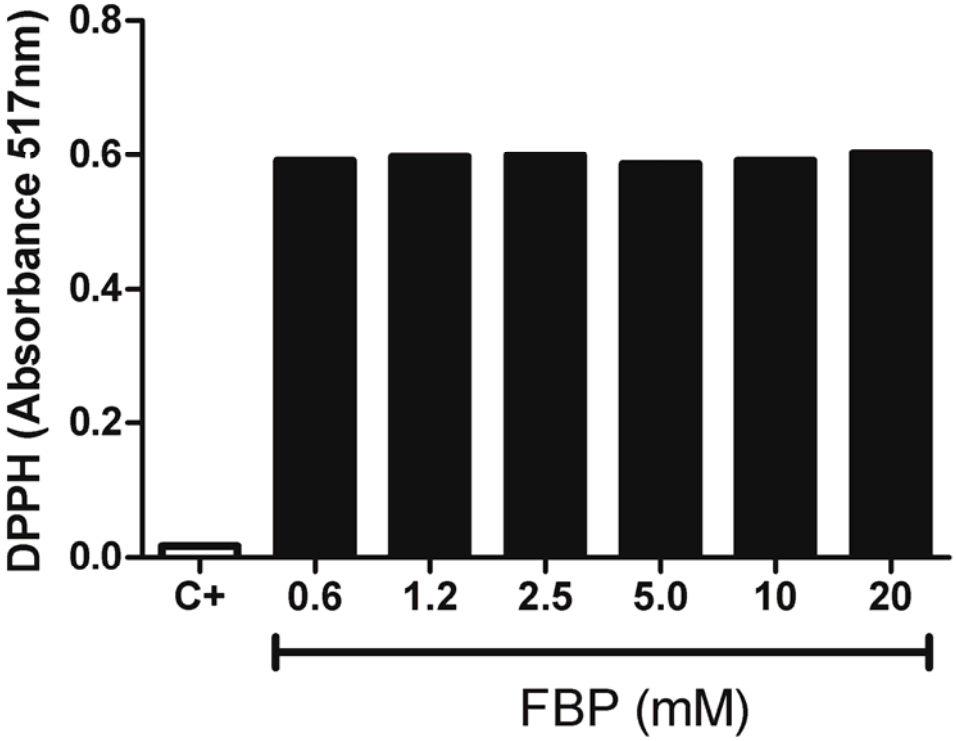
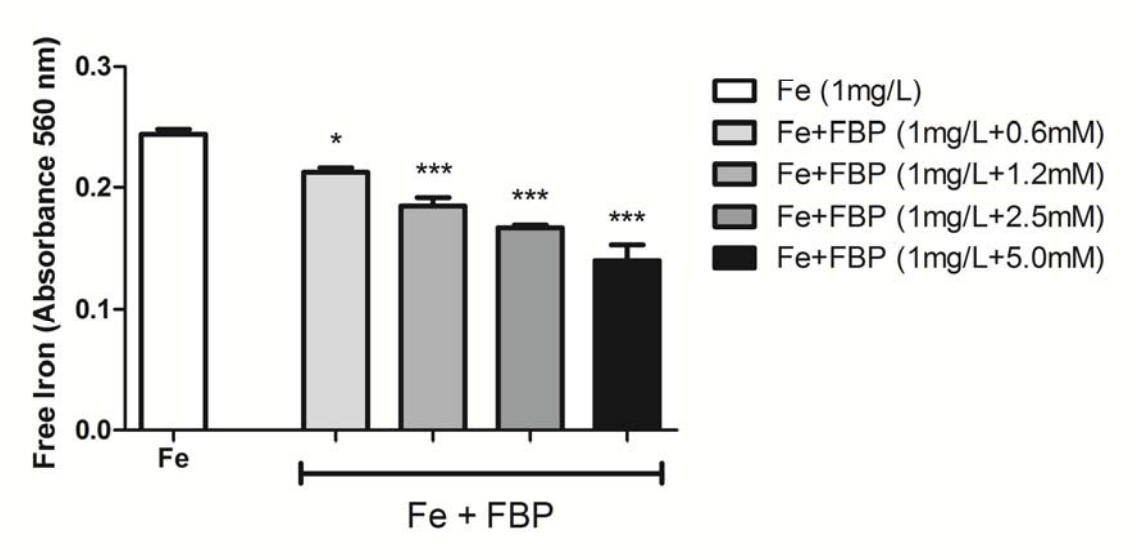


Figure 10


Free Iron (Absorbance 560 nm)
0.3
0.2
0.1
0.0
*
***
***
***
Fe
Fe + FBP
Fe (1mg/L)
Fe+FBP (1mg/L+0.6mM)
Fe+FBP (1mg/L+1.2mM)
Fe+FBP (1mg/L+2.5mM)
Fe+FBP (1mg/L+5.0mM)

5. CONSIDERAÇÕES FINAIS

A hemocromatose é uma doença de alta prevalência e incidência, sendo a doença de caráter genético mais comum em caucasianos. Tem como consequência o aumento da absorção de ferro no intestino, o que causa uma sobrecarga de ferro nos tecidos, principalmente no fígado, podendo levar à fibrose, cirrose e câncer. A fibrose é caracterizada por um aumento da deposição de componentes de matriz extracelular, que tem como principais características a ativação e proliferação de células estreladas hepáticas (HSC). Essas células são responsáveis pela produção e síntese de componentes da matriz extracelular, como colágeno tipo I (Col-1). Essa produção leva a uma resistência das HSC a entrar em apoptose, contribuindo para perpetuação do estado fibrótico, levando a extensas áreas cicatriciais e à perda da função hepática e, em um prognóstico pior, cirrose e câncer. Além disso, essas células são responsáveis por secretar o fator de crescimento transformante β1 (TGF- β1), que é uma citocina altamente fibrogênica, que estimula a produção de Col-1, ativa HSC e inibe a transcrição de fatores relacionados à síntese de lipídios, como o receptor ativado por proliferadores do peroxisoma γ (PPARγ). Esse receptor está expresso em HSC quiescentes, que possuem um fenótipo de lipócito. Ele não é expresso quando a HSC está ativada. Além disso, a ativação de PPARγ diminui a síntese de Col-1 e (TGF- β1).

Na fibrose experimental, o uso de quelantes de ferro e antioxidantes é capaz de reverter o quadro fibrótico, inibir a ativação e proliferação de HSC, a síntese de matriz e a liberação de TGF- β1. Por essa razão, utilizamos a frutose-1,6-bisfosfato, que possui efeitos antioxidantes e propriedades quelantes. Assim, nosso objetivo foi avaliar o efeito da FBP em células GRX que foram expostas ao ferro na tentativa de simular o que pode acontecer com o fígado durante a hemocromatose. Demonstramos que a FBP diminui a proliferação celular e que isso não foi causado por morte, necrose ou apoptose(apesar do grupo Fe + FBP ter apresentado uma tendência a aumentar a apoptose), nem por regulação do ciclo celular. Mesmo na presença do ferro, a FBP foi capaz de reverter o fenótipo celular das células GRX, normalizando os níveis de expressão de PPARγ, Col-1 e diminuindo significativamente a liberação de TGF- β1. Demonstramos, também,que a FBP não possui efeito antioxidante direto, mas foi capaz de quelar o ferro de modo dose-dependente.

O ferro, como esperado, induziu o aumento da proliferação e a FBP conseguiu impedir o crescimento celular. Sugere-se que um dos mecanismos responsáveis por esse efeito observado da FBP seja a diminuição dos níveis de TGF- β1 e a reversão fenotípica das células GRX, mesmo quando expostas a ferro livre. Ainda, sugerimos que a FBP não age como um antioxidante direto, mas indireto, através do sequestro de ferro, inibição da reação de Fenton e a consequente falha na geração de radicais livres. Por fim, estudos *in vivo* devem ser realizados para saber se a FBP pode ser usada de forma terapêutica em portadores de hemocromatose hereditária que, por causa do diagnóstico realizado tardiamente, possuem uma alta incidência de fibrose hepática.

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