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THE EFFECTS OF MACROPHAGE MIGRATORY INHIBITORY FACTOR AND BETAINE ON MORPHOFUNCTONAL, CELLULAR AND TISSUE CHANGES IN LIVER FIBROSIS: EPIDEMIOLOGICAL RELEVANCE OF CHRONIC LIVER DISEASE

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DEJSTVA MAKROFAGNOG MIGRATORNOG INHIBITORNOG FAKTORA I BETAINA NA MORFOFUNKCIONALNE, ĆELIJSKE I TKIVNE PROMENE U FIBROZI JETRE: EPIDEMIOLOŠKI ZNAČAJ HRONIČNE BOLESTI JETRE

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The effects of macrophage migratory inhibitory factor and betaine on morphofunctonal, cellular and tissue changes in liver fibrosis: epidemiological relevance of chronic liver disease

Liver diseases are prevalent worldwide, causing significant morbidity and mortality. The incidence and prevalence of chronic liver disease (CLD) and its progression to fibrosis, cirrhosis and hepatocellular carcinoma (HCC) are very high. Out of a total of 2 million deaths a year from liver disease worldwide, about 45% of all deaths are caused by liver fibrosis. Therefore, the most important goal of preventive and therapeutic strategies is to alleviate the progression of the disease from steatosis to fibrosis, cirrhosis and HCC. In this regard, this is indicated by the importance of numerous experimental models of CLD that are a good basis for examining the mechanisms of fibrosis progression and testing of antifibrogenic agents, and thus further contribute to the prevention and treatment of liver disease.

Hepatic fibrogenesis is a dynamic and reversible process, involving molecular, cellular, and tissue changes responsible for excess accumulation and remodeling of the extracellular matrix (ECM). Oxidative stress, inflammation and hepatic stellate cells (HSC) activation are the key mechanisms that contribute to liver tissue remodeling and fibrosis.

Macrophage migration inhibition factor (MIF) is a multifunctional cytokine that is released from immune cells and contributes to the control of the innate and immune systems. In chronic liver disease, inflammation and oxidative stress stimulate, in addition to inflammatory cells and endothelial cells, resident macrophages and hepatocytes to increase MIF expression. However, its role varies depending on the cause and stage of chronic liver disease.

Thioacetamide (TAA) -induced hepatotoxicity is a suitable animal model of both acute and chronic liver injury. Chronic administration of TAA leads to altered redox homeostasis, inflammation, hepatocyte necrosis, and liver fibrosis. MIF-mediated TAA-induced hepatotoxicity depends on the interaction of numerous factors and signaling pathways. MIF exhibits its proinflammatory and antiinflammatory effects depending on the etiology and stage of liver disease. However, its exact role during liver fibrogenesis is not clear enough.

Betaine (trimethyl-glycine) is an oxidative metabolite of choline that acts as an antioxidant by increasing the levels of S-adenosylmethionine and methionine. The use of betaine as a dietary supplement can alleviate oxidative stress and the harmful effects of alcoholic and non-alcoholic fatty liver disease, as well as liver fibrosis and necrosis. However, the precise antioxidant, anti-inflammatory and antifibrotic effects of betaine on MIF in the onset and development of liver fibrosis are still unknown.

The aims of the dissertation were to examine the effect of MIF on liver functional changes as well as the effects of MIF on oxidative stress, inflammation and fibrogenesis in the liver of mice; to examine the effects of MIF on liver morphological changes and matrix metalloproteinases-2 and 9 (MMP-2 and -9), tissue inhibitor matrix metalloproteinase-1 (TIMP-1) and dimer-9; to study the modulatory effects of betaine on MIF-mediated liver fibrosis; to evaluate epidemiological relevance of chronic liver disease.

To establish a liver fibrosis animal model, 8-week-old wild type C57BL/6 male mice (n=40) and MIF knockout C57BL/6 mice ($MIF^{-/-}$) (n=40) were divided into the following groups (n=10): 1. C group- continuously on a standard diet; 2. Bet group- mice on a standard diet that were treated with betaine solution in drinking water (2% solution *ad libitum*) for 6 weeks; 3. $MIF^{-/-}$ group - $MIF^{-/-}$ mice continuously on a standard chow diet: 4. $MIF^{-/-}$+Bet group- $MIF^{-/-}$ mice on standard diet and supplemented with betaine; 5. TAA group- mice on standard diet and treated with TAA (200 mg/kg TAA dissolved in 200μL PBS, i.p.3x/week/8weeks; 6. TAA+Bet group - wild type mice treated with TAA and supplemented with betaine; 7. $MIF^{-/-}$+TAA group - $MIF^{-/-}$ mice treated with TAA; 8. $MIF^{-/-}$+TAA+Bet group- $MIF^{-/-}$ treated with TAA and supplemented with betaine. ALT, AST, ALP, γGT, CRP, bilirubin and albumin, were measured in serum, while oxidative stress parameters, markers of inflammation (interleukin /IL/-6, interferon /IFN/-γ) and fibrogenic mediators (TGF-β1, PDGF, MMPs (2 and 9) and tissue inhibitor of matrix metalloproteinases /TIMP1/) were determined in liver samples. For histological analysis of the liver, liver tissue was stained with Hematoxylin-Eosin (HE), Masson's trichrome and reticulin, and was examined by using a light microscope. Methods used in this dissertation were spectrophotometry, ELISA, SDS-PAGE reverse electrophoretic zymography and zymography and light microscopy.

After 8 weeks of TAA administration in wild mice, alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP, gamma-glutamyl transpeptidase (γGT), bilirubin, markers oxidative stress (malondialdehyde /MDA/, advanced oxidation protein products /AOPP/ and total oxidative status /TOS/), proinflammatory cytokines (IL-6 and IFN-γ) and fibrogenic mediators (TGF-β1, PDGF, MMPs and TIMP) were increased, while thiols, total antioxidative status (TAS), superoxide dismutase (SOD) and catalase (CAT) were decreased compared to the control group ($p < 0.01$). Quantification of liver fibrosis (Masson's trichrome and reticulin staining) in the TAA group showed a significantly increased accumulation of ECM when they make up collagen type I and III (coll1 and Coll3) compared to control animals ($p < 0.01$).

However, in $MIF^{-/-}$ mice treated with TAA, the activity of ALT and AST, bilirubin, MDA, AOPP, TOS, IL-6, IFN-γ was significantly lower, while thiol and TAS were increased compared to the TAA group ($p < 0.01$). On the other hand, the liver values of profibrogenic mediators (TGF-β1 and PDGF, MMPs, and TIMP were significantly higher in MIF-deficient mice compared to wild-

type (TAA group) ($p<0.01$). Histologically, Masson's trichrome and reticulin staining showed an increase in the fibrous score in MIF-deficient mice.

Betaine supplementation of mice treated with thioacetamide, ALT, AST, γGT, bilirubin, MDA, AOPP, CRP, IL-6, IFN-γ, TGF-β1, PDGF, MMPs and TIMP were significantly lower, while SOD, CAT, TAS, and thiol concentration were increased compared to TAA group ($p<0.01$). The histological structure of the liver was less irregular with scanty fibrosis and a mild inflammatory infiltrate in mice of the TAA+Bet group. Also, mice of the TAA+Bet group had a lower fibrous score, less Coll1 and Coll3 compared to the TAA group ($p<0.01$).

Betaine modulates the prooxidative effect of MIF by reducing MDA and TOS, and by increasing the concentration of thiol in liver fibrosis caused by TAA. Also, betaine modulates the pro-inflammatory effect of MIF by reducing CRP, IL-6, and IFN-γ.

Betaine supplementation in $MIF^{-/-}$ mice treated with TAA, significantly reduced ALT and AST activity compared to $MIF^{-/-}$ + TAA and TAA + Bet group ($p<0.01$). Also, betaine decreased the activity of γGT in comparison with the $MIF^{-/-}$ + TAA group and bilirubin in compared to TAA + Bet ($p<0.01$). Besides, betaine significantly reduced MDA and TOS in $MIF^{-/-}$ mice compared to $MIF^{-/-}$ + TAA and TAA + Bet group ($p<0.01$). Also, betaine reduced AOPP in comparison to TAA + Bet group ($p<0.01$). In comparison to the MIF-/-+ TAA group, betaine supplementation increased SOD, CAT, and TAS in MIF-/-mice treated with TAA ($p<0.01$). However, after betaine supplementation of $MIF^{-/-}$ mice, irregular histological structures of liver tissue remained with mild fibrosis and mixed inflammatory infiltrate. Betaine reduced ECM deposition (reduction of fibrous score) compared to the $MIF^{-/-}$ + TAA group ($p<0.01$). However, compared to the TAA + Bet group, this score was significantly higher ($p<0.01$).

TAA-induced liver injury was mediated through the prooxidative and proinflammatory effects of MIF. MIF exerts prooxidative effects by increased lipid peroxidation and oxidative stress (MDA, AOP, and TOS), as well as decreasing activity of an antioxidant enzyme (SOD and CAT), thiol, and TAS in the liver. Also, MIF exerts anti-inflammatory effects through increased CRP and pro-inflammatory cytokines (IL-6 and IFN-γ). However, MIF has antifibrotic effects on TAA-induced liver fibrosis through decreased liver TGF-β and PDGF-BB, as well as decreased activity of MMP-2 and 9 and TIMP1. The antifibrogenic effects of MIF can be explained by the inhibition of HSC.

Betaine exerts hepatoprotective effects by reducing ALT, AST, ALP, γGT and bilirubin in TAA-induced liver fibrosis. Also, betaine improved redox imbalance by reducing lipid peroxidation and oxidative stress as well and increasing the activity of antioxidant enzyme and thiol in the liver. Besides hepatoprotective and antioxidative effects, betaine exerts anti-inflammatory effects through

a decrease in CRP and proinflammatory cytokines (IL-6 and IFN-γ). Betaine supplementation reduces fibrogenesis by decreasing TGF-β1 and PDGF-BB levels in the liver. Potential additional mechanisms of betaine in TAA-induced liver fibrosis were decreased activity of MMP-2 and 9, as well TIMP-1.

Betaine modulates the prooxidative, proinflammatory, and antifibrogenic effects of MIF in TAA-induced liver fibrosis. Betaine attenuates the prooxidative effects of MIF by decreasing MDA and AOPP, as well as by increasing liver thiols and TAS. Also, betaine mitigates the proinflammatory effects of MIF by reducing liver IL-6 and IFN-γ. On the other hand, betaine stimulates antifibrogenic effects of MIF by decreasing TGF-β1, PDGF-BB, MMP-2, MMP-9, TIMP-1, and deposition of ECM (Coll1 and Coll3) in the liver.

Keywords: Liver, thioacetamide, macrophage migratory inhibitory factor, oxidative stress, inflammation, fibrogenesis, betaine

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Dejstva makrofagnog migratornog inhibitornog faktora i betaina na morfofunkcionalne, ćelijske i tkivne promene u fibrozi jetre: epidemiološki značaj hronične bolesti jetre

Uvod

Akutne i hronične bolesti jetre su rasprostranjene širom sveta sa značajnim morbiditetom i mortalitetom. Incidencija i prevalencija hroničnih bolesti jetre i njihova progresija do fibroze, ciroze i hepatocelularnog karcinoma (HCC) je veoma visoka. Od ukupno 2 miliona smrtnih slučajeva godišnje od bolesti jetre širom sveta, oko 45% svih smrtnih slučajeva uzrokovano je fibrozom jetre. Stoga je najvažniji cilj preventivnih i terapijskih strategija ublažavanje progresije bolesti od steatoze do fibroze, ciroze i HCC. S tim u vezi, na ovo ukazuje značaj brojnih eksperimentalnih modela hronične bolesti jetre, koji su dobra osnova za ispitivanje mehanizama progresije fibroze i ispitivanje antifibrogenih sredstava, a samim tim i dalje doprineti prevenciji i lečenju bolesti jetre.

Fibrogeneza jetre je dinamičan i reverzibilan proces koji uključuje molekularne, ćelijske i tkivne promene odgovorne za deponovanje i remodelovanje ekstracelularnog matriksa (ECM). Oksidativni stres, inflamacija i aktivacija zvezdastih ćelija jetre (HSC) su ključni mehanizmi koji doprinose remodelovanju tkiva jetre i fibrozi.

Faktor inhibicije migracije makrofaga (MIF) je multifunkcionalni citokin koji se oslobađa iz imunih ćelija i igra važnu ulogu u kontroli urođenog i imunog sistema. U hroničnim bolestima jetre, inflamacija i oksidativni stres stimulišu, pored inflamatornih ćelija i endotelnie ćelije, rezidentne makrofage i hepatocite na povećanu ekspresiju MIF. Međutim, njegova uloga varira u zavisnosti od uzroka i stadijuma hronične bolesti jetre.

Hepatotoksičnost izazvana tioacetamidom (TAA) je pogodan animalni model akutne i hronične bolesti jetre. Hronična primena TAA dovodi do izmenjene redoks homeostaze, inflamacije, nekroze hepatocita i fibroze jetre. Uloga MIF u oštećenju jetre izazvane TAA zavisi od interakcije brojnih faktora i signalnih puteva. MIF ispoljava svoja proinflamatorna i anti-inflamatorna dejstva u zavisnosti od etiologije i stadijuma bolesti jetre Međutim, njegova tačna uloga tokom fibrogeneze jetre nije dovoljno jasna.

Betain (trimetil-glicin) je oksidativni metabolit holina koji deluje kao antioksidans povećavajući nivo S-adenozilmetionina i metionina. Upotreba betaina kao dijetetskog suplementa može ublažiti oksidativni stres i štetne efekte alkoholne i nealkoholne masne bolesti jetre, kao i fibrozu i nekrozu jetre. Međutim, precizni antioksidativni, antiinflamatorni i antifibrotični efekti betaina na MIF u nastanku i razvoju fibroze jetre još uvek nisu poznati.

Ciljevi istraživanja

Ispitati uticaj MIF-a na funkcionalne promene jetre kod ispitivanih miševa; ispitati efekte MIF-a na oksidativni stres, inflamaciju i fibrogenezu u jetri; istražiti efekte MIF na morfološke promene jetre i matriks metaloproteinaze-2 i -9 (MMP-2 i -9), tkivni inhibitor matriks metaloproteinaza-1 (TIMP-1) i dimer 9; ispitati modulatorne uticaje betaina na MIF-posredovanu fibrozu jetre i proceniti epidemiološki značaj hroničnih bolesti jetre.

Materijali i metode

Eksperiment je izveden na mužjacima miševa divljeg soja C57BL/6 i na miševima kojima je uklonjen gen za MIF ($MIF^{-/-}$ knockout C57BL/6 mice) starosti 8 nedelja, težine 21-25g, koje su bile podeljene u sledeće grupe po 10 životinja (n = 10): 1. Kontrolna grupa; 2. Bet-grupa (životinje su dobijale betain u vodi za piće; 3. $MIF^{-/-}$ grupa miševa kojima je uklonjen gen za MIF; 4. $MIF^{-/-}$+Bet grupa ($MIF^{-/-}$ miševi su dobijali betain u vodi za piće); 5. TAA grupa (životinje su tretirane tioacetamidom; 6. TAA+Bet grupa (miševa divljeg tipa tretirani tioacetamidom uz suplementaciju betainom u vodi za piće; 7. $MIF^{-/-}$+TAA grupa ($MIF^{-/-}$miševi tretirani sa TAA; 8. $MIF^{-/-}$+TAA+Bet grupa ($MIF^{-/-}$ miševa koji su tretirani TAA uz suplementaciju betainom u vodi za piće).

Fibroza jetre je izazvana TAA (200 mg/kg) rastvorenim u 200µL PBS (Phosphate Buffered Saline) intraperitonealno (i.p.) tri puta nedeljno u periodu od 8 nedelja. Betain (MP Biomedicals) je rastvoren u vodi za piće (2% wt/v) i životinje su imale slobodan pristup vodi *ad libitum*. Posle tretmana, životinje su žrtvovane iskrvarenjem u ketaminskoj (100 mg/kg i.p.) anesteziji i za analizu su uzeti uzorci krvi i jetre.

U serumu su određivani sledeći parametri: alanin aminotransferaza (ALT), aspartat aminotransferaza (AST), alkalna fosfataza (ALP), γ-glutamil-transferaza (γ-GT), ukupni bilirubin, albumini i C-reaktivni protein (CRP). U tkivu jetre određivani su: parametri oksidativnog stresa-malondialdehid (MDA), oksidacioni produkti proteina (advanced oxidation protein products, AOPP), aktivnost ukupne superoksid dizmutaze (SOD), katalaze (CAT), ukupna količina tiola, ukupni antioksidativni status (TAS) i ukupni oksidativni status (TOS); markeri zapaljenja-interleukin-6 (IL-6) i interferon-γ (INF-γ); markeri fibroze-transformišući faktor rasta-beta1 (TGF-β1) i faktor rasta oslobođen iz trombocita (plateled derived growth factor, PDGF-BB), matriks metaloproteineza 2 i 9 (MMPs), dimer 9 i tkivni inhibitor matriks metaloproteinaza 1 (TIMP-1). Histopatološke promene u tkivu jetre ispitivane su svetlosnom mikroskopijom.

Određivanje biohemijskih parametara u serumu

Aktivnost enzima ALT, AST, ALP, γ-GT, kao koncentracija bilirubina, albumina i C-raektivnog proteina (CRP) u serumu je određivana spektrofotometrijski.

Određivanje parametara oksidativnog stresa, inflamatornih i fibrogenih citokina u jetri

Uzorci jetre za određivanje oksidativnih/antioksidativnih parametara, proinflamatornih citokina i fibrogenih faktora su homogenizovani po standardnoj proceduri. U dobijenom supernatantu spektofotometrijski su određivani parametri oksidativnog stresa (MDA, AOPP, ukupna SOD, CAT i tioli), a TOS i TAS su mereni pomoću komercijalnih setova (Rel Assay). ELISA *(ELISA kit, BD Bioscience, San Diego, California, SAD)* metodom su određivani proinflamatorni citokini (IL-6 i IFN-γ) i fibrogeni faktori (TGF-β1 i PDGF-BB).

Zimografija za određivanje MMP-2 i MMP-9, dimer 9 i TIMP-1 u jetri

Aktivnosti MMP-2, MMP-9, dimer 9 I TIMP-1 su određivane SDS-PAGE zimografijom i reverznom elektroforetskom zimografijom prema već prethodno ustanovljenoj i opisanoj proceduri.

Histološka analiza

Uzorci tkiva jetre su fiksirani u 10% rastvoru formaldehida na sobnoj temperaturi. Nakon fiksacije, isečci debljine 5 μm se prožimaju rastopljenim parafinom, zatim se boje hematoksilinom i eozinom (HE), Masson's trichrome i retikulin prema uputstvima proizvođača. Preparati su analizirani i fotografisani pomoću svetlosnog mikroskopa *(Olympus BX51 opremljenim Artcore 500 MI artray, Co. Ltd. Japan kamerom).* Stepen fibroze je kvantifikovan pomoću ImageJ 1.42 softvera, izračunavanjem procenta pozitivno obojenih površina u odnosu na ukupnu površinu ispitivanog tkiva jetre

Rezultati

Smanjenje telesne težine, a povećanje težine jetre, kao i povećanje odnosa težina jetre/telesna težina, ispoljilo se nakon osam nedelja tretiranja miševa divljeg soja sa TAA. Takođe, dokazano je hepatotoksično dejstvo TAA koje se ogleda povećanjem aktivnosti ALT, AST, APL, γ-GT, kao i koncentracije ukupnog bilirubina u poređenju sa kontrolom ($p<0.01$). TAA je doveo do lipidne peroksidacije i oksidativnog stresa u jetri povećanjem koncentracije MDA, AOPP i TOS, a smanjenjem ukupnih tiola, TAS, kao i aktivnosti SOD i CAT u poređenju sa kontrolnom grupom ($p<0.01$). Pored poremećaja redoks homeostaze, TAA je značajno povećao koncentraciju CRP i proinflamatornih citokina (IL-6 i IFN-γ) i profibrogenih medijatora (TGF-β1 i PDGF-BB), kao i aktivnost MMPs i TIMP u odnosu na kontrolnu grupu ($p<0.01$). Takođe, histološkom analizom tkiva jetre uočena je umerena fibroza jetre sa inflamatornim infiltratom. Kvantifikacija fibroze jetre (Masson's trichrome i retikulin bojenje) u TAA grupi pokazala je značajno povećanu akumulaciju ECM koga čine kolagen tip I i III (Coll1 and Coll3), u poređenju sa kontrolnim životinjama ($p<0.01$).

Za razliku od dobijenih rezultata kod miševa divljeg soja koji su tretirani TAA, TAA u MIF deficijentnih ($MIF^{-/-}$) miševa izazvao je manju redukcije telesne težine i odnosa težina jetre/telesna težina. Dalje, kod $MIF^{-/-}$ miševa tretiranih TAA, aktivnost ALT i AST, kao i koncentracija

bilirubina bila je značajno niža u poređenju sa TAA grupom ($p<0.01$). U poređenju sa TAA grupom, uočeno je i značajno sniženje markera lipidne proksidacije i oksidativnog stresa, MDA, AOPP ($p<0.01$) i TOS ($p<0.05$), a povećanje antioksidativne zaštite, tiola i TAS ($p<0.01$). Takođe, koncentracija CRP u krvi, kao i nivo IL-6 i INF-γ u tkivu jetre $MIF^{-/-}$ miševa su bili značajno sniženi u poređenju sa divljim sojem miševa tretiranih TAA ($p<0.01$). Ovakvi rezultati ukazuju na oksidativno i proinflamatorno dejstvo MIF. S druge strane, koncentracija profibrogenih medijatora (TGF-β1 i PDGF-BB), kao i aktivnost MMP-2 and -9, dimer MMP-9 i TIMP-1 su bili su značajno viši u MIF deficijentnih miševa u poređenju sa divljim sojem (TAA grupa) ($p<0.01$). Dalje, histološkom analizom tkiva jetre uočena je izrazito nepravilna struktura jetre sa mešovitim inflamatornim infiltratom. Izražena premošćujuća (bridging porto-portal) fibroza i mikronodularna ciroza jetre je bila prisutna kod $MIF^{-/-}$ miševa u odnosu na divlji soj (TAA grupu). Histološko Masson's trichrome i retikulin bojenje je pokazalo izraženu fibrozu (povećanje Coll1 i Coll3). Osam nedelja nakon administracije TAA, fibrozni skor je bio značajno veći u jetri miševa sa nedostatkom MIF u poređenju sa divljim sojem miševa ($p<0.01$). Poređenjem histopatološkg nalaza tkiva jetre $MIF^{-/-}$ miševa, koncentracije profibrogenih medijatora (TGF-β1 i PDGF-BB), kao i aktivnosti MMP-2 and -9, dimer MMP-9 i TIMP-1, sa miševima divljeg soja se indirekto ukazuje na antifibrogeno dejstvo MIF.

Suplementacija betainom miševa TAA grupe u trajanju od 6 nedelja smanjila je oštećenje hepatocita izazvano TAA s obzirom da je aktivnost ALT, AST, γGT i koncentracija bilirubina bila značajno niža u odnosu na životinje koje nisu dobijale betain ($p<0.01$). Slično ovim nalazima, u poređenju sa TAA grupom, suplementacija betainom redukovala je oksidativno oštećenje jetre izazvano TAA, smanjenjem lipidne peroksidacije i oksidativnog stresa, MDA i AOPP ($p<0.01$), kao smanjenjem TOS ($p<0.05$). S druge strane, u TAA+Bet grupi u poređenju sa TAA grupom u jetri je dokazano značajno povećanje aktivnosti SOD, CAT, TAS ($p<0.01$) i koncentracije tiola ($p<0.01$). Pored antioksidativnog dejstva, betain je pokazao i antiinflamatorno dejstvo smanjenjem koncentracije CRP, IL-6 i INF-γ, kao i antifibrogeno, smanjenjem koncentracije profibrogenih medijatora (TGF-β1 i PDGF-BB), kao i aktivnosti MMP-2 and -9, dimer MMP-9 i TIMP-1 u poređenju sa TAA grupom ($p<0.01$). Suplementacija betainom miševa TAA grupe poboljšala je histološki nalaz tkiva jetre i smanjila oštećenje hepatocita. Histološka struktura jetre bila je manje nepravilna sa oskudnom fibrozom i blagim inflamatornim infiltratom. Kvantifikacijom fibroze (Masson i retikulin bojenje) dobijen je značajno manji fibrozni skor, odnosno manje Coll1 i Coll3 u poređenju sa TAA grupom ($p<0.01$). Betain modulira prooksidativno dejstvo MIF smanjenjem MDA i TOS, a povećanjem koncentracije tiola u fibrozi jetre izazvane TAA. Takođe, betain modulira i proinflamatorno dejstvo MIF smanjenjem CRP, IL-6 i INF-γ.

Suplementacija betainom MIF$^{-/-}$ miševa značajno je redukovala aktivnost ALT i AST u poređenju sa MIF$^{-/-}$+TAA i TAA+Bet grupom ($p<0.01$). Takođe, kod istih životinja betain je smanjio aktivnost γGT u poređenju sa MIF$^{-/-}$+TAA grupom i koncentraciju bilirubina u odnosu na TAA+Bet ($p<0.01$). Osim ovog, betain je kod MIF$^{-/-}$ miševa značajno smanjio MDA i TOS u poređenju sa MIF$^{-/-}$+TAA i TAA+Bet grupom ($p<0.01$). Takođe, betain je smanjio oksidaciju proteina čiji je parametar AOPP u odnosu na TAA+Bet grupom ($p<0.01$). Suplementacija betainom MIF$^{-/-}$ miševa ne samo da je dovela do smanjenja oksidativnog stresa i lipidne peroksidacije, već i do povećanja antioksidativne zaštite značajnim povećanjem aktivnosti ukupne SOD i katalaze kao i povećanje TAS u poređenju sa MIF$^{-/-}$+TAA grupom ($p<0.05$; $p<0.01$). Međutim, nakon suplementacije betainom MIF$^{-/-}$ miševa ostale su nepravilne histološke strukture tkiva jetre sa blagom fibrozom i mešovitim inflamatornim infiltratom. Betain je smanjio taloženje ECM (smanjenje fibroznog skora) u poređenju sa MIF$^{-/-}$+TAA grupom ($p<0.01$), međutim u odnosu na TAA+Bet grupu taj skor je bio značajno viši ($p<0.01$).

Betain modulira prooksidativne, proinflamatorne i antifibrogene efekte MIF u TAA-indukovanoj fibrozi jetre. Betain ublažava prooksidativne efekte MIF smanjenjem MDA i AOPP, kao i povećanjem tiola i TAS u jetri. Takođe, betain ublažava proinflamatorne efekte MIF smanjujući koncentraciju IL-6 i IFN-γ. S druge strane, betain stimuliše antifibrogene efekte MIF smanjenjem TGF-β1, PDGF-BB, MMP-2, MMP-9, TIMP-1 i taloženjem ECM (Coll1 I Coll3) u jetri

Ključne reči: Jetra, tioacetamid, fibrogeneza, makrofagni migratorni inhibitorni faktor, oksidativni stres, inflamacija, betain

Naučna oblast: Medicina

Uža naučna oblast: Epidemiologija

UDK:

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1. INTRODUCTION

1.1. Epidemiology of chronic liver disease

The incidence and prevalence of chronic liver diseases (CLD) are very high worldwide. The progression of CLD to fibrosis, cirrhosis and hepatocellular carcinoma (HCC) is characterized by high morbidity and mortality. Out of a total of 2 million deaths a year from chronic liver disease in the world, about 45% of all deaths are caused by liver fibrosis (1). Therefore, the most important goal of preventive and therapeutic strategies is to alleviate disease progression from steatosis to fibrosis, cirrhosis, and HCC. In this regard, recent clinical data have confirmed that eradicating or reducing the causes of CLD, such as alcoholism, obesity or hepatotoxicity caused by drugs and toxins, leads to the alleviation of fibrosis in the initial stage of liver cirrhosis (1).

In patients with chronic non-communicable liver disease, low economic standards increase the risk of morbidity and mortality. In that context, according to world data, about 70 million people who consume alcohol develop ALD, which can turn into cirrhosis and fibrosis (1). Besides, approximately 2 billion adults have metabolic syndrome or are overweight, which is a risk factor in the pathogenesis of NAFLD, NASH and HCC. Also, the prevalence of drug-induced toxic hepatitis is increasing (1).

However, depending on gender, race or ethnicity, the manifestation of liver disease is different. On the other hand, epidemiological studies of the incidence and prevalence of CLD are limited due to various factors, such as population composition, methods used to assess disease and to diagnose or differently define certain pathological liver conditions. Therefore, the frequency and prevalence of chronic non-communicable diseases can not be accurately determined (3).

1.2. Pathogenesis of liver fibrosis

Liver fibrosis is characterized by the deposition and remodeling of extracellular matrix (ECM) proteins (3, 4, 5, 6). The most common causes of progressive liver fibrosis are chronic hepatitis, ALD, nonalcoholic steatohepatitis /NASH/, as well as some congenital disorders such as hemochromatosis and Wilson’s disease (4).

Liver fibrosis is currently at the focus of significant scientific research, bearing in mind that cirrhosis as the final stage of progressive liver fibrosis affects between 1% and 2% of the global population. Out of a total of 2 million deaths a year from CLD in the world, about 45% of all deaths are caused by liver fibrosis (4, 7). Therefore, the most important goal of preventive and therapeutic strategies is to alleviate disease progression from steatosis to fibrosis, cirrhosis and HCC. In this regard, experimental models of liver fibrosis are necessary to better understand the complex interaction between chronic liver inflammation and oxidative distress, fibrosis and tissue

remodeling and testing of antifibrogenic agents, and thus further contribute to the prevention and treatment of liver disease (3, 6, 8, 9).

Liver fibrogenesis is a dynamic and reversible process, which includes molecular, cellular and tissue changes responsible for the excess accumulation and remodeling of ECM (8, 9). Major signaling pathways in hepatic fibrosis are fibrogenic signaling pathway, growth factor signaling, as well as chemokines, adipokines and neuroendocrine pathways (10). Oxidative stress, inflammation, activation of hepatic stellate cells (HSC) which produce collagen, platelet-derived growth factor (PDGF), matrix metalloproteinases (MMPs) and tissue inhibitor of matrix metalloproteinases (TIMPs), as well as other proteins of ECM, have a key role in hepatic fibrogenesis (11, 12, 13, 14).

1.2.1. Oxidative stress in liver fibrogenesis

Reactive oxygen species (ROS) are chemical metabolites that play a role in various physiological functions (11, 12, 15). Thus, the activity of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase 2 (NOX2) in Kupffer cells and neutrophils is key in protection against microorganisms (11, 15). For the difference from mild stress, which induces hormesis, excessive ROS generation alter redox homeostasis resulting in oxidative stress (15, 16). It is known that altered redox homeostasis contributes to the development of many diseases, such as diabetes, neurological disease, liver diseases, cancer and other disorders (17, 18, 19, 20). ROS promotes the progression of chronic liver disease to fibrosis, cirrhosis and HCC.

In the liver, sources of ROS can be organelles, such as mitochondria, endoplasmic reticulum (ER) and peroxisomes. Mitochondrial remodeling and ER stress contribute to the progression of ALD and NAFLD to steatohepatitis and fibrosis progression (11, 12, 16). Also, ROS can be generated in reactions catalyzed by enzymes such as cytochrome P450 (CYP) 2E1, NADPH oxidase (NOX), cyclooxygenases xanthine oxidase and others (12, 16).

The liver has a very important role in detoxification and metabolic homeostasis. The CYP450 family members are enzymes that include in the generation of ROS and bioactivated intermediates during drug, toxin and alcohol-induced liver injury (21). In chronic liver diseases, altered redox homeostasis and persistent production of ROS and reactive nitrogen species (RNS) contribute to hepatic fibrogenesis. Besides oxidative burst, a decrease in the capacity of antioxidant systems aggravates liver fibrosis (16). Therefore, inhibition of ROS/RNS production and increased antioxidative defense are targets for mitigating mechanisms of fibrosis progression (11, 22).

Similarly, NASH progression includes oxidative stress and increase of ROS which induce the activation of nuclear factor-kappaB (NF-κB) and the increased production of proinflammatory cytokines and profibrogenic mediators, accompanied by the depletion of endogenous antioxidants such as glutathione (GSH). It is known that NF-kB is important for regulating antioxidant enzymes

that mediate ROS signaling. Activation of NF-kB by ROS regulates the expression of genes, transforming quiescent HSCs into proliferative cells (14). Kupffer cells contribute to the progression of fibrosis through increasing ROS-mediated NF-kB activation, reduction of GSH level, as well as increased production of inflammatory and fibrogenic cytokines such as TNF-α, IL-1ß, IL-6 and TGF-ß1 (33). ROS act as inducers of the TGF-β1 signaling pathway through activation of matrix metalloproteinases, induction of TGF-β1 expression, and increasing of TGF-β1 release. On the other hand, TGF-β1 increases mitochondrial ROS production (34).

1.2.2. Inflammation in liver fibrogenesis

Chronic inflammation, together with disturbed redox homeostasis and oxidative stress, is known to be a key pathogenetic mechanism involved in liver fibrosis. It has been suggested that interaction between etiological factors, proinflammatory cytokines, ROS/RNS, and immune responses of liver cells contribute to maintaining chronic liver inflammation (14). Still, the mechanisms are not completely understood.

ROS and oxidative stress may be responsible for initiating the inflammatory response by increasing the production of pro-inflammatory cytokines, particularly IL-1α, IL-1β, and TNF-α, chemokines and other mediators in Kupffer cells and other cells of the inflammatory response (35, 36). Besides, many other etiological factors trigger inflammation in the liver through inflammatory mediators that are released from damaged hepatocytes, macrophages, epithelial or endothelial cells and activate liver sinusoidal endothelial cells (LSEC). Chronic or low-grade inflammation contributes to the activation and phenotype transformation of HSC, the main ECM-producing cells with deposition of collagens and matrix proteins resulting in liver fibrosis (37).

Many hepatotoxic agents (drugs, toxins, alcohol metabolite viruses, bacteria, etc.) that can induce hepatocyte death (apoptosis and necrosis) act as a signal of extracellular or intracellular cell death. The binding of TNF-α to its receptors, as a signal of extracellular cell death, is accompanied by ROS generation, NF-κB activation and inflammatory response. Intracellular signals of cell death can be triggered by the accumulation of saturated fatty acids, such as NASH, where lipotoxicity causes ER stress. ROS and damage-related molecular patterns (DAMPs) released from necrotic or apoptotic hepatocytes bind to Toll-like receptor-4 (TLR4) in Kupffer cells which release proinflammatory cytokines and chemokines. Furthermore, these mediators can activate hepatocytes, HSC or have an autocrine effect. DAMPs can also activate HSC that produce ECM components, type I collagen, (Coll1), type III collagen (Coll3), fibronectin and other fibrogenic markers (38).

Besides Kupffer cells, monocyte-derived macrophages, Th17 cells, mucosal-associated invariant mucosal associated invariant (MAI) T cells, dendritic, natural killer (NK), NKT cells and neutrophils are included in maintaining the inflammatory response and hepatic fibrosis modulation

(38, 39). Namely, hepatic macrophages are the most important cells in the progression of chronic non-communicable liver disease-inducing fibrosis and ECM remodeling. It has been suggested that macrophages may be classified into M1 and M2 types of macrophages. When stimulated by interferon-gamma (IFN-γ), lipopolysaccharides (LPS) and TNF-α, M1 cells secrete TNF-α, IL-1, IL-6 and ROS involving phagocytosis, inflammation and liver fibrogenesis. M1 favors the Th1 response. Unlike the proinflammatory M1 cell type, immunosuppressive M2 cells favor the Th2 response and secrete anti-inflammatory cytokines (TGF-β1, PDGF, IL-4, IL-10, and IL-13) (38, 40, 41).

However, this classification in M1 and M2 is not fully applicable and therefore a functional classification into defensive, regulatory and restorative macrophages has been proposed. Proinflammatory cytokines from defensive macrophages cause T cell differentiation to Th17 cells, while NK cell activation is included in the differentiation of hepatic restorative macrophages which secrete IL4. Besides, NK cells have an antifibrogenic role, by promoting HSC apoptosis, unlike NKT cells that have a profibrogenic phenotype. Regulatory macrophages of the liver are similar to M2 cells and secrete TGF-β1 and IL-10 (24, 40, 41, 42).

Kupffer cells, as antigen-presenting cells, are responsible for maintaining and progression of inflammatory and fibrotic response, activating HSC in chronic liver disease. Also, the proinflammatory effects of Kupffer cells are amplified by producing large amounts of nitric oxide (NO) through inducible NO synthetases (iNOS) and generating very reactive peroxynitrite. Peroxynitrite and other RNS, together with ROS, activate HSC, ECM production and fibrosis progression (12). Also, ROS/RNS and other reactive metabolites can activate Kupffer cells which modulate the recruitment of inflammatory cells, produce proinflammatory cytokine (IL-6, TNF-α, and TGF-β), activate HSC, leading to ECM accumulation and liver fibrosis. In the late stage of fibrosis, ROS-activated Kupffer cells significantly contribute to the activation of NF-kB and the synthesis of proinflammatory and profibrogenic cytokines and chemokines, as well as decreasing GSH (38). It is known that NF-κB is the major transcription factor that regulates the immune system and stress response. Their activation in the various liver cells promotes inflammation and fibrosis by increasing the production of proinflammatory cytokines (IL-1β, TNF-α, and IL-6) and activation of NF-kB/c-Jun N-terminal kinases (JNKs) signaling pathways (14, 43).

On the other hand, Nrf2 signaling pathways promote antiinflammatory response by increasing the antioxidative capacity and decreasing the recruitment of proinflammatory cells. This indicates that there is a crosstalk between the proinflammatory NF-κB and antiinflammatory Nrf2 signaling pathways (32, 44).

Activated LSEC also contribute to inflammation in the liver by inducing the expression of selectins, vascular cell-1 adhesion molecules (VCAM-1) and intercellular adhesion-1 molecules (ICAM-1). Also, these cells secrete cytokines, chemokines and other pro-inflammatory mediators that contribute to the maintaining of a chronic inflammatory process (13, 45). In support of this is the fact that LSEC dysfunction mitigates inflammation by decreasing Kupffer cells activation and NF-kB signaling pathway activation (13).

Besides Kupffer cells and LSEC, activation of HSC contribute to maintaining a pro-inflammatory state in chronic liver disease. These cells produce various chemokines that promote adhesion and migration of lymphocytes and other inflammatory cells. Additionally, activated HSC express the innate immune receptor TLR4, to interact with bacterial LPS and like, Kupffer cells, HSC phagocytose apoptotic bodies from hepatocytes (42, 46).

1.2.3. The role of hepatic stellate cells in liver fibrosis

In a healthy liver, HSC are cells in a resting state and serve as storage of vitamin A (36). They account for about 10% of liver cells and are found in the Disse space, where they can communicate directly with other types of liver cells. HSC, as resting myofibroblasts which are a major integral element of the ECM in both the healthy and diseased liver. Also, these cells play a pivotal role in hepatic fibrogenesis by producing collagen and other ECM components.

Activation of HSC is a critical process in hepatic fibrogenesis (10) (Fig.1x). HSC activation comprises two major phases, the initiation and the perpetuation (3, 4, 5, 10). TGF-β1, PDGF and ROS are key mediators in the initiation phase which activate HSC. Various autocrine and paracrine signaling increase HSC activation and expression of receptors for mitogenic and fibrogenic factors (46). TGF-β1, PDGF, ROS and various cytokines cause the transdifferentiation of HSC into myofibroblasts that produce ECM proteins, such as Coll1 and Coll3, other matrix proteins, cytokines, MMPs and TIMPs, the key enzymes that are responsible for ECM degradation and remodeling in liver fibrosis (3, 4, 5, 8). Persistent hyperactivation of HSC contributes progression of fibrosis to cirrhosis and eventually HCC. The second phase of HSC activation is perpetuation that leads to proliferation, chemotaxis, fibrogenesis, contractility, ECM remodeling, and proinflammatory cytokines release. Many mediators, including TGF-β1, PDGF, TNF-α, and vascular endothelial growth factor (VEGF) lead to HSC proliferation. Among these mediators, TGF-β1 and PDGF are the crucial factors for HSC activation, proliferation and ECM production. HSC migrate towards growth factors and chemokines and chemokine receptors contribute to hepatic fibrogenesis (36, 47, 48). Due to HSC activation hepatic sinusoidal remodeling, characterized by matrix deposition and capillarization of hepatic sinusoids, an increase in resistance in the sinusoids is caused. Increased intrahepatic sinusoidal resistance together with stimulation of HSC contraction

caused by various mediators (endothelin-1 /ET1/, angiotensin II, decrease NO and other) contribute to portal hypertension in liver fibrosis (36, 48). Besides, HSC by releasing various chemokines, contribute to liver immune cell infiltration, promoting recruitment of neutrophils, NK/NKT cells, T cells and other immune cells (36).

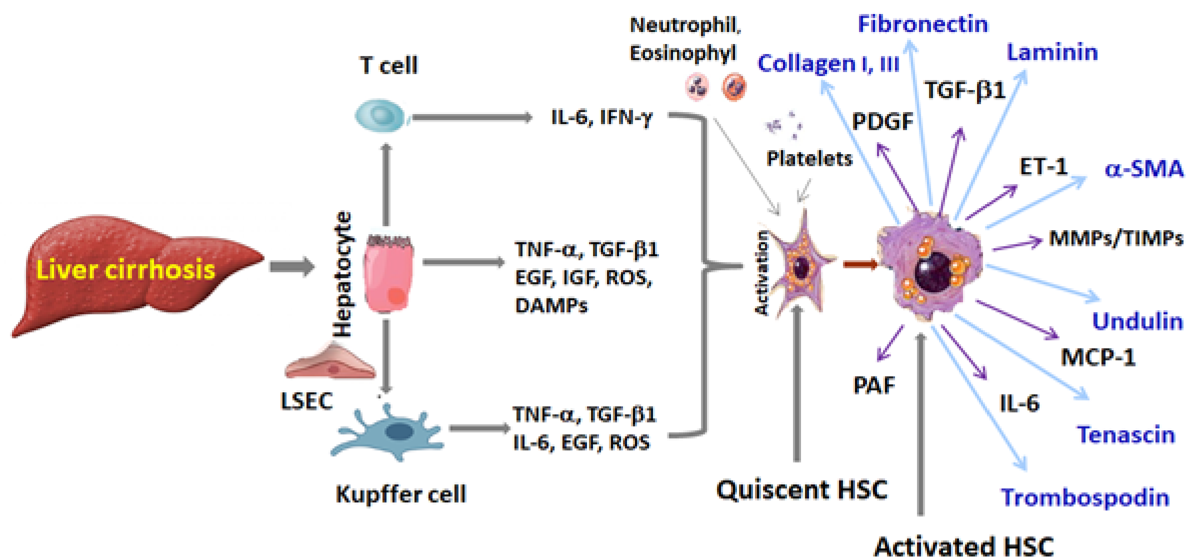


Fig. 1x. Mechanisms of hepatic stellate cells activation and perpetuation. During the CLD, damaged hepatocytes release ROS, DAMPs, proinflammatory cytokines and other mediators. Also, they activate other cells (Kupffer cell, LSEC, T-cells) that produce Il-6, TNF-α, TGF-β1, IFN-γ and ROS. Together with inflammatory cells and platelets lead to activation and proliferation of HSC. Activated HSC produce proinflammatory mediatory, fibrogenic factors, collagens and various ECM proteins that promote hepatic fibrogenesis.

1.2.3.1. TGF-β1 and PDGF in liver fibrosis

Among many fibrogenic mediators, TGF-β1 and PDGF are the two most important growth factors (49, 50, 51).

TGF-β proteins in three isoforms are one of the major profibrogenic cytokines which stimulate HSC activation which promote hepatic fibrogenesis (49, 34, 52). In addition to HSC activation, TGF-β1, a multifunctional cytokine, affects many biological processes associated with fibrogenesis, such as oxidative stress, metabolism, epigenetics, circadian rhythm and others (49). On the one hand, TGF-β1 alters redox homeostasis, increasing ROS production (through mitochondrial damage and NADPH oxidases induction) and suppresses the antioxidant system, leading to redox imbalance (49). ROS induce profibrogenic effects of TGF-β1 such as fibroblast

activation, apoptosis and synthesis of other fibrogenic cytokines through different mechanisms such as increasing the TGF-β1 expression and release, and MMPs activation. Namely, ROS act as a vicious cycle for fibrosis. Knowing this, antioxidant therapy can be effective in mitigating fibrosis (34). Etiological factors leading to CLD progression stimulate TGF-β1 production from monocytes and Kupffer cells. In addition to these cells, HSC, LSEC, platelets and hepatocytes may be important sources of TGF-β1 in liver fibrogenesis. Many proinflammatory cytokines regulate TGF-β1, which is involved in the activation and proliferation of HSC (53). TGF-β1, the main profibrogenic factor which induces HSC activation, activates their transdifferentiation into myofibroblast (MFB) and stimulates ECM synthesis (49, 38). ROS, PDGF and connective tissue growth factor (CTGF) also contribute to HSC activation and proliferation. TGF-β1 plays a very important role in modulating cell plasticity, not only in HCS cells transdifferentiation into collagen-producing MFB, but in the dedifferentiation of hepatocytes and liver tumor cells (52).

PDGF are peptide growth factors with varying combinations of four polypeptide chains (A, B, C, and D). Kupffer cells, platelets, vascular endothelial cells, and pericytes of PDGF. PDGF-BB produced by Kupffer cells plays a key role in HSC activation, proliferation, chemotaxis, migration, portal fibroblast proliferation and ECM production in liver fibrosis (51, 10). Activated HSC migrate to tissue repair sites and secrete large amounts of ECM and regulate ECM degradation (51). It has been shown that in experimental models of liver fibrosis increased production of PDGF or TGF-β1 and that antagonists of TGF-β1 or PDGF significantly mitigate hepatic fibrogenesis (54, 55). A recent study has shown the existence of an interaction between PDGF and TGF-β1 signaling. PDGF increases TGF-β1 expression and HSC cell proliferation and ECM matrix production (56), while TGF-β1 increases the expression of the PDGFβ receptor (57, 58, 42).

1.2.3.2. Extracellular matrix (ECM) in liver fibrosis

Liver ECM is a complex network of different macromolecules that acts as structural support and modulates liver tissue homeostasis and remodeling (39). Normal liver ECM is composed of collagens (Col1, 2, 3, 4, 5, 6, 14, 18), elastin, structural glycoproteins, proteoglycans glycosaminoglycan and matricellular proteins. Different liver cell types such as HSC, hepatocytes, Kupffer cells, LSEC produce ECM components, MMPs and TIMPs and contribute to ECM remodeling and liver regeneration. Numerous growth factors, cytokines, and chemokines are bound to ECM and contribute to their composition and remodeling (39, 36, 59).

Disruption in the balance between ECM injury and repair, due to the effect of different pathological factors, can promote hepatic fibrogenesis with an accumulation of ECM components, particularly of Coll1 and Coll3, fibronectin, and laminin, forming a dense matrix and liver stiffening

(42). In contrast to earlier findings in which ECM plays a passive role in liver fibrosis, recent research indicates an active, immunomodulatory role of ECM. In addition to the immunomodulatory role of many proinflammatory and profibrogenic cytokines, chemokines and growth factors, ECM constituents can directly modulate immune responses interacting with a ligand on immune cells and promoting chronic inflammation and fibrogenesis (39, 59).

1.2.3.3. MMPs and TIMPs in liver fibrosis

MMPs are calcium-dependent endoproteinases that are secreted as pro-enzymes from different cells such as HSC, Kupffer cells, hepatocytes, endothelial cells, fibroblasts and inflammatory cells (60, 61). It has been discovered that there are 24 different MMPs in humans. Based on the specificity of the substrate for MMPs, they can be classified into six groups: 1. collagenases 2. stromelysins 3. gelatinases (MMP-2, -9); 4. matrilysins; 5. membrane-type MMPs; and 6. others (60). The crucial role of MMPs are the regulation of ECM composition and remodeling. Moreover, these endoproteinases regulate cell behavior, proliferation, differentiation, migration, adhesion and apoptosis, as well as inflammation, fibrosis and tissue remodeling in pathological processes (60).

Maintaining the interaction between MMPs and TIMPs is the key process in the turnover of liver ECM and a normal liver structure (62). It has been found that dysregulation of MMPs expression contributes to the progression of liver fibrosis. Besides, degradation and deposition of ECM, MMPs/TIMPs contribute to liver carcinogenesis and regeneration (60).

Two key MMPs secreted from HSC are MMP-2 and MMP-9 (60, 61, 62). MMP-2 expression is increased in liver fibrosis (63), alcoholic liver cirrhosis (64), chronic viral hepatitis (65) and HCC. MMP2 is a serum marker for alcoholic liver disease (64) and the progression of liver fibrosis. Besides, MMP-2 acts as an autocrine proliferative and migratory factor and accelerates the activation of HSCs (66). MMP-9, or gelatinase-B, is secreted by numerous cell types, including leukocytes, macrophages, Kupffer cells, and fibroblasts. Its role in ECM remodeling is to degrade ECM components, such as Coll4, elastin and fibronectin (62, 60). Numerous studies related to MMP-9 show that there is a dual role of MMP-9 in liver fibrosis (60, 62, 64, 67). MMP-9 expression has been detected in the early stages of hepatic fibrogenesis (67, 68). On the other hand, MMP-9 can cause HSC apoptosis (68, 62) and fibrosis resolution (69).

1.3. Macrophage infibitory factor (MIF)

1.3.1. MIF and MIF receptors

The initial role of MIF was discovered in 1966. was to inhibit macrophage migration associated with macrophage phagocytosis (70). However, by cloning the gene for MIF in 1989. (71)

and studying it, a wide range of MIF roles has been demonstrated. MIF, as a key proinflammatory cytokine, exhibits both cytokine and chemotactic properties and regulates the innate immune response (72, 73, 74, 75, 76, 77). In inflammation processes and stress stimulation, MIF releases from various immune cell types, endothelial cells, tissue macrophages, some parenchymal and endocrine cells (74, 78).

MIF stimulates the production of numerous inflammatory cytokines, such as interleukins, TNF-α, IFN-γ, prostaglandin (PG) E2 and MMPs (79, 80, 81). In addition to these effects, MIF affects the directed migration of leukocytes and macrophages into the inflammatory sites. Also, MIF counter-regulates the inhibitory effects of glucocorticoids on proinflammatory cytokine and increases IFN-γ production, controlling immunity and inflammation (82, 83). Furthermore, the endocrine role of MIF explained their release from the adrenocorticotropin cells of the pituitary together with ACTH in response to stress or inflammatory stimuli (86, 87, 88).

The variety of biological functions of MIF can be explained by the interactions of MIF with different receptors. Receptors for MIF are CD74, CXCR2 and CXCR4. CD74, a MIF receptor discovered in 2003 (89), is a type II transmembrane protein expressed on the surface of various cell types. MIF interacts with the CD74 ligand-binding protein to activate extracellular signal pathways that regulate cell proliferation, inflammation, apoptosis, and differentiation (90).

MIF also binds to the chemokine receptors CXCR2 and CXCR4 promoting cell adhesion, chemotaxis, migration and proliferation (91). These receptors are crucial in acute inflammation stimulated by leukocytes and monocytes/neutrophils and T cells recruitment and chemotaxis (92, 93). Besides, MIF stimulates the production of proinflammatory cytokines, nitric oxide and prostaglandin E2, promoting a pro-inflammatory tumor microenvironment (94, 95). Furthermore, MIF plays a key role in angiogenesis and neovascularization causing the overexpression of VEGF, IL-8 and MMPs (96, 97, 98).

MIF consists of two catalytic activities, tautomerase (MIF-2) and a thiol-protein oxidoreductase (TPOR). The possible effects of tautomerase are the regulation of inflammation and redox homeostasis, although its function is not clear enough. The TPOR activity of MIF is dependent on a sequence of Cys-Ala-Leu-Cys, similar to that found in thioredoxin (99, 100, 101). A recent study reported that MIF occurs in reduced MIF (redMIF) that is detected in healthy subjects, and oxidized MIF (oxMIF) was detected in patients with inflammatory diseases (98).

1.3.2. MIF in disease

MIF is a key cytokine in chronic inflammatory diseases, sepsis and infectious diseases (78, 102). It has been indicated that IFN-γ action, as a key cytokine to both innate and adaptive immunity, increased CD74 receptor expression in various tissue injuries (103, 104). Its expression

is upregulated with the progression of atherosclerosis (105). Besides, MIF was included in numerous autoimmune diseases (17, 106), asthma (108) and kidney diseases (109). High expression of MIF indicated in type 2 diabetes mellitus in both mice (110, 111) and humans (112). Also, the proinflammatory effects of MIF are proven in high fat diet-induced obese mice (113). On the other hand, *Gligorovska et al*. have shown an increase in obesity and insulin resistance in fructose-diet $MIF^{-/-}$ mice (114).

MIF is associated with a variety of human tumors, such as breast, colon, lung, and liver malignancy (115, 116, 117, 118). In contrast, MIF regulates myocardial ischemia/reperfusion injury triggering the signaling pathway and protects cardiomyocytes during ischemia-reperfusion injury (119, 120, 121). Similarly, MIF acts as a protective factor in a model of chronic injury-mediated renal fibrosis (122, 123).

1.3.3. MIF in liver disease

Chronic liver disease is characterized by MIF overexpression in endothelial cells, Kupffer cells, HSC and hepatocytes, as well as from various immune cells (74, 124). In patients with chronic virus hepatitis, ALD, NAFLD, liver cirrhosis and HCC, high serum levels of MIF were observed (118, 125, 126, 127).

In the experimental model of NASH-induced fibrosis progression, MIF exerted the pro-fibrotic effect due to a direct effect on NKT cell polarization towards type I NKTI which activates HSC on ECM production (123). Also, type I NKT cells cause hepatocyte damage which releases numerous proinflammatory and profibrotic cytokines resulting in the progression of hepatic fibrosis in NASH (123). Also, MIF overexpression was detected in fibrotic hepatic tissue of thioacetamide (TAA)-treated rats (128). A recent study shows that in contrast to the above-mentioned data, MIF might also exert antifibrotic effects in experimental hepatotoxin-induced liver fibrosis, decreasing myofibroblast activity of HSC, without altered immune cell infiltration (74). Namely, it has been shown that MIF exhibits hepatoprotective effects in experimental models of carbon tetrachloride (CCl_4) and TAA-induced liver fibrosis (74, 123). Similarly, in NAFLD mice-induced high-fat diets, MIF exerts an antiinflammatory effect also through the CD74/AMPK signaling pathway (adenosine monophosphate dependent kinase), which inhibits TNF-α and IL-6 synthesis, and macrophage change from M1 to M2 phenotype (40). M2 macrophages produce much more antiinflammatory cytokines than M1, which promote tissue regeneration (129).

MIF exerts pro- and anti-inflammatory effects depending on the etiology and stage of the liver disease (73, 130). Furthermore, whether MIF as an antioxidant protein or stimulates pro-inflammatory cytokine production depends on the concentration of MIF in the tissue (73).

1.4. Animal models of liver fibrosis

Due to the high prevalence of chronic liver disease worldwide, animal models are crucial to further elucidating the molecular mechanisms of hepatic fibrogenesis, discovering therapeutic targets, and developing novel diagnostic and therapeutic modalities investigating new drugs (131, 132, 133). When using animal models to study liver fibrosis, the number, species or strain of animals must be considered, and also the reproducibility and feasibility of the experiment. Using genetically modified animals in experimental models is very important for the examination of specific genes. The most commonly used species of experimental animals for studying fibrogenesis are rodents (131, 132, 133). Different strains of mice are the most suitable because of their remarkable genetic similarity to humans. There are several animal models of liver fibrosis: *chemical-, diet-, surgical- and genetic-induced liver fibrosis* (131, 132, 133, 134).

Chemical-induced liver fibrosis

The most widely used hepatotoxic drug-induced fibrosis models are CCl4-induced fibrosis, TAA-induced fibrosis, and dimethyl or diethylnitrosamine-induced fibrosis.

CCl4-induced liver fibrosis. CCl4 is the most common hepatotoxin used for experimental induction of chronic liver injury in rodents. CCl4 is metabolized in the liver by cytochrome P450 generating highly reactive trichloromethyl radicals, causing oxidative stress, inflammation, severe centrilobular hepatocellular necrosis and bridging fibrosis (131, 135).

TAA-induced fibrosis. Besides CCl4, TAA-induced hepatotoxicity is used as an animal model of acute liver injury, and liver fibrosis, cirrhosis and HCC (132, 136, 137, 138, 139). Acute exposure to high-dose TAA is an excellent experimental model of acute liver failure and hepatic encephalopathy, while chronic administration to low-dose TAA (intraperitoneally or orally) is a reliable experimental model for fibrosis and cirrhosis in rats and mice. Optimal protocols recommended for experimental TAA-induced liver fibrosis are protocol in Sprague-Dawley rats, administration of TAA twice weekly at a dose of 250 mg/kg for 12 weeks, and protocol in mice, administration of TAA at a dose of 200 mg/kg twice weekly for 8–10 weeks (140). Also, chronic exposure to TAA can be achieved by a non-invasive method by adding TAA to drinking water at a concentration of 300 mg/ml, for 12 weeks (131, 134).

TAA is bioactivated in the liver, leading to the creation of its S-oxide and reactive SS-dioxide, which is responsible for TAA hepatotoxicity (141). These reactive metabolites of TAA covalently bind to macromolecules in the liver and cause hepatocellular necrosis of periportal hepatocytes (132, 145). Additionally, cytochrome P450 2E1 (CYP2E1) and flavin-adenine-dinucleotide (FAD)-containing monooxygenases induce ROS generation, altered redox homeostasis which promotes hepatocellular injury, oxidative stress, inflammation, and lipotoxicity (142,

143). The effects of chronic TAA exposure on liver damage depend on the route of administration, dosage, length of exposure and animal species. Bridging fibrosis develops after prolonged TAA administration and lasts for several weeks after TAA withdrawal. Moreover, long-term administration of TAA induced the development of cholangiocarcinoma and HCC. Unlike CCl4, TAA displays a longer latency between the administration of the toxin and the development of liver injury. TAA also leads to more periportal inflammatory infiltrates, more pronounced ductal proliferation and hepatocyte vacuolization (134).

Dimethyl or diethyl nitrosamine-induced liver fibrosis. Dimethyl or diethylnitrosamine is metabolized by CYP2E1 in the liver, generating high bioactive metabolites. These metabolites cause DNA mutations that lead to hepatocellular necrosis and malignancy (133). This model is very suitable for studying the cellular and molecular mechanism progression of liver fibrosis to HCC (144, 145).

It is known that chronic alcohol consumption leads to ALD. Since alcohol alone is not sufficient to induce the progression of steatosis to fibrosis in mouse models, it is combined with other toxins, such as CCl4 or a specific diet (145, 146).

Diet-induced liver fibrosis

Many specific diets, such as a high-fat diet, methionine-choline-deficient diet (MCD), and amino acid-deficient diet, can induce liver fibrosis. MCD in mice is the most commonly used diet model for the development of NASH (131, 145).

The most common *surgical-induced liver fibrosis model* is the common bile duct which causes cholestatic injury and periportal biliary fibrosis in both rats and mice (131, 145).

Genetically modified models are very important for the examination of specific genes and signaling pathways in liver fibrosis and the identification of potential new target therapy (145).

1.5. Betaine

Betaine (trimethyl glycine), an oxidative metabolite of choline, is found in plants, animals and microorganisms and dietary sources including bran, vegetables, and seafood (147, 148, 149). In mammalian metabolism, betaine is a vital methyl donor in the methionine-homocysteine cycle (15, 151) which occurs in the mitochondria of liver and kidney cells. Betaine-homocysteine methyltransferase is an enzyme that catalyzes the reaction between betaine and homocysteine (152).

Betaine exerts antioxidant effects by regulating the metabolism of SAA, such as homocysteine, methionine, S-adenosine methionine (SAM) and others (153). These sulfur containing-amino acids (SAA) are involved in the synthesis of GSH and polyamine. Moreover, betaine acts as an osmoprotectant that protects cells against stress inducers (150, 154). Betaine has been shown to inhibit cytokines and ROS production in Kupffer cells. Also, betaine exerts

antiinflammatory effects through its inhibition of the NF-kB signaling pathway and inflammasome activation (155, 156), alleviates ER stress (157) and inhibition of apoptosis (158).

The hepatoprotective effects of betaine have been reported in different experimental models (148, 159, 160, 161). Thus, betaine alleviates redox homeostasis, inflammation in ALD (159, 161) and NAFLD (160, 161), as well as fibrotic and necrotic lesions in the liver (148). Triglyceride (TG) accumulation in the liver (steatosis) may be triggers for progression to NASH and cirrhosis. It has been shown that betaine attenuates the dysregulated lipid metabolism through the regulation of adipokines (adiponectin, leptin and resistin) and reduces TG accumulation. The protective effect of betaine in alcoholic-induced liver injury may be associated with the inhibition of oxidative stress by reducing SAA breakdown disease (162). An ethanol-induced liver disease characterized by suppressing methionine synthase (MS) activity and inducing hyperhomocysteinemia with ER stress (163). Betaine, as a methyl donor, converts homocysteine to methionine and SAM, which act as antioxidants. However, the precise antioxidative, antiinflammatory and antifibrotic effects of betaine on MIF in TAA-induced liver fibrosis development and related mechanisms are not yet clear enough.

2. OBJECTIVES

The objectives in the dissertation were:

- to examine the effect of MIF on liver functional changes in investigated mice;
- to examine the effects of MIF on oxidative stress, inflammation and fibrogenesis in the liver of investigated mice;
- to examine the effects of MIF on liver morphological changes and matrix metalloproteinases 2 and 9, (MMP-2 and -9), dimer MMP-9 and tissue inhibitor matrix metaloproteinase -1 (TIMP-1) in investigated mice;
- to study the modulatory effects of betaine on MIF-mediated oxidative stress, inflammation and fibrogenesis in the liver of investigated mice;
- to evaluate the epidemiological relevance of chronic liver disease

3. MATERIAL AND METHODS

3.1. Experimental design

3.1.1. Setting an animal model of liver fibrosis

The experiment was performed on wild-type C57BL/6 male mice and Macrophage Migration Inhibitory Factor knockout C57BL/6 mice ($MIF^{-/-}$) 8 weeks old, weighing 21-25 g, that were raised at the Military Medical Academy, Belgrade. Animals were kept under standard laboratory conditions (temperature 22 ± 2°C, relative humidity 50 ± 10%, 12/12 light-dark cycle with lights turned on at 9.00 am) with free access to tap water and a standard chow diet during the experiment. All experimental procedures were in full compliance with the Directive of the European Parliament and the Council (2010/63EU) and approved by the Ethical Committee of the University of Belgrade (Permission No 6600/2).

Before the experiment, all animals (n=80) were fed with a control diet. At the age of 8 weeks, they were divided into the following groups: 1. C group, control, had free access to tap water and a standard chow diet (n=10); 2. Bet group fed with standard chow diet and supplemented with betaine (n=10); 3. $MIF^{-/-}$ group consisted of $MIF^{-/-}$ mice continuously fed with standard chow diet (n=10); 4. $MIF^{-/-}$+Bet group consisted of $MIF^{-/-}$ mice fed with standard chow diet and supplemented with betaine (n=10). 5. TAA group fed with standard chow diet and treated with thioacetamide (TAA) (n=10); 6. TAA+Bet group consisted of wild type mice treated with TAA and supplemented with betaine (n=10)); 7. $MIF^{-/-}$+TAA group consisted of $MIF^{-/-}$ mice continuously fed with standard chow diet and treated with TAA (n=10); 8**.** $MIF^{-/-}$+TAA+Bet group consisted of $MIF^{-/-}$ mice continuously fed with standard chow diet, treated with TAA and supplemented with betaine (MIF; n=10). Chronic liver inflammation was induced by TAA (200 mg/kg) dissolved in 200μL PBS, intraperitoneally three times a week during an 8 week period. The working solution was stored at 4°C. The fresh solution was prepared every week. Simultaneously, C, Bet, $MIF^{-/-}$ and $MIF^{-/-}$+Bet group received vehicle (saline, 0.9% NaCl) in the same manner. Betaine (MP Biomedicals) was dissolved in drinking water (2% wt/v) and animals had free access to drink *ad libitum*. On the day before, sacrifice, mice were fasted overnight. After the treatment, animals were sacrificed by exsanguination in ketamine (100 mg/kg intraperitoneally /i.p./) anesthesia.

3.1.2. Blood and tissue samples

Blood samples were taken from the right side of the heart by cardiac puncture under ketamine anesthesia (100 mg/kg intraperitoneally /i.p./). Blood was collected in dry tubes and serum was obtained by centrifugation at 1500g for 10 min. Serum samples were stored at -80°C until required for analysis. Liver samples for biochemical analysis were homogenized on ice, in cold buffered 0.25 M sucrose medium (Serva, Feinbiochemica, Heidelberg, New York), 10 mM phosphate buffer (pH 7.0) and 1mM ethylenediaminetetraacetic acid (EDTA, Sigma Chem. co. St.

Louis, USA). The homogenates were centrifuged at 2000 x g for 15 minutes at 4°C. Crude sediments were dissolved in a sucrose medium and centrifuged. The supernatants were transferred into the tubes and centrifuged at 3200 x g for 30 minutes at 4°C. Obtained sediments were dissolved in deionized water. After one hour of incubation, the samples were centrifuged at 3000 x g for 15 minutes at 4°C, and supernatants were stored at -70°C (164). Liver samples were taken for the determination of oxidative/antioxidative parameters and pathohistology analysis.

3.2. Biochemical analysis

3.2.1. Determinations of serum biochemical parameters

Biochemical evaluation of liver injury was performed by determination of the activity of serum alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP). Activities of these enzymes were measured spectrophotometrically at photometer BTS-330 according to the manufacturer's instruction using special kits containing 2-oxoglutarate (Sigma Aldrich, St. Louis, MO) for ALT and AST and a kit containing 4-nitrophenyl phosphate (Sigma Aldrich) for ALP activity. γ-Glutamyl transferase (γ-GT) measurements were conducted using a commercially available kit (Olympus Diagnostika, Hamburg). Total bilirubin level was determined by a photometric method (Hitachi 911, DIASys Diagnostic Systems GmbH&Co.KG, Holzheim, Germany). Albumin concentration was determined using commercial kits from Bio-diagnostic, Egypt. The concentration of C-reactive protein (CRP) was determined turbidimetrically, based on agglutination of latex particles coated with antibodies on CRP (BioSystems, Barcelona, Spain).

3.2.2. Determination of hepatic oxidative/antioxidative parameters

Lipid peroxidation, measured as malondialdehyde (MDA) level, was determined spectrophotometrically in a reaction with thiobarbituric acid as described by Girotti et al. (165). The results are expressed as nmol of MDA per milligram of proteins (nmol/mg protein).

Spectrophotometric determination of advanced oxidation protein products (AOPP) level was performed by Witko's method (166). Samples were prepared in the following way: Two hundred microliters of serum were diluted 1/5 in PBS, then 10 l of 1.16 M potassium iodide and 20 l of acetic acid were added to each tube. The absorbance of the reaction mixture was read immediately at 340 nm, against a blank, containing 1000 mL of PBS, 10 mL of potassium iodide and 20 mL of acetic acid. Chloramine T solution (0-100 μmol/L) was used as a calibrator. The chloramine T absorbance at 340 nm is linear within a range of 0-100 μmol/L, and AOPP concentrations were expressed asμmol/L of chloramine T equivalents (167).

The activity of total superoxide dismutase (EC1.15.1.1.; SOD) in the liver was measured spectrophotometrically, as inhibition of epinephrine autooxidation at 480 nm. After the addition of

10mM epinephrine (Sigma, St. Louis, USA), analysis was performed in the sodium carbonate buffer (50mM, pH 10.2; Serva, Feinbiochemica, Heidelberg, New York) containing 0.1 mM EDTA (Sigma, St. Louis, USA) (168).

Catalase (CAT) activity in the liver was determined spectrophotometrically based on the decrease in absorbance of hydrogen peroxide at 240 nm, and it is expressed in units of activity per milligram protein present in a given sample (169). One unit of the enzyme activity was defined as 1.0 mmol hydrogen peroxide reduced per minute at pH 7.0 at 25°C.

A spectrophotometric assay based on 2,2-dithiobisnitrobenzoic acid (DTNB or Elman's reagent) is used for total thiol assay (170). An aliquot of serum is mixed with Tris-EDTA buffer, then DTNB is added. After 15-minute incubation at room temperature, the absorbance is measured at 405 nm. A reagent blank without sample and a sample blank with methanol instead of DTNB were prepared similarly. Glutathione (GSH;50-100 μmol/L) solution is used as a calibrator. Total thiol levels were expressed as μmol/L.

Total antioxidant status (TAS) levels were measured using commercially available kits (Rel Assay). The method is based on the bleaching of the characteristic color of a more stable ABTS (2,2'-azino- bis [3-ethylbenzothiazoline-6-sulfonic acid]) radical cation by antioxidants (171). The results were expressed as mmol Trolox (Rel Assay) equivalent/L.

Total oxidant status (TOS) levels were measured using commercially available kits (Rel Assay). In the new method, oxidants present in the sample oxidized the ferrous ion-o-dianisidine complex to ferric ion (172). The ferric ion produced a colored complex with xylenol orange in an acidic medium. The color intensity, which could be measured spectrophotometrically, was related to the total amount of oxidant molecules present in the sample. The assay was calibrated with hydrogen peroxide and the results were expressed in terms of micromolar hydrogen peroxide equivalent per liter (μmol H_2O_2 equivalent/L).

3.3. ELISA method

3.3.1. Determination of the liver cytokines (IL-6 and IFN-γ)

For the determination of cytokines in liver tissue, samples were homogenized in 10 volumes of PBS. After centrifugation (10 min at 12 000g, 4 °C), the supernatants were carefully collected through the fat cake and diluted to 1/40 000 in PBS (Osei-Hyiaman 2008). To the determine concentration of cytokines (IL-6 and IFN-γ, we applied ELISA kits from BD Bioscience (San Diego, California, USA), according to the manufacturer's instructions.

3.3.2. Determination of the liver fibrogenic factors (TGF-β1 and PDGF-BB)

For the determination of TGF-β1 and PDGF-BB in liver tissue, samples were homogenized in 10 volumes of PBS. After centrifugation (10 min at 12 000g, 4 °C), the supernatants were carefully collected through the fat cake and diluted to 1/40 000 in PBS. To determine liver tissue concentration of TGF-β1 and PDGF-BB we applied ELISA kits from ELABSCIENCE (Houston, Texas, USA), according to the manufacturer's instructions.

3.4. Determination of liver MMP-2, MMP-9, dimer MMP-9 and TIMP-1 by zymography

Activities of gelatinase A (proMMP-2 and MMP-2) and gelatinase B (proMMP-9 and MMP-9) and TIMP-1 were determined with SDS-PAGE reverse electrophoretic zymography and zymography (173, 174). For each sample, equal total tissue homogenate protein concentration was loaded, after protein concentration determination. The tissue homogenates were diluted with 20 % sucrose solution (10 μL homogenate and 1190 μL sucrose solution, v/v) and incubated in a thermostatically controlled water bath at 37 0C for 45 min. After incubation, homogenates were mixed with 2 x zymography sample buffer [0.125 M Tris–HCl, pH 6.8, 20 % (v/v) glycerol, 4 % (w/v) SDS, and 0.005 % bromophenol blue], and then loaded into SDSPAGE that was performed on 8 % acrylamide gels containing 0.1 % (w/v) gelatin (Sigma–Aldrich, St. Louis, MO, USA). The negative control consisted of tissue homogenates incubated with 5 mmol/l EDTA. Human recombinant gelatinases A and B (*R&D Systems, Minneapolis, MN*) were employed as standard. The negative control contained a mixture of standard human recombinant gelatinases A and B, and 5 mmol/l EDTA. After electrophoresis, the gel was washed twice for 30 min in zymography renaturing buffer (2.5 % Triton X-100) with a gentle shake to remove SDS, then incubated for 24 h at 37 0C in reaction buffer (50 mM Tris–HCl, pH 7.4, 200 mM NaCl, and 5 mM CaCl2). The gels were then stained for 2 hours with Coomossie brilliant blue before being destain with destain solution (50% methanol, 10% acetic acid, and 40% ddH2O). Finally, gelatinolytic activities on the gels were presented as transparent bands on the blue background. Gelatinolytic activities were identified as clear zones and calculated the densitometric value of the lyses against a dark blue background on zymography gels using ImageJ 1.42q software package (*National Institutes of Health, Bethesda, MD, USA*), which quantified both the surface and the intensity of the lysis bands after scanning of the gels. Relative activities of gelatinases A and B were expressed as percentages of total activity, which was taken as 100 %. The relative activities of the gelatinases were compared using Student's t-test.

3.5. Histology analysis

Liver tissue was sectioned and incubated in 10% formalin solution at room temperature. After fixation, the liver samples were processed by the standard method. Tissues were incorporated in paraffin, sectioned at 5 μm and then stained with Hematoxylin-Eosin (HE), Masson's trichrome and reticulin, according to the manufacturer's instructions. The sections were analyzed and photographed using an Olympus BX51 (Olympus, Tokyo, Japan) light microscope equipped with an Artcore 500 MI (Artray, Co. Ltd., Tokyo, Japan) camera.

Fibrosis quantification was performed by analyzing liver tissue samples stained with Masson's trichrome and Reticulin with a digital image camera. The surface of the blue-stained area at the a microscope magnification x200 was calculated in 10 random fields on each section of each animal, and presented as a percentage of the total liver cross-sectional area using the ImageJ software.

3.6. Statistical analysis

All results are expressed as means ± SD. As the normal distribution of parameters was confirmed by Kolmogorov-Smirnov test, one-way analysis of variance (ANOVA) with Tukey`s *post hoc* test was used for testing the difference among groups. The difference was considered statistically significant if $p<0.05$. Computer software SPSS 15.0 was used for the statistical analysis.

4. RESULTS

4.1. Effects of MIF and betaine on body and liver weight and liver/body ratio

Thioacetamide induced a significant increase in liver/body ratio in TAA group in comparison with C group ($p<0.01$). However, the liver/body weight ratio was significantly higher in $MIF^{-/-}$+TAA and TAA+Bet group compared to TAA group ($p<0.01$, respectively). The liver/body weight ratio was decreased in $MIF^{-/-}$+TAA+Bet group compared to $MIF^{-/-}$+TAA and TAA+Bet group ($p<0.05$, respectively) (Table 1).

Table 1. Body and liver weight and liver/body ratio of mice with TAA-induced liver fibrosis

Group	Body weight (g)	Liver weight (g)	Liver/body ratio (%)
C	28.17±2.55	1.32±0.24	4.68±0.75
Bet	25.50±1.52	1.28±0.17	5.04±0.72
MIF-/-	27.90±2.88	1.39±0.17	4.99±0.52
MIF-/-+Bet	26.60±2.46	1.44±0.20	5.43±0.95
TAA	24.90±1.91	1.49±0.06	6.02±0.42$^{\#\#}$
TAA+Bet	25.00±1.31	1.70±0.09	6.81±0.40$^{\#\#}$
MIF-/-+TAA	24.56±1.42	1.82±0.56	7.45±2.44**
MIF-/-+TAA+Bet	24.89±1.54	1.54±0.16	6.19±0.69††

Significance of the difference was estimated by using one-way analysis of variance (ANOVA) with Tukey´s post hoc test; (**$p<0.01$ vs. C; $^{\#\#}p<0.01$ vs. TAA; $^{\dagger\dagger}p<0.01$ vs. $MIF^{-/-}$+TAA and TAA+Bet).

Abbreviations: C, control group; $MIF^{-/-}$ group, mice knockout for macrophage migration inhibitory factor (MIF); Bet, betaine group; $MIF^{-/-}$+Bet group, knockout MIF mice who have received betaine; TAA group, animals who have received thioacetamide (TAA); $MIF^{-/-}$+TAA group, knockout MIF mice who have received thioacetamide; TAA+Bet group, animals who have received thioacetamide and betaine; $MIF^{-/-}$+TAA+Bet group, knockout MIF mice who have received thioacetamide and betaine.

4.2. Effects of MIF and betaine on serum ALT, AST, ALP, γGT activities, and concentrations of albumin and bilirubin in TAA-induced liver fibrosis

Our study has shown that serum ALT activity was significantly increased in TAA group (157.00 ± 13.16 U/L) in comparison with control values (43.00 ± 5.35 U/L) ($p<0.01$). On the other hand, Serum ALT activity was significantly decreased in TAA+Bet and $MIF^{-/-}$+TAA group (118.23±12.21; 125.12±4.78 U/L; $p<0.01$, respectively) compared to TAA group. A similar decrease in ALT activity was observed in $MIF^{-/-}$+TAA+Bet group (101.16 ±18.48 U/L) when compared to $MIF^{-/-}$+TAA (125.12 ± 4.78 U/L) and TAA+Bet group ($p<0.01$, respectively) (Fig. 1A).

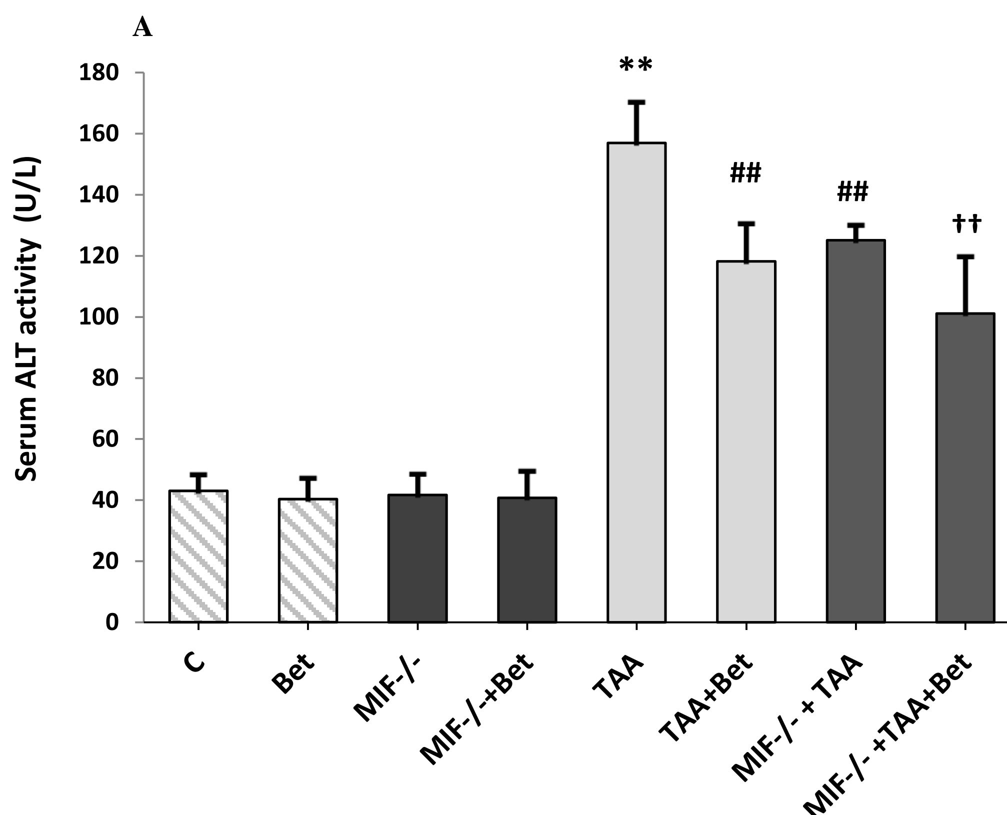


Fig. 1A. Effects of MIF and betaine on serum ALT activity in TAA-induced liver fibrosis. Liver fibrosis was induced by TAA (200 mg/kg dissolved in 200µL PBS) i.p. three times a week during on 8 week period. Betaine was dissolved in drinking water (2% wt/v) and animals had free access to drink *ad libitum.*

Significance of the difference was estimated by using one-way analysis of variance (ANOVA) with Tukey´s post hoc test; (**$p<0.01$ vs. C; $^{\#\#}p<0.01$ vs. TAA; $^{\dagger\dagger}p<0.01$ vs. $MIF^{-/-}$+TAA and TAA+Bet).

For abbreviations see Tab. 1. ALT, AST, alanine-, aspartate aminotransferase;

Serum AST activity was significantly increased in TAA group (381.1 ± 23.81 U/L) in comparison with control values (72.1 ± 7.68 U/L) ($p<0.01$). These results show a significant decrease in AST activity in TAA+Bet group and $MIF^{-/-}$+TAA (295.12 ± 26.95; 326.75±47.13 U/L, $p<0.01$, respectively) compared to TAA group. A similar decrease in AST activity was observed in $MIF^{-/-}$+TAA+Bet group (250.50±28.13 U/L) when compared to $MIF^{-/-}$ +TAA (326.75 ± 47.13 U/L) and TAA+Bet group ($p<0.01$, respectively) (Fig. 1B).

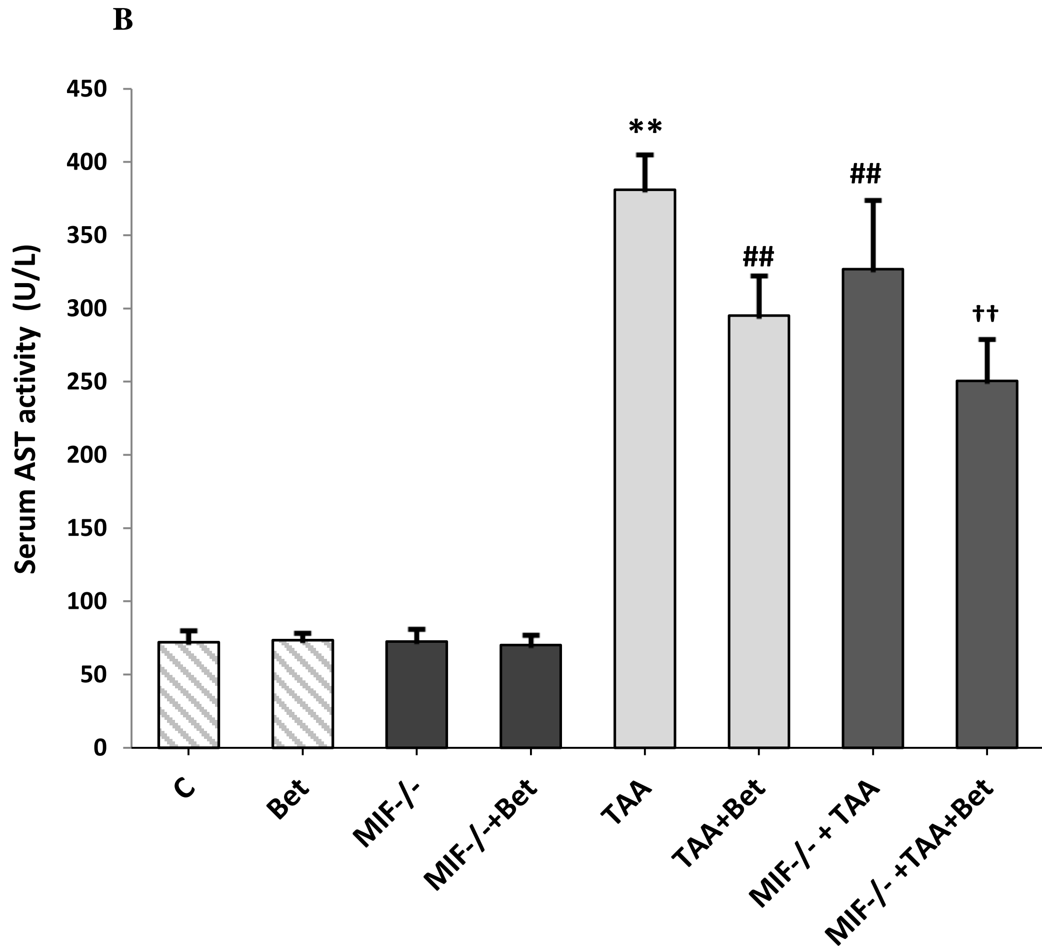


Fig. 1B. Effects of MIF and betaine on serum AST activity in TAA-induced liver fibrosis after an 8-week experimental period

Significance of the difference was estimated by using one-way analysis of variance (ANOVA) with Tukey´s post hoc test; (**$p<0.01$ vs. C; $^{\#\#}p<0.01$ vs. TAA; $^{\dagger\dagger}p<0.01$ vs. $MIF^{-/-}$+TAA and TAA+Bet).

For abbreviations see Tab. 1. ALT, alanine aminotransferase; AST, aspartat aminotransferase.

Thioacetamide treatment significantly increased serum ALP activity in TAA group (109.63 ± 14.2 U/L) in comparison with control (59.5 ± 8.38 U/L) ($p<0.01$). There was no statistically significant difference in serum ALP activity between other tested groups ($p>0.05$) (Fig. 1C).

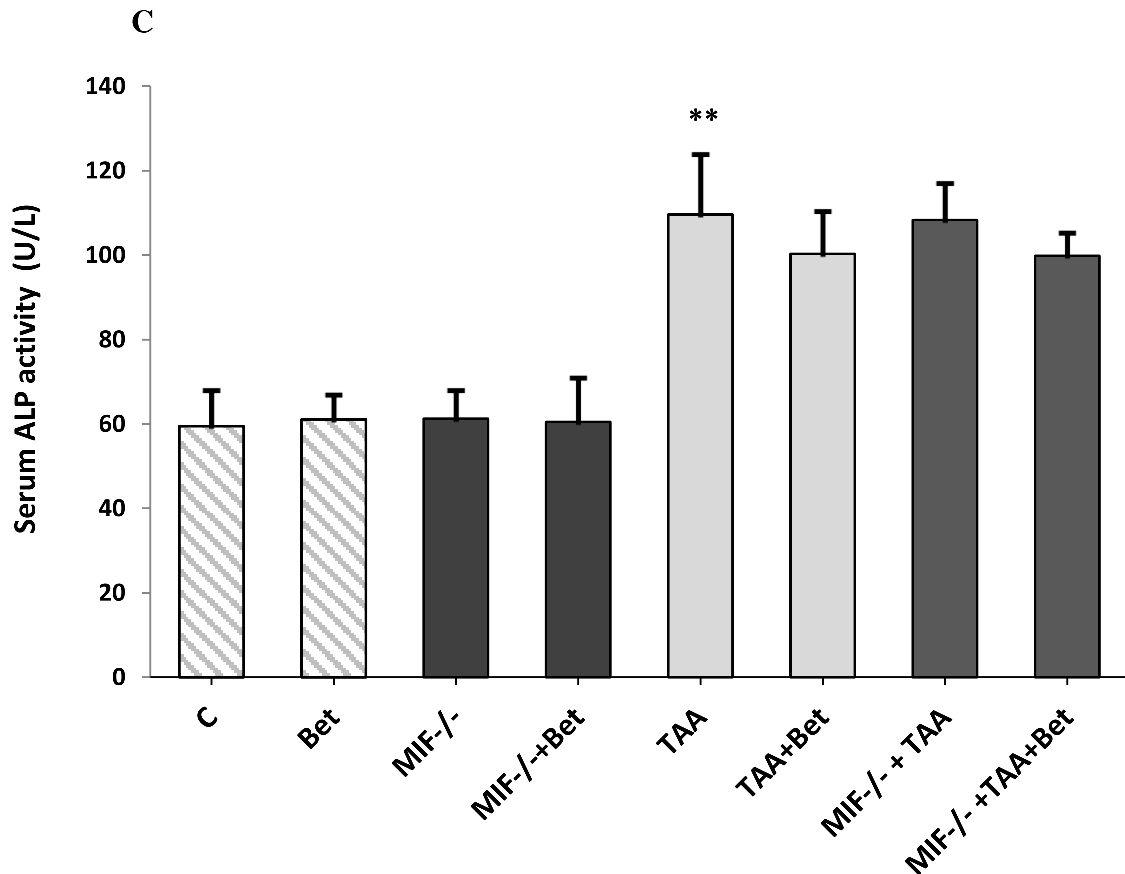


Fig. 1C. Effects of MIF and betaine on serum ALP activity in TAA-induced liver fibrosis after an 8-week experimental period

Significance of the difference was estimated by using one-way analysis of variance (ANOVA) with Tukey´s post hoc test; (**$p<0.01$ vs. C)

For abbreviations see Table. 1. ALP, alkaline phosphatase

Serum γGT activity was higher in TAA group (21.89 ± 4.11 U/L) in comparison with control group (8.28 ± 1.00 U/L) ($p<0.01$). However, γ-GT activity was significantly lower in TAA+Bet 17.74 ± 2.31 compared to TAA ($p<0.01$), as well as in MIF$^{-/-}$+TAA+ Bet group (16.56 ± 2.12 mg/L) compared to MIF$^{-/-}$ +TAA group (21.44 ± 3.73 U/L) ($p<0.01$) (Fig. 1D).

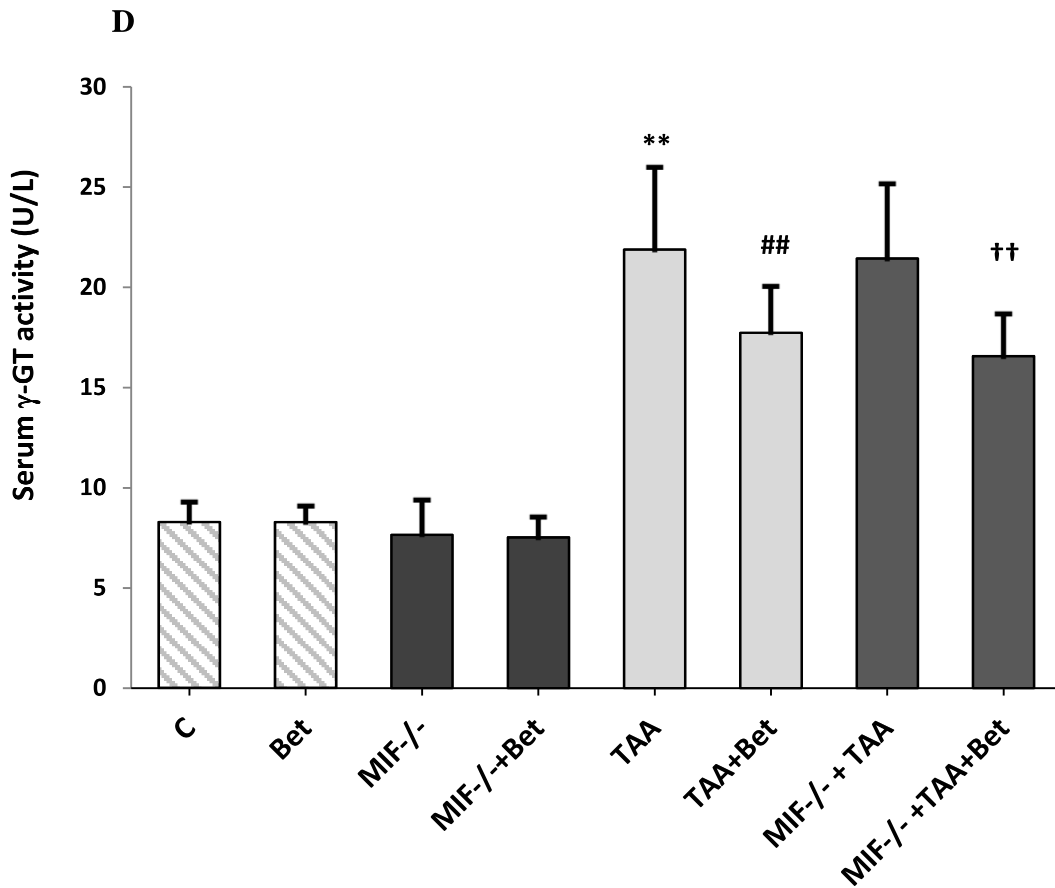


Fig. 1D. Effects of MIF and betaine on serum γGT activity in TAA-induced liver fibrosis after an 8-week experimental period

Significance of the difference was estimated by using one-way analysis of variance (ANOVA) with Tukey´s post hoc test; (**$p<0.01$ vs. C; $^{\#\#}p<0.01$ vs. TAA; $^{\dagger\dagger}p<0.01$ vs. MIF$^{-/-}$+TAA and TAA+Bet).

For abbreviations see Table. 1. γGT, gama glutamyl transpeptidase.

Further serum analysis revealed that bilirubin concentration was significantly higher in TAA group (8.24 ± 2.92 μmol/L) in comparison with control group (2.54 ± 0.52 μmol/L) ($p<0.01$). On the other hand, bilirubin concentration was significantly decreased in TAA+Bet and MIF$^{-/-}$+TAA group (3.60 ± 2.55; 7.20 ± 1.83 μmol/L; $p<0.05$, $p<0.01$, respectively) compared to TAA group. Bilirubin concentration was significantly lower in MIF$^{-/-}$+TAA+Bet group (3.43 ± 0.9 μmol/L) compared to TAA+Bet group ($p<0.01$) (Fig. 1F).

E

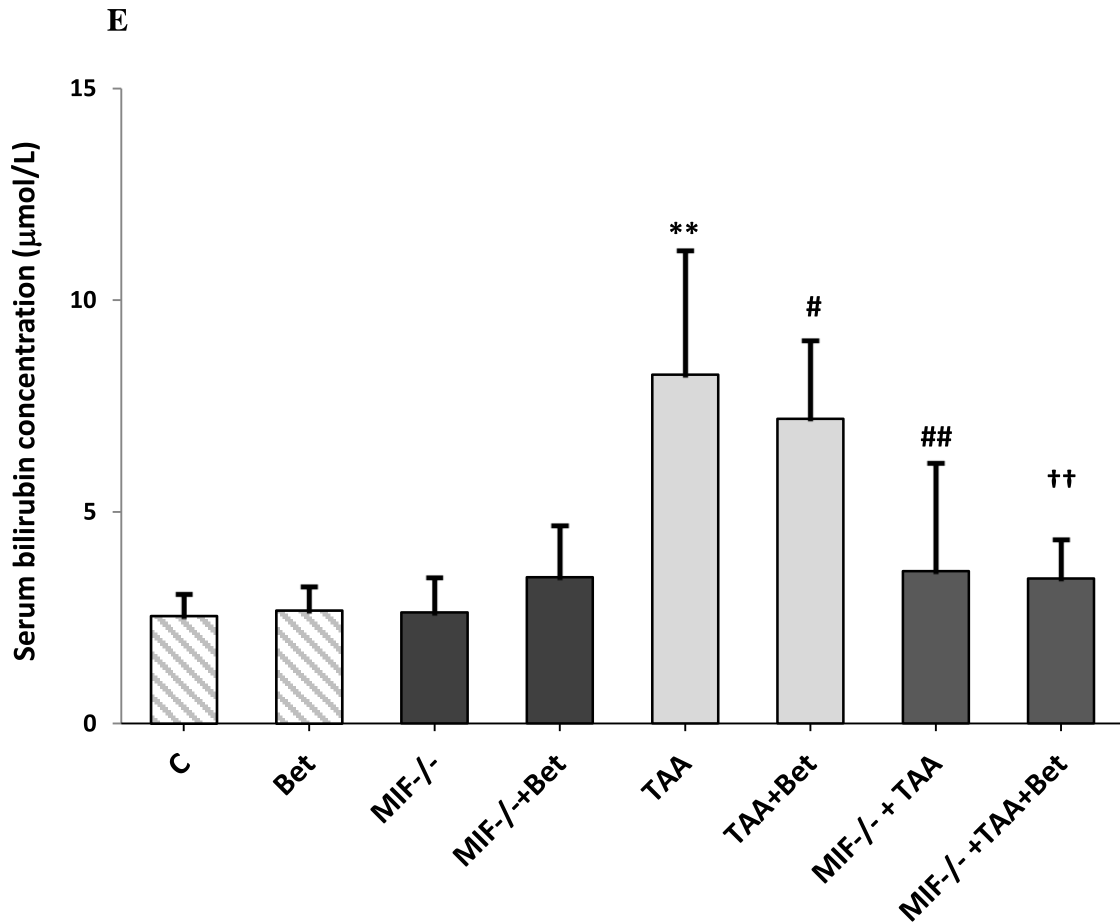


Fig. 1E. Effects of MIF and betaine on serum bilirubin concentration in TAA-induced liver fibrosis after an 8-week experimental period

Significance of the difference was estimated by using one-way analysis of variance (ANOVA) with Tukey´s post hoc test; (**$p<0.01$ vs. C; $^{\#}p<0.05$ vs. TAA; $^{\#\#}p<0.01$ vs. TAA; $^{\dagger\dagger}p<0.01$ vs. TAA+Bet).

For abbreviations see Table. 1.

There was no statistically significant difference in serum albumin concentration between all tested groups ($p>0.05$) (Fig. 1E).

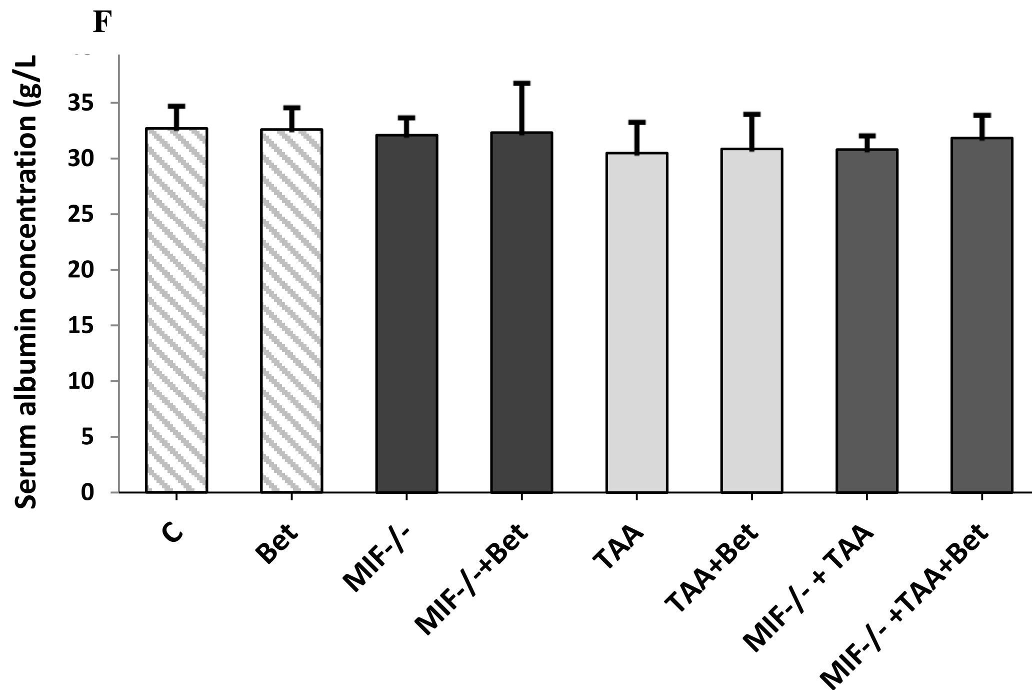


Fig. 1F. Effects of MIF and betaine on serum albumin concentration in TAA-induced liver fibrosis after an 8-week experimental period

Significance of the difference was estimated by using one-way analysis of variance (ANOVA) with Tukey´s post hoc test ($p>0.05$)

For abbreviations see Table. 1.

For all parameters, there was no significance in Bet, $MIF^{-/-}$, and $MIF^{-/-}$+Bet group compared to control group ($p>0.05$, respectively) (Fig. 1).

4.3. Effects of MIF and betaine on hepatic prooxidant and antioxidant parameters in the TAA-induced liver fibrosis

MDA concentration was significantly increased in TAA group (62.82 ± 5.1 μmol/mg prot.) compared to control (19.53 ± 1.84 μmol/mg prot.). Hepatic MDA concentrations decreased significantly in the TAA+Bet and MIF-/-+TAA groups (47.78 ± 4.28; 42.33 ± 3.86 μmol/mg prot.; $p<0.01$, respectively) when compared to the TAA group. Besides, the level of liver MDA was significantly decreased in $MIF^{-/-}$+TAA+Bet (36.23+3.23 μmol/mg prot.) compared with TAA+Bet and $MIF^{-/-}$+TAA ($p<0.01$, respectively) (Fig. 2A).

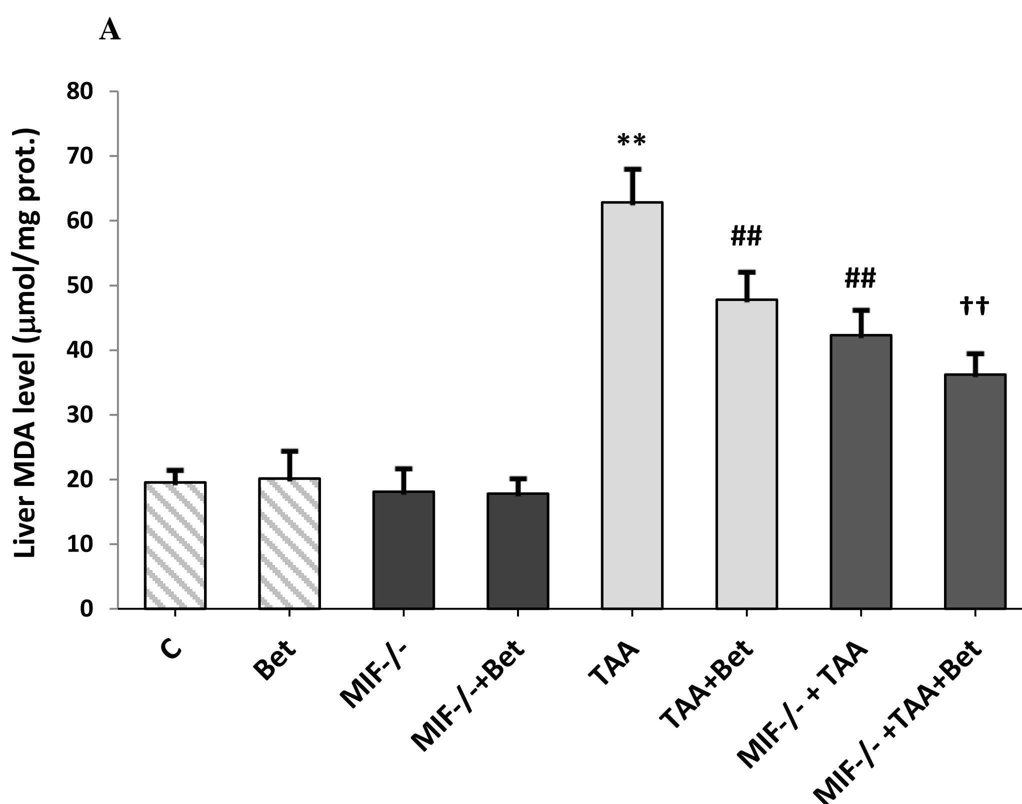


Fig. 2A. Effects of MIF and betaine on liver MDA level in TAA-induced liver fibrosis after an 8-week experimental period

Significance of the difference was estimated by using one-way analysis of variance (ANOVA) with Tukey´s post hoc test; (**$p<0.01$ vs. C; $^{\#\#}p<0.01$ vs. TAA; $^{\#\#}p<0.01$ vs. TAA; $^{\dagger\dagger}p<0.01$ vs. $MIF^{-/-}$+TAA and TAA+Bet).

For abbreviations see Table. 1. MDA, malondialdehyde.

AOPP level in serum was significantly increased in TAA group (120.57 ± 7.10 μmol/L chloramin-T eq/mg prot.) compared to C group (61.30 ± 2.08 μmol/L chloramin-T eq/mg prot.) ($p<0.01$). In contrast, the significant decrease in serum AOPP level was also registered in TAA+Bet and MIF$^{-/-}$+TAA group (93.72 ± 4.30; 80.40 ± 4.21 μmol/L chloramin-T eq/mg prot.; $p<0.01$, respectively) compared to TAA group. Betaine supplementation significantly decreased liver AOPP level in MIF$^{-/-}$+TAA+Bet (79.44 ± 3.38 μmol/mg prot.) group compared with TAA+Bet group ($p<0.01$) (Fig. 2B).

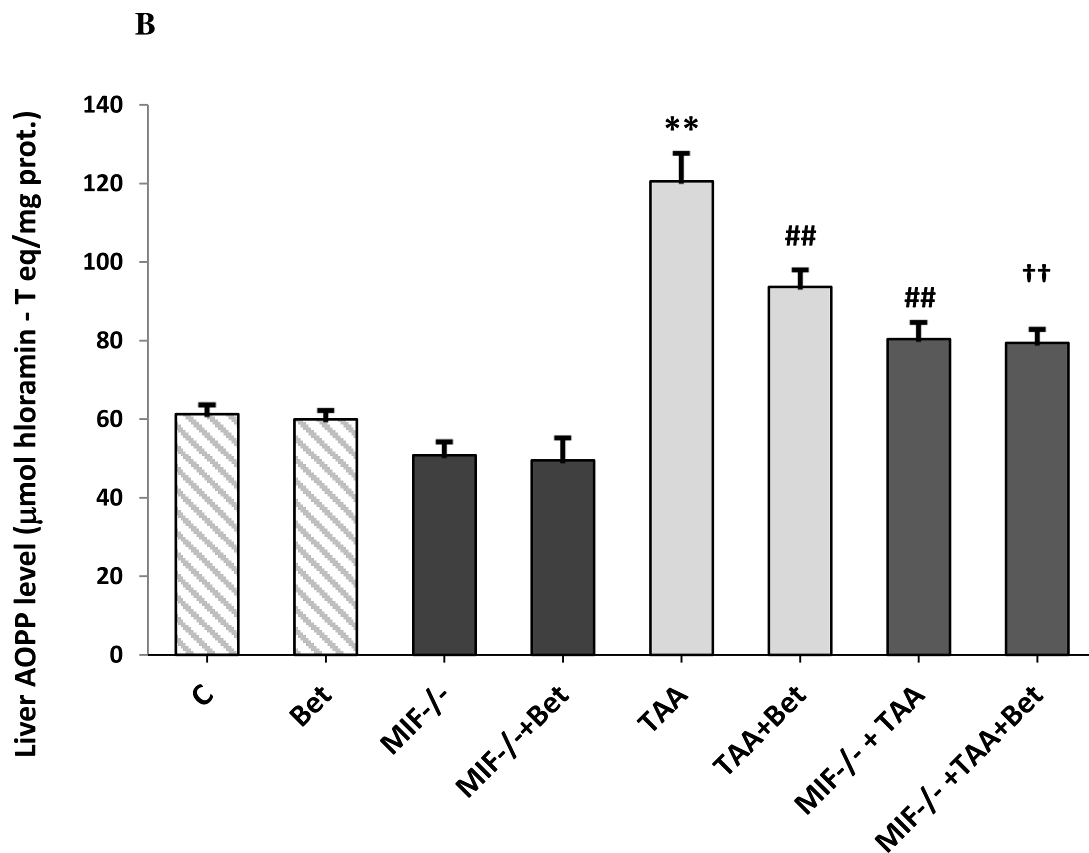


Fig. 2B. Effects of MIF and betaine on liver AOPP level in TAA-induced liver fibrosis after an 8-week experimental period

Significance of the difference was estimated by using one-way analysis of variance (ANOVA) with Tukey´s post hoc test; **$p<0.01$ vs.C; ##$p<0.01$ vs. TAA; ††$p<0.01$ vs. TAA+Bet)

For abbreviations see Fig. 1. AOPP, advanced oxidation protein products

TAA treatment significantly decreased total liver SOD activity in TAA group (99.67 ± 8.74 U/mg prot.) in comparison with control (210.14 ± 7.14 U/mg prot.) ($p<0.01$). On the other hand, treatment with betaine induced a significant increase in SOD activity in TAA+Bet (128.26 ± 16.79 U/mg prot.) compared to TAA group ($p<0.01$). Also, a significant increase in activity of this enzyme was registered in $MIF^{-/-}$+TAA+Bet group (135.83 ± 23.62 U/mg prot.) then in $MIF^{-/-}$+TAA group (121.12 ± 10.86 U/mg prot.) ($p<0.05$) (Fig. 2C).

C

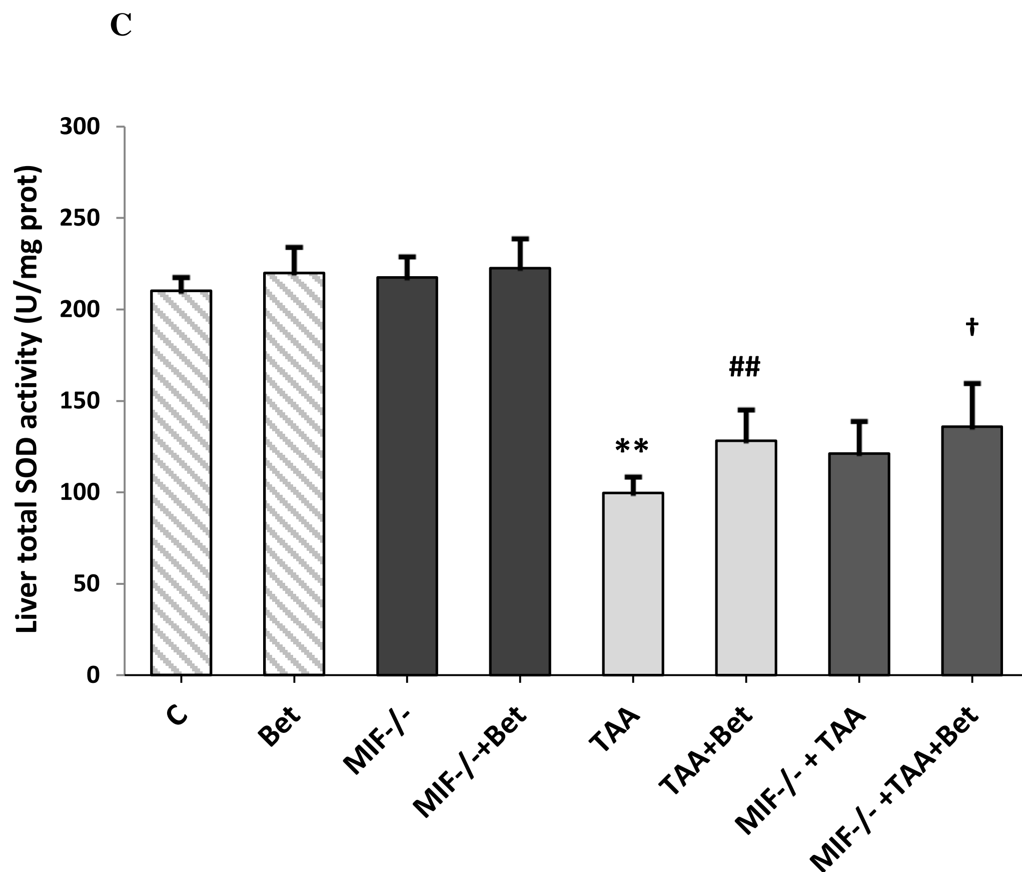


Fig. 2C. Effects of MIF and betaine on liver total SOD activity in TAA-induced liver fibrosis after an 8-week experimental period

Significance of the difference was estimated by using one-way analysis of variance ANOVA) with Tukey´s post hoc test; **p<0.01 vs.C; $^{##}$p<0.01 vs. TAA; †p<0.05 vs. $MIF^{-/-}$ +TAA.

For abbreviations see Fig. 1. SOD, superoxide dismutase

Further analysis of hepatic antioxidant capacity revealed that CAT activity was significantly decreased in TAA group (7.20 ± 1.68 U/mg prot.) when compared to control values (29.11 ± 2.62 U/ mg prot.) ($p<0.01$). CAT activity was significantly increased in TAA+Bet (10.25 ± 2.04 U/ mg prot.) compared to TAA group ($p<0.01$). Also, CAT activity was significantly increased in MIF$^{-/-}$ +TAA+Bet group (11.20 ± 2.39 U/mg prot.) compared to MIF$^{-/-}$+TAA group (7.94 ± 1.34 U/mg prot.) ($p<0.05$, respectively) (Fig. 2D).

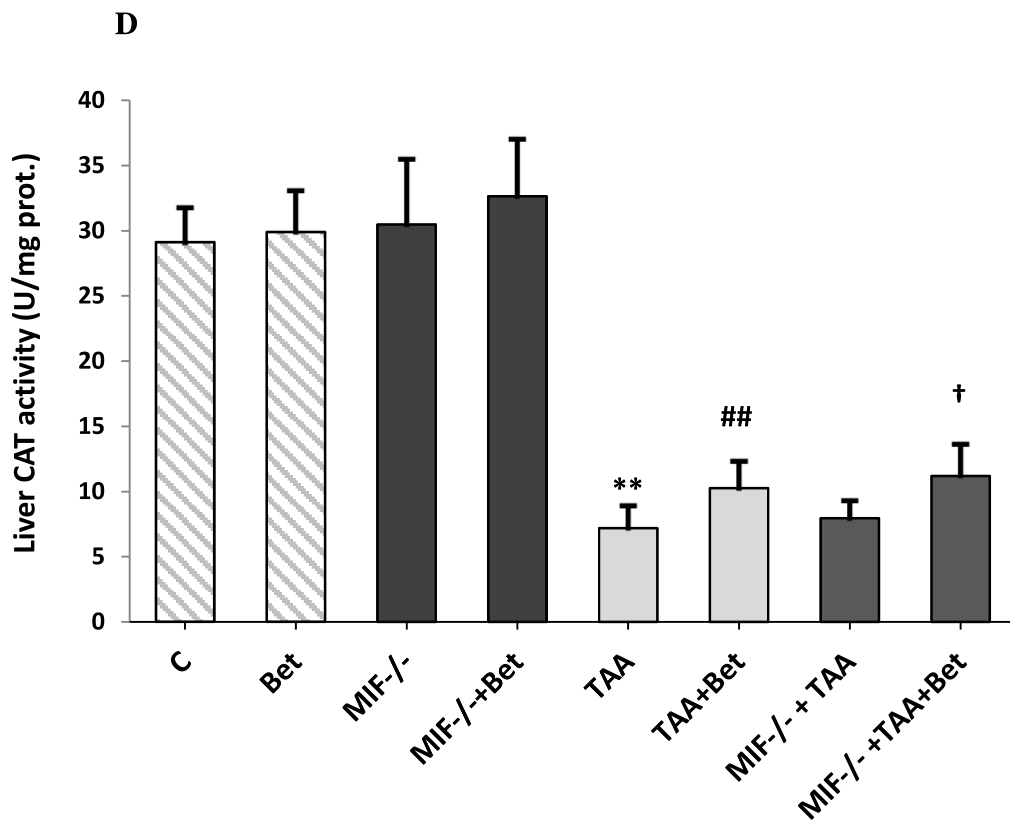


Fig. 2D. Effects of MIF and betaine on liver CAT activity in TAA-induced liver fibrosis after an 8-week experimental period

Significance of the difference was estimated by using one-way analysis of variance (ANOVA) with Tukey´s post hoc test; **$p<0.01$ vs. C; ##$p<0.01$ vs. TAA; †$p<0.05$ vs. MIF$^{-/-}$+TAA.

For abbreviations see Fig. 1. CAT, catalase

Thiol concentration in the liver was significantly lower in TAA group (3.15 ± 0.58 μmol/mg prot.) in comparison with control (5.39 ± 0.25 μmol/mg prot.) ($p<0.01$). Contrast, liver thiols concentration was significantly increased in TAA+Bet and $MIF^{-/-}$+TAA group (3.75 ± 0.21; 4.09 ± 0.25 μmol/mg prot.; $p<0.05$; $p<0.01$, respectively) compared to TAA group. Moreover, level of liver thiol was significantly increased in $MIF^{-/-}$+TAA+Bet group (4.99 ± 0.73 μmol/mg prot.) in comparison with TAA+Bet and $MIF^{-/-}$+TAA group (3.75 ± 0.21; 4.09 ± 0.25 μmol/mg prot.; $p<0.01$, respectively) (Fig. 2E).

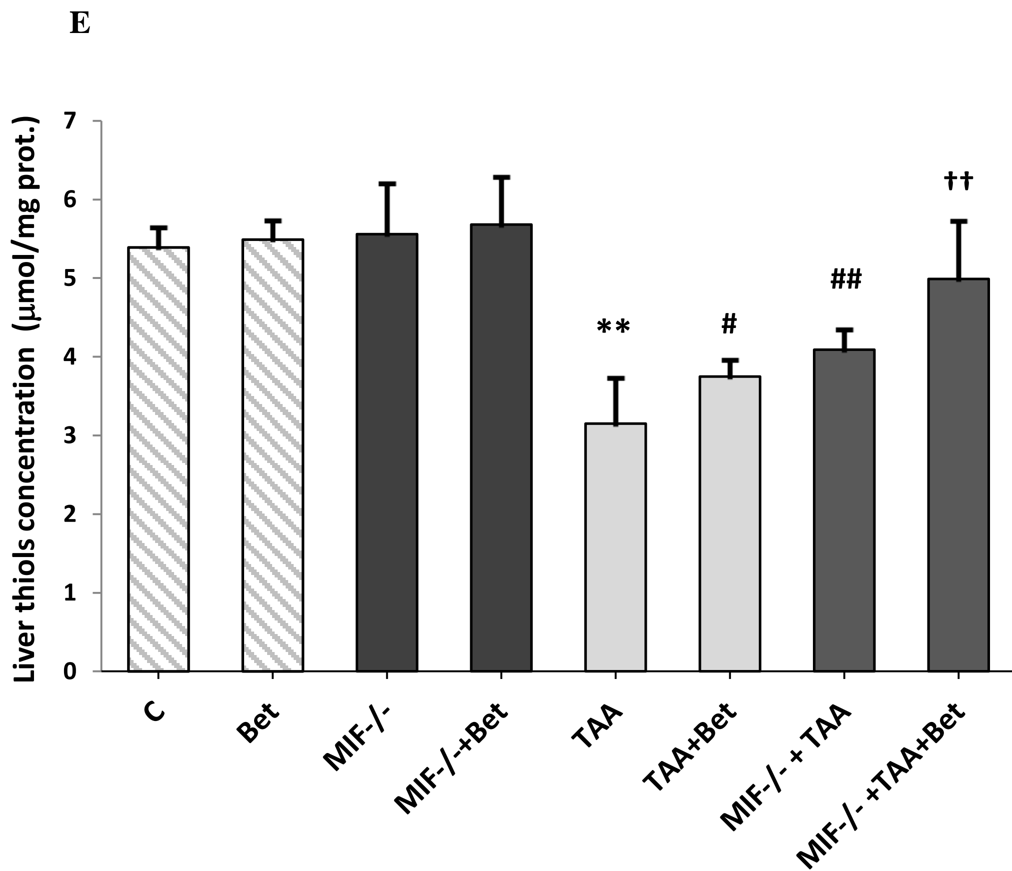


Fig. 2E. Effects of MIF and betaine on liver thiol concentrations in TAA-induced liver fibrosis after an 8-week experimental period

Significance of the difference was estimated by using one-way analysis of variance (ANOVA) with Tukey´s post hoc test; **$p<0.01$ vs.C; $^{\#}p<0.05$, $^{\#\#}p<0.01$ vs. TAA; $^{\dagger\dagger}p<0.01$ vs. $MIF^{-/-}$+TAA and TAA+Bet.

For abbreviations see Fig. 1.

Our study has shown that liver TAS level was significantly decreased in TAA group (0.51 ± 0.12 µmol Trolox eq./mg prot.) in comparison with control (1.59 ± 0.51 µmol Trolox eq./mg prot.) ($p<0.01$). On the other hand, liver TAS level was significantly increased in TAA+Bet and MIF$^{-/-}$ +TAA group (1.13 ± 0.32; 0.82 ± 0.20 µmol Trolox eq./mg prot. $p<0.01$, respectively) compared to TAA. Moreover, liver TAS level was significantly increased in MIF$^{-/-}$+TAA+Bet group (1.20 ± 0.38 µmol Trolox eq./mg prot.) compared to MIF$^{-/-}$+TAA group (0.82 ± 0.20 µmol Trolox eq./mg prot.). (Fig. 2G). For all parameters, there was no significance in Bet, MIF$^{-/-}$, and MIF$^{-/-}$+Bet group compared to the control group ($p>0.05$, respectively) (Fig. 2).

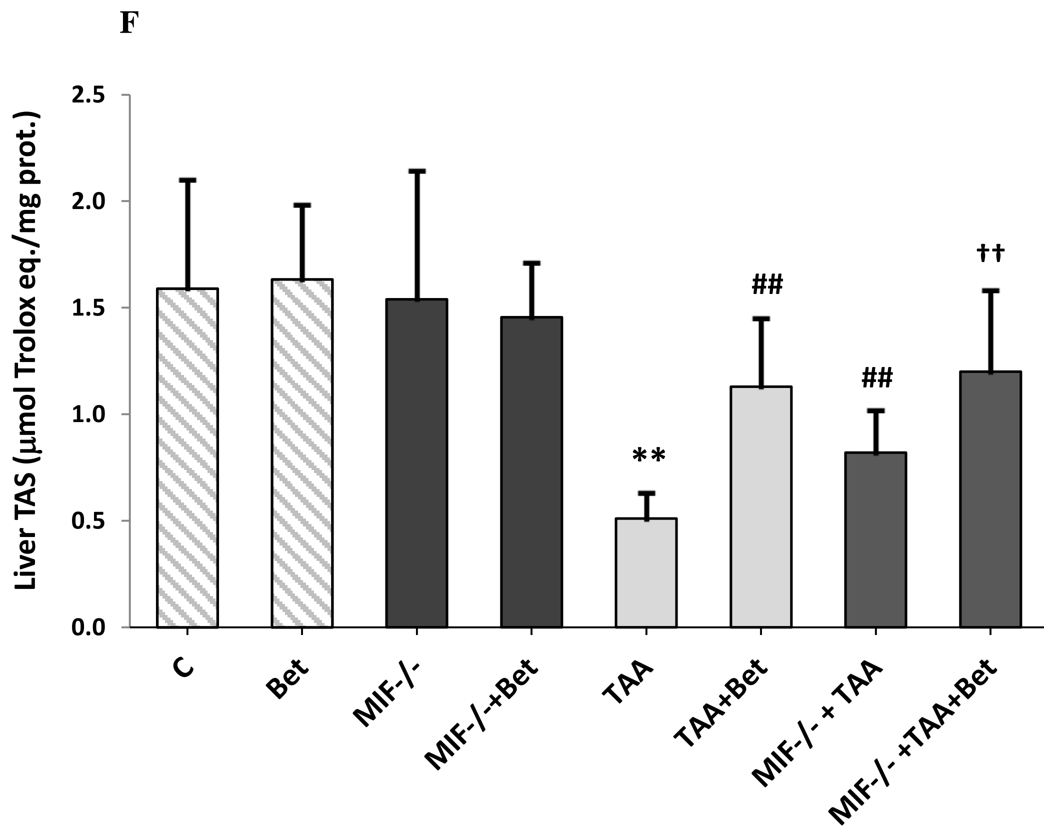


Fig. 2F. Effects of MIF and betaine on liver TAS concentrations in TAA-induced liver fibrosis after the 8-week experimental period

Significance of the difference was estimated by using a one-way analysis of variance (ANOVA) with Tukey´s post hoc test; **p<0.01 vs.C; ##p<0.01 vs. TAA; ††p<0.01 vs. MIF$^{-/-}$+TAA and TAA+Bet.

For abbreviations see Fig. 1. TAS, total antioxidative status

In contrast, thioacetamide-induced significant increase in liver TOS level in TAA group (19.32 ± 3.89 µmol H_2O_2 equivalent/mg prot.) when compared to control (5.56 ± 0.73 µmol H_2O_2 equivalent/mg prot.) ($p<0.01$). On the other hand, liver TOS level was significantly decreased in TAA+Bet and MIF$^{-/-}$+TAA group (17.50 ± 3.94; 17.15 ± 2.89 µmol H2O2 equivalent/mg prot.; $p<0.05$, respectively) compared to TAA group. Moreover, liver TOS level was significantly decreased in MIF$^{-/-}$+TAA+Bet group (13.92 ± 1.62 µmol H_2O_2 equivalent/mg prot.) compared to MIF$^{-/-}$+TAA (17.15 ± 2.89 µmol H_2O_2 equivalent/mg prot.) and TAA+Bet ($p<0.01$, respectively) (Fig. 2F).

G

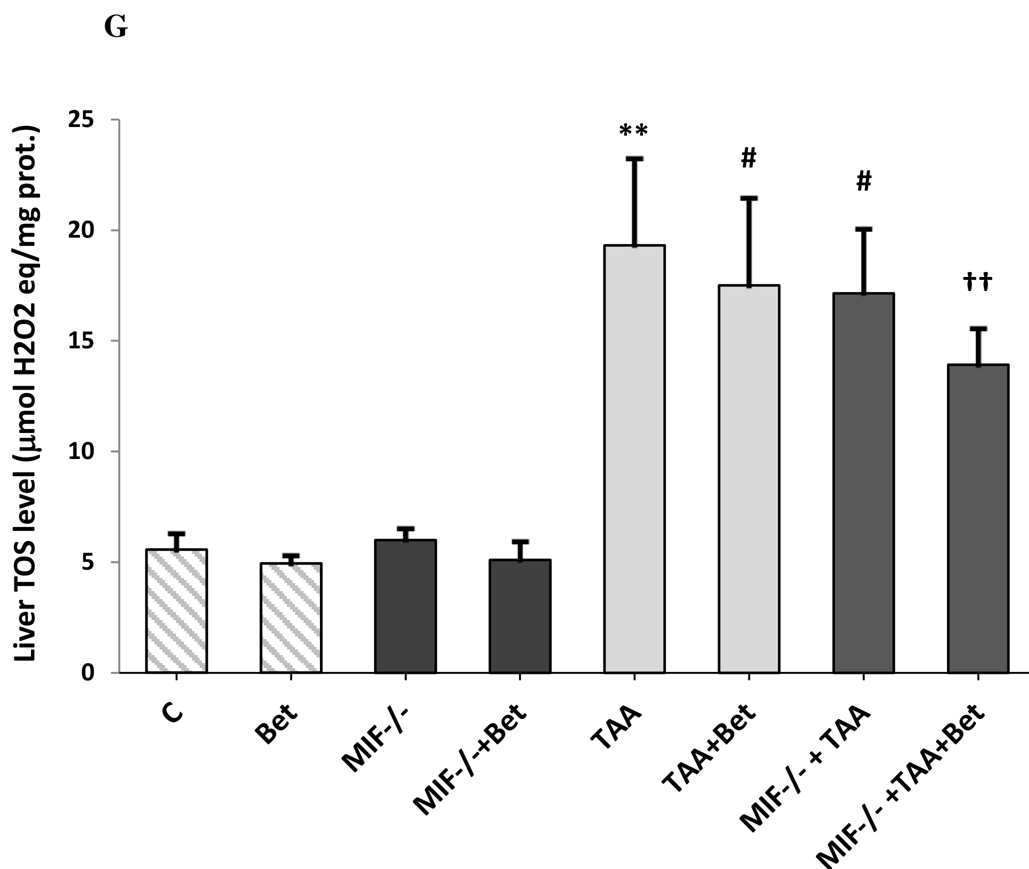


Fig. 2G. Effects of MIF and betaine on liver TOS concentrations in TAA-induced liver fibrosis after the 8-week experimental period

Significance of the difference was estimated by using a one-way analysis of variance (ANOVA) with Tukey´s post hoc test; **$p<0.01$ vs.C; #$p<0.05$ vs. TAA ††$p<0.01$ vs. MIF$^{-/-}$+TAA and TAA+Bet.

For abbreviations see Fig. 1. TOS, total oxidative status

4.4. Effects of MIF and betaine on CRP and proinflammatory cytokines (IL-6 and IFN-γ) in TAA-induced liver fibrosis

Serum CRP concentration was significantly higher in TAA group in comparison with the control values ($p<0.01$). However, serum CRP concentration was significantly decreased in TAA+Bet and $MIF^{-/-}$+TAA compared to TAA ($p<0.01$, respectively). Also, serum CRP was significantly decreased in $MIF^{-/-}$+TAA+Bet group compared to TAA+Bet and $MIF^{-/-}$+TAA group ($p<0.01$, respectively) (Table 2).

Our study has shown that liver IL-6 concentration was significantly higher in TAA group in comparison with the control values ($p<0.01$). However, liver IL-6 concentration was significantly decreased in TAA+Bet and $MIF^{-/-}$+TAA compared to TAA group ($p<0.01$, respectively). Moreover, level of IL-6 in the liver was significantly decreased in $MIF^{-/-}$+TAA+Bet group compared to TAA+Bet and MIF+TAA group ($p<0.01$, respectively) (Table 2).

Results of our study have shown that liver IFN-γ concentration was significantly higher in TAA group in comparison with the control values ($p<0.01$). However, liver IFN-γ concentration was significantly decreased in TAA+Bet and $MIF^{-/-}$+TAA group compared to TAA group ($p<0.01$, respectively). Moreover, the level of IFN-γ in the liver was significantly decreased in $MIF^{-/-}$+TAA+Bet group compared to TAA+Bet and $MIF^{-/-}$+TAA group ($p<0.01$, respectively) (Table 2). For all parameters, there was no significance in Bet, $MIF^{-/-}$, and $MIF^{-/-}$+Bet group compared to the control group ($p>0.05$, respectively) (Table 2).

Table 2. Effects of MIF and betaine on serum concentration of CRP, and liver IL-6 and IFN-γ level in TAA-induced liver fibrosis after the 8-week experimental period

	PARAMETERS		
Group	**CRP (mg/L)**	**IL-6 (pg/mL)**	**IFN-γ (pg/mL)**
C	2.55 ± 0.15	34.33 ± 0.85	124.18 ± 6.12
Bet	2.62 ± 0.17	32.48 ± 1.40	120.18 ± 5.59
$MIF^{-/-}$	2.58 ± 0.16	31.80 ± 1.29	121.44 ± 6.88
$MIF^{-/-}$+Bet	2.57 ± 0.15	31.81 ± 1.85	122.05 ± 5.25
TAA	14.25 ± 1.45**	215.08 ± 10.38**	234.68 ± 17.30**
TAA+Bet	8.43 ± $1.31^{\#\#}$	146.68 ± $12.93^{\#\#}$	186.97 ± $10.45^{\#\#}$
$MIF^{-/-}$+TAA	10.28 ± $1.68^{\#\#}$	158.78 ± $18.05^{\#\#}$	190.67 ± $13.14^{\#\#}$
$MIF^{-/-}$+TAA+Bet	5.85 ± 1.26^{ff}	109.53 ± 7.60^{ff}	148.40 ± 9.08^{ff}

Significance of the difference was estimated by using a one-way analysis of variance (ANOVA) with Tukey´s post hoc test; **p<0.01 vs.C; $^{\#\#}$p<0.01 vs. TAA; ^{ff}p<0.01 vs. $MIF^{-/-}$+TAA and TAA+Bet.

For abbreviations see Table 1. CRP, C-reactive protein; IL-6, interleukin-6; IFN-γ, interferon-γ

4.5. Effects of MIF and betaine on profibrogenic mediators (TGF-β1 and PDGF-BB) in TAA-induced liver fibrosis

Our study has shown that liver TGF-β1 concentration was significantly higher in TAA group (170.12 ± 24.89 pg/mL) in comparison with the control values (85.60 ± 10.92 pg/mL) ($p<0.01$). Liver TGF-β1 concentration was significantly higher in $MIF^{-/-}$+TAA (230.49 ± 34.45 pg/mL) and significantly decreased in TAA+Bet group (126.16 ± 20.76 pg/mL) compared to TAA group ($p<0.01$). Moreover, level of TGF-β1 in the liver was significantly decreased in $MIF^{-/-}$+TAA+Bet group (172.15 ± 26.25 pg/mL) compared to $MIF^{-/-}$+TAA group ($p<0.01$), and significantly higher compared to TAA+Bet group ($p<0.01$) (Fig. 3A).

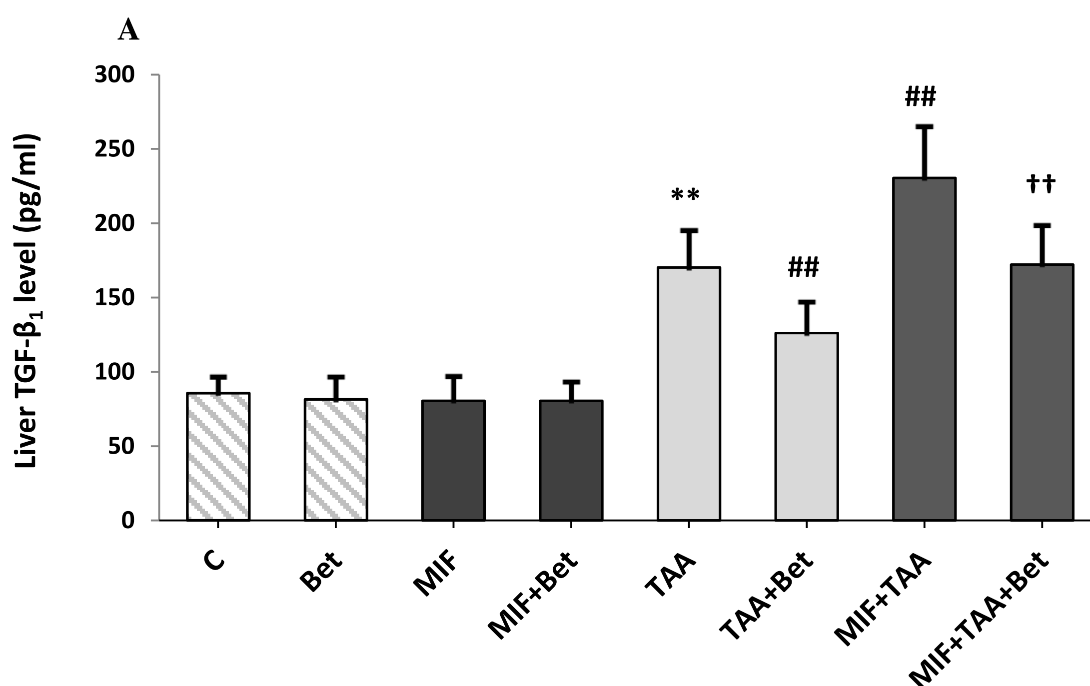


Fig. 3A. Effects of MIF and betaine on the liver TGF-β1 level in mice with liver fibrosis. Liver fibrosis was induced by TAA (200 mg/kg dissolved in 200μL PBS) i.p. three times a week during an 8 week period. Betaine was dissolved in drinking water (2% wt/v) and animals had free access to drink *ad libitum.*

Significance of the difference was estimated by using one-way analysis of variance (ANOVA) with Tukey´s post hoc test; $^{**}p<0.01$ vs.C; $^{\#\#}p<0.01$ vs. TAA; $^{\dagger\dagger}p<0.01$ vs. $MIF^{-/-}$+TAA and TAA+Bet.

For abbreviations see Table 1.

Results of our study have shown that liver PDGF-BB concentration was significantly higher in TAA group (2199.86 ± 322.63 pg/mL) in comparison with the control values (760.53 ± 101.41 pg/mL) ($p<0.01$). Liver PDGF-BB concentration was significantly higher in $MIF^{-/-}$+TAA (2452.72 ± 129.63 pg/mL) and significantly decreased in TAA+Bet group (1135.65 ± 144.49 pg/mL) compared to TAA group ($p<0.05$; $p<0.01$, respectively). Moreover, level of PDGF-BB in the liver was significantly decreased in $MIF^{-/-}$+TAA+Bet group (1859.66 ± 347.43 pg/mL) compared to $MIF^{-/-}$+TAA group ($p<0.01$), and significantly higher compared to TAA+Bet group ($p<0.01$) (Fig. 3B).

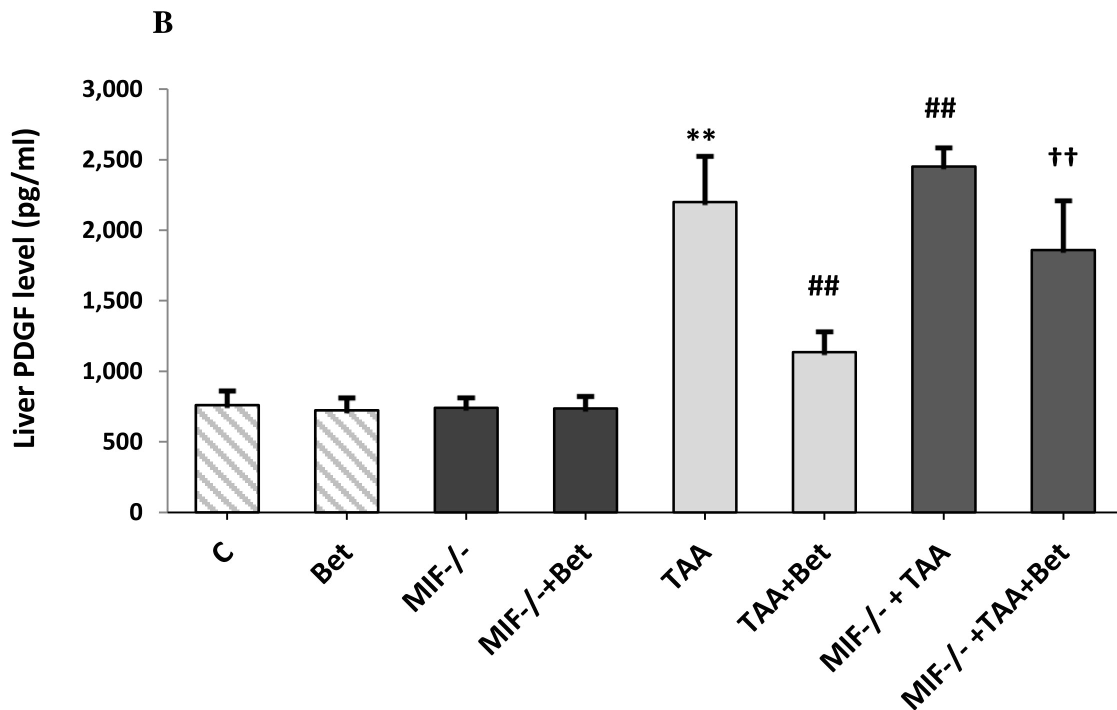


Fig. 3B. Effects of MIF and betaine on the liver PDGF-BB level in mice with liver fibrosis. Liver fibrosis was induced by TAA (200 mg/kg dissolved in 200µL PBS) i.p. three times a week during an 8 week period. Betaine was dissolved in drinking water (2% wt/v) and animals had free access to drink *ad libitum.*

Significance of the difference was estimated by using a one-way analysis of variance (ANOVA) with Tukey´s post hoc test; $^{**}p<0.01$ vs.C; $^{\#\#}p<0.01$ vs. TAA; $^{\dagger\dagger}p<0.01$ vs. $MIF^{-/-}$+TAA and TAA+Bet.

For abbreviations see Table 1

For TGF-β1 and PDGF-BB parameters, there was no significance in Bet, $MIF^{-/-}$, and $MIF^{-/-}$ +Bet group compared to the control group ($p>0.05$, respectively) (Fig. 3A, 3B).

4.6. Effects of MIF and betaine on MMP-2, MMP-9, dimer MMP-9 and TIMP-1 activity in TAA-induced liver fibrosis

Gelatin zymography revealed that tissue homogenates contained both MMP-2 (72 kDa) and MMP-9 (92 kDa). MMP-9 appeared also in a dimer form (~220 kDa) in all samples of liver tissue.

Densitograms of gelatin zymography of MMPs in tissue homogenates of TAA-induced liver fibrosis in mice are presented in Fig. 4A.

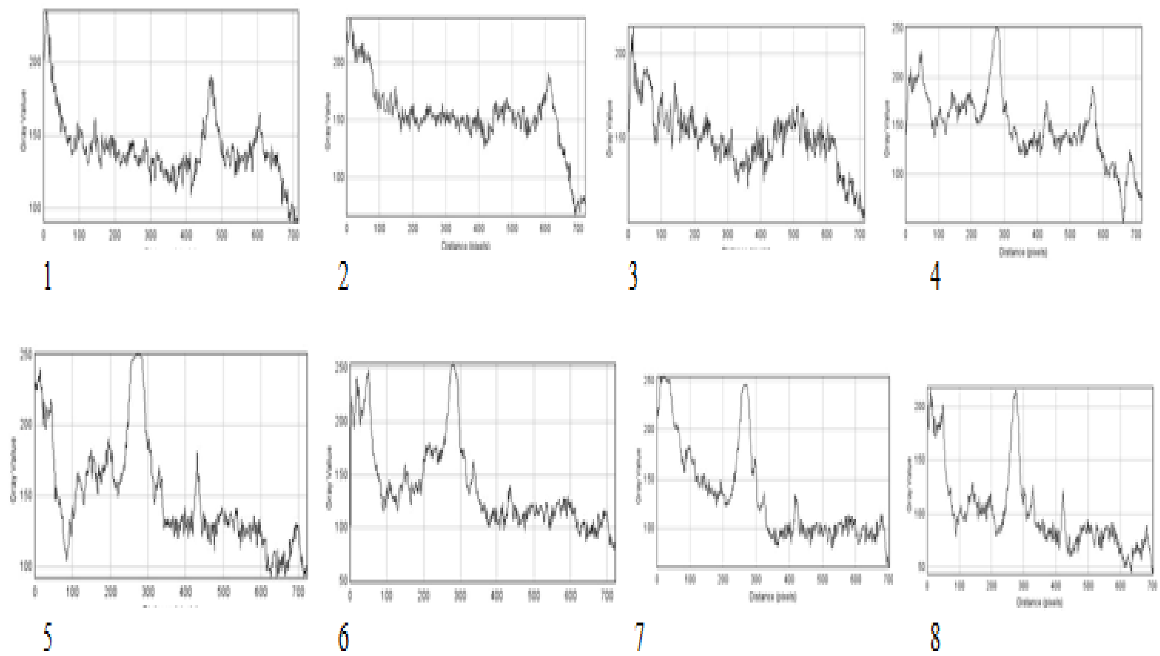


Fig. 4A. Densitograms of gelatin zymography of MMPs in tissue homogenates of TAA induced liver fibrosis in mice. Liver tissue homogenates (LTH) were processed as described and analyzed by gelatin zymography. LTH samples of all groups shown above each lane: 1-C, 2-Bet, 3-MIF$^{-/-}$, 4-MIF$^{-/-}$; 5- TAA; 6-TAA+Bet; 5- MIF$^{-/-}$+TAA, 8- MIF$^{-/-}$ + TAA + Bet.

Fig. 4B shows an electrophoregram and corresponding densitogram of human recombinant MMP-9 and-2. Gelatin zymography of MMPs in tissue homogenates of thioacetamide-induced liver fibrosis in mice and MMP-9 and MMP-2 molecular weight calibration markers were presented in Fig. 4C.

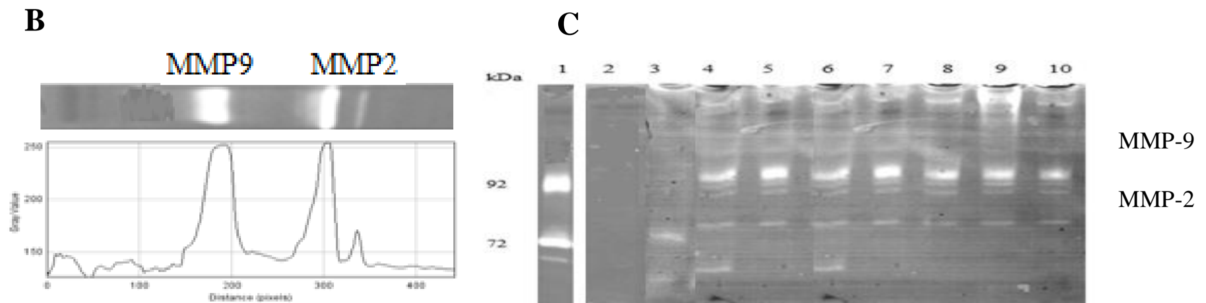


Fig. 4B. Gelatin zymography electrophoregram and corresponding densitogram of standard human recombinant MMP-9 and -2. **Fig. 4C**. Gelatin zymography of MMPs of tissue homogenates of TAA-induced liver fibrosis in mice. Liver tissue homogenates (LTH) were processed as described and analyzed by gelatin zymography. Position 1 represents a zymogram of MMP-9 and MMP-2 molecular weight calibration markers: MMP-9 is at a position of 92 kDa, MMP-2 is at a position of 72 kDa. Line 2 shows the absence of both MMPs which were incubated with 5 mmol/L EDTA. LTH samples shown above each lane: 1-C, 2-Bet, 3-MIF$^{-/-}$, 4-MIF$^{-/-}$; 5- TAA; 6-TAA+Bet; 5- MIF$^{-/-}$+TAA, 8- MIF$^{-/-}$+ TAA + Bet.

Densitometric area (Arbitary Units) of MMP-2, MMP-9, dimer MMP-9 and TIMP-1 in liver tissue homogenates were significantly increased in TAA group in comparison with the control values ($p<0.01$). Densitometric area of MMP-2, MMP-9 and TIMP-1 were significantly increased in MIF$^{-/-}$+TAA and significantly decreased in TAA+Bet group compared to TAA group ($p<0.05$; $p<0.01$), while densitometric area of dimer MMP-9 was significantly decreased in MIF$^{-/-}$+TAA compared to TAA+Bet and TAA group ($p<0.01$). Moreover, MMP-2, MMP-9 and TIMP were significantly decreased in MIF$^{-/-}$+TAA+Bet group compared to MIF$^{-/-}$+TAA group ($p<0.01$), and significantly increased compared to TAA+Bet group ($p<0.01$). However, the densitometric area of dimer MMP-9 in MIF$^{-/-}$+TAA+Bet group was significantly decreased compared to TAA+Bet group ($p<0.01$) (Fig. 5a, Fig. 5b).

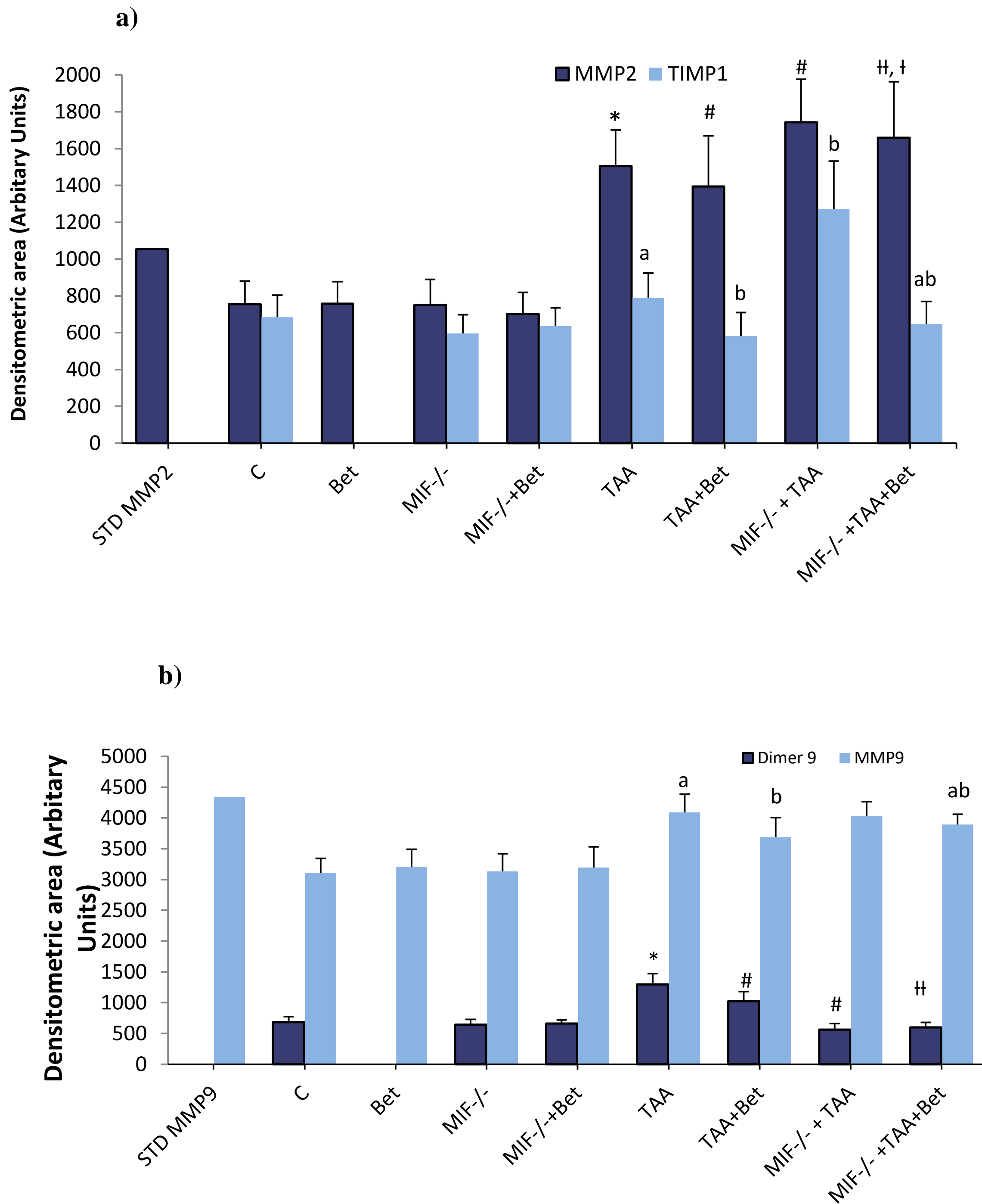


Fig. 5. Effects of MIF and betaine on liver activity of MMP-2 and TIMP-1 **(a),** and dimer MMP-9 and MMP-9 **(b),** in TAA-induced liver fibrosis

Significance of the difference was estimated by using one-way analysis of variance (ANOVA) with Tukey´s post hoc test; *p<0.01, ^{a}p<0.01, vs.C;$^{\#}$p<0.01, ^{b}p<0.01, vs. TAA;††p<0.01, ^{ab}p<0.01 vs. TAA+Bet; MIF$^{-/-}$+TAA; †p<0.05, ^{ab}p<0.05 vs. MIF$^{-/-}$ +TAA

For abbreviations see Tab.1.MMP, matrix metaloproteinase; TIMP, tissue inhibitor MP

4.7. Effects of MIF and betaine on liver tissue morphology in TAA-induced liver fibrosis

Table 3. presents a comparison review of liver tissue changes between investigated animal groups after an 8-week experimental period.

Histology findings of liver tissue in animals of C, Bet, $MIF^{-/-}$ and $MIF^{-/-}$+Bet (H&E, Masson and Reticulin) **(Fig. 6 a, b, c, d)** have shown the regular histological structure of the liver without inflammation, fibrosis and cirrhosis. Coll1 was found in the adventitial tissue of larger blood vessels. Reticular fibers (Coll3) were distributed around individual hepatocyte beds and perivascularly. Moreover, individual fat droplets were present in hepatocytes. Dinuclear hepatocytes were present, individual nuclei of hepatocytes were polyploid with multiple nucleoli. Mitosis was not present (0/10 HPF)

Table 3. Comparison of liver tissue characteristics of investigated animals

Characteristics	C	Bet	$MIF^{-/-}$	$MIF^{-/-}$+Bet	TAA	TAA+Bet	$MIF^{-/-}$+TAA	$MIF^{-/-}$+TAA+Bet
Fibrosis	-	-	-	-	+	+	+	+
Cirrhosis	-	-	-	-	-	-	+	-
Inflammation	-	-	-	-	+	+	+	+
Microvesicular fatty changes	-	-	-	-	+	+	+	+
Macrovesicular fatty changes	-	-	-	-	-	-	-	+
Polyploid hepatocytes with multiple nuclei	+	+	+	+	+	+	+	+
Bizarre polyploid nuclei of hepatocytes	-	-	-	+	+	+	-	+
Mallory bodies	-	-	-	-	+	+	-	+
Hemosiderophages	-	-	-	-	+	+	-	+
Focal necrosis of hepatocytes	-	-	-	-	+	+	+	+
Kupffer cells hyperplasia	-	-	-	-	+	-	+	+
Number of mitoses / HPF (max.)	0	0	0	2	1	5	1	5
Pathological mitoses	-	-	-	-	-	-	-	+

Table summarize significant findings in groups of animals “+“ marks the significant presensce of the finding, while “-“ minimal or absent changes.

For abbreviations see Table 1.

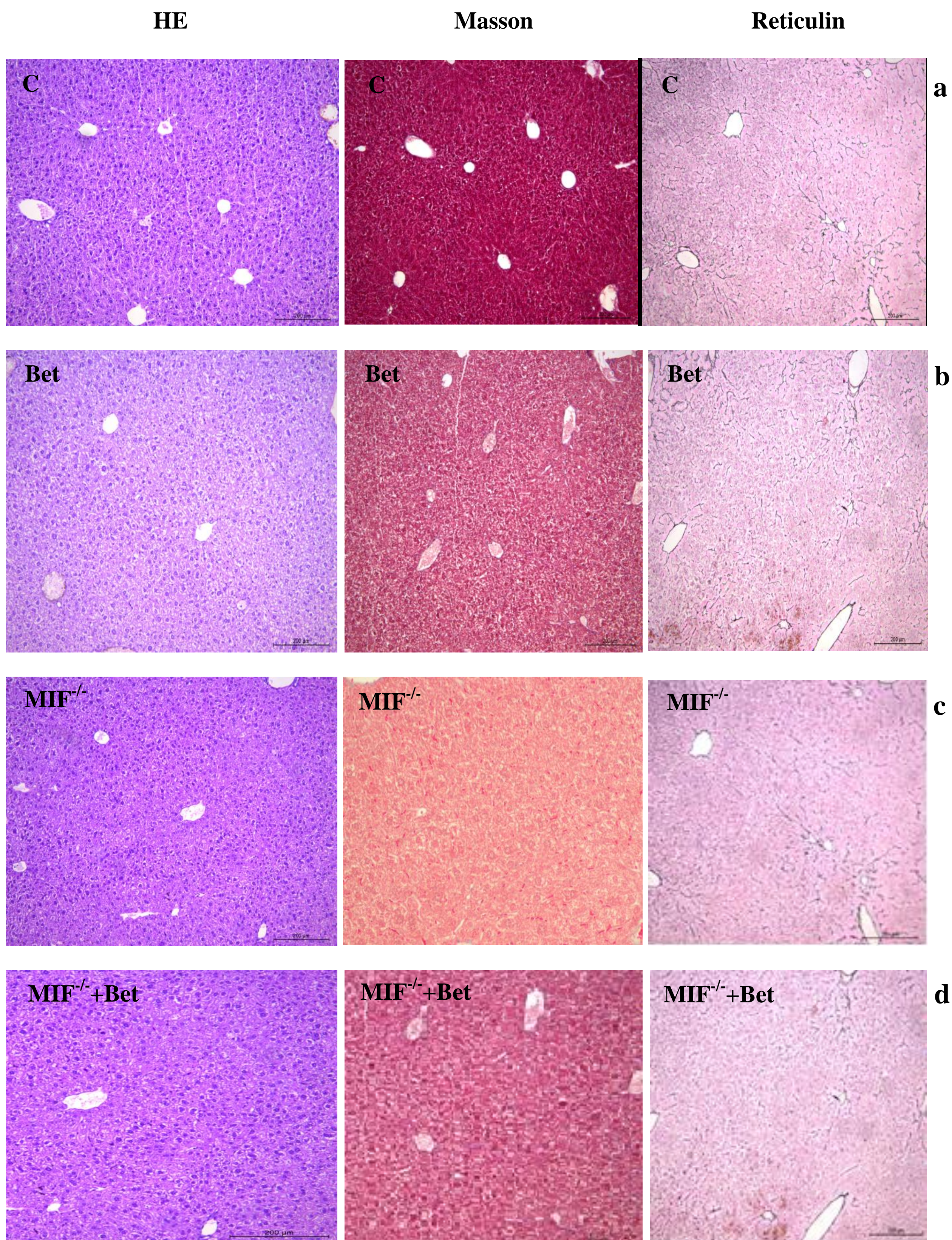


Fig. 6. Liver tissue in mice of C **(a)**, Bet (b), MIF$^{-/-}$**(c)** and MIF$^{-/-}$+Bet **(d)** groups (H&E, Masson and Reticulin)

In contrast to these groups, histology findings of liver tissue in TAA-treated animals showed the irregular structure of the liver with mononuclear inflammatory infiltrate in the portal and perivascular spaces. Mild liver fibrosis was present. The microvesicular fatty change was present. Numerous hepatocytes with strange, polyploid nuclei were found in TAA-treated animals. Nuclei contain multiple nucleoli. Some nucleoli were eosinophilic. Mallory's body and rare hemosiderophages were also observed. Rare focal hepatocyte necrosis was present. The number of mitoses was 0/10 HPF to 1/10HPF (High Power Field) **(Fig. 7a).** Masson-stained Coll1 bridges the liver tissue from one port space to another (bridging) were located perilobularly **(Fig. 7b)** and reticular fibers (Coll3) monitored the distribution of Coll1 **(Fig. 7c).**

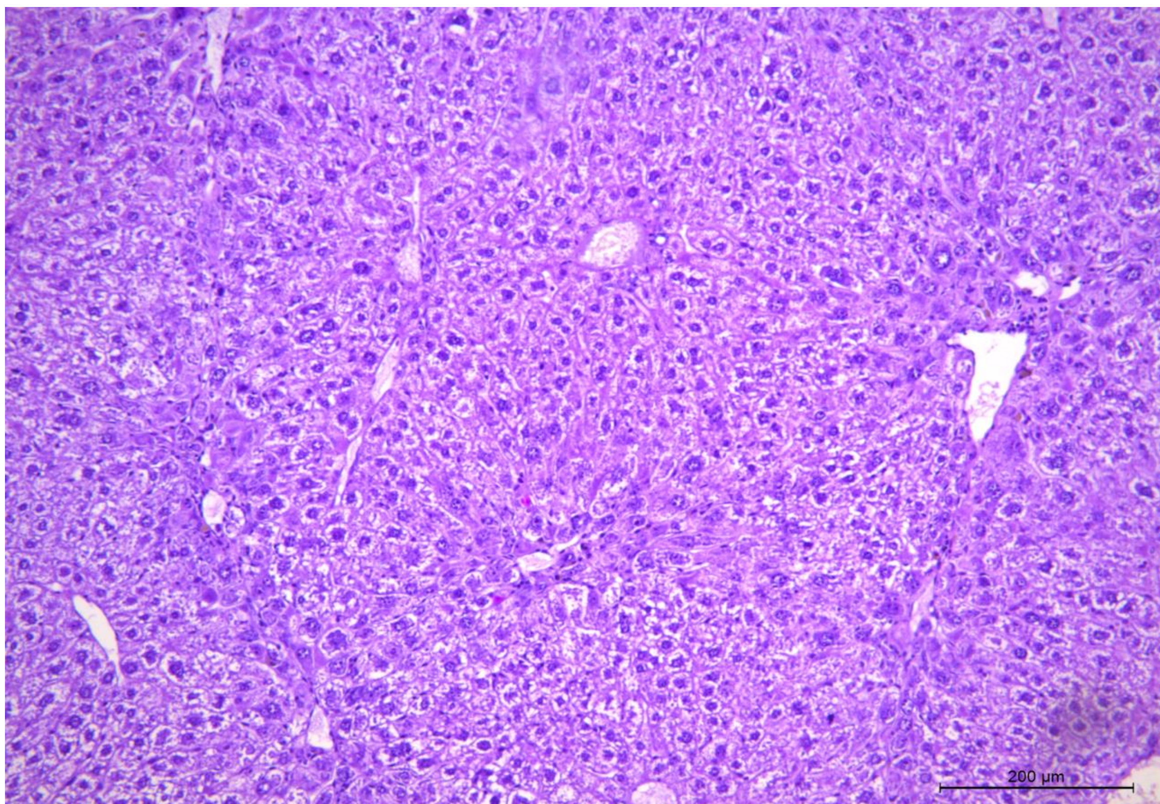


Fig. 7a. Liver morphology of TAA-treated animals (Hematoxylin & Eosin, scale bar 200µm). The irregular structure of the liver with mononuclear inflammatory infiltrate in the portal and perivascular spaces with mild liver fibrosis and microvesicular fatty change were present. Mallory's body, rare hemosiderophages and focal hepatocyte necrosis were also observed.

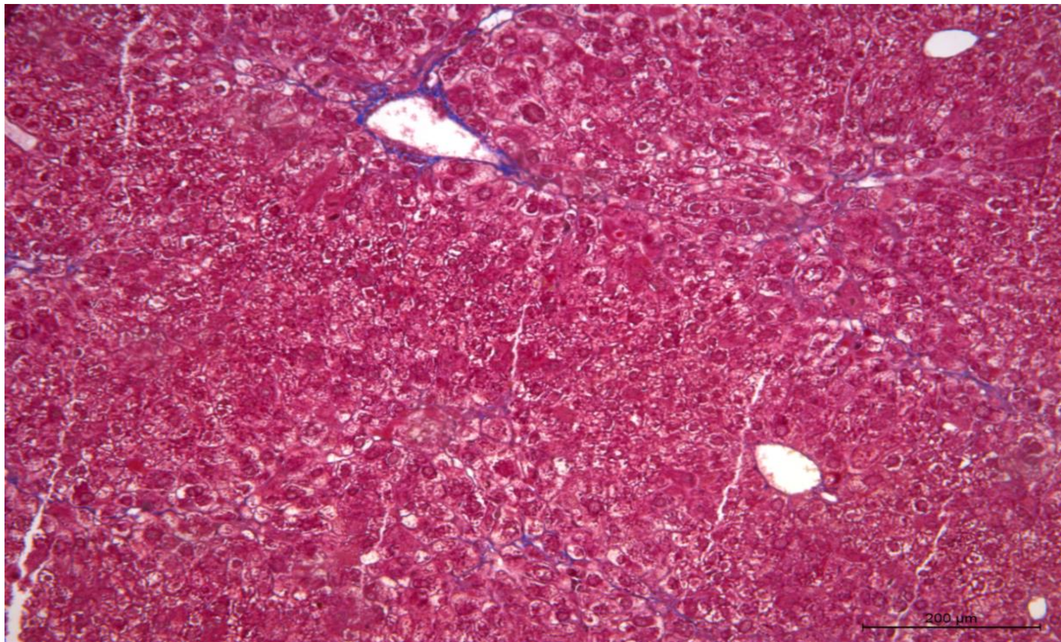


Fig. 7b. Liver morphology of TAA-treated animals (Masson's trichrome staining, scale bar 200µm). Masson-stained Coll1 bridges the liver tissue from one port space to another (bridging) were located perilobulary. Masson staining showed that collagen staining was blue in liver sections.

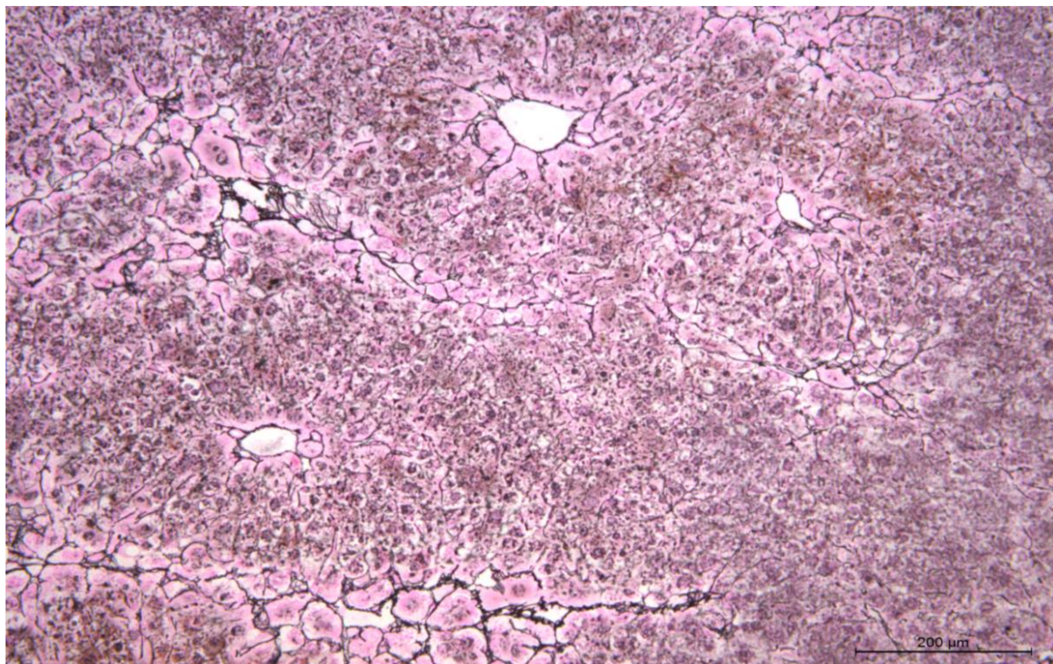


Fig. 7c. Liver tissue of TAA-treated animals *(*Reticulin staining*,* scale bar 200µm). Reticular fibers (Coll3) monitored the distribution of Coll1.

In contrast to wild-type C57BL/6 mice, MIF knockout mice treated with TAA had a more pronounced irregular liver structure with a mixed inflammatory infiltrate in the disse spaces and perivascularly along large blood vessels. Pronounced fibrosis (bridging porto-portal) and micronodular cirrhosis were also present. Moreover, micro- and macrovesicular fatty changes were present. Hepatocytes with bizarre, polyploid nuclei were numerous and contained multiple nucleoli. Some nucleoli were eosinophilic. Fat drops were found in some giant, bizarre nuclei. Mallory's body and numerous hemosiderophages were also observed. Focal necrosis and apoptosis of hepatocytes were present. The number of mitoses was 0/10 HPF to 4/10HPF **(Fig. 8a).** Masson-stained Coll1 was found perilobulary in the form of thin strips bridging liver tissue from one port space to another **(Fig. 8b).** Reticular fibers (Coll3) monitored the distribution of Coll1. **(Fig. 8c).**

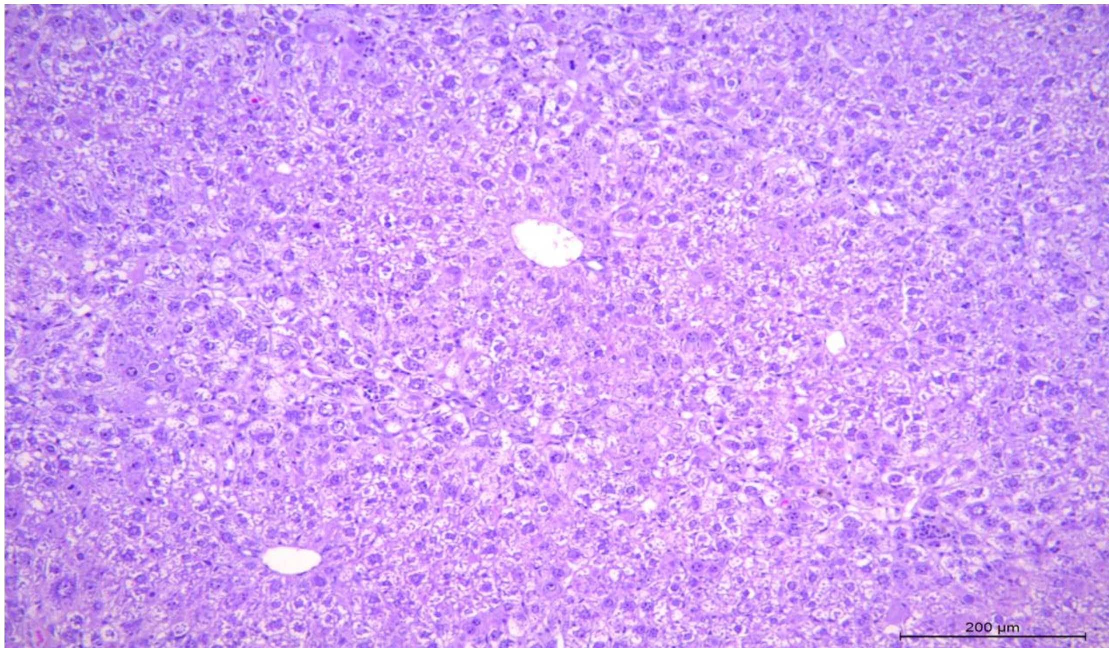


Fig. 8a. Liver tissue of $MIF^{-/-}$ TAA-treated animals (Hematoxylin & Eosin, scale bar 200μm). The liver has a more pronounced irregular structure, with a mixed inflammatory infiltrate in the Disse spaces and perivascularly along large blood vessels. Pronounced fibrosis (bridging porto-portal) and micronodular cirrhosis were also present. Micro- and macrovesicular fatty change, focal necrosis and apoptosis of hepatocytes were present. Mallory's body and numerous hemosiderophages were also observed.

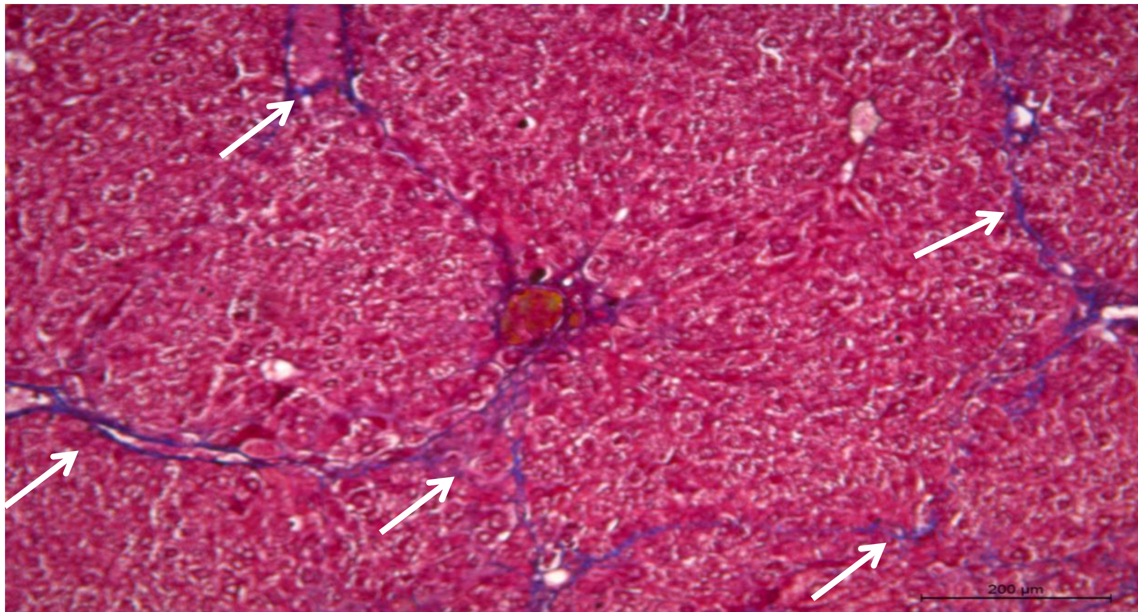


Fig. 8b. Liver tissue of TAA-treated MIF$^{-/-}$animals (Masson's trichrome staining, scale bar 200µm). Masson-stained Coll1 bridges the liver tissue from one port space to another (bridging) located perilobulary. Masson staining showed that collagen staining was blue in liver sections (white arrows).

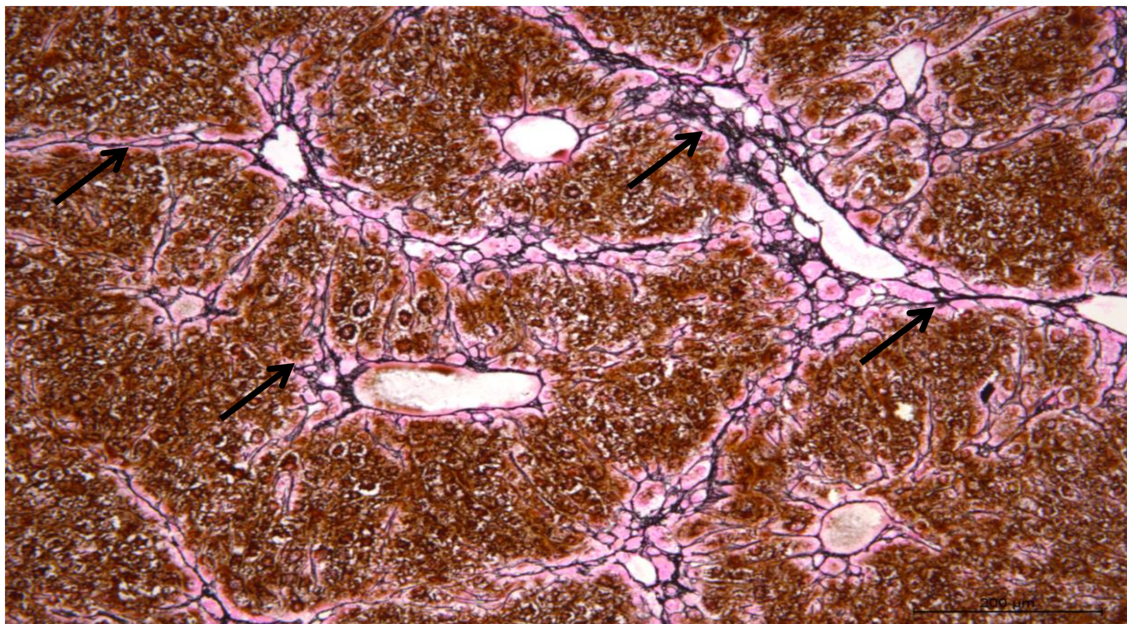


Fig. 8c. Liver tissue of MIF$^{-/-}$-TAA-treated animals (Reticulin staining, scale bar 200µm). Reticular fibers (Coll3) monitored the distribution of Coll1 *(dark arrows)*

Betaine supplementation of TAA-treated wild-type animals improved the histological finding of liver tissue. The histological structure of the liver was less irregular with scanty fibrosis (bridging porto-portal) and mild inflammatory infiltrates. Mallory bodies, rare hemosiderophages and rare focal hepatocyte necrosis were observed. The number of mitoses was 0/10 HPF to 5/10HPF **(Fig. 9a).** Masson-verified Coll1 bridged the liver tissue from one port space to another (perilobulary) **(Fig. 9b).** Reticular fibers (Coll3) monitored the distribution of Coll1 **(Fig. 9c).**

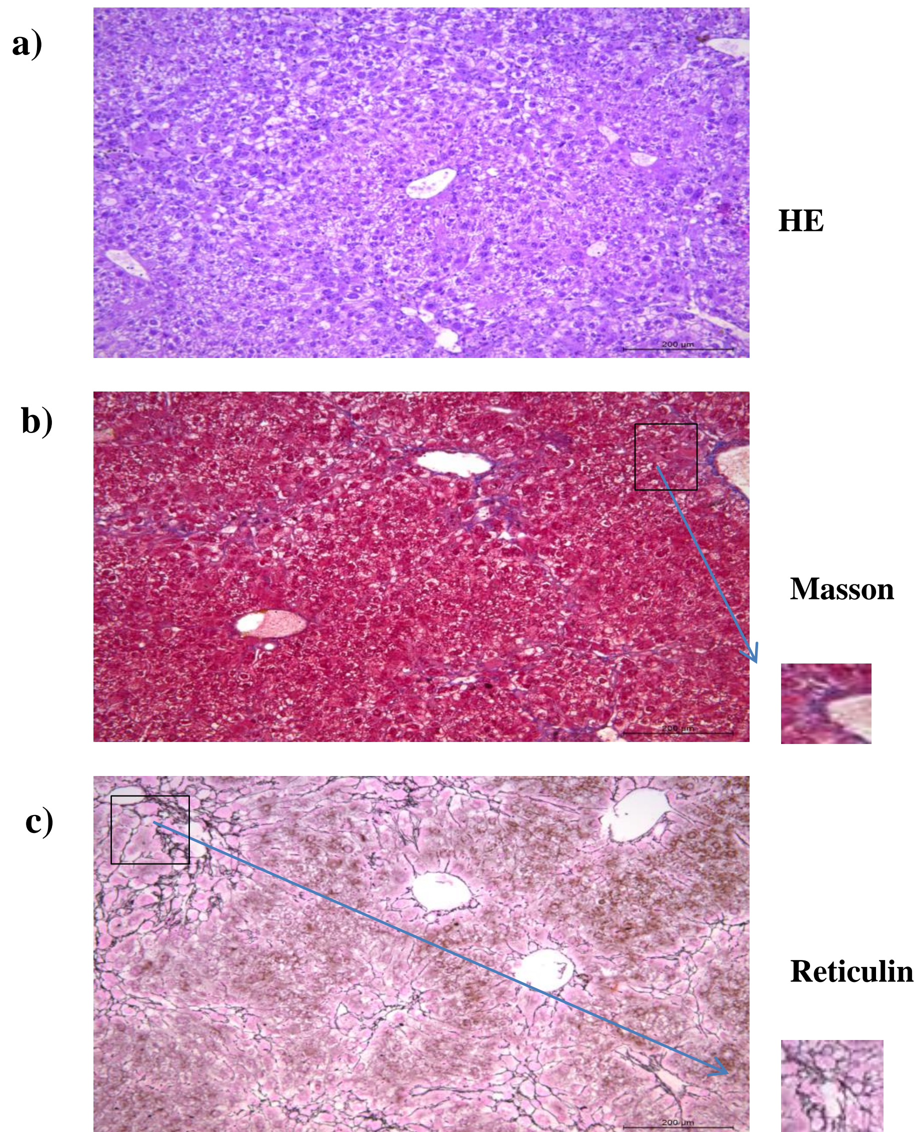


Fig. 9a,b,c. Effect of betaine supplementation on liver tissue histopathology in the TAA-induced liver fibrosis (H&E, Masson, Reticulin, scale bar 200μm). Pathohistological findings of liver tissue are described in the text.

After betaine supplementation, MIF knockout C57BL/6 mice had irregular histological structure of the liver tissue with mild fibrosis (bridging porto-portal) and mixed inflammatory infiltrates. Micro- and macrovesicular fatty changes were present. Mallory bodies and rare apoptotic bodies were observed. Kupffer cell hyperplasia and rare focal necrosis of hepatocytes were present. The number of mitoses 2/10 HPF to 5/10HPF and pathological mitoses were present **(Fig. 10a).** Coll1 stained with Masson bridged the liver tissue from one port space to another located perilobulary **(Fig. 10b).** Reticular fibers (Coll3) monitored the distribution of Coll1. **(Fig. 10c).**

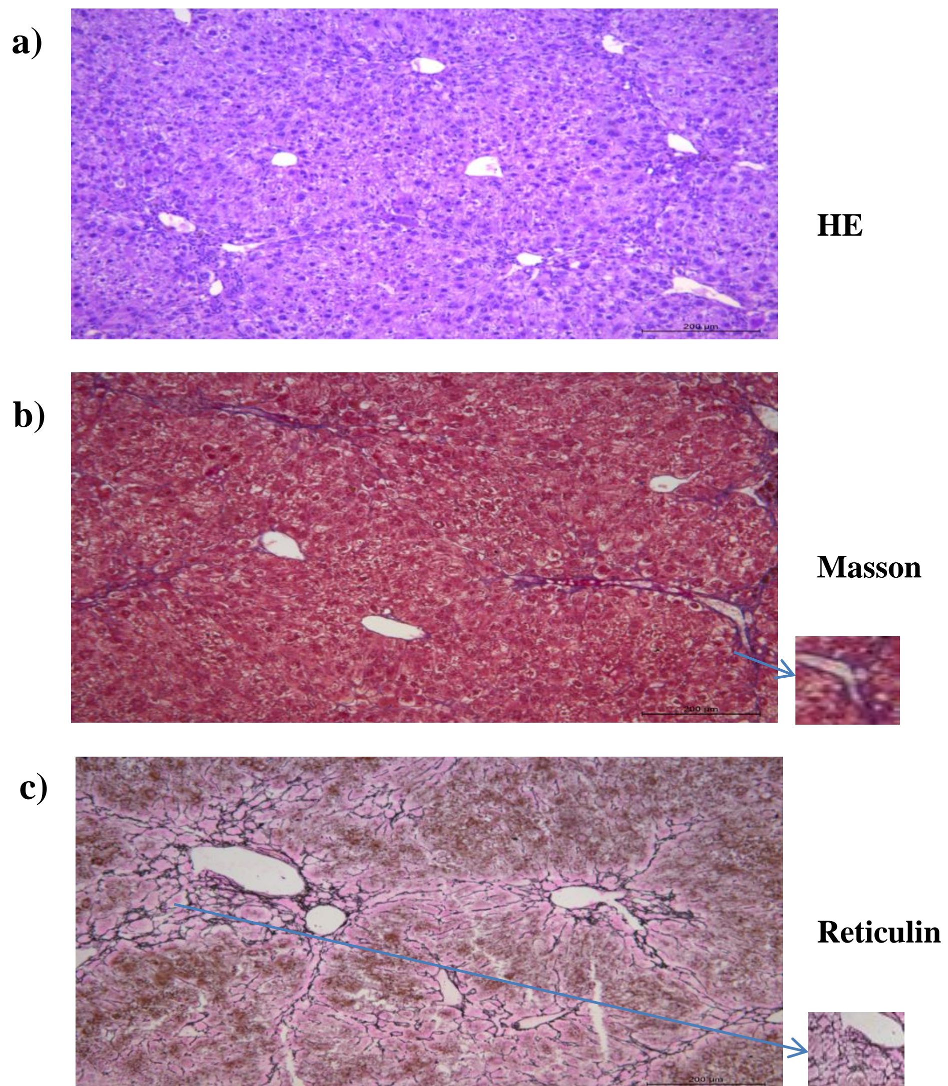


Fig. 10a,b,c. Effect of betaine supplementation on liver tissue histopathology in the TAA-induced liver fibrosis of $MIF^{-/-}$ mice (H&E, Masson, Reticulin, scale bar 200μm). Pathohistological findings of liver tissue are described in the text.

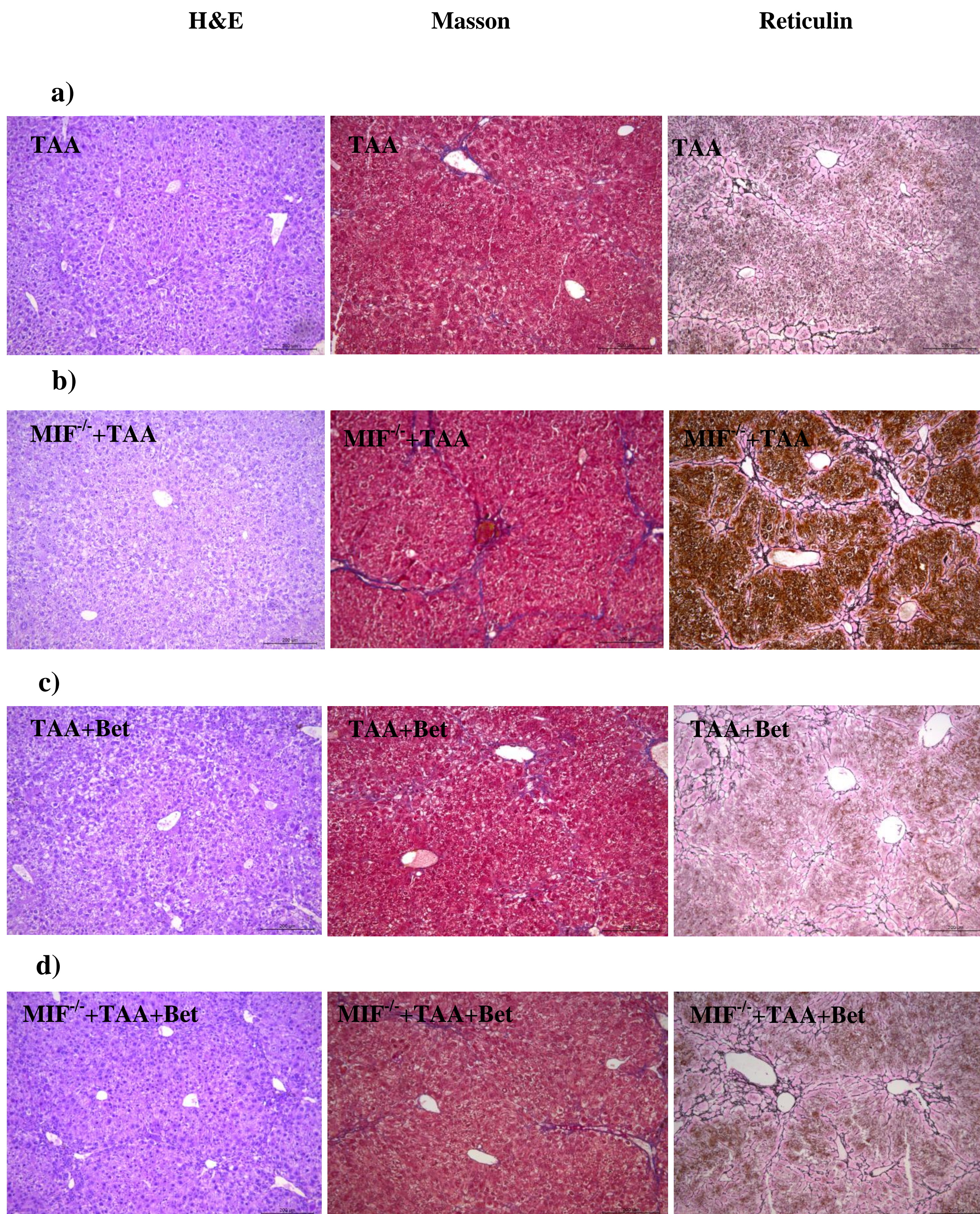


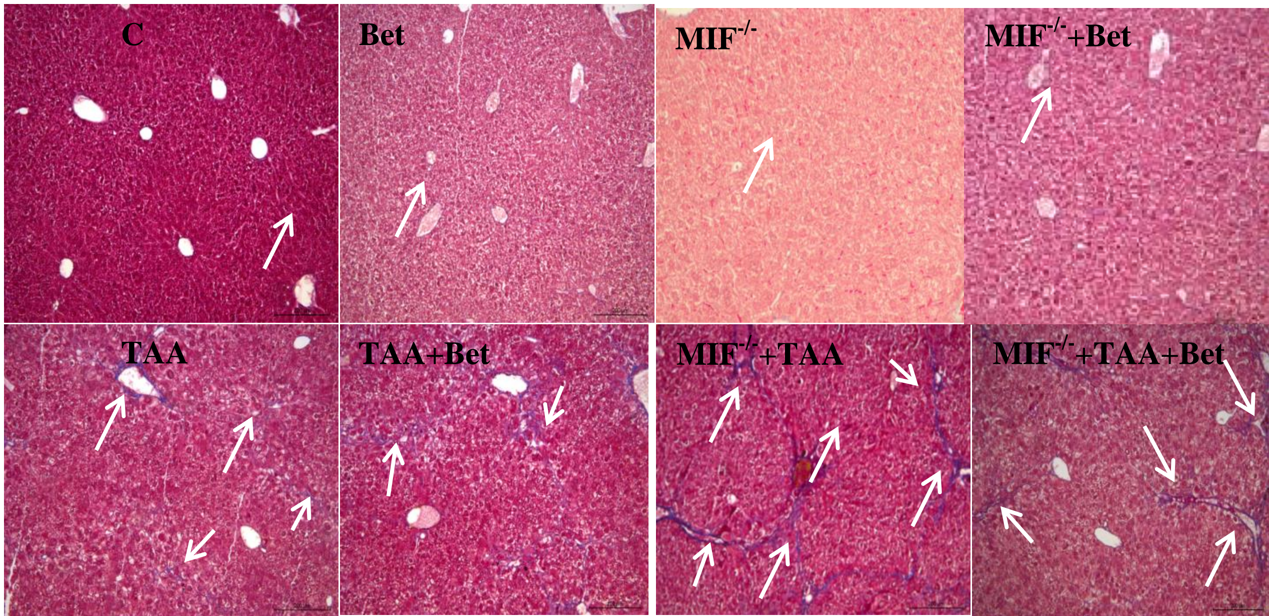
Fig. 11. The effects of betaine and MIF on liver morphology in TAA-induced liver fibrosis in mice **(a)**, MIF$^{-/-}$+TAA **(b)**, TAA+Bet **(c)** and MIF$^{-/-}$+TAA+Bet **(d)** groups: Comparation review of pathohistology of liver tissue (H&E, Masson, Reticulin, scale bar 200µm.) of investigated animals. Pathohistological findings are described in the text.

For abbreviations see Table 1. H&E, Hematoxilyn&Eosin.

4.7.1. The effects of MIF and betaine on ECM deposition inTAA-induced liver fibrosis

TAA-induced liver fibrosis in mice was characterized by accumulation of ECM (Coll1 and Coll3) which was detected with Masson's trichrome (Fig. 12A) and reticulin staining (Fig. 13A). Quantification of liver fibrosis in TAA group (2.15 ± 0.20 %) showed significantly increased accumulation of ECM (Coll1) compared to control (0.15 ± 0.02 %) ($p<0.01$). However, the fibrosis score was significantly higher in the liver of TAA-treated MIF deficient mice (5.45 ± 0.65 %) compared to wild-type TAA-treated mice ($p<0.01$). Betaine supplementation TAA-treated mice mitigated liver fibrosis (1.45 ± 0.15%) in comparison with the TAA group ($p<0.05$). Furthermore, betaine supplementation TAA-treated MIF deficient mice reduced ECM accumulation (3.23 0.41%) compared to the $MIF^{-/-}$+TAA group, but increased compared to the TAA+Bet group ($p<0.01$) (Fig. 12B). Similar to this, quantification of liver fibrosis in the TAA group (2.35 ± 0.21%) showed significantly increased accumulation of ECM (Coll3) compared to the control (0.14 ± 0.03%) ($p<0.01$). However, the fibrose score was significantly higher in the liver of TAA-treated MIF deficient mice (5.15 ± 0.67%) compared to wild-type TAA-treated mice ($p<0.01$). Betaine supplementation TAA-treated mice mitigated liver fibrosis (1.25 ± 0.15%) in comparison with the TAA group ($p<0.05$). Furthermore, betaine supplementation TAA-treated MIF deficient mice reduced ECM accumulation (3.72 0.40%) compared to the $MIF^{-/-}$+TAA group, but increased compared to the TAA+Bet group ($p<0.01$) (Fig. 13B).

A



B

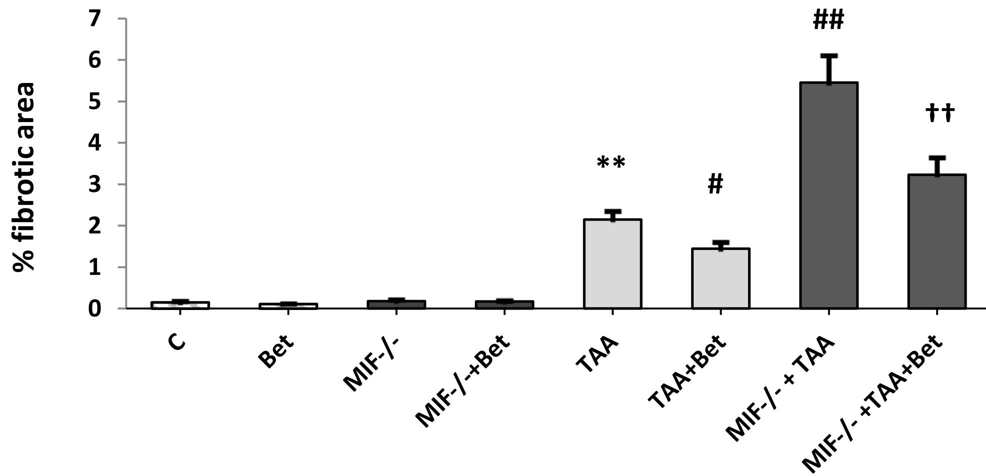
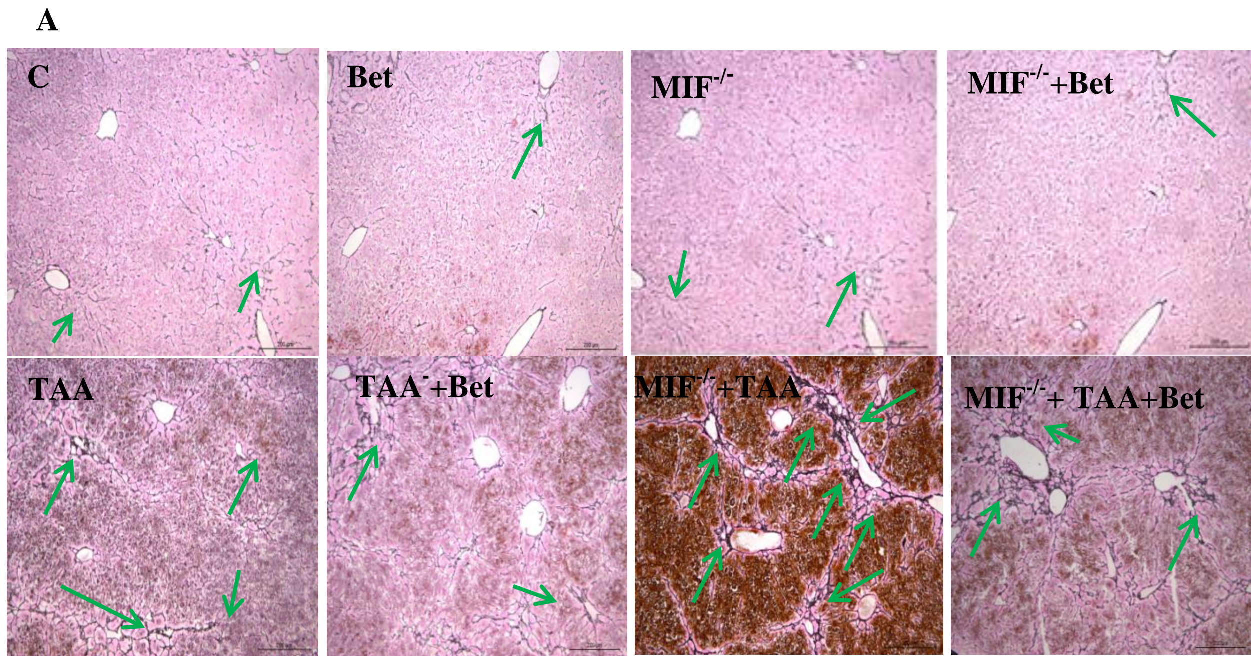


Fig. 12. A) Effects of MIF and betaine on ECM deposition in TAA-induced liver fibrosis (Masson's Trichrome staining, scale bar 200µm). **White arrows show Coll1. B)** Quantification of the percentage of fibrotic area in the liver (scoring systems with morphometric analysis of liver tissue fibrosis).

Significance of the difference was estimated by a two-way analysis of variance (ANOVA) with Tukey's post hoc test (** p<0.01). with Tukey´s post hoc test; **p<0.01 vs.C; $^{\#}$p<0.05, $^{\#\#}$p<0.01, vs. TAA;††p<0.01 vs. MIF$^{-/-}$+TAA and TAA+Bet. The data are presented as mean±SD.

For abbreviations see Table 1.

A



B

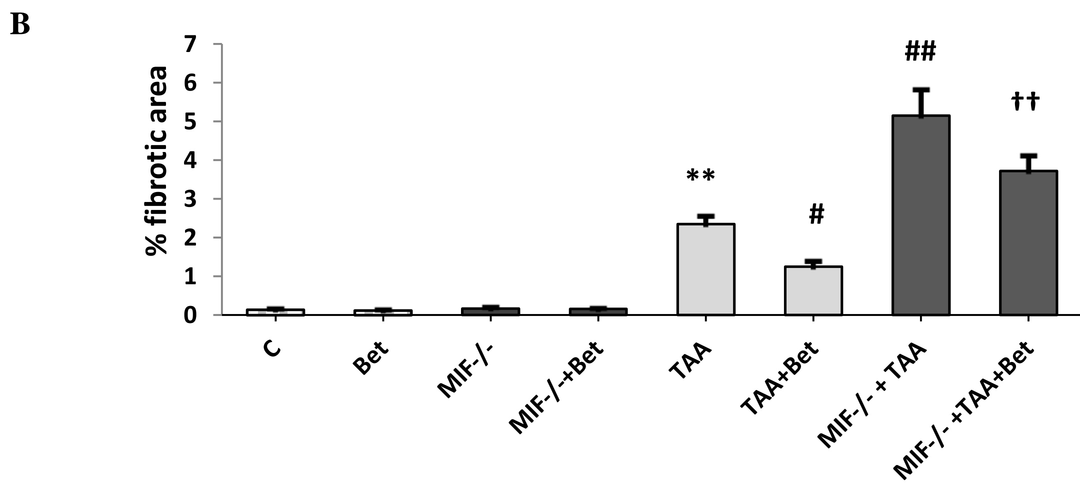


Fig. 13. A) Effects of MIF and betaine on ECM deposition in TAA-induced liver fibrosis (Reticulin staining, scale bar 200µm). **Green arrows show Coll3. B)** Quantification of the percentage of fibrotic area in the liver (scoring systems with morphometric analysis of liver tissue fibrosis).

Significance of the difference was estimated by a two-way analysis of variance. (ANOVA) with Tukey's post hoc test (** $p<0.01$). with Tukey´s post hoc test; **$p<0.01$ vs.C; $^{\#}p<0.05$, $^{\#\#}p<0.01$, vs. TAA; $^{\dagger\dagger}p<0.01$ vs. MIF$^{-/-}$+TAA and TAA+Bet. The data are presented as mean±SD.

For abbreviations see Table 1

5. DISCUSSION

Acute and chronic liver injury occurs as a consequence of various etiological factors, such as viruses, alcohol, drugs, or other xenobiotics (175, 176). Numerous toxic agents cause hepatocellular necrosis, apoptosis, releasing dangerous signals from damaged hepatocytes, Kupffer cells and other inflammatory cells. As a consequence of cell injury or activation, inflammatory cytokines and ROS/RNS are generated which lead to altered redox homeostasis and amplification of inflammation. Then, proinflammatory cytokines, chemokines and other pathological factors released from these cells induced HSC activation and its transdifferentiate into myofibroblast-like cells which are responsible for matrix accumulation (38, 177). TGF-β1 and PDGF-BB are the major profibrogenic cytokines in liver fibrogenesis.

TAA, particularly especially its highly reactive metabolite, is a powerful hepatotoxin that causes a redox imbalance, inflammatory response and consequent fibrosis, so it is an excellent model of liver fibrosis because it mimics chronic liver disease in the human population (131, 132, 133). Oxidative stress and inflammation are the most common mechanisms involved in triggering liver damage and, depending on the cause and duration of action, lead to fibrosis, cirrhosis and hepatocellular carcinoma (178, 179).

Experimental models of liver fibrosis are necessary to better understand the complex interaction between chronic liver inflammation and oxidative distress, fibrosis and tissue remodeling in chronic non-communicable liver diseases and may pave the way toward novel strategies in prevention and treatment (132, 178).

Our study has shown an increase in liver/body ratio (liver index) in mice treated with TAA compared to the control mice (Table 1). This weight loss and liver weight gain may be due to the toxic effects of TAA (180). Additionally, the increased deposition of ECM in liver fibrosis is associated with an elevation of the liver index in TAA-treated mice (181). However, the liver/body weight ratio was significantly higher in $MIF^{-/-}$ mice treated with TAA, indicating the MIF has the effect of decreasing deposition of ECM and reducing this ratio (Table 1). In this study, we investigated the role of MIF in hepatic function, oxidative stress, inflammation and fibrogenesis in TAA-induced liver injury in mice, and the protective effect of betaine. The hepatotoxicity of TAA was confirmed in our study by significant increases of serum activity in ALT, AST, ALP and γGT, (Fig. 1A, 1B, 1C, 1D) can be considered as sensitive biomarkers enzymes of liver injury reflecting membrane cell injury, hepatocellular necrosis, and epithelial cells of biliary duct injury (180). ALT is the most important marker of hepatocellular injury and disease progression, but AST is a better predictor of histological activity (182). Also, TAA caused an increase in serum bilirubin levels (Fig. 1E), while the concentration of albumin remained unchanged (Fig. 1F). An increase in serum enzyme aminotransferases activity together with more than double the increase in the total bilirubin concentration is considered as a marker index of hepatotoxicity (180, 183, 184). However, in TAA-

treated $MIF^{-/-}$mice, there was a significant decrease in enzyme activity aminotransferases and bilirubin concentration, which shows that MIF contributes to an increase in serum ALT, AST activity, and total bilirubin level (Fig. 1A, 1B, 1E), indicating that MIF is an important factor in the hepatotoxic effects of thioacetamide.

Betaine is the key component of the methionine–homocysteine cycle (151). It exerts antioxidant effects by increasing the synthesis of glutathione (151,153). The protective effect of betaine has been investigated in ALD and NAFLD (159, 164, 161, 185), drug- and toxin-induced liver injury (186, 187, 188, 189, 190, 191), as well as bile acid-induced liver injury (189). However, the effects of betaine on prooxidative and proinflammatory effects of MIF in TAA-induced hepatotoxicity are not clarified.

Our results have shown that betaine supplementation in TAA-treated wild and $MIF^{-/-}$ mice have caused a significantly lower liver/body weight ratio (Table 1). Betaine supplementation in TAA-treated animals caused a significant decrease in serum of ALT and AST activities, γGT, without affecting ALP activity. Besides, betaine prevented the TAA-induced hepatotoxicity in $MIF^{-/-}$ mice by significantly decreasing ALT and AST activity, compared to TAA+Bet and $MIF^{-/-}$+TAA groups (Fig. 1A, 1B). This indicates that the protective effects of betaine are achieved through oxidative and inflammatory effects of MIF, as well as independent from it. Also, betaine decreased the total bilirubin concentration, but only by the inhibition of MIF effects (Fig. 1E). Betaine achieves this by preventing the contact of MIF-mediated ROS and the membrane, thus preventing cell damage. This is accomplished by the formation of a protective membrane around cells (153). The other mechanism scavenging intracellular ROS includes antioxidant mechanisms of defenses via the methionine-homocysteine cycle (153). On the contrary, betaine decreases γGT activity independent of MIF (Fig.1D), so the other effects of betaine are included in hepatoprotection. These results can be explained by the hepatoprotective and anti-apoptotic effects of betaine, as well as the stimulation of autophagy, mitochondrial protection and regulation of energy metabolism which ameliorates liver function (155, 161, 189). Due to all of these effects, together with suppression of HSC activation, betaine exerts antifibrogenic action, not only in TAA toxicity but also in CCl4- and alcohol-induced liver fibrosis or NAFLD (148, 159, 160). Similar to our study, betaine supplementation decreases the ALT and AST activity and improves the histological findings in the liver mice fed with a methionine-choline deficient diet (161). The hepatoprotective effect of betaine is shown by a decrease in insulin resistance and aminotransferase activities, which was also confirmed in mice with high-fat-diet-induced NAFLD (185).

TAA is a hepatotoxin that is rapidly bioactivated by cytochrome P450 oxidation processes, resulting in TAA-intermediates, S-oxide and SS-dioxide, which are responsible for TAA hepatotoxicity (141). We observed that chronic administration of TAA/eight weeks in mice elevated

MDA, AOPP and TOS in the liver tissue (Fig. 2A, 2B, 2G). On the other hand, TAA induced the depletion of the antioxidative potential of hepatocytes via a decrease in enzyme activity of SOD, CAT, reduced concentration of thiols and TAS (Fig. 2C, 2D, 2E, 2F). Excessive ROS production induced by TAA causes lipid peroxidation, oxidative damage of proteins and DNA, inducing hepatocellular necrosis, apoptosis, inflammation and fibrosis (136, 141). Our study has shown that, besides an increase in serum aminotransferases activity (Fig. 1), MIF contributes to an increase in the concentration of MDA and AOPP (Fig. 2A, 2B). The prooxidant effect of MIF is reflected in the reduction of lipid peroxidation and oxidation, protein damage, in the liver of thioacetamide-treated $MIF^{-/-}$ mice compared to the wild strain (Fig. 2A, 2B). This suggests that MIF plays a role in the hepatotoxic and prooxidative effects of thioacetamide. MIF regulates cellular redox processes and inhibits oxidative stress-induced apoptosis (192). Moreover, the pro-oxidative effect of MIF in TAA-induced liver injury can be partially explained by a decrease in thiols in the liver, without changes in SOD and CAT activity (Fig. 2C, 2D). In contrast to our results, *Israelson et al.* showed that MIF suppressed misfolded SOD1 accumulation in experimental amyotrophic lateral sclerosis (193). Recent research shows that MIF has an oxidative effect in the triggering of epithelial-mesenchymal change and promotes the pathogenesis of proliferative vitreoretinopathy (194).

On the other hand, results in some studies evident of the antioxidant potential of MIF (121, 195, 196). The mechanisms of the antioxidant role of MIF may be different depending on its redox state, receptor-coupled signaling pathways, and the organ itself. MIF has a cardioprotective effect on radiation-induced cardiac senescence (195). As a stress-regulating cytokine, MIF plays the main role in protecting the heart and kidney from I/R injury (196, 197). MIF contains oxidoreductase activity of thiol protein, determined by a characteristic sequence of Cys-Ala-Leu-Cys, similar to that found in thioredoxin (99, 100), and that this enzyme activity is protective against oxidative injury induced by I/R injury (99, 100). The enzymatic activities of MIF contribute to the regulation of cellular redox stress. MIF occurs in two isoforms, reduced MIF (redMIF) and oxidized MIF (oxMIF). Expression of oxMIF in various inflammatory disorders is the disease-related isoform of MIF (101,196). Also, MIF exhibits antioxidant and anti-apoptotic effects due to its catalytic thiol protein oxidoreductase activity (100, 192) and gene induction of antioxidant enzymes (198), increases the ratio of reduced and oxidized glutathione, decreases oxidative protein damage, and ameliorates mitochondrial function in cardiomyocytes (199). *Yukitake et al.* demonstrated that macrophage MIF, a regulator of antioxidant response element- (ARE-) mediated gene transcription, induces expression of heme oxygenase-1, glutathione-S-transferases, -glutamylcysteine synthetase, uridine diphosphate glucoronyl transferase, and other enzymes and cytoprotective proteins that protect cells from oxidative stress. According to this, regulation of redox homeostasis via the MIF-

ARE axis can be a possible target in the therapy of many diseases that have oxidative stress at its pathogenesis (198). However, MIF in TAA-induced hepatotoxicity exerts a pro-oxidative effect. Its mechanism on an antioxidant defense system is not clear, so this MIF/ARE axis cannot be a universal therapeutic target in oxidative organ damage.

TAA-induced hepatotoxicity is characterized by mitochondrial dysfunction and remodeling, severe oxidative stress and inflammation (189, 200). It is known that betaine exerts its antioxidative effect through S-adenosylmethionine (SAM), as a "ROS scavenger", maintaining the integrity of the biomembrane (153, 201), as well as increasing the activity of antioxidative enzymes (161, 202, 203,). Results of this study have shown that betaine supplementation exerts protective effects, indicated by a decrease in MDA, AOPP, and TOS concentration in TAA-treated animals (Fig. 2A, 2B, 2G) as well as an increase in antioxidant activity of SOD, CAT and level of thiol groups, and TAS (Fig. 2C, 2D, 2E). Thus, betaine significantly decreases liver oxidative stress in MIF$^{-/-}$ deficient TAA-treated mice, reducing the lipid peroxidation and TOS, compared to the TAA+Bet and MIF+Bet groups (Fig. 2A, 2G). Betaine reduces MIF-mediated lipid peroxidation and increases antioxidant capacity. These findings show that betaine inhibits oxidative MIF pathways via the detoxification of ROS generated from the metabolism of TAA in the liver. Betaine inhibits NF-κB the and thus inhibits the association between stress and inflammation (155). Furthermore, betaine's hepatoprotective effect can reduce endogenous DAMP generation, thereby inhibiting the transcription factor NF-B. An increase in the intracellular pool of MIF acts as DAMPs, and induces ROS generation, ER stress, and suppresses activation-induced apoptosis p53 (153, 204). In contrast, betaine exerts antioxidative and antiinflammatory effects by alleviating ER stress, apoptosis (155), and reducing lipid peroxidation (205). Besides reducing the oxidative protein damage, betaine increases the antioxidative activity of SOD, CAT and TAS, via mechanisms that do not include MIF (Fig. 2C, 2D, 2F). The antioxidative mechanism of betaine is to control the metabolism of SAA (26, 153, 155). Betaine converts homocysteine to methionine, which is a very important antioxidant, through the GSH synthesis and the reduction of oxidative stress. Also, betaine exerts a hepatoprotective effect by inducing the SAM synthesis in hepatocytes (206), which plays a crucial role in restoring the biomembrane (153, 207). SAM is a valuable antioxidant for ROS scavenging and a modulator of GSH metabolism (206). The results of *Okada et al*. have shown that the 1% betaine treatment has an antioxidative effect which is caused by an increase in the GSH concentration and related enzymes, following D-galactosamine-induced hepatotoxicity (208). GSH has a very important role in the detoxification of chemically reactive metabolites (209). Impaired GSH synthesis is responsible for altered redox homeostasis and oxidative injury in many liver diseases including ALD, NAFLD and drug-induced liver injury (DILI) (210). Also, a decrease in GSH level induces activation of HSC, leading to liver fibrosis (211), while restoration in hepatic

GSH level mitigates hepatic fibrosis (212). GSH contributes to the redox regulation of cellular thiol. Our results have shown that betaine restores thiol groups after prooxidative effects of MIF in TAA-induced liver injury (Fig. 2E). Betaine inhibits ROS production and NF-κB activity by maintaining thiol level, particularly GSH (213). Also, betaine exerts an antioxidant effect by diffusing through the lipid bilayer membrane into the cells (214) and increases CAT activity, which protects the cell against the excess accumulation of hydrogen peroxide (H_2O_2) (153). Moreover, betaine acts as an osmoprotectant that protects cells against stress inducers (150). Also, betaine has been shown to inhibit cytokine and ROS production as an important organic osmolyte in Kupffer cells (154). In our investigation, betaine supplementation increased the activities of liver SOD and CAT (Fig. 2C, 2D). SOD converts superoxide anion (O_2^-) to the less toxic H2O2, which metabolizes into the water by the activity of glutathione peroxidase (GPx) and CAT. SOD prevents cell injury caused by this highly toxic O_2^-, and the other enzyme, CAT, has detoxifying effects against the excessive accumulation of H_2O_2. Also, it has been shown that betaine protects the mitochondrial function and thus reduces the oxidative stress in the liver and other organs, increasing the antioxidative capacity (189, 215). Previous studies have shown that betaine exerts antioxidative effects against CCl4-induced hepatotoxicity down-regulation of CYP2E1 expression and restored SOD, CAT and GPx activities and TAS (216).

The results of our study have shown an increase in serum concentration of CRP and cytokines, IL-6 and IFN-γ in the liver TAA-treated mice (Table 2). TAA-induced redox imbalance and oxidative stress in the liver are crucial triggers of inflammation and fibrosis. Increased ROS/RNS production and oxidative stress cause the lipid peroxidation of cell membranes, and oxidative protein and DNA damage, inducing hepatocellular necrosis, apoptosis, amplified inflammation and promotes HCS activation (216). The proinflammatory effect of MIF includes the release of proinflammatory cytokines and iNOS, from monocytes/macrophages and Th1 cells. It also includes the stimulation of chemokine functions and the inhibition of immunosuppressive and antiinflammatory effects of glucocorticoids (73).

MIF was found to contribute to an increase in the concentrations of CRP, IL-6, and IFN-γ (Table 2), MDA, AOPP (Fig. 2A, 2B), and transaminase activity (Fig. 1A, 1B), indicating that MIF plays an important role in the proinflammatory, prooxidative, and hepatotoxic effects of TAA. The proinflammatory effect of MIF can at least partly be explained by its pro-oxidative effect, which is reflected in the reduction of lipid peroxidation in the liver in TAA-treated $MIF^{-/-}$ mice compared to the wild strain (Fig. 2A). The reactive metabolites of TAA (S-oxide and the highly reactive SS-dioxide) can induce the secretion of MIF from intracellular pools, and its transcription (217). The link between ROS, MIF and inflammation can be explained by stimulating the activation of NF-kB by ROS. Activated NF-kB is a major transcription factor that increases the production of IL-1β, IL-

6, TNF-α, chemokines, growth factors, cyclooxygenase-2, CRP, and other mediators (218). *Nicholas et al.* discovered that MIF increases acute-phase protein synthesis in hepatocytes, as well as the stimulation of acute-phase proteins by other cytokines. The MIF increases the synthesis of CRP by primary human hepatocytes (219). Similar to our results, other studies have also shown the proinflammatory effect of MIF mediated by oxidative stress (220, 221). The results of our study showed that MIF caused a significant increase in IFN-γ concentrations in liver TAA-treated mice (Table 2). *Eissa et al*. observed that berberine decreased of IFN-γ level in TAA-treated rats, but that decrease was less in the protection group than in the berberine-treatment animals (222). These results indicate that a high concentration of IFN-γ may be a safety factor in protecting immunity and possible infections (223). It is known that IFN-γ is used as a therapy for hepatitis viral infections (224). IFN-γ is a proinflammatory cytokine that contributes to innate and adaptive immunity. MIF counter-regulates the inhibitory effects of glucocorticoids, increasing IFN-γ production, controlling immunity and inflammation (82). MIF stimulates IFN-γ-producing NK cells which promotes the effector mechanisms to control immunity to T. gondii infection (225). Moreover, *Wang et. al.* have shown an increased level of IFN-γ in patients with hepatic fibrosis (226).

MIF is a multipotent proinflammatory cytokine, which contributes to the progression of many chronic inflammatory disorders (105, 108, 119, 118, 220). In obesity, as a chronic condition of low-grade inflammation, the secretion of MIF and IL-6 is increased, which contributes to insulin resistance (73). Recent studies show that liver MIF induces ER stress with unfolded protein response activation (227, 228), and stimulation of inflammasomes (229), and thus contributes to chronic low-grade inflammation. High expression of MIF was indicated in type 2 diabetes mellitus in both mice (110, 111) and humans (112). Also, the proinflammatory effects of MIF are proven in high fat diet-induced obese mice (113). On the other hand, *Gligorovska et al*. have shown an increase in obesity and insulin resistance in fructose-diet $MIF^{-/-}$ mice (114). Similar to, in NAFLD mice-induced high-fat diets, MIF exerts an anti-inflammatory effect through the CD74/AMPK signaling pathway, which inhibits TNF-α and IL-6 synthesis, and macrophage change from M1 to M2 phenotype. M2 macrophages produce much more anti-inflammatory cytokines than M1, which promote tissue regeneration (129). This indicates that the action of MIF in the liver has a dual effect (either promoting or protecting the liver inflammation), depending on the etiology of the liver disease, stage of disease, altered MIF expression and complexity of the MIF signal (73, 130). Also, MIF may play a protective role in resolving fibrosis, which suggests a complex interaction between MIF and other factors in the progression of liver disease (230). In TAA-induced liver fibrosis, MIF has been shown to exhibit anti-fibrogenic action by directly inhibiting HSC activation via the CD74/AMPK signaling pathway, rather than by modulating the inflammatory response (231, 232).

MIF exerts inflammatory effects mediated by the MIF/CXCR2 or MIF/CXCR4 signaling pathways (75).

It has been shown that betaine, as a natural substance, exerts anti-inflammatory effects in diseases, such as ALD and NAFLD (159, 185). Since inflammation is one of the mechanisms of liver damage caused by toxins, our study has shown that betaine significantly decreases the serum concentration of CRP and hepatic IL-6 and IFN-γ levels in TAA-treated mice (Table 2). Additionally, betaine supplementation in $MIF^{-/-}$+TAA-treated mice causes a significant decrease of these cytokines compared to the $MIF^{-/-}$+TAA and TAA+Bet group (Table 2). These results have shown that betaine inhibits MIF-mediated inflammatory mechanisms induced by TAA much more in $MIF^{-/-}$ deficient mice, because of the additional anti-inflammatory effect of betaine. Similar to our results, *Tsai et al*. observed that betaine significantly reduced IL-6 production in the CCl4-induced liver fibrosis (148). Betaine reduced the proinflammatory effects of MIF through the inhibition of expression of NF-kB (155, 233), NLRP3 inflammasome activation (155, 234), ER stress, and apoptosis (155, 227, 228). Also, betaine additionally stimulates the anti-inflammatory effect of MIF via AMPK (155, 235, 236). Other anti-inflammatory and hepatoprotective mechanisms of betaine include osmoprotection since it is one of the most powerful organic osmolytes, and regulation of the methionine-homocysteine cycle, as a methyl group donor (150, 151). Recent studies highlight that betaine supplementation protects the mitochondrial function in experimental models of liver injury (193) by regulating lipid and energy metabolism. The regulation is achieved by increasing lipolysis, tricarboxylic acid cycle and mitochondrial oxidative phosphorylation (237, 238, 239). Similar to other bioactive compounds against liver damage by hepatotoxins, betaine exerts hepatoprotective effects by increasing the antioxidative defense, mitigating inflammation, apoptosis and ER stress, as well as regulating mitochondrial function (155,189, 240).

Liver fibrosis is a complex process characterized by increased production of fibrogenic and inflammatory mediators, and modulation of MMPs and TIMP activity that promote disbalance in the production and degradation of ECM with liver tissue remodeling (8, 9, 38, 177). Activated HSC are master cells in liver fibrogenesis (38, 73, 177). In the physiological state, HSC participate in vitamin A storage, retinol metabolism and the synthesis of ECM components. During chronic liver disease, the redox homeostasis is altered. Excessive production of free radicals together with decreased antioxidative capacity causes oxidative stress. Oxidative stress acts on HSC either directly or indirectly through paracrine stimulation of other cells including Kupffer cells activation and hepatocellular injury. Then, proinflammatory cytokines, chemokines and other pathological factors released from these cells induced HSC activation and transdifferentiate into myofibroblast-like cells (38, 177). TGF-β1 and PDGF-BB are the major profibrogenic cytokines in liver fibrogenesis. TGF-β1 stimulates HSC transdifferentiation into myofibroblasts which produce and

release collagen, other components of ECM and particularly fibronectin which include cell growth, migration, adhesion and differentiation (52). PDGF-BB is the most important mitogen for HSC, causing their proliferation, chemotaxis, migration, transdifferentitation, as well as collagen synthesis and deposition of ECM (51).

The results of our study have shown an increase in hepatic TGF-β1 and PDGF-BB levels in the liver TAA-treated mice compared to control (Fig.3A, 3B). Similar to our results, in the same model on rats, the expression of α-SMA, TGF-β1, collagen, and other ECM components was increased due to the increased activity of HSC (222). Furthermore, *Ghafoory et. al.* have shown an increase in TGF-β1 level in CCl4-induced liver fibrosis which is derived from platelets (241). PDGF antagonists mitigate hepatic fibrogenesis in TAA-, CCl4- and bile ductal ligation (BDL)- models of liver fibrosis (242, 243, 244). TAA is bioactivated in the liver, leading to the creation of its S-oxide and reactive SS-dioxide, which is responsible for TAA hepatotoxicity (141). These reactive metabolites of TAA covalently bind to macromolecules in the liver and cause hepatocellular necrosis of periportal hepatocytes (132, 145). Additionally, cytochrome P450 2E1 (CYP2E1) and flavin-adenine-dinucleotide (FAD)-containing monooxygenases induce ROS generation, altered redox homeostasis which promotes hepatocellular injury, oxidative stress, inflammation, and lipotoxicity (142, 143). The effects of chronic TAA exposure on liver damage depend on the route of administration, dosage, length of exposure and animal species. Bridging fibrosis develops after prolonged TAA administration and lasts for several weeks after TAA withdrawal. TAA- and CCl4- induced ROS production in the liver promotes inflammation and fibrosis (131). Leptin signaling also contributes to fibrogenesis via TGF-β1 which is released from Kupffer cells following liver damage (10, 241). TGF-β1 leads to redox imbalance by increasing ROS generation or reduction in the synthesis of GSH and other antioxidant enzymes. ROS production induced by TGF-β1 originates from mitochondrial disfunction and NADPH oxidases activity. On the other hand, ROS mediate many of TGF-β1's profibrogenic effects, leading to ECM synthesis and deposition that lead to the progression of fibrosis. PDGF is released from numerous cells, such as platelets, endothelial cells, myofibroblasts and macrophages (9, 51, 246, 247,) and stimulates HSC proliferation and ECM production. PDGF-BB promotes HSC proliferation and activation through PDGF receptors. PDGF-BB may be the major cytokine for HSC activation in TAA-induced liver fibrosis (248). Also, PDGF-BB is released from activated HSC (9). PDGF-BB overexpression in liver macrophages also contributes to liver fibrogenesis (249). Besides these cells, recent studies have shown that bone marrow fibroblast cells express the fibrogenic mediators TGF-β1 and PDGF (250, 251, 252), which are crucial for the activation and proliferation of myofibroblasts (252, 253).

As it is known, activated HSC, whose main marker α-SMA, play a pivotal role in hepatic fibrogenesis by producing collagen and other ECM components. The main pathohistological characteristic in the fibrotic liver is an accumulation of ECM, predominantly of Coll1 and Coll3 (42). Maintaining the interaction between MMPs and TIMPs is the key process in the turnover of liver ECM and a normal liver structure (62). It has been found that dysregulation of MMP expression contributes to the progression of liver fibrosis. Besides, degradation and deposition of ECM, MMPs/TIMP contribute to liver carcinogenesis and regeneration (60).Two key MMPs secreted from HSCs are MMP-2 and MMP-9 (60, 61, 62).

Fig. 4A and 4B show densitograms of gelatin zymography of MMPs in tissue homogenates of TAA-induced liver fibrosis in mice, as well as densitograms of standard human recombinant MMP-2 and-9. Our results indicated increased densitometric area of MMP-2, MMP-9, dimer MMP-9, and TIMP-1 in the liver TAA-treated mice compared to control, after the 8-week experimental period (Fig. 5a, 5b). Similar to our results, *Bingul et al.* (160) indicated that HFD + CCl4- and ethanol + CCl4 induced liver fibrosis resulted in HSC cell activation and increased expression of α-SMA, Coll1A1, and TGF-β1 proteins, as well as expression of TIMP-1 and MMP-2 mRNA. Besides, TAA-induced liver fibrogenesis in male Sprague-Dawley rats increased transcription of both MMPs and TIMPs, but only TIMPs showed increased translation (254).

MMP-2 expression is increased in liver fibrosis, especially in the progressive phase where it is involved in ECM degradation (63). Also, increased MMP-2 activity was found in alcoholic liver cirrhosis (64), chronic viral hepatitis (65), IRI (255) and BA (256). MMP-2 is a serum marker for alcoholic liver disease and its activity and its activity is maintained during illness, while MMP-8 and MMP-9 activity were only increased in an advanced stage of liver cirrhosis (64). Besides, MMP-2 acts as an autocrine proliferative and migratory factor and accelerates the activation of HSC (66). On the other hand, it has been reported that MMP-2 can be reduced in liver fibrosis by inhibiting Coll1 expression instead of its degradation (257, 258). Toxin-induced fibrosis resolves quickly, which may be explained by an increase in MMP-2 and a decrease in TIMP-1 (259). Furthermore, the results of some studies showed increased MMP-2 mRNA expression in all stages of fibrosis, while only MMP-2 activity was increased during the resolution of IRI fibrosis (260, 261).

MMP-9, or gelatinase-B, is secreted by numerous cell types, including leukocytes, macrophages, Kupffer cells, and fibroblasts. Its role in ECM remodeling is to degrade ECM components, such as Coll4, elastin, and fibronectin (60, 62). The mechanism of the activation of liver fibrogenesis, as well as excessive increases of MMP-9 activity, is only partially understood. ECM degradation by MMP-9 is a potential mechanism that contributes to the activation of HSC. Besides, MMP-9 contributes to inflammatory response in liver fibrogenesis through activation of

proinflammatory mediators or activation of iNOS (262). Numerous studies related to MMP-9 show that there is a dual role of MMP-9 in hepatic fibrogenesis (60, 62, 64). This enzyme promotes ECM remodeling via releasing profibrogenic or protective factors (60). Results of some studies have shown that MMP-9 may release/activate TGF-β1 from ECM in the early stages of hepatic fibrogenesis (70, 71). Similar to our results, MMP-9 expression is increased in liver fibrogenesis (60), alcoholic liver cirrhosis (64) and IRI (255). Also, in mice with the progression of fulminant liver failure and development of hepatic encephalopathy, inhibition of MMP-9 activity alleviated the symptoms of disease (266). Further, inhibition of MMP-9 activity ameliorates IRI (263) and promotes hepatocellular regeneration (264). On the other hand, MMP-9 can reduce fibrosis by accelerating HSC apoptosis and thus indirectly contributes to fibrinolysis at low TIMP-1 activity (62, 71, 72, 265). According to the findings of *Feng et al.,* Kupffer cell-derived MMP-9 is the most important in matrix remodeling and repair during fibrosis regression in TAA-induced liver fibrosis, and the resolution process is triggered by TAA withdrawal (267). *Sun et al.* discovered that MMP-9 is secreted by fibroblasts and endothelial cells, and plays a very important role in neoangiogenesis, invasion, and metastasis (60). It, therefore, remains unclear, whether MMP-9 promotes fibrosis or leads to its resolution. On the other hand, during fibrosis resolution, monocytes differentiate into scar-associated macrophages (SAMs) with overexpression of MMPs (MMP-9, MMP-12, MMP-13), a decrease of cytokines, chemokines, TGF-β and enhanced expression of CD74 and MIF. Furthermore, SAMs which produce MMPs promote fibrosis resolution (268, 269).

MIF is a multipotent pro-inflammatory cytokine, which contributes to the progression of many chronic inflammatory disorders (27, 271, 272, 273, 274, 275). The proinflammatory effect of MIF includes the release of cytokines (IL1β, IL-6, IFN-γ) and iNOS, from monocytes/macrophages and Th1 cells. The inflammatory mediators activate HSC, which produce numerous cytokines, chemokines that participate in the maintenance of chronic inflammation and progression to liver fibrosis (276). Besides MIF-mediated HSC effects in liver fibrosis, it activates other cells of the liver, such as hepatocytes and inflammatory cells (277).

Since hepatocytes are responsible for the metabolism of numerous hepatotoxic factors, their damage causes oxidative stress and the release of proinflammatory and fibrogenic cytokines. Overexpression of MIF in hepatocytes indicates that hepatocytes are an important source of MIF in liver fibrosis. Besides hepatocytes, resistant liver macrophages known as Kupffer cells are a possible source of MIF. Kupffer cells are very sensitive to ROS and numerous proinflammatory cytokines. They are activated and release various factors, as well as MIF, which contribute to oxidative stress, inflammation, and hepatic fibrogenesis. These processes are key pathogenetic mechanisms in CCl4- and TAA-induced liver injury. Activated HSC plays a crucial role in liver

fibrogenesis by producing collagens, other ECM proteins, such as growth factors and cytokines. However, the role of HSC in MIF production is not clear enough (123).

Results of our study showed that hepatic TGF-β1 and PDGF-BB levels in TAA-treated MIF-deficient mice were significantly increased compared to TAA-treated wild type of mice (Fig. 3A, 3B). These findings indicate the MIF exerts an anti-fibrogenic effect that can be explained by inhibiting HSC activation, stimulation of ECM degradation and fibrosis resolution (74, 278). Similar to our results, *Heinrichs et al.* showed that in CCl4- and TAA-induced liver fibrosis in MIF- or receptor CD74- deficient mice, enhanced the expression of profibrogenic genes such as TIMP-1, TGF-β1, and coll1A1 (74). MIF exerts its antifibrogenic effect through the CD74 receptor on HSC (74). Furthermore, MIF-mediated CD74 inhibition of HSC contributes significantly to PDGF-BB function migration and proliferation of HSC. These results suggest that MIF decreases ECM and progression of liver fibrosis. MIF-induced CD74/AMPK inhibits TGF-β-induced Coll1 expression (279, 280). Similarly, in NAFLD mice-induced high-fat diets, MIF exerts an antiinflammatory effect also through the CD74/AMPK signaling pathway, which inhibits TNF-α and IL-6 synthesis (40). Namely, enhanced fibrogenesis in MIF-deficient mice is probably a consequence of the role of MIF in HSC biology, but not altered immune cell infiltration (74). Due to the undefined effects of MIF on fibrosis, further study is needed to elucidate the effects of MIF via CD74 receptors on HSC, thereby reducing their activation and fibrogenic response.

In the rat model of TAA-induced fibrosis in rats, the source of MIF was damaged hepatocytes adjacent to fibrotic areas (128). Although in most liver diseases increased MIF is associated with inflammation, its effect on fibrogenesis in the liver is not clear enough. The proinflammatory and fibrogenic effects of MIF have been demonstrated in experimental models of ALD, as well as in the human population, and it correlates with patient mortality in alcoholic hepatitis (280, 281, 282) In ALD, hepatocytes produce MIF which reacts with the CD74 receptor and its co-receptors CXCR2, CXCR4, and CXCR7 expressed both on resident hepatic macrophages and peripheral monocytes (283, 284). The results of some studies have shown that in acute CCl4 toxicity (285) and CCl4-induced liver fibrosis there is an increase of MIF in the liver and increased concentrations in the blood, which indicates the local and systemic effect of MIF in the progression of chronic liver disease (286, 277). Unlike chronic ethanol feeding, MIF mediates protective effects following chronic-binge ethanol feeding (277). Also, MIF exerts an anti-steatotic effect on MCD-induced NAFLD (127) while promoting hepatic fibrogenesis in MCD-induced NASH (236). Similarly, in NAFLD/NASH progression, MIF contributes to liver fibrogenesis by the profibrotic phenotype of NKT cells (123) but alleviates liver fibrosis in toxic liver injury models (77, 283). The role of MIF in the liver depends on the etiology of the liver disease, stage of disease, a specific pattern of intrahepatic MIF receptor expression, and complexity of the MIF signal (73, 123, 287).

Further examination of the role of MIF in both liver and neurodegenerative diseases (288) is of great importance for finding the target MIF diagnosis and therapy. For example, recent research shows that patients with high serum MIF levels, and low levels of its CD74 binding receptor in the blood, indicate an increased risk of mortality in patients with advanced liver cirrhosis (289).

MMPs/TIMP regulation is of particular importance in ECM decomposition and remodeling (60). In liver fibrosis, MMP-1 and MMP-9 are negatively correlated with histopathological findings (60). MMP-2 activity in patients with liver fibrosis was increased 2.4 fold compared with controls and represents a significant diagnostic marker of liver fibrosis (63). As liver fibrosis progresses, serum TIMP levels increase so that TIMP-1 interacts with all three MMPs (MMP-1, 2, and 9) (290). In support of this, our results indicated increased densitometric area of MMP-2, MMP-9, and TIMP-1 in TAA-treated MIF-deficient mice compared to wild-type TAA-treated mice, after the 8-week experimental period (Fig. 5a, 5b). Besides, the key role of MIF in inflammation, MIF stimulates the secretion of MMPs (291, 292), which explains the increased degree of fibrosis in MIF-deficient mice. Synthesis and remodeling of ECM is a key event in progressive CLD. TGF-β1, together with PGDF-BB is the most important profibrogenic mediator that stimulates the synthesis of Coll1 and Coll3, laminin, fibronectin, and α-SMA (49). On the other hand, increased TIMP-1 expression and decreased MMPs activity contribute to ECM remodeling (8, 9). Besides, TIMP-1 is anti-apoptotic for HSC, which may result in an increased number of activated stellate cells (8). Also, increased expression of TIMP-1 has been demonstrated in CCl4- and BCL-induced liver fibrosis (293). Increased TIMP-1 activity leads to excessive accumulation of ECM. Also, the findings of some studies show that inhibition of TIMP-2 reduces liver fibrosis, increasing degradation of ECM and inhibiting of HSC (294, 295). Our results showed increased relative activity and densitometric area of TIMP1 than MMP-2 in TAA-induced liver fibrosis $MIF^{-/-}$ mice (Fig. 5a), which may indicate a more significant role of TIMP in maintaining fibrosis. Given that the crucial factor for liver fibrogenesis is the balance of MMPs and TIMPs, the findings of our study show that the antifibrogenic activity of MIF is more pronounced through a decrease in TIMP activity than MMP-2. Moreover, increased fibrogenesis in $MIF^{-/-}$ mice contributes to the increased relative activity of MMP-9 (Fig. 5b). On the other hand, reduced relative activity and densitometric area of dimer MMP-9 in the $MIF^{-/-}$+TAA group compared to TAA-treated wild mice (Fig. 5b) can be explained by the absence of prooxidative and proinflammatory effects of MIF.

Changes in the hepatic level of the fibrogenic factors (TGF-β1 and PDGF-BB) (Fig.3A, 3B), as well as in the activity of MMPs and TIMPs (Fig. 5a, 5b) were confirmed by histological examination of liver tissue of TAA-treated mice (Fig. 7; Fig. 8). MIF knockout mice treated with TAA had a more pronounced irregular structure of the liver tissue (Fig. 8a, 8b, 8c; Fig. 11b)

compared to TAA-treated wild mice (Fig. 7a, 7b, 7c; Fig. 11a), with a mixed inflammatory infiltrate located in the Disse spaces and perivascularly along large blood vessels. Focal necrosis and apoptosis of hepatocytes were present. Several mitoses were 0/10 high-power field /HPF/ to 4/10HPF. Moreover, micro- and macrovesicular fatty changes were present. Hepatocytes with bizarre, polyploid nuclei were numerous. Mallory bodies and numerous hemosiderophages are also observed (Table 3). Bridging porto-portal pronounced fibrosis and micronodular cirrhosis were also present. Masson-stained Coll1 bridges the liver tissue from one portal space to another (bridging) and was located perilobularly in the form of thin strips (Fig. 8b; Fig. 12a). Reticular fibers (Coll3) monitored the distribution of Coll1 (Fig. 8c; Fig. 13a). The quantitative analysis of Masson's trichrome and reticulin-stained liver tissue showed that the accumulation of collagen was significantly increased in liver MIF-deficient mice compared to TAA-treated wild-type, expressed as % fibrotic area (Fig. 12b; Fig. 13b). Accumulation of ECM and % fibrotic area was increased compared to liver tissue of TAA-treated wild type mice (Fig. 7b, 7c; Fig. 12a, 12b; Fig. 13a, 13b) Similar to our pathohistological findings, *Heinrichs et al.* showed an increased percentage of Coll1in CCl4-induced liver fibrosis (74).

The most important role of TGF-β1 in hepatic fibrogenesis is activating HSC (49). Activated HSC produces PGDF, MMPs, collagen, and other matrix components, contributing to ECM deposition. Given the complexity of pathogenesis of hepatic fibrosis, the study of antifibrotic therapy is a very important potential therapeutic approach. Altered redox homeostasis and persistent inflammation trigger liver fibrosis. Since TGF-β1 and PDGF-BB play a major role in hepatic fibrogenesis, inhibition of its activity may be a target for antioxidative, antiinflammatory and antifibrotic therapy. Numerous studies have shown the protective effect of various natural bioflavonoids that can inhibit the fibrogenic potential of these cytokines (181).

Betaine (trimethyl glycine), an oxidative metabolite of choline, is found in plants, animals and microorganisms and dietary sources including bran, vegetables, and seafood (147, 148, 149). In mammalian metabolism, betaine is a vital methyl donor in the methionine-homocysteine cycle (15, 151). Betaine exerts antioxidant effects by regulating the metabolism of SAA, such as homocysteine, methionine, SAM and others (153). Moreover, betaine acts as an osmoprotectant that protects cells against stress inducers (150, 154). The hepatoprotective effects of betaine have been reported in different experimental models (148, 159, 160, 161). However, this is the first study that put into attention the modulatory effects of betaine on MIF in TAA-induced hepatotoxicity.

In addition to the anti-oxidative and anti-inflammatory effects of betaine, our study also showed that betaine has anti-fibrogenic effects. Namely, betaine supplementation significantly decreases the hepatic TGF-β1 and PDGF-BB levels (Fig. 3A, 3B) as relative activities of MMP-2, MMP-9, dimer 9, and TIMP-1 (Fig. 5a, 5b) in TAA-treated mice. Additionally, betaine

supplementation in $MIF^{-/-}$+TAA-treated mice causes a significant decrease of these fibrogenic mediators compared to the $MIF^{-/-}$+TAA group (Fig. 3A, 3B).

TAA-induced hepatotoxicity leads to the release of ROS, chemokines, and cytokines, as well as an influx of inflammatory cells into the liver tissue. Besides, inflammatory cells secrete many cytokines such as TGF-β and PDGF, which activate HSC. It has been shown that TGF-β1 is the main cytokine that activates HSC (34, 49, 47, 297). Recent research shows that that TGF-β1 stimulates HSC activation through activation of methionine adenosyltransferase 2A (MAT2A), leading to reduction of SAM concentration (298). These findings indicate a novel TGF-β1 signal pathway in HSC activation, proliferation and differentiation in the myofibroblast-like cells, which are pivotal cells in hepatic fibrogenesis (298). MAT is the key enzyme that synthesizes SAM, a precursor of GSH (299). This indicates that betaine, as the regulator of hepatic metabolism of SAA, increases SAM synthesis (300), and possibly inhibits the TGF-ß1/p65/MAT2A signaling pathway. Similarly, in ALD where methionine synthetase activity is reduced, betaine supplementation maintains methionine and SAM levels (301).

Our findings showed that betaine regulated redox state (Fig. 2), attenuation of inflammation, reduced the inflammation in the liver tissue and the levels of proinflammatory cytokines such as IL-6 and IFN-γ in TAA-induced liver fibrosis (Table 2). Also, we showed that betaine supplementation reduced the hepatic level of TGF-β1 and PGDF-BB (Fig. 3A, 3B), as well as the relative activity of MMP-2, MMP-9, dimer MMP-9 and TIMP-1 (Fig. 5a, 5b). This indicates that betaine, in addition to its antioxidative and antiinflammatory effects, inhibits the activation and function of HSC.

Similar to this, in CCl4-induced liver fibrosis, TGF-β1, Coll1A1, and Coll3A1 RNA overexpression were suppressed by betaine treatment (148). Furthermore, in HFD-CCl4-induced liver fibrosis, betaine treatment suppresses HSC activation by reducing α-SMA, Coll1A1, and TGF-β1 as well as TIMP-1, TIMP-2, and MMP-2 mRNA expressions (160). In mice with NASH induced by a MCD diet, betaine-regulated redox status reduced inflammation and apoptosis and stimulated autophagy (161). Inhibition of MMP-2 activity or blockade of MMP-2 synthesis with morin (296) or chrysin (297) inhibits activation and proliferation of HSC. Also, due to the inhibition of HSC cell proliferation, the synthesis and secretion of MMPs, TIMPs, numerous components of the ECM and the accumulation of collagen are reduced. Our findings demonstrated betain's ability to reduce densitometric area and relative activity of MMP-2, MMP-9, and TIMP-1 in liver fibrosis, with TIMP reduction being the most pronounced (Fig. 5a, 5b). It is known that ROS can activate MMPs by oxidation or modification of amino acids (302). The decrease in MMPs activity can be explained

by the antioxidant action of betaine. Thus, silymarin, as a bioactive component, reduces the expression of MMP-2 and MMP-9 in human melanoma cells (303).

In different experimental models, betaine also alleviated liver fibrosis (296, 304, 305). Based on our results, it can be observed that the antifibrotic properties of betaine could be mediated through its antioxidative and antiinflammatory effects, decreasing lipid peroxidation, protein oxidative damage, as well as IL-6 and IFN-γ level. The antioxidative and antiinflammatory effects of betaine contribute to its hepatoprotective properties in TAA-induced liver fibrosis. Preservation of antioxidant defense capacity by increasing thiol and decreasing TOS and MDA may explain the hepatoprotective activity of betaine in this study. Furthermore, betaine has been shown to regulate lipid metabolism and reduce the accumulation of TG and cholesterol in ALD/NAFLD/NASH (161, 202, 203, 305). Since one of the main signs of liver fibrogenesis is the accumulation of cholesterol in HSC, which stimulates cells to TGF-β1-induced activation (306), betaine supplementation can suppress liver fibrosis. The more precise effects and mechanisms of betaine on suppressing hepatic fibrogenesis are still not clear enough.

However, as mentioned, compared to the TAA+Bet group, betaine supplementation of TAA-treated MIF-deficiency mice results in a weakened antifibrogenic response to hepatic levels of TGF-β1 and PDGF-BB (Fig. 3A, 3B), as relative activities of MMP-2, MMP-9 and TIMP-1 (Fig. 5a, 5b). These findings can be explained by the lack of antifibrogenic action of MIF. The antifibrogenic activity of betaine in MIF$^{-/-}$ mice is less pronounced compared to the TAA + Bet group, indicating that MIF exhibits antifibrogenic activity that is stimulated by betaine (Fig. 5a, 5b). However, supplementation betaine of MIF$^{-/-}$ mice did not change dimer MMP-9 activity (Fig. 5a, 5b), indicating that the antifibrogenic effect of MIF via dimer MMP-9 is not mediated by betaine. MIF exerts an antifibrogenic effect via CD74 receptors on HSC by inhibiting their activation and PDGF-mediated proliferation (74). Results of our study indicated that in MIF-deficient mice treated with TAA, oxidative stress and inflammation were reduced, while liver fibrosis was exacerbated. MIF in TAA-induced liver fibrosis exhibits prooxidative and proinflammatory effects, but it suppresses fibrosis, ie it has an antifibrogenic effect.

Although chronic liver inflammation promotes fibrosis, the mechanisms of regulation of fibrogenesis and inflammation are different. Numerous inflammatory cells, especially Th1 lymphocytes, secrete lymphokines (IFN-γ, TNF-α) which activate M1 macrophages. Cytokines from activated macrophages (TNF-α, IL-6, IL-12) and MMPs further accelerate inflammation. Cytokines (IL-4 and IL-13) that produce Th2 lymphocyte stimulate M2 macrophages, which have an anti-inflammatory effect (307). Unlike macrophages in other tissues, liver macrophages consist of two different populations: Kupffer cells and recruited monocytes derived from bone marrow.

Kupffer cells stimulate inflammation (like M1) and attract macrophages derived from monocytes. Monocyte-derived macrophages produce profibrogenic factors (TGF-β and PDGF) and contribute to HSC activation (such as M2). However, these cells are involved in fibrinolysis, through MMPs activation, which is responsible for ECM remodeling, fibrous tissue degradation, and resolving fibrosis (286, 308).

This behavior of MIF in experimental liver fibrosis can be explained by the different mechanisms, signaling pathways, and receptors through which this pleiotropic cytokine acts (74, 75, 126, 129, 130). The mechanisms of MIF-induced oxidative stress and inflammation in the liver have already been described earlier in the discussion. The antifibrogenic effect of MIF can be explained as achieved through the CD74 receptor on HSC (74). These findings indicate that betaine as a natural, non-toxic component and specific MIF/CD74 agonists can be used in the prevention and antioxidative therapy of liver fibrosis. Further research should focus on the study of betaine stimulation of the MIF-mediated antifibrogenic effect.

Changes in the hepatic level of the fibrogenic factors (TGF-β1 and PDGF-BB) (Fig. 3A, 3B), as well as in the activity of MMPs and TIMPs in betaine's groups treated with TAA (Fig.5a, 5b) were confirmed by histological examination of liver tissue (Fig. 9; Fig. 10; Fig. 11c, 11d).

Histopathological assessment of fibrosis is the "gold standard" for determining the degree of fibrosis, as the most important diagnostic assessment in chronic liver disease (309). Betaine supplementation of TAA-treated wild-type animals improved the histological findings of liver tissue (Fig. 9). The HE staining of the liver tissue showed that the histological structure of the liver was less irregular with scanty fibrosis and mild inflammatory infiltrates (Fig. 9a; Table 3). Under Masson's trichrome staining of the liver tissue, the quantitative analysis showed the markedly decreased Coll1 deposition, i.a % of fibrosis area is reduced (Fig. 12a, 12b). (160). Also, under reticulin staining, the markedly decreased Coll3 deposition and morphometric analysis of liver tissue fibrosis showed a decreased percentage of the fibrotic area was detected in the liver tissue of TAA-Bet animals (Fig.13a, 13b). Accordingly, betaine supplementation can be used in the treatment of liver fibrosis. Betaine mitigated liver fibrogenesis due to its antioxidant, anti-inflammatory, and hepatoprotective effects. Also, due to HSC inhibition, the synthesis of profibrogenic mediators, MMP, and Coll1 and Coll3 deposition was reduced.

However, after betaine supplementation, MIF knockout C57BL/6 mice had irregular histological structure of the liver tissue with portal-portal bridging mild fibrosis and mixed inflammatory infiltrates. Micro- and macrovesicular fatty change, focal necrosis and Kupffer cell hyperplasia were detected. Some of the mitoses were 2/10 HPF to 5/10HPF as well as pathological mitoses were present (Fig. 10a; 11d; Table 3). The quantitative analysis of Masson's trichrome and reticulin-stained liver tissue revealed that MIF-deficient mice treated with betaine accumulated

more collagen than the TAA-Bet group (Fig. 12a, 12b; Fig. 13a, 13b), whereas betaine supplementation TAA-treated MIF deficient mice accumulated less ECM than the MIF-/-+TAA group. Markedly, pathohistological changes of liver tissue, as well as a higher percentage of fibrosis in the MIF$^{-/-}$+TAA+Bet group compared to the TAA+Bet group, is probably due to the lack of MIF. Our study has shown that in TAA-induced hepatic fibrogenesis, MIF exerts prooxidative, proinflammatory and antifibrogenic effects. Similar to our results, *Heiners et. al* have shown that in TAA- and CCl4 induced liver fibrosis, MIF exerts antifibrogenic effects which are realized through CD74 receptors on HSC. On the other hand, recent research shows that MIF exhibits profibrogenic activity in NASH (123). Our findings showed that the antioxidative, antiinflammatory and antifibrogenic effects of betaine are MIF-mediated in TAA-induced liver fibrosis.

6. CONCLUSION

1. TAA induces liver fibrosis in both wild and $MIF^{-/-}$ knockout mice after an 8-week experimental period. Histological analysis of liver tissue showed an irregular structure of the liver with bridging (peri-central and peri-portal) fibrosis and inflammatory infiltrate. TAA is associated with the development of periportal fibrosis to a state resembling human cirrhosis. Chronic administration of low-dose TAA (intraperitoneally) is a reliable experimental model of liver fibrosis in $MIF^{-/-}$ mice for investigating the role of MIF in liver fibrosis and for a better understanding of human liver fibrogenesis.
2. MIF promotes hepatocellular injury in TAA-induced liver fibrosis by increasing serum transaminases (ALT and AST), ALP, γGT activity and bilirubin concentration.
3. MIF altered redox homeostasis by increasing MDA, AOPP and TOS, as well by decreasing antioxidative defense of hepatocytes (thiol groups and TAS)
4. MIF aggravates inflammation in TAA-induced liver hepatotoxicity by increasing serum CRP and liver IL-6 and IFN-γ concentration**.**
5. MIF aggravates morphological changes of liver tissue induced by TAA. More irregular structure of liver tissue with an inflammatory infiltrate, micro- and macrovesicular fatty change, necrosis and apoptosis of hepatocytes were detected.
6. MIF exerts an antifibrotic effect by decreasing profibrogenic cytokines (TGF-β1 and PDGF-BB), MMP-2, MMP-9, dimer MMP-9, TIMP-1 and ECM deposition (Coll1 and Coll3) in the liver.
7. Betaine alleviates liver function in TAA-induced hepatotoxicity by decreasing serum transaminases (ALT and AST), ALP, γGT activity and bilirubin concentration
8. Betaine exerts antioxidative effects by decreasing lipid peroxidation (MDA), AOPP and TOS in TAA-induced hepatotoxicity. Also, betaine increases the antioxidative capacity of the liver (SOD, catalase, thiol groups, and TAS).
9. Betaine alleviates liver inflammation in TAA-induced liver fibrosis by decreasing CRP and liver proinflammatory cytokines (IL-6 and IFN-γ)
10. Betaine supplementation improves the histological finding of liver tissue. Less irregular structure of liver tissue with scanty fibrosis, mild inflammatory infiltrates and rare focal hepatocyte necrosis.
11. Betaine supplementation mitigates liver fibrogenesis by decreasing TGF-β1, PDGF-BB, MMP-2, MMP-9, dimer MMP-9, TIMP-1 and ECM deposition (Coll1 and Col3) in the liver.

12. Betaine modulates the prooxidative, proinflammatory and antifibrogenic effects of MIF in TAA-induced liver fibrosis. Betaine attenuates the prooxidative effects of MIF by decreasing MDA and AOPP, as well as by increasing liver thiols and TAS. Also, betaine mitigates the proinflammatory effects of MIF by reducing liver IL-6 and IFN-γ. On the other hand, betaine stimulates antifibrogenic effects of MIF by decreasing TGF–β1, PDGF-BB, MMP-2, MMP-9, TIMP-1 and deposition of ECM (Coll1 and Coll3) in the liver.

Liver fibrosis is the hallmark of chronic liver disease. Effective therapy for patients with liver fibrosis is still being investigated. Since the incidence and prevalence of diseases associated with liver fibrosis have acquired an epidemic character, our research could contribute to a preventive and therapeutic approach to liver fibrosis. The results of this study imply MIF as possible targets in the treatment of chronic liver disease in humans. Also, these results suggest that betaine may be used as an important bioactive component for the prevention and treatment of MIF-mediated chronic liver injury.

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ABBREVIATIONS

ALD - alcoholic liver disease

ALP - alkaline phosphatase

ALT - alanine aminotransferase

AMP - adenosine monophosphate

AMPK - adenosine monophosphate dependent kinase

ANOVA - analysis of variance

AOPP- advanced oxidation protein products

AST - aspartate aminotransferase

BDL - bile duct ligation

CAT - catalase

CCl4 - carbon tetrachloride

CLD - chronic liver disease

Coll1,3 - type I and III collagen

CCL2- chemokine 2

CXR - chemokine receptors

CRP - C-reactive protein

CTGF-connective tissue growth factor

CYP450 -cytochrome P450

DAMPs- damage-related molecular patterns

ECM - extracellular matrix

EDTA - ethylenediaminetetraacetic acid

EGFR - epidermal growth factor receptor

EGF- epidermal growth factor

ER - endoplasmic reticulum

ERKs - Extracellular signal-regulated protein kinases

ET1- endothelin-1

γGT- gama-glutamyl transpeptidase

GPx - glutathione peroxidase

GSH - glutathione

HCC - hepatocellular carcinoma

HIF-1α - hypoxia-inducible factor 1-alpha

HPA - hypothalamic–pituitary axis

HPF - High Power Field

HSC - hepatic stellate cell

ICAM- 1- intercellular adhesion-1 molecules

IFN-γ- interferon -gama

IL - interleukin

IRI - ichemia/reperfusion injury

LPS- lipopolysaharides

JNK - C-jun N-terminal kinase

LSEC - liver sinusoidal endothelial cells

MAPK- mitogen-activated protein kinase

MCD - methionine choline deficiency

MCP-1- monocyte chemoattractant protein-1

MDA - malondialdehyde

MIF - Macrophage migration inhibitory factor

$MIF^{-/-}$ mice - MIF knockout C57BL/6 mice

MFB - myofibroblast

MMP - matrix metalloproteinase

NADPH - nicotinamide adenine dinucleotide phosphate oxidase

NAFLD - nonalcoholic fatty liver disease

NASH - nonalcoholic steatohepatitis

NF - nuclear factor

NK - Natural killer

Nrf2 - Nuclear factor-E2-related factor-2

NO - nitric oxide

NOX2- nicotinamide adenine dinucleotide phosphate (NADPH) oxidase 2

iNOS- inducible NO synthetasis

O_2^-- superoxide anion

PBS - phosphate buffered saline

PDGF - platelet derived growth factor

PI3K - phosphatidylinositol 3-kinase

PDGF - platelet derived growth factor

RNS - reactive nitrosative species

ROS - reactive oxygen species

SAM - S-adenosylmethionine

SAMs - scar-associated macrophages

SMA - smooth muscle actin

SOD - superoxide dismutase

TAA - thioacetamide

TAS - total antioxidative status

TG - triglyceride

TGF-β - transforming growth factor beta

TIMP - tissue inhibitor metalloproteinase

TLR - toll like receptor

TNF-α – tumor necrosis factor alpha

TOS- total oxidative status

TPOR - thiol-protein oxidoreductase

VCAM-1 -vascular cell-1 adhesion molecules

VEGF- vascular endothelial growth factor

CURICULUM VITE

Resident Doctor Dušan Vukićević (June 26, 1991.), MD, was born and raised in Belgrade, where he completed his elementary and high school education with honors. In 2010/2011, he started his studies at the Belgrade University, Faculty of Medicine, during which he was engaged in teaching activities as well as research. He graduated in 2016 with an average grade of 9.89/10.00.

In 2016/2017, he enrolled in PhD studies at the Belgrade University, Faculty of Medicine, the module - Epidemiology. In 2017/2018, he began working as a research on a scientific project funded by the Serbian Ministry of Education, Science, and Technological Development. Dr. Dušan Vukiević successfully passed all of the exams required by the study program after completing the required work at the PhD level.

In August 2020, he continued his career in Germany. He took the position of resident doctor at the University Clinic Mannheim, Clinic for Neurology, where he currently works.

Dr Dušan Vukićević is the author of four papers published *in extenso* in journals

(1 M21a, 2M21 and 1 M23; total IF = 12.32) indexed in the *Science Citation Index*.

образац изјаве о ауторству

Изјава о ауторству

Име и презиме аутора Душан Вукићевић

Број индекса 5122/16

Изјављујем

да је докторска дисертација под насловом

"Дејства макрофагног миграторног инхибиторног фактора и бетаина на морфофункционалне, ћелијске и ткивне промене у фибрози јетре: епидемиолошки значај хроничне болести јетре"

"The effects of macrophage migration inhibitory factor and betaine on morphofunctonal, cellular and tissue changes in thioacetamide-induced liver fibrosis: epidemiological relevance of chronic liver diseases"

- резултат сопственог истраживачког рада;
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образац изјаве о истоветности штампане и електронске верзије докторског рада

Изјава о истоветности штампане и електронске верзије докторског рада

Име и презиме аутора Душан Вукићевић

Број индекса 5122/16

Студијски програм Епидемиологија

Наслов рада "Дејства макрофагног миграторног инхибиторног фактора и бетаина на морфофункционалне, ћелијске и ткивне промене у фибрози јетре: епидемиолошки значај хроничне болести јетре"

"The effects of macrophage migration inhibitory factor and betaine on morphofunctonal, cellular and tissue changes in thioacetamide-induced liver fibrosis: epidemiological relevance of chronic liver diseases"

Ментор: Проф. др Татјана Радосављевић и Проф. др Наташа Максимовић

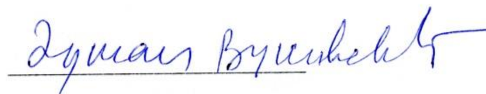
Изјављујем да је штампана верзија мог докторског рада истоветна електронској верзији коју сам предао/ла ради похрањивања у **Дигиталном репозиторијуму Универзитета у Београду.**

Дозвољавам да се објаве моји лични подаци везани за добијање академског назива доктора наука, као што су име и презиме, година и место рођења и датум одбране рада.

Ови лични подаци могу се објавити на мрежним страницама дигиталне библиотеке, у електронском каталогу и у публикацијама Универзитета у Београду.

Потпис аутора

У Београду, 14. 04. 2021.



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Изјава о коришћењу

Овлашћујем Универзитетску библиотеку „Светозар Марковић“ да у Дигитални репозиторијум Универзитета у Београду унесе моју докторску дисертацију под насловом:

“Дејства макрофагног мигрaторног инхибиторног фактора и бетаина на морфофункционалне, ћелијске и ткивне промене у фибрози јетре: епидемиолошки значај хроничне болести јетре”

“The effects of macrophage migration inhibitory factor and betaine on morphofunctonal, cellular and tissue changes in thioacetamide-induced liver fibrosis: epidemiological relevance of chronic liver diseases”

која је моје ауторско дело.

Дисертацију са свим прилозима предао/ла сам у електронском формату погодном за трајно архивирање.

Моју докторску дисертацију похрањену у Дигиталном репозиторијуму Универзитета у Београду и доступну у отвореном приступу могу да користе сви који поштују одредбе садржане у одабраном типу лиценце Креативне заједнице (Creative Commons) за коју сам се одлучио/ла.

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6. Ауторство – делити под истим условима (CC BY-SA)

(Молимо да заокружите само једну од шест понуђених лиценци.
Кратак опис лиценци је саставни део ове изјаве).

Потпис аутора

У Београду, 14. 04.2021