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THE EFFECTS OF BETAINE AND NEUREGULIN-1 ON TISSUE, CELLULAR AND MOLECULAR CHANGES ON *IN VIVO* AND *IN VITRO* MODELS OF NONALCOHOLIC FATTY LIVER DISEASE AND FIBROSIS

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DEJSTVO BETAINA I NEUREGULINA-1 NA TKIVNE, ĆELIJSKE I MOLEKULARNE PROMENE NA *IN VIVO* I *IN VITRO* MODELIMA NEALKOHOLNE MASNE BOLESTI I FIBROZE JETRE

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The effects of betaine and neuregulin-1 on tissue, cellular and molecular changes on *in vivo* and *in vitro* models of nonalcoholic fatty liver disease and liver fibrosis

Nonalcoholic fatty liver disease (NAFLD) is one of the most common causes of chronic liver diseases in the general population. NAFLD consists of three major entities including steatosis, nonalcoholic steatohepatitis (NASH) and cirrhosis. The principal causes of steatosis are insulin resistance and hyperinsulinemia, which lead to increased lipolysis in adipose tissue with subsequent inflow of free fatty acids (FFA) to the liver and stimulation of hepatic *de novo* lipogenesis. Lipotoxic effects of FFAs cause mitochondrial dysfunction and oxidative stress that lead to the activation of inflammatory response and progression of steatosis to NASH. Autophagy is impaired in NAFLD and contributes to the accumulation of damaged organelles and proteins in hepatocytes, ultimately leading to apoptosis. Chronic liver injury and inflammation stimulate fibrogenic pathways and ultimately may result in liver cirrhosis. Hepatic stellate cells (HSCs) play a central role in hepatic fibrogenesis and are a major source of collagen and other extracellular matrix proteins. Transforming growth factor beta (TGF-β) induces a transdifferentiation of HSCs into myofibroblast-like cells which express collagen I gene and alpha-smooth muscle actin. Neuregulin-1 (NRG-1) belongs to epidermal growth factor family and is involved in cell differentiation, proliferation, growth and survival. Recombinant human NRG-1 (rhNRG-1) exerts antifibrotic effects in the heart, lungs, kidneys and skin. However, the exact role of NRG-1 in liver fibrogenesis has not been elucidated.

Betaine, 3-methyl glycine is a nontoxic amino acid, a methyl-group donor and exerts antioxidative effects by increasing the amount of sulfur-containing amino acids. Additionally, betaine may induce epigenetic silencing of genes involved in lipogenesis contributing to alleviation of steatosis. On the other hand, the effects of betaine on inflammation, autophagy and apoptosis, as well as on signaling pathways involved in NAFLD pathogenesis are still not clarified.

The aims of the dissertation were to define the model of NAFLD using methionine-choline deficient (MCD) diet in mice; to examine the effects of betaine on liver morphological and ultrastructural changes, as well as on oxidative stress, apoptosis,

autophagy and inflammation in the liver; to study the effects of NRG-1 on connective tissue accumulation, gene expressions for collagen type I and III, and matrix metalloproteinases 2 and 9 (MMP-2 and -9) in murine models of liver fibrosis induced by CCl_4 and bile duct ligation; to examine effects of NRG-1 on TGF-β gene expression in isolated hepatocytes and on ErbB3 and ErbB4 receptors phosphorylation in the liver.

To set NAFLD animal model, 8 weeks old male C57BL/6 mice (n=28) were randomly divided into the following groups (n=7 per group): 1. control, continuously fed with standard chow; 2. groups fed with MCD diet for two (MCD2), four (MCD4) or six weeks (MCD6). After the treatment blood samples were taken determination of transaminase activity and lipid profile. Liver samples were used for pathohistology and determination of oxidative stress parameters as well as FFA profile.

To investigate the effects of betaine on NAFLD, animals (n=28) were on standard diet until the age of 8 weeks, and then they have been divided into the following groups (n=7 per group): 1. control – continuously on standard diet; 2. BET group – animals on standard diet that were treated with betaine solution in drinking water (1.5% solution *ad libitum*) for 6 weeks; 3. MCD group – animals on MCD diet during 6 weeks, and 4. MCD+BET group – animals on MCD diet treated with betaine in drinking water. Transaminase activities were measured in serum, while oxidative stress parameters, autophagy markers (LC3II, p62, mTOR, Beclin 1, Atg4b, Atg5, Atg7), apoptosis markers (Bax, Bcl2, Bbc3, Bak1) and markers of inflammation (IL-6, IL-10, TNF-α) were determined in liver samples. Ultrastructural changes in liver tissue were examined by using transmission electron microscopy.

The effects of NRG-1 on liver fibrosis were examined on two different animal models. In the first model, male C57BL/6j mice (n=32) were divided into the following groups (n=8 per group): 1. control –corn oil i.p. 3x per week; 2. NRG-1 group –rhNRG-1 (20 μg/kg i.p.) 5x per week; 3. CCl_4 group –20% CCl_4 solution (1mg/kg body weight i.p.) diluted in corn oil 3x per week and 4. CCl_4+NRG-1 group –both CCl_4 and NRG-1 in combination in a previously described manner. The duration of treatment in all groups was 4 weeks. The second liver fibrosis model has been established in mice of the same strain by

bile duct ligation. Animals (n=32) were divided into four groups (n=8 per group): 1. control –phosphate buffered saline (PBS) i.p.; 2. NRG-1 group –rhNRG-1 (20 μg/kg i.p.) 5x per week; 3. BDL group – bile duct ligation and PBS, and 4. BDL+NRG-1 group – bile duct ligation and rhNRG-1 (20 μg/kg i.p) 5x per week starting 24 hours after the surgery. After 4 weeks, liver tissue was stained with Masson's Trichrome to quantify fibrosis and determine gene expression for collagen type I and III, and MMP-2 and -9. The presence and phosphorylation of ErbB3 and ErbB4 receptors was determined in the liver of control and rhNRG-1-treated animals. The expression of TGF-β gene was determined i*n vitro* on isolated primary hepatocytes before and 16 hours after NRG-1 stimulation.

MCD diet for 2 weeks induced mild steatosis with focal fatty change, while after 4 weeks steatosis was more pronounced with diffuse fatty change. However, MCD diet for 6 weeks caused diffuse micro- and macro vesicular steatosis accompanied with inflammatory infiltrate indicating nonalcoholic steatohepatitis (NASH). MCD diet after 6 weeks induced decrease in serum total cholesterol, triglycerides and HDL and increase in LDL vs. control. Increase in liver oleic and linoleic acid, and a decrease in saturated and docosohexaenoic acid (DHA) were evident in MCD6 group ($p<0.01$ *vs.* control).

Betaine supplementation reduced steatosis and inflammation evident on histology, and improved serum lipid profile by decreasing total cholesterol, LDL and increasing HDL level compared to MCD group ($p<0.01$). Betaine reduced lipid peroxidation and nitrosative stress that were increased in MCD group, and also improved the activity of antioxidant enzymes such as paroxonase, arylesterase, superoxide dismutase, catalase and glutathione peroxidase vs. MCD group. Besides, betaine increased liver glutathione concentration and reduced the activity of glutathione reductase by comparison with MCD diet-treated group.

MCD diet for 6 weeks induced an increase in liver tumor necrosis factor alpha (TNF-α), interleukin (IL)-6 and TGF-β expression while betaine treatment prevented this increase. Betaine also increased the expression of anti-inflammatory IL-10 compared to MCD group ($p<0.05$). mRNA expression of Bax was decreased and Bcl2 was increased in MCD+BET *vs.* MCD group ($p<0.05$). Betaine in combination with MCD diet increased mRNA expression of autophagy genes Atg4, Atg5, Atg7 and Beclin1 by comparison with

control ($p<0.05$) and MCD group ($p<0.05$). Levels of SQSTM1/p62 were increased in MCD diet-fed mice compared to control ($p<0.05$), while in MCD+BET group its level was significantly reduced compared both with control and MCD group ($p<0.05$). Electron microscopy showed increased presence of autophagosomes and reduced apoptotic bodies in betaine treated mice fed with MCD diet when compared to control and MCD group.

Histology showed severe liver fibrosis in BDL model, while CCl_4 induced mild fibrotic changes after 4 weeks. NRG-1 treatment significantly increased fibrotic area in liver tissue from 1.86 to 2.65 % in CCl_4 model and from 5.03 to 8.25 % in BDL-induced liver fibrosis ($p<0.01$). In the BDL model, NRG-1 treatment increased expression of col1a1 and col3a1 vs. BDL group ($p<0.05$). NRG-1 treatment resulted in a significant decrease of MMP2 expression and there was a trend towards attenuated induction of MMP9 mRNA compared to group treated only with CCl_4 ($p<0.05$). In the livers of BDL-treated animals, MMP 9 expression was significantly increased compared with control ($p<0.01$). NRG-1 treatment increased phosphorylation of liver ErbB3 receptors to a greater extent in relation to the ErbB4 receptors ($p<0.01$). Additionally, NRG-1 treatment induced increased TGF-β expression *in vitro* on isolated hepatocytes, compared to untreated cells ($p<0.01$).

Betaine exerts hepatoprotective effects by reducing lipid peroxidation and nitrosative stress in MCD diet-induced NAFLD through an increase in liver antioxidant enzyme activities and by restoring glutathione content. Betaine alleviated NAFLD by increasing autophagy processes in Beclin 1- and Akt/mTOR-dependent manner, but independently on AMPK activation. Potential additional mechanisms of betaine in MCD-diet induced NAFLD in mice are antiapoptotic and anti-inflammatory effects. Betaine treatment prevents initial fibrogenesis by decreasing TGF-β expression in the MCD diet-induced NAFLD. NRG-1 aggravates liver fibrosis in CCl_4 and BDL mouse model by increasing extracellular matrix accumulation. The pro-fibrotic effects of NRG-1 could be the result of ErbB3 receptor phosphorylation and activation of the NRG-1/ErbB3 signaling pathway in the liver. Betaine delays the progression of steatosis into NASH and may be used as a hepatoprotective agent in NAFLD.

Keywords: NAFLD, betaine, oxidative stress, autophagy, inflammation, apoptosis, liver fibrosis, neuregulin-1

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UDC:

Dejstvo betaina i neuregulina-1 na tkivne, ćelijske i molekularne promene na *in vivo* i *in vitro* modelima nealkoholne masne bolesti i fibroze jetre

Uvod

Nealkoholna masna bolest jetre (*eng*. *non-alcoholic fatty liver disease*; NAFLD) je jedan od najčešćih uzročnika hroničnih oboljenja jetre u razvijenim zemljama, s obzirom na sve veću učestalost gojaznosti i dijabetes melitusa tipa 2 pre svega u mlađoj populaciji. NAFLD predstavlja hepatičnu manifestaciju metaboličkog sindroma, koja se može ispoljiti u tri oblika: kao masna promena jetre, nealkoholni steatohepatitis (*eng*. *non-alcoholic steatohepatitis*; NASH) ili ciroza jetre, sa mogućnošću progresije u hepatocelularni karcinom.

Patogeneza NAFLD je kompleksna i još uvek nedovoljno rasvetljena. Savremena teorija ukazuje na značaj interakcije brojnih mehanizama na ćelijskom i molekularnom nivou u patogenezi NAFLD, tzv.teorija višestrukih udara. Smatra se da centralnu ulogu u njenom razvoju ima insulinska rezistencija, što uslovljava povećanje *de novo* lipogeneze u jetri, povećanje lipolize u masnom tkivu, povećan priliv masnih kiselina u jetru i nastanak steatoze. Slobodne masne kiseline ispoljavaju lipotoksično dejstvo i uzrokuju mitohondrijsku disfunkciju, oksidativni stres, kao i stres endoplazmatskog retikuluma. Usled povećanja permeabilnosti sluznice u digestivnom traktu i prolaska endotoksina bakterija crevne flore u cirkulaciju, dolazi do pokretanja zapaljenskog odgovora i oslobađanja proinflamatornih citokina, kao što su interleukini (IL-1, IL-6, IL-8) i faktor tumorske nekroze alfa (TNF-α). Oštećenja ćelija usled zapaljenja i oksidativnog strresa dovode do apoptoze i nekroze hepatocita. Ovi mehanizmi dovode do pogoršanja steatoze usled oksidativne modifikacije apoB100 i smanjenog efluksa lipoproteina veoma male gustine (*eng. very low density lipoprotein*; VLDL), kao i do progresije steatoze u NASH kod genetski predisponiranih osoba.Skorašnje studije ukazuju da su izmenjeni procesi apoptoze i autofagije verovatno uključeni u patogenezu NAFLD.

Hronično oštećenje jetre i inflamacija podstiču razvoj fibroze posredstvom brojnih proinflamatornih citokina, faktora rasta i slobodnih kiseoničnih radikala. Centralna uloga u fibrogenezi pripada aktiviranim hepatičnim zvezdastim ćelijama, koje su glavni izvor

kolagena i drugih proteina ekstracelularnog matriksa. Kupferove ćelije i u manjoj meri hepatociti i neparenhimske ćelije oslobađaju transformišući faktor rasta beta (*eng. transforming growth factor-β;* TGF-β), slobodne kiseonične radikale i citokine (IL-1, TNF-α), koji udruženo sa oksidovanim lipoproteinima male gustine (*eng. low-density lipoprotein*; oxLDL) uzrokuju aktivaciju i transdiferencijaciju zvezdastih ćelija u miofibroblastima slične ćelije. Aktivirane zvezdaste ćelije eksprimiraju gen za alfa-aktin glatkih mišića i gen za kolagen tipa I. Faktor rasta poreklom iz trombocita (*eng. platelet-derived growth factor;* PDGF) stimuliše proliferaciju i hemotaksu zvezdastih ćelija i sinergistički sa TGF-β podstiče fibrogenezu. Miofibroblasti takođe oslobađaju faktore rasta i citokine, koji autokrinim mehanizmom podstiču autoaktivaciju i vode razvoju ciroze jetre.

Neuregulin-1 (NRG-1) pripada familiji epidermalnog faktora rasta i uključen je u procese ćelijske diferencijacije, proliferacije, rasta i preživljavanja. Dejstvo NRG-1 je posredovano ErbB3 i ErbB4 receptorima, koji nakon vezivanja liganda grade heterodimere sa ErbB2. Dosadašnje studije su pokazale da rekombinantni humani NRG-1 (rhNRG-1) igra važnu ulogu u razvoju i održanju strukture kardiomiocita i ispoljava antifibrogeno dejstvo u srcu, plućima, bubrezima i koži. Međutim, uloga NRG-1 u procesu fibrogeneze u jetri još uvek nije ispitivana.

Za eksperimentalno izučavanje NAFLD koriste se različiti životinjski modeli, genetski, nutritivni kao i njihova kombinacija. Korišćenje dijete deficijentne u metioninu i holinu (MCD) je jedan od najčešće korišćenih nutritivnih modela, pre svega zbog ponovljivosti i kratkog vremenskog roka za razvoj masnih promena u jetri. Nedostatak holina u ishrani, remeti stvaranje fosfatidilholina u jetri, koji je neophodan za sintezu VLDL i time doprinosi akumulaciji triglicerida u jeti izazivajući steatozu, praćenu inflamacijom i oksidativnim stresom.

Betain ili 3-metilglicin je netoksična amino kiselina koja se dobija iz različitih nutritivnih izvora (školjke, spanać) ili se endogeno sintetiše iz holina. Betain učestvuje u remetilaciji homocisteina kao donor metil grupe i ispoljava antioksidativno dejstvo u alkoholnoj bolesti jetre kod miševa povećanjem sadržaja aminokiselina koje sadrže sumpor. Osim ovog, betain ispoljava i antiinflamatorno dejstvo i smanjuje stepen steatoze u jetri mehanizmima nezavisnim od metabolizma homocisteina. Takođe je pokazano da betain

povećava osetljivost tkiva na insulin kod dijabetičnih životinja delimično smanjenjem stresa endoplazmatskog retikuluma.S druge strane, dejstvo betaina na procese autofagije i apoptoze kao i na signalne puteve uključene u patogenezu NAFLD još uvek nisu u potpunosti razjašnjeni.

Ciljevi istraživanja

Definisati model NAFLD na miševima korišćenjem MCD dijete

Ispitati uticaj betaina na morfološke i ultrastrukturne promene u jetri miševa sa NAFLD

Ispitati uticaj betaina na oksidativni stres, apoptozu, autofagiju i inflamaciju u jetri miševa sa NAFLD

Ispitati dejstvo neuregulina-1 na akumulaciju vezivnog tkiva, ekspresiju gena za kolagen tip I i III kao i na gensku ekspresiju matriksmetaloproteinaza 2 i 9, na modelima fibroze izazvanim podvezivanjem duktusa holedohusa i primenom ugljen tetra hlorida (CCl_4)

Ispitati *in vitro*dejstvo neuregulina-1 na ekspresiju gena za TGF-β na izolovanim hepatocitima

Ispitati uticaj neuregulina-1 na fosforilaciju ErbB3 i ErbB4 receptora u tkivu jetre

Materijal i metode

Eksperimentalni dizajn

Eksperimenti su izvođeni na mužjacima miševa C57BL/6 soja, prosečne telesne mase 23±3g, uzgajanih na Vojnomedicinskoj akademiji u Beogradu. Životinje se čuvaju pod standardnim laboratorijskim uslovima (temperatura 22±2^{o}C, relativna vlažnost 50±10%, ciklus svetlost/tama 12/12 h), i pre početka eksperimenta imaju *ad libitum* pristup standardnoj hrani i vodi.

U prvom delu istraživanja, u cilju definisanja modela NAFLD, životinje (n=28) uzrasta 8 nedelja, su randomizacijom bile podeljene u sledeće grupe: 1. kontrola (n=7) – kontinuirano na standardnoj dijeti; 2. MCD2, MCD4 i MCD6 (n=7 po grupi) – grupe životinjana MCD dijeti u trajanju od dve, četiri i šest nedelja. Nakon tretmana uzorci krvi su uzeti za određivanje aktivnosti transaminaza (alanin- i aspartat-animotransferaza, ALT i AST) spektrofotometrijskom metodom i lipidnog profila (holesterol, trigliceridi, LDL i

lipoproteini velike gustine /*eng. high-density lipoprotein*; HDL/).Uzorci jetre su korišćeni za patohistološku analizu stepena razvoja masnih promena u jetri i prisustva zapaljenskog infiltrata. Pored toga, određivani su parametri oksidativnog stresa i antioksidativne zaštite, a gasnom hromatografijom je određen profil zasićenih i nezasićenih masnih kiselina u tkivu jetre.

Drugi eksperiment je sproveden u cilju ispitivanja efekata betaina na tok NAFLD. Životinje (n=28) su do 8 nedelja starosti bile na standardnoj ishrani, a potom podeljene u sledeće grupe: 1. kontrolna grupa (n=7) - kontinuirano na standardnoj ishrani, 2. BET grupa (n=7) – životinje na kontrolnoj ishrani, koje su dobijale betain rastvoren u vodi za piće (1,5% rastvor *ad libitum*), 3. MCD grupa – na MCD dijeti tokom 6 nedelja, i 4. MCD+BET grupa – životinje na MCD dijeti uz suplementaciju betainom u vodi za piće. Nakon 6 nedelja, životinje su žrtvovane i za analizu su uzeti uzorci krvi i jetre. U serumu je određivana aktivnost transaminaza, a u tkivu jetre su određivani:

Markeri oksidativnog stresa - koncentracija lipidnih peroksida, sadržaj redukovanog glutationa (GSH), aktivnost glutation reduktaze i peroksidaze (GRed i GPx), aktivnost superoksid dizmutaze (SOD) i njenih izoenzima, katalaze, paroksonaze (PON1), arilesteraze (ARE) kao i sadržaj nitrita i nitrata (NOx).

Markeri autofagije –nivoi proteina LC3II, p62, mTOR i genska ekspresija Beclin 1, Atg4b, Atg5 i Atg7

Markeri apoptoze – Bax, Bcl2, Bbc3, Bak1

Markeri zapaljenja – IL-10, IL-6, TNF-α i profibrogeni marker TGF-β

Transmisionom elektronskom mikroskopijom ispitivaće se ultrastrukturne promene u jetri

U trećem delu istraživanja je ispitivan uticaj NRG-1 na fibrozu jetre na dva eksperimentalna modela. U prvom modelu mužjaci miševa C57BL/6j soja (n=32) su podeljeni u sledeće grupe (8 životinja po grupi): 1. kontrola –tretirana kukuruznim uljem i.p. 3x nedeljno; 2. NRG-1 grupa–tretirana rhNRG-1 (20 μg/kg i.p.) 5x nedeljno; 3. CCl_4 grupa –tretirana 20% rastvorom CCl_4(1 mg/kgi.p.) u kukuruznom ulju 3x nedeljno i 4. CCl_4+NRG-1 grupa – tretirana kombinacijom CCl_4 i rhNRG. Drugi model fibroze je bio postavljen podvezivanjem ductusa choledochusa (*eng. bile duct ligation*, BDL) miševima

istog soja. Životinje (n=32) su bile podeljene u četiri grupe: 1. kontrola –tretirane PBS-om (*eng. phosphate saline buffer*) i.p.; 2. NRG-1 grupa –tretirana rhNRG-1 (20 μg/kg i.p.) 5x nedeljno, 3. BDL – životinje kojima je podvezan žučni kanal i tretirane PBSom i 4. BDL+NRG-1 – životinje kojima je bio podvezan žučni kanal i tretirane su rhNRG-1 (20 μg/kg i.p.) 5x nedeljno počev od prvog dana nakon operacije. Nakon četiri nedelje, životinje su bile žrtvovane u opštoj anesteziji, a uzorci krvi i jetre su bili uzeti za analizu. U krvi je određivana aktivnost transaminaza, a tkivo jetre bojeno Masson's Trichrome bojenjem u cilju kvantifikacije fibroze. Pored toga u jetri je bila određivana ekspresija gena za kolagen tipa I i III i matriksmetaloproteinaze (MMP) 2 i 9. U tkivu jetre kontrolne i životinje stimulisane rhNRG-1, je određivano prisustvo i fosforilacija ErbB3 i ErbB4 receptora za NRG-1. U *in vitro* uslovima ekspresija gena za TGF-β je određivana na izolovanim hepatocitima pre i 16 h nakon stimulacije NRG-1.

Patohistološka analiza

Uzorci tkiva se fiksiraju u 10% rastvoru formaldehida na sobnoj temperaturi. Isečci debljine 5 μm se prožimaju rastopljenim parafinom, potom boje hematoksilinom i eozinom (HE) odnosno Masson's Trichrome bojenjem i pripremaju se za analizu svetlosnim mikroskopom. Preparati su analizirani i fotografisani pomoću svetlosnog mikroskopa (*OlympusBX51 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 analiziranog tkiva.

Transmisiona elektronska mikroskopija

Uzorci tkiva jetre se periodu od 2h fiksiraju potapanjem u 4% rastvor paraformaldehida i u 2% rastvor glutaraladehida. Uzorci tkiva se zatim ponovo fiksiraju u 1% rastvoru osmijum tetroksida, pa dehidriraju u rastvorima etanola rastuće koncentracije, da bi zatim ultramikrotomom bili pravljeni preseci tih tkivnih blokova (*Leica Ultracut UCT, Beč, Austrija*). Polutanki isečci (0,5 μM) se posle sečenja na ultramikrotomu, boje toluidin plavim i koriste se za identifikaciju oblasti od interesa. Ultratanki isečci (70-80 nm) se dobijaju serijskim sečenjem polutankih isečaka i boje se olovo-citratom i uranil-acetatom radi dobijanja kontrasta. Na kraju, uzorci se posmatraju transmisionom

elektronskom mikroskopijom (*Fei, Morgagni 268D, Ajndhoven, Holandija*) radi detektovanja ultrastrukturnih promena.

Određivanje parametara oksidativnog stresa

Uzorci jetre su homogenizovani na ledu u 0,25 M hladno puferovanom saharoznom medijumu, u 10 M fosfatnom puferu (0,25 M saharoze, 10 mM K/$NaPO_4$ pufer, pH 7,0 i 1 mmol/L EDTA). Homogenati tkiva su centrifugirani na 2000 obrtaja tokom 15min na 4^o C. Supernatanti (nadtalozi)se prebacuju u epruvete i potom centrifugiraju na 3200 obrtaja tokom 30 min na 4^o C. Dobijeni supernatanti (nadtalozi)se čuvaju na - 70˚C i u njima je merena koncentracija lipidnih peroksida, GSH, GRed i GPx, SOD i njenih izoenzima, katalaze, PON1, ARE kao i sadržaj NOx.

Određivanje lipidnog profila u plazmi i profila slobodnih masnih kiselina u jetri

Nivo ukupnog holesterola u plazmi je određivan merenjem vodonik peroksida koji nastaje pod desjtvom holesterol oksidaze u reakciji sa 4-aminotriptinom i fenolom na 500nm. Koncentracija LDL je određivana kombinacijom ultracentrifugiranja i precipitacije opisanim po Friedewaldu, dok je HDL odeđivan korišćenjem enzimske kolorimetrijske metode, a ukupni trigliceridi korišćenjem modifikovanog reagensa 2,4,6,-tribromo-3-hidroksibenzoične kiseline.

Slobodne masne kiseline u uzorcima jetre su određivane gasnom hromatografijom sa plamenim jonizacionim detektorom.

Western Blot

Uzorci tkiva jetre su najpre homogenizovani u RIPA puferu koji sadrži koktel inhibitora proteaza i fosfataza. Nakon centrifugiranja na 14000G tokom 5 minuta, supernatant (nadtalog) se uzima i koristi za dalju analizu. Iste količine proteina za svaki uzorak se radvajaju elektroforezom, a zatim prenose na nitrocelulozne membrane i inkubirajupreko noći na 4°C sa odgovarajućim primarnim antitelima: antitelo specifično za protein mikrotubule 1 lakog lanca 3II (LC3 II), sekvestozom (SQSTM1/p62), AMPKα1/2, fosfo-AMPKα1/2 (Thr172), Akt, fosfo-Akt (Ser473), mTOR, fosfo/mTOR (Ser2448), p70S6 kinase (S6K), fosfo-S6K (Thr389), ErbB3, ErbB4, fosfo-ErbB3, fosfo-ErbB4, GAPDH i aktin. Nakon toga membrane su bile inkubirane sa kozjim ili zečjim sekundarnim

antitelima konjugovanim peroksidazom tokom 1h na sobnoj temperaturi. Specifični proteini su vizuelizovani korišćenjem pojačavajućih hemi-luminscentnih reagenasa i ChemiDoc MP sistema. Nivoi proteina su bili kvantifikovani pomoću denzitometrije korišćenjem ImageJ softvera i izraženi u odnosu na kontrolni protein aktin ili GAPDH.

Real time PCR

Nakon izolacije, 1μg RNK je bio korišćen za reakciju reverzne transkripcije, korišćenjem reverzne transkriptaze i oligo(dt) prajmera prema uputstvu proizvođača. Real time PCR analiza se izvodila na pločama sa 96 bunarića, pomoću TaqMan Universal Master Mix-a i TaqMan prajmerima za TNF-α, IL-6, IL-10, TGF-β, Bax, Bak1, Bbc3, Bcl2, Atg4b,Atg5, Atg7, beclin-1, kolagen tip I (col 1a1), kolagen tip III (col3a1), MMP-2 i MMP-9. Genska ekspresija traženih gena je bila normalizovana u odnosu na ekspresiju 18S ribozomalne RNK (Rn18s) ili β-aktina. Real-time PCR analize su izvođene naRealplex^2 Mastercycler (Eppendorf, Hamburg, Germany) korišćenjem ploča sa 96 polja.

Rezultati

MCD dijeta je izazvala blagu steatozu sa fokalnim masnim promenama nakon 2 nedelje, koja je bila izraženija nakon 4 nedelje. Difuzna makro- i mikrovezikularna steatoza sa inflamatornim infiltratom bila je prisutna u grupi miševa na MCD dijeti tokom 6 nedelja ukazujući na razvijen NASH. MCD dijeta je uzrokovala promeneu lipidnom profilu u serumu miševa, značajno smanjujući ukupni holesterol, tigliceride i HDL i povećavajući koncentraciju LDLa. Značajne promene su uočene i u hepatičnom profilu slobodnih masnih kiselina, gde je MCD dijeta za 6 nedelja uzrokovala porast oleinske i linolne kiseline, a smanjenje zastupljenosti zasićenih i polinezasićenih (C 22:6n3, C 22:5n3)masnih kiselina ($p<0,01$ u odnosu na kontrolu).

Suplementacija betainom je vidljivo ublažila steatozu i inflamaciju na histološkom nalazu i poboljšala lipidni profil u serumu smanjujući nivo ukupnog holesterola i LDLa, a povećavajući nivo HDLa u poređenju sa MCD grupom ($p<0,01$). Betain je takođe smanjio lipidnu peroksidaciju i nitrozativni stres čiji su parametri MDA i NOx bili značajno povišeni u MCD grupi. Takođe, betain je povećao aktivnost antioksidativnih enzima PON1, ARE, ukupnu SOD, katalazu i GPx u poređenju sa MCD grupom. Pored toga, betain je

značajno povećao sardžaj glutationa u jetri i smanjio aktivnost GRed u odnosu na grupu hranjenu MCD dijetom ($p<0,01$).

MCD dijeta u trajanju od 6 nedelja je uzrokovala porast u mRNK ekspresiji TNF-α, IL-6 i TGF-β, dok je suplementacija betainom sprečila ovaj porast. Betain je, takođe, povećao ekspresiju antiinflamatornog IL-10 u odnosu na grupu tretiranu MCD dijetom, koja je dovela do sniženja njegove ekspresije ($p<0,05$). Ekspresija proapoptotskog Bax je bila snižena, a expresija Bcl2, koji ima antiapoptotsko dejstvo je bila povišena u MCD+BET grupi u odnosu na MCD ($p<0,05$).

Suplementacija betainom je dovela po povećanja expresije gena za autofagiju Atg4, Atg5, Atg7 i Beclin1 u poređenju sa kontrolnom i MCD grupom ($p<0,05$). MCD dijeta je izazvala povećanje nivoa SQSTM1/p62 u odnosu na kontrolu ($p<0,05$), dok je betain izazvao značajno smanjenje nivoa SQSTM1/p62 u odnosu i na kontrolu i MCD grupu ($p<0,05$). Analiza elektronsom mikroskopijom je pokazala povećano prisustvo autofagozoma, kao i smanjeno prisustvo apoptotskih telašaca kod miševa na MCD dijeti koji su bili tretirani betainom u odnosu na kontrolnu i MCD grupu.

Histološko Masson's trichrome bojenje je pokazalo iraženu fibrozu u BDL modelu, dok je CCl_4izazvao blaži stepen fibroze nakon četiri nedelje. NRG-1 tretman je statistički začajno pogoršao fibrozu jetre od 1,86 na 2,65% u CCl_4– izazvanoj fibrozi, od 5,03 na 8,25% u fibrozi izazvanoj podvezivanjem žučnih puteva ($p<0,01$). Ekspresija gena za col1a1 i col3a1 nije bila promenjena pod dejstvom NRG-1 u CCl_4 grupi, dok je na modelu izražene fibroze NRG-1 doveo do povećanja genske ekspresije za oba tipa kolagena ($p<0,05$). Pored toga, NRG-1 je indukovao značajno smanjenje ekspresije za MMP2 u odnosu na CCl_4 grupu ($p<0,05$). U BDL grupi, genska ekspresija za MMP9 u jetri je bila značajno povišena u odnosu na kontrolu ($p<0,01$), dok tretman NRG-1 nije uzrokovao promene u ekspresiji gena za MMP2 i MMP9 ($p>0,05$).

NRG-1 je izazvao povećanu fosforilaciju ErbB3 receptora u jetri miševa u mnogo većoj meri nego ErbB4 ($p<0,01$). Pored toga, NRG-1 je povećao gensku ekspresiju za TGF-β in vitro na izolovanim hepatocitima, u poređenju sa netretiranim ćelijama ($p<0,01$).

Betain ispoljava antioksidativno dejstvo smanjenjem lidpidne peroksidacije i nitrozativnog stresa u NAFLD izazvanoj MCD dijetom, kroz povećanje aktivnosti

antioksidativnih enzima u jetri i obnavljanjem sadržaja GSH. Dodatno, betain snižava nivo triglicerida i LDL u serumu, a ujedno dovodi do povećanja nivoa HDL holesterola.

Betain ublažava tok NAFLD potencijalno indukcijom procesa autofagije kroz povećanje ekspresije gena Atg4, Atg5, Atg7 i Beclin 1, nezavisno od AMPK/mTOR signalnog puta. Smanjenje apoptoze i inflamacije u NAFLD izazvanoj MCD dijetom, je još jedan potencijali mehanizam protektivog dejstva betaina. Suplementacija betainom je smanjila ekspresiju gena za TGF-β, ublažavajući time inicijalnu fibrozu jetre na modelu NAFLD.

NRG-1 pogoršava fibrozu jetre miševa naCCl_4 i BDL modelu, povećanjem ekspresije gena za kolagen tip I i III, potencijalno dovodeći po povećane sinteze ekstracelularnog matriksa. Profibrogeni efekti NRG-1 su rezultat povećane fosforilacije ErbB3 receptora i aktivacije NRG-1/ErbB3 signalnog puta u jetri.

Ključne reči: NAFLD, betain, oksidativni stres, autofagija, fibroza jetre, neuregulin-1
Naučna oblast: Medicina
Uža naučna oblast: Fiziološke nauke
UDK:

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1. INTRODUCTION

1.1. Definition and epidemiological data

NAFLD is the hepatic manifestation of metabolic syndrome, and represents a wide spectrum of liver diseases, ranging from simple steatosis, nonalcoholic steatohepatitis (NASH), and fibrosis with the potential to progress into cirrhosis and hepatocellular carcinoma (HCC) (1).

NAFLD is the most common liver disease in the Western countries and is strongly associated with metabolic syndrome (MetS) and insulin resistance. Nowadays, NAFLD is considered as emerging public health issue worldwide with an increasing prevalence among general population, that is in the range of 20-40% (2,3). Over past two decades, the prevalence of type 2 diabetes mellitus (T2DM) has been increasing rapidly accompanied with obesity, especially among children (4). Global prevalence of NAFLD is 25.24% with highest values in the Middle East and South America, and the lowest in Africa. Metabolic comorbidities that are associated with NAFLD include obesity, T2DM, hyperlipidemia, hypertension and MetS (3). All these comorbidities represent risk factors for cardiovascular diseases, which are one of the leading cause of death in NAFLD patients (2).

NAFLD is usually described as accumulation of fat in hepatocytes, predominantly in the form of triglycerides that occurs in the absence of alcohol intake. It is often without symptoms, and can be present for years undetected (5).

1.2. Pathogenesis of NAFLD

According to “two-hit” hypothesis, steatosis makes liver vulnerable to various second-hits that lead to inflammation and further hepatocytes damage (6). Lipid accumulation is described as a first hit in NAFLD development. However, it is now evident that two-hit theory is too simplistic to describe the complexity of NAFLD pathogenesis, where multiple parallel factors are acting synergistically and contribute to disease development and progression (Figure 1.1.). That is why “multiple-hit” hypothesis is used today to cover all molecular mechanisms and signaling pathways that occurs in NAFLD (7). Steatosis is usually associated with increased secretion of hepatokines, decreased

glycogen synthesis, increased gluconeogenesis and inhibition of insulin signaling (8,9). Accumulation of excess lipids in hepatocytes causes insulin resistance that is followed by chronic inflammation which increases the risk of NAFLD progression into fibrosis, cirrhosis or eventually HCC. Besides dyslipidemia, today it is well known that adipose tissue dysfunction/inflammation is crucial in NAFLD pathogenesis, as well as oxidative stress and dysbiosis of the gut microbiota accompanied with increased absorption of bacterial products, such as short-chain fatty acids, lipopolysaccharide and endotoxins (8–11).

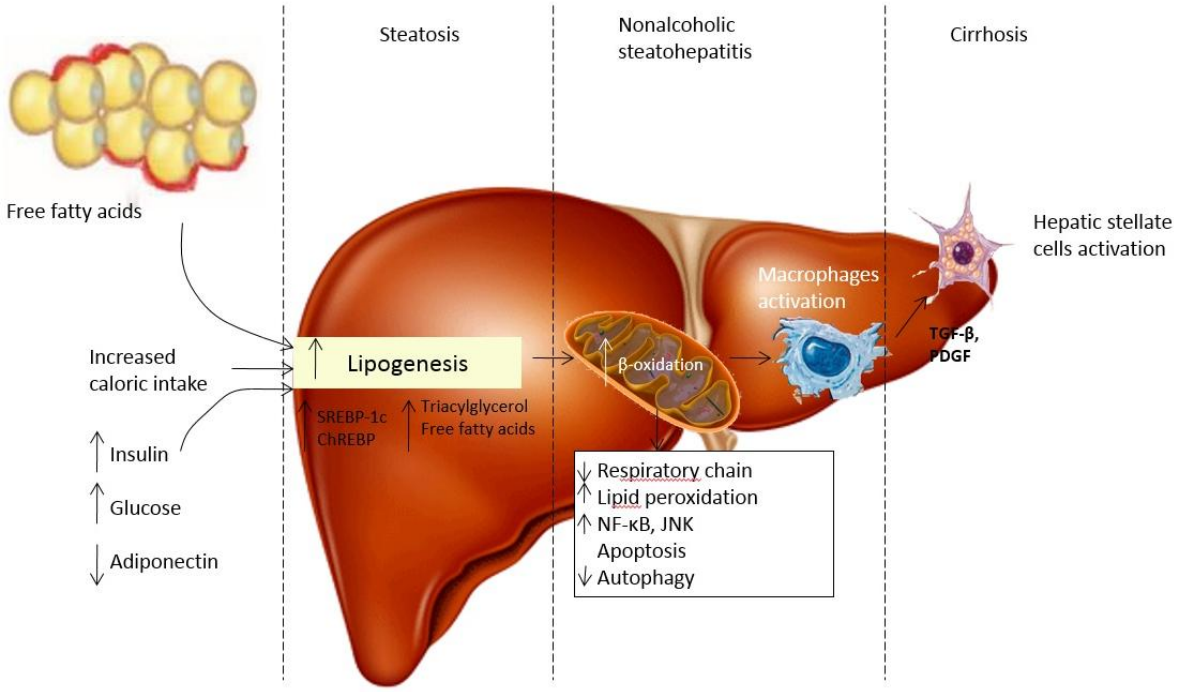


Figure 1.1. Schematic diagram showing the course and progression of liver steatosis to steatohepatitis and cirrhosis. Various pathogenetic mechanisms are involved as described by "multiple-hit" theory, where first hit represents lipid accumulation and lipotoxicity, making liver more vulnerabile to second hit factors such as oxidative stress, inflammation and apoptosis.

Visceral obesity is closely linked with NAFLD, which occurs in 60-95% of obese people. The importance of visceral fat has been shown in many experimental studies on obese animals (12). Visceral adipose tissue is a metabolically active and inflammatory organ, that can modulate the metabolism and function of liver, muscles, brain and cardiovascular system (13). The production of pro- and anti-inflammatory adipokines is imbalanced in patients with NAFLD (14). Adiponectin and leptin are the most important adipokines involved in NAFLD pathogenesis and have been studied the most. Leptin is an anorexigenic hormone that prevents lipid accumulation in non-adipose tissues, and in the liver this is achieved by decreasing the expression of sterol regulatory element-binding protein-1 (SREBP-1). In obese people, leptin levels increase as a consequence of leptin resistance, and it exerts pro-fibrogenic characteristics through activation of stellate cells via the hedgehog and mTOR pathway, as well as by stimulating Kupffer cells to produce transforming growth factor (TGF)-β (7,15). Adiponectin has hepatoprotective effects by its ability to reduce inflammation inhibiting the release of proinflammatory cytokines such as interleukin (IL)-6 and tumor necrosis factor (TNF)-α (15) and improving insulin resistance. Antifibrotic effect of adiponectin is potentially mediated by the AMP-activated protein kinase (AMPK) activation (7,16). Besides these two well-characterized adipokines, recent research revealed other adipokines with a potential role in NAFLD such as visfatin, grelin, resistin, chemerin, apelin, lipocalin, and retinol-binding protein (15,17).

1.2.1. Lipotoxicity in NAFLD pathogenesis

Fat accumulates in the liver predominantly in the form of triglycerides, which are derived from glycerol esterification and from free fatty acids. Sources of free fatty acids are dietary intake, lipolysis in adipose tissue and hepatic *de novo* lipogenesis. When free fatty acids reach hepatocytes, they undergo acyl-CoA synthases activity and form fatty acyl-CoA that can enter β-oxidation cycle or undergo esterification (7,18). Accumulation of triglycerides *per se* does not exert hepatotoxic effects. On the contrary, it can represent a protective mechanism to balance the excess of free fatty acids in the liver that has been shown in mouse models of fatty liver disease (19). However, in NAFLD, inhibition of triglyceride incorporation in very low-density lipoprotein (VLDL) followed by decreased

free fatty acid oxidation occurs simultaneously with lipotoxicity and toxic metabolites generation and therefore leads to more severe liver damage and worsening of steatohepatitis (20). *De novo* lipogenesis in the liver is increased by activation of transcription factors such as SREBP-1, carbohydrate response element-binding protein (ChREBP) and peroxisome proliferator-activated receptor (PPAR)-γ (21). Free fatty acids in liver cells induce disruption in insulin signaling pathways and contribute to insulin resistance. Insulin resistance is described as a condition with higher insulin levels required to achieve normal metabolic response and circulating glucose regulation. Insulin resistance is manifested as a reduced ability of insulin to inhibit glucose production in the liver and to stimulate the utilization of glucose in adipose tissue and skeletal muscles (22). In the state of insulin resistance, lipolysis in adipose tissue is impaired, which further increases free fatty acids efflux to the liver. Insulin resistance is a cardinal feature of NAFLD and is more prevalent in patients with NASH compared to simple steatosis (7). Insulin resistance increases fat tissue lipolysis, circulating levels of free fatty acids and decreases glycogen storage in the liver, which stimulates gluconeogenesis. Insulin resistance can be central (hepatic) and peripheral (skeletal muscle and adipose tissue). Peripheral is manifested as a reduced uptake of glucose from blood in muscles and fat tissue with increased efflux of free fatty acids, while central is manifested as uncontrolled production of hepatic glucose resulting from impaired suppression of gluconeogenesis and glycogen synthesis (22). Lipotoxicity impairs insulin signaling by promoting oxidative damage and inflammation, leading to steatosis progression to NASH, fibrosis and cirrhosis (23,24). It is known that lipotoxicity promotes cell death in NAFLD and is called hepatocytes lipoapoptosis, whose degree correlates with severity of NAFLD (25,26). When the lipid influx to the liver cannot be handled by mitochondrial or peroxisome function, respiratory oxidation processes may be altered and collapse with impairment of lipid homeostasis, increased generation of toxic lipid metabolites and reactive oxygen species (ROS). Molecular oxygen, which accepts electrons, is the main source of radicals. The most important are hydroxyl radical (•OH), nitric oxide radical (NO•) and the superoxide anion (O_2•−). These unstable and reactive radicals are generated as products of intracellular metabolic reactions and have the ability toreact with proteins, free fatty acids, and DNA. Mitochondria represents the main

endogenous intracellular sources of ROS, but ROS are being produces in endoplasmic reticulum (ER), and peroxisomes as well. On the other hand, superoxide anion radicals (O_2•−) are produced as a result of enzymatic activity, such as cytochrome P450 metabolism and xanthine oxidase (27).

1.2.2. Oxidative stress in NAFLD pathogenesis

Oxidative stress is recognized as a key mechanism responsible for liver damage in general, and is result of imbalance between prooxidant and antioxidant mechanisms in the favor of prooxidation. Usually it is a consequence of excessive ROS production combined with reduced activity of antioxidant enzymes. Altered function of mitochondria results in ROS overproduction leading to increased lipid and protein peroxidation that consequently alters lipid metabolism in the liver, playing thus significant role in steatosis and steatohepatitis development (28). Long-chain fatty acids contribute to generation and release of ROS by lipooxigenation through activity of microsomal cytochromes, CYP2E1 and CYP4A. Microsomal and peroxisomal oxidation are normally involved in fatty acids metabolism, but their activity is increased when long-chain fatty acids are accumulated due to the decrease in CYP2E1 (28). ROS may interact with polyunsaturated fatty acids. The result of intracellular lipid peroxidation leads to formation of 4- hydroxy-2-nonenal (4-HNE) and malondialdehyde (MDA). These products freely diffuse outside of cells causing the damage of distant cells, therefore amplifying the toxic effects of oxidative stress. All sources of ROS, including mitochondria, xanthine oxidase, endoplasmic reticulum, CYP2E1 and peroxisome can contribute to NAFLD development and progression (28).

The major enzymes which serve as antioxidants are copper/zinc superoxide dismutase (Cu/ZnSOD) and manganese-superoxide dismutase (MnSOD) which promote reduction of O_2•− toH_2O_2. Besides, glutathione peroxidase (GPx) is another important antioxidant enzyme that facilitates conversion of H_2O_2 into water (28). It has been proven that decreased antioxidant defense plays a significant role in oxidative damage in NASH, predominantly decreased hepatic glutathione (GSH) and decreased activity of SOD, GPx and glutathione transferase, which correlates with disease severity (28,29). It was observed

that in patients with NAFLD decreased activity of antioxidants is accompanied with increased serum markers of oxidative stress, while in animal models of NAFLD these findings are confirmed with low levels of mitochondrial GSH (28,30).

Damaged function of mitochondrial respiratory chain is the main source of ROS, superoxide, hydrogen peroxide, and hydroxyl radicals. Acute oxidative stress can be a result of inflammation, organ infarction, reperfusion injury and shock. In the stressed condition, oxidative reactions such as β-oxidation, tricarboxylic acid (TCA) cycle turn oxidized (NAD+ and FAD) into reduced (NADH and FADH2) cofactors. After the oxidation of these reduced cofactors, through the respiratory chain electrons are being transferred to molecular oxygen. During prolonged oxidative stress, mitochondria lose their function of detoxification superoxide and their capacity to control the oxidative balance is being reduced or completely collapsed (27). Injured mitochondria become an additional source of excess superoxide due to electron leakage and conversion of superoxide into hydrogen peroxide by SOD. Hydrogen peroxide can be metabolized by GPx or catalase to water. Fenton and/or Haber-Weiss reactions generate highly reactive and toxic ROS and are mediated by iron, an inducer of oxidative stress which is found to be elevated in NASH (27,31,32). High plasma levels of citrate promote oxidative stress mediated by iron, by increasing hydroxyl radical formation. Besides, fatty acid and glucose in higher concentrations elevate pyruvate and acetyl-CoA increasing citrate formation. Furthermore, ROS can interact with polyunsaturated fatty acids and initiate lipid peroxidation and MDA or 4-HNE formation which can spread oxidative stress (33). β-oxidation in mitochondria is not inhibited until the respiratory chain is severely damaged resulting in accelerated ROS production, making mitochondria the major source of oxidative stress. However, ER stress can induce production of superoxide and hydrogen peroxide by activity of monooxygenases (cytochrome P450 and peroxisomes). Antioxidant defense in mitochondria is not powerful enough to deal with continuous and prolonged oxidative stress that results in mitochondrial DNA and respiratory chain damage (27,34). Mitochondrial DNA damage results in accumulation of mutations that cannot be repaired completely. Causing this chain reaction, oxidative stress is more and more pronounced and large amounts of ROS continue to be generated and further damage other mitochondria or organelles inducing cellular apoptosis.

Highly reactive ROS and lipid peroxidation products are able to diffuse into the extracellular space affecting neighboring Kupffer cells and hepatic stellate cells (HSC). Kupffer cells directly uptake free fatty acids and free cholesterol, and together with oxidative injury of hepatocytes induce activation of nuclear transcription factor (NF)- κB, that consequently increase the synthesis of TNF-α and proinflammatory cytokines such as IL-6 or IL-8 (27,35). Kupffer cells produce TGF-β resulting in HSCs activation and acquiring a fibrogenic myofibroblast-like phenotype. Exposing HSC to hydroxyl radicals lead to an increase in ER stress that further stimulate autophagy and HCS activation and transformation to fibrogenic phenotype (27,36). Autophagy, an adaptive response to cellular stress is up-regulated in activated HSC in the state of liver damage (27,37).

ROS consequently activate an inflammatory response contributing to hepatocytes necroinflammation and worsening of mitochondrial damage (38,39). There is a strong connection between the degree of oxidative stress and severity of NASH. Peroxidation of phospholipids on mitochondrial membranes lead to alteration of electron transportation system and ROS overproduction. Besides, high blood glucose level is associated with increased glucose autooxidation, glycation, inhibition of antioxidant enzymes, and mitochondrial dysfunction. In NAFLD insulin resistance contributes to the increase in CYP2E1 expression, which can potentiate liver injury (29).

Increased ROS production is accompanied with a decrease in antioxidant defense, which is strong factor promoting oxidative stress in NASH. Decrease in SOD, catalase and GSH correlate with severity of the liver injury. Several mechanisms are described by which ROS contribute to NASH development. Free radicals cause lipid peroxidation, as well as oxidative modification of hepatocytes DNA and cell proteins, and mitochondrial electron transportation chain leading to even more increase in ROS production. Not only ROS are involved in oxidative damage of hepatocytes, but also it has been show that nitric oxide (NO) level is also increased leading to formation of reactive nitrogen species (RNS) that can be involved in NAFLD pathogenesis as well (40).

1.2.3. Inflammation in NAFLD pathogenesis

ROS have strong potential to activate inflammatory cascade by the activation of the NF-κB followed by the increase in IL-1, IL-6, IL-8, IL-10 and IL-17 expression as well as TGF-β and platelet-derived growth factor (PDGF) (41). Two main inflammatory signaling pathways C-Jun N-terminal kinase (JNK)-AP-1 and IKK-NK-κB are predominantly involved in chronic inflammation process in NAFLD. Various animal studies support the key role of hepatic cytokines in NAFLD progression, suggesting that increased levels of cytokines cause typical histological changes such as necrosis and apoptosis of hepatocytes, neutrophil infiltration, HSCs activation and production of Mallory bodies (7). Since inflammation, increased TNF-α and NF-κB activation can promote liver disease progression, the chronic inflammatory state present in NAFLD may play an important role in HCC development (7,23,42).

Recent studies have demonstrated that activity of Kupffer cells represents an important factor in initiation and progression of NAFLD. Kupffer cells, liver macrophages, are located in sinusoids, and can exhibit an M2-like phenotype in normal conditions and express several toll-like receptors (TLR) (43,44). After TLR ligand binding, Kupffer cells can induce T cell activation and cytotoxic T-lymphocytes response followed by neutrophil and monocytes infiltration. There is a known interaction between Kupffer cells and various immune cells, such as T lymphocytes, dendritic cells, HSCs and innate lymphocytes. Besides M2, TLRs have ability to activate Kupffer cells towards the M1 phenotype. Signals leading to macrophage activation converge on two main downstream pathways, NF-κB and JNK(43,45). The JNK pathway is activated by ROS, saturated free fatty acid, and cholesterol (43–45), while NF-κB acts as a key regulator of inflammation and cell death and is activated by various stimuli, such as TLRs, IL-1β, and TNF-α (43,46). When activated towards M1 phenotype, Kupffer cells produce various factors which are involved in inflammation and fibrosis development. IL-1 has a strong inflammatory effect and also can promote triglycerides synthesis in the liver by decreasing PPAR-α activity. Activated Kupffer cells secrete TNF superfamily ligands such as TNF-α and TNF-related apoptosis-inducing ligand (TRAIL), therefore stimulating apoptosis and inflammation of hepatocytes.

Production of TNF-α contributes to monocyte infiltration, hepatocytes apoptosis and plays important role in NASH development (43,46,47).

1.2.4. Apoptosis and autophagy in NAFLD pathogenesis

Apoptosis represents controlled and organized process of cell death. During apoptosis, cells are fragmented into small apoptotic bodies that contain cleaved DNA and cell fragments and are removed by phagocytosis. Apoptotic processes are triggered by two pathways: Initiaation of extrinsic pathway is mediated by death receptors (Fas, TNF receptor, TRAIL) and activates proteolytic enzymes, especially caspases; and the intrinsic apoptotic pathway that is initiated by mitochondrial dysfunction, ER stress, lysosomal permeabilization and damage of DNA. In hepatocytes, the main apoptotic trigger is mitochondrial dysfunction, when proapoptotic proteins, such as cytochrome c and caspase 9, are released in cytosol, where this complex activates caspases 3, 6 and 7 leading to final cell death. In mitochondria, apoptotic triggers are regulated by Bcl-2 proteins family that includes antiapoptotic proteins Mcl-1, Bcl-2ad Bcl-xL, and proapoptotic multi-domain Bax-like proteins, such as Bax, Bak and BH3. In NAFLD, the key trigger of apoptosis is lipotoxicity. Products of lipid metabolism are capable to initiate apoptotic signaling pathways in hepatocytes, these includes cholesterol, free fatty acids, phospho- and sphingolipids. Lipotoxicity is closely associated with chronic inflammatory processes that occurs during NASH development following obesity, diabetes and metabolic syndrome (48). Toxic products of lipid metabolism can damage cells through several mechanisms, by impairment function of endoplasmic reticulum and mitochondria, as well as other cellular organelles. Well-defined targets of toxic lipids are predominantly adipose tissue, skeletal muscle, heart and pancreas, and modulation of their response to lipid metabolites contribute to presentation of the complex picture of metabolic syndrome (49).

Autophagy is an intrinsic pathway for the degradation of damaged organelles, and initially is a protective mechanism that is initiated by cell starvation or damage. There are three types of autophagy depending on the way that damaged substrate is transported to the lysosomes: macroautophagy, microautophagy and chaperone-mediated autophagy. The

major pathway is macroautophagy. The autophagosome is formed at first as a double membrane that enclose degradation target, then it fuses with a lysosome to form an autophagolysosome where recycling occurs. Lipophagy is the mechanism of lipid content regulation via processes of autophagy (49). Since abnormal lipid accumulation, stored in lipid droplets in hepatoctytes is main characteristic of NAFLD (50), it may implicate that autophagy processes are misfunctional. Additionally, steatosis may contribute to NASH development through secondary insults from oxidants and cytokines triggering oxidative stress and chronic inflammation (51–53).

Macroautophagy is known as a homeostatic mechanism responsible for recycling of organelles and long-lived proteins (54). Lipases that are contained in lysosomes are able to degrade organelle-associated lipids and exogenous lipoproteins, but intracellular lipids were not previously considered to be a substrate for autophagic degradation (55). However, recent studies are suggesting that macroautophagy could have a role in the regulation of hepatic lipid stores (53). By genetic or pharmacological inhibition of autophagy gene Atg5, it was shown that cellular triglycerides content significantly increased in cultured hepatocytes. High amounts of triglycerides and cholesterol are still stored in lipid droplets because of a decreased lipolysis and reduced β-oxidation of fatty acids in cells with inhibited macroautophagy. Studies on laboratory animals showed that during starvation, macroautophagy is stimulated and recognized by increased presence of lipid autophagosomes (53,56). Therefore, increasing autophagy can potentially reduce the fat content in fatty liver disease.

1.3. Pathogenesis of liver fibrosis

Chronic liver injury can often result in more severe stage of liverdisease, liver fibrosis which precedes to the development of cirrhosis and HCC (57). The usual causes of liver fibrosis are viral hepatitis C and B (HCV and HBV), alcoholic steatohepatitis and NASH. Autoimmune hepatitis, cholestasis, metabolic disorders, primary biliary cirrhosis, drugs and toxins contribute to liver fibrosis development as well (58,59).

Fibrosis represents an excess accumulation of ECM proteins as a wound-healing response to chronic, repeated injury and hepatocytes death, due to failure of the regeneration process. In fibrosis processes, synthesis of ECM proteins is increased and accompanied with its decreased degradation. It results in accumulation of collagen (type I, III and IV), fibronectin, elastin, laminin and proteoglycans up to six times more than normal. Fibrotic tissue accumulates in portal area, pericentral and perisinusoidal spaces, with the tendency to form bridging structures and nodules in cirrhosis (60). Loss of normal tissue architecture followed by impaired function leads to liver insufficiency, with the only remaining therapy being organ transplantation. However, there is evidence suggesting that liver fibrosis is reversible, and it represents good therapy-targeting point to attenuate excessive ECM accumulation, and prevent irreversible liver damage (59,61).

In the pathogenesis of liver fibrosis both parenchymal and nonparenchymal cells are involved. Activation of myofibroblasts that produce ECM proteins plays a critical role in the pathogenesis of liver fibrosis. Three types of cells may be transdifferentiated into hepatic myofibroblasts: HSC, portal fibroblasts and bone marrow-derived collagen producing cells, fibrocytes. Major source of myofibroblasts, and therefore ECM proteins are activated HSCs. In liver injury, damaged hepatocytes and Kupffer cells trigger inflammatory response, release ROS and fibrotic mediators such as TGF-β (62,63). TGF-β is a key mediator in liver fibrogenesis and is the strongest stimulus for HSCs activation, their transformation into myofibroblasts and increased ECM proteins synthesis (63). So far, the most efficient therapy for liver fibrosis is removal of etiological agent. However, there is still no standard therapy for liver fibrosis. Recent studies are searching for the best therapeutic approach that will reduce inflammatory response, collagen amount in the liver, and still do not disturb normal balance of ECM proteins synthesis and degradation (64).

1.3.1. Neuregulin-1

Neuregulin belongs to epidermal growth factor family and is encoded by one of four genes (from *NRG1* to *NRG4*). Neuregulin acts through activation of ErbB tyrosine kinase receptors (ErbB2, ErbB3 and ErbB4) and is involved in cellular proliferation,

differentiation, development and survival in various tissues (65). Recent studies suggest that neuregulin-1 may play and important role in glucose metabolism by increasing GLUT4 content in skeletal muscles cells, indicating that prolonged treatment with neuregulin-1 regulates glucose metabolism in muscles. However, the effects of neuregulin-1 in liver have not been investigated in detail, even it is well known that liver plays an important role in glucose metabolism. It has been shown that expression of ErbB3 receptors is more pronounced than ErbB4 in the liver of adult rats (66). The NRG-1/ErbB signaling is proven to be indispensable for normal development and growth of various organ systems including nervous and cardiovascular systems (67,68) . To date, many studies have shown that NRG-1 is crucial for the maintenance of structural and functional integrity of the adult heart (69). Recombinant human NRG-1 (rhNRG-1) is currently being examined in clinical trials (phase II and III) in patients with chronic heart failure. Recent studies indicate that NRG-1 has anti-fibrotic effects in the heart, kidney, lung, and skin (70–73). Although NRG-1 seems to have anti-fibrotic properties in different organs, the role of NRG-1 in fibrotic liver diseases has not been elucidated.

1.4. Animal models of NAFLD and liver fibrosis

Various animal models are used for studying NAFLD and liver fibrosis. An ideal animal model of NAFLD should represent all manifestations that are present in human population. This model should demonstrate steatosis, inflammatory infiltrate near hepatic blood vessels and ballooning of hepatocytes. Further, it would be appropriate if a model develops metabolic abnormalities such as dyslipidemia, insulin resistance, obesity and altered adipokine profile (74). Animal models that are frequently used for studying NAFLD can be classified as dietary models and genetic models. Also, combination of those two is used as well. The complexity of NAFLD pathogenesis is the main reason why are there so many different models, since none of those are ideally matching human condition.

1.4.1. Dietary models for NAFLD

The best described dietary model is methionine-choline deficient (MCD) diet model. This diet is usually rich in sucrose and the fat content is about 40%. The deficiency in methionine and choline results in altered β-oxidation of free fatty acids and impaired production of VLDL, that consequently lead to fat accumulation in the liver, hepatocytes death, oxidative stress and inflammation (75,76). On the other hand, methionine plays important role as intermediate in S-adenosylmethionine (SAM) and GSH synthesis, two important antioxidants. The methionine deficiency contribute to oxidative stress at first, and afterwards to inflammation and fibrosis (77,78). This animal model is most used due to its reproducibility and short time required for NASH histological phenotype development, around six weeks, compared to high-fat diet model, that need much more time to develop (75). The MCD diet model induces more severe oxidative stress, inflammation, apoptosis and fibrosis than other nutritional NAFLD models (75). However, this model has its disadvantages because of its association with weight loss (around 20% after three weeks) and lack of insulin resistance and metabolic syndrome manifestations (74).

High-fat diet is second widely used nutritional model that contribute to NASH phenotype more similar to the human disease. It is characterized by the presence of obesity, insulin resistance, dyslipidemia and inflammation, but without hepatocytes ballooning and less pronounced fibrosis after prolonged period of use (76,78). High-fat diet provides approximately 60% energy from fat, 20% from carbohydrates and 20% from proteins, but there are also many variations of this diet composition. To gather fully developed NASH manifestations, with complete metabolic alteration and histological changes, this model requires prolonged usage, around 20 weeks or even more, depending on animal strain that is used (75,76,79). Taken together, in rodents, high-fat diet replicates altered metabolic status quite similar to metabolic changes observed in humans, but histopathological outcome is not always fully manifested (75).

The addition of high fructose syrup to high-fat diet has showed the development of all features of NASH. High-carbohydrate diet increases oxidative stress in the mice liver, followed my macrophage infiltration and fibrosis. This diet contribute to metabolic

syndrome with obesity, insulin resistance and fatty liver. Adding fructose promotes the development of fibrosis, adipose tissue inflammation and hepatocellular damage in mouse models of NASH (75).

Besides these nutritional models, cholesterol rich diet, also called atherogenic diet induces progressive steatosis, inflammation and fibrosis with severe hepatocytes damage and ballooning after 24 weeks. Also, in this model, dyslipidemia, lipid peroxidation and oxidative stress are well manifested. In contrast to high-fat diet, these animals on atherogenic diet are more sensitive to insulin due to decreased amount of adipose tissue, and metabolic changes are different from human NASH (75).

For better understanding of NASH, various genetically modified rodents are developed. In the most of them, genetic alteration result in fat accumulation in the liver, but without inflammation or fibrosis, so usually it is necessary to combine knockout mice model with modified diet to induce all manifestations of NASH.

1.4.2. Genetic models for NAFLD

Among the genetic models, mice with leptin deficiency (the ob/ob mice) have spontaneous point mutation in the gene coding the leptin synthesis. Leptin has role in food intake regulation and increases the energy storage. These mice are obese, hyperphagic, inactive and develop hyperglycemia, hyperinsulinemia and insulin resistance accompanied with hyperlipidemia (80). In this model, metabolic alterations are fully developed, but the inflammation in the liver tissue may be absent, so the additional stimuli are required for complete NASH presentation. Db/db knockout mice are carriers of leptin receptor gene mutation, and develop resistance to the leptin effects despite its normal or elevated circulating level. These mice exert similar phenotype like ob/ob mice that include obesity and metabolic disturbances with insulin resistance. With an additional stimulus, such as MCD diet, NASH can be observed with all histological signs with high potential for significant fibrosis development (75).

SREBP-1c transgenic mice overexpress SREBP-1c in adipose tissue that consequently have impaired development and function leading to severe insulin resistance

and hepatic steatosis. Liver steatosis is followed by inflammation, ballooning hepatocytes and pericellular fibrosis. This model is a suitable model for studying lipodystrophy associated with NASH (75). KK-Ay/a mice that have mutation in the agouti gene, manifested as hyperphagia and impaired suppression of food intake. Phosphatase and tensis homolog null mice results in hepatomegaly accompanied by steatosis due to increased fatty acid synthesis (81). There are also PPAR-α null mice, Acyl-coenzyme A oxidase null mice, Methionine adenosyl transferase null mice etc. (75).

1.4.3. Liver fibrosis animal models

In vivo models of liver fibrosis can be divided in several groups: Chemical-based models, diet-based models, surgery-based models and genetically modified animal models (82). The most used models are chemical ones that require short time of intraperitoneal application of specific chemicals. Ethanol is known to cause chronic liver disease that starts with steatosis and may progress into fibrosis and cirrhosis. However in rodent models, mere alcohol consumption is not enough to induce the progression of alcoholic liver disease into fibrosis. Therefore, it is necessary to combine alcohol intake with some specific diet, pharmacological agents or genetic modification. Carbon tetrachloride (CCl_4) is most commonly used hepatotoxin in order to induce and study liver fibrosis and cirrhosis in rodents. In the liver, CCl_4 biotransformation relies on CYP2E1 and produces trichloromethyl radical which is involved in lipid peroxidation and free radicals generation. These changes contribute to an acute phase reaction with centrilobular necrosis of hepatocytes, activation of Kupffer cells and inflammatory response (82,83). This model can be applied to both mice and rats, but using mice is more preferable due to their higher metabolic rate of CCl_4. Since this liver fibrosis model is easy reproducible, requires 4 to 6 weeks of administration and has low mortality rate, it is widely accepted and one of the most commonly used.

Besides these two, thioacetamide, dimethylnitrosamine (DMN) and dietylnitrosamine (DEN) are frequently used to experimentally induce liver fibrosis in rodents. After the biotransformation, reactive oxygen species are produced and react with lipids, proteins and nucleic acid causing cell dysfunction and consequent necrosis. Numerous dietary models

are usually used to induce progression of NAFLD to NASH and afterwards to more severe stages of liver damage, such as fibrosis (82).

Surgery-based model such as bile duct ligation (BLD) is well known to induce cholestatic liver injury and periportal fibrosis. The obstruction of ductus choledochus lead to bile accumulation in the intrahepatic bile ducts and increases biliary pressure. Afterwards, secretion of cytokines increases and inflammatory processes occur. This results in biliary epithelial cells proliferation, increased expression of fibrogenic markers such as TGF-β, collagen type 1, α-smooth muscle actin (SMA) and tissue inhibitor metalloproteinase (TIMP)-1 as well as in increased ROS production and oxidative damage of hepatocytes (82). Fibrosis can be studied on genetically modified models, such as multidrug resistance-associated protein 2 (MDR2) deficient mice and *Alms1Fat ausi* mutant mice.(foz/foz), as well as on *in vitro* models using HSC culture (82).

1.5. Betaine

Betaine, known as trimethylglicine (N,N,N-trimethylammonioacetate) is a methyl derivate of glycine (Figure 1.2.) .

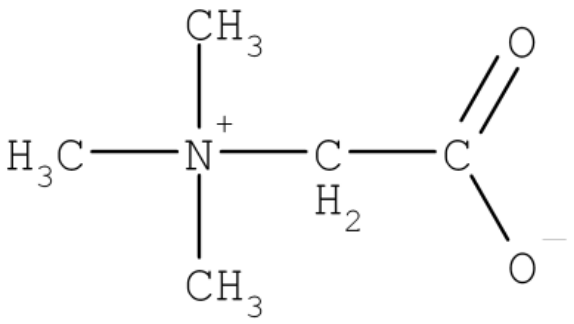


Figure 1.2. The chemical structure of betaine

It represents an important nutrient that can be found in grains, spinach, shrimps and beet, and dietary intake is an important source of betaine, even there is endogenous synthesis from choline, especially in the liver and kidney (84,85). Betaine has few important roles in mammalian organism, especially in kidney and liver. In the kidney, primary role of betaine is osmoprotection in the inner medulla cells. It includes betaine

accumulation from blood via the Na^+- and Cl^--dependent betaine-GABA transporter (BGT1) on the basal plasma membrane. Betaine balances high extracellular osmolarity and regulates cell volume. In the liver, betaine primarily functions as a methyl group donor. One-carbon metabolism is metabolic network of biosynthetic pathways in cytoplasm, mitochondria and cell nucleus where betaine is involved. In cell nucleus, betaine plays an important role in DNA replication and repairmen and in DNA methylation. In the cytoplasm, primary role of betaine is remethylation of homocysteine to methionine, a precursor to SAM, that is universal methyl group donor to more than 80 biological reactions of methylation in organism. Methyl groups are transferred from betaine to homocysteine through the methionine cycle that is catalyzed by enzyme called betaine-homocysteine methyltranferase (BHMT). Betaine is widely used in animals and humans nutrition. In human population it has been used to reduce cardiovascular risk and complications as well as neurodegenerative diseases by lowering increased homocysteine levels (84). In animals, betaine is added to animal diet to increase the lean muscle mass and decrease the fat (86). Recently, the role of betaine in alcoholic and nonalcoholic fatty liver disease has been investigated. In mice that developed NAFLD by feeding with high fat diet, betaine supplementation increased SAM and prevented and reversed insulin resistance and liver steatosis by modulating pathways involved in glycogen synthesis, gluconeogenesis and insulin signaling (84). Similar study suggested that one of the mechanism responsible for betaine action was in part due to relief of ER stress (86). Effects of betaine in NAFLD seem to include multiple metabolic pathways directly or indirectly, but those are still not completely investigated.

2. OBJECTIVES

The objectives of the dissertation were:

- To develop a NAFLD model in mice using MCD diet.
- To examine the effects of betaine on liver morphological and ultrastructural changes in mice with NAFLD.
- To examine the effects of betaine on oxidative stress, apoptosis, autophagy and inflammation in the liver in mice with NAFLD.
- To examine the effects of neuregulin-1 on connective tissue accumulation, gene expression for collagen type I and III, and matrix metalloproteinases 2 and 9 in animal models of liver fibrosis induced by CCl_4 and bile duct ligation.
- To examine *in vitro* effects of neuregulin-1 on TGF-β gene expression in isolated hepatocytes.
- To examine the effects of neuregulin-1 on ErbB3 and ErbB4 receptors phosphorylation in the liver.

3. MATERIAL AND METHODS

3.1. Experimental design

3.1.1. Setting animal model of NAFLD

The experiments were performed on male C57BL/6 mice weighing on average 23±3 g raised at the Military Medical Academy in Belgrade. Animals were housed in controlled laboratory environment (temperature 22 ±2°C, relative humidity 50±10%, 12/12 h light-dark cycle with lights turned on at 9:00 a.m.) and had free access to water and standard chow before the experiment.

Experimental animals (n=28) at the age of 8 weeks were randomly divided into the following groups: 1. Control (n =7) continuously fed with standard chow; 2. Groups fed with MCD diet for two (MCD2; n=7), four (MCD4; n=7) or six weeks (MCD6; n= 7). After the treatment, blood samples were taken for spectrophotometric determination of aminotransferase (alanine- and aspartate aminotransferase, ALT and AST), alkaline phosphatase (ALP) activities and lipid profile (cholesterol, triglycerides, low-density lipoprotein /LDL/, and high-density lipoproteins/HDL/). Liver samples were used for pathohistological evaluation of fatty liver change and the presence of inflammatory infiltrate. Besides, the parameters of oxidative stress and activities of antioxidative enzymes, as well as free fatty acids profile have been determined in the liver. The MCD diet composition is showed in Table 1.

Table 1. Methionine/Choline deficient diet composition (960439-MP Biomedical, CA, USA). Control diet had the same composition as methionine/choline deficient diet except 2g/kg choline and 3g/kg DL-methionine were added at the expense of sucrose.

Nutrient	**gm/kg**
Alphacel non-nutritive bulk	10
Calcium Phosphate dibasic	250
Corn Oil	50
Corn Starch	100
Cupric carbonate	1.15
Di-calcium phosphate	1.5
Ferric citrate	3
L-amino acids	85.2
Magnesium oxide	12
Manganese carbonate	1.7
Mineral mix	14.45
Potassium citrate monohydrate	110
Potassium sulphate	26
Sodium Chloride	37
Sucrose	225
Sucrose, finely powdered	55
Vitamin mix	10
Zinc Carbonate	8
Total	1000

3.1.2. The effects of betaine on NAFLD

This experiment will be performed in order to investigate the effects of betaine on NAFLD development in mice fed with MCD diet. Animals (n=28) will be on standard diet until the age of 8 weeks, and then they will be divided into the following groups: 1. control (n=7) – continuously on standard diet, 2. BET group (n=7) – animals on standard diet that will be treated with betaine solution in drinking water (1.5% solution *ad libitum*), 3. MCD group (n=7) – animals on MCD diet during 6 weeks, and 4. MCD+BET group (n=7) – animals on MCD diet treated with betaine in drinking water. After 6 weeks, animals will be sacrificed and blood and liver samples will be taken for further analysis. Aminotransferase activities will be measured in serum, while oxidative stress parameters, autophagy markers (LC3II, p62, mTOR, and gene expression for Beclin 1, Atg4b, Atg5, Atg7), apoptosis markers (Bax, Bcl2, caspase 3) and markers of inflammation (IL-1β, IL-6, TNF-α) will be determined in liver samples. Ultrastructural changes in the liver will be examined by using transmision electron microscopy.

3.1.3. The role of NRG-1 on liver fibrosis on two in vivo models

For this experiment, adult male C57BL/6j mice (Charles River Laboratories) average weight 25g were used. Animals have been housed in controlled laboratory environment (temperature 22 ±2°C, relative humidity 50±10%, 12/12 h light-dark cycle with lights turned on at 9:00 a.m.) and had free access to water and standard chow before the experiment.

In the first model, mice (n=32) were divided into the following groups (n=8 per group): 1. control –treated with corn oil (Sigma Aldrich) i.p. 3x per week; 2. NRG-1 group – treated with rhNRG-1 (20 μg/kg i.p., Peprotech) 5x per week; 3. CCl_4 group – treated with 20% CCl_4 solution (1mg/kg body weight i.p., Sigma Aldrich) diluted in corn oil 3x per week and 4. CCl_4+NRG-1 group – treated with both CCl_4 and NRG-1 in combination in a previously described manner for 4 weeks.

The second liver fibrosis model was performed by ligation of the bile duct (BDL), as described by Tag C. et al. (87). Animals (n=32) were divided into four groups (n=8 per

group): 1. control – treated with phosphate buffered saline (PBS) i.p.; 2. NRG-1 group – treated with rhNRG-1 (20 μg/kg i.p.) 5x per week; 3. BDL group – animals that had bile duct ligated and treated with PBS, and 4. BDL+NRG-1 group – animals that had bile duct ligated and treated with rhNRG-1 (20 μg/kg i.p) 5x for week. For bile duct ligation animals were anesthetized with i.p. injections of ketamine/xylazine (100mg/10mg/kg body weight). After making an incision at the middle abdominal line, the peritoneal cavity was opened and organs were exposed. Liver lobes were lifted, then the bile duct was separated from portal vein and ligated with two sutures. Animals were left to recover overnight before starting rhNRG-1 treatment. After four weeks, animals will be sacrificed in general phenobarbital anesthesia (150mg/kg), and blood and liver samples have been taken for analysis. Transaminase activities were measured in serum, while liver tissue was stained with Masson's trichrome in order to determine and quantify the fibrosis. In the liver tissues, gene expression for collagen type I and III, and matrix metalloproteinases 2 and 9 will be determined. The presence and phosphorylation of ErbB3 and ErbB4 receptors will be determined in the liver of control and rhNRG-1-treated animals by Western blot.

3.1.4. The effects of NRG-1 on TGF-β expression in vitro on isolated hepatocytes

Primary hepatocytes were isolated from mouse liver by two-step liver perfusion as previously described (88) with minor modifications. Liver was perfused by cannulating inferior cava vein, while portal vein was cut for drainage. Solution 1 was PBS without calcium and magnesium (6 min perfusion at 5mL/min); Solution 2 contained collagenase P (Roche, Germany) dissolved in Dulbecco's Modified Eagle Medium, DMEM (Invitrogen) in concentration 40mg/mL (7 min perfusion at 5mL/min). Liver was collected in 30mL of solution 2 on ice, and transferred in Petri dish under sterile conditions. 20mL of DMEM were added in Petri dish, and hepatocytes were scraped from liver. Tissue was held with tweezers and shaken well to release more cells. The solution got blurry, and the rest of the tissue was discarded. Cell suspension was filtered through 75μm cell strainer into 50mL tube and centrifuged on 50G for 4 minutes, then washed twice with 30 mL of DMEM (4 min on 50G centrifugation). Hepatocytes were cultured in DMEM containing 10% heat-inactivated fetal bovine serum (Invitrogen), 100 U/mL penicillin, and 100 μg/mL

streptomycin in two 6-well plates, in a humidified 5% CO2 incubator at 37°C. Cells were used 24 hours after culturing and were serum-starved for 12 hours prior to experiments. Primary hepatocytes were stimulated with rhNRG-1β (20 ng/mL) for 16 hours. Cells were collected for mRNA isolation and relative mRNA expression for TGF-β was measured by quantitative polymerase chain reaction (qPCR).

The expression of TGF-β gene will be determined *in vitro* on isolated primary hepatocytes before and 16 hours after NRG-1 stimulation.

3.2. Histopathology 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). The sections were analyzed and photographed using an Olympus BX51 (Olympus, Tokyo, Japan) light microscope equipped with Artcore 500 MI (Artray, Co. Ltd., Tokyo, Japan) camera.

The right liver lobe was fixed in 4% buffered formalin and embedded in paraffin. Sections were stained with Masson's trichrome, according to the manufacturer's instructions. Images were acquired with an Olympus U-TU1X-2 microscope and analyzed with ImageJ 1.42 software. Liver fibrosis was quantified by calculating the percentage of positively stained ECM area to total tissue area in digitalized microscopic images. Quantification was performed by a person blinded to the treatment protocol.

3.3. Transmission electron microscopy (TEM)

The liver tissue was fixed by immersion in a 3% solution of glutaraldehyde (Agar Scientific Ltd., Stansted, UK) in a 0.1 mol/L cacodylic buffer (pH = 7.4) over night at $+4^{o}$C. The tissue was then postfixed with 1% solution of osmium tetraoxide (Merck, Darmstadt, Germany) in a 0.1 mol/L cacodylic buffer, followed by overnight incubation at +4°C in a 4.8% aqueous solution of uranyl acetate (Serva, Heidelberg, Germany). The samples were embedded in a four-component embedding medium made up of the epoxy

embedding medium, dodecenyl succinic anhydride, epon hardener methyl nadic anhydride (all from Fluka Chemika, Buchs, Germany), and epon hardener accelerator N-benzildimethylamine (Agar Scientific Ltd., Stansted, UK). Semi-thin sections were cut from the blocks of embedded tissue, stained with toluidine blue, and analyzed under the Olympus BX41 microscope (Olympus GmbH, Hamburg, Germany). All slides were photo-documented with the Olympus C5060-ADU wide zoom camera and the Olympus DP-Soft Image Analyzer program (Olympus GmbH, Hamburg, Germany). Ultra-thin sections were treated with uranyl acetate and lead citrate (Alkaloid, Skopje, FYRM), and analyzed on a transmission electron microscope (Fei Morgagni 268D, Eindhoven, the Netherlands) equipped with a MegaViewIII Soft Imaging System digital camera (Olympus Soft Imaging Solutions GmbH, Münster, Germany).

3.4. Biochemical analysis

3.4.1. Determination of hepatic enzymes in the serum

Liver damange was biochemically confirmed by determination of the activity of serum alanine aminotransferase, aspartate aminotransferase and alkaline phosphatase. 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 determination.

3.4.2. Determination of acute phase proteins

The concentration of C-reactive protein (CRP) and ferritin was determined turbidimetrically, based on agglutination of latex particles coated with antibodies on CRP and ferritin (BioSystems, Barcelona, Spain). The concentration of serum transferrin was determined turbidimetrically, based on precipitation in the reagent contains antitransferin antibodydisolved in imidazole buffer (1 ml/L) and sodium azide (0.95 g/L, pH 7.5). All analyses were carried out on BTS-330 photometer according to the manufacturer's instructions.

3.4.3. Serum lipid profile determination

Lipid concentrations in plasma (total cholesterol, triacylglycerol [TAG] and HDL) were measured by enzymatic colorimetric methods. Cholesterol and triglyceride assay kits were purchased from Abcam (Cambridge, UK, Cholesterol Assay Kit, ab65390; Triglyceride Quantification Kit, ab65336). Total plasma cholesterol concentration was determined by measuring hydrogen peroxide generated by cholesterol oxidase in oxidative coupling reaction with 4-aminoantipyrine and phenol at 500 nm (89). Plasma LDL concentration was obtained by a combination of ultracentrifugation and precipitation procedure according to Friedewald et al (90). Plasma HDL concentration was determined by using enzymatic colorimetric method (Roche Diagnostics, Basel, Switzerland). In the presence of double positively charged anions, the HDL fraction was measured after selective LDL precipitation by polyanion sulfates (dextran sulfate and magnesium sulfate) in accordance with the Centers of Disease Control and Prevention (Atlanta, GA: Lipid Reference Section). After centrifugation, HDL fraction was determined directly in the supernatant by the PAP method. Modified cholesterol-oxidase reagent by inclusion of 2,4,6-tribromo-3-hydroxybenzoic acid was used to measure plasma TAG concentration by enzymatic colorimetric method.

3.4.4. Free fatty acid analysis

Lipids for fatty acid analysis were extracted from the liver according to the Bligh and Dyer method (91). The fatty acid methyl esters (FAMEs) from the lipid extract were transesterified with HCl in methanol according to the method as described by Ichihara and Fukubayashi (92). FAMEs were quantified using an Agilent Technologies 7890A Gas Chromatograph with a flame ionization detector. Separation of the FAMEs was performed on a 112-88A7, HP-88 capillary column (100 m x 0,25 mm x 0,2 μm) using He as a carrier gas at a flow rate of 105 ml/min. The samples were injected at a starting oven temperature of 175˚C, injector temperature was 250˚C and detector temperature was 280˚C. The oven temperature was programmed to increase from 175˚C to 220˚C at 5˚C/min. Fatty acids were

identified by their retention time with reference fatty acid standards (SupelcoTM FAME Mix, USA) and were expressed as a percentage of total fatty acids in the liver.

3.4.5. Oxidative stress parameters determination

Lipid peroxidation in liver homogenates was measured as MDA production, assayed in the thiobarbituric acid reaction as described by Girotti et al (93). The results are expressed in nanomoles per milligram of proteins in liver homogenates.

Nitrite concentration (NO_2^-) in liver homogenates was determined by using Griess reagent on ELISA reader at 540 nm (Plate reader, Mod. A1, Nubenco Enterprises, ICN)(94). Results are shown in nmol/mg proteins.

SOD activity was determined in liver homogenates according to the method of Sun and Zigman by measuring the absorbance change during epinephrine autooxidation into adrenochrome at 340 nm (95). Commercial SOD from human erythrocytes (Sigma Aldrich, Germany) was used as a standard for the evaluation of total SOD activity. One unit (U) of enzyme activity changes the absorbance for 0.001 per minute (ΔA/min) under the test conditions. Samples for manganese SOD (MnSOD) activity determination were previously treated with 8 mM KCN (Sigma Aldrich) and then analyzed as previously described (95). The activity of copper-zinc-SOD (Cu/Zn SOD) was calculated as a difference between the activities of total SOD and MnSOD.

Activity of GRed in liver homogenates was determined spectrophotometrically according to the method of Carlberg and Mannervik (96). Absorbance change was measured during oxidation of NADPH in the presence of dithiobis (2-nitrobenzoic acid) at 340 nm. Human recombinant GRed (Sigma Aldrich) was used as a standard for the evaluation of total GRed activity. One unit (U) of enzyme activity catalyzes the transformation of one μmol of DTNB per minute under the test conditions.

Activity of GPx in liver homogenates was determined spectrophotometrically according to the method of Wendel (97). Absorbance change was measured during oxidation of NADPH

at 340 nm. Commercial GPx from human erythrocytes (Sigma Aldrich) was used as a standard for the evaluation of total GPx activity. One unit (U) of enzyme activity catalyzes the transformation of one μmol of hydrogen peroxide per minute under the test conditions.

Catalase (CAT) activity in liver homogenates was assayed by means of UV-kinetic method in the presence of H_2O_2 (98). Activity was expressed as U/mg proteins.

Reduced glutathione (GSH) was determined spectrophotometrically with DTNB following the Ellman´s method at 412 nm (99). Results were shown in nmol/mg proteins.

Proteins were determined by the Lowry method using bovine serum as the standard (100). All spectrophotometric measurements were performed with Cecil CE 2021UV/VIS spectrophotometer.

PON1 activity was assayed by using synthetic paraoxon (diethyl-p-nitrophenyl phosphate) as a substrate in the assay mixture containing 2.0mM paraoxon, 2.0mM $CaCl_2$ and 40μL of liver homogenates in 100mM Tris-HCI buffer (pH 8.5). PON1 activity was determined by measuring the initial rate of substrate hydrolysis to p-nitrophenol, which absorbance was monitored at 412nm (101).

Phenylacetate was used as a substrate for the determination of arylesterase activity by using the molar extinction coefficient of phenol (1310 M^{-1} cm^{-1}) produced in this reaction (101).

3.5. Western Blot

The liver tissue was homogenized on ice in RIPA buffer containing protease/phosphatase inhibitor cocktail (all from Sigma-Aldrich, St. Louis, MO). The homogenates were centrifuged at 14000 g for 15 min at 4°C, and the supernatants were collected. Protein concentrations were determined using the method described by Bradford (102), using bovine serum albumin as a standard (Sigma). Equal protein amounts from each sample (30 μg) were separated by SDS-PAGE and transferred to nitrocellulose membranes.

After membrane blockade with 5% powder milk or 5% bovine serum albumin in Tris-buffered saline (TBS) Tween 10, membranes were incubated with primary antibodies at 4°C overnight. The membranes were then washed and incubated with secondary antibodies at room temperature for 1h. Rabbit polyclonal primary antibodies used were anti-microtubule-associated protein 1 light chain 3B (anti-LC3B), anti-sequestosome 1 (anti-SQSTM1/p62), anti-AMPKα1/2, anti-phospho-AMPKα1/2 (Thr172), anti-Akt, anti-phospho-Akt (Ser473), anti-mTOR, anti-phospho/mTOR (Ser2448), anti-p70S6 kinase (S6K), anti-phospho-S6K (Thr389), anti-ErbB3, anti-ErbB4, anti-phospho-ErbB3, anti-phospho-ErbB4 (all from Cell Signaling Technology, Beverly, MA), anti-actin and anti-GAPDH (Santa Cruz Biotechnology). Anti-actin and anti-GAPDH were diluted in 1:3000, while other primary antibodies were diluted in 1:1000. Goat anti-rabbit peroxidase-conjugated IgG was used as a secondary antibody (Jackson IP Laboratories, West Grove, PA) in a dilution 1:3000. After washing, specific protein bands were visualized using enhanced chemiluminescence reagents (Amersham Pharmacia Biotech, Piscataway, NJ) and ChemiDoc MP System (Bio-Rad, Hertfordshire, UK). The protein levels were quantified by densitometry using ImageJ software (NIH, Bethesda, MD) and expressed relative to actin or GAPDH as a loading control. The results were presented as fold change in signal intensity, which was set to 1 in untreated controls.

3.6. Real time PCR

Total RNA from liver tissue was extracted using TRIZOL reagent according to the manufacturer's instructions. Approximately 1 μg of RNA was used in the reverse transcription reaction using M-MuLV reverse transcriptase and random hexamers according to the manufacturer's instructions. Real-time PCR was performed in a Realplex^{2}Mastercycler using 96-well reaction plates, TaqMan Universal PCR Master Mix, and TaqMan primers/probes for mouse TNF-α, IL-6, IL-10, TGF-β, Bax, Bak1, Bbc3, Bcl2, Atg4b, Atg5, Atg7, beclin-1, collagen type I (col 1a1), collagen type III (col3a1), MMP-2, MMP-9, Rn18s, and β-actin. mRNA levels were expressed relative to Rn18s or β-

actin as a loading control and the results were presented relative to the control value, which was arbitrarily set to 1.

3.7. Statistical analysis

Normal distribution of parameters was checked by Kolmogorov-Smirnov test. Depending on distribution, the statistical difference among groups was determined using analysis of variance (ANOVA) with Tukey *post hoc* test, Student's t-test or Kruskall-Wallis with Mann-Whitney nonparametric test. All statistical analysis were performed using GraphPad Prism 6 or SPSS 15.0 software. The difference was considered statistically significant if $p<0.05$.

4. RESULTS

4.1 The effects of MCD diet on biochemical and histological changes in mice liver

4.1.1. The effects of MCD diet on mice body/liver weight ratio

MCD diet induced a significant decrease in body weight after two (17.29 ± 1.41g; $p<0.01$), four (15.86 ± 1.16g; $p<0.01$) and six weeks (15.00 ± 1.00g; $p<0.01$) by comparison with control (22.86 ± 1.77g). The lowest body weight was observed in MCD6 group. Also, the liver/body weight ratio was significantly lower in all MCD diet-treated groups in comparison with control. However, the most prominent decrease in this ratio was evident in MCD2 (4.08±0.45%) and MCD4 group (4.21±0.47%) (Figure 4.1).

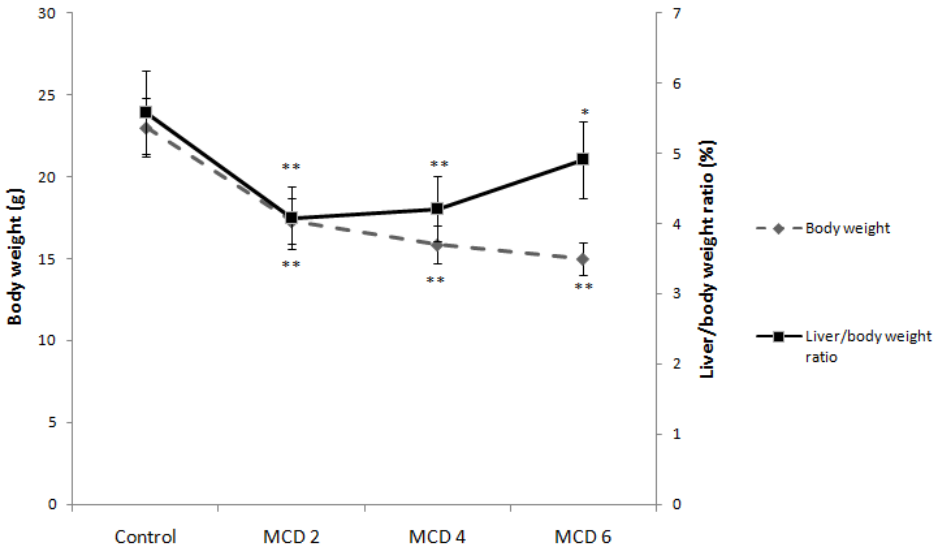


Figure 4.1. The effects of methionine/choline-deficient (MCD) diet on animal body weight and body/liver weight ratio 2, 4 and 6 weeks after beginning of treatment (MCD2, MCD4 and MCD6 groups respectively).

The significance of the difference was estimated by using one-way analysis of variance (ANOVA) with Tukey's *post hoc* test (*$p<0.05$, **$p<0.01$ vs. control). The data are presented as mean ±SD.

4.1.2. The effects of MCD diet on histological changes

No pathological changes were found in control group (Figure 4.2.A). MCD diet caused hepatic steatosis in mice, which was evident in all experimental groups. Mild hepatic steatosis with focal fatty change was found in MCD2 group in the form of microvesicular steatosis (Figure 4.2.B). Within four weeks MCD diet induced moderate steatosis with diffuse fatty change in mice liver (Figure 4.2.C). In MCD6 group the presence of severe macrovesicular steatosis with intralobular inflammatory infiltrate indicates NASH. Ballooning degeneration of hepatocytes and cell swelling were observed as a result of fat accumulation. Apoptotic bodies may be occasionally seen in MCD6 group (Figure 4.2.D).

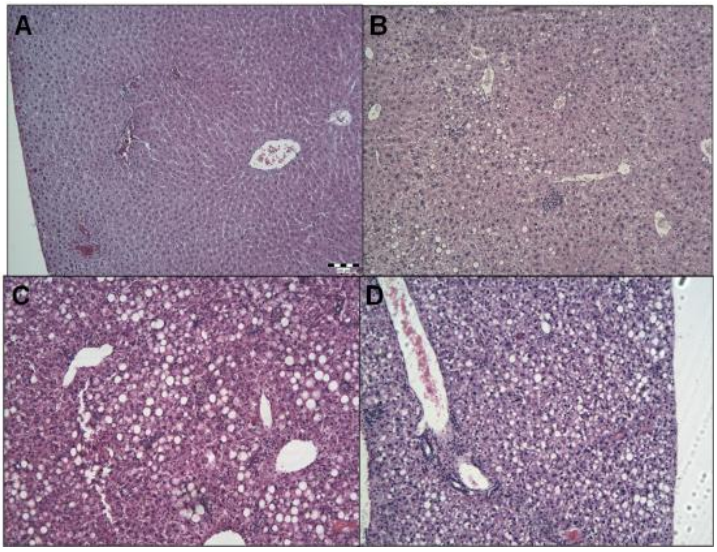


Figure 4.2. Histological findings of liver tissue in MCD diet-fed mice (Hematoxylin & Eosin, magnification x 40).

A. Control group shows normal morphology of the liver.
B. In MCD2 group focal fatty changes are evident (mild steatosis).
C. In MCD4 group diffuse fatty change is evident (more severe steatosis).
D. In MCD6 group steatosis with prominent inflammatory infiltrate, cell swelling and ballooning degeneration of hepatocytes are evident. Apoptotic bodies may also be seen.

4.1.3. The effect of MCD diet on serum biochemical parameters

Our study has shown that serum ALT activity was significantly increased in all experimental groups (MCD2: 492.32±61.10 U/L, MCD4: 510.15±63.51 U/L and MCD6: 537.09±77.32 U/L) in comparison with control group (66.05±19.22 U/L) ($p<0.01$). No significant difference in ALT activity was evident among MCD diet-treated groups. AST activity was significantly increased in MCD2 (642.87±42.24 U/L), MCD4 (669.65±86.21 U/L) and MCD6 (896.45±121.22 U/L) group in comparison with control (69.43±16.01 U/L). However, the most pronounced increase was observed in MCD6 group, while

between MCD2 and MCD4 no significant difference was evident. On the other hand, MCD diet did not cause the change in serum ALP activity in all experimental groups when compared with control (Figure 4.3.A). Accordingly, AST/ALT ratio was significantly increased in MCD diet-treated groups (MCD2: 1.30±0.04, MCD4: 1.31±0.05) in comparison with control (1.04±0.05) ($p<0.05$). However, the highest ratio was evident in MCD6 group (1.67±0.08) ($p<0.01$) (Figure 4.3.B).

Serum total cholesterol level was significantly decreased in MCD diet-treated group at all time points in comparison with control (2.34±0.25 mmol/L) ($p<0.01$). The most prominent decrease was observed in MCD6 group (1.16±0.14 mmol/L) compared with MCD2 (1.57±0.15 mmol/L) and MCD4 groups (1.33±0.16 mmol/L) ($p<0.01$).

Analysis of serum cholesterol fractions has shown that MCD diet induced a progressive decline in serum HDL level. While an initial decline was evident in MCD2 (1.17±0.09 mmol/L) when compared with control group (1.57±0.20 mmol/L) ($p<0.01$), serum HDL level was significantly lower in MCD4 (0.28±0.07 mmol/L) and MCD6 (0.18±0.08 mmol/L) groups in comparison with MCD2 group ($p<0.01$).

In contrast, serum LDL level was significantly elevated in MCD4 (0.49±0.10 mmol/L) and MCD6 (0.73±0.10 mmol/L) groups in comparison with control (0.07±0.03 mmol/L) ($p<0.01$). Difference in serum LDL level between MCD6 and MCD4 group was also highly significant ($p<0.01$) (Figure 4.4.A).

Serum triglyceride level was significantly decreased in all experimental groups (MCD2: 0.75±0.10 mmol/L, MCD4: 0.7±0.14 mmol/L and MCD6: 0.48±0.05 mmol/L) in comparison with control group (1.52±0.12 mmol/L) ($p<0.01$). The decrease was more prominent in MCD6 group compared to MCD4 and MCD2 groups ($p<0.05$) (Figure 4.4.B).

Concentration of CRP and ferritin was significantly increased ($p<0.01$), while the concentration of transferrin was significantly lower ($p<0.05$) in MCD6 group when compared with control group (Table 2).

Table 2. Concentration of acute-phase proteins in serum of mice fed with MCD diet during 2, 4, and 6 weeks (MCD2, MCD4, MCD6, respectively)

	Group			
Parameter	Control	MCD2	MCD4	MCD6
CRP	0.17±0.04	0.16±0.06	0.21±0.03	0.43±0.05**
Ferritin (g/L)	87.20±6.83	92.62±10.10	95.84±10.40	135.02±23.94**
Transferrin (g/L)	0.09±0.02	0.07±0.02	0.08±0.01	0.04±0.01*

CRP: C-reactive protein

Significance of the difference was estimated by using one-way analysis of variance (ANOVA) with Tukey's post hoc test was used (*$p<0.05$, **$p<0.01$ vs. control).

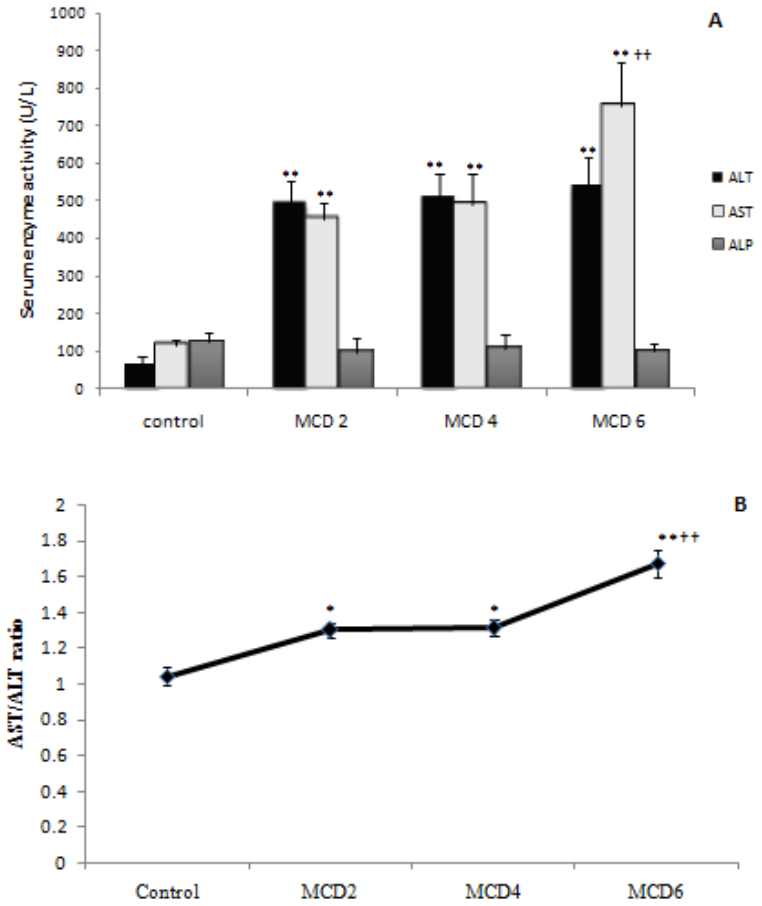


Figure 4.3. The effects of MCD diet on serum enzymes (ALT, AST and ALP) activity (A), and AST/ALT ratio (B).

Significance of the difference was estimated by using one-way analysis of variance (ANOVA) with Tukey's post hoc test was used (*p<0.05, **p<0.01 vs. control; ††p<0.01 vs. MCD4).

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALP, alkaline phosphatase

For further information see Fig. 1.

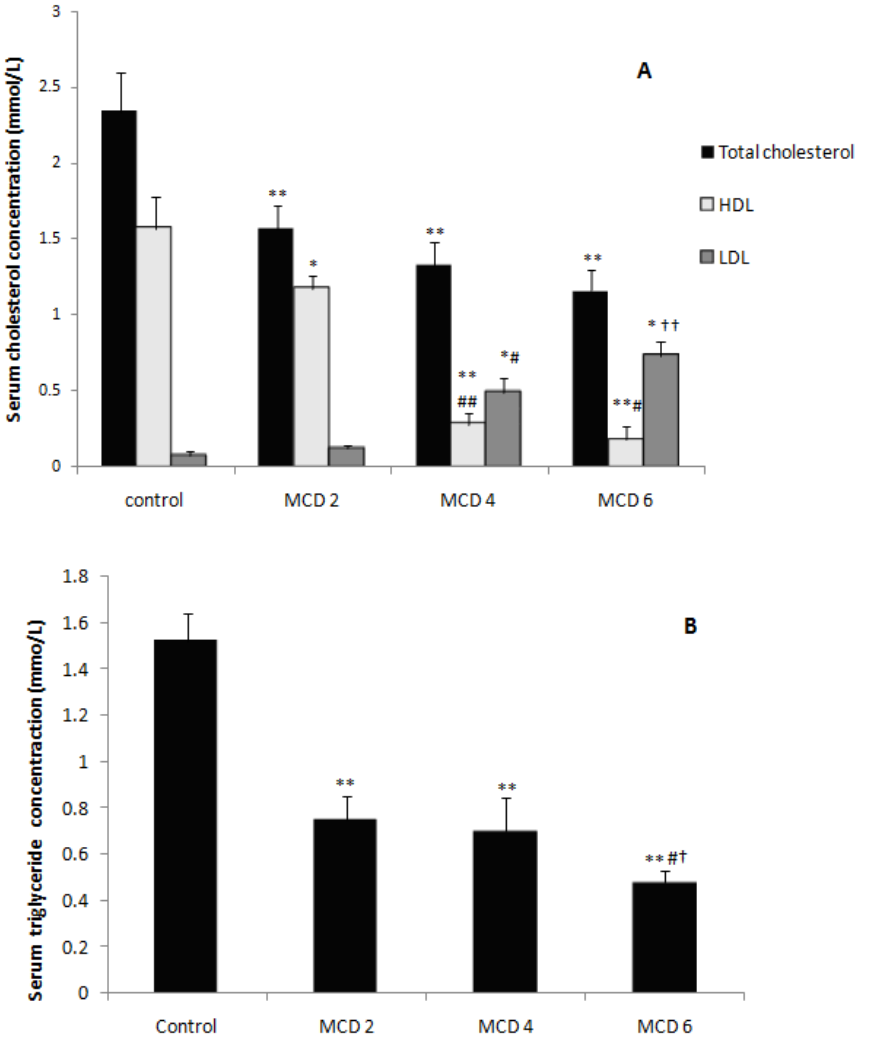


Figure 4.4. Time dependent effects of MCD diet on serum lipid profile: total cholesterol, high density lipoprotein (HDL) and low density lipoprotein (LDL) (A) and triglyceride level (B).

Significance of the difference was estimated by using one-way analysis of variance (ANOVA) with Tukey's post hoc test was used (*$p<0.05$, **$p<0.01$ vs. control; #$p<0.05$, ##$p<0.01$ vs. MCD2; †$p<0.05$, ††$p<0.01$ vs. MCD4).

For further information see Fig. 1.

4.1.4. The effects of MCD diet on free fatty acid profile in the liver

MCD diet induced a significant decrease in liver palmitic acid (C16:0) proportion at all time points and in stearic acid (C18:0) proportion only 2 and 4 weeks after beginning of the diet in comparison with control group ($p<0.01$). While oleic acid (C18:1 n9) was significantly higher, oleic acid isomer (C18:1 n7) was significantly lower in all experimental groups when compared with control ($p<0.01$). Linoleic acid (C18:2 n6) was significantly higher in all experimental groups vs. control with the highest proportion in MCD2 group. After this period linoleic acid (C18:2 n6) proportion progressively declined and in MCD6 group it was significantly lower than in MCD2 group ($p<0.05$). When compared with control group, arachidonic acid (C20:4 n6) was significantly lower ($p<0.05$) and docosapentaenoic acid (DPA, C22:5 n3) was significantly higher ($p<0.01$) only 2 weeks after the beginning of the MCD diet. At other time points no significant change was found. In contrast, liver docosahexaenoic acid (DHA, C22:6 n3) proportion was significantly decreased in MCD4 and MCD6 group in comparison with control ($p<0.01$) (Table 3).

Table 3. The effect of MCD diet on liver free fatty acid profile. The duration of MCD diet was 2, 4 or 6 weeks (MCD2, MCD4 and MCD6 respectively)

Group	Free fatty acid (%)							
	C 16:0	C 18:0	C 18:1 n9	C 18:1 n7	C 18:2 n6	C 20:4 n6	C 22:5 n3	C 22:6 n3
Control	21.9±0.78	13.24±0.73	10.11±0.67	2.33±0.37	18.47±0.76	15.53±0.62	0.54±0.05	10.09±0.44
MCD2	16.66±1.17**	9.00±1.65**	14.44±1.94**	0.91±0.19**	26.07±2.54**	12.20±2.21*	1.23±0.24**	9.13±0.76
MCD4	15.66±0.49**	10.47±1.08**	15.43±2.22**	0.89±0.27**	22.86±1.87*	14.51±1.90	0.44±0.15	7.16±0.95**
MCD6	16.74±0.68**	12.29±0.68	13.44±1.80**	0.90±0.18**	21.77±1.20*#	16.00±1.02	0.43±0.08	6.46±0.73**

Significance of the difference was estimated by using one-way ANOVA with Tukey's post hoc test

(**$p<0.01$, *$p<0.05$ vs. control; #$p<0.05$ vs. MCD2 group)

Abbreviations: C 16:0 – palmitic acid; C 18:0 – stearic acid; C 18:1 n9 – oleic acid; C 18:1 n7 – oleic acid isomer; C 18:2 n6 – linoleic acid; C 20:4 n6 – arachidonic acid; C 22:5 n3 - docosapentaenoic acid (DPA); C 22:6 n3 - docosahexaenoic acid (DHA).

4.1.5. Association between free fatty acid and lipid status

Assessments by the Pearson correlation coefficients revealed significant correlation between liver DHA proportion and serum LDL (r = -866, p<0.001), total cholesterol (r=813, p<0.001) and triglyceride level (r=695, p<0.001), (Figure 4.5.).

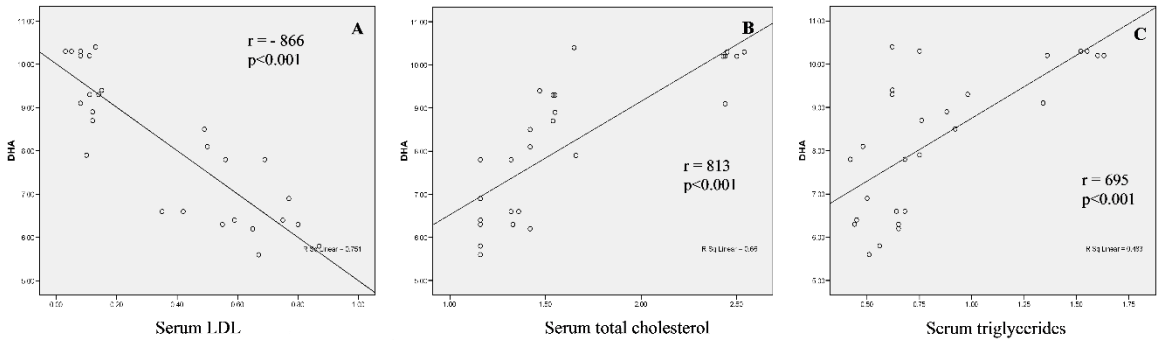


Figure 4.5. Association between hepatic DHA and serum LDL (A), total cholesterol (B) and triglycerides (C). Correlation was estimated by using Pearson's correlation coefficient *r*.

4.1.6. The effects of MCD diet on oxidative stress parameters in the liver

Liver MDA concentration was significantly increased in all experimental groups (MCD2: 364.30 ± 63.8, MCD4: 332.14 ± 32.00, and MCD6: 48.57 ± 49.98 nmol/mg proteins) compared with control group (189.3 ± 43.9 nmol/mg protein; p<0.01). However, no significant difference in MDA concentration was observed between groups (p>0.05; Figure 4.6.A).

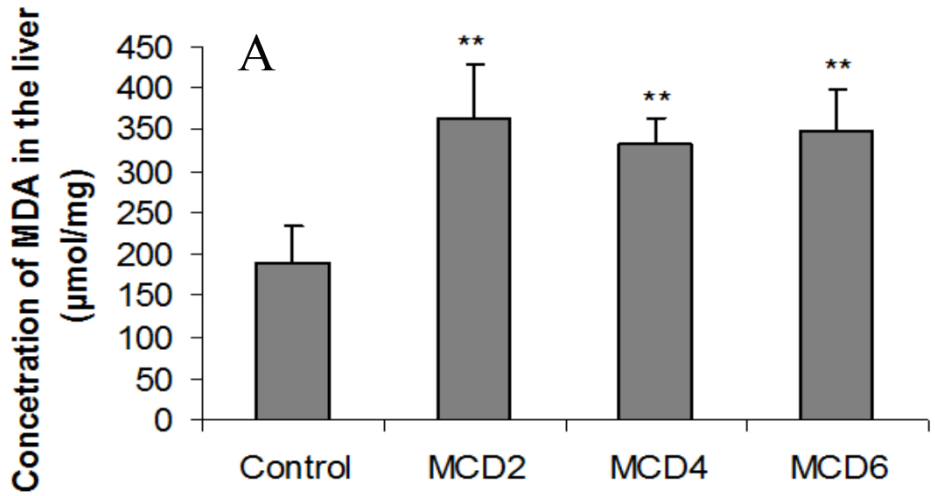
The MCD diet induced a significant increase in liver NOx levels in all experimental groups in comparison with control (19.15 ± 5.95 nmol/mg protein, p<0.01). NOx level was significantly higher in MCD6 (52.4 ± 11.45 nmol/mg protein) compared to MCD2 (33.03

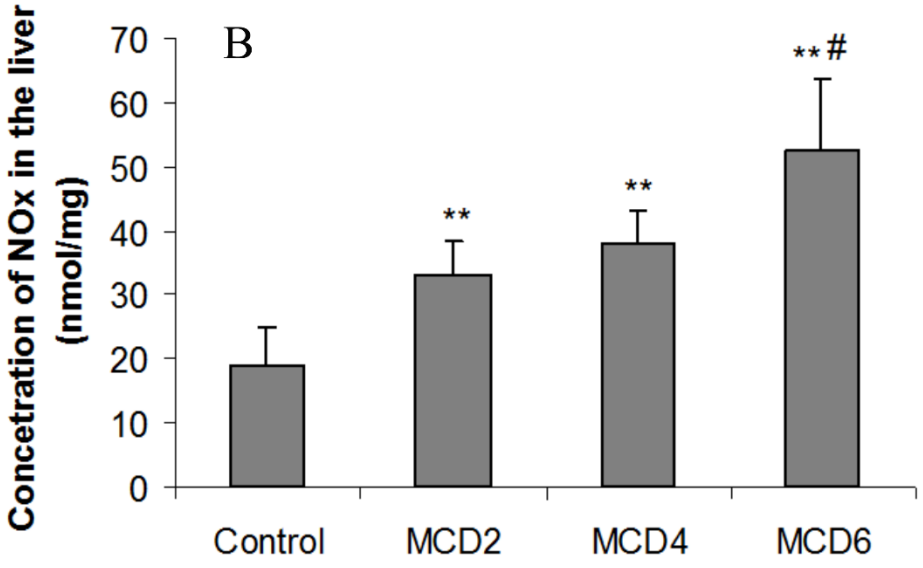
± 15.18 nmol/mg protein) and MCD4 (37.98 ± 5.05 nmol/mg protein, $p<0.05$; Figure 4.6.B).

The activity of total SOD activity in the liver was significantly decreased in MCD2 (3.42 ± 0.77 U/mg protein) as compared to control (27.5 ± 2.67 U/mg protein, $p<0.01$). During time, the total SOD activity continue to decrease in MCD4 (1.32 ± 0.18 U/mg protein) and MCD6 (1.68 ± 0.32 U/mg protein) and it was significantly lowerc in comparison with MCD2, $p<0.01$ (Figure 4.6.C). Activities of MnSOD and Cu/ZnSOD followed the changes of total SOD in all groups, where the values in MCD4 and MCD6 groups were significantly lower than in MCD2 ($p<0.05$).

Catalase activity in the liver was significantly reduces in all MCD diet- treated groups in comparison with control (147.14 ± 22.25 U/mg protein, $p<0.01$). However, the most pronounced decrease in CAT activity was observed in MCD4 group (31.07 ± 6.93 U/mg protein) compared to MCD2 (55.71 ± 14.00 U/mg protein, $p< 0.05$, Figure 4.6.D).

MCD diet induced a significant decrease in liver GSH level in MCD4 (95.3 ± 25.11 nmol/mg protein) in comparison with control group (123.34 ± 32.12 nmol/mg protein, $p<0.05$). The most prominent decrease was evident in MCD6 (78.08 ± 28.13 nmol/mg protein) compared to control, $p<0.01$; Figure 4.6.D)


A
Concetration of MDA in the liver (µmol/mg)
450
400
350
300
250
200
150
100
50
0
**
**
**
Control
MCD2
MCD4
MCD6


B
Concetration of NOx in the liver (nmol/mg)
70
60
50
40
30
20
10
0
**
**
**#
Control
MCD2
MCD4
MCD6

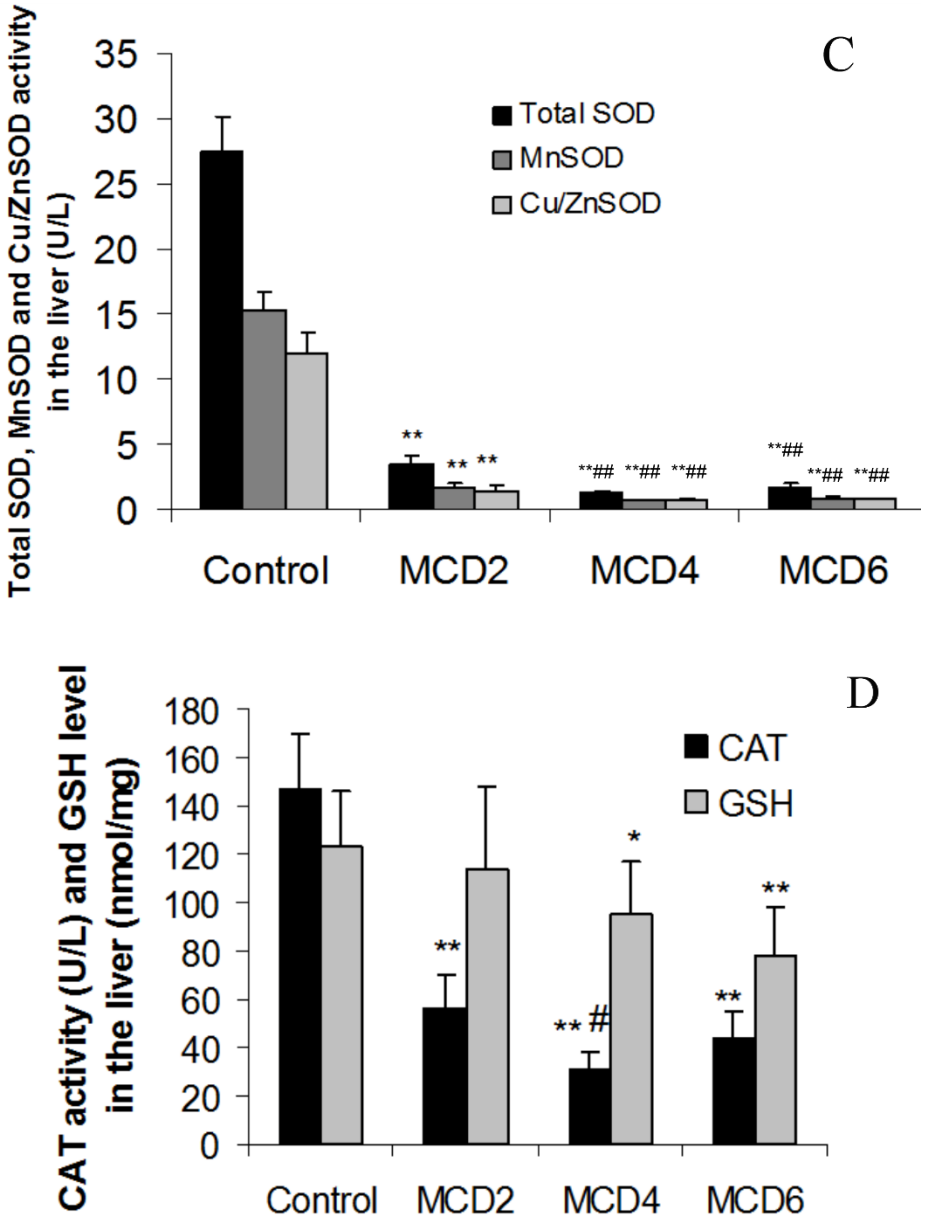


Figure 4.6. The effects of MCD diet on liver MDA level (A), level of NOx (B), activity of total SOD, MnSOD and Cu/ZnSOD (C), and the catalase activity and GSH content (D) in mice liver after 2, 4, and 6 weeks.

Significance of the difference was estimated by using one-way analysis of variance (ANOVA) with Tukey's post hoc test was used (*p<0.05, **p<0.01 vs. control; #p<0.05, ##p<0.01 vs. MCD2; †p<0.05).

SOD: superoxide dismutase, MnSOD: manganese superoxide dismutase, Cu/ZnSOD: cooper/zinc superoxide dismutase, CAT: catalase.

4.2. The effect of betaine on biochemical, molecular and morphological changes in NAFLD mouse model

4.2.1. The effects of betaine on animal body and liver weight

Animal body weight was measured at the beginning of experiment, and animals weighted average 23±3g. After six weeks of experiment, in control and BET groups, animal weight was significantly increased (30.10±2.86g and 28.65±3.69g, respectively) by comparison with initial weight ($p<0.05$). In contrast, in MCD group animal weight was significantly decreased (16.43±1.82g) when compared to the weight at the beginning of the experiment ($p<0.01$). Also, body weight at the end of experiment was significantly reduced in MCD+BET group by comparison with initial weight ($p<0.01$; Figure 4.7.). On the other hand, body/liver weight ratio did not significantly differ between all experimental groups (data not shown).

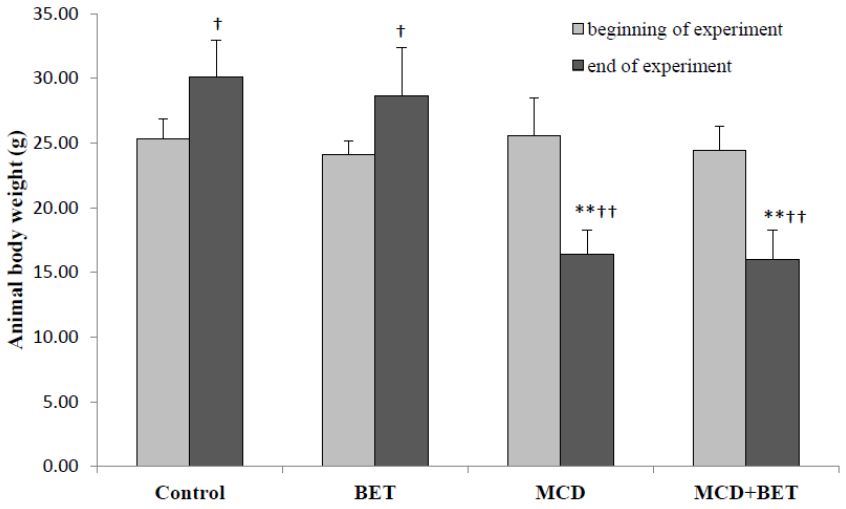


Figure 4.7. Animal body weight before and after the experiment. Animals were on MCD diet and treated with betaine for 6 weeks. Betaine was dissolved in drinking water (1.5% w/v) and animals had free access to betaine-contaning water during the whole experiment.

Significance of the difference was estimated by using one-way analysis of variance (ANOVA) with Tukey's post hoc test was used (**$p<0.01$ vs. control, †$p<0.05$, ††$p<0.01$ vs. the same group at the beginning of experiment). The data are presented as means ±SD.

Groups: Control – mice fed with standard chow, BET – mice fed with standard chow and was on betaine supplementation, MCD – mice on methione, choline-deficient diet, MCD+BET – mice on methionine, choline-deficient diet with betaine supplementation.

4.2.2. The effects of betaine on serum biochemical parameters

MCD diet induced a significant increase in serum ALT activity (304.19±14.59 U/L) by comparison with control (29.71±4.72 U/L; $p<0.01$). Betaine alone did not cause a significant change in ALT activity when compared to control ($p>0.05$), while in combination with MCD diet, betaine caused a significant decrease in ALT activity (29.33±8.33 U/L) vs. MCD group ($p<0.01$). Similar to ALT, serum AST activity was

significantly increased in MCD group (321.74±27.01 U/L) when compared with control (130.00±30.16 U/L) ($p<0.01$), while in MCD+BET group this enzyme activity (71.33±7.31 U/L) was significantly reduced vs. MCD group ($p<0.01$). Betaine alone did not induce significant change in AST activity (Figure 4.8.A).

While total serum cholesterol level was significantly decreased by MCD diet (0.69±0.19 mmol/L) when compared with control (2.41±0.20 mmol/L; $p<0.01$), betaine supplementation induced a significant increase in MCD+BET group (1.14±0.18 mmol/L) by comparison with MCD group ($p<0.01$). Analysis of serum cholesterol fractions has shown that MCD diet induced a significant decrease in HDL level (0.36±0.20 mmol/L) when compared with control (2.27±0.16 mmol/L) ($p<0.01$). Serum HDL level was significantly higher in MCD+BET (1.07±0.14 mmol/L) when compared with MCD group ($p<0.01$).

On the other hand, serum LDL level was significantly increased by MCD diet (1.25±0.13 mmol/L) by comparison with control (0.08±0.01 mmol/L; $p<0.01$), while betaine treatment in combination with MCD diet significantly reduced LDL level (0.08±0.01 mmol/L) vs. MCD group ($p<0.01$). Similar to LDL, MCD diet induced a significant increase in serum triglyceride level (0.36±0.05 mmol/L) by comparison with control group (0.74±0.08 mmol/L; $p<0.01$). Triglyceride level in MCD+BET group (0.80±0.11 mmol/L) was significantly decreased by comparison with MCD group ($p<0.01$). However, betaine administered alone did not induce significant changes in serum lipid concentrations (total cholesterol, HDL, LDL, triglycerides) when compared with control (Figure 4.8. B and C).

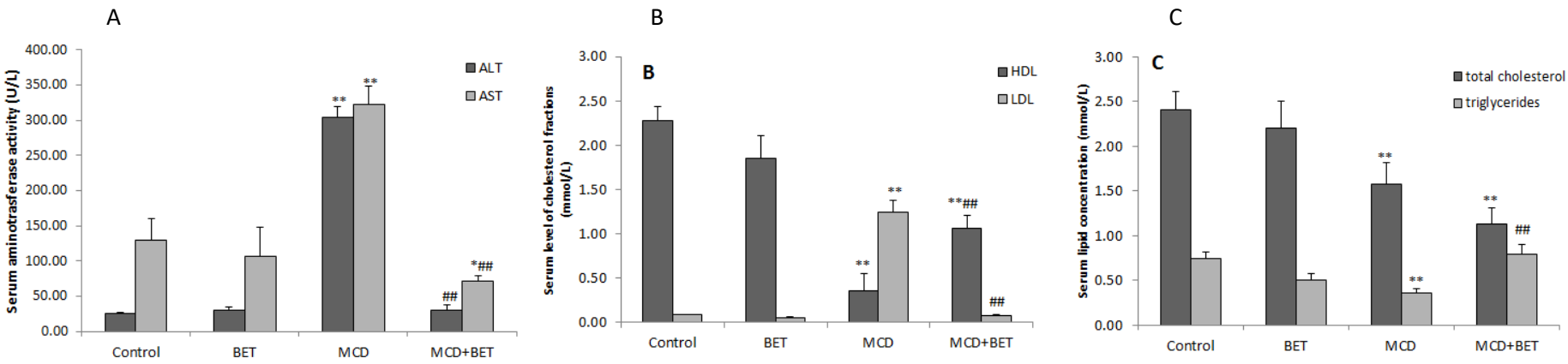


Figure 4.8. Effect of betaine on serum biochemical analysis in mice with NAFLD induced by MCD diet: A) aminotransferase activities (ALT, AST); B) HDL and LDL level and C) cholesterol and triglycerides level.

Significance of the difference was estimated by using one-way analysis of variance (ANOVA) with Tukey's *post hoc* test (*$p<0.05$, **$p<0.01$ *vs.* control, ##$p<0.01$ *vs.* MCD). The data are presented as means ±SD.

For further information see Figure 1

4.2.3. Pathohistological findings

No pathological changes were found in control group (Figure 4.9.A), as well as in BET group (Figure 4.9.B). Hepatic steatosis with diffuse fatty change in the form of micro- and macrovesicular steatosis was found in MCD group, accompanied with mild perivascular inflammation (Figure 4.9.C). However, in MCD+BET group steatosis and inflammation were less prominent than in MCD group with few fat droplets seen occasionally in hepatocytes (Figure 4.9.D).

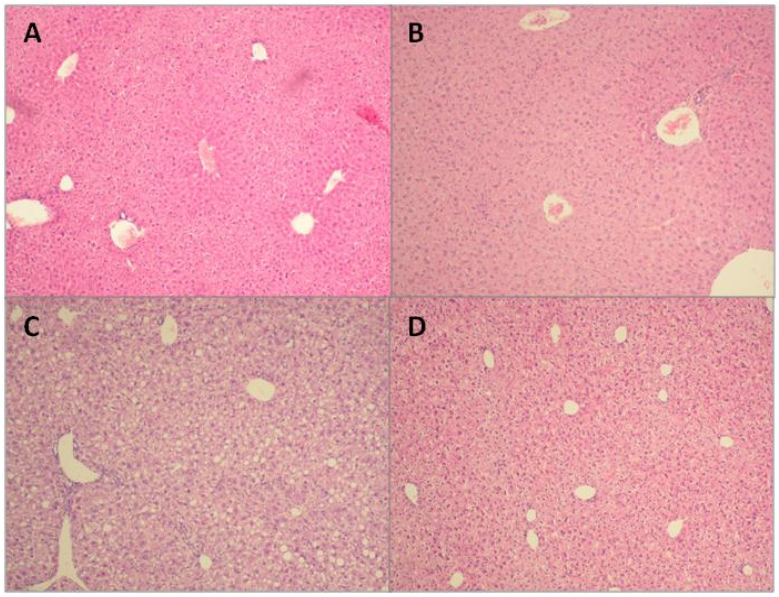


Figure 4.9. The effects of betaine and MCD diet on liver morphology (Hematoxylin-eosin staining, magnification x10)

(A) No pathological changes were found in control group

(B) Normal liver morphology with preserved tissue architecture and lobular organization in BET group

(C) Hepatic steatosis with diffuse fatty change in the form of micro- and macrovesicular steatosis associated with mild perivascular neutrophil infiltration in MCD group.

(D) In MCD+BET group steatosis and inflammation were less prominent than in MCD group with few fat droplets seen occasionally in hepatocytes without inflammation

For further information see Figure 1.

4.2.4. The effects of betaine on oxidative stress parameters

Our results have shown that MDA concentration was significantly increased in MCD group (581.80±153.40 nmol/mg prot.) by comparison with control (160.70±49.87 nmol/mg prot.; $p<0.05$). However, in MCD+BET group, MDA concentration (192.00±104.4 nmol/mg prot.) was significantly decreased vs. MCD group ($p<0.05$).

Liver nitrites concentration was significantly increased in MCD diet-treated group (30.32±8.96 nmol/mg prot.) in comparison to control group (11.87±0.71 nmol/mg prot.; $p<0.05$). Similar to MDA, significant decrease in liver nitrites was evident in MCD+BET (12.36±1.7 nmol/mg prot.) when compared to MCD group ($p<0.05$; Figure 4.10.A and B).

Liver PON1 activity was significantly decreased in MCD group (58.20±4.12 U/mg protein) by comparison with control (118.60±33.48 U/mg protein; $p<0.05$). However, betaine supplementation caused an increase of its activity in MCD+BET (94.59±23.26 U/mg protein) vs. MCD group ($p<0.05$).

Betaine and MCD diet induced similar changes of liver ARE activity, and MCD diet significantly reduced its activity (465.80±75.78 U/mg protein) vs. control group (797.80±210.70 U/mg protein; $p<0.05$). In MCD+BET group ARE activity (692.50±187.40 U/mg protein) was significantly higher compared with MCD group ($p<0.05$; Figure 4.10. C and D).

Liver tSOD activity was significantly reduced in MCD (65.11±12.09 area/mg protein) by comparison with control group (94.36±8.11 area/mg protein) ($p<0.01$). Betaine in combination with MCD diet induced a significant increase in tSOD activity (111.10±15.58 area/mg protein) when compared to MCD group ($p<0.01$). Similar increase was evident in BET group (114.20±7.27 area/mg protein) vs. control ($p<0.01$). Apart from

total SOD, activities of its isoenzymes were significantly reduced in MCD diet-treated group (Cu/Zn SOD: 24.50±5.88, and MnSOD: 40.60±6.80 area/mg protein) by comparison with control (34.64±4.40 and 58.95±5.62 area/mg protein, respectively) ($p<0.01$). Betaine administered alone, as well as in combination with MCD diet induced a significant increase in MnSOD activity (BET: 74.98±3.95 and MCD+BET: 73.00±3.41 area/mg protein) by comparison with control ($p<0.01$). On the other hand, no significant difference was evident in Cu/Zn SOD activity between MCD and MCD+BET group ($p>0.05$; Figure 4.11.).

Liver CAT activity was significantly decreased in MCD diet-treated group (91.50±18.23 U/mg prot.) by comparison with control (159.90±4.79 U/mg prot.; $p<0.01$). However, in MCD+BET group, activity of this enzyme was significantly increased (124.60±32.96 U/mg prot.) when compared with MCD group ($p<0.01$), but still it was lower by comparison with control ($p<0.05$). Similar changes in liver GSH level were observed in experimental groups. MCD diet significantly reduced liver GSH content (97.48±11.08 nmol/mg prot.) by comparison with control (126.70±20.25 nmol/mg prot.; $p<0.01$), while betaine supplementation accompanied with MCD diet induced a significant increase in GSH content (165.20±33.92 nmol/mg prot.) vs. MCD group ($p<0.01$) and control ($p<0.05$). On the other hand, GRed activity was significantly increased in BET and MCD group (12.28±3.02 and 15.70±1.83 U/mg prot., respectively) by comparison with control (7.06±0.53 U/mg prot.; $p<0.01$). In MCD+BET group, GRed activity was significantly decreased (8.38±1.56 U/mg prot.) compared to MCD ($p<0.01$). Liver GPx activity was significantly decreased in MCD group when compared to control ($p<0.01$). Betaine administered alone significantly increased liver GPx activity vs. control ($p<0.01$). Liver GPx activity in MCD+BET group was significantly increased by comparison with MCD and control group ($p<0.01$; Table 4).

Correlations

By using Pearson correlation test, we found that in MCD group PON1 significantly correlated with tSOD (r=0.864; $p<0.05$), GSH (r=0.866; $p<0.05$) and MDA (r= - 915; $p<0.05$). Similarly, ARE was found to correlate significantly with tSOD (r=0.894), GSH

(r=0.864) ($p<0.05$), MDA (r= -0.964) ($p<0.01$), but also with GR (r=0.818) ($p<0.05$), CAT (r=0.962), and nitrites (r= -0.947) ($p<0.01$).

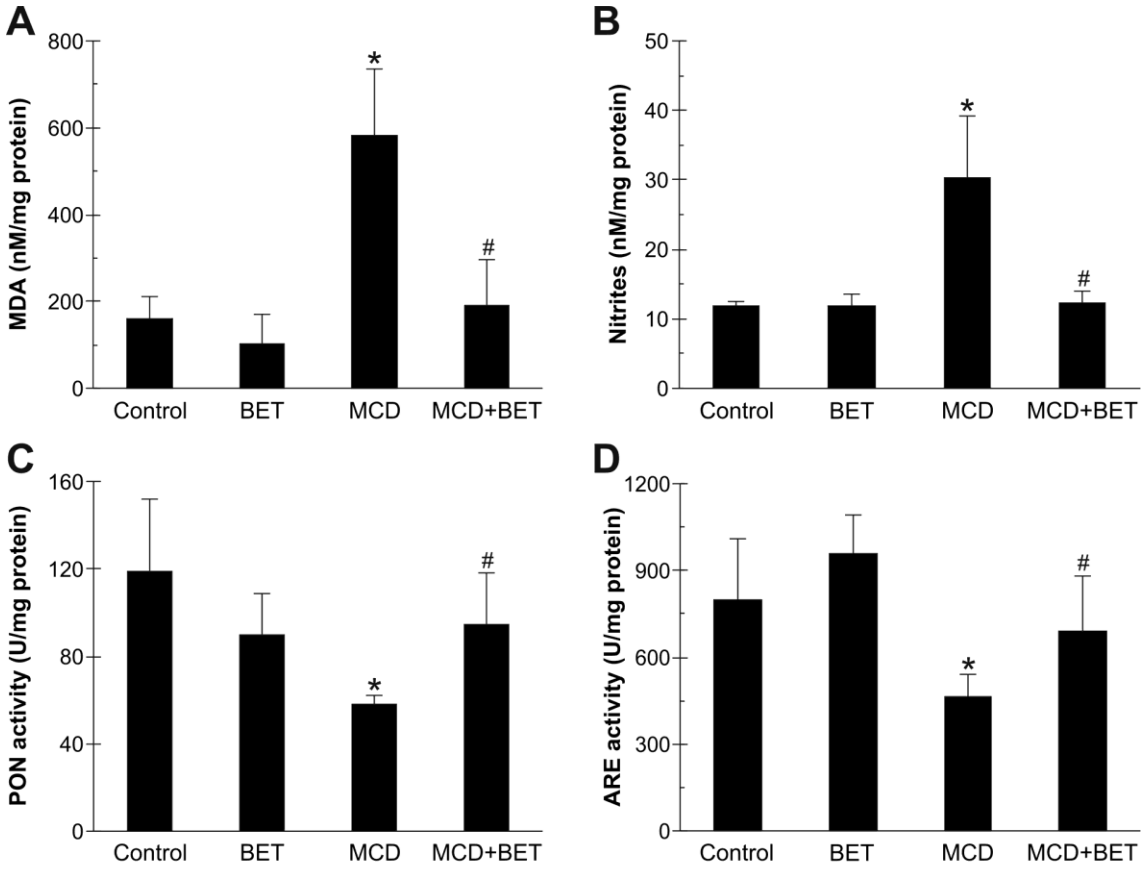


Figure 4.10. Effect of betaine on liver MDA, marker of lipid peroxidation (A), nitrites concentration that represents nitrosative stress (B), PON1 activity (C) and ARE activity (D) in NAFLD induced by MCD diet.

Significance of the difference was estimated by using one-way analysis of variance (ANOVA) with Tukey's post hoc test was used (*$p<0.05$ vs. control, #$p<0.05$ vs. MCD). The data are presented as mean ±SD.

MDA – malondialdehyde, PON1 – paroxonase-1, ARE – arylesterase. For further information see Figure 1.

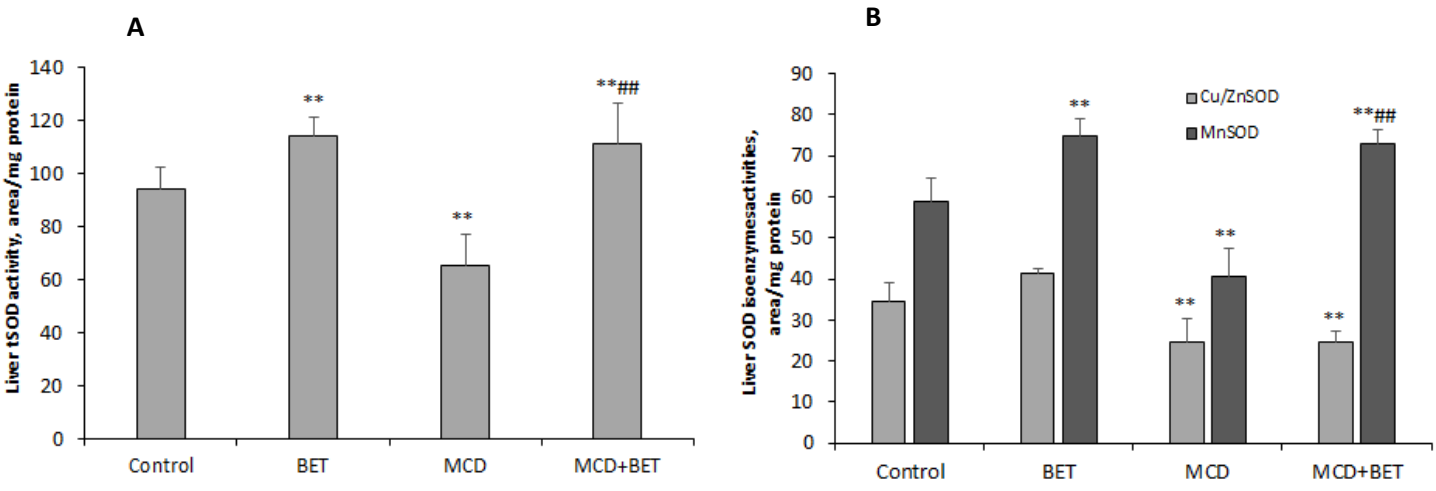


Figure 4.11. Effect of betaine on liver total SOD activity (A) and its isoenzymes activities (B) in NAFLD induced by MCD diet.

Significance of the difference was estimated by using one-way analysis of variance (ANOVA) with Tukey's post hoc test was used (**p<0.01 vs. control, ##p<0.01 vs. MCD). The data are presented as mean ±SD.

For further information see figure 1.

Table 4. Effect of betaine on liver GSH content, GRed, GPx and CAT activity in MCD diet

	Groups			
	Control	**BET**	**MCD**	**MCD+BET**
GSH (nmol/mg prot.)	126.70±20.25	136.70±12.71	97.48±11.08**	165.20±33.92*##
GRed (U/mg prot.)	7.06±0.53	12.28±3.02**	17.70±1.83**	8.38±1.56##
GPx (U/mg prot.)	20.83±5.46	74.39±14.98**	12.87±3.33**	67.34±9.64**##
CAT (U/mg prot.)	159.90±4.79	127.60±29.17*	91.50±18.23**	124.60±32.96*##

induced NAFLD

Significance of the difference was estimated by using one-way analysis of variance (ANOVA) with Tukey's post hoc test was used (*$p<0.05$, **$p<0.01$ vs. control, ##$p<0.01$ vs. MCD). The data are presented as mean ±SD.

GSH – Glutathione; GRed – Glutathione reductase; GPx – Glutathione peroxidase; CAT – Catalase.

4.2.5. The effects of betaine in hepatic expression of inflammation and apoptosis markers in MCD diet-induced NAFLD

Our results indicate that MCD diet induced a significant increase in expression of inflammatory marker genes TNF ($p<0.05$) and IL-6 ($p<0.05$) vs. control with more prominent increase in TNF. Besides, in MCD group expression of anti-inflammatory cytokine IL-10 and fibrogenic mediator TGF-β were increased by comparison with control group ($p<0.05$).

The expression of TNF and IL-6 mRNA was significantly lower in MCD+BET compared to MCD group ($p<0.05$). Betaine treatment in combination with MCD diet further increased expression of IL-10 mRNA compared to MCD group ($p<0.05$). In contrast to MCD group, no significant difference in TGF-β expression was evident between MCD+BET and control group ($p>0.05$; Figure 4.12.A.).

The mRNA levels of both proapoptotic protein Bax and antiapoptotic protein Bcl-2 were significantly upregulated in MCD group by comparison with control group ($p<0.05$). On the other side, betaine supplementation in combination with MCD diet significantly reduced expression of Bax mRNA ($p<0.05$) and increased the expression of Bcl-2 mRNA when compared with MCD group ($p<0.05$). The levels of mRNA encoding the proapoptotic factors Bbc3 and Bak1 did not display significant differences between thecontrol and experimental groups (Figure 4.12.B).

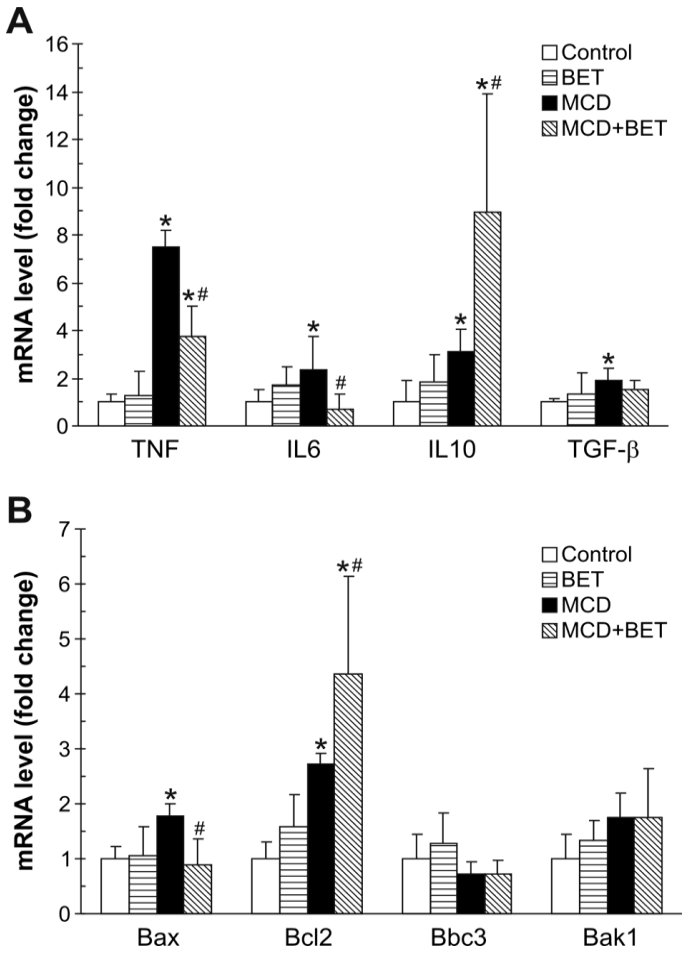


Figure 4.12. Effect of betaine on liver mRNA levels of (A) cytokines and (B) apoptotic proteins in MCD-fed mice.

The mRNA expression of selected cytokines and apoptotic mediators was analyzed by quantitative RT-PCR in liver tissue from different treatment groups (n = 7 per group). Animals in control group were fed with control diet. MCD group was fed with MCD diet for six weeks. BET group was on control diet followed by betaine supplementation (1.5% solution in drinking water) and MCD+BET that was on MCD diet combined with betaine treatment for six weeks.

Significance of the difference was estimated by using one-way analysis of variance (ANOVA) with Tukey's post hoc test was used (*$p<0.05$, vs. control, #$p<0.05$ vs. MCD). The data are presented as means ±SD.

4.2.6. The effects of betaine supplementation on autophagy and Akt/mTOR signaling in MCD diet-induced NAFLD

The protein levels of LC3-II, the lipidated form of LC3 that is incorporated in, and degraded by autophagosomes (103) were increased in all three treatment groups (BET, MCD, and MCD + BET) compared to control ($p<0.05$) without the difference among those three groups. While the level of SQSTM1/p62, a protein selectively degraded by autophagy (104), was increased in MCD-fed mice by comparison with control ($p<0.05$), betaine supplementation significantly reduced its levels compared to both control and MCD group ($p<0.05$).

MCD diet did not affect the mRNA levels of autophagy activators Atg4b, Atg5, Atg7, and beclin 1. On the other hand, betaine treatment alone significantly increased Atg4 and beclin 1 mRNA expression by comparison with control ($p<0.05$). Betaine treatment in mice fed with MCD diet significantly increased the mRNA levels of Atg4b, Atg5, Atg7, and beclin 1 by comparison with control ($p<0.05$).

The phosphorylation of mTOR, its substrate S6K, and its upstream activator Akt, was significantly upregulated in BET compared to control group ($p<0.05$). While no difference in p-S6K, p-mTOR and p-Akt expression was found in MCD vs. control group ($p>0.05$), in MCD + BET group the expression of phosphorylated forms of S6K, mTOR and Akt was significantly increased compared to both control and MCD group ($p<0.05$). However, no significant differences were observed between the four groups in the phosphorylation of the mTOR inhibitor AMPK (Figure 4.13.). These data indicate that betaine-mediated increase in autophagic response and Akt/mTOR signaling in the liver might contribute to its beneficial action in MCD-induced NAFLD.

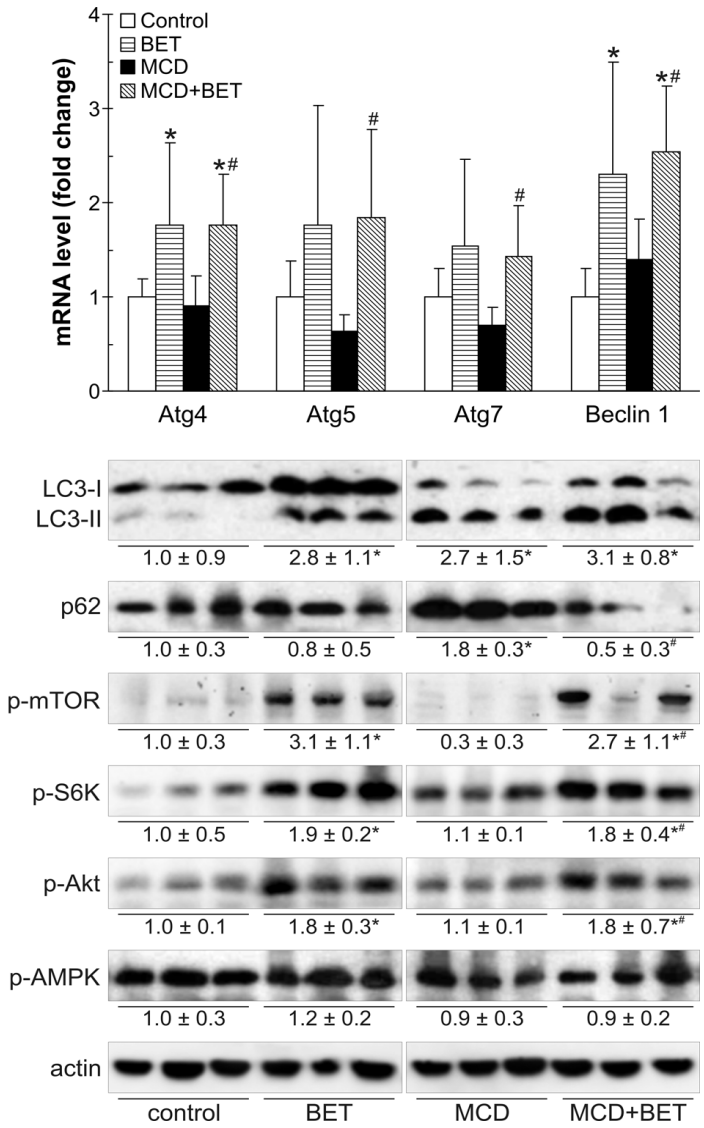


Figure 4.13. Effect of betaine on autophagy and mTOR signaling in MCD-fed mice.

The expression of Atg4, Atg5, Atg7, and beclin 1 mRNA was analyzed by quantitative RT-PCR in liver tissue from different treatment groups, while LC3 conversion, p62 accumulation, and the phosphorylation of mTOR, S6K, Akt, and AMPK were assessed by immunoblotting. Significance of the difference was estimated by using one-way analysis of variance (ANOVA) with Tukey's post hoc test was used (n = 7 per group; *$p < 0.05$ compared to control; $^{\#}p < 0.05$ compared to MCD group; representative blots are shown). For further information see Figure 4.11.

4.2.7. The effects of betaine on ultrastructural changes in the liver

In the control group, the analysis with electronic microscope showed normal liver ultrastructure, while in BET group normal liver tissue architecture was found with evident autophagosomes and mitochondria of different sizes within HSCs. In MCD diet-treated group diffuse fatty change that disrupts normal liver architecture was found with occasional apoptotic bodies in endothelial cells, enlarged mitochondria, hepatocytes and myofibroblasts surrounded with collagen fibers. In MCD+BET group steatosis and autophagosomes were clearly evident and accompanied with mitochondria of different sizes with rare mega-mitochondria and several apoptotic bodies in hepatocytes (Figure 4.14.)

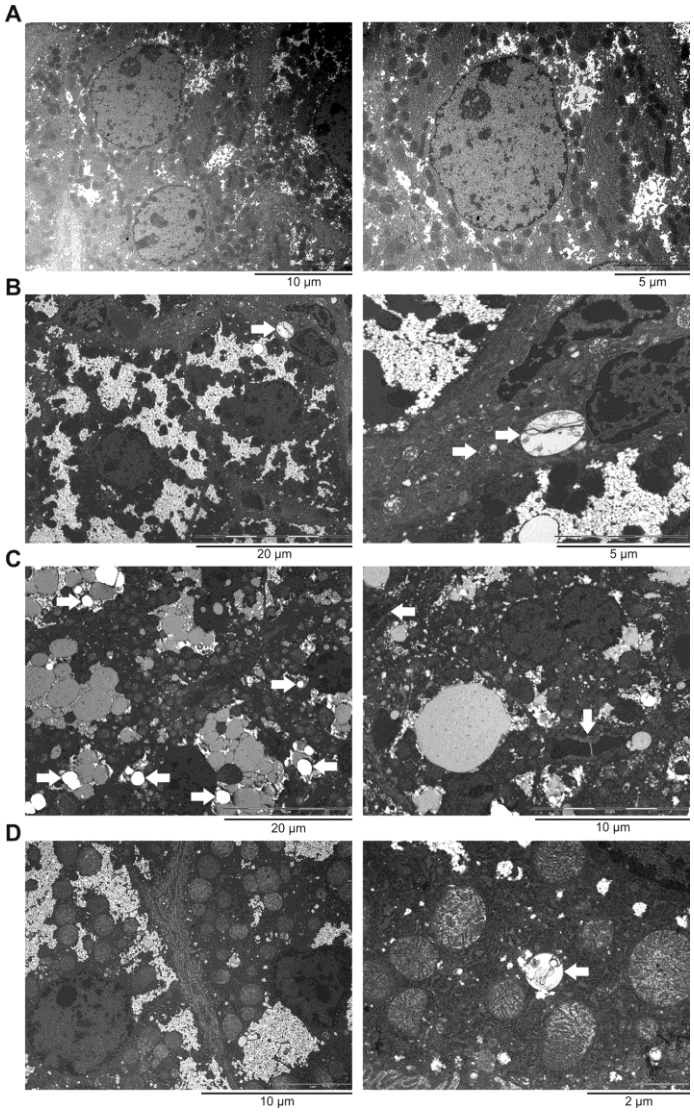


Figure 4.14. Effect of betaine on liver ultrastructure in MCD-fed mice evaluated on TEM.

Liver tissue sections from control (A), betaine-treated (B), MCD-fed (C), and MCD + betaine-treated mice (D) were analyzed by TEM. White arrows point at autophagic vesicles with cytoplasmic content (B, D), lipid droplets (C, left), or apoptotic endothelial cells (C, right).

4.3. The effects of NRG-1 on animal models of liver fibrosis and isolated hepatocytes

4.3.1. The effects of NRG-1 on hepatocyte damage in CCl_4 and BDL-induced liver fibrosis

Serum aminotransferase activities, ALT (CCl_4, 96.96±40.0 U/L and BDL, 239.17±103.46 U/L) and AST (CCl_4, 81.87±25.81 U/L and BDL 437.30±171.58 U/L) were significantly increased in both liver fibrosis models, compared to control group (ALT, 57.98±39.00 and AST 71.35±36.42 U/L) ($p<0.01$). However, NRG-1 treatment did not induce any significant changes in transaminase activities, (Figure 4.15.).

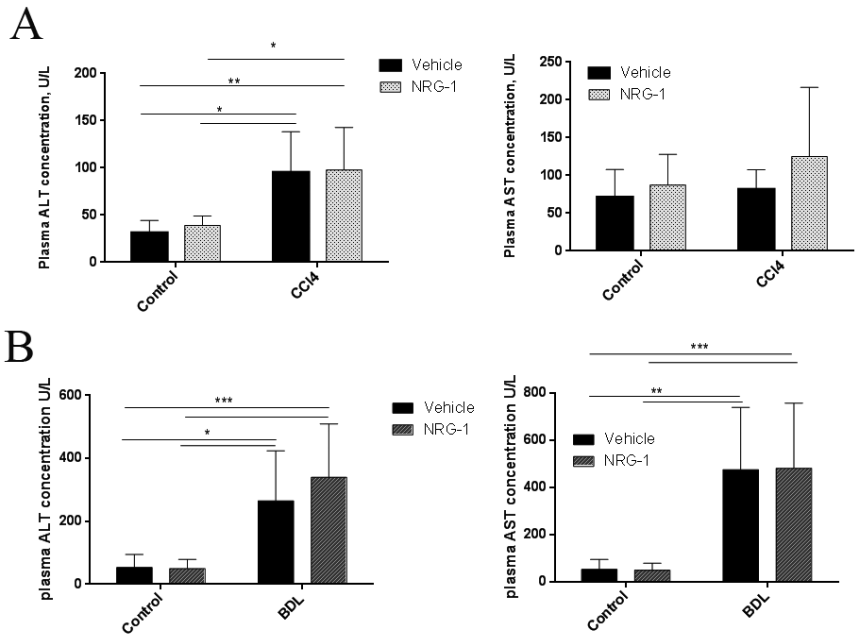


Figure 4.15. The effects of neuregulin-1 (NRG-1) on serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activity in mice with liver fibrosis.

A) Mild liver fibrosis model induced by CCl_4 i.p. injections for 4 weeks and B) severe liver fibrosis developed 4 weeks after bile duct ligation (BDL). NRG-1 was administered 5 times a week i.p. (20µg/kg body weight). Significance of the difference was estimated by two way analysis of variance (ANOVA) with Tukey's post hoc test (* $p<0.05$, ** $p<0.01$).Data are presented as mean±SD.

4.3.2. The effects of NRG-1 treatment on fibrotic changes in the liver tissue

Masson trichrome staining showed significantly increased accumulation of ECM proteins in both models by comparison with control group ($p<0.01$). However, NRG-1 treatment significantly increased liver fibrosis from 1.86 to 2.65 % in CCl_4 model and from 5.03 to 8.25 % in BDL-induced liver fibrosis ($p<0.01$) (Figure 4.16.).

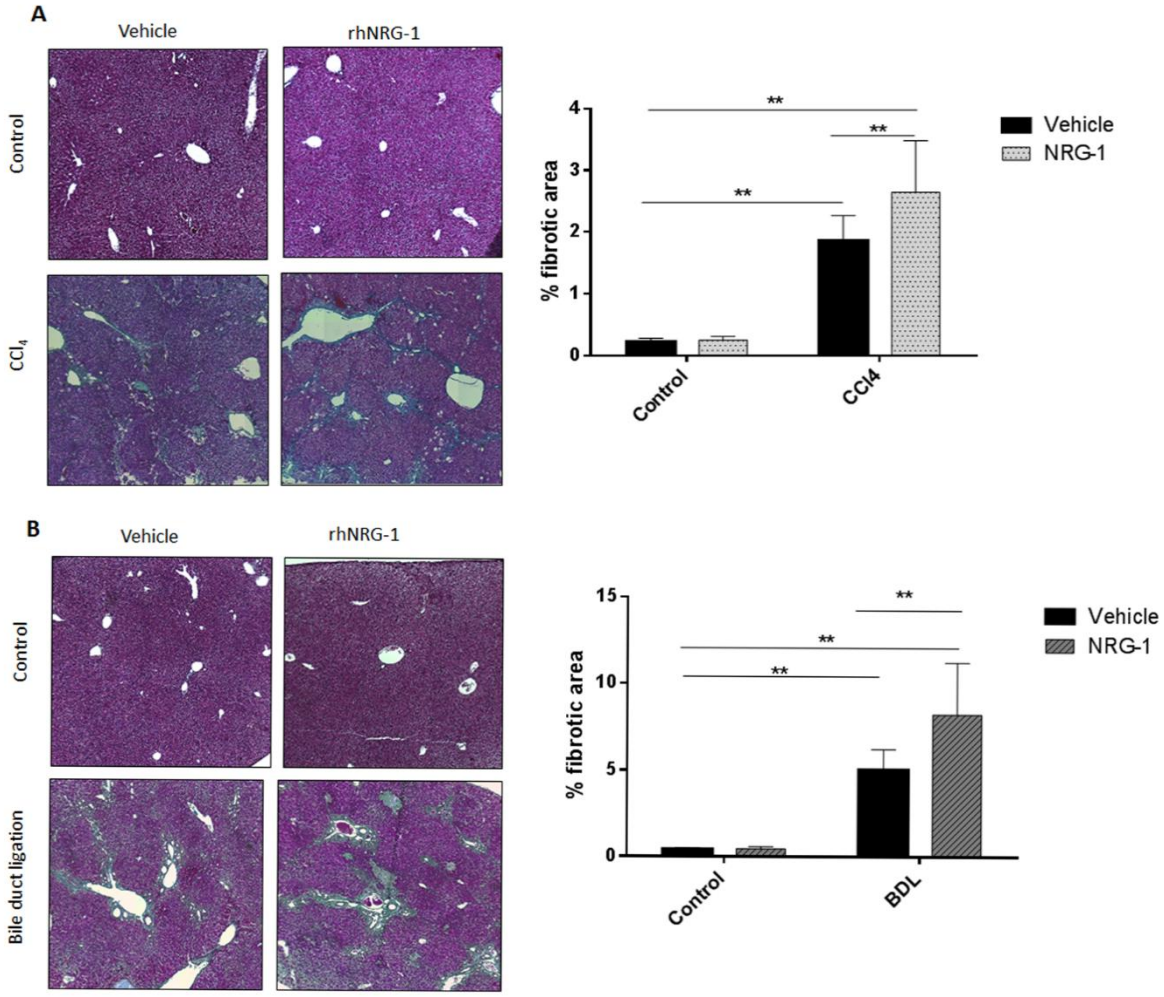


Figure 4.16. Histological findings in liver tissue (Masson's trichrome staining, scale bar 100µm, quantification of percentage of fibrotic area). Effects of neuregulin-1 (NRG-1) on fibrotic tissue accumulation in A. Mild liver fibrosis model induced by CCl_4 i.p. injections for 4 weeks and B. severe liver fibrosis developed 4 weeks after bile duct ligation (BDL). NRG-1 was administered 5 times a week i.p. (20µg/kg body weight). Significance of the difference was estimated by two way analysis of variance (ANOVA) with Tukey's post hoc test (** $p<0.01$). Data are presented as mean±SD.

4.3.3. The effects of NRG-1 on collagen 1, collagen 3, MMP2 and MMP9 mRNA expression in the liver fibrosis

In addition to histological findings, relative mRNA expression of collagen type 1 and 3 (Col1a1 and Col1a3) was significantly increased in CCl_4 group when compared with control, while in CCl_4+NRG group no significant difference was observed when compared to CCl_4 group. On the other hand, in BDL group there was no change in mRNA expression for collagen type 1 and 3 compared to control. In contrast to CCl_4 model, NRG-1 treatment significantly increased mRNA expression for both types of collagen in BDL+NRG-1 group, compared to BDL group ($p<0.05$, Figure 4.17.A).

In CCl_4-induced liver fibrosis, relative MMP2 and MMP9 mRNA expression was significantly increased by comparison with control ($p<0.05$). NRG-1 caused a decrease in these MMPs expressions, but only significant is the decrease in MMP2 mRNA expression compared with CCl_4 group ($p<0.05$). Besides, MMP-9 expression was significantly higher in BDL-treated vs. control group ($p<0.01$). However, NRG-1 did not cause a significant change in MMP2 and MMP9 expression either in control or BDL group by comparison with vehicle-treated groups ($p>0.05$, Figure 4.17.B).

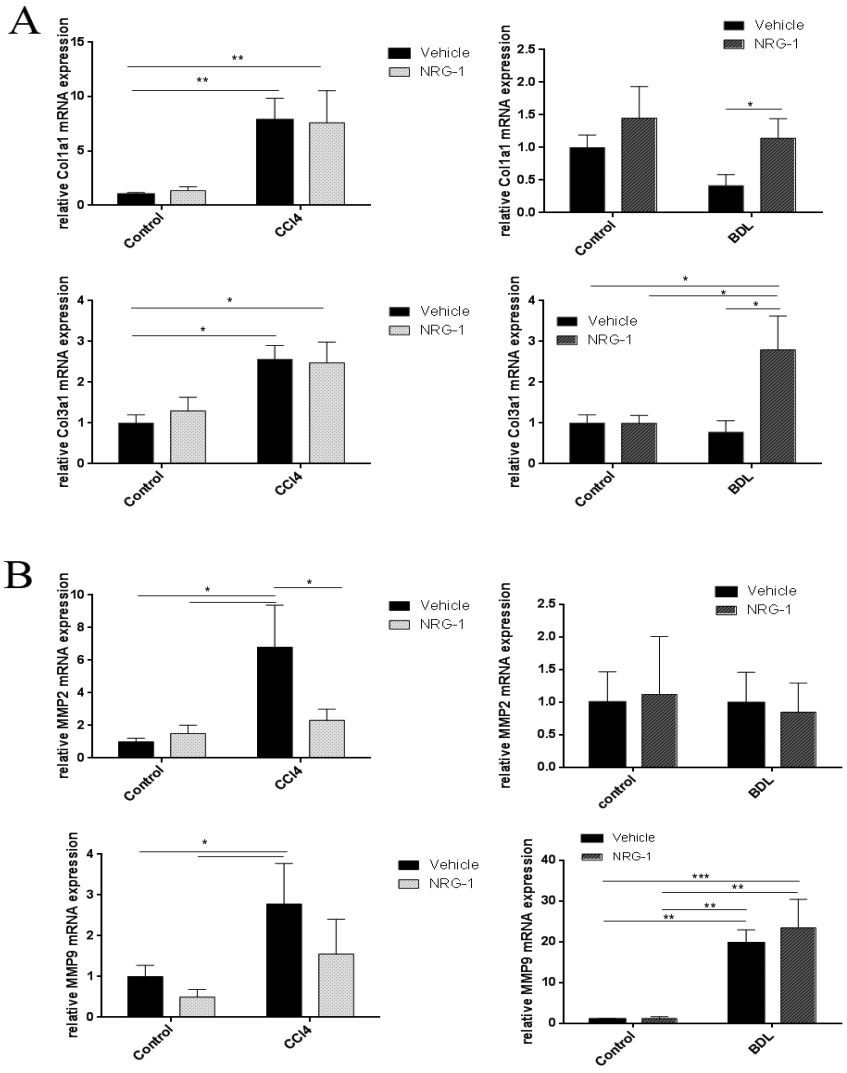


Figure 4.17. Effects of neuregulin-1 (NRG-1) on relative collagen 1 and 3 (col1a1 and Col3a1) expression (A) and matrix-metalloproteinases (MMP) 2 and 9 (B) in both liver fibrosis models.

Mild liver fibrosis model induced by CCl_4 i.p. injections for 4 weeks and severe liver fibrosis developed 4 weeks after bile duct ligation (BDL). NRG-1 was administered 5 times a week i.p. (20µg/kg body weight). Significance of the difference was estimated by two way analysis of variance (ANOVA) with Tukey's post hoc test (*$p<0.05$, **$p<0.01$).Data are presented as mean±SD.

4.3.4. The effects of NRG-1 on total and phosphorylated ErbB3 and ErbB4 receptors in the liver

Western blot analysis did not show significant differences in total amount of ErbB3 and ErbB4 NRG-1 receptors in healthy mouse liver. However, 30 minutes after NRG-1 stimulation, Western blot showed a significantly increased phosphorylation of ErbB3 receptors when compared with control ($p<0.01$), while ErbB4 phosphorylation was not significantly different between NRG-1 and control group ($p>0.05$; Figure 4.18.).

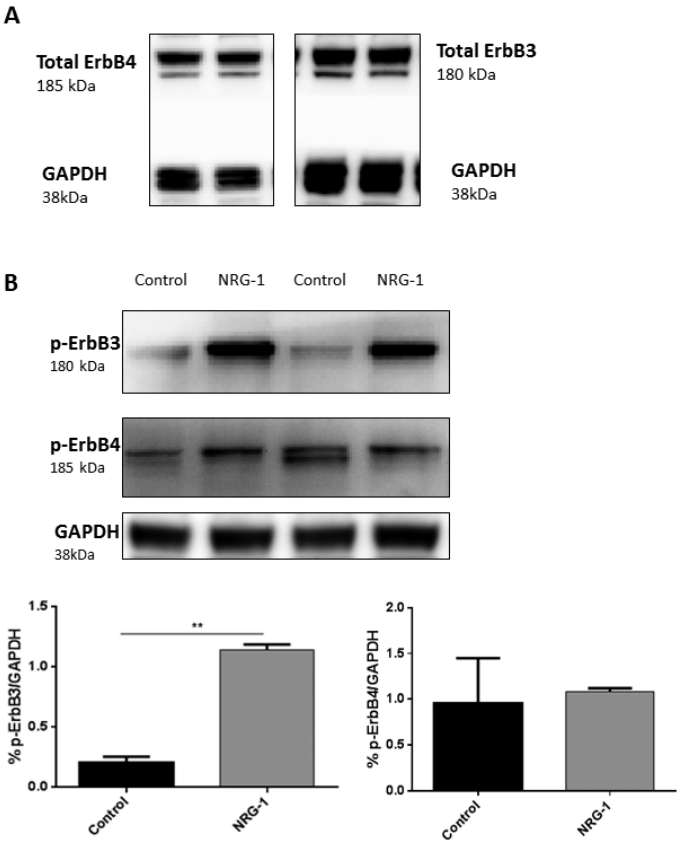


Figure 4.18. Neuregulin-1 (NRG-1) receptors in mouse healthy liver (Western blot analysis with primary antibodies ErbB3, ErbB4, phosphor-ErbB3, phospho-ErbB4 and GAPDH, all diluted at 1:1.000). A. Total amount of ErbB3 and ErbB4 receptors in mouse liver. B. Liver ErbB3 and ErbB4 receptors phosphorylation 30 minutes after NRG-1 i.p injection.

Significance of the difference was estimated by two way analysis of variance (ANOVA) with Tukey's post hoc test. Data are presented as mean±SD.

4.3.5. The effects of NRG-1 treatment on TGF-β mRNA expression on isolated hepatocytes

In vitro NRG-1 (20ng/ml) stimulation lasting 16 hours induced a significant increase in relative TGF-β mRNA expression in primary isolated mouse hepatocytes, compared to control cells ($p<0.01$). After the same period of stimulation with three different doses of NRG-1, Western blot did not show a significant difference in total amount of TGF-β in hepatocytes that were collected after the treatment ($p>0.05$, Figure 4.19.).

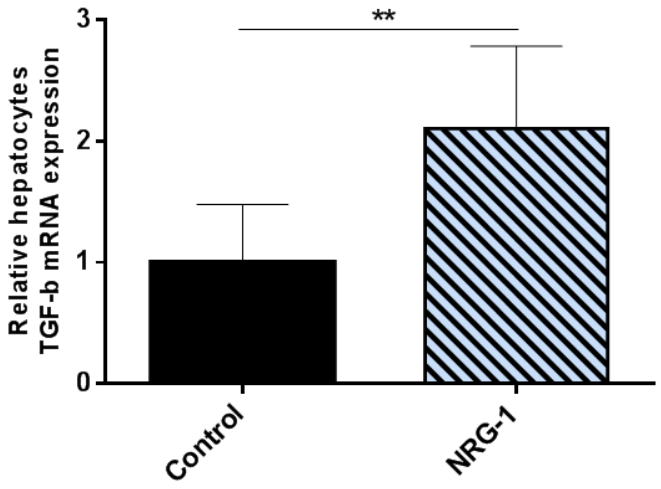


Figure 4.19. Effects of NRG-1 on TGF-β mRNA expression on isolated hepatocytes

RT-PCR analysis of relative TGF-β mRNA expression in primary isolated hepatocytes treated with NRG-1 (20ng/ml) for 16 h. Significance of the difference was estimated by Student's t test (**$p<0.01$). Data are presented as mean±SD.

5. DISCUSION

NAFLD represents one of the leading health problems in the world, predominantly in well-developed countries with prevalence of 20-30% due to rapidly increasing prevalence of obesity and diabetes mellitus type 2 especially among young population (4). Despite all the efforts and numerous groups of research to find an adequate therapeutic solution, NAFLD still remains too complex for targeting all pathogenetic pathways at the same time. Therefore, the best solution is to improve prevention, or to reduce all risk factors before the development of the disease. In order to test all substances that could potentially be used for prevention and treatment of NAFLD, it is necessary to use an adequate animal model that can meet all the requirements in order to cover all changes and disturbances that occurs in human NAFLD.

Various rodent models of NASH have been described, but no single animal model can completely reflect the range of pathohistological and pathophysiological features associated with human NASH. Ob/ob mice that are obese and diabetic develop with increased leptin levels develop severe type 2 diabetes and also obesity due to a functional defect in the long-form leptin receptor. High fat diet-fed mice develop MetS, are obese with hyperinsulinemia and glucose intolerance with developed insulin resistance, similar to NAFLD in humans, but histological changes are often not completely developed (74,105).

NAFLD induced by using MCD diet is a commonly used animal model since it is reproducible, changes in the liver develop rapidly and are quite similar to those observed in humans (106). In MCD diet-induced NAFLD, animals do not develop insulin resistance, and lipid profile is different than in human population. This model of NAFLD is associated with characteristic changes in serum lipid profile. Serum triglycerides progressively decreased and showed the lowest value after six weeks of MCD diet when compared to control and MCD2 and MCD4 groups (Fig. 4.4B). Similar finding was evident in study by Park et al. (107) where plasma triglycerides decreased even after one week of MCD diet with more pronounced decrease after six weeks. Low triglyceride level in plasma, as a measure of liver VLDL secretion, can be a consequence of increased hepatic fatty acid uptake and decreased VLDL secretion from the liver (108). Besides, total serum cholesterol

decreased, but it does not imply an anti-atherogenic effect of MCD diet, since LDL cholesterol was increased and HDL decreased. This indicates that MCD-induced model can be potentially used for studying the link between atherogenic dyslipidemia and NAFLD.

However, this model is associated with changes in liver structure and function, thus it is appropriate for studying pathogenetic mechanisms of NAFLD progression. In our study, MCD diet after two weeks induced initial fatty changes in mice liver with progressive intensification and the development of inflammation with ballooning degeneration of hepatocytes after 6 weeks of feeding with MCD diet. Fat accumulation in liver tissue was evident histologically (Figure 4.2.B) as a simple steatosis after 2 weeks. Although inflammation was not developed, oxidative stress and lipid peroxidation in the liver associated with reduced activity of antioxidative enzymes was evident 2 weeks after MCD diet (109). These findings indicate that MCD treatment for two weeks may be a suitable model for investigation of early fatty changes and oxidative damage in initial stage of NAFLD.

In contrast to early changes in liver tissue, 6 week-treatment with MCD diet induced liver steatosis accompanied with inflammation and apoptosis that were evident on histology (Figure 4.2.D). The presence of inflammation in 6 weeks of MCD diet model was also confirmed by increased levels of acute phase proteins in serum, CRP and ferritin (Table 2). Fat accumulation in the liver was obvious even macroscopically and it caused the increase in the liver weight that made liver/animal weight ratio even higher, since MCD diet caused weight loss in experimental animals (Figure 4.1.). The increase in serum transaminases (Figure 4.3.A) also confirmed hepatocyte damage in this animal model. Histological and biochemical analysis confirm that feeding with MCD diet for 6 weeks is an appropriate model for studying pathogenetic mechanisms that may contribute to NASH development or its progression.

It is well known that FFAs, especially saturated FFAs exert hepatotoxic effects and contribute to the progression of steatosis to NASH (110–113)(114,115). Mechanisms involved in the lipotoxicity of FFAs include mitochondrial dysfunction and increased ROS production as well as ER stress and activation of inflammatory and cell death pathways

(116). Saturated fatty acids can act as a ligand for TLR-4, that leads to the activation of proapoptotic and proinflammatory pathways (117). Intracellular FFA accumulation is associated with increased synthesis of IL-6, IL-8 and TNF-α (112) as well as profibrogenic growth factors (113). Also, fatty acids can destabilize lysosomal membrane and cause a release of cathepsin B that also activate apoptotic pathway (118). Lipotoxic effects of FFAs have been also confirmed in cultured hepatocytes which exibit inflammatory and fibrogenic response similar to NAFLD in human population when exposed to high concentration of free fatty acids (110). Also, Jun et al. (111) showed that stimulation of HepG2 cells with free fatty acids resulted in hepatic lipotoxicity due to disrupted fatty acid oxidation in hepatocytes. In this context esterification of FFAs in the form of triglycerides as well as intracellular fatty acid binding to specific fatty acid binding protein (FABP) can be considered a protective response that prevents lipotoxic injury of the liver.

Based on previous data, this study is one of the first that determined fatty acid profile in MCD diet model of NAFLD. Our study has shown a decrease in hepatic palmitic and oleic acid isomer proportions, whereas the proportions of oleic and linoleic acid were elevated. In addition, increase in hepatic linoleic acid proportion was most pronounced in MCD2, whereas the highest oleic acid proportion was evident in MCD4 group (Table 2). Other fatty acids showed characteristic time-dependent changes. Stearic acid was decreased after 2 and 4 weeks of MCD treatment, while after 6 weeks it returned to basal values. Since the hepatotoxic effects of saturated fatty acids are well known (117), accumulation of palmitic and stearic acid clearly did not contribute to the liver injury caused by MCD diet in the present study. On the other hand, DPA level was increased and arachidonic acid level was decreased after 2 weeks of MCD diet, and both fatty acids returned to basal values after 4 and 6 weeks of MCD diet. The significance of this finding is still not established. Lipid mediators derived from n-6 PUFAs are bioactive and may exert proinflammatory, atherogenic and pro-thrombotic effects. Linoleic acid is the direct precursor for generation of its oxidized metabolites (OXLAMs) that are actually bioactive lipids. Similarly, arachidonic acid can also serve as a substrate for oxidized metabolites, which together with OXLAMs are associated with steatosis markers, liver oxidative injury and insulin resistance

(119). However, only linoleic acid, but not arachidonic acid seems to contribute to the NASH development and progression in the present study.

On the other hand, lipid metabolites generated from n-3 PUFAs (protectins, maresins and resolvins) are anti-inflammatory and pro-resolving mediators which are also able to decrease lipogenesis (116,120). It has been shown that high n-6/n-3 PUFA ratio in hepatocytes and in circulation correlates with NAFLD severity (119). MCD diet used in present study caused the decrease in n-3 PUFA such as DHA (Table 2), and the ratio between n-6 and n-3 PUFAs was increased in liver tissue. These changes in hepatic free fatty acid profile in NAFLD, even in the initial phase, may represent an important triggering factor for promotion of oxidative stress and inflammation that will contribute to NAFLD progression. Supplementation with DHA and diet with lower n-6 PUFAs content may potentially be used for alleviation of liver steatosis and prevention of its further progression.

The relevance of this finding in human NAFLD remains questionable, since MCD diet does not induce insulin resistance and metabolic syndrome. Other human and experimental studies support this claim, since they have shown contradictory findings related to FFA profile in the liver and serum. In contrast to our model, high-fat diet was found to induce an increase in liver DHA level (121). Studies on patients with NAFLD and NASH have shown that hepatic FFAs were unchanged across the spectrum of liver injury (122), whereas plasma FFA levels were significantly increased in NAFLD and were suggested to be the primary source for TAG synthesis in hepatocytes. Within TAGs and diacylglycerols, the saturated palmitic acid and the monounsaturated oleic acid were elevated, reflecting, perhaps, their greater availability in the circulating FFA pool (123,124). Other studies have demonstrated elevated levels of circulating oleic and palmitic acid in patients with NAFLD (110). The precise effect of DHA supplementation should be further investigated.

There is a possible association between serum free fatty acids and cardiovascular risk factors such as dyslipidemia, insulin resistance and body mass index, that are also present in NAFLD (125). However, in our animal NAFLD model, a significant negative correlation between hepatic DHA and serum LDL level may indicate a potential role of DHA

supplementation in prevention of atherogenic lipid profile in patients with NAFLD. In addition, DHA+EPA diet was found to decrease hepatic total cholesterol content (126), whereas our study suggests that DHA may have an opposite influence on serum cholesterol level because there was a significant positive correlation found. Supplementation with n-3 polyunsaturated fatty acids in humans decreases serum triglyceride level (127). A significant decrease in serum triglycerides in our animal NAFLD model probably is not caused directly by decreased hepatic DHA despite their positive correlation, but due to specific lipid metabolism in rodents than in a human population.

MCD diet after 6 weeks of consummation induced an increase in unsaturated fatty acids such as oleic acid, and n-6 polyunsaturated fatty acids (PUFA), linoleic and arachidonic acid.

An inflammatory response that occurs in the liver is an important driving force of NAFLD progression, since inflammation processes may contribute to hepatic fibrogenesis which ultimately leads to cirrhosis (116). In our study we showed increased expression of proinflammatory cytokines TNF and IL-6, which confirm the presence of inflammatory processes evident on histology, making this model suitable for NASH studying. Inflammation is the process that occurs after the second hit, oxidative stress and free radicals accumulation that cause cell damage. MCD diet for six weeks caused increased lipid peroxidation that was evident as an increase in MDA as well as in nitrosative stress (Figure 4.10.). Besides, the activity of enzymes responsible for antioxidant defense was decreased (Figure 4.10. and 4.11., Table 3). Oxidative injury and damage of cells and organelles is responsible for triggering inflammation and subsequent the autophagy to remove damaged cell particles or cell death processes apoptosis and necrosis (29). Our results indicate that there was an increase in pro-apoptotic markers expression suggesting that there is an increase in cell apoptosis in NASH, as suggested in previous studies, both clinical and experimental (128–130). In contrast to increased apoptosis, autophagy markers were significantly reduced in MCD group, due to decreased removal of damaged organelles, and more pronounced cell death.

Betaine represents an important human nutrient and is present in almost all tissues, but the liver, kidney and testes are organs with the highest concentration of betaine. Dietary intake is a major source of betaine, but it can also be synthesized from choline in the liver and kidney (131). Recent data highlight the potential therapeutic use of betaine, with the specific focus on its mechanisms which could possibly attenuate liver injury. Our results showed that betaine supplementation improved histological findings in the liver in animals fed with MCD diet. Steatosis was reduced and inflammation less prominent than in MCD group (Figure 4.9.). This finding was also confirmed by significant decrease in ALT and AST activities in serum (Figure 4.8.), thus indicating that betaine has aprotective role in NAFLD. Other studies that investigated betaine effects on fatty liver, have obtained similar results (132). Methyl donors are described to have the ability to reduce fat deposition in the liver by decreasing free fatty acid levels and preventing further triglyceride accumulation. Additionally, it has been shown that betaine can potentially increase insulin sensitivity in mouse models of NAFLD (133,134). On the other hand, betaine supplementation significantly increased serum HDL and decreased LDL concentration, and also increased serum triglyceride content by comparison with MCD group. Wang et al. (135) suggested that betaine alleviated hepatic fat accumulation by the activation of AMPK system and inhibition of *de novo* lipogenesis in the liver. Besides, betaine may reduce liver steatosis by improving adipose tissue function and stimulation of adiponectin secretion. Study on high-fat diet-induced NAFLD showed that betaine improved insulin sensitivity and adipokine production what is considered as one of the protective effects of betaine (136). Hepatoprotective effect of betaine evident as a decerease in serum aminotransferase activities was also confirmed in human NAFLD (136). Altered lipid profile in patients with NAFLD usually increases the risk of cardiovascular diseases, suggesting that betaine treatment may reduce this risk by improving serum HDL level and reducing LDL.

These mechanisms of steatosis and lipid profile improvement are not sufficient to explain all beneficial effects of betaine in NAFLD. Therefore we examined the effects of betaine supplementation on oxidative stress, inflammation, apoptosis and autophagy in the

liver in MCD-diet induced NAFLD. An imbalance between oxidative stress and antioxidative protection has been implicated in the etiology of various disorders such as cancers, renal diseases, atherosclerosis, diabetes mellitus and liver diseases (137). Oxidative stress has been described as the major component of the "second hit" that is a key point in aggravation of fatty liver and its progression (28,29,138–140) Our study confirmed this finding, since MCD diet induced oxidative damage of the liver with increased lipid peroxidation evident as an elevation of MDA level (Figure 4.10.A). In our model oxidative stress could be explained by decreased activity of liver SOD and its isoenzymes, especially MnSOD in mitochondria that will lead to accumulation of superoxide anion, which has an ability to build peroxynitrite in reaction with NO. Similar to this, in MCD group catalase activity was decreased. These findings are in accordance with one of our previous studies (41) where dynamics of oxidative stress was observed in MCD diet-induced NAFLD. Liver GSH decreased in MCD group with increased nitrites (Figure 4.10.). This decline could be a consequence of methionine deficiency, and subsequent decreased cysteine production which is necessary for GSH synthesis (141). Also, there is increased utilization of GSH during the neutralization of free radicals, which can additionally explain low GSH level in our model (41). GSH decrease in MCD group was followed by reduced GPx activity in the absence of substrate, while activity of glutathione reductase was increased, probably in order to restore GSH. MnSOD was decreased in MCD group by comparison with control, and this was confirmed in various studies, both experimental and clinical (142,143). It has been shown that MnSOD expression negatively correlates with steatosis grade and MnSOD amount negatively correlates with triglycerides, indicating the relationship between fat accumulation in the liver and low levels of MnSOD (142).

Betaine exerted antioxidative effects in the present study, evident as a decrease in lipid peroxidation (Fig.4.10.A) and nitrozative stress (Fig.4.10.B), as well as an increase in antioxidative enzymes. Previous *in vivo* studies have shown that betaine exerts its antioxidative effect by two mechanisms: directly as a "free radical scavenger" and indirectly by improving the antioxidant defense, including enzymatic antioxidants like GPx, catalase, SOD, and nonenzymatic GSH (41,143). Betaine supplementation in our study also

significantly increased the activities of SOD, catalase, GPx, and GSH content in the liver. Superoxide anion can be detoxified by SOD in mitochondria into hydrogen peroxide, which can be further metabolized into water by the activity of GPx. For the normal function of GPx, the adequate amount of GSH is necessary in mitochondrial matrix. On the contrary, depletion of GSH content leads to mitochondrial dysfunction and cell death. Additionally, catalase has detoxifying effects against excess accumulation of hydrogen peroxide, and therefore represents another mechanism of antioxidant defense in the cell. Additionally, betaine increases the supply of substrate for GSH synthesis, by taking part in SAM synthesis, therefore making betaine an important factor for regulation of the metabolism of sulfur-containing amino acid (143,144).

Study by Ganesan et al. (145) investigated antioxidant effects of betaine in lymphoid organs during induced restraint stress in rats. This research group showed that betaine supplementation reduces lipid peroxidation that is in accordance with our results in the liver. It can potentially be explained by inhibition of ROS production that damage proteins, DNA and lipids. Due to its lipotropic properties, betaine can easily pass through lipid bilayer in cell membrane and diffuse into all parts of the cell and exert its antioxidant activity. Balkan et al. (146) suggested that betaine prevented lipopolysaccharide-induced necrosis in the liver by inhibition of Kupffer cell activity and through antioxidative effect.

On the other hand, betaine exert its antioxidant properties by increasing GSH content in the liver that was accompanied with increased GPx activity (Table 3). Recent study showed that 1.8% betaine solution increased activities of SOD and the GSH content in blunt snout fish fed with high-fat diet (147). Study by Ganesan et al. (145) showed that betaine supplementation prevented depletion in GSH in spleen of rats as well as activity of GPx and glutathione S transferase. It is suggested that betaine play role in membrane stabilization and protect membrane proteins from denaturation (148). In hydroxyl free radical, there is an unpaired electron that is mostly responsible for membrane damage and lipid peroxidation. With betaine, this electron might be trapped and dismuted by betaine, which may represent one of its antioxidant mechanism (149,150). Besides, betaine

indirectly increases GSH content by restoration of SAM that is a contributor to an increase in substrate required for GSH synthesis (145).

Main antioxidant enzyme that is present in cell cytoplasm and mitochondria is SOD with its isoenzymes Cu/ZnSOD and MnSOD and together with catalase they play an important role in neutralization of $O2^-$ into H_2O_2. Study by Alirezaei et al. (151) showed that betaine reduced lipid peroxidation and increased the activity of SOD, catalase and GPx in ethanol-induced oxidative stress in rat brain. In the same study it has been showed that betaine treatment reduced the content of H_2O_2 by elevation of catalase activity in betaine treated group as well as in ethanol + betaine group (151). In our study, we have notice the same changes in catalase activity. Its activity has been decreased in MCD diet-treated group, while betaine administered alone, and in combination with MCD diet significantly improved catalase activity (Table 3), suggesting that this can potentially be additional mechanism for ROS neutralization besides SOD. Comparing the activities of Cu/ZnSOD and MnSOD, it can be noticed that betaine exerts its antioxidant activity by increasing MnSOD activity in a higher extent than Cu/ZnSOD. This makes MnSOD predominant antioxidant that is being activated after betaine supplementation. MnSOD has been increased in betaine group, as well as in betaine+MCD, while that is not the case with Cu/ZnSOD. (Figure 4.11.). Liu et al. (152) conducet a study that examined effects of betaine on oxidative stress and survival of yeast exposed to H_2O_2. Their results suggest that betaine treatment alone and in the state of oxidative stress have ability to improve activity of SOD, catalase and GPx. Similar results have been reported in man studies. Hagar et al. showed that betaine improved SOD activity in cisplatin-induced nephrotoxicity, as well as the activity of catalase, GPx and the GSH. Total antioxidant status was also increased by betaine (153). Total oxyradical scavenging capacitywas significantly reduced in high-fat diet-induced NAFLD in rats, while betaine supplementation increased this capacity in the reaction with hydroxyl and peroxyl radicals (154). Similar study on animal model of alcoholic liver injury showed that betaine supplementation improved total oxyradical scavenging capacity in the liver by regulation the metabolism of sulfur amino acid (155). Recent study showed that betaine attenuated oxidative stress, reduced CYP450 activity, improved GSH-transferase acitivity in liver of radiation-exposed animals (156).

In addition, this study examined the effects of betaine on liver PON1 activity, which was significantly reduced in MCD diet model of NAFLD. Many studies assert the role of PON1 in atherogenesis and its correlation with increased serum lipids (157,158), while fewer studies have investigated the role of this enzyme in liver diseases such as NAFLD (159–161). Serum PON1 is liver-produced HDL-associated peroxidase and it was shown to possess antioxidative properties, which are probably attributed to the enzyme's capability to protect HDL and LDL from oxidation (162–164). In PON1 deficient mice, higher serum oxidative stress was shown and HDL was no longer able to protect LDL from oxidation (165). In the present study, betaine supplementation in NAFLD mice prevented triglyceride accumulation in the liver, reduced steatosis and also improved serum lipid status by decreasing LDL and increasing HDL content. This finding can be in part explained by improved PON1 activity in betaine-supplemented mice, since some studies have found significant correlation between PON1 activity and serum lipids (166,167). Elevated serum LDL and decreased HDL might have a causal role in the pathogenesis of NASH (160), and beneficial effects of betaine on serum lipid profile might be one of the mechanisms by which betaine alleviates fatty liver disease. Study on *db/db* mice has shown that betaine increased fatty acid oxidation by increasing PPARα and carnitine palmitoyltransferase 1α (CPT1α) mRNA levels, indicating a possible mechanism of betaine action in the alleviation of hyperlipidemia (168).

Activity of liver PON1 in the present study was significantly reduced in mice with MCD diet-induced NAFLD with negative correlation with liver MDA, which has also been confirmed by similar studies (169,170). In addition, it has been shown that oxidative stress can affect PON1 activity and its expression (171). However, it has been shown that mRNA and protein expression of PON1 were both elevated in human NASH livers (161). This discrepancy could be related to the differences in investigated species or to the diffrences in oxidized lipids that are able to inactivate PON1 (172). Interestingly liver PON1 activity was found to positively correlate with SOD activity and GSH content. This finding may suggest that betaine improves GSH and SOD directly, and probably to a lesser extent by modulating liver PON1 activity. Similar correlations were found in study on diabetic patients where serum PON1 was inversely correlated with parameters of insulin resistance,

chronic inflammation and with markers of oxidative stress, inversely with MDA, and directly with SOD and GPx (173). The same study has shown significantly increased GRed activity in group of subjects with metabolic syndrome (173), what is also in accordance with our results in animal model of NAFLD. We observed that PON1 activity was significantly decreased in NAFLD compared to control group, while betaine improved its activity in the liver. PON1 plays a role in the lipid metabolism and generation of VLDL in the liver (174), and liver dysfunction can potentially be caused by decreased PON1 activity. The putative function of PON1 in the liver is to provide hepatic protection against oxidative stress (172), while low activities of PON1 are associated with diabetes mellitus,oxidative stress, increased cholesterol levels, cardiovascular diseases, and sepsis (175,176). Our results are in accordance with these findings, since decreased PON1 activity and increased oxidative stress were evident as well as correlations between PON1 and MDA and nitrites.

Inflammatory response in the liver is an important factor leading to NAFLD progression and development of steatohepatitis. However, as in other chronic liver diseases the presence of chronic inflammation precedes NASH and is the key mechanism contributing to liver fibrosis (177). Oxidative stress is considered to be an important trigger for inflammatory processes in NAFLD. Earlier studies showed that H_2O_2 is able to rapidly and potently activate NF-kB in human T-cells. Further studies reported that various oxidants may directly activate NF-kB, while antioxidants have ability to inhibit its activity (178). NF-kB is an important transcription factor involved in immune response and inflammation. Under normal conditions, this factor is in the cytoplasm and is bound to inhibitory kB (I-κB), which then inhibits translocation of NF-kB into cell nucleus. Increased ROS production in cells activates redox sensitive kinase that phosphorylates I-kB, leading to its degradation and NF-kB translocation to the nucleus with subsequent upregulation of proinflammatory genes, such as TNF-α and IL-6 (24,179). In our study apart from increased oxidative stress, in the liver tissue, the mRNA expressions of inflammatory mediators were increased. Inflammation in the NAFLD may be induced by few mechanisms, but oxidative stress is considered to be the main factor contributing to inflammation and NASH development (23,24,180). Therefore, increased inflammation verified on histology and by increased expression of proinflammatory cytokines could be

the consequence of prolonged oxidative injury of hepatocytes. In the present study the mRNA expression of TNF-α and IL-6 were significantly increased in MCD diet-fed mice, as well as IL-10 (Figure 4.12.). Increased expression of anti-inflammatory IL-10 is probably a compensatory response of liver tissue on increased inflammation. Similar to oxidative stress, free fatty acids are also triggers for synthesis of proinflammatory cytokines, and in our model, we have shown increased long chain fatty acids in the liver (Table 2). It has been shown that increased levels of linoleic acid promotes the synthesis of tissue arachidonic acid and both are able to induce synthesis of inflammatory eicosanoids and reduce conversion of alpha linoleic acid into EPA and DHA (181), which were significantly reduced in our NAFLD model (Table 2). Impaired free fatty acid profile, together with oxidative stress potentially represent important mechanisms leading to inflammation and steatosis progression to NASH.

TNF-α represents a proinflammatory cytokine derived from various organs, including adipose tissue, skeletal muscles and liver, and its increased production occurs as a consequence of metabolic disturbances and is closely related to insulin resistance in obese animals. TNF-α is expressed to a higher extent in patients with NASH than with simple steatosis. Recent study by Ceccarelli et al. (182) have showed that lipopolysaharide-induced TNF-α synthesis represents a key regulator of proinflammatory and profibrogenic response in the liver in NAFLD examined in clinical study and in HSC cell line. Betaine supplementation reduced inflammation that was evident on histology (Figure 4.9.), but also confirmed by decreased mRNA expression of TNF-α and IL-6, as well as TGF-β (Figure 4.12.) in the liver. Betaine was confirmed to decrease the mRNA expression of TNF-α and IL-6 in colonic mucosa in mice and also down-regulated lipopolysaharide-induced inflammatory response (183). Prevention of inflammation by betaine treatment may be partly explained by suppression of NF-kB activation that was confirmed in the aging and oxidative injury (184). In addition, betaine was shown to inhibit generation of ROS and activation of NF-kB in endothelial cells, and it is capable of restoring the redox balance by moderation thiol homeostasis (183–185). Increase in IL-10 mRNA expression contributes to the anti-inflammatory effects of betaine in our model of NAFLD.

Betaine has been shown to attenuate alcoholic liver disease by elevation of SAM concentration in liver tissue (186). Previous studies (186) suggest that SAM may attenuate liver injury by increasing IL-10 concentration and decreasing TNF-α concentration. Investigation conducted on macrophage cell line obtained that SAM and its metabolite 5'-methylthioadenosine (MTA) significantly decreased lipopolysaharide-inducible NF-kB transcriptional activity and consequently the expression of TNF-α. Besides, SAM leads to the increase in intracellular adenosine concentration that may increase IL-10 expression. Examination SAM role on macrophage cell line showed that SAM binding to the adenosine A2 receptor is the mechanism of IL-10 expression modulation (187–189). High-fat diet for 6 weeks in IL-$10^{-/-}$ mice induced a significant increase in hepatic triglyceride content compared to control animals, which suggest that IL-10 can protect liver against steatosis. Besides, inhibition of IL-10 in another NAFLD animal model aggravated hepatic inflammation by increasing IL-6 and TNF-α expression in liver (190).

Apoptosis represents an important mechanism that contributes to the progression of various liver diseases and if uncontrolled it can trigger liver fibrogenesis (128,130,191). Numerous studies confirmed a significant role of apoptosis in NAFLD development and progression to liver fibrosis (129,180,192,193).

Oxidative injury and increased influx of free fatty acids are considered to be main mechanisms that trigger hepatocyte apoptosis in NAFLD. Oxidative stress can damage cell structure and function on every level, but the most significant is mitochondrial dysfunction. Since mitochondria are the major source of ROS, they are most susceptible to oxidative injury and peroxidation of membrane lipids that have PUFAs as essential components of phospholipids (194). Free radicals react with PUFAs damaging mitochondrial membrane and also inactivate antioxidant enzymes such as SOD. Oxidative injury leads to the inhibition of electron-transport chain in mitochondria, which ultimately increases production of ROS. It is known that ROS damage mitochondria DNA, so taken together prolonged oxidative stress leads to mitochondrial dysfunction starting vicious cycle in ROS production and oxidative damage (180,195–197). Altered electron transport chain accompanied with defective ATP production and disrupted ultrastructure such as

mitochondrial enlargement, membrane crystalline inclusions and loss of cristae are indicators of mitochondrial dysfunction. Under normal conditions, damaged cell components and organelles are being removed by degradative processes such as autophagy. However, prolonged oxidative injury without adequate repair lead to cell death by apoptosis and necrosis (180). Initiation of apoptosis by dysfunctional mitochondria starts with cytochrome C release, which forms an activation complex with apoptotic protein activation factor-1 and caspase 9. This complex further activates effector caspases 3, 6 and 7, which execute final apoptotic changes. Proapoptotic protein Bax can integrate into outer mitochondria membrane following apoptotic stimuli, while antiapoptotic protein Bcl-2 is localized predominantly in mitochondria (128,180,193).

Our results presented on Figure 4.12.B showed increased mRNA expression of both proapoptotic Bax and antiapoptotic Bcl-2 protein in NAFLD model. This paradoxical finding can be explained by different time frame when these proteins are expressed in hepatocytes after proapoptotic stimulus. However, histological and TEM analysis suggest increased apoptosis in NAFLD as a presence of apoptotic bodies (Figure 4.9. and 4.14.). Figure 4.14. shows the ultrastructure of hepatocytes, where enlarged and mega-mitochondria were observed suggesting their impaired structure, and probably the function as well. Apoptosis in NAFLD can be partly explained by increased oxidative stress and lipid peroxidation (Figure 4.10. and 4.11.) and consequent mitochondrial dysfunction. On the other hand, several studies point to the role of free fatty acid profile in apoptotic processes in NASH. Lipotoxicity mediators, saturated fatty acids and free cholesterol are important triggering factors that activate specific signaling pathways leading to apoptotic cell death (128,198). Accumulation of free fatty acids, especially saturated, induces ER stress in hepatocytes, that activates signaling pathways causing apoptotic cell death (199).

Betaine supplementation in MCD diet-fed mice significantly reduced apoptosis by decreasing expression of Bax and increasing Bcl-2. Besides, by decreasing levels of lipid peroxidation and oxidative stress, betaine can indirectly reduce apoptosis through its antioxidant effects. Similar to our results, apoptosis induced by bile duct ligation in rats, was significantly reduced after betaine supplementation in study by Graf et al. (200). In

animals exposed to ionizing radiation, betaine treatment significantly reduces the activity of caspase-3 in the liver (156). Betaine effects on apoptosis were examined on isolated hepatocytes where betaine prevented the activation of caspase 3, 8 and 9, as well as an increase of cytochrome C in the cytoplasm, suggesting that antiapoptotic effect of betaine is predominantly at the mitochondria level (200). Another *in vitro* study on isolated hepatocytes treated with adenosine to induce cell damage analogous to alcoholic liver injury showed that betaine reduced the caspase-3 activity in hepatocytes via methylation reaction catalyzed by betaine-homocysteine methyltransferase (201).

Autophagy represents a "housekeeper" process, which major role is to remove damaged or dysfunctional cellular contents and organelles and to provide substrates for energy production during starvation. So far known facts about the autophagy make it an important factor in maintaining normal cell function (202,203). Autophagy can be induced in stressed conditions such as oxidative or metabolic stress (202). In NASH, which is characterized with hepatocyte injury and inflammation, degradation and dysfunction of cells and organelles is increased. Since damaged organelles are removed by autophagy, alteration in this process could lead to aggravation of cellular injury. First described forms of autophagy in the liver were mitophagy and lipophagy (202). Autophagy has an important role in removing dysfunctional mitochondria, which occurs in aging, neurodegenerative disease, terminal differentiation of blood cells or prolonged oxidative stress, and is called mitophagy (202,204). Besides cytosolic lipases, autophagy is involved in regulation of cell content of lipids, by process called macrolipophagy (205,206), which represents the most important role of autophagy in fatty liver disease, since it could regulate the process of lipid accumulation in hepatocytes (202). Study on $Atg7^{-/-}$ mice showed a significant increase in triglyceride accumulation in the liver indicating that defective autophagy promotes development of steatosis (202,203). This is in accordance with our results, where autophagy was decreased in MCD diet fed mice. Similarly, inhibition of Atg5 in hepatocytes also results in lipid droplets accumulation in the liver (203). MCD diet in our NAFLD model increased the levels of SQSTM1/p62 in the liver, suggesting that autophagic flux is decreased. This finding is followed by increased amount of LC3-II protein, marker of autophagosomes, which is degraded by autophagy. The study on patients

with steatosis and NASH showed accumulation of p62 and LC3-II that may indicate that autophagy was decreased (207), which is in accordance with our results. In clinical studies on patients with liver steatosis, accumulation of autophagy cargo p62/SQSTM1 was evident suggesting decrease autophagy activity. This finding support the proposition that level of autophagy inversely correlate with liver fat content (208). The role of autophagy in the progression of steatosis is not completely elucidated. Alteration in lipid metabolism, increased oxidative stress, inflammation and apoptosis are usually associated with the modulation of autophagy process as a protective response against stress and cell injury (207,209). Pharmacological inhibition of autophagy induced an elevation of triglyceride content in the liver, possibly due to impaired lipolysis and not by increased synthesis of lipids (203). It has been shown that autophagy is an important mechanism in regulation of hepatic intracellular lipid metabolism and participates in lipid droplet degradation, therefore, dysfunctional autophagy is a potential pathogenic mechanism involved in fatty liver disease (210). Our results are in accordance with these findings, since in our NAFLD model, there was increased fat accumulation in the liver and decreased autophagy.

On the other hand, betaine increased the expression of Atg4, Atg5 and Atg7 in mice on MCD diet (Figure 4.13.A), suggesting that another mechanism of protective effect of betaine against fat accumulation is the stimulation of lipophagy. Besides, betaine supplementation increased the content of LC3II accompanied with low levels of p62, suggesting that betaine increased autophagy (Figure 4.13.B). Increased LC3-II levels was probably due to increase in LC3-II synthesis, rather than decrease in its degradation, since betaine stimulated degradation ofp62 and the expression of Atg4b, Atg5, Atg7, and beclin 1. Besides, there were increased autophagic vesicles in the mice liver on MCD diet, evident on TEM. Ultrastructural analysis using TEM showed decreased steatosis, presence of autophagosomes in MCD+BET group, with rare megamitochondira and few apoptotic bodies (Figure 4.14.D) confirming increased autophagy and decreased steatosis after betaine supplementation.

It has been reported that ROS are important inducers of autophagy during starvation and nutrient deficiency. It is proposed that O_2^- is the primary radical involved in autophagy

(211). However, increased oxidative injury can reduce autophagic function in hepatocytes and contribute to ROS accumulation due to increasing number of dysfunctional mitochondria. Our results have shown that betaine exerts antioxidant activity by reducing lipid peroxidation and increasing the activity of antioxidant enzymes, we may suggest that modulation of oxidative stress by betaine supplementation, can be additional mechanism by which betaine stimulates autophagy leading to diminished lipid content in hepatocytes and removal of dysfunctional mitochondria. There are evidence that increased oxidation of GSH is able to induce autophagy even there are not present any other autophagy stimuli (212).With decreased lipid peroxidation, we may assume that betaine treatment increases oxidation of GSH to reduce the amount of hydrogenperoxide, catalyzed by GPx, suggesting that this antioxidative effect of betaine is also trigger mechanism of autophagy (211).

Recent studies in the past five years suggest that NO and nitrosylation process have the role in autophagy inhibition. It is demonstrated that NO donors or an increase in NO synthase in cell culture results in autophagy prevention by inhibition of c-Jun-N-terminal kinase 1 and IkB kinase β that regulate Beclin 1 and AMPK activation (211,213). Increased production of reactive nitrogen species, evident in our MCD model of NAFLD (Figure 4.10.B) are at least partly responsible for decreased autophagy. Sarkar et al. (213) have showed that NO donors can also inhibit autophagy by upregulation classical mTOR pathway with increased p-s6K and mTOR activity in HeLa cells, suggesting that NO impairs autophagy by two independent mechanisms via JNK1-Bcl2-Beclin1 and AMPK/mTOR pathways. In our model, reactive nitrogen species didn't modulate autophagy via classical AMPK/mTOR signaling pathway, but possibly by activating JNK1-Bcl2-Beclin1 pathway. Similar to ROS, betaine treatment also diminished the content of reactive nitrogen species, which could be another linking mechanism between nitrosative stress and autophagy. Sine it has been shown that NO donors may inhibit autophagy, decrease in reactive nitrogen species by betaine may be additional probable cause of increased autophagy in our study.

Apoptosis, process that is important for cell and organelles turnover and is in tight interaction with autophagy (214). Apoptosis and autophagy usually occurs in the same cell,

with the autophagy preceding the apoptosis. Initial stress stimulates and autophagic response, but when stress exceeds a critical duration or intensity, apoptotic pathways of cell death are activated. In the most cases, autophagy tends to suppress apoptosis or increase stress threshold before apoptosis occurs and therefore to reduce tendency of cells to die. This autophagy-apoptosis crosstalk seems to be anti-apoptotic rather than pro-apoptotic. However, in the condition of intense and prolonged cell injury that reach the cell limit, adaptive mechanisms, including autophagy are being overwhelmed and apoptotic cell death pathway is activated. Activated caspases digest proteins that are essential for autophagy, and hence inhibit the autophagic programme, probably in order to interrupt its protective function and accelerate cell death. The main targets for caspases are Atg3 and Beclin 1, making them to lose their ability to stimulate autophagyand convert proautophagic proteins into pro-apoptotic ones (215–217). This interaction between apoptosis and autophagy is perhaps additional mechanism of cell damage in our MCD-diet induced NAFLD. Our results indicate increased mRNA expression of pro-apoptotic Bax and Bcl-2 (Figure 4.12.) and the presence of apoptotic bodies in MCD-diet fed mice liver (Figure 4.14.). Increased apoptotic response in the hepatocytes may be the possible inhibitor of autophagy in our model. Caspase 10 is also being showed to degrade Bcl-2-associated transcriptional factor (BCLAF1), preventing BCLAF1 to interact with Bcl-2 and cause disruption of Bcl2-Beclin 1 complex that will formation of Beclin-1-C fragments that further promote release of pro-apoptotic factors, activating autophagy. Besides, activation of caspases inhibits Beclin 1-induced autophagy by as cytochrome c by mitochondria (215,216,218,219). Increased autophagy flux (Figure 4.13.) by betaine supplementation is one possible mechanism that protected cells from apoptotic death. The major mechanisms by which autophagy reduces chances of cells to undergo apoptosis is mitophagy (215). Damaged and dysfunctional mitochondria are particularly able to activate apoptosis, so their removal by autophagy can postpone cell death by increasing the threshold for the induction of apoptosis (215). Another protective role of autophagy is removal of pro-apoptotic proteins in cytoplasm and decreasing their amount. It has been shown that autophagy mediates the removal of caspase 8 in colon cancer cells (220). Study on Atg7 knockout animals registered increased caspase 8 activity in TNF-induced apoptosis in hepatocytes (221). In addition, autophagy decreases

the SQSTM1 abundance, a protein that stimulate ROS production and cell death if it overexpressed (222).

Since autophagy occurs before the apoptosis, it plays an important role in suppression of inflammation activated by death cells by increased recruitment of macrophages and removal of death cells. With inhibition of autophagy in $Atg5^{-/-}$ and $Beclin1^{-/-}$, death cells with defective exposure of lysophosphatidylcholine on its surface accumulate and promote inflammation (215,223). This may be the linking mechanism between autophagy and inflammation, and may be applied to our results, where betaine supplementation reduced inflammation, that can be at least partly by increased autophagy.

Through stimulation of autophagy, betaine exert additional hepatoprotective effects by dereasing fat content in the liver, reducing oxidative stress, inflammation and apoptosis, besides modulating these processes directly.

Our results indicate that betaine may potentially have antifibrotic effects by down-regulation of TGF-β expression. MCD diet induced an increase in TGF-β expression, while betaine prevented this increase. (Figure 4.12.). Similar results were obtained in high-fat+CCl_4 model of liver fibrosis in rats. Bingul et al. (224) showed that betaine supplementation reduced the expression in profibrotic markers such as TGF-b, a-SMA and Col1a1 in the liver, suggesting that betaine may directly affect the activity of HSCs. However, our model is predominantly NAFLD model, and the process of fibrogenesis is probably in early initial phase. Therefore, betaine could potentially be used for prevention of fibrosis development by modulation of TGF-β mRNA expression.

Recent studies reported benefitial effects of NRG-1, especially in heart failure but also in lung fibrosis, nephropathy, nerve regeneration processes and insulin resistance (71,225–228). Clinical trials in patients with chronic heart failure showed that rhNRG-1 treatment caused improvements in cardiac function (65,229–232). The effects of rhNRG-1 on liver physiology or pathopysiology is currently unknown. Previous studies have shown that rhNRG-1 prevents cardiac, kidney, skin, and lung fibrosis (71,72). The anti-fibrotic effects

of rhNRG-1 on fibroblasts are mainly mediated by the ErbB4 receptor (71). In this study, we examined the effects of NRG-1 on liver fibrosis. Two different murine models for liver fibrosis were used; CCl_4 induced mild fibrosis and BDL induced severe liver fibrosis (Figure 4.16.). Surpisingly, the present study shows increased liver fibrosis in mice that were treated with NRG-1. Furthermore, we showed that the expression of the pro-fibrotic factor TGF-β is increased in isolated hepatocytes after stimulation with NRG-1 (Figure 4.19). Therefore, the increased ECM accumulation in the liver in the NRG-1-treated animals could be due to increased TGF-β expression in hepatocytes by NRG-1.

MMPs represent extracellular endopeptidases that are involved in ECM regulation and degradation and may have both inhibitory and stimulatory role in fibrogenesis (233). In the CCl_4 model, our results indicated increased expression of liver MMP2 and MMP9 (Figure 4.17.). This increase is a consequence of a compensatory tissue response to hepatocyte damage, and overproduction of ECM proteins consistent with other studies (234–236). In the NRG-1 treated group, a significant decrease in liver MMPs expression was observed, which may represent an additional pro-fibrotic mechanism of NRG-1. On the other hand, in BDL model with severe liver fibrosis, no significant change in MMP2 and MMP9 mRNA expression between BDL and BDL+NRG-1 groups was evident, that may suggest that fibrogenesis in BDL occurs via a different mechanism.

So far, various studies pointed out the importance of NRG-1/ErbB4 signaling pathway, suggesting that in cardiovascular system, NRG-1 exerts its effects predominantly by phosphorylation of ErbB4 receptors (65,237). ErbB2 and ErbB4 receptors are required for normal cardiac function, since their absence leads to embryonal lethality because of deficient cardiac morphogenesis (238). In contrast to the heart, ErbB3 receptors are known to be highly expressed in the liver, while ErbB2 and ErbB4 are expressed in small amounts (239,240). Pro-fibrotic effects of NRG-1 in the liver could be explained by predominantly activating ErbB3 in hepatocytes. Indeed, we observed a significant increase in ErbB3 phosphorylation in liver tissue after NRG-1 exposure (Figure 4.18.), while ErbB4 was not phosphorylated by NRG-1. In contrast to the anti-fibrotic effects of NRG-1 in heart, kidney, lung and skin through NRG-1/ErbB4 signaling, in the liver NRG-1 exerts its activity

predominantly by activation of ErbB3 receptors. In addition to our research, Scheving et al. (240) pointed out a significant role of ErbB3 receptors in liver fibrosis development, and therefore pro-fibrotic effects of NRG-1 in the liver can be explained by NRG-1/ErbB3 pathway. They showed that mice lacking ErbB3 and both ErbB3/EGF receptors develop less pronounced fibrosis after CCl_4 injections compared to wild type mice, which indicates that activation of ErbB3 and EGF receptors are involved in the progression of fibrosis.

6. CONCLUSIONS

1. Based on our results, it can be concluded that MCD die tinduces initial fatty change within 2 weeks with progression to more diffuse fatty change at 4 weeks and NASH in 6 weeks accompanied by apoptosis and inflammation. MCD diet is an appropriate model for investigation of the histological, biochemical and molecular changes where metabolic syndrome, and the role of adipose tissue are not reflected.
2. Betaine exerts antioxidative effects by reducing lipid peroxidation and nitrosative stress in MCD diet-induced NAFLD. Anttioxidative capacity of the liver is enhanced by an increase in GSH level and activity of PON1, total SOD and its isoenzymes, CAT and GPx.
3. Additionally, betaine improved serum lipid profile by reducing serum triglyceride and LDL level, and increasing HDL concentration.
4. Betaine potentially alleviates the course of NAFLD by stimulation of autophagy through an increase in Atg4, Atg5, Atg7 in Beclin 1- and Akt/mTOR-dependent manner, but independently on AMPK activation.
5. Betaine exerts antiapoptotic effect in MCD-diet induced NAFLD by lowering Bax and by increasing Bcl2 mRNA expression.
6. Betaine alleviates inflammatory response in MCD-diet fed mice, by reducing the expression of TNF, IL-6 and by increasing anti-inflammatory IL-10.
7. Betaine treatment prevents initial fibrogenesis by decreasing TGF-β expression in the liver that was increased in NAFLD model.
8. NRG-1 aggravates liver fibrosis in CCl_4 and BDL mouse model by increasing ECM accumulation. The pro-fibrotic effects of NRG-1 could be mediated by ErbB3 receptor phosphorylation and activation of the NRG-1/ErbB3 signaling pathway in the liver.

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ABBREVIATIONS

4-HNE – 4-hydroxy-2-nonenal

ALP – alkaline phosphatase

ALT – alanine aminotransferase

ANOVA – analysis of variance

ARE – arylesterase

AST – aspartate aminotransferase

BCLAF-1 - Bcl-2-associated transcriptional factor

BDL – bile duct ligation

BGT - betaine-GABA transporter

CAT – catalase

CCl_4 – carbon tetrachloride

ChREBP - carbohydrate response element-binding protein

DEN – dietylnitrosamine

DHA – docosohexaoenoic acid

DMN – dimethylnitrosamine

DPA – docosopentaenoic acid

ECM – extracellular matrix

EGFR – epidermal growth factor receptor

ER – endoplasmic reticulum

FABP – fatty acid binding protein

GPx – glutathione peroxidase

GRed – glutathione reductase

GSH – glutathione

HBV – hepatitis B virus

HCC – hepatocellular carcinoma

HCV – hepatitis C virus

HDL – high density lipoprotein

HSC – hepatic stellate cell

IL – interleukin

JNK – C-jun N-terminal kinase

LDL – low density lipoprotein

MCD – methionine choline deficiency

MDA – malondialdehyde

MetS – metabolic syndrome

MMP – matrix metalloproteinase

MTA- 5'- methylthioadenosine

NAFLD – nonalcoholic fatty liver disease

NASH – nonalcoholic steatohepatitis

NF – nuclear factor

NO – nitric oxide

NRG-1 – neuregulin -1

PBS - phosphate buffered saline

PDGF – platelet derived growth factor

PON1 – paroxonase-1

PPAR - peroxisome proliferator-activated receptor

ROS – reactive oxygen species

RT-PCR – real time polymerase chain reaction

SAM – S-adenosylmethionine

SMA – smooth muscle actin

SOD – superoxidedismutase

SREBP-1 - sterol regulatory element-binding protein-1

T2DM – diabetes mellitus type 2

TAG – triacyl glycerol

TBARS – thiobarbituric acid reactive substances

TCA – tricarboxyl acid

TGF-β – transforming growth factor beta

TIMP – tissue inhibitor metalloproteinase

TLR – toll like receptor

TNF-α – tumor necrosis factor alpha

TRAIL - TNF-related apoptosis-inducing ligand

VLDL – very low density lipoprotein

CURICULUM VITAE

Teaching Assistant Milena Vesković, MD was born in Leskovac in 1987, where she completed her elementary and high school education with honors. In 2005, she started her studies at Faculty of Medicine, University of Belgrade. Over the course of her studies, she was engaged as a student teaching assistant at the Institute of Pathophysiology at the Faculty of Medicine, University of Belgrade. She graduated from the School of Medicine University of Belgrade in 2011, with average grade 9.04/10.00. In 2011, Milena Vesković enrolled Specialistic Academic Studies in Experimental physiology and pathophysiology, and finished it in 2013.

In 2012/2013, Milena Vesković enrolled in her PhD studies at the School of Medicine, University of Belgrade attending the module – Physiological sciences. In the same year, she became a Teaching Associate at the Department of Pathophysiology. In 2014 she was promoted to Teaching Assistant and started working as a Research Trainee on scientific project No. 175015 entitled "The role of the neuroendocrine-inflammatory axis in the pathogenesis of non-alcoholic fatty liver disease ", financed by the Serbian Ministry of Education and Science. Since than she showed special interest in studying pathophysiology of liver diseases, and so far she presented the research results on numerous domestic and international conferences where her presentations were awarded.

During 2016, dr Vesković spent six months at Physiopharmacology laboratory at University of Antwerp in Belgium, winning ERASMUS scholarship for scientific research that was a part of her doctoral dissertation.

Milena Vesković is the author of 14 papers published *in extenso* in journals indexed in the Science Citation Index (SCI), and her papers have been cited 81 times, according to SCOPUS.

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