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Some aspects of molecular mechanisms of xenobiotics' hepatotoxicity and hepatoprotection

Modulatory roles of natural polyphenols

Určité aspekty molekulárních mechanismů hepatotoxicity a hepatoprotekce cizorodých látek

Modulátorové úlohy přírodních polyfenolů

PhD Thesis

Supervisor: Prof. Dr.Hassan Farghali, DrSc.

Prague, 2013

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ABSTRACT

Background & Aims: Oxidative stress and apoptosis are proposed mechanisms of cellular injury in studies of xenobiotic hepatotoxicity. The aim of this work is to find early signal markers of drug-induced injury of the liver by focusing on select antioxidant/oxidant and apoptotic genes. As well, to address the relationship between conventional liver dysfunction markers and the measured mRNA and protein expressions in the D-galactosamine/lipopolysaccharide and tert-butylhydroperoxide hepatotoxicity models. Furthermore, potential hepatoprotective capabilities of antioxidant polyphenols quercetin and curcumin were evaluated in relation to its modulation of the oxidative stress and apoptotic parameters in the given xenobiotic hepatotoxicity models.

Methods: Biochemical markers testing the hepatic function included aminotransferases (ALT, AST) and bilirubin. Measurements of TBARS and conjugated dienes were used to assess lipoperoxidation. Plasma levels of catalase and reduced glutathione were used as indicators of the oxidative status of the cell. Real time PCR was used to analyse the mRNA expressions of the inducible nitric oxide synthase (NOS-2), heme oxygenase-1 (HO-1), superoxide dismutase (SOD-1), glutathione peroxidase (Gpx-1), caspase 3 (Casp3), BH3 interacting domain death agonist (Bid) and Bcl-2 -associated X protein (Bax), and tumor necrosis factor α (TNF- α)mRNAs. Additionally, the protein expressions of HO-1 and NOS-2 were assessed with the use of Western blot method. Morphometric evaluation of hepatocytes at the light microscopical level was done on semithin epon sections stained by toluidine blue using Leica IM 500 program for digital recording and measurements. Statistical analysis was performed using ANOVA and post hoc Boneffori, Tukey- Kramer comparison test or unpaired T-test with Welch correction.

Results: Overall, the results of this study have revealed the early activation of oxidative stress and apoptosis in the given hepatotoxic models as seen by the relevant changes in the tested parameters. Hepatoprotective effects of curcumin and quercetin were demonstrated, where the induction of the antioxidant enzyme HO-1 and its products played the most important cytoprotective role. In case with curcumin, this effect was paralleled with the concomitant reduction of NOS-2 and TNF-α expressions.

Conclusion: Understanding the mutual regulatory mechanisms of the tested parameters in hepatocyte injury should provide important clues to the diagnosis and treatment of liver damage. The research data from the present study paves a way for those interested in further research of these dietary polyphenols, curcumin and quercetin as it gives an overview of the potential cytoprotective mechanisms and effective doses in the given models.

Key words: liver, xenobiotic hepatotoxicity, D-galactosamine, lipopolysaccharide, tert-butylhydroperoxide, hepatoprotection, curcumin, quercetin, heme oxygenase 1, nitric oxide synthase 2, catalase, superoxide dismutase 1, glutathione peroxidase, tumor necrosis factor-alpha, Bid, Bax, caspase 3.

SOUHRN

Zdůvodnění a cíle: Oxidační stres a apoptóza jsou navrhovanými mechanismy buněčného poškození v studiích hepatotoxicity vyvolané cizorodými látkami. Cílem této práce bylo nalézt časné signální markery jaterního poškození cizorodými látkami soustředěním na vybrané antioxidační/oxidační a apoptotické geny. Cílem mé práce dále bylo oslovit vztah mezi obvyklými markery jaterní dysfunkce a mRNA a proteinovou expresí v modelech hepatotoxicity s D-galaktozamin/lipopolysacharidem a tert-butylhydroperoxidem. Kromě toho, byly vyhodnocené hepatoprotekční možnosti antioxidačních polyfenolů quercetinu a kurkuminu v souvislosti s jejich modulacemi oxidačního stresu a apoptotických parametrů v stanovených modelech hapatoxicity způsobené cizorodými látkami.

Metody: Biochemické markery zkoumající jaterní funkce zahrnují aminotransferázy (ALT, AST) a bilirubin. Meření TBARS a konjugovaných dienů bylo použito k stanovení peroxidace lipidů. Hladiny katalázy a redukovaného glutathionu byly použity jako indikátory oxidačního stavu v buňce. Pomocí 'real-time PCR' byly analyzovány mRNA exprese inducibilní syntázy oxidu dusnatého (NOS-2), hemoxygenázy-1 (HO-1), superoxiddismutázy (SOD-1), glutathionperoxidázy (Gpx-1), kaspázy 3 (Casp3), BH3 interacting domain death agonist (Bid) and Bcl-2 -associated X protein (Bax), a tumor necrosis factor α (TNF- α)mRNAs. Proteinové exprese HO-1 a NOS-2 byly stanoveny pomocí metody Western blot. Morfometrické hodnocení hepatocytů použitím světelného mikroskopu bylo uděláno na polotenkých segmentech, které byly obarveny toluidinovou modří. Pro tento účel byl použit Leica IM 500 program pro digitální záznamy a měření. Statistická analýza byla udělána pomocí ANOVA a post hoc Boneffori, Tukey- Kramer porovnávácího testu nebo nepárového T-testu s Welch korekcí.

Výsledky: Výsledky této studie nám odhalují časnou aktivaci oxidačního stresu a apoptózy v daných modelech hepatotoxicity, což je vidět z příslušných změn měřených parametrů. Hepatoprotekční účinky kurkuminu a quercetinu byly prokázany, přičemž indukce antioxidačního enzymu HO-1 a jejich produkty hrály nejdůležitější cytoprotekční roli. V případě kurkuminu, paralelně s tímto účinkem, jsme pozorovali i průvodní redukci NOS-2 a TNF-α expressí.

Závěr: Porozumění vzájemných regulačních mechanismů testovaných parametrů při poškození hapatocytů by mělo poskytnout důležité stopy k diagnóze a léčbě jaterní nemoci. Data vyplývající z této studie připravují cestu pro ty, kteří mají zájem o další výzkum těchto dietních polyfenolů, kurkuminu a quercetinu, neboť dávají přehled potenciálních cytoprotekčních mechanismů a účinných dávek v daných modelech.

Klíčová slova: játra, cizorodé látky, hepatotoxicita, D-galactozamin, lipopolysacharid, tert-butylhydroperoxid, hepatoprotekce, kurkumin, quercetin, hemoxygenáza 1, syntáza oxidu dusnatého 2, kataláza, superoxiddismutáza 1, glutathionperoxidáza, tumor necrosis factor-alfa, Bid, Bax, kaspáza 3.

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2 LIST OF ABBREVIATIONS

Ac-DEVD-AMC	acetyl-Asp-Glu-Val-Asp-7-amido-4-methylcoumarin
ADP	adenosine diphosphate
ALT	alanine aminotransferase
AMC	acetyl-Asp-Glu-Val-Asp-7-amido-4-methylcoumarin
ANOVA	analysis of variance
Apaf-1	apoptotic protease activating factor 1
AP-1	activator protein-1
ARE	antioxidant response element
AST	aspartate aminotransferase
ATP	adenosine triphosphate
Bad	Bcl-2-associated death promoter
Bak	Bcl-2 homologous antagonist/killer
Bax	Bcl-2-associated X protein
Bcl-2	B-cell lymphoma 2
BH_4	tetrahydrobiopterin
Bid	BH3 interacting domain death agonist
CaM	calmodulin
Casp3	caspase 3
CAT	catalase
CD	conjugated dienes
cGMP	cyclic guanosine monophosphate
CHOP	DNA damage-inducible transcript 3
CO	carbon monoxide
COX-2	cycloxygenase 2
C_T	threshold value
Cyt C	cytochrome C
D-GalN	D-galactosamine
DISC	death inducing signaling complex
DNA	deoxyribonucleic acid

EDTA	ethylenediaminetetraacetic acid
eNOS	endothelial nitric oxide synthase
ERK	extracellular signal-regulated protein kinase
FAD	flavin adenine dinucleotide
$FADH_2$	flavin adenine dinucleotide hydroquinone form
Fas/CD95	fas ligand
FMN	flavin mononucleotide
FOXO	forkhead box protein O
GPx	glutathione peroxidase
GSH	reduced glutathione
GS-SG	oxidized glutathione
HBV	hepatitis B virus
H_2O_2	hydrogen peroxide
HO-1	heme oxygenase-1
H_3PO_4	phosphoric acid
HSP70	heat shock protein 70
INF-γ	interferon gamma
IL	interleukin
i.p.	intraperitoneally
iNOS	inducible nitric oxide synthase
I/R	ischemia and reperfusion injury
JNK	c-Jun N-terminal kinase
LPS	lipopolysaccharide
MAPK	mitogen-activated protein kinases
MDA	malondialdehyde
MLL	myeloid/lymphoid gene
MPT	permeability transition pore
mRNA	messenger ribonucleic acid
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NAD+	nicotineamide adenine dinucleotide

NADH	reduced nicotineamide adenine dinucleotide
NADP	nicotinamide adenine dinucleotide phosphate
NAPQI	*N*-acetyl-*p*-benzo-quinone imine
NF-κB	nuclear factor kappa B
NO	nitric oxide
N_2O_3	dinitrogen trioxide
NO_2^-	nitrite
NO_3^-	nitrate
NOS-2	nitric oxide synthase-2
nNOS	neuronal nitric oxide synthase
NOXA	phorbol-12-myristate-13-acetate-induced protein 1
Nrf2	nuclear (erythroid-derived)-like 2 factor
NTB^-	2-nitro-5-thiobenzoate
O_2	molecular oxygen
O_2^-	superoxide radical
$ONOO^-$	peroxynitrite
PBS	phosphate buffered saline
PI3K	phosphatidylinositide 3-kinases
PKC	protein kinase
PUMA	p53 upregulated modulator of apoptosis
RNS	reactive nitrogen species
ROS	reactive oxygen species
RT-PCR	real-time polymerase chain reaction
SEM	standard error of mean
SIRT1	NAD-dependent deacetylase sirtuin-1
SMAC/DIABLO	second mitochondria-derived activator of caspases
SOD-1	superoxide dismutase-1
STAT-3	signal transducer and activator of transcription 3
TBA	thiobarbituric acid
TBARS	thiobarbituric acid reactive substances
TBOOH	*tert*-butyl hydroperoxide

TNF-α	tumor necrosis factor-alpha
TNF-R1	tumor necrosis factor receptor
TRAIL-R1	tumor necrosis factor receptor superfamily member 10A
UDP	uridine diphosphate
UTP	uridine triphosphate

3 INTRODUCTION

Xenobiotic induced hepatotoxicity, such as those caused by medications, has been an ongrowing problem largely attributable to the increasing amounts of new drugs entering the market (Jaeschke *et al*. 2012, Rangnekar and Fontana 2011). Additionally, the growing number of xenobiotics found in our environment increases the chance of exposure of liver to these toxic substances. Growing scientific evidence attributes oxidative stress and increased reactive oxygen species production as the main mediators of pathology of both acute and chronic liver injury caused by xenobiotics. Oxidative stress can be induced by toxins as it causes accumulation of reactive oxygen/nitrogen species, further causing a misbalance in pro-oxidant/antioxidant steady state. Protein crossslinking, lipid peroxidation, mitochondrial dysfunction and induction of cell death pathways are some of the proposed mechanisms of cellular damage due to this misbalance (Jaeschke 2011).

Activation of nuclear transcription factors by hepatotoxins may be an important mechanism in liver cell defence and survival in toxic injury. Activation of transcription factor, such as antioxidant response element, leads to increased transcription of antioxidant enzymes including heme oxygenase-1, superoxide dismutase-1 and glutathione peroxidise. These antioxidant enzymes are important for maintaining a homeostatic redox state and their deficiency or over-expression may cause irreversible damage to the tissues of the body. One such example of cellular damage is a resultant increase in cytokine TNF-α level. This cytokine has been shown to induce apoptosis mediated by executive caspases and the members of Bcl-2 family proteins, Bid and Bax (Morgan *et al*. 2010, Van Herreweghe *et al*. 2010). Interestingly, enzymes such as nitric oxide synthase-2 exhibit both pro-oxidant and antioxidant characteristics, depending on the current cell conditions and may exhibit relationship with other antioxidant enzymes. Elucidation of the steps involved in this complex interaction between the oxidative stress and apoptotic mechanisms has yet to be determined. For this reason, studies on the oxidative stress mechanisms as pertaining to liver damage and clarification of exact mechanisms involved in different settings are of crucial importance. In addition,

complete understanding of the role of antioxidant system pathways as well as their inter-relationship and their modulation will be of great benefit.

Fortunately, in the recent years scientific field has seen a growth in research studies done on a genomic scale, which help further our understanding of the toxicity mechanisms. Specifically, the field of toxicogenomics involves identification of genomic biomarkers of toxicity through analysis of mRNA profiling. The information obtained from these molecular analyses discloses gene changes that have important effects on cell function, without necessarily exhibiting changes in the morphology of the cell. As well, there exists a possibility of induction of target genes by xenobiotics without involving any changes in the DNA sequence. These epigenetic effects include chromatin modifications such as alteration in histones, as well as post-transcriptional changes (Heijne *et al.* 2005). In the future, the combination of application of epigenetics, genomics and proteomics will lead to more accurate assessments of the risks associated with xenobiotic exposure.

Substances isolated from natural sources have been used traditionally in the pharmacotherapy of various diseases, as they are relatively inexpensive and widely available. Silymarin and reservatrol are some of many examples of natural substances that have shown a strong hepatoprotective potential due to their antioxidant, anti-inflammatory and liver regeneration capabilities (Pradhan and Girish 2006, Glauert *et al.* 2010). On the other hand, substances such as curcumin and quercetin have shown to exhibit antioxidant and cytoprotective properties, but their use as hepatoprotectants has not been extensively investigated (Zhou *et al.* 2011, Chirumbolo 2010). The need for seeking other novel potential hepatoprotective substances is important in regards to the aim to reduce the occurrence of xenobiotic mediated liver injury. Conversely, the hepatotoxic potential of natural supplements should also be taken into account.

This research work will try to encompass all of these before mentioned concepts with the overall goal to evaluate mechanisms of xenobiotic induced hepatotoxicity as well as to seek and assess novel potential hepatoprotective substances.

3.1 Hepatotoxicity

Liver is a vital organ responsible for the detoxification and clearance of majority of xenobiotics that enter the body. Due to its high blood perfusion rate and high metabolic capacity, liver is continuously exposed to high levels of xenobiotics and to their reactive metabolites. Fortunately, this organ has a high capacity of regeneration and ability to repair any underlying damage. Generally speaking, hepatotoxicity occurs when liver regeneration capabilities are exhausted and cell damage ensues. Hepatic injury due to xenobiotic exposure involves inflammatory, oxidative stress and liver peroxidation reactions that can result in mitochondrial failure and cell death. The resulting pathological alterations of the endogenous substances such as proteins, nucleic acids, and lipids can harm the proper functioning of the liver (Hong *et al* 2009).

It is well established that the oxidative stress and apoptotic cell death pathways play the most important role in the underlying mechanisms of xenobiotic hepatocyte injury (Jaeschke *et al* 2012, Kaplowitz 2000). However, elucidating the molecular and cellular mechanisms mediating these two pathways is important for predicting preventable hepatotoxicity caused by xenobiotics. The following sections summarize these two important pathways and their mediators in greater detail.

3.1.1 *Oxidative stress: Pro-oxidant and Antioxidant Parameters*

Oxidative stress is described as a state of an imbalance between the production of reactive oxygen and nitrogen species, and the ability of the body's detoxifying mechanisms to remove these reactive species and repair the consequent cell damage (Ha *et al.* 2010). At states of severe oxidative stress, cytoprotective abilites of the antioxidant system are exhausted, where the accumulating amounts of reactive oxygen and nitrogen species cause extensive damage to cell lipids, proteins and DNA (Gu and Manautou, 2012).

Reactive oxygen species (ROS) are byproducts of reactions of the electron transport chain occuring at the inner mitochondrial membrane under normal physiological conditions. These molecules play an important role in cell redox signaling,

where a balance between their production and consumption is maintained (Mari *et al* 2010). During the electron transport chain, majority of the available molecular oxygen molecules accept electrons from the cofactors such as NADH and FADH2 in turn being reduced to water. However, a small amount of electrons can exit the respiratory chain and directly convert oxygen into a highly reactive superoxide molecule. This molecule can in turn react with reactive nitrogen species, such as nitric oxide, to form a powerful oxidant peroxynitrite that is capable of attacking tyrosine residues of proteins and inducing structural and functional changes (Ha *et al.* 2010). The resulting protein-protein crosslinking causes a permanent loss of protein function and its degradation. Furthermore, the accumulating intracellular ROS cause oxidative degradation of lipids, potentiating a free radical chain reaction where free radicals attack cell membranes and impose cellular damage (Gu and Manautou 2012).

Fortunately, the cell contains several antioxidant enzymes including superoxide dismutase, catalase, glutathione peroxidase and heme oxygenase among may others, that are able to detoxify these highly reactive species and stabilize the intracellular equilibrium. Upon small imbalance in the normal redox state, cell‘s redox-sensing system is able to detect the oxidative stress and induce the anti-oxidant defense mechanisms. This cellular response to oxidative stress occurs at the transcriptional level, where the antioxidant response element is responsible for the enhancement of expression of antioxidant enzyme genes and restoration of the previous redox balance (Jaeschke 2011, Scandalios 2002).

In the case of xenobiotic induced liver injury, the cellular antioxidant enzymes‘ detoxifying capacities may be exhausted resulting in intracellular accumulation of reactive oxygen and nitrogen species. These molecules may specifically target mitochondria to cause structural alterations of mitochondrial proteins, leading to permeability transition pore (MPT) formation. This may lead to disruption of mitochondrial membrane potential, halting of of ADP synthesis and nuclear DNA fragmentation all leading to initiation of necrotic cell death. Additionally, the release of proapoptotic factors such as cytochrome C can result in caspase activation and apoptosis (Jaeschke *et al*. 2012). Furthermore, the inflammatory response may be initated during the xenobiotic insult and it involves mediators such as the resident macrophages of the

liver, Kupffer cells, and neutrophils. The inflammatory response could further be amplified upon additional xenobiotic insult and/or due to necrotic and apoptotic cell death (Jaeschke 2011). All of the above mentioned alterations in the redox state of the cell and the induction of cell death pathways together trigger the miryad of pathophysiological processes which could be detrimental to the proper functioning of the liver. (Gu and Manautou 2012, Malhi and Gores 2008).

The following sections will summarize in greater detail the current knowledge of parameters involved in oxidative stress response that are part of our research interest and their involvement in regulation of hepatotoxicity

3.1.1.1 Heme Oxygenase-1 and Bilirubin

Heme-oxygenase (HO) is an essential antioxidant enzyme that catalyzes the degradation of heme in NADPH dependent oxidation reaction to produce carbon monoxide (CO), biliverdin and iron (Fe^{2+}) (Otterbein *et al.* 2000). Biliverdin is then subsequently converted into yellow product bilirubin by the enzyme biliverdin reductase.

The net reaction is as follows:

$$\text{Heme} + 3O_2 + 3½\text{NADPH} + 3½H^+ + 7e^- \rightarrow \text{biliverdin} + Fe^{2+} + CO + 3½\text{NADP}^+ + 3H_2O$$

The following figure (Fig. 1) clearly illustrates the heme degradation pathway by the enzyme heme oxygenase 1 and the major properties of its products.

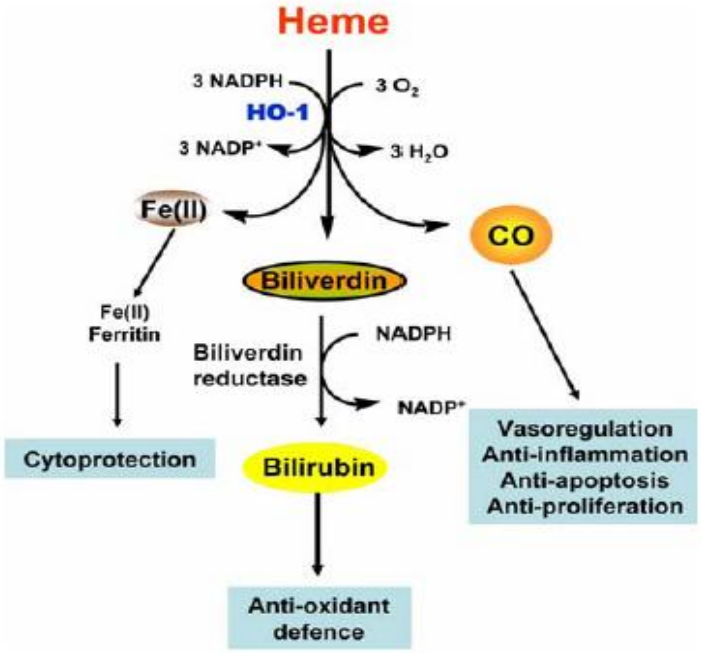


Fig. 1. *Schematic representation of HO-1 activity. HO-1 degradation of heme into products biliverdin (subsequent conversion to bilirubin by biliverdin reductase), CO and iron. Sequestration of iron by ferretin. Bilirubin and CO have wide variety of cytoprotective effects. (Source: Farombi et al. 2006)*

So far, three isoforms of this enzyme have been discovered, the inducible HO-1 (also known as heat shock protein 32) and constitutive isoforms HO-2 and HO-3. Spleen is the organ with the highest concentration of HO-1, while HO-2 is found mainly in the brain and testes, as well as other tissues in lower levels. However, not much is known about the function of HO-3 and this enzyme is actively investigated (Lingyun and Wang, 2005).

In the liver HO-1 is ubiquitously expressed and is activated in response to stimuli such as oxidative stress, hypoxia, heavy metals, NO and NO donors and inflammatory cytokines (IL-1, IL-6, IL-10, TNF-α) (Flores *et al.* 2005, Ryter *et al.* 2006, Song *et al.* 2003). Induction of HO-1 activity is under extensive regulation by several intracellular molecules and enzymes. As seen in the figure 2, the upstream signaling kinases that regulate HO-1 transcription include extracellular signal-regulated protein kinase (ERK), c-Jun N-terminal kinase (JNK), p38 MAPK, protein kinase (PKC), protein kinase A

(PKA) and phosphatidyl inositol 3-kinase (PI3K). Several transcription factors are also involved in HO-1 induction, including activator protein-1 (AP-1), nuclear factor-κB (NF-κB), nuclear E2-related factor -2 (Nrf2) and electrophile/stress response elements. In physiological conditions, these before mentioned transcription factors and protein kinases are located in the cytosol. In response to external stimuli, such as oxidative stress, they enter the nucleus to regulate HO-1 transcription by binding to the specific DNA sequence of the promoter of HO-1 gene (Farombi *et al.* 2006, Paine *et al.* 2010). It is important to note that the upstream kinases and transcription factors regulating the HO-1 activity are the active targets of many potential cytoprotective agents that are extensively investigated nowdays (Ryter and Choi 2009, Sun Jang *et al.* 2009). For example, hepatoprotective properties of curcumin may be attributed to enhanced activation of Nrf2 and consequent induction of antioxidant response element (ARE) which results in upregulation of the antioxidant enzyme HO-1 (Farombi *et al*. 2008).

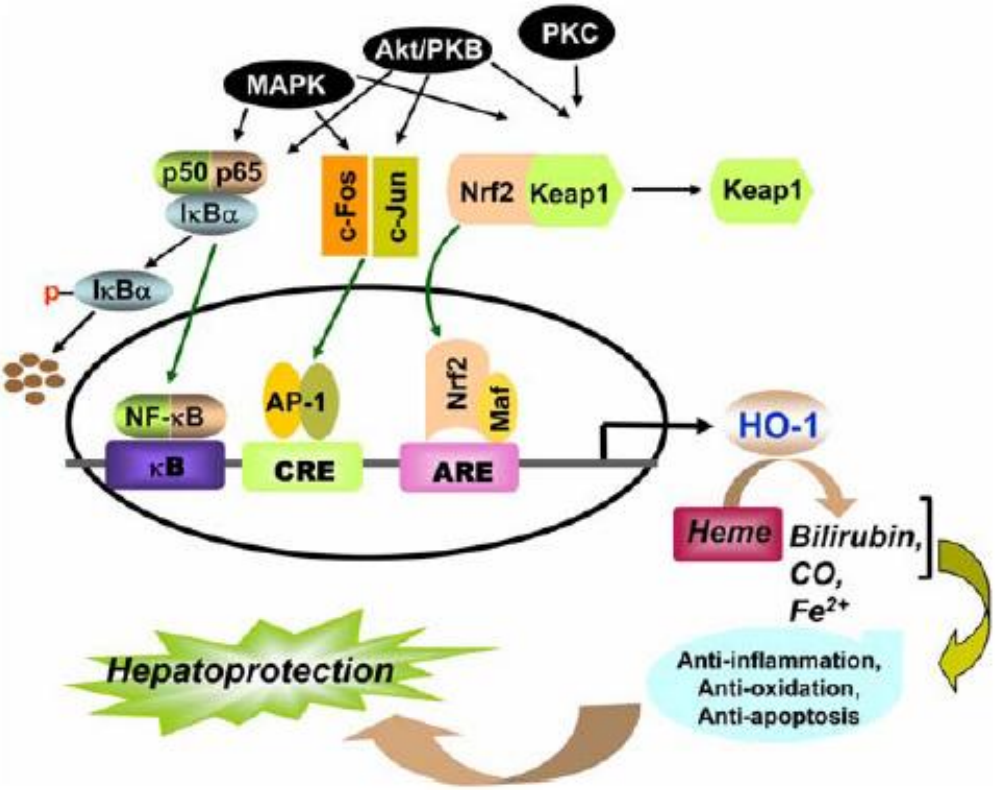


Fig. 2. Regulation of HO-1 is under control of several transcription factors and upstream kinases. (Farombi et al. 2006)

Increased activity of HO-1 plays an active role in protective mechanisms of the cell upon induced liver damage. The exact molecular mechanism of HO-1 cytoprotection is incompletely understood. Nevertheless, products biliverdin/bilirubin and CO are currently thought to be the key mediators of HO-1 mediated cytoprotection as they help restore intracellular homeostatic balance under oxidative stress conditions (Paine *et al.* 2010). For instance, in platelets CO has anti-aggregating function through the decreases in thrombotic formation and subsequent inflammatory damage. When applied exogenously at low concentrations CO mimics the cytoprotective potential of HO-1 (Wen *et al.* 2012). In the damaged liver, rising levels of CO enhance anti-inflammatory function of macrophages and inhibit production of pro-inflammatory cytokines, through a mechanism involving modulation of p38 MAPK (Bauer and Pannen 2009).

The other products of heme degradation pathway, biliverdin and bilirubin, are some of the most potent endogenous antioxidants known. These molecules exhibit anti-proliferative and anti-inflammatory properties. It was shown that an increase in bilirubin/biliverdin levels has a potential to ameliorate hepatic ischemia reperfusion injury and reduce hydrogen peroxide damage (Vardanian *et al*. 2008). Furthermore, at low physiological levels, unbound conjugated bilirubin scavenges peroxyl radicals and prevents oxidation of fatty acids and proteins. Other beneficial protective effects of bilirubin have been shown in the cardiovascular system, where bilirubin prevents oxidation of low-density lipoproteins thus reducing the risk of atherosclerosis. A meta-analysis of clinical studies has proven an inverse relationship between plasma bilirubin levels and the severity of atherosclerosis (Novotny and Vitek, 2003). Similarily, the individuals who have higher than normal bilirubin levels (eg. Gilbert syndrome patients) tend to have a lower risk of developing ischemic heart disease. Recent findings have also linked bilirubin and biliverdin as scavengers for reactive oxygen species (ROS) as well as reactive nitrogen species (RNS), thus counteracting the induced oxidative stress and resulting cellular damage (Kapitulnik and Maines 2009). It must be stressed that despite numerous cytoprotective effects of these two powerful antioxidants, their beneficial effects are exceeded at their higher serum levels. High levels of unbound bilirubin can cause cytotoxicity targeting mainly the central nervous system. The end result is the

condition called bilirubin encephalopathy where motor, sensory and cognitive functions are impaired (Kapitulnik 2004).

The third product of HO-1 catalyzed reaction is Fe^{2+}, which is considered cytotoxic even at low concentrations due to its ability to produce hydroxyl radicals. However, upon the HO-1 induction the level of Fe^{2+} sequestering protein ferritin is also increased. This protein acts as an additional antioxidant and cytoprotectant by actively pumping Fe^{2+} out of the cell (Immenschuh *et al* 2010).

Overall, induction of HO-1 pathway has been shown to have cytoprotective characteristics. Studies dealing with acetaminophen toxicity have demonstrated that a pretreatment with HO-1 inducer (hemin) prevented hepatotoxicity, while application of the HO-1 inhibitor (protoporphyrin) worsened the damage (Bauer and Bauer 2003). Considering the hepatoprotective properties of HO-1 induction, modulation of its activity is of great value in battling disease states that involve extensive inflammation and oxidative stress. New research focus is geared towards finding pharmaceuticals that may induce HO-1 therapeutically and thus amelliorate human pathological conditions.

3.1.1.2 Nitric Oxide Synthase-2 and Nitric Oxide

Nitric oxide synthase (NOS) is a complex enzyme that converts molecular oxygen (O_2), arginine and NADPH into the products nitric oxide (NO), citrulline and $NADP^+$ as shown in the equation below.

L-arginine + 3/2 NADPH + H^+ + 2 O_2 → citrulline + NO + 3/2 $NADP^+$

This enzyme requires five cofactors including flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), tetrahydrobiopterin (BH_4), iron protoporphyrin IX (heme) and calmodulin (CaM), to carry out the catalytic conversion (Fig. 3) (Alderton *et al.* 2002).

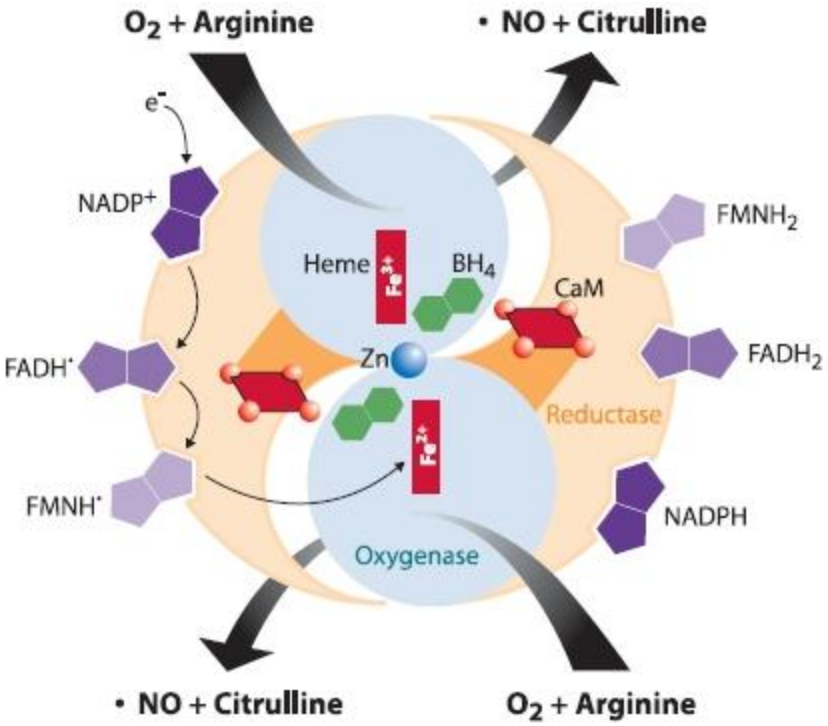


Fig. 3. *Nitric oxide synthesis by nitric oxide synthase and its co-factors (Source: Cayman's Nitrate/Nitrite Colourometric Assay Kit booklet).*

Up to date, there are three forms of NOS recognized. These include two types that are constitutively expressed, one in neurons (NOS-1 or nNOS) and the other in endothelial cells (NOS-3 or eNOS). Other cells including macrophages, fibroblasts, Kuppfer cells and hepatocytes express the NOS type 2 (NOS-2 or iNOS); the isoform that is inducible in response to inflammatory cytokines such as IL-1, TNF- α and INF-γ.

In the liver, NOS-3 is uniformly distributed and the regulation of NO production by this constitutive isoform is highly dependent on the fluctuating intracellular calcium levels. This maintains the adequate perfusion of the organ and cell integrity.

In the healthy liver tissue, NOS-2 is localized in the periportal zone of the liver acinus. Upon cell damage (infection, hepatitis, endotoxemia etc.) the activity of NOS-2 is upregulated at the transcriptional level and its distribution is localized to the areas of liver injury (McNaughton *et al.* 2002). The resulting amplification of the NO levels results in complex interactions with a variety of molecules. This can lead to alteration of function

of key target molecules and pathways, that can be either beneficial or harmful to the cell. The differing roles of NO is thought to depend on the general redox balance of the cell, source of the NO production and type of cellular damage (Chen *et al*. 2003, Ryter and Choi 2007, Lingyun and Wang 2005).

At physiological conditions, nitric oxide is a short lived free radical that mediates most of its functions by binding to the heme group of the enzyme guanylate cyclase resulting in its activation and consequent increase in the levels of cGMP (Martinez-Ruiz, 2011). In pathological conditions, there are several different proposed mechanisms of NO in liver injury. This small molecule tends to react with molecular oxygen (O_2) to produce several damaging reactive nitrogen species, such as the dinitrogen trioxide (N_2O_3) and a strong pro-oxidant peroxynitrite ($ONOO^-$) (Espey *et al.* 2002). Dinitrogen trioxide undergoes lipid peroxidation reaction with lipids and it reacts with endogenous amines to form carcinogenic N-nitrosamines. As well, N_2O_3 can undergo S-nitrosation reactions, where it reacts with cysteine portions of proteins to modify their function. One such example is interaction of N_2O_3 with cysteine residues of active sites of caspases which results in their inhibition and thus modulation of apoptotic activity (Vodovotz *et al.* 2004). On the other hand, peroxynitrite oxidates DNA, lipids and proteins resulting in modification of biological function that has been observed in conditions such as liver ischemia and reperfusion injury (I/R), hemorrhagic shock, endotoxemia, acetaminophen toxicity and alcoholic hepatitis (Vardanian *et al.* 2008).

In addition to direct contribution to tissue damage by formation of reactive nitrogen species, NO generated by NOS-2 also upregulates the inflammatory response through specific signaling mechanisms. In oxidative stress, increased level of inflammatory cytokines, such as TNF-α, leads to generation of NO and further amplification of the inflammatory damage (Fonseca *et al.* 2003). Furthermore, NO reversibly competes with O_2 for the binding site on cytochrome oxidase to inhibit its activity and ensuing production of ATP (Cooper 2003). Because of its ability to bind to heme group, this molecule can also alter the activity of enzymes that contain this group, including cytochrome P450, NOS-2 and catalase.

On the contrary, there have been several studies indicating cytoprotective effect of NO and thus challenging the claim that this molecule is toxic to the cell. Studies on liver

regeneration have shown increased activity of NOS-2 results in decrease of TNF-α, increase of Nf-kB, inhibition of Bid cleavage, inhibition of Cyt C release and induction of HSP70, which together demonstrate potential anti-apoptotic properties of this molecule (Muntane *et al.* 2010, Want *et al.* 2002). Upregulation of the antioxidant enzyme hemoxygenase-1 by higher NO levels is thought to occur through the activation of the Nrf2/ARE complex. The consequent increase in production of carbon monoxide and biliverdin, have exhibited a potential cytoprotective mechanism of NO by increasing antioxidant and anti-apoptotic defense systems of the cell (Liu *et al.* 2007).

Despite much of the conflicting data, it is presently believed that there exist a fine balance between the detrimental and beneficial effects of NO that is largely dependent on the current oxidant status of the cell. The actual role of NO and NOS-2 within the liver as pertaining to hepatotoxicity and hepatoprotection mechanisms continues to be actively investigated. The pharmacological strategies aimed at selectively enhancing the protective and reducing the deletirious effects of NO offer possibilities of beneficial therapeutic potentials.

3.1.1.3 Antioxidant Enzymes: Superoxide Dismutase, Catalase and Glutathione Peroxidase

Generation of reactive oxygen species is highly deleterious to the proper functioning of the cell. These potent molecules can oxidize physiological substances including proteins, lipids and nucleic acids, thus modifying their functions that may result in cell death. The antioxidant enzymes superoxide dismutase (SOD), glutathione peroxidase and catalase work together to remove reactive oxygen species from the cell in order to prevent oxidation of biological material (Yuan and Kaplowitz 2008). In the normal physiological state, small percentage of molecular oxygen undergoes incomplete reduction to form the superoxide anion. The enzyme SOD then reduces the superoxide anion into hydrogen peroxide, which is further reduced to water by the actions of catalase and glutathione peroxidase (Valdivia *et al.* 2009).

Under normal physiological conditions the pathways of these three antioxidant enzymes are complemented by the additional hepatocellular antioxidants, glutathione

(GSH), vitamin E and ascorbate. However, upon the exposure to hepatotoxic substances the capacity of these protective pathways may be exceeded. Reactive oxygen species may in turn cause peroxidation of the phospholipids bilayer, induction of the permeability transition pore formation and the release of cytochrome-C, with the consequent activation of cell death pathways (Yuan and Kaplowitz 2008).

The modulation of the antioxidant potentials of these three important enzymes by pharmacological substances as well as their involvement in hepatoxicity is under active investigation. Each of these three important antioxidant enzymes are discussed in greater detail in the following sections.

3.1.1.3.1 Superoxide Dismutase

Superoxide dismutase 1 (SOD1), or also known as a copper-zinc dismutase (Cu-Zn-SOD), is a member of superoxide dismutase family of enzymes. This enzyme destroys free superoxide radicals by binding copper and zinc ions in the presence of its Cu/Zn co-factors. SOD1 is located in the cytoplasm and mitochondrial intermembrane and it catalyzes the conversion of superoxide radicals into molecular oxygen and hydrogen peroxide as seen below (McCord and Fridovich 1969)**.**

$$O_2^{\cdot -} + O_2^{\cdot -} + 2H^+ \xrightarrow{SOD} H_2O_2 + O_2$$

SOD is a cytoprotective enzyme as it overcomes all the deleterious effects of superoxide radical (O_2^-) that undergoes reactions with itself or with other biological radicals (ROS, RNS) or metals. Interestingly the deletion of SOD1 gene causes a famililal amyotrophic lateral sclerosis (Lou Gehrig's disease) a form of motor neuron degenerative disease. As well, the depletion of this enzyme in the liver has been linked to carcinogenesis in viral liver disease (Inagaki *et al.* 1992). Similarly, the over expression of SOD1 is linked to neuronal disorders that are seen in Down syndrome patients.

The high antioxidant potential of SOD1 makes this enzyme a target for modulation of its activity in diseases involving oxidative stress. Exogenous application of SOD has been recently shown to reduce oxidative stress in diabetic rats and in those with inflammatory bowel disease (Di Naso *et al*. 2011, Segui 2004). Modulation of SOD-1

activity by natural substances such as flavonoids enhances the antioxidant and cytoprotective potential of this enzyme, thus making it an attractive target for studies on hepatoprotection (Puiggros *et al.* 2009).

3.1.1.3.2 Catalase

The final end-product of enzymatic reaction catalyzed by SOD, hydrogen peroxide (H_2O_2), is a deleterious to the cell and it needs to be rapidly converted into the less dangerous molecule. This is accomplished by the two important antioxidant enzymes, catalase and glutathione peroxidase, which are responsible for reduction of H_2O_2 into water and oxygen as seen below

$$2\ H_2O_2 \rightarrow^{CAT/GPx} \rightarrow 2\ H_2O + O_2$$

Catalase is located intracellularly and it is widely distributed throughout the body with the highest concentration found in the liver. The structure of catalase is composed of four polypeptide chains and a heme group that reacts with hydrogen peroxide (H_2O_2). In addition to reducing H_2O_2, this enzyme is able to oxidize various toxins including alcohols, consuming the H_2O_2 in the process. Any molecule containing the heavy metals is a potent inhibitor of this enzyme, due to its interaction with the heme group. In the liver, presence of catalase in mitochondria has shown to have important implications in prevention of reactive oxygen species generation and consequent activation of pro-apoptotic pathways induced by the toxin TNF-α (Salvi *et al.* 2007). Thus modulation of these pathways may have beneficial effects on treatment of inflammatory liver conditions such as hepatitis.

3.1.1.3.3 Glutathione Peroxidase and Glutathione

As before mentioned, the antioxidant enzyme glutathione peroxidase (GPx) is another enzyme responsible for the detoxification of hydrogen peroxide and thus has an adequate capacity to prevent deletirious oxidation of other cellular molecules by this

molecule. There are eight different isoenzymes of GPx, of which GPx1 is the most abundant type. This isoenzyme is widely distributed in the cytoplasm and mitochondria of most cells and its overexpression has been shown to increase protection against oxidative stress in animal models (Handy *et al.* 2009). As well, GPx1 helps prevent lipid oxidation by converting lipid hydroperoxides into the alcohol derivatives that are less toxic.

The antioxidant potential of GPx involves the use of reduced glutathione (GSH) molecule to oxidize hydrogen peroxide resulting in generation of oxidized glutathione (GS-SG) and water.

$$2GSH + H_2O_2 \rightarrow^{GPx} \rightarrow GS–SG + 2H_2O$$

This cycle is then completed by the action of glutathione reductase (GSR) that reduces the oxidized glutathione and replenishes the GSH levels.

$$GS–SG + NADPH + H^+ \rightarrow^{GSR} \rightarrow 2\ GSH + NADP^+$$

Reduced glutathione is a powerful antioxidant that protects hepatocytes from oxidative injury by scavenging reactive oxygen and nitrogen species. As well, GSH helps clear xenobiotics from the body acting as a conjugate in reactions catalyzed by glutathione S-transferase. Furthermore, GSH is able to reverese oxidative modifications due to its capability to remove disulfide bonds and nitrothiols from proteins. Thus, a homeostatic decrease in the GSH pool can make cells more vulnerable to further damage by toxins, as seen in conditions involving inflammation, infection or any intracellular disbalance where the production of ROS is greatly increased (Ballatori *et al*. 2009). The resulting depletion of GSH levels causes a disruption of the proper functioning of the cell by inducing the mitochondrial permeability transition pore opening (Mari *et al*. 2010). This event makes the cell more susceptible to the oxidative stress and apoptotic processes. Depletion of GSH can also occur due to acute exposure to pro-oxidants whose electrophilic metabolites conjugate GSH, as is the case with *N*-acetyl-*p*-benzo-quinone imine (NAPQI) metabolite in acetaminophen induced hepatotoxicity. Once GSH is depleted, NAPQI begins attacking cysteinyl residues of cellular proteins, modifying their

function and causing cellular dysfunction that may result in acute liver failure (Saito *et al* 2010).

Overall, decreased levels of GSH greatly increase the potential risk of oxidative damage due to limitation of the GPx activity. Conversely, the elevated levels of GSH increase the antioxidant capacity and resistance to oxidative stress. These processes have been implicated in pathology of liver diseases and further investigation on this potent endogenous antioxidant is needed.

3.1.2 *Apoptosis*

Cell death is part of the normal physiological function, important for cell renewal and elimination of non-functional cells. The process of apoptosis is a form of cell death that is programmed and highly regulated. It is characterized morphologically by rounding of the cells, shrinkage of cytoplasm, plasma blebbing, condensation of chromatin and nuclear fragmentation (Elmore 2007). The dying fragmented cell is enclosed into the membrane bound vesicles (apoptotic bodies), which are further engulfed by the local macrophages. The presence of apoptotic bodies is one of the main features that distinguishes apoptosis from other form of cell death, necrosis. Furthermore, in pathological conditions constant apoptosis results in inflammatory reactions due to lysis of apoptotic bodies and consequent release of the inflammatory cytokines (Elmore 2007). In the liver, chronic stimulation of apoptosis and accompanying inflammation have been attributed to several hepatic diseases including fulminant hepatitis, cholestatic liver disease, viral hepatitis and liver cancer (Guicciardi and Gores 2005, Hacker *et al*. 2011). The mechanisms of initiation and mediators of apoptosis, that are of interest in this research study, are further described in greater detail.

3.1.2.1 Apoptotic Pathways: Involvement of Caspases

Apoptosis involves activation of specific molecular cell death pathways that are tightly controlled and can be triggered by various stimuli. Currently, there are two main pathways attributed to apoptosis. These include the extrinsic pathway, that involves

stimulation of the death receptors found on the plasma membrane, and the intrinsic pathway that focuses on the mitochondrial dysfunction (Fig. 4). Furthermore, there is an additional pathway that involves T-cell mediated cytotoxicity and perforin-granzyme dependent apoptosis. All of these three pathways influence each other and merge on the common end pathway termed the extrinsic pathway. This final pathway involves processes of activation of intracellular proteases such as caspases (cysteine-dependant aspartate-directed proteases) and endonuclases. Caspases require proteolytic cleavage at their aspartase residues to be activated as they are constitutively expressed as inactive proenzymes. As well, these proteases specifically cleave aspartate residues of their substrates and are able to activate each other in a cascade-like process resulting in the amplification of the damaging response. Caspases 2,8,9 and 10 are termed upstream caspases as they are activated by binding of adaptors, which results in self association and autocatalytic activation. Upstream caspases are responsible for activation of the downstream caspases, including caspase 3, 6 and 7. These caspases are unable to self associate and are the main cause of the degradation of intracellular components resulting in morphological changes typical for apoptosis (Hacker *et al.* 2011, Malhi and Gores 2008).

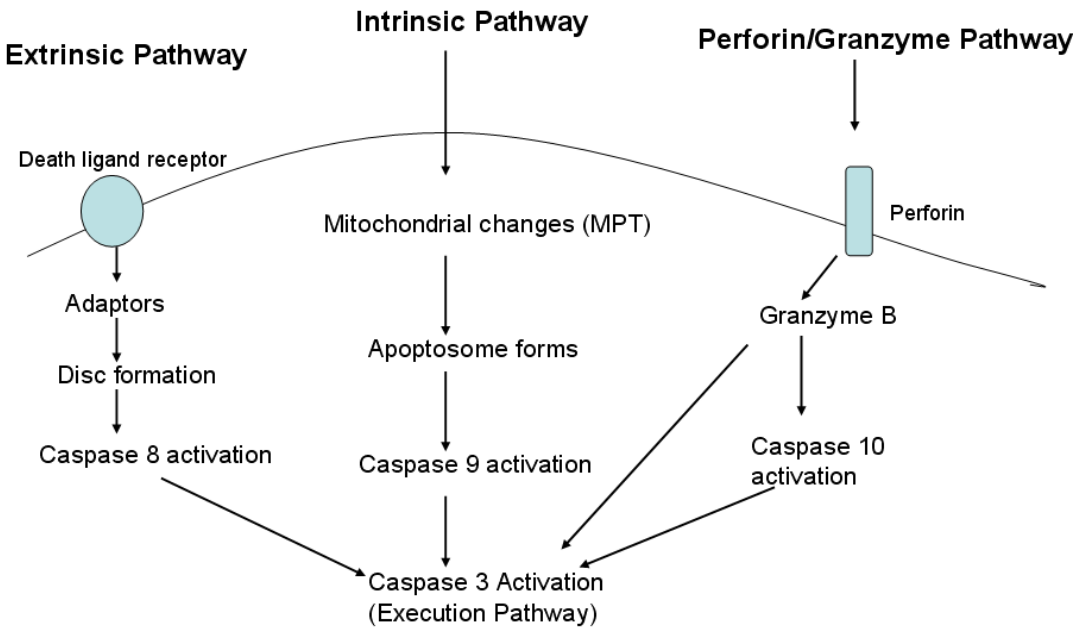


Fig. 4. *Schematic representation of apoptotic events (Smart and Hodgston 2008)*

Extrinsic pathway involves recognition of several death receptors at the plasma cell membrane, all of which are the members of the tumor necrosis factor (TNF) receptor gene superfamily. These include tumor necrosis factor receptor (TNF-R1), Fas/CD95, TRAIL-R1, TRAIL-R2 to name a few. Upon binding of the appropriate ligand, such as TNF-α, to their corresponding receptor induction of adapter proteins and proteases results in formation of intracellular death inducing signaling complex (DISC). This complex then activates caspases 8 further commiting the pathway to the cell death executioner pathway (Fulda and Debatin 2006).

On the other hand, various extracellular and intracellular signals such as irradiation, oxidative stress, toxins and xenobiotics are all capable of activating the intrinsic cell death pathway that involves mitochondrial dysfunction and is free of death receptor interaction. This pathway is characterized by the alteration of the mitochondrial membrane, formation and opening of the MPT, and the consequent release of mitochondrial proteins such as cytochrome c, SMAC/DIABLO, apoptosis inducing factor and endonuclease G. Formation of apoptosomes depends on release of cytochrome c due

to its binding to Apaf-1 and procaspase 9, leading to activation of the Caspase 9 and further downstream execution pathway (Malhi and Gores 2008). It must be noted that the intrinsic and extrinsic pathways are not independent of each other. The amplification of the apoptotic signal from death receptors may require the mitochondrial involvement and the two pathways are closely intertwined.

As previously mentioned, both extracellular and intracellular signaling pathways merge onto the common executioner pathway that is initiated by the activation of the execution caspases. These include caspase 3, 6 and 7 that function in cleaving polypeptide substrates such as endonucleases, cytokeratins, cytoskeletal and nuclear proteins among many others. Out of these executioner caspases, caspase 3 is the most important as it cleaves endonuclases, which in turn degrade chromosomal DNA and cause chromatin condensation. The formation of the apoptotic bodies can also be attributed to the action of the caspase 3, as it induces cytoskeletal reorganization and disintegration (Hacker *et al.* 2011). The uptake of the apoptotic cell bodies is the last step in the apoptotic cell death pathway.

3.1.2.2 Bcl Family of Proteins: Bid and Bax

Bcl-2 family of proteins are responsible for the control and regulation of the mitochondrial outer membrane permeabilization, governing the intrinsic pathway of apoptosis. This group of proteins includes members that are either pro-apoptotic (Bid, Bax) or anti-apoptotic (Bcl-2xL). Pro-apoptotic proteins act upstream at the level of mitochondria and determine whether the intrisic pathway is initiated. The pro-apoptotic proteins are further divided into mutli-domain Bax like proteins (Bax and Bak) and the BH3 only domain (Bid, Bad, Bim, Noxa and Puma), all of which are required for the execution of apoptosis (Garcia-Saez *et al.* 2010).

Bid interacts with Bax causing its insertion into the outer mitochondrial membrane. Bax then interacts with the voltage anion channel and facilitating its opening. As well, Bax and/or Bak are able to form a pore called mitochondrial apoptosis-induced channel, resulting in the release of cytochrome c, SMAC/DIABLO and other pro-apoptotic mitochondrial factors (Malhi and Gores 2008). This action triggers the

activation of caspases. Furthermore the anti-apoptotic Bcl-2 proteins are able to prevent Bax activation by Bid, due to sequestering of Bid. Growing evidence has shown that the tumor suppressor p53 is responsible for the upregulation of Bid expression . However, p53 is also able to directly interact with Bax, without involving any transcriptional changes. Additionally, Bid cleavage by the upstream caspase 8 results in cytochrome c release (Guicciardi and Gores 2005). Despite the abundance of the known facts regarding the pro-apoptotic Bcl-2 involvement in apoptosis, none of the pathways elucidative are definite and need to be further explored.

3. 2 Pharmacological Substances

3.2.1 ***Pharamacological Hepatotoxic Substances***

This research work on hepatotoxicity was focused on the following substances, which in experimental models can induce oxidative stress, apoptosis and fulminant hepatic failure.

3.2.1.1 *D-Galactosamine (D-GalN)*

Endogenously, D-Galactosamine (D-GalN) is a constituent of glycoprotein hormones, such as follicle stimulating hormone and lutenizing hormone. It is an amino sugar derived from galactose with the molecular structure shown below (Fig. 5).

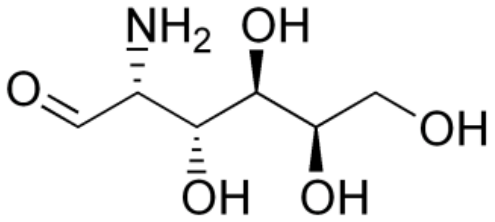


Fig. 5. *D-galactosamine*

This substance has a wide application in the experimental hepatology, where it is used as an experimental model of fulminant hepatic failure. Following an administration of high D-GalN concentrations, uridine becomes sequestered as uridine diphosphate (UDP)-GalN resulting in a decrease of uridine triphosphate (UTP) pool. The same enzymatic mechanism is responsible for transferring galactosyl moiety from UDP-galactose to proteins and its catalytic function diminishes in presence of increasing D-GalN concentrations. Consequently, the hepatocyte RNA and protein synthesis are severely affected until the supply of UTP can be replenished (Silverstein 2004, Stachlewitz *et al.* 1999).

D-GalN also induces reactive oxygen species (ROS) generation and cell death in hepatocytes. The release of inflammatory cytokines, such as tumor necrosis factor (TNF)-α, IL-1, IL-6 and IL-10 from Kupffer cells and activation of NF-κB has been shown to play the greatest role in mediation of cell death by both apoptotic and necrotic pathways (Choi *et al*. 2011, Gujral *et al.* 2003, Sun *et al.* 2003). Moreover, an increase in mitochondrial hyperpolarization and dysfunction leading to early generation of reactive oxygen species and induction of cell death in human hepatocytes upon D-GalN administration was reported (Gonzalez *et al.* 2009).

Involvement of induction of both oxidative stress and apoptosis by D-GalN cell injury makes this an excellent model for the study of these processes, as the exact mechanism of cellular damage in fulminant hepatic failure remains unclear.

3.2.1.2 *Lipopolysaccharide (LPS)*

The outer membrane of Gram-negative bacteria contains covalently bonded large molecules of lipid and polysaccharide, also known as lipopolysaccharide (LPS). This proteogylcan greatly contributes to the structural integrity of the bacteria and helps stabilize the overall structural membrane. The lipid portion of the molecule is a derivative of two N-acetyl glucosamine units and several amino acids attached to it. The conserved polysaccharide core contains simple sugars such as heptose, glucose and glucosamine (Fig. 6). The rest of the polysaccharide consists of repeating sugar units, also called the

O-antigen. This unit is highly variable among species, ensuring the protection of bacteria from the host antibodies.

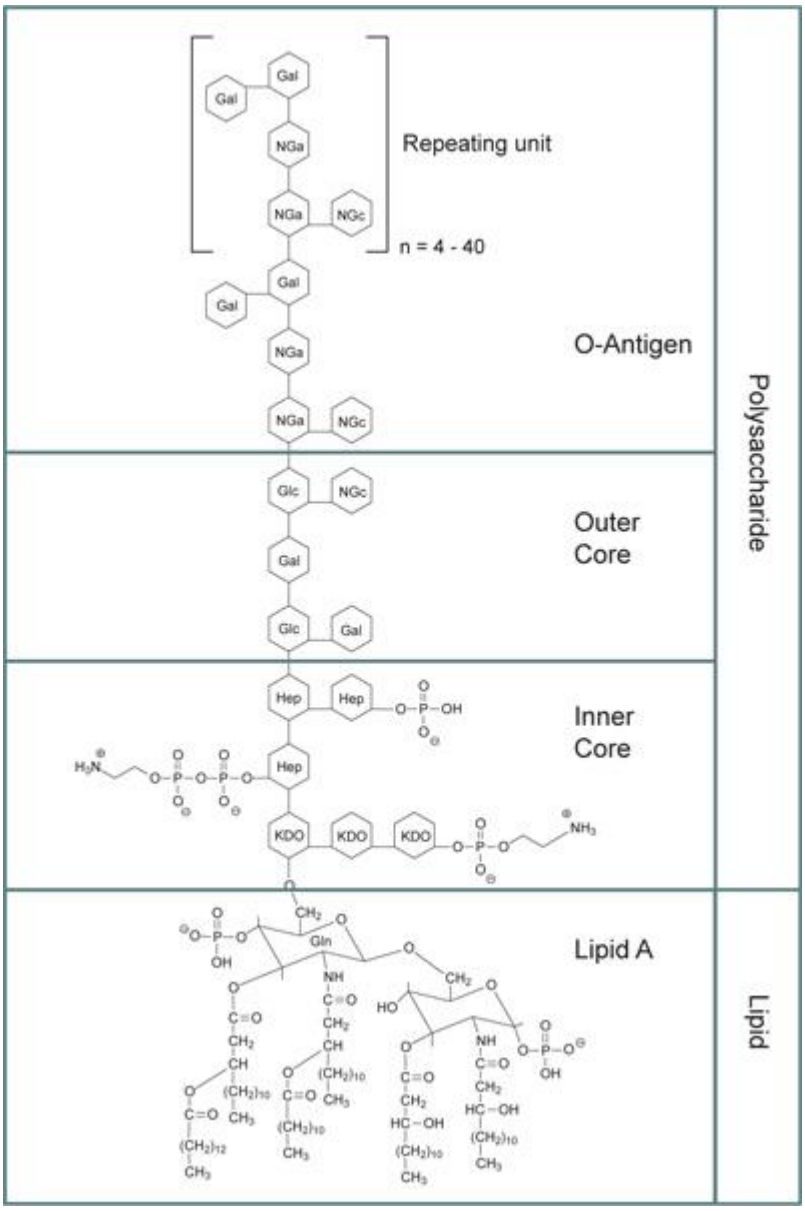


Fig. 6. *General structure for bacterial ipopolysaccharide (Source: Sigma Aldrich catalogue)*

In animals, bacterial LPS is responsible for eliciting a strong immune response, acting as an endotoxin and promoting secretion of several pro-inflammatory cytokines. Kuppfer cells, resident macrophages in the liver, are responsible for producing several cytokines, such as TNF- α and several interleukins. Clinically, it was shown that

endotoxin binding proteins are protective in severe alcoholic liver disease, by decreasing levels of TNF- α, IL-6 and IL-8 (Fukui 2005). Experimental studies in knockout mice have shown that TNF-α is the key mediator of LPS induced cell damage (Marino *et al*. 1997). Other mechanisms including increased nitric oxide production, increased caspase 3 activity and mitochondrial membrane destabilization have also been shown to play an important role in LPS hepatocyte damage (Chakravortty *et al.* 2001, Hamada *et al.* 1999, Nolan 2010).

Lipopolysaccharide in lower doses is often combined with D-GalN in experimental models of hepatotoxicity, to sensitize animals to TNF-α lethality. Interestingly, studies in knockout mice for two different TNF-α receptors (TNFR1 and TNFR2) have shown that at higher doses LPS lethality is not dependent on these two receptors, suggesting that at higher doses of LPS other mechanisms other than TNF-α are involved (Silverstein 2004).

Given the current knowledge regarding the postulated mechanisms of LPS hepatotoxicity, further studies are needed to assess the involvement of mediators other than TNF-α and interleukins.

3.2.1.3 Tert-Butyl hydroperoxide (t-BOOH)

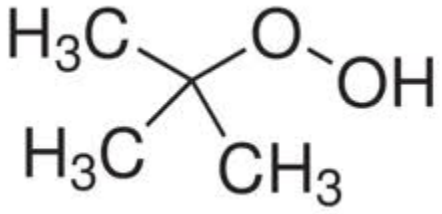


Fig. 7. *Tert-butyl hydroperoxide*

Tert-butyl hydroperoxide (*t*-BOOH) is a precursor of malondialdehyde and a membrane oxidant that has been extensively used as a model of oxidative stress in different systems (Fig.7). In hepatocytes, this molecule has been shown to reduce cell viability and cellular antioxidant status, increase intracellular ROS production and lipoperoxidation, activate NF-κB and caspase 3 pathways, alter Bcl-2/Bad ratio, reduce

mitochondrial membrane potential and increase cytochrome C release (Ghosh *et al*. 2011, Vidyashankar *et al.* 2010). These varying mechanism of action make t-BOOH a convenient hepatotoxicity model for studies of hepatoprotective potential of various antioxidant substances *in vitro* (Cerny *et al*. 2009, Kim *et al.* 2007, Mingcang *et al.* 2010).

3.2.2 *Potential Hepatoprotective Substances*

In this research work, the following phytochemicals were tested with regards to their hepatoprotective properties. The background information regarding each of these phytochemials as well as their role in amellioration of diseases is given in the following sections.

3.2.2.1 Curcumin

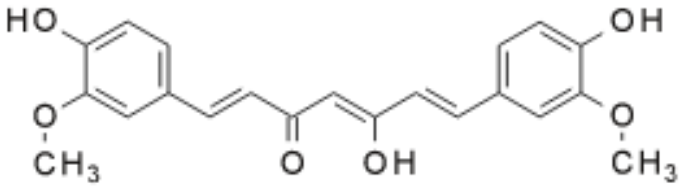


Fig. 8. *Curcumin*

Curcumin belongs to a class of natural phenols called curcuminoids that are isolated from the Indian plant *Curcuma longa* (Fig. 8). This phytochemical is highly consumed worldwide as a food colouring agent, food additive and it is a main component of the popular yellow spice turmeric.

For many years this substance has been largely reserved as a healing agent in much of the Indian and Chinese traditional medicine. Recently, however, curcumin has attracted attention of Western researchers due to its effective anti-cancerous, anti-oxidant and anti-inflammatory properties (Aggarwal *et al.* 2007, Darvesh *et al.* 2012). Currently this drug is undergoing clinical trials for amellioration of various cancers and neurodegenerative diseases, particularly Alzheimer's disease (Hatcher *et al.* 2008). Its

application in treatment of Alzheimer's disesase is based on its ability to inhibit myeloid plaque formation, as well it has been shown to improve cognitive abilities of the elderly in small scale populations (Ng *et al.* 2006, Sikora *et al.* 2010).

The exact mode of action of curcumin is under intense study, but is thought to involve several different molecular pathways including p53, STAT-3, CHOP, Nrf2, NF-κB, glutathione S-transferase, heme-oxygenase-1, cyclooxygenase-2 (COX-2) among many others. As an antioxidant substance, curcumin has been shown to be an effective scavenger of reactive oxygen and nitrogen species. Its action on inhibition of inflammatory and pro-oxidant mediator NF-κB, involves consequent inhibition of production of many pro-inflammatory cytokines, such TNF-α, IL-1β and IL-6 (Lima *et al.* 2011). Furthermore, this substance has been shown to trigger Nrf2/ARE signaling pathway, initiating activation of several important antioxidative enzymes, including heme oxygenase and sirtuins (Lima *et al.* 2011, Sikora *et al.* 2010). Experimental hepatotoxicity studies have also shown curcumin as an inducer of heme oxygenase-1 with concurrent involvement of nitric oxide (Farombi *et al.* 2008). This molecule thus pre-conditions the cell to increase its own antioxidant systems in combat of further oxidant challenge.

Despite its beneficial effects in treatment of several diseases, cytoprotective effects of curcumin have not been extensively studied in the liver. To date, curcumin has been shown to be effective in amelliorating livery injury by ethanol, thioacetamide, iron overdose and cholestasis in animal models (Rivera-Espinoza and Muriel 2009). However, further research in both experimental and clinical models is needed, as this agent shows a great potential as a therapeutic agent in amellioration of hepatic disorders.

3.2.2.2 Quercetin

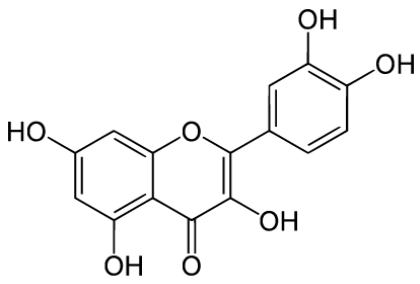


Fig. 9. *Quercetin*

Quercetin (Fig. 9) is a flavonol isolated from natural sources including fruits (apples, cranberries), vegetables (onion, broccoli), tea leaves (*Camellia sinensins*) and grains (buckwheat). By itself, quercetin is the aglycone portion of several other flavonoid glycosides, including rutin and quercitrin, where it is bound to sugars such as rhamnose and rutinose. These polyphenols are responsible for several health benefits of consuming fresh fruits and vegetables and are attractive focus of the current investigations.

Several studies have shown quercetin to posess anti-inflammatory, anti-oxidant, immunosuppressive and anti-cancerous properties involving several different mechanisms (Chirumbolo 2010). Cardiovascular studies have demonstrated that quercetin is a potent antioxidant and it prevents depletion of potent vasodilator NO by scavenging free O_2^- , thus lowering hypertension. Furthermore, quercetin has been shown to down-regulate p47 NOX subunit, increase eNOS and decrease NOX activity (Galleano *et al.* 2010). In small scale epidemiological studies done on hypertensive patients, quercetin improved blood pressure control and decreased oxidised LDL concentrations (Edwards et al. 2007, Egert *et al.* 2009).

Given the encouraging findings of quercetin as a potent antioxidant in cardiovascular studies, it is equally imporant to test this substance in its potential to amelloriate hepatic diseases. To date, a small number of studies have demonstrated quercetin's antioxidant potential in experimental studies on hepatocytes. For example, in hepatocytes quercetin was able to modulate nitric oxide levels by downregulating Nf-kB (Bharrhan *et al.* 2012, Jo *et al.* 2008). Furthermore, quercetin increases antioxidant

capacity of the hepatocytes, decreases pro-oxidant and inflammatory mediators, as well it modulates expression of several antioxidant genes (Ghosh *et al.* 2011, Weng et al. 2011, Zhao *et al.* 2011).

However, further research is needed to test the hepatoprotective potential of this potent antioxidant flavonol and to clarify its exact mechanisms of protection.

4 RESEARCH AIM & HYPOTHESIS

Understanding the exact mechanism of xenobiotic hepatotoxicity is one of the major challenges hepatologists are faced with today. Several mechanisms have been shown to play the major role in liver toxicity, including oxidative stress and apoptosis. Despite the fact that these processes have been extensively studied in the past, further research is needed to clarify their involvement in different hepatotoxicity models. Moreover, agents that have potential hepatoprotective properties have shown promising initial results in amellioration of hepatic diseases and further research on these substances is of great value.

It can be hypothesized that during the events of oxidative stress, there will be an increase in gene expression of both antioxidant (eg. HO-1, SOD-1, GPx, Cat) and pro-oxidant (eg.NOS-2) enzymes. Extensive oxidative stress will lead to activation of cell death mediators (eg.Bid, Bax, Casp3) and inflammatory cytokines (eg.TNF-alpha).

Furthermore, agents which are able to alter expression of anti-/pro-oxidant enzymes and apoptotic mediators have a potential in modulation of hepatotoxicity. Administration of hepatotoxic substances will result in increase in oxidative stress, lipid peroxidation and apoptosis. However, agents with potential hepatoprotective properties will increase expressions of antioxidant enzymes, decrease expressions of pro-oxidant enzymes and decrease levels of pro-apoptotic and inflammatory mediators.

Thus, the aim of this research work is:

- To elucidate some underlying molecular mechanisms of xenobitic hepatotoxicity in the given *in vitro* (Tert –butyl hydroxyperoxide) and *in vivo* (D-GalN/LPS) models, in regards to the involvement of oxidative stress and apoptotic cell death pathways.

- To evaluate the activities of specific antioxidant enzymes (HO-1, SOD-1, GPx, CAT), pro-oxidant enzyme (NOS-2), the inflammatory cytokine

TNF-α and apoptotic mediators (Bid, Bax, Casp3) involved in the given hepatotoxicity models

- By evaluating gene expression to see whether measurable changes in gene expression are early signals of toxicity and if this takes place before the gene is translated into a protein (confirmation by western blot and biochemistry).

- To see whether there exists a potential mutual relationship between the given parameters. If there is an increase in gene expression of cytotoxic mediator whether there is a corresponding increase in antioxidant genes expressions.

- To assess the relationship between oxidative stress and apoptosis.

- To evaluate the potential hepatoprotective properties of the natural substances, curcumin and quercetin, as pertaining to their abilities to ameliorate oxidative stress and apoptosis.

5 METHODS

5.1 Animals and Experimental Design

5.1.1 *Animals*

This study was performed on male Wistar rats of 200–300 g body weight obtained from Velaz-Lysolaje, Czech Republic. They were given water and standard granulated diet ad libitum and were maintained under standard conditions; light (i.e. 12 h light and 12 h dark); temperature (22 ± 2ºC); relative humidity (50 ± 10%). All rats received humane care according to the general guidelines and approval of the Ethical Committee of the First Faculty of Medicine, Charles University in Prague.

5.1.2 *Rat Hepatocyte Isolation*

For the purposes of *in vivo* experiments rats were injected intraperitoneally with a dose of specific substance of interest (eg. D-GalN, curcumin) dissolved in the vehicle of choice (eg.dimethyl sulfoxide, physiological solution). The number of animals was kept constant for each of the tested group. The rats were killed at twenty four hours following injection under light ether anasthesia and the blood samples were collected. For the purposes of liver extraction, rats were injected with heparin into the vena cava, followed by liver perfusion with specific buffer for several minutes. Subsequently, hepatocytes were isolated using the standard two-phase colagenase perfusion method that allows for disruption of intercellular connection in liver tissue. Purified hepatocyte suspension was obtained by centrifugation. The total count of cells using Burker chamber cells in one mililiter homogenous suspension was performed. Next, a small amount of hepatocytes were stained by trypan blue solution for measurement of total cell viability. Number of average coloured (dead cells) was included in total cell count and results expressed in percentage. Suspensions having a cell count greater than 85% were used in the subsequent steps (Moldeus *et al.* 1978).

5.1.3 Cell Culture Preparation

Initially, the polystyrene Nunclon™ culture plates were covered with collagen suspension diluted in acetic acid and washed with Williams E medium. Plates were covered with an adequate amount of hepatocyte suspension having the density of 104 000 viable cells/cm^2. This was in accordance with the cell count of pipeted hepatocyte suspenson. Incubation of plates for the first 24 hours was carried out in complete medium containing Willams E medium, penicilin (1%), streptomycin (1%), glutamine (1%), insulin (0.06%) and fetal bovine serum (5%). In the following 24 hours plates were incubated with a mix of complete medium and/or appropriate testing substances.

All cell cultures were incubated in an incubator with a high relative humidity of ± 90% in atmosphere containing 5% CO_2 at 37°C. The non-adherent cells together with the old complete medium were removed and the fresh complete medium was applied, indicating the time point of 0h. The cells were further cultivated for different amounts of time and treatments as indicated in the results section. The hepatocytes and medium were analyzed at the end of the appropriate time period. (Berry *et al.* 1991, Canova *et al.* 2008).

5.2 Biochemical Parameters Measurements

5.2.1 *Alanine aminotransferase (ALT)*

Determination of plasma alanine aminotransferase (ALT) was carried out using Fluitest® GPT ALT kit according to the specified manufacturer's instructions. This method is based on the conversion of 2-oxoglutarate and L-alanine into the L-glutamate and pyruvate by ALT, as seen in the following reaction

$$\text{2-oxoglutarate + L-alanine} \xrightarrow{\text{ALT}} \text{L-glutamate + Pyruvate}$$

Furthermore, pyruvate and NADH are converted by lactate dehydrogenase (LD) into L-lactate and NAD^+, as illustrated below

$$\text{Pyruvate} + \text{NADH} + H^+ \xrightarrow{\text{LD}} \text{L-lactate} + NAD^+$$

Decrease in the levels of NADH was measured with UV spectrophotometry, which is in direct relation to the activity of ALT (Bergmeyer *et al.* 1986).

5.2.2 Aspartate aminotransferase (AST)

Fluitest® GOT AST kit by Analyticon test was used in determination of aspartate aminotransferase (AST) plasma levels.This method is based on the AST conversion of the 2-oxoglutarate and L-aspartate into glutamate and oxalacetate respectively in the following reaction.

$$\text{2-oxoglutarate} + \text{L-aspartate} \xrightarrow{\text{AST}} \text{Glutamate} + \text{Oxalacetate}$$

A subsequent indicator reaction which is catalyzed by malate dehydrogenase is used to measure the oxalacetate increase. In the second reaction, NADH is oxidized to NAD as seen below

$$\text{Oxalacetate} + \text{NADH} + H^+ \xrightarrow{\text{MDH}} \text{L-malate} + NAD^+$$

The rate of decrease in NADH as measured by photometry is directly proportional to the rate of formation of oxalacetate, and thus reflects the measure of AST activity (Bergmeyer *et al.* 1986).

5.2.3 ***Bilirubin***

Quantitative determination of direct and total bilirubin in plasma was measured using the Fluitest® BIL T/D kit according to the specified manufacturer's instructions. This method is based on the Jendrassik-Gróf principle that involves the presence of caffeine accelerator, that allows total bilirubin to couple with sulfanilic acid to form a red azobilirubin dye.

$$\text{Sulfanilic Acid} + NaNO_2 \xrightarrow{HCl} \text{Diazotized Sulfanic Acid}$$

$$\text{Bilirubin} + \text{Diazotized Sulfanic Acid} \xrightarrow{HCl} \text{Azobilirubin}$$

Photometrical measurement of the color intensity of the red azo dye formed is directly proportional to the total bilirubin concentration. Determination of direct bilirubin is performed without caffeine additive. The addition of alkaline tartrate causes a transformation from the red azobilirubin dye to a blue dye and the absorbance maximum (546nm to 578nm). The color intensity of this blue coloured product is proportional to the direct bilirubin concentration (Doumas and Wu, 1991)

5.2.4 *Urea*

Concentration of urea in the medium was directly measured using the diagnostic Fluitest® Urea Kit manufacturer‘s instructions. The method of measurement is based on hydrolysis of urea by enzyme urease to produce ammonia and CO_2.

$$\text{Urea} + H_2O \xrightarrow{\text{Urease}} 2\ NH_4^+ + CO_2$$

In the subsequent step ammonia combines with 2-oxoglutarate and NADH to yield glutamate and NAD in a reaction catalyzed by urease/glutamate dehydrogenase GLDH.

$$NH_4^+ + \text{NADH} + \text{2-oxoglutarate} \xrightarrow{\text{GLDH}} H_2O + NAD^+ + \text{glutamate}$$

The measured decrease in absorbance due to decrease in NADH levels is proportional to the urea concentration (Chaney and Marbach 1962).

5.2.5 *Glutathione (GSH)*

Assessment of reduced gluathione in homogenate is based on the method that depends on a reaction between thiol group with Ellman’s reagent (5,5-dithio-2-nitrobenzoic acid). This reagent is used for measuring low-molecular mass thiols such as glutathione in biological samples (Sedlak and Lindsay 1968).

Thiol Ellman's reagent Yellow product

Fig. 10. *Reaction of thiols with the Ellman's reagent. (Source: Sedlak and Lindsay 1968)*

Thiols cleave the disulfide bonds of the Ellman's reagent to yield 2-nitro-5-thiobenzoate (NTB^-), which ionizes to the yellow coloured NTB^{2-} dianion in water at neutral and alkaline pH. The measured absorbance at 412 nm was used to measure directly the levels of NTB^{2-} and thus corresponds to the levels of glutathione.

5.2.6 Catalase

The measurement of catalase in plasma was performed using a spectrophotometric assay of hydrogen peroxide based on formation of its stable yellow coloured complex with ammonium molybdate (Goth 1991, Korolyuk *et al.* 1988). The absorbance is measured spectrophotometrically and related to the catalase activity.

$$H_2O_2 + \text{ammonium molybdate} \xrightarrow{\text{Catalase}} \text{Yellow coloured complex} + 2\ H_2O$$

5.2.7 NO_2^-/NO_3^-

Assessment of plasma NO_2^-/NO_3^- was carried out using a nitrate/nitrite colourimetric assay kit of Cayman Chemical Company manufacturer's instruction. In short, this method is based on a colourimetric conversion of nitrate (NO_3^-) to nitrite (NO_2^-) by nitrate reductase. The addition of the Griess reagent (1% sulfanilamide, 0.1% naphtylethylendiamine, 2.5% H_3PO_4) converts nitrite into a coloured azo compound.

Spectrophotometrical measurement of absorbance at 540 nm determines the nitrite concentration, using the appropriate standard curve (Green *et al.* 1982).

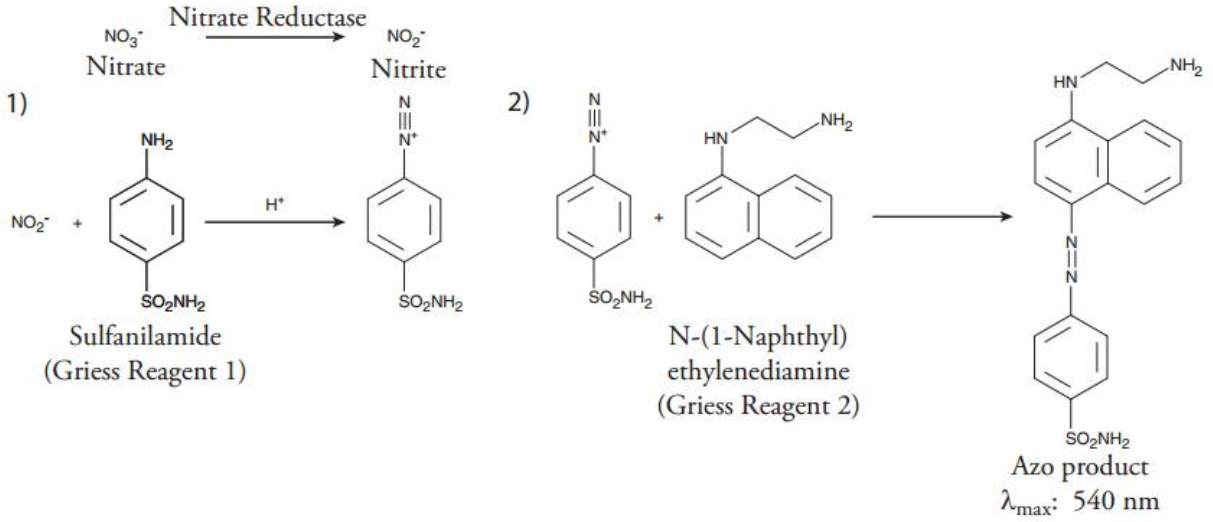
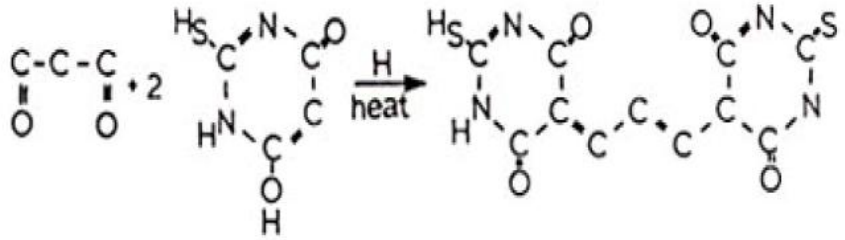


Fig. 11. Chemistry of the Griess reagents (Green *et al.* 1982)

5.3. Lipid Peroxidation Measurements

5.3.1 *The Thiobarbituric Acid Reacting Substances (TBARS)*

Products of lipid peroxidation, also termed thiobarbituric acid–reactive substances (TBARS), are assayed by the thiobarbituric acid method (Yagi 1976). The basic principle of the method is the reaction of one molecule of malonaldehyde and two molecules of thiobarbituric acid (TBA) to form a red malonaldehyde-TBA complex, which can measured by spectrophotomety at 532 nm. Lipoprotein fractions are first acid precipitated and removed from the sample by centrifugation, thus removing any interfering TBARS. The remaining TBARS in sample form a coloured product. Malondialdehyde standard is used for plotting a standardization curve from which concentration of the TBARS in sample can be determined.



MDA TBA Coloured Product

Fig. 12. Thiobarbituric acid method (Yagi 1976).

5.3.2 *Conjugated Dienes (CD)*

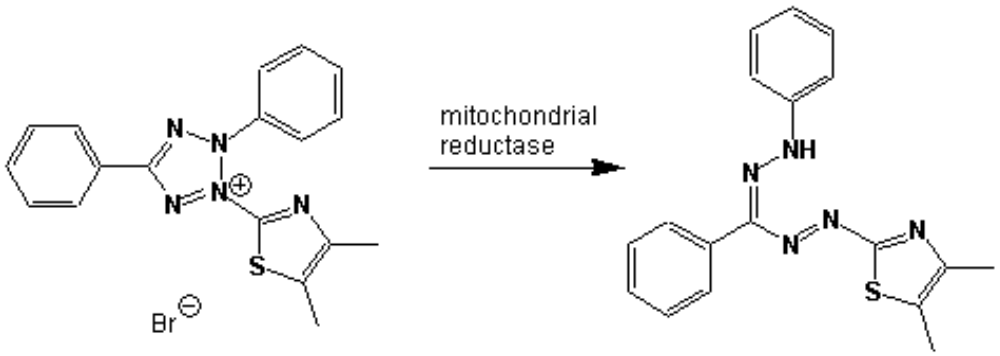
Additional evidence for lipid peroxidation can be measured by assay for the formation of conjugated dienes, which arise from the rearrangement of double bonds on the polyunsaturated fatty acid side chain (Ward *et al.* 1985). The resulting conjugated dienes strongly absorb ultraviolet light at 233 nm that is measured by spectroscopy. A reaction mixture containing EDTA is added to prevent further lipoperoxidation.

5.4 Measurements of Apoptotic/Necrotic Parameters

5.4.1 *MTT Test*

The MTT test is a colorimetric assay used for measuring the cell viability in a given sample *in vitro*, which can also be applied in the determination of cytotoxicity of the given hepatotoxic agents (Mosmann 1983).

MTT or 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide, is a yellow compound which is reduced to the purple formazan in the living cells by the action of the mitochondrial reductases. A solubilization solution of dimethyl sulfoxide is added to dissolve the insoluble purple formazan product into a coloured solution. The absorbance of the coloured solution is measured spectrophotometrically at the wavelength of 540 nm.



MTT Formazan product

Fig. 13. MTT assay (Mosmann 1983).

5.4.2 *Caspase 3*

Cell lysates were prepared according to the instructions of Sigma-Aldrich fluorometric caspase 3 assay kit. The principle of this assay is based on the hydrolysis of the peptide substrate acetyl-Asp-Glu-Val-Asp-7-amido-4-methylcoumarin (Ac-DEVD-AMC) by the action of caspase 3 resulting in the release of fluorescent 7-amino-4-methylcoumarin (AMC) moiety, as shown below.

$$\text{Ac-DEVD-AMC} \xrightarrow{\textit{Casp-3}} \text{Ac-DEVD + AMC}$$

The excitation and emission wavelengths of AMC (360 nm and 460 nm respectively) were measured by the microplate fluorometer. The concentration of the released AMC was calculated from the calibration curve performed with defined AMC concentration solutions. The results are expressed as percentage of caspase 3 activity in the treated group relative to the control.

5.5 Molecular Measurements

5.5.1 *Real time PCR*

5.5.1.1 Sample Isolation

All of the liver samples were isolated at the end of the experiment as specified in each protocol. Total cell count of viable cells was carried out for each sample, and the constant number of viable cells was used throughout the experiments. Each sample was placed in the cold RNA stabilizing buffer with the addition of beta-mercaptoethanol (Moldeus *et al.* 1978).

5.5.1.2 Total RNA Isolation

The total RNA isolation from the hepatocytes was performed according to the instructions of the RNeasy handbook by QIAGEN company. In short, the lysis of hepatocytes was carried out in RNA stabilizing solution together with lysating agent and beta-mercaptoethanol. Cell lysates were then homogenized using the QIAshredder high pressure membrane by the centrifugation at 20 000 g. The elimination of unwanted genome DNA was executed using a specified high affinity chromatography column (gDNA Eliminator spin column). The following step involved washing the sample with diluted 70% ethanol solution and retention of total RNA in the specific high affinity chromatographic column. The selective binding properties of a silica-based membrane and the use of high-salt buffers allows for the binding of up to 100 µg of RNA longer than 200 bases. The column was then washed several times by different buffers to remove the contaminants. The final step involved elution of the purified total RNA, that was further stored in deep freezer at temperatures below -70°C.

5.5.1.3 Reverse Transcription from RNA to cDNA

Following the total RNA isolation, the reverse transcription from total RNA to cDNA was processed by universal kit GeneAmp® RNA PCR using a murine leukemia virus (MuLv) reverse trancriptase (RT). In summary, the reverse transcriptase creates single-stranded DNA from a double stranded RNA template. Normal transcription involves the synthesis of RNA from DNA, thus the term reverse transcription is the *reverse* of normal transcription. Reverse transcription includes the following three phases: 10 min at 25ºC for RT enzyme activation, 30 min at 48ºC for PCR amplification and 5 min at 95ºC for denaturation. Following this procedure, all cDNA samples were then refrigirated at temperature below -20°C.

5.5.1.4 Real-time PCR

Real-time polymerase chain reaction (RT-PCR**)** is used to amplify and quantify DNA of interest (target DNA). This involves the use of a pair of primers that are complementary to a defined sequence on each of the two strands of the DNA. Initially, during the PCR annealing stage, the primers are extended by a DNA polymerase so that a copy of the designated sequence is made, thus leading to exponential amplification. Upon reaching the probe an enzyme called 5'-3'-exonuclease degrades the probe, physically separating the fluorescent reporter from the quencher. This results in an increase in fluorescence that corresponds to the exponential increase of the product and is analyzed by the real-time PCR thermo cycler (Kubista *et al.* 2006). The cycle at which the fluorescence of a sample crosses the threshold value (C_T) is determined. The principle of relative quantification was used that is based on comparison of C_T values between an internal gene (whose expression is similar across all the samples) to expression of the target gene. Sooner the threshold value is reached (lower C_T) the larger the amount of target gene has been expressed compared to the endogenous control gene (Schefe *et al*. 2006).

In this study, RT-PCR was carried out with the help of ABI PRISM 7900, and TagMan ® Gene Expression master mix (Applied Biosystems). Total of eight genes were evaluated according to manufacturer's instructions of the TagMan ® Gene Expression

Assays Kit. Target genes in our analysis included nitric oxide synthase-2 (NOS-2), heme oxygenase-1 (HO-1), glutathione peroxidase 1 (Gpx1), superoxide dismutase 1 (SOD1), BH3 interacting domain death agonist (Bid), Bcl-2 -associated X protein (Bax), caspase 3 (Casp 3), tumor necrosis factor-α (TNF- α) and housekeeping gene used was glyceraldehyde 3-phosphate dehydrogenase (Gapdh). Housekeeping gene-expression was stable and constant during the experiments and was used in comparison with target gene-expression (Pfaff *et al.* 2005).

Thermal cycling conditions for primer and probes optimization were 10 min at 90–95 ºC for Taq polymerase activation, followed by 15 s at 95–99 ºC for DNA denaturation and 1 min at 60 ºC for annealing. The obtained Ct values were used in relative quantification of gene expression relative to the endogenous gene control Ct measurements, and the results were calculated using the $\Delta\Delta C_T$ method (Arocho *et al.* 2006).

5.5.2 Western Blot

In this study, the use of the Western blot analysis allowed for the identification of the target proteins of the underlying toxicity processes. Western blot method involves the use of gel electrophoresis to separate denatured proteins by the length of the polypeptide or by their three dimensional structure. The separated proteins are then transferred on to a nitrocellulose membrane, where they are detected using antibodies specific to the target protein and visualized using chemiluminescence and photometric instruments (Burnette 1981).

In this study, isolated liver tissue was lysed to release proteins of interest (HO-1 and NOS-2) using a 20 mM Tris-HCl, pH 7.5, lysis buffer and homogenized with an electric homogenizer. Bradford assay was performed to determine the protein concentration of the lysed samples. The samples were then centrifuged for 20 min at 12000 rpm at 4°C and supernatant was collected. The cell lysates were mixed with sample buffer consisting of b-mercaptoethanol, 10 % sodium dodecyl sulphate (SDS), glycerol, bromophenol blue and 0.5M Tris HCl (pH 6.8) at dilution factor of 1:1 and heated for 5 minutes at 95°C. Treatment of samples with SDS allowed for proteins to be

reduced and denatured, while as bromophenol blue aided in visualization of the migration of proteins.

SDS polyacrylamide gel electrophoresis (SDS-PAGE) was performed in the next steps. The polyacrylamide gels are neutral, hydrophilic and three dimensional networks of long hydrocarbons crosslinked by methylene groups that allow for separation of proteins within a gel. In short, 20µl of sample containing 30µg of protein was loaded on to of 8% and 10% SDS-acrylamide separating gels. As well, the molecular weigh marker (NAME) was loaded on to the gel in order to enable determination of the protein size and to visualize the progress of electrophoretic run. Electrophoresis run was carried out at 100V for couple of hours, until the proteins separated and migrated to the front line. Following this step, the separated proteins were transferred by electrophoresis from the SDS-PAGE gels on to a nitrocellulose membrane overnight at 4°C (Towbin *et al.* 1979).

The following day, membranes were blocked for 1 h with 5% non-fat milk in tris-buffered solution, at room temperature. Membranes were then washed in Tween phosphate buffer saline (TPBS) consisting of NaCl, KCl, Na_2HPO_4, KH_2PO_4, Tween and H_2O. They were then incubated with rabbit primary antibody against NOS-2 (1:1000), mouse HO-1 primary antibody (1: 500) or mouse beta actin primary antibody (1:5000). After eluting the unconjugated primary antibody with the wash buffer, the membranes were then incubated with corresponding secondary antibody, anti-rabbit IgG HRP conjugate (1:20,000) or anti-mouse IgG HRP conjugate (1:100,000). This step was followed by chemiluminescence labeling with Super Signal West Pico Chemiluminescent Substrate for 2 minutes. Bands were detected with the use of Molecular Imager VersaDoc™ MP 5000 System and analyzed by Quantity One 1-D Analysis Software (Bio-Rad, Prague). Optical densities of NOS-2 and HO-1 bands were normalized by the corresponding loading control (beta-actin) and then to the mean of the corresponding control group (Bolt and Mahoney 1997, Renart *et al.* 1979).

5.6 Morphological Evaluation

Cells were washed with cold PBS solution and stabilized with 4% paraformaldehyde. Morphological evaluation of hepatocytes at the light microscopical

level was done on semithin epon sections (1 – 2 μm thick) stained by toluidine blue using Leica IM 500 program for digital recording and measurements.

In short, toluidine blue is a cationic (basic) dye that is used commonly for staining semin thin sections of resin-embedded tisusue. At alkali conditions the dye binds the nucleic acids and all proteins blue, allowing for clear visualization of structural details (Mercer 1963).

5.7 Statistical Evaluation

All of the executed experiments were performed using a minimum of five rats per tested group. The collected data was adjusted using the Dixon test that excluded the outliers. The data was then processed using the GraphPad statistical program. All of the data passed the normality test. Thus the statistical significance of the mean scores was performed using the One-way Analysis of Variance (ANOVA) with the use of appropriate post tests (post hoc Boneffori, Tukey- Kramer comparison test, unpaired T-test with Welch correction) to evaluate the plasma, tissue, protein and/or genetic alterations between the treated vs. untreated animals (negative control). In each case, there was just one independent measurement of each parameter from each animal. The p-values less than 0.05 were considered significant.

6 RESULTS

6.1 *In vitro* tert-butylhydroperoxide hepatotoxicity model: The effects of quercetin

6.1.1 *The effects of tert-butylhydroperoxide and quercetin treatments on hepatocyte function and cell viability*

In this *in vitro* xenobiotic hepatotoxicity model, hepatocytes were treated with two differing concentrations of tert-butylhydroperoxide (TBOOH), 0.25 and 0.50 mM. As can be seen from Table 1, treatment with both concentrations of TBOOH resulted in significantly higher levels of ALT and AST production, which is indicative of hepatocyte damage. Addition of quercetin to TBOOH treated cells ameliorated the hepatocyte damage as it decreased ALT and AST levels compared to TBOOH groups. However, when given alone, quercetin did not have any influence on AST and ALT production compared to control group.

Table 1 : *The effect of quercetin treatment on ALT and AST leakage into culture medium, in tert-butylhydroperoxide hepatotoxicity model.*

	ALT [U/l]	**AST** [U/l]
Control	42.40 ± 0.61	106.08 ± 23.74
TBOOH 25	83.65 ± 0.86*	134.33 ± 19.22*
TBOOH 50	105.42 ± 2.56*	154.12 ± 14.44*
Q 25	48.98 ±1.29*	98.20 ± 18.38
Q 50	45.48 ±0.85	98.94 ±15.08
TBOOH 25 + Q 25	61.91 ± 4.74#	86.47 ± 18.37#
TBOOH 25 + Q 50	45.06 ± 3.81#	96.03 ± 19.43#
TBOOH 50 + Q 25	68.86 ± 2.56^	93.47 ± 19.89^
TBOOH 50 + Q 50	40.45 ± 3.51^	92.68 ± 14.9^

*Control: negative control group receiving complete medium only; TBOOH 25 & TBOOH 50: 0.25 mM and 0.50 mM tert-butylhydroperoxide respectively; Q25 and Q50: 250 μM and 500 μM quercetin respectively; TBOOH+Q: combination of tert-butylhydroperoxide and quercetin. * indicates significant values (p≤0.05) compared to the control group (complete medium); # indicates significant values (p≤0.05) compared to TBOOH 25 group; ^ indicates significant values (p≤0.05) compared to TBOOH 50 group. The results are expressed as means ± SEM, n=10.*

Cell viability was assessed using an MTT test, where mitochondrial dehydrogenase of viable hepatocytes reduces 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide to an insoluble purple formazan, that is measured spectrophotometrically. Application of tert-butylhydroperoxide to the cell culture, has significantly decreased viability of hepatocytes by 3-fold in 0.25 mM, and 5.5-fold in

0.50 mM concentrations (Fig. 14). Quercetin significantly improved cell viability in TBOOH treated cells, which is shown by significant increases in all quercetin treated hepatocytes. It must be noted that the lower concentration of quercetin (250 μM) preserved cell viability when compared to control (complete medium only), whereas the higher concentration of quercetin (500 μM) significantly increased the cell viability when compared to the control (Fig. 14).

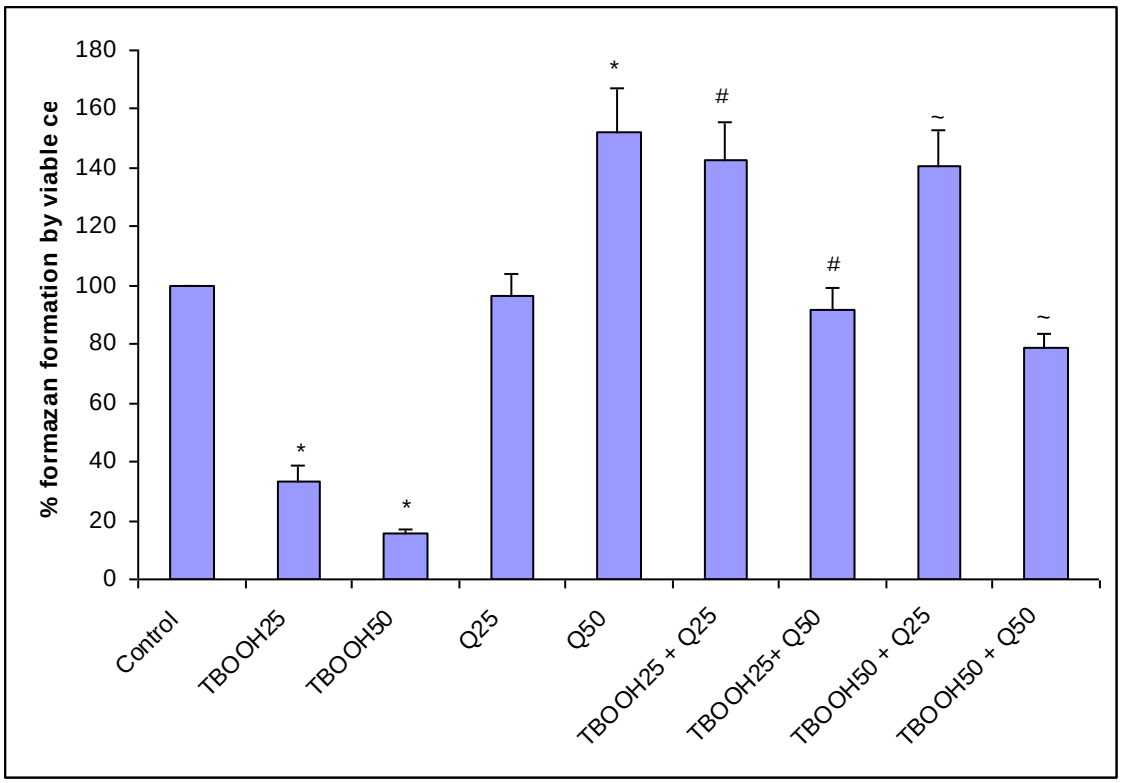


Fig. 14. *The effects of tert-butylhydroperoxide and quercetin treatment on mitochondrial dehydrogenase activity (MTT test), expressed as a percentage of formazan formation by viable hepatocytes. Control: negative control group receiving complete medium only; TBOOH 25 & TBOOH 50: 0.25 mM and 0.50 mM tert-butylhydroperoxide respectively; Q25 and Q50: 250 μM and 500 μM quercetin respectively; TBOOH +Q: combination of tert-butylhydroperoxide and quercetin. * indicates significant values (p≤0.05) compared to the control group (complete medium); # indicates significant values (p≤0.05) compared to TBOOH 25 group; ~ indicates significant values (p≤0.05) compared to TBOOH 50 group. The results are expressed as means ± SEM, n=10.*

6.1.2 *The effects of tert-butylhydroperoxide and quercetin treatments on oxidative stress parameters*

The mRNA expressions measurements of HO-1, NOS-2 and inflammatory mediator TNF-α, as well as the plasma levels of nitrites were used as determinants of oxidative status of the cells.

As can be seen from Fig. 15, TBOOH had no effect on HO-1 mRNA expressions, whereas quercetin treatment (without TBOOH) of hepatocytes increased HO-1 mRNA expressions significantly. Interestingly, treatment with 250 μM quercetin significantly increased HO-1 mRNA expressions in both of the TBOOH groups, but 500 μM had no effect.

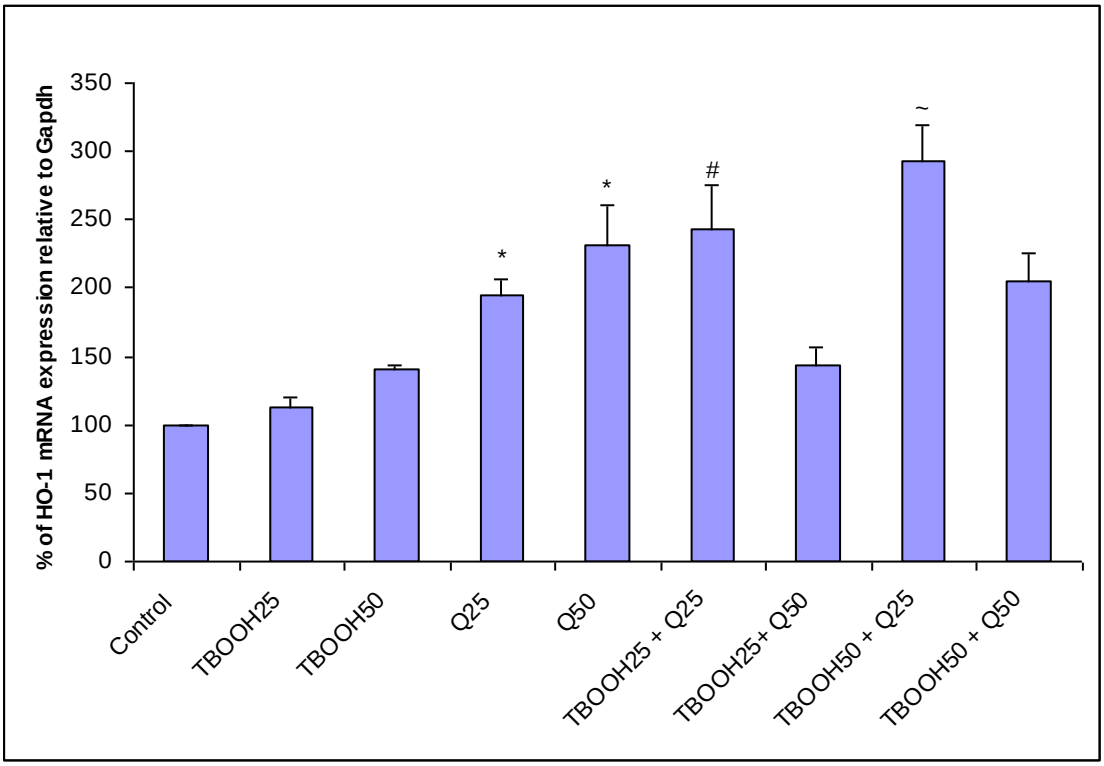


Fig. 15. *The effects of tert-butylhydroperoxide and quercetin treatment on HO-1 mRNA expression. The results are expressed as a relative percentage of gene expression against Gapdh as the endogenous control. Control: negative control group receiving complete medium only; TBOOH25 & TBOOH50: 0.25 mM and 0.50 mM tert-butylhydroperoxide respectively; Q25 and Q50: 250 μM and 500 μM quercetin respectively; TBOOH+Q: combination of tert-butylhydroperoxide and quercetin. * indicates significant values (p≤0.05) compared to the control group (complete medium); # indicates significant values (p≤0.05) compared to TBOOH25 group; ~ indicates significant values (p≤0.05) compared to TBOOH50 group. The results are expressed as means ± SEM, n=10.*

The administration of TBOOH to hepatocytes increased mRNA expressions of NOS-2 significantly. Mostly, quercetin administration had no effect on NOS-2 mRNA expressions, with the exception of 500 μM concentration given alone, which significantly increased NOS-2 mRNA expression (Fig. 16).

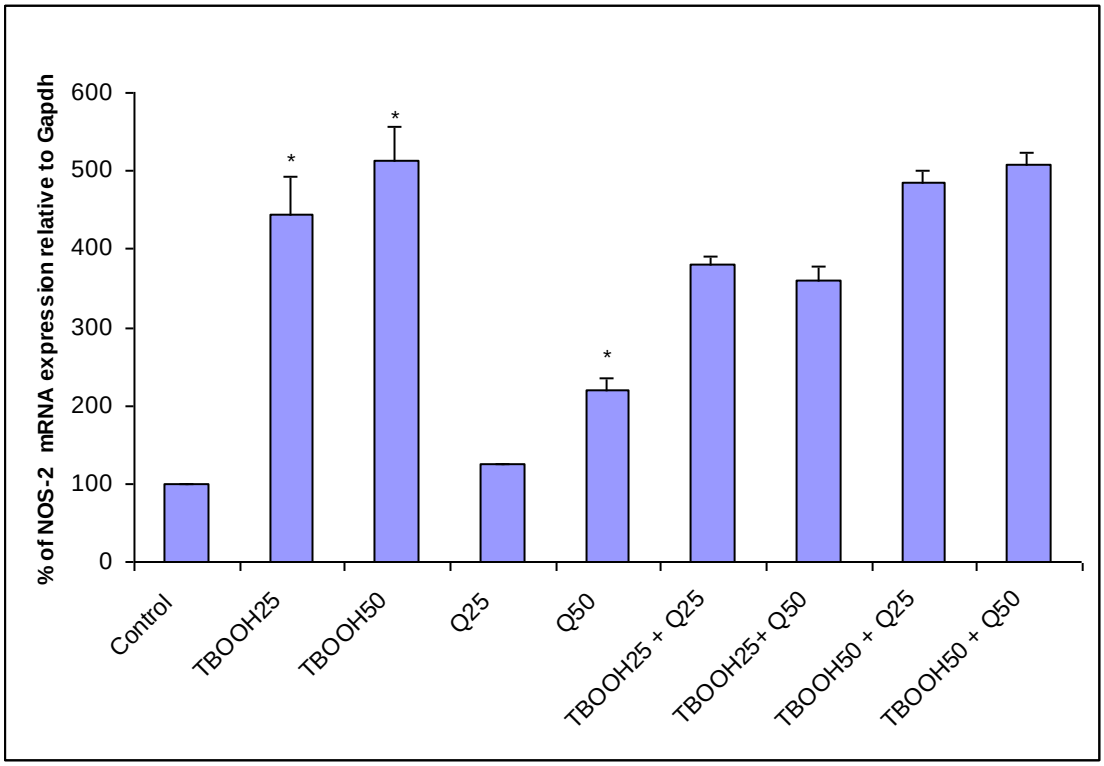


Fig. 16. *The effects of tert-butylhydroperoxide and quercetin treatment on NOS-2 mRNA expression. The results are expressed as a relative percentage of gene expression against Gapdh as the endogenous control. Control: negative control group receiving complete medium only; TBOOH25 & TBOOH50: 0.25 mM and 0.50 mM tert-butylhydroperoxide respectively; Q25 and Q50: 250 μM and 500 μM quercetin respectively; TBOOH+Q: combination of tert-butylhydroperoxide and quercetin. * indicates significant values (p≤0.05) compared to the control group (complete medium);. The results are expressed as means ± SEM, n=10.*

The inflammatory cytokine TNF-α mRNA gene expressions were increased in TBOOH 0.5 mM group, but not in 0.25 mM TBOOH group. Furthermore, quercetin alone, and combination of TBOOH 0.25 mM and quercetin 500 µM had no effect on TNF-α mRNA gene expressions. Addition of quercetin in other combination groups (Q+TBOOH) significantly reduced TNF-α mRNA gene expressions (Fig. 17).

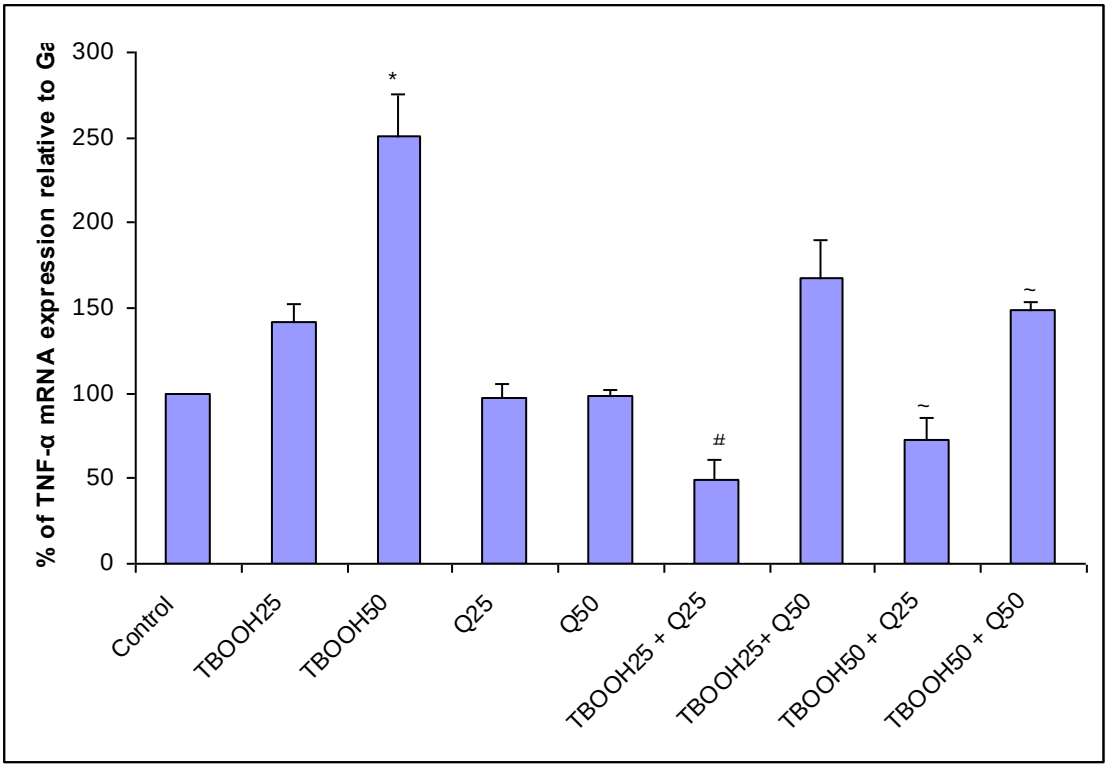


Fig. 17. *The effects of tert-butylhydroperoxide and quercetin treatment on TNF-α mRNA expression. The results are expressed as a relative percentage of gene expression against Gapdh as the endogenous control. Control: negative control group receiving complete medium only; TBOOH25 & TBOOH50: 0.25 mM and 0.50 mM tert-butylhydroperoxide respectively; Q25 and Q50: 250 µM and 500 µM quercetin respectively; TBOOH+Q: combination of tert-butylhydroperoxide and quercetin. * indicates significant values (p≤0.05) compared to the control group (complete medium); # indicates significant values (p≤0.05) compared to TBOOH25 group; ~ indicates significant values (p≤0.05) compared to TBOOH50 group. The results are expressed as means ± SEM, n=10.*

6.2 *In vivo* xenobiotic hepatotoxicity model (D-Galactosamine and Lipopolysaccharide): Involvement of oxidative stress and apoptosis

6.2.1 The effects of D-GalN/LPS treatment on liver function and lipid peroxidation

The combination of D-GalN (i.p., 400 mg/kg) and LPS (i.p., 50 μg/kg) treatment in rats has produced hepatic failure, which can be seen by significant ($p < 0.05$) increases in levels of aminotransferases in plasma. A two hundred fold increase in AST level and one hundred fold increase in ALT level compared to those of the control group was observed (Table 2). The extent of lipid peroxidation as measured by formation of thiobarbituric acid reactive substances (TBARS) and conjugated dienes (CD) did not show any statistically significant differences between the two groups ($p > 0.05$). Furthermore, Table 2 shows significant ($p < 0.05$) increases in plasma levels of antioxidant enzyme catalase (CAT) and statistically non-significant increase ($p > 0.05$) in levels of reduced glutathione (GSH) in homogenate of D-GalN/LPS treated rats.

Table 2 : *The effects of lipopolysaccharide-induced hepatitis in D-Galactosamine sensitized rats (D-GalN/LPS) on levels of ALT, AST, CAT, CD, GSH and TBARS 24h after injection.*

	Control	**D-GalN/LPS**
ALT Plasma [μcat/l]	0.82 ± 0.05	166.94 ± 12.42*
AST Plasma [μcat /l]	2.016 ± 0.09	254.80 ± 4.85*
CAT Plasma [μg/ml]	51.24 ± 6.55	156.00 ± 1.88 *
CD Homogenate [nmol/mg protein]	2.45 ± 0.57	2.94 ± 0.54
TBARS Homogenate [nmol/mg protein]	197.84 ± 22.24	261.12 ± 6.10
GSH Homogenate [μmol/mg protein]	458.36 ± 19.35	513.06 ± 49.89

Control: negative control group receiving vehicle only. D-GalN/LPS: D-Galactosamine 400 mg/kg with lipopolysaccharide 50 μg/kg;-; Values are mean ± SEM, n = 8;

** Indicates value significant compared to CONTROL $p \leq 0.05$.*

6.2.2 *The effects of D-GalN/LPS treatment on oxidative stress parameters*

Antioxidant, Gpx1 and SOD1, mRNA expressions were related to Gadph as the endogeneous control, and measured in both D-GalN/LPS and the control groups (Fig. 18). The increase of Gpx1 gene expression in the treated group is non significant as $p > 0.05$. However, D-GalN/LPS treatment has caused a highly significant decrease of SOD1 gene expression in comparison to the untreated control group.

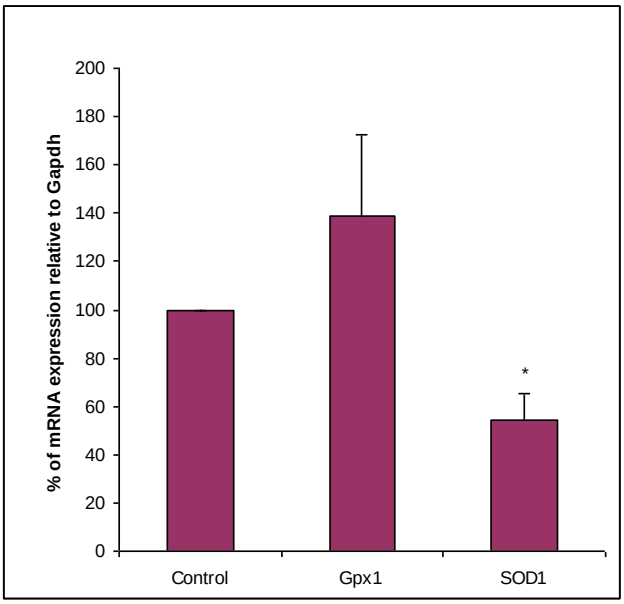


Fig. 18. *The effect of lipopolysaccharide-induced hepatitis in D-Galactosamine sensitized rats (D-GalN/LPS) on Gpx1 and SOD1 gene expressions relative to Gapdh as the endogenous control 24h after injection. Control: saline injection only; D-GalN/LPS: D-Galactosamine 400 mg/kg with lipopolysaccharide 50 µg/kg; Values are mean ± SEM., n = 8; * indicates value significant compared to Control $p \leq 0.05$.*

The extent of heme catabolism as measured by bilirubin levels illustrates significantly higher levels of bilirubin in plasma (Fig. 19a) of D-GalN/LPS treated rats compared to that of the control group. The same trend is observed in the inducible HO-1 gene expression (Fig. 19b) relative to the Gapdh, where the seven fold increase in the D-GalN/LPS treated group is highly significant.

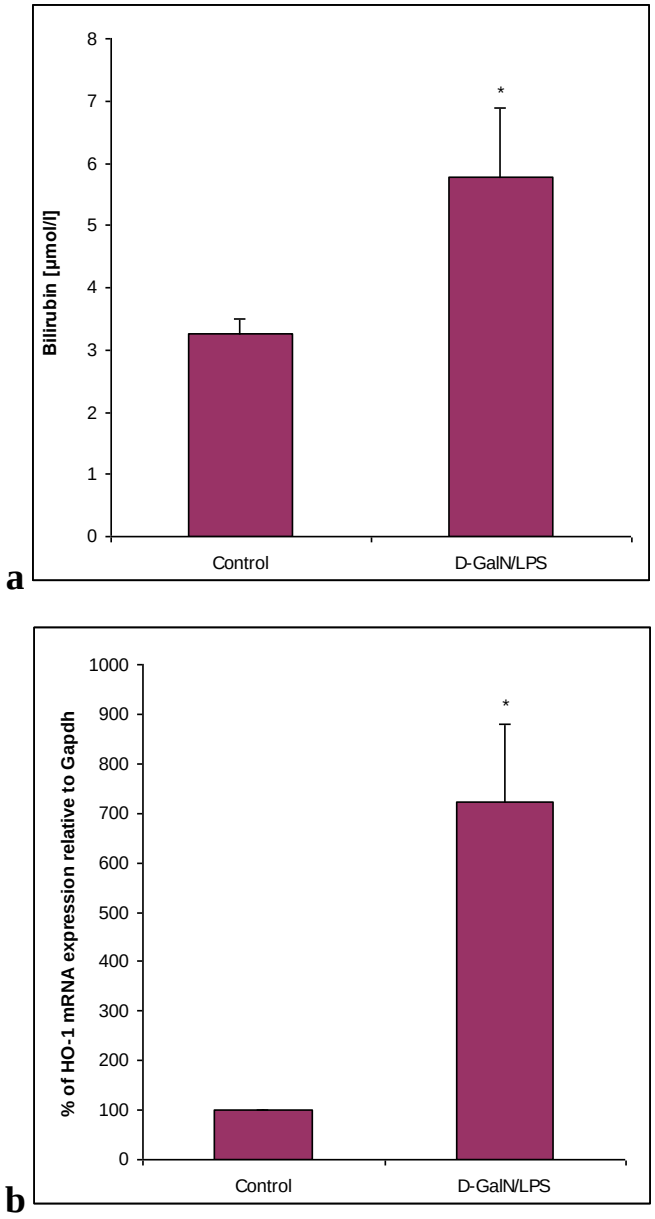


Fig. 19. *The effect of lipopolysaccharide-induced hepatitis in D-Galactosamine sensitized rats (D-GalN/LPS) on plasma bilirubin (a) and on HO-1 gene expression relative to Gapdh as the endogenous control (b) 24h after injection. Control: saline injection only; D-GalN/LPS: D-Galactosamine 400 mg/kg with lipopolysaccharide 50 µg/kg; Values are mean ± SEM., n = 8; * indicates value significant compared to Control p ≤ 0.05.*

In comparison to the untreated control animals, D-GalN/LPS treatment induced simultaneous statistically significant increase in both plasma NO_2^- levels (Fig. 20a) and NOS-2 gene expression relative to Gapdh as endogeneous control (Fig. 20b).

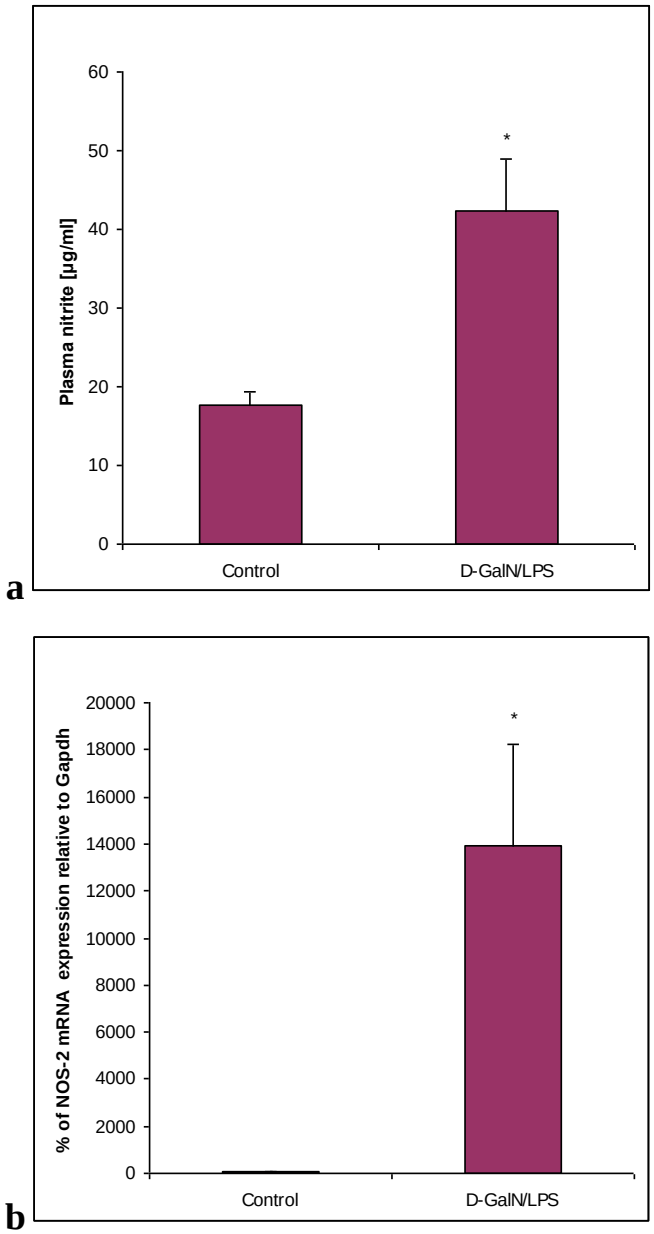


Fig. 20. *The effect of lipopolysaccharide-induced hepatitis in D-Galactosamine sensitized rats (D-GalN/LPS) on plasma NO_2^- (a) and on NOS-2 gene expression relative to Gapdh as the endogenous control (b) 24h after injection. Control: saline injection only; D-GalN/LPS: D-Galactosamine 400 mg/kg with lipopolysaccharide 50 µg/kg; Values are mean ± SEM., n = 8; * value significant compared to Control p ≤ 0.05*.*

6.2.3 *The effects of D-GalN/LPS treatment on apoptotic markers and morphological findings*

Selected apoptotic parameters including Casp3, Bid and Bax gene expressions relative to the Gapdh as endogenous control are illustrated in the Fig. 21. The expression of Casp3 gene did show a significant increase. The same trend is also observed in the expressions of Bid and Bax genes, where the increase in the D-GalN/LPS treated group was statistically significant. Bax gene expression was more than two-fold and thus the highest of the three apoptotic genes that were measured.

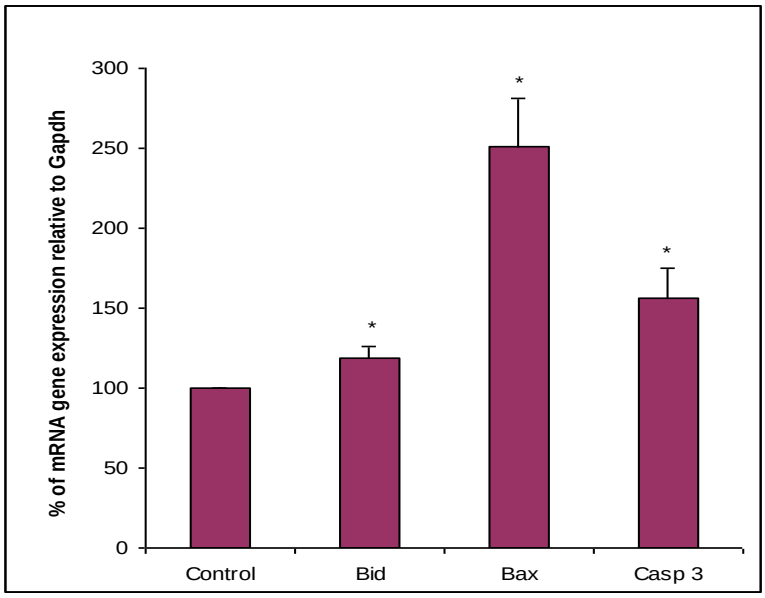


Fig. 21. *The effect of lipopolysaccharide-induced hepatitis in D-Galactosamine sensitized rats (D-GalN/LPS) Bid, Bax and Casp3 gene expressions relative to Gapdh as the endogenous control 24h after injection. Control: saline injection only; D-GalN/LPS: D-Galactosamine 400 mg/kg with lipopolysaccharide 50 µg/kg; Values are mean ± SEM., n = 8; * indicates value significant compared to control $p \leq 0.05$*.*

The morphological evaluation has shown the well preserved cytological features of the control liver tissues. Specifically, the peripheral region of the central vein lobules consists of radial arranged cords of hepatocytes with more or less comparable cytological features, such as stainability of the cytoplasm and distribution of cell organelles (Fig. 22a). The administration of D-GalN/LPS has significantly affected morphological parameters of the rat livers. Even at the lower magnification (Fig. 22b) striking necrotic lesions can be observed in the peripheral and intermediate regions of the central vein lobules. Aggregation of heterochromatin near the nuclear envelope confirms the apoptosis in some hepatocytes (Fig. 22c). At the peripheral region of some injured lobules transitional stages of aponecrosis can be detected and the presence of pycnotic nuclei is clearly visible (Fig. 22d).

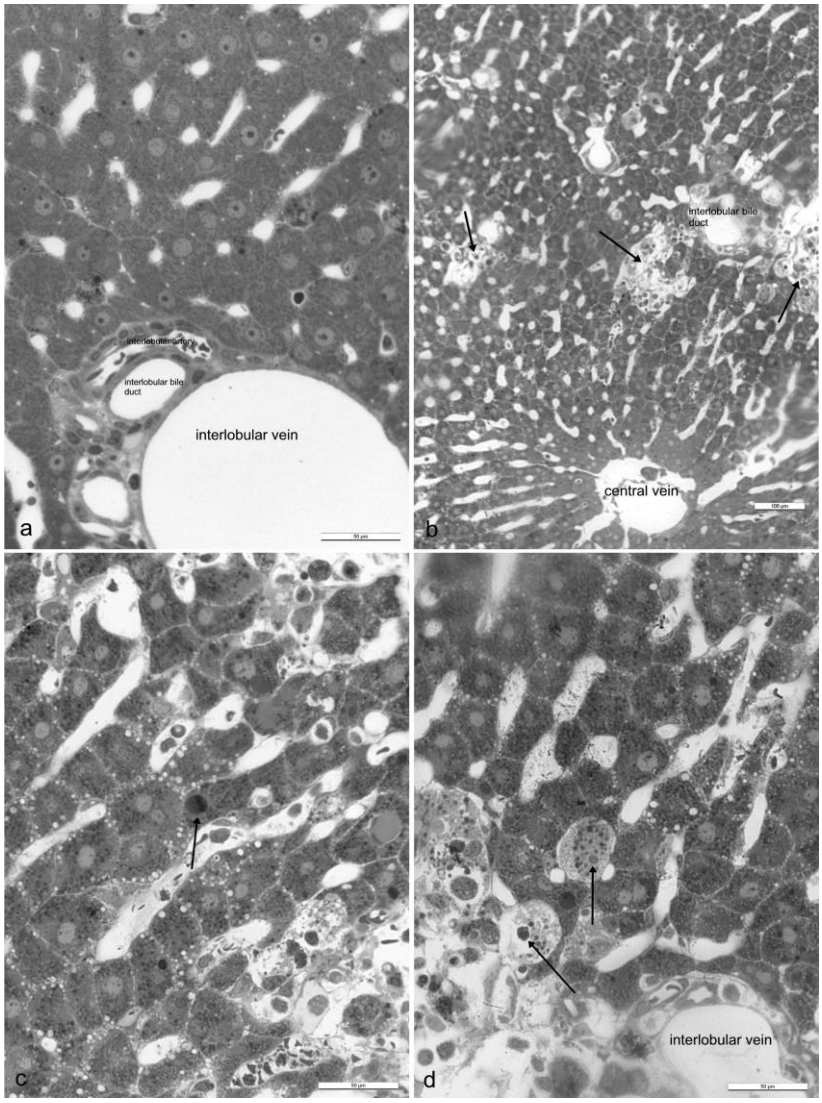


Fig. 22. *Light microscopy morphological findings of rat liver of control and D-GalN/LPS treated samples: a) control hepatocyte liver- peripheral region of the central vein lobule; bar 50 μm. b) the effect of D-GalN/LPS treatment; low magnification, bar 100 μm; c) the effect of D-GalN/LPS treatment; higher magnification, typical changes of demilunar apoptotic heterochromatin arrangement in the nucleus are indicated by an arrow; bar 50 μm; d) peripheral region of injured lobule- some transitional stages of aponecrosis can be detected; presence of pycnotic nuclei (arrows); marked disintegration of the cell cytoplasm; bar 50 μm; All samples prepared by semithin epon section, toluidine blue.*

6.3 The effects of curcumin treatment on *in vivo* D-GalN/LPS xenobiotic hepatotoxicity model

6.3.1 The ameliorative effects of curcumin on D-GalN/LPS hepatotoxicity

In this study, the influence of curcumin treatment (*i.p.*, 100 mg/kg) on D-GalN (*i.p.*, 400 mg/kg) and LPS (*i.p.*, 10 μg/kg) model of hepatotoxicity was tested. The results show that male Wistar rats treated with the combination of D-GalN/LPS developed acute hepatotoxicity within 24 hours of the insult. This can be confirmed by a several-fold increase in plasma levels of transaminases (AST and ALT) that are indicative of failing liver function (Table 3). Curcumin treatment alone did not have any significant effect on these parameters. However, pretreatment with curcumin in D-GalN/LPS rats significantly decreased the levels of both ALT and AST.

The plasma level measurements of the antioxidant molecule and product of heme degradation pathway, bilirubin, and antioxidant enzyme catalase were used as indicators of the oxidative status. In the groups treated with D-GalN/LPS, we observed significant 5.2-fold increase in bilirubin and 2.4-fold increase in catalase plasma levels. Curcumin pre-treatment significantly increased the levels of these two important in D-GalN/LPS treated rats, but had no effect when given alone (Table 3).

The extent of lipid peroxidation was determined by measuring the levels of CD in homogenate samples. It was shown that D-GalN/LPS treatment resulted in 3.5-fold significantly higher levels of CD compared to that of control. Curcumin pre-treatment significantly reduced the levels of conjugated dienes in D-GalN/LPS rats, but had no effect when administered alone.

Table 3 : *The effects of curcumin pre-treatment on plasma levels of ALT, AST, bilirubin, catalase, nitrites and homogenate levels of conjugated dienes (CD) in D-GalN/LPS hepatotoxicity model.*

	Control	**CUR**	**D-GalN/LPS**	**D-GalN/LPS + CUR**
ALT [U/l]	27.77 ± 3.02	35.89 ± 4.79	247.31± 54.79*	113.05 ± 31.92#
AST [U/l]	56.80 ± 4.48	57.90 ± 9.79	127.87 ± 10.63*	63.70 ± 9.39#
Bilirubin [μmol/l]	3.68 ± 1.78	4.88 ± 0.82	19.38 ± 3.48*	36.89 ± 2.97#
Catalase [μg/ml]	55.43 ± 6.45	41.22 ± 3.23	132.93 ± 10.20*	164.35 ± 9.11#
CD [nmol/mg protein]	2.19 ± 0.37	1.77 ± 0.42	7.76 ± 1.49*	3.43 ± 0.60#
Nitrites [μg/ml]	20 ± 3.65	19.67 ± 2.76	55.89 ± 6.89*	30.55 ± 2.90#

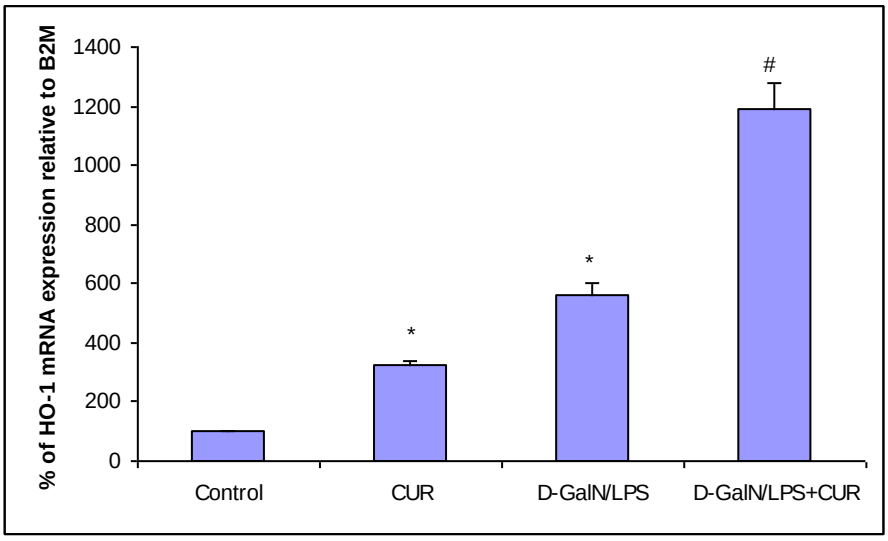
Control: negative control group receiving vehicle only; CUR: curcumin 100 mg/kg; D-GalN/LPS: D-Galactosamine 400 mg/kg with lipopolysaccharide 10 μg/kg; D-GalN/LPS +CUR: combination of D-galactosamine, lipopolysaccharide and curcumin treatment;

** indicates significant values (p≤0.05) compared to the control group (vehicle only); # indicates significant values (p≤0.05) compared to the respective positive control group (D-GalN/LPS). The results are expressed as means ± SEM, n=7.*

6.3.2 The effects of curcumin treatment on oxidative stress parameters in D-GalN/LPS hepatotoxicity model

The mRNA expressions measurements of antioxidant enzymes HO-1 and SOD1, as well as NOS-2 enzyme were used as determinants of oxidative status of the liver. Administration of D-GalN/LPS has resulted in significant 5-fold increase in the HO-1 mRNA expression (Fig. 23a), as well as a 240-fold increase in NOS-2 mRNA expression (Fig. 23b). Conversely, D-GalN/LPS administration resulted in a 1.6-fold decrease in SOD1 mRNA expression (Fig. 23c). Pre-treatment with curcumin of D-GalN/LPS rats resulted in further increase in HO-1 mRNA expression (2.2-fold) (Fig. 23a); a decrease in NOS-2 mRNA expression (4.7-fold) (Fig. 23b); while it had no significant effect on SOD1 mRNA expression (Fig. 23c). However, curcumin alone had

no effect on the mRNA expressions of these important enzymes when compared to saline control.



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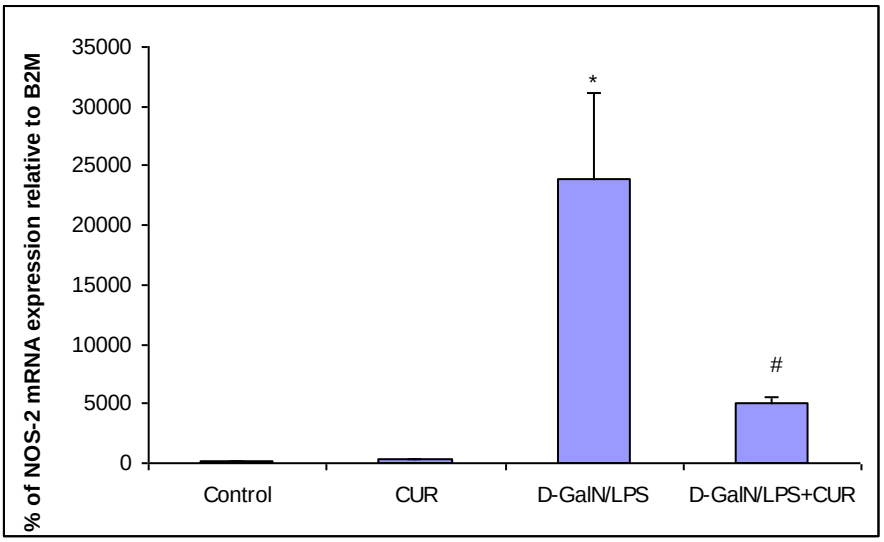

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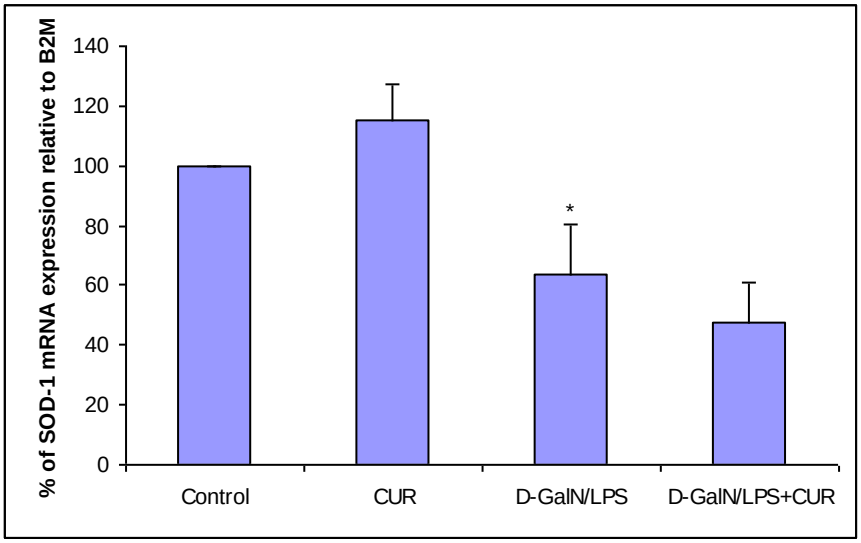


Fig. 23. *The effects of curcumin pre-treatment on HO-1(a), NOS-2 (b) and SOD1 (c) mRNA expressions in D-GalN/LPS hepatotoxicity model. The results are expressed as a relative percentage of gene expression against beta-2 microglobulin as the endogenous control. Control: vehicle only; CUR: curcumin 100 mg/kg; D-GalN/LPS: D-galactosamine 400 mg/kg + lipopolysaccharide 10 μg/kg; D-GalN/LPS + CUR: combination of D-galactosamine, lipopolysaccharide and curcumin pre-treatment. * Indicates significant values (p≤0.05) compared to the control group (vehicle only); # Indicates significant values (p≤0.05) compared to the respective positive control group (D-GalN/LPS). The results are expressed as means ± SEM, n=7.*

Additionally, the effects of curcumin on the pro-inflammatory cytokine TNF-α mRNA expression levels were evaluated. It can be seen that D-GalN/LPS toxicity significantly increases the expression of this important cytokine. As might be expected, curcumin significantly decreased the mRNA level of this cytokine in the D-GalN/LPS rats, indicating the potential anti-inflammatory effect (Fig. 24.).

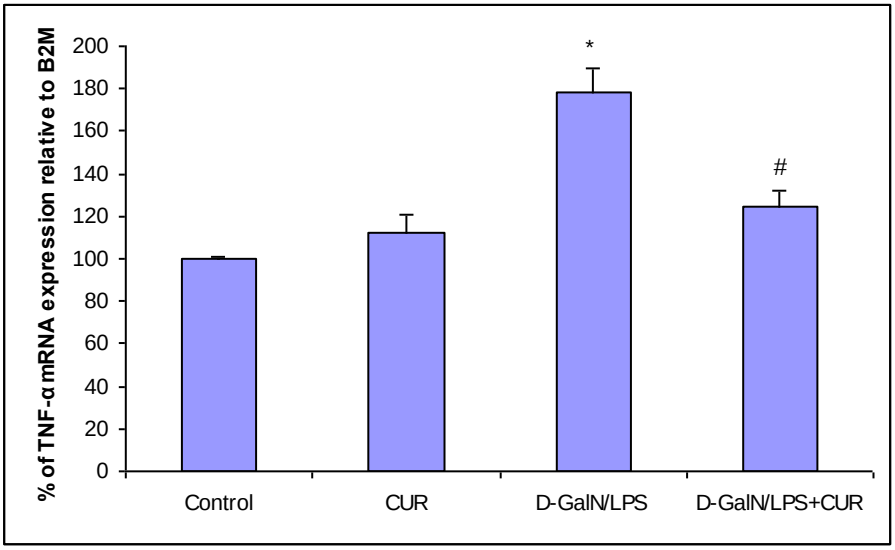
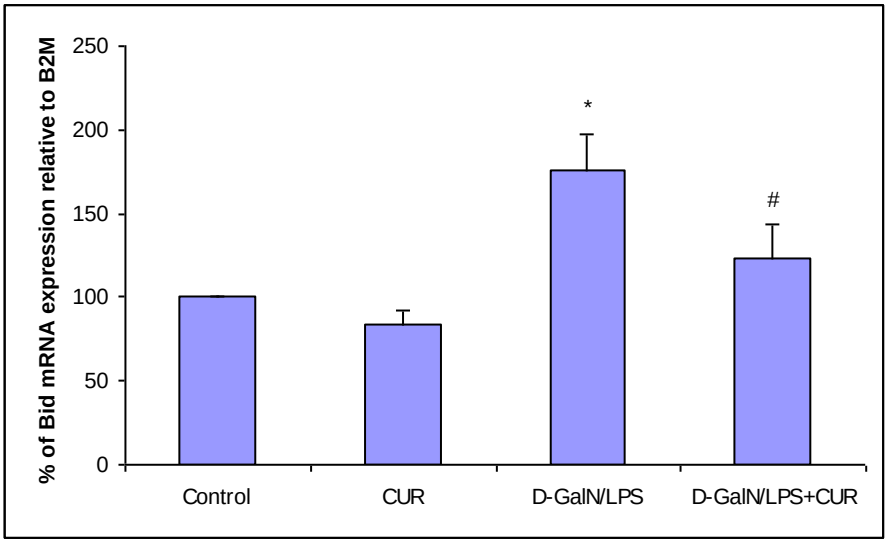


Fig. 24. *The effects of curcumin pre-treatment on TNF-α mRNA expressions in D-GalN/LPS hepatotoxicity model. The results are expressed as a relative percentage of gene expression against beta-2 microglobulin as the endogenous control.* ***Control:*** *vehicle only; CUR: curcumin 100 mg/kg; D-GalN/LPS: D-galactosamine 400 mg/kg + lipopolysaccharide 10 μg/kg; D-GalN/LPS + CUR: combination of D-galactosamine, lipopolysaccharide and curcumin pre-treatment. * Indicates significant values (p≤0.05) compared to the control group (vehicle only);); # Indicates significant values (p≤0.05) compared to the respective positive control group (D-GalN/LPS). The results are expressed as means ± SEM, n=7.*

6.3.3 The effects of curcumin treatment on apoptotic parameters in D-GalN/LPS hepatotoxicity model

The mRNA expressions measurements of Bid, Bax and Caspase 3 (Casp3) were used as determinants of potential apoptotic process. As might be expected, the administration of D-GalN/LPS has resulted in significant increases in all three parameters; Bid (1.5-fold) Fig. 25a; Bax (2-fold) Fig 25b; and Casp3 (1.4-fold) Fig 25c. Pre-treatment with curcumin reduced the apoptotic process, as can be seen by statistically significant decreases of both Bid and Bax mRNA expressions in D-GalN/LPS treated rats. There was no statistically significant difference in Casp3 mRNA expression, despite the noted decrease upon curcumin pre-treatment in D-GalN/LPS rats. Importantly, curcumin alone had no effect on the mRNA expressions of these apoptotic markers when compared to saline control.



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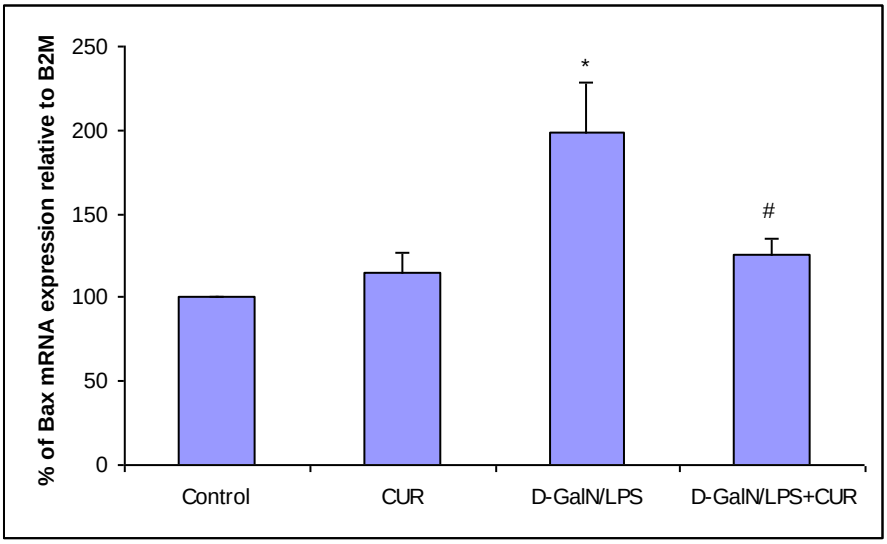

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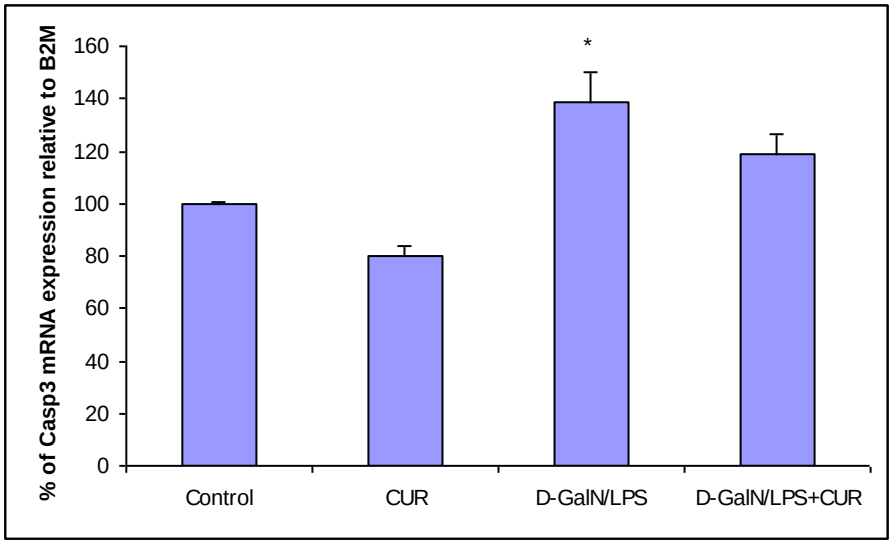


Fig. 25. *The effects of curcumin pre-treatment on Bid (a), Bax (b) and Casp3 (c) mRNA expressions in D-GalN/LPS hepatotoxicity model. The results are expressed as a relative percentage of gene expression against beta-2 microglobulin as the endogenous control.* *Control: vehicle only; CUR: curcumin 100 mg/kg; D-GalN/LPS: D-galactosamine 400 mg/kg + lipopolysaccharide 10 µg/kg; D-GalN/LPS + CUR: combination of D-galactosamine, lipopolysaccharide and curcumin pre-treatment. * Indicates significant values ($p \leq 0.05$) compared to the control group (vehicle only); # Indicates significant values ($p \leq 0.05$) compared to the respective positive control group (D-GalN/LPS). The results are expressed as means ± SEM, n=7.*

6.4 The effects of quercetin treatment on *in vivo* D-GalN/LPS xenobiotic hepatotoxicity model

6.4.1 The ameliorative effects of quercetin on D-GalN/LPS hepatotoxicity

In this study, the influence of quercetin treatment (*i.p.*, 50 mg/kg) on D-GalN (*i.p.*, 400 mg/kg) and LPS (*i.p.*, 10 μg/kg) model of hepatotoxicity was tested. The results show that male Wistar rats treated with the combination of D-GalN/LPS developed acute hepatotoxicity within 24 hours of the insult. This can be confirmed by a several-fold increase in plasma levels of transaminases (AST and ALT) that are indicative of failing liver function (Table 4).

Table 4 : *The effects of quercetin treatment on plasma levels of ALT, AST, bilirubin and catalase in D-GalN/LPS hepatotoxicity model.*

	Control	**Q**	**D-GalN/LPS**	**D-GalN/LPS + Q**
ALT [U/l]	10.35 ± 0.11	29.08 ± 3.68	291.09 ± 35.53 *	51.59 ± 3.64 #
AST [U/l]	74.53 ± 4.91	60.51 ± 7.29	896.56 ± 135.64 *	151.85 ± 20.79 #
Bilirubin [μmol/l]	3.03 ± 0.41	3.23 ± 0.49	19.66 ± 1.13 *	20.18 ± 1.99
Catalase [μg/ml]	61.21 ± 4.88	49.52 ± 10.20	336.50 ± 14.92 *	273.46 ± 33.24

Control: negative control group receiving vehicle only; Q: quercetin 50 mg/kg; D-GalN/LPS: D-Galactosamine 400 mg/kg with lipopolysaccharide 10 μg/kg; D-GalN/LPS + Q: combination of D-galactosamine, lipopolysaccharide and quercetin treatment;
** indicates significant values ($p \leq 0.05$) compared to the control group (vehicle only); # indicates significant values ($p \leq 0.05$) compared to the respective positive control group (D-GalN/LPS). The results are expressed as means ± SEM, n=6.*

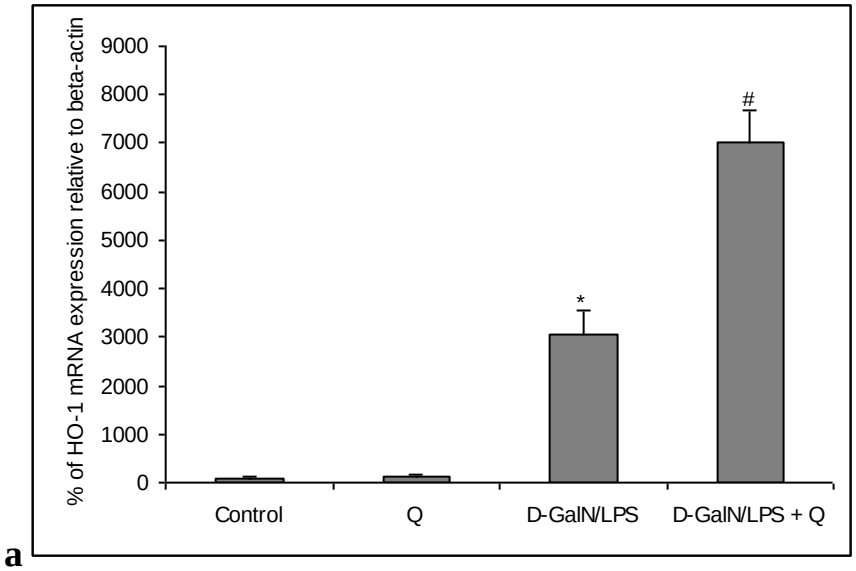
Quercetin alone had no influence on the plasma levels of these two liver enzymes. Interestingly, quercetin (Q) treatment in D-GalN/LPS rats resulted in significant lowering of the ALT (5.6-fold) and AST (5.9-fold) plasma levels, compared to the group receiving

the hepatotoxic D-GalN/LPS combination. Thus, this data suggests a clear hepatoprotective effect as a result of quercetin treatment (Table 4).

The plasma level measurements of the antioxidant molecule and product of heme degradation pathway, bilirubin, and antioxidant enzyme catalase were used as indicators of the oxidative status. In the groups treated with D-GalN/LPS, we observed significant 6.4-fold increase in bilirubin and 5.5-fold increase in catalase plasma levels. Quercetin treatment had no effect on the levels of these two antioxidants as can be seen by comparable values between D-GalN/LPS and D-GalN/LPS+Q groups (Table 4).

6.4.2 The effect of quercetin on HO-1, NOS-2 mRNA and protein expressions in D-GalN/LPS model of hepatotoxicity

The second question to be addressed in this study was to determine whether, and how, quercetin influences the activity of the potent antioxidant enzyme HO-1, as well as that of the enzyme NOS-2 in D-GalN/LPS model of hepatotoxicity. As postulated, both HO-1 mRNA and protein expressions were significantly increased (30.6-fold and 2-fold respectively, $p \leq 0.05$) in D-GalN/LPS group compared to control (Fig. 26). Conversely, quercetin alone did not exert any significant changes in HO-1 mRNA nor protein expressions, as the levels were comparable to those seen in the control group. However, when given to D-GalN/LPS treated rats, quercetin significantly ($p \leq 0.05$) increased the levels of HO-1 mRNA expression (by 2.3-fold) and protein expression (by 1.2-fold) in comparison to the group receiving hepatotoxic D-GalN/LPS combination (Fig. 26). These results clearly indicate the induction of HO-1 under hepatotoxic conditions, where quercetin further increased its activity under hepatotoxic, but not under physiological, conditions.



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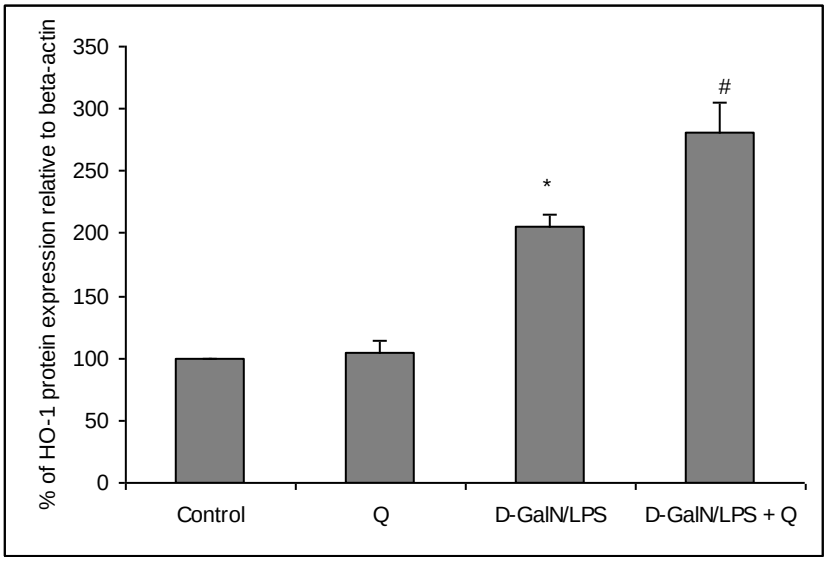

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Fig. 26. *Increase in HO-1 gene and protein expressions by quercetin treatment in D-GalN/LPS hepatotoxicity model. Gene expression of HO-1 (a). The results are expressed as relative percentage of HO-1 gene expression against beta-actin as the endogenous control. Protein bands (b) and optical density (c) of HO-1 protein expression. The results are expressed as relative percentage of optical densities of HO-1 against beta-actin as*

*the reference; Control: vehicle only; Q: quercetin 50 mg/kg; D-GalN/LPS: D-galactosamine 400 mg/kg + lipopolysaccharide 10 µg/kg; D-GalN/LPS + Q: combination of D-galactosamine, lipopolysaccharide and quercetin treatment. * Indicates significant values (p≤0.05) compared to the control group (vehicle only); # Indicates significant values (p≤0.05) compared to the respective positive control group (D-GalN/LPS). The results are expressed as means ± SEM, n=6*

Furthermore, D-GalN/LPS challenge resulted in a significant 12.3-fold increase in NOS-2 mRNA and 2.4-fold increase in NOS-2 protein expressions in comparison to the control group ($p \leq 0.05$) (Fig. 27). Interestingly, however, there was no significant difference in NOS-2 mRNA and protein expressions between the D-GalN/LPS and D-GalN/LPS+Q groups, despite the notice of slight decrease in these levels upon addition of quercetin (Fig. 27). Moreover, quercetin did not show to have any influence on NOS-2 mRNA and protein expressions when given under normal physiological conditions (Fig. 27).

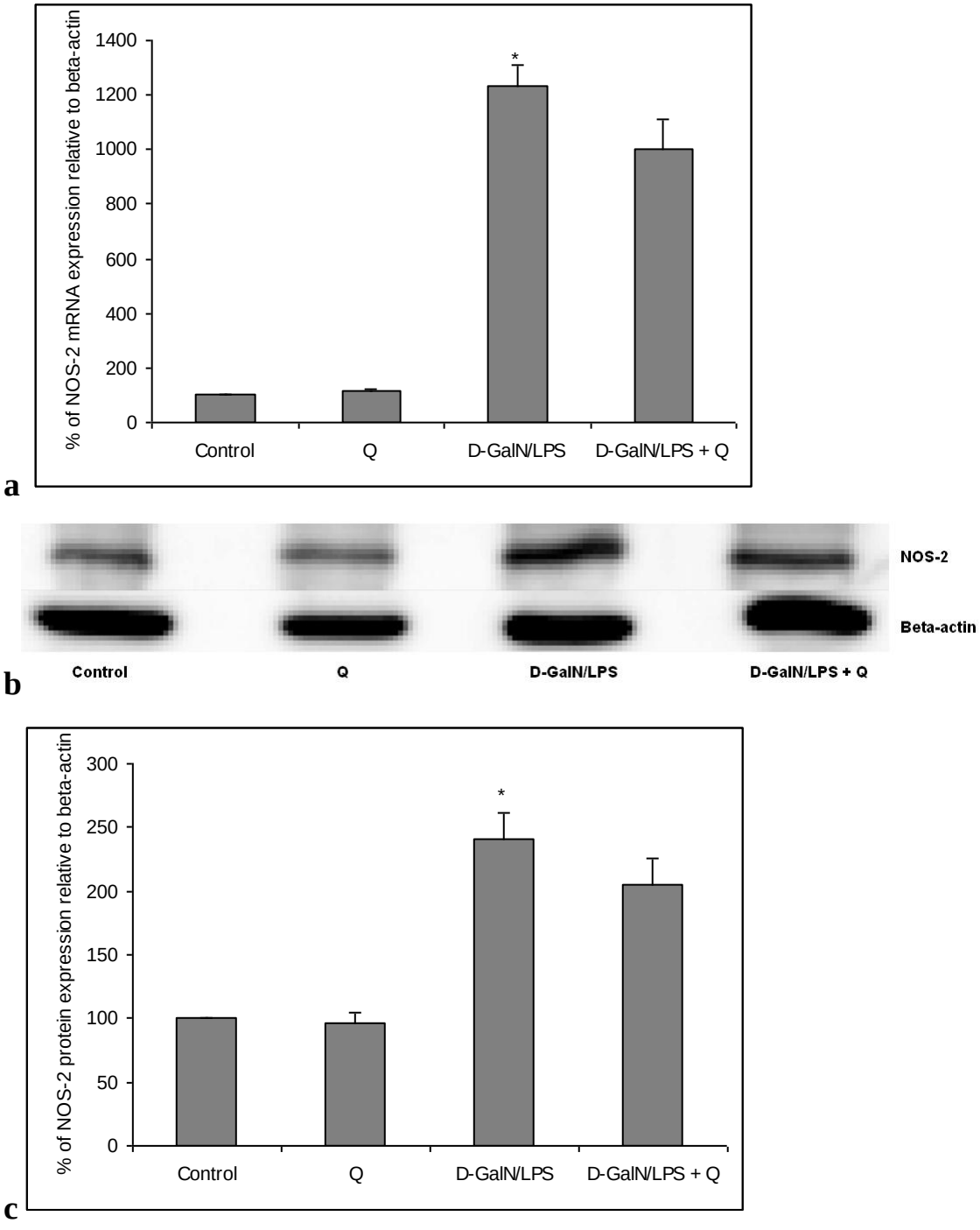

Fig. 27. *The effects of quercetin treatment on NOS-2 on gene and protein expressions in D-GalN/LPS hepatotoxicity model*. Gene expression of NOS-2 (A). *The results are expressed as relative percentage of NOS-2 gene expression against beta-actin as the endogenous control. Protein bands (B) and optical density (C) of NOS-2 protein expression. The results are expressed as relative percentage of optical densities of HO-1*

*against beta-actin as the reference; Control: vehicle only; Q: quercetin 50 mg/kg; D-GalN/LPS: D-galactosamine 400 mg/kg + lipopolysaccharide 10 μg/kg; D-GalN/LPS + Q: combination of D-galactosamine, lipopolysaccharide and quercetin treatment. * Indicates significant values (p≤0.05) compared to the control group (vehicle only); The results are expressed as means ± SEM, n=6.*

Additionally, the levels of nitrites measured in plasma closely correlated to both NOS-2 mRNA and protein expression in the tested groups as was expected (Fig. 28).

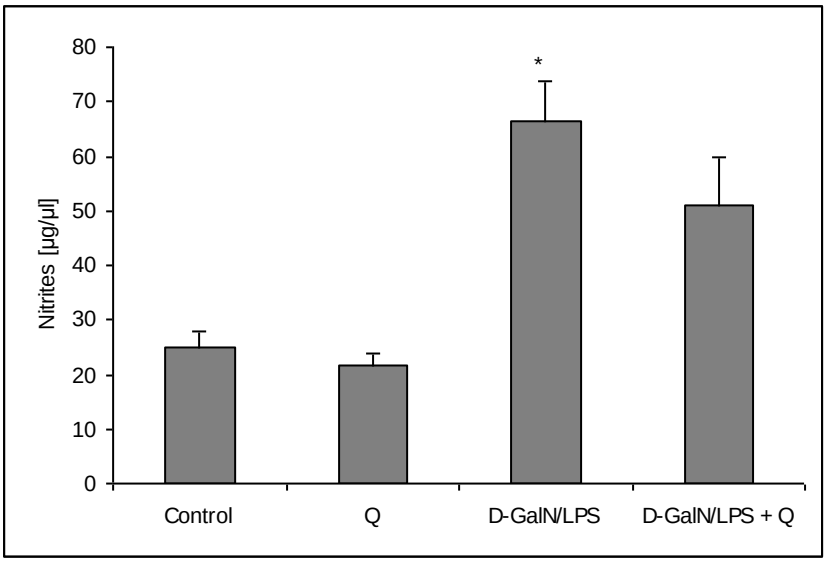

Fig. 28. *The effects of quercetin treatment on NOS-2 on plasma nitrite levels in D-GalN/LPS hepatotoxicity model. Control: vehicle only; Q: quercetin 50 mg/kg; D-GalN/LPS: D-galactosamine 400 mg/kg + lipopolysaccharide 10 μg/kg; D-GalN/LPS + Q: combination of D-galactosamine, lipopolysaccharide and quercetin treatment. *Indicates significant values (p≤0.05) compared to the control group (vehicle only); The results are expressed as means ± SEM, n=6.*

7 DISCUSSION

Xenobiotic hepatotoxicity is a widely studied subject and recent advances in the studies of toxicogenomics have been useful in elucidating various pathways of hepatotoxicity. Despite this, there is still information missing in regards to the underlying processes that are responsible for the ensuing cell damage. It has been widely accepted that the oxidative stress and apoptosis play a major role, however the precise details in regards to the mechanisms involved remains to be explored. It is hoped that once these mechanisms are identified, they can act as targets of potential therapeutic drugs that would act to ameliorate the underlying damage. In the recent years there was a growing interest in substances isolated from dietary sources as they were shown to exhibit several cytoprotective properties and are widely available. Among those are quercetin, found in various fruits and vegetables, and curcumin, a component of the popular Indian spice turmeric. Further research is needed to confirm these results as well to gain a mechanistic understanding of toxic changes that occur in the liver.

In this scientific work, two different models of hepatotoxicity were employed, *in vitro* TBOOH and *in vivo* D-GalN/LPS model. The involvement of various oxidative stress and apoptotic parameters was evaluated. The study focused on testing the hypothesis that during the events of oxidative stress, there will be an increase in gene expression of both antioxidant (eg. HO-1, SOD1, GPx, CAT) and pro-oxidant (eg.NOS-2) enzymes. Extensive oxidative stress will lead to activation of cell death mediators (eg.Bid, Bax, Casp3) and/or inflammatory cytokines (eg.TNF-α). Furthermore, this study also focused on the hypothesis that agents which are able to alter the expression of these before mentioned anti-/pro-oxidant enzymes and apoptotic mediators have a potential in modulation of hepatotoxicity. Thus, these agents would be able to increase expressions of antioxidant enzymes, decrease expressions of pro-oxidant enzymes and decrease levels of pro-apoptotic and inflammatory mediators. The influence of two dietary substances curcumin and quercetin as potential hepatoprotectants has been explored in this study.

The following paragraphs will aim to explicate the results of this scientific work with the special focus on the two hypotheses that were raised, along with the set aims of the study.

7.1. The molecular mechanisms of oxidative stress and apoptosis in xenobiotic hepatotoxicity models

To test the first aim, the elucidation of the underlying molecular mechanism of xenobiotic hepatotoxicity in regards to the involvement of oxidative stress and apoptotic cell death pathways, two models of hepatotoxicity were explored, one *in vitro* (Tert – butyl hydroxyperoxide) and the other *in vivo* (D-GalN/LPS). The study specifically focused on the evaluation of activities of antioxidant enzymes (HO-1, SOD1, GPx, CAT), pro-oxidant enzyme (NOS-2), inflammatory cytokine (TNF-α) and apoptotic mediators (Bid, Bax, Casp3) involved in the given hepatotoxicity models. As well, whether there exists a potential mutual relationship between the given parameters.

The varying mechanism of action make TBOOH an excellent hepatotoxicity model for studies focused on understanding oxidative stress and cell death processes (Cerny *et al*. 2009, Kim *et al.* 2007, Mingcang *et al.* 2010). In short, TBOOH is able to decrease cellular antioxidant status, increase intracellular ROS production and lipoperoxidation, activate caspase 3, reduce mitochondrial membrane potential and increase cytochrome C release resulting in initiation of cell death pathways (Ghosh *et al*. 2011, Vidyashankar *et al.* 2010). As well, this substance is soluble in Williams E media making it suitable for conducting *in vitro* experiments.

In the *in vitro* study, 24 hours following the treatment of hepatocytes with TBOOH resulted in severe cytotoxicity as noted by significantly increased levels of ALT and AST, and more than half reduction of cell viability. This was measured by the levels of formazan formation by the viable cells, also known as MTT test. Moreover, there were significant parallel increases in the mRNA expressions of the NOS-2 and pro-inflammatory cytokine TNF-α, but there were no significant changes in the HO-1 mRNA expressions as a result of TBOOH insult. The increased expression of NOS-2 may trigger an augmented production of nitric oxide, and consequently result in increase in reactive nitrogen species (RNS) formation. These highly reactive molecules are able to directly attack various cellular components, thus inducing nitrosative stress which is detrimental to proper functioning of the cell. It can be concluded that the noted decrease in cell

viability of TBOOH treated cells is due to the accumulation of RNS and pro-inflammatory cytokine TNF-α, all of which contribute to the initiation of the cell death pathways. Despite the noted increases in the cytoprotective HO-1 mRNA expressions, there were no significant results that would indicate a potential relationship in this model of hepatotoxicity between the cytoprotective HO-1 and the proinflammatory NOS-2 and TNF- α.

The combination of D-GalN/LPS is a useful *in vivo* model of hepatotoxicity, as it resembles hepatitis of various etiologies. In short, D-galactosamine exerts its damaging actions on the cell through inhibition of protein synthesis by depleting uridine triphosphate pool, resulting in early generation of reactive oxygen species and apoptosis (Choi *et al.* 2011, Gonzalez *et al.* 2009, Sun *et al*. 2003). On the other hand, lipopolysaccharide increases the release of pro-inflammatory cytokines (mainly TNF-α), induces the release of reactive nitrogen species and inflammatory prostaglandins due to the activation of NOS-1 and cycloxygenase 2 (COX-2) respectively (Chakravortty *et al.* 2001, Devaux *et al*. 2001, Nolan 2010).

In this study, 24h following the D-GalN/LPS insult there was a noted increase in the levels of ALT and AST, which are indicative of failing liver function and are cardinal features of liver toxicity. On the other hand, the extent of lipid peroxidation was of no significance in this model, as the levels of conjugated dienes and the measured TBARS in plasma of D-GalN/LPS treated animals were not different from those of the control group.

One of the important physiological antioxidants is the reduced glutathione (GSH), which protects cells from oxidative injury by scavenging reactive oxygen/nitrogen species. Interestingly, a homeostatic decrease in the GSH pool can make cells more vulnerable to further damage by toxins (Ballatori *et al*. 2009). In this study, the GSH pool has remained intact as the measured GSH levels were similar between the control and D-GalN/LPS group. In addition to the antioxidant action of GSH, the antioxidant enzymes SOD1, GPx1 and catalase work together to counteract the oxidation of proteins, lipids and DNA, by removing reactive oxygen species from the cell (Yuan and Kaplowitz 2008). Specifically, SOD reduces the harmful superoxide into hydrogen peroxide, which is further reduced to water by the action of catalase and glutathione peroxidase (Valdivia

et al. 2009). It is noteworthy that in this study, gene expression of SOD1 decreased significantly, while that of the GPx1 remained unchanged. Catalase was significantly induced by D-GalN/LPS as was seen by significantly increased levels in plasma. It might be expected that under the present experimental conditions, the responses of these three parameters would be increased in parallel, however, under D-GalN/LPS toxicity the expected mutual relationship of these antioxidant enzymes was not seen. Differential response of these enzymes may be dependent on the dictating cellular needs in fight against increased levels of reactive oxygen species in induced oxidative stress states (Djordjevic *et al*. 2010).

One of the ways lipopolysaccharide exerts its cellular harm is by the stimulation of production of pro-inflammatory cytokine TNF-α by the Kupffer cells (Lichtman *et al.* 1994). It is well known that the high levels of TNF-α increase the expression of NOS-2 enzyme, whose product nitric oxide can in turn stimulate additional production of TNF-α and amplify the inflammatory damage (Sass *et al.* 2001). However, not everything is clear cut, as nitric oxide plays a complex role in both oxidative stress and cell death responses. For instance, this small molecule is thought to play a dual role in apoptosis acting as both pro-apoptotic and anti-apoptotic mediator depending on various cellular conditions and cell types (Brune 2005, Chung *et al.* 2001). Several studies have shown that NO stimulates the pro-apoptotic caspase 3 activity and thus commits the cell to apoptosis (Obara *et al.* 2010). On the other hand, earlier studies on hepatocytes have shown that nitric oxide exerts anti-apoptotic action through direct inhibition of caspase activity by S-nitrosylation, resulting in prevention of Bcl-2 cleaveage and cytochrome C release (Kim *et al*. 1998, Li *et al*. 1997). The activity of nitric oxide in this study was reflected in the measurement of its oxidation end product nitrite in plasma. The levels of nitrites were significantly increased upon the D-GalN/LPS treatment which was paralleled with the gene expression of NOS-2 enzyme as was expected. As well, there was a noted parallel increase in both NOS-2 and TNF-α mRNA expressions due to D-GalN/LPS administration as noted in the second part of this study dealing with hepatoprotective effects of curcumin.

Moreover, the pathophysiological conditions of the liver involving oxidative stress initiate the upregulation of HO-1 and cause an increase in products of heme

degradation pathway (Ryter and Choi 2012, Vardanian *et al.* 2008). The products, biliverdin/bilirubin and CO, are the key mediators of HO-1 mediated cytoprotection for the reason that they help restore intracellular homeostatic balance and reduce the release of pro-inflammatory cytokines (Gomes *et al.* 2010, Paine *et al.* 2010, Wu and Wang 2005). For instance, it has been shown that the cooperative action of biliverdin/bilirubin and CO is responsible for the prolonged survival of mice due to TNF-α reduction in D-GalN/LPS toxicity model (Sass *et al*. 2004). Additionally, the induced enzyme heme oxygenase-1 has the ability to exert its cytoprotective effect through the inhibition of inflammatory NOS-2 induction and a decrease in Caspase 3 activity as is noted in some studies (Sass *et al.*, 2003, Wen *et al.* 2007). One study has shown that exogenous addition of CO gas helped ameliorate D-GalN/LPS induced acute liver injury in mice by reducing inducible NOS/NO production, inhibiting hepatocyte apoptosis and reducing the release of pro-inflammatory cytokines (Wen *et al.* 2012). This is in accordance to the present findings of this analysis, in so far as the relationship between HO-1 and billirubin from one hand and NOS-2 and TNF- α gene expression on the other hand. Thus the observed increase in HO-1 expression in this study is a result of the cellular adaptive response in times of oxidative stress, as this enzyme and its products are potent antioxidants and anti-inflammatory mediators (Lehmann *et al*. 2010, Pae and Chung 2009, Zhu *et al.* 2008).

Interdependence of members of Bcl-2 pro-apoptotic and antiapoptotic proteins through their action on mitochondrial permeability pores, cytochrome C release and activation of caspases plays an important role in apoptotic cell death (Chipuk and Green 2008, Garcia-Saez *et al.* 2010). The data from the past and current literature indicates that both D-Galactosamine and lipopolysaccharide cell death is involves TLR-mediated inflammatory pathway that results in increased levels of TNF-α, NF-κB, IFN-β, c-Jun N-terminal kinases, as well the activation of executioner caspases, which further commits the hepatocytes to cell death. (Ben *et al*. 2012, Kang *et al*. 2013, Morikawa *et al*. 1996, Yan *et al.* 2013). Similarly in this study, we have observed the measured increases in the gene expressions of Bcl-2 pro-apoptotic members, Bid and Bax, as well as that of Casp 3 that D-GalN/LPS treated rats. This signifies initiation of the apoptotic pathways. This was further confirmed by the presence of pycnotic nuclei as seen in the morphological evaluation of the liver tissue of D-GalN/LPS treated rats. In addition, marked

disintegration of hepatocyte cytoplasm shows a necrotic continuation following process of apoptotic cell death. Typical changes and aggregation of heterochromatin near the nuclear envelope testify an occurrence of apoptosis in some hepatocytes. These findings support the concept of the presence of apoptosis which was followed by necrotic changes, in other words aponecrotic cell death.

Overall, the given results demonstrate complex involvement and potential interrelationships between several different mediators of oxidative stress and apoptosis. Given the known and expected functions of the mentioned parameters, it can be seen that the induction of NOS-2, TNF-α, and pro apoptotic Bid, Bax and Casp3 is associated with cellular damage in the models tested. As well, the parallel increase in antioxidants HO-1, bilirubin, catalase and glutathione peroxidase is due to the induction of cellular protective mechanism that are triggered as an adaptive response in the D-GalN/LPS hepatotoxicity model. The acquired knowledge from the results of these hepatotoxic studies paves a way for studying substances that are able to modulate these selected parameters in regards to further enhancing cytoprotective actions and halting the cytotoxic processes, therefore acting as potential hepatoprotective agents.

7.2 The molecular mechanisms of cytoprotective dietary agents, curcumin and quercetin

The second part of this study focused on evaluating dietary substances quercetin and curcumin as potential hepatoprotective agents in regards to their ability to ameliorate hepatotoxicity. It was hypothesized that the hepatoprotective agents would be able to increase expressions of antioxidant enzymes, decrease expressions of pro-oxidant enzymes and decrease levels of pro-apoptotic and inflammatory mediators.

Quercetin has several hepatoprotective properties including a strong antioxidant potential in ameliorating alcoholic liver disease; it possesses anti-HBV activity; it is able to downregulate expression of inflammatory mediators in cirrhotic rats and induce cell death in hepatoma cell lines (Chang *et al* 2006, Cuevas *et al* 2011, Liu *et al* 2010, Nussler *et al* 2010, Oliva *et al* 2011, Tian *et al* 2010). Despite these known beneficial effects the exact mechanisms of quercetin's cytoprotection need to be further explored. Given our experience with dietary polyphenols, as well as those of others, it has been seen that the

induction of HO-1 and concomitant reduction of NOS-2 activities are responsible for the cytoprotective action of these substances (Cerny *et al.* 2011, Chen *et al.* 2005, Farghali *et al.* 2009, Zhang *et al.* 2011). Thus, indicating a kind of crosstalk between the two protein molecules including other cascade of effects which led to cytoprotection. Therefore, this study focused on the assessment of potential hepatoprotective effects of quercetin in regards to its modulation of these two inducible enzymes that play important roles in oxidative stress in both aforementioned *in vitro* and *in vivo* models.

In the *in vitro* model of hepatotoxicity, a range of quercetin and TBOOH concentrations were used to gain a better understanding of the parameters tested. Quercetin significantly decreased the levels of aminotransferases and increased the cell viability of the cultured cells treated with cytotoxic TBOOH, clearly demonstrating a cytoprotective action. As for the influence of quercetin on mRNA expressions of HO-1, NOS-2 and TNF-α, the results showed that quercetin had an HO-1 inducing effect; it effectively lowered TNF-α mRNA expressions; whereas it did not have any effect on NOS-2 mRNA expressions in TBOOH treated cells. These results confirm the previous discussion that the induction of the antioxidant HO-1 is associated with increased cellular defense. Surprisingly this cytoprotective effect was not associated with the parallel inhibition of NOS-2 enzyme as might be expected given the findings of the aforementioned studies. On the other hand, the observed parallel decrease in TNF-α mRNA expression is a contributing factor to quercetin's cytoprotective mechanisms in this model. Several other studies have also confirmed cytoprotective effect of quercetin in the TBOOH model of toxicity, involving mechanisms that include decrease in ROS generation, increase in total glutathione levels, decrease in lipid peroxidation and increase in radical scavenging activity among others (Akhlaghi and Bandy 2012, Moretti *et al.* 2012, Oidovsambuu *et al.* 2013). Hence, our findings give an additional insight into the mechanisms of quercetin's cytoprotection in this experimental model of hepatotoxicity.

As for the *in vivo* model of hepatotoxicity that resembles clinical hepatitis, quercetin treatment effectively improved the liver damage by lowering the levels of the aminotransferases (ALT and AST) that were raised initially due to D-GaLN/LPS insult. Measurements of the plasma levels of the enzyme catalase were used as an indicator of the physiological antioxidant status of the cell. Quercetin did not have any significant

effect on lowering the increased plasma levels of catalase as a result of D-GalN/LPS administration, indicating that the increased antioxidant status of the cell was maintained in order to combat the undergoing oxidative stress processes. For instance, the presence of catalase in mitochondria of hepatocytes is known to have important implications in prevention of reactive oxygen species generation and the consequent activation of pro-apoptotic pathways induced by TNF-α (Salvi *et al.* 2007).

As was expected, the administration of D-GalN/LPS significantly increased both HO-1 gene and protein expressions, including the plasma levels of its product bilirubin. The augmented levels of bilirubin are of importance since this potent antioxidant ameliorates oxidative stress by scavenging peroxyl radicals, consequently preventing oxidation of fatty acids and proteins (Paine *et al.* 2010). Treatment with quercetin resulted in a further increase of HO-1 gene and protein activities in D-GalN/LPS+Q rats, which is in agreement to our findings as well as to other studies demonstrating the cytoprotective effect of quercetin by induction of HO-1 (Chen *et al.* 2005, Yao et al. 2007, Zhang *et al.* 2011). However, this increase in HO-1 activity was not paralleled by a simultaneous increase in bilirubin plasma levels, as might have been expected. This could be explained by a postulated fine balance in bilirubin production, as the beneficial effects of this potent antioxidant is bypassed at higher serum levels resulting in cytotoxicity that targets the central nervous system (Kapitulnik 2004).

In regards to the second enzyme of interest, NOS-2, its gene and protein expressions and the nitrite levels were significantly increased by D-GalN/LPS treatment, which could be attributed to the LPS-induced NO production (Chakravortty *et al*. 2001, Mu *et al.* 2001). Some studies have reported that quercetin mediates its cytoprotective actions by inducing HO-1 which has suppressive actions on NO production (Chen *et al.* 2005, Zhang *et al.* 2011). However, in contrast to the results of this research work on curcumin and those of others on dietary polyphenols (Cerny *et al.* 2011, Chen *et al.* 2005, Farghali et al 2009, Zhang *et al.* 2011), quercetin did not have a significant reducing effect on NOS-2 activity or nitrate levels in both hepatotoxicity models. Thus, it could be postulated that there seems to be an alternative mechanism of HO-1 induction protection that is not related directly to NOS-2 activity reduction in these models among other possible mechanisms.

One possible explanation is that the products of HO-1 pathway, bilirubin and CO are mainly responsible for the quercetin mediated cytoprotection given their aforementioned cytoprotective actions. It could also be postulated that the mechanism of HO-1 cytoprotection is due to its effect on the upregulation of anti-inflammatory mediators such as IL-10, and inhibition of production of pro-inflammatory cytokines such as TNF- α, as has been noted in some studies (Park *et al.* 2010, Ryter *et al.* 2006). IL-10 acts to inhibit COX-2 activity resulting in a decrease in production of inflammatory prostaglandin E_2 that contributes to much of the damage of LPS insult (Berg *et al.* 2001). However, until now not much is known whether there exists a potential cross-talk between these two enzymes. On the other hand, the relationship between COX-2 and NOS-2 is such that the peroxynitrite radicals resulting from increased NOS activity are able to inhibit the activation of COX-2 by tyrosine nitration (Devaux *et al.* 2001). Thus, high NOS-2 activity could contribute to reduction of pro-inflammatory damage caused by COX-2. The discussed possible mechanism of the quercetin cytoprotection are summarized in the figure 29.

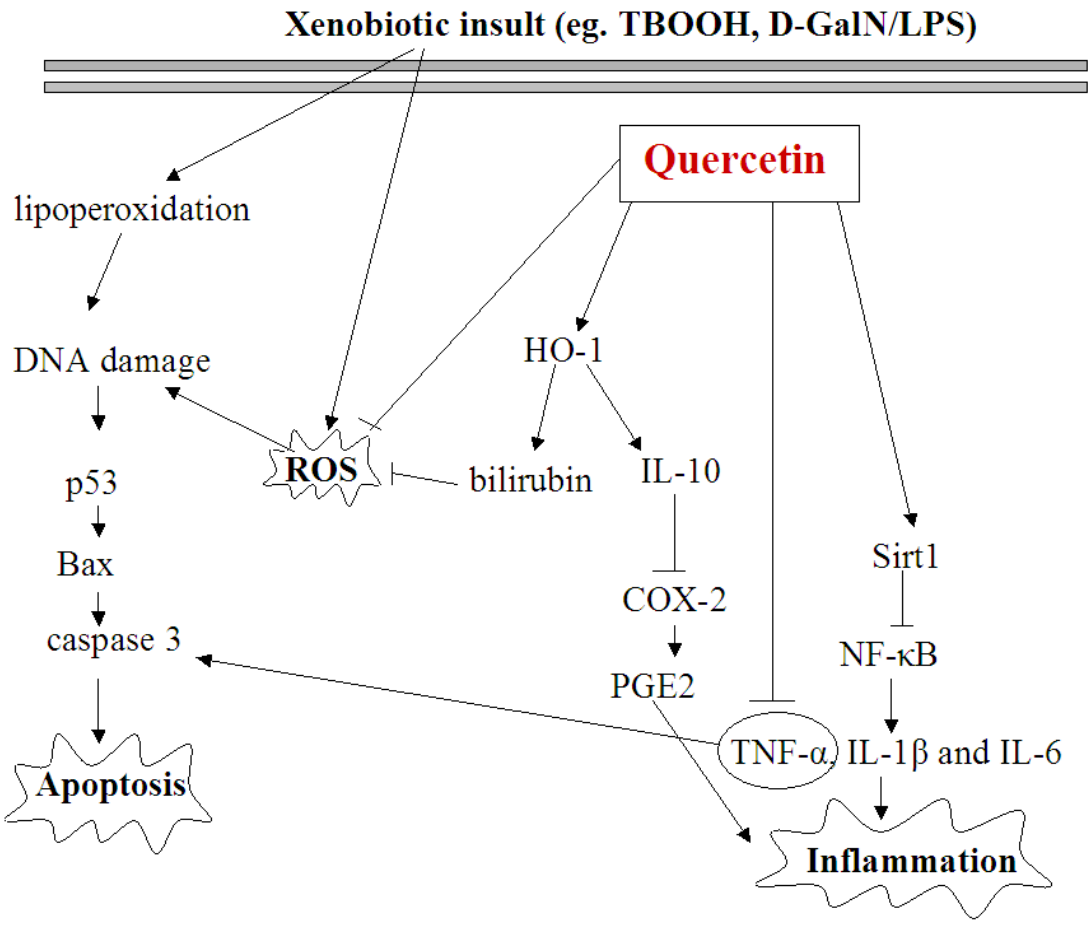

Fig. 29. Molecular mechanisms of potential cytoprotective effects of quercetin.

Another presumptions is that quercetin was extensively metabolized into its metabolite quercetin-3'-sulfate, which does not possess NOS-2 and NO lowering properties (Chen *et al.* 2005, Cho *et al.* 2012). Moreover, an important finding is that quercetin administration to healthy rats without a presence of a toxicant did not produce any significant changes on the tested parameters. It could be assumed, therefore, that there exist different regulation mechanisms which are responsible for the differences seen under physiological versus pathological conditions.

Moving on to our second dietary substance of interest, curcumin, its cytoprotective, anti-inflammatory and antitumor properties are based on several mechanisms, none of which have been extensively explored in amelioration of liver diseases. Activation of important antioxidative enzymes such as heme oxygenase,

induction of sirtuin activity, inhibition of pro-inflammatory cytokines such as TNF-α are just some of the many proposed beneficial properties of this polyphenol (Farombi *et al.* 2008, Lima *et al.* 2011, Sikora *et al.* 2010).

As might have been anticipated, curcumin pretreatment notably improved liver function and decreased lipoperoxidation. This is reflected by a significant decreases in plasma levels of aminotransferases (ALT & AST) and homogenate levels of conjugated dienes in the given *in vivo* model of hepatotoxicity.

In view of the curcumin's effect on the oxidative status of the cell, pre-treatment with curcumin elevated the plasma levels of potent antioxidants catalase and bilirubin, and increased the HO-1 mRNA expression in D-GalN/LPS rats. This indicates an increase in the antioxidant status of the cell as a result of hepatotoxic injury, as curcumin when given alone did not significantly raise the levels of these potent antioxidants. On the other hand, curcumin pre-treatment had no effect on the SOD1 gene expression. It might be expected that under the present experimental conditions, the responses of these antioxidant parameters would be increased in parallel, which was demonstrated in regards to bilirubin, catalase and HO-1, but not SOD1.

Moreover, the potential relationship between mRNA expressions of HO-1, NOS-2 and TNF-α was also explored in this model. As previously discussed, HO-1 and its products bilirubin and carbon monoxide, exert much of their cytoprotective effect by decreasing the production of the pro-inflammatory cytokines (Sass *et al*. 2004), by inhibiting inflammatory NOS-2 induction and decreasing caspase 3 activity (Sass *et al.*, 2003, Wen *et al.*2003), which was in agreement with the results of this study. Similarly, a study done on macrophages demonstrated that curcumin's beneficial effects are due to an over-expression of HO-1, that is paralleled by inhibition of NOS-2 expression and NO production via the inactivation of the NF-κB (Kim *et al.* 2008).

The oxidative stress induced by lipopolysaccharide leads to TNF- α mediated apoptosis that involves activation of the executive caspases as well as proteins Bid and Bax (Morgan *et al.* 2010, Van Herreweghe *et al.* 2010). Our results have shown that curcumin pre-treatment resulted in a significant decrease in both Bid and Bax, but it had no significant effect on the Casp3 mRNA expressions in the D-GalN/LPS rats. It could be postulated that the those cells that have committed themselves to apoptosis due to the

activation of execution caspases were not affected by quercetin; Whereas curcumin had an inhibitory effect only on the early activators of apoptosis, Bid and Bax, thus preventing the potential activation of the downstream executioner caspases and the resulting cell death. However, it must be noted that when given alone curcumin had no significant effect on these parameters, suggesting that curcumin's effect is different in physiological versus pathological conditions. This molecule therefore pre-conditions the cell to increase its own antioxidant systems in combat of further oxidant challenge. This is relevant to the observed increase in HO-1 mRNA expressions, and concominant reduction in NOS-2, Bid and Bax mRNA expressions, demonstrating a potential relationship between these tested parameters. The following figure nicely summarizes the discussed cytoprotective molecular mechanisms of curcumin.

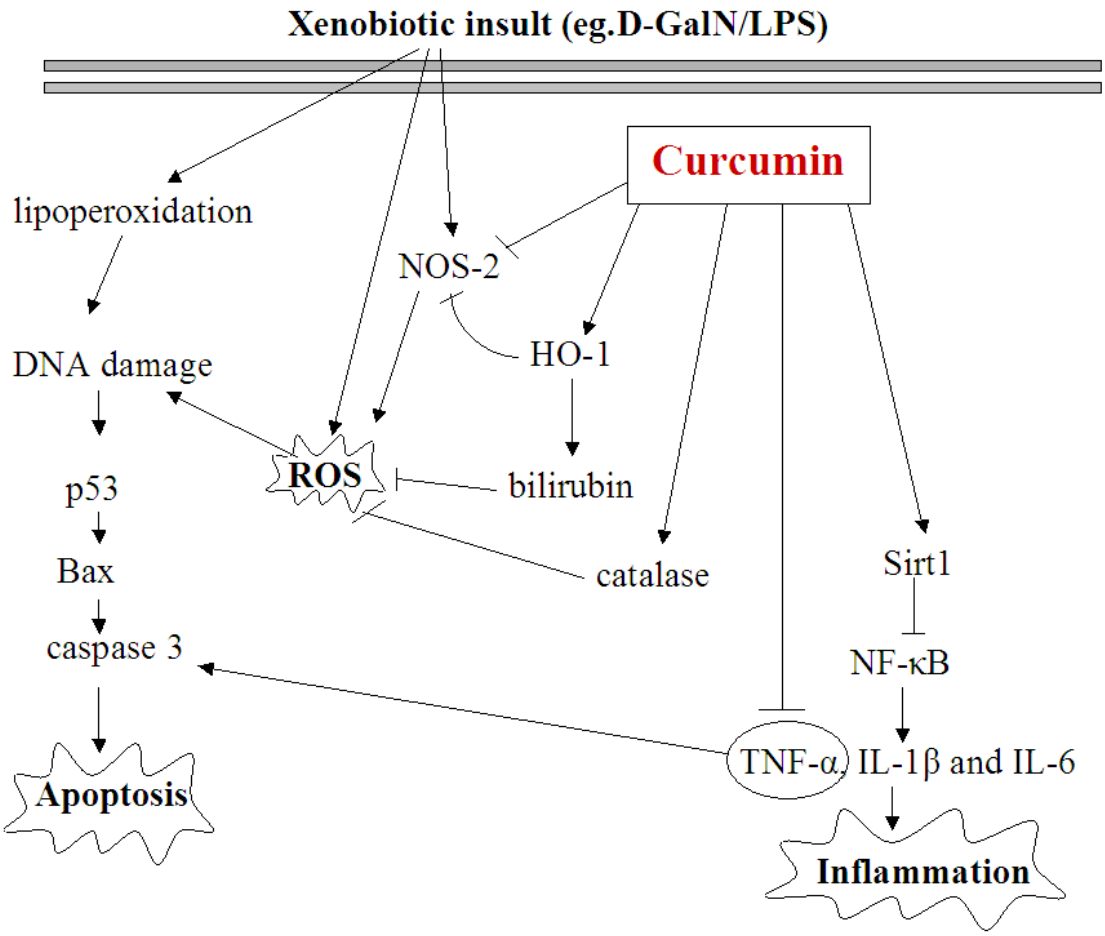

Fig. 30. Molecular mechanisms of potential cytoprotective effects of curcumin.

Another valuable cytoprotective effect of curcumin is the activation of Nrf2/ARE signaling pathway resulting in induction of several important antioxidative enzymes and sirtuins, a NAD+ dependent deacetylase responsible for multiple beneficial effects (Farghali *et al.* 2013, Lima *et al.* 2011, Sikora *et al.* 2010). The most widely studied sirtuin is the human sirtuin 1 (SIRT1) whose levels are reduced in several chronic diseases, while its enhanced activity is associated with improvement of these disease states. One of the beneficial effects is SIRT1 downregulation of NF-κB, a protein responsible for inflammatory cytokine production, including TNF-α, IL-1β and IL-6 (Chung *et al.* 2010). Interestingly, curcumin is also known to inhibit NF-κB, which may actually be attributed to its SIRT1 enhancing effect. In regards to its influence on apoptosis, enhanced SIRT1 activity results in negative regulation of transactivation factors leading to attenuation of pro-apoptotic mediators including Bcl-2 proteins, p53 and FOXO, while in response to TNF-α it inhibits the transcriptional activity of antiapoptotic factor NF-κB (Rajendran *et al.* 2011). Considering the role of SIRT1 in regulation of the aforementioned mechanisms, modulation of its activity by activators such as dietary polyphenols provides an attractive area of future research.

However, we must remain cautious in regards to many studies demonstrating various cytoprotective effects of these substances, as these dietary polyphenols can also be toxic in some conditions. Such is the example of quercetin's potent inhibiting effect on topoisomerase II that may cause double-strand DNA lesions at topoisomerase binding sites including the MLL gene, which can lead to development of secondary leukemias (Skibola and Smith 2000, Vanhees *et al.* 2011). Furthermore, the current studies focused on therapeutic potential of dietary polyphenols lack clinical data, as not much is known about their modes of action, target mechanisms and their pharmacokinetic profile. Another thing to keep in mind is that these polyphenols are usually poorly absorbed, rapidly and extensively metabolized, which may lead to their inactivation or formation toxic products. Thus the range of therapeutic doses of these substances must be established in clinical trials.

Overall, the results of this study have shown that both quercetin and curcumin exhibit cytoprotective effects through modulation of HO-1 and NOS-2 systems and inhibition of early initation of cell death. Both of these polyphenols induce HO-1,

however curcumin relies on concomitant NOS-2 activity reduction, while this is not the requirement with quercetin. The research data from the present study paves a way for those interested in further research of these dietary polyphenols as it gives an overview of the potential cytoprotective mechanisms and effective doses in the given models.

8 CONCLUSION

This research work aimed to evaluate various mechanisms of xenobiotic hepatotoxicity with the focus on the select parameters of oxidative stress and apoptosis.

In summary, both xenobiotic hepatotoxicity models resulted in differential oxidative stress response as reflected by the alterations in expressions of certain oxidant/antioxidant genes, while the expression of others remained unchanged. Even though our findings were not able to confirm a direct relationship between the oxidative and apoptotic parameters that were tested, a parallel relationship between selected enzymes' gene expressions and their respective biochemical markers was seen. The enzyme HO-1 has been shown to be the main regulator of cytoprotection in all tested models.

Moreover, the results of this study have shown that both quercetin and curcumin exhibit cytoprotective effects through modulation of HO-1, NOS-2 and/or TNF-α systems and inhibition of early initation of cell death. Both of these polyphenols induce HO-1, however curcumin relies on concomitant NOS-2 activity reduction, while this is not the requirement with quercetin. Thus quercetin's significant induction of HO-1 alone, without concomitant NOS-2 activity reduction, might be sufficient in combating cellular damage.

The research data from the present study paves a way for those interested in further research of these dietary polyphenols as it gives an overview of the potential cytoprotective mechanisms and effective doses in the given models.

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APPENDIX

Physiol. Res. 60: 549-558, 2011

Differential Oxidative Stress Responses to D-Galactosamine-Lipopolysaccharide Hepatotoxicity Based on Real Time PCR Analysis of Selected Oxidant/Antioxidant and Apoptotic Gene Expressions in Rat

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Summary

Oxidative stress and apoptosis are proposed mechanisms of cellular injury in studies of xenobiotic hepatotoxicity. This study is focused on addressing the mutual relationship and early signals of these mechanisms in the D-galactosamine and lipopolysaccharide (D-GalN/LPS) hepatotoxicity model, with the help of standard liver function and biochemistry tests, histology, and measurement of gene expression by RT-PCR. Intraperitoneal injection of 400 mg/kg D-GalN and 50 μg/kg LPS was able to induce hepatotoxicity in rats, as evidenced by significant increases in liver enzymes (ALT, AST) and raised bilirubin levels in plasma. Heme oxygenase-1 and nitric oxide synthase-2 gene expressions were significantly increased, along with levels of their products, bilirubin and nitrite. The gene expression of glutathione peroxidase 1 remained unchanged, whereas a decrease in superoxide dismutase 1 gene expression was noted. Furthermore, the significant increase in the gene expression of apoptotic genes Bid, Bax and caspase-3 indicate early activation of apoptotic pathways, which was confirmed by histological evaluation. In contrast, the measured caspase-3 activity remained unchanged. Overall, the results have revealed differential oxidative stress and apoptotic responses, which deserves further investigations in this hepatotoxicity model.

Key words

Hepatotoxicity • D-galactosamine/Lipopolysaccharide • Apoptosis • Oxidative stress • RT-PCR

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Introduction

Liver is vulnerable to cellular damage, due to its extensive exposure to high concentrations of xenobiotics. Fulminant hepatic failure (FHF) can be induced by viral infection or xenobiotic injury and its incidence in population is low: however, unless a liver transplantation is carried out the rates of mortality are high (Chan *et al.* 2009). Combination of D-galactosamine and lipopolysaccharide (D-GalN/LPS) is a well established experimental model for studies of FHF (Feng *et al.* 2007, Silverstein 2004). Administration of D-GalN/LPS causes cytokine release that contributes to increased oxidative stress and formation of reactive oxygen species, which are fatal to the cell and result in hepatocyte death (Liu *et al.* 2008, Oberholzer *et al.* 2001). In addition, D-GalN inhibits mRNA and protein synthesis as it depletes the uridine triphosphate pool (Stachlewitz *et al.* 1999). The exact mechanism of cellular damage in FHF remains unclear. Identifying novel and sensitive early markers in this model of hepatotoxicity that can be used to complement conventional liver function tests is still needed.

Furthermore, the oxidative stress causes a

misbalance in pro-oxidant/antioxidant steady state due to generation of increased amount of oxidants resulting in cellular damage as manifested by apoptosis and/or necrosis (Hong *et al.* 2009). Oxidative stress can be induced by toxins and it causes accumulation of reactive oxygen/nitrogen species, by activation of inducible nitric oxide synthase (NOS-2) (Diesen and Kuo 2010). Heme oxygenase-1 (HO-1), superoxide dismutase 1 (SOD1), glutathione peroxidase 1 (Gpx1) and catalase are major antioxidant enzymes, which along with the reactions that they catalyze, play important roles in defense against oxidative stress induced by toxins (Farombi and Surh 2006, Mari *et al.* 2009, Valdivia *et al.* 2009). Oxidative stress can induce a TNF-α mediated apoptosis that involves the activation of executive caspases and the members of Bcl-2 family proteins BH3 interacting domain death agonist (Bid) and Bcl-2-associated X protein (Bax) (Morgan *et al.* 2010, Van Herreweghe *et al.* 2010). Clarifying the steps involved in the complex interaction between the oxidative stress and apoptotic mechanisms is of great value in identifying early markers of cell injury.

One of the approaches to methods in toxicity research that has gained popularity in recent decades is study of toxicogenomics, which focuses on gene and protein activity responses to toxic substances (Gatzidou *et al.* 2007). Real time PCR analysis is one of the methods that has been proven reliable in verification of gene expressions in this field. As well, this method in combination with histopathology and biochemistry provides a further mechanistic approach to research in toxicology (Harril and Rusyn 2008). Our previous research work addressed the mutual cross talk of CO/HO-1 and NO/NOS-2 systems in the D-galactosamine (D-GalN)/lipopolysaccharide (LPS) hepatotoxicity (Farghali *et al.* 2009) with the use of these three before mentioned methods. The aim of this study is to provide further insight into the mechanisms of cellular injury in this model, by focusing on involvement of several other major antioxidant enzymes and apoptotic mediators. By analysis of their gene expressions we will attempt to address potential early signals of cell injury and existence of a relationship between conventional liver dysfunction markers and the select gene expression responses.

Materials and Methods

Animals and experimental design

This study was performed on male Wistar rats of 200-300 g body weight obtained from Velaz-Lysolaje, Czech Republic. They were given water and standard granulated diet ad libitum and were maintained under standard conditions; light (i.e. 12 h light and 12 h dark); temperature (22±2 ºC); relative humidity (50±10 %). All rats received humane care according to the general guidelines and approval of the Ethical Committee of the First Faculty of Medicine, Charles University in Prague. Rats in the D-GalN/LPS group were injected intraperitoneally with a dose of 400 mg/kg D-GalN (D-galactosamine hydrochloride) and 50 μg/kg LPS (lipopolysaccharide from Escherichia coli K-235) dissolved in dimethyl sulfoxide (DMSO), the control group received the equal volume of vehicle only. Eight animals of each group were killed at twenty four hours after injection under light ether anasthesia, after which the blood samples were collected. Following this, livers were excised quickly and perfused for morphological evaluation, preserved in liquid nitrogen for RT-PCR studies, and homogenized for biochemical study.

Measurements of liver enzymes and bilirubin

Determination of plasma alanine aminotransferase (ALT) was carried out using Fluitest® GPT ALT kit and/or BioLATest® ALT UV Liquid 500 tests. Fluitest® GOT AST kit by Analyticon and/or BioLATest® AST UV Liquid 500 tests were used in determination of aspartate aminotransferase (AST) plasma levels. Total bilirubin in plasma was measured using Fluitest® BIL-Total kit.

Determination of NO_2^-/NO_3^-, reduced glutathione (GSH) and catalase levels

Assessment of plasma NO_2^-/NO_3^- was carrried out using a nitrate/nitrite colourimetric assay kit of Cayman Chemical Company (An Arbor, MI) and a microplate reader according to manufacturer's instructions. In short, this method is based on a colourimetric conversion of nitrate (NO_3^-) to nitrite (NO_2^-) by nitrate reductase. The addition of the Griess reagent (1 % sulfanilamide, 0.1 % naphtylethylen-diamine, 2.5 % H_3PO_4) converts nitrite into a coloured azo compound. Spectrophotometrical measurement of absorbance at 540 nm determines the nitrite concentration, using the appropriate standard curve. Assessment of reduced gluathione in homogenate was based on the method that depends on a reaction between thiol group with 5,5-dithio-2-nitrobenzoic acid (DTNB) which can be measured spectrophotometrically (Sedlak

and Lindsay 1968). The measurement of catalase in plasma was performed according to the reaction between H_2O_2 and molybdenium ammonium as previously reported (Aebi 1984).

Lipid peroxidation: the thiobarbituric acid reacting substances (TBARS) and conjugated dienes (CD) measurement

D-GalN/LPS lipid peroxidation of the rat liver was assayed by the thiobarbituric acid (TBARS) method, and the spectrofluorometric assay for conjugated dienes (CD) was carried out as described earlier (Yokode *et al*. 1988). The results were expressed in nmol/mg of total protein.

Select gene expression measurements with the use of real-time PCR method

Twenty-four hours following drug administration, the liver samples were obtained to be used for total RNA isolation according to the manufacturer's instructions of the Qiagen® RNeasy plus kit (Bio-Consult Laboratories). Following total RNA isolation, the reverse transcription from total RNA to cDNA was processed by universal kit GeneAmp® RNA PCR using a murine leukemia virus (MuLv) reverse trancriptase (RT). Reverse transcription included the following three phases: 10 min at 25 ºC for RT enzyme activation, 30 min at 48 ºC for PCR amplification and 5 min at 95 ºC for denaturation.

Expressions of select genes were evaluated using real-time polymerase chain reaction (RT-PCR) of cDNA originating from total RNA, with the help of ABI PRISM 7900, and TagMan® Gene Expression master mix (Applied Biosystems). Total of eight genes were evaluated using the TagMan® Gene Expression Assays Kit – nitric oxide synthase-2 (NOS-2), heme oxygenase-1 (HO-1), glutathione peroxidase 1 (Gpx1), superoxide dismutase 1 (SOD1), BH3 interacting domain death agonist (Bid), Bcl-2 -associated X protein (Bax), caspase 3 (Casp 3) – as genes of interest (target genes) and glyceraldehyde 3-phosphate dehydrogenase (Gapdh) gene as a control (endogenous or housekeeping) gene, using the FAM coloured primes and probes. Housekeeping gene-expression was stable and constant during the experiment and was used in comparison with target gene-expression. Thermal cycling conditions for primer and probes optimization were 10 min at 90-95 ºC for Taq polymerase activation, followed by 15 s at 95-99 ºC for DNA denaturation and 1 min at 60 ºC for annealing. The obtained Ct values were used in relative quantification of gene expression measurements relative to the endogenous gene control Ct measurements, and the relative gene expression was calculated using the ΔΔCt method. (Arocho *et al.* 2006).

Measurement of caspase 3 activity and morphological evaluation

Cell lysates were prepared according to the instructions of Sigma-Aldrich (Prague, Czech Republic) fluorometric caspase 3 assay kit. The results were expressed as percentage of caspase 3 activity in the treated group relative to the control. The protein concentration in the supernatant was determined using an Bio-Rad protein assay kit according to the manufacturer's instructions.

Morphological evaluation of hepatocytes at the light microscopical level was done on semithin epon sections (1-2 μm thick) stained by toluidine blue using Leica IM 500 program for digital recording and measurements.

Statistical examinations

All experiments were performed in two groups of eight rats with the reported results stated as ± standard error of mean. The statistical analysis was performed using unpaired T-test with Welch correction. The p-values less than 0.05 were considered significant.

Results

Effects of D-GalN/LPS treatment on liver function, lipid peroxidation and oxidative stress parameters

The combination D-GalN/LPS treatment in rats has produced hepatic failure, which can be seen by highly significant ($p<0.001$) increases in levels of aminotransferases in plasma. A two hundred fold increase in AST level and one hundred fold increase in ALT level compared to those of the control group was observed (Table 1). The extent of lipid peroxidation as measured by formation of thiobarbituric acid reactive substances (TBARS) and conjugated dienes (CD) did not show any statistically significant differences between the two groups ($p>0.05$). Furthermore, Table 1 shows highly significant ($p<0.001$) increase in antioxidant enzyme catalase (CAT) in plasma of D-GalN/LPS treated rats compared to control: however, there was no measurable change in its level in homogenate (data not shown). There was no significant ($p>0.05$) difference between the two groups in the measurement of reduced glutathione (GSH) level in homogenate.

Table 1. Effects of lipopolysaccharide-induced hepatitis in D-galactosamine sensitized rats (D-GalN/LPS) on levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), catalase (CAT), conjugated dienes (CD), reduced glutathione (GSH) and formation of thiobarbituric acid reactive substances (TBARS) 24 h after injection.

	CONTROL	**D-GalN/LPS**
ALT		
Plasma	0.8225 ± 0.05	166.948 ± 12.42***
AST		
Plasma	2.016 ± 0.09	254.802 ± 4.85 ***
CAT		
Plasma	51.24 ± 6.55	156.00 ± 1.88 ***
CD		
Homogenate	2.45 ± 0.57	2.94 ± 0.54 *ns*
GSH		
Homogenate	458.36 ± 19.35	513.06 ± 49.89 *ns*
TBARS		
Homogenate	197.84 ± 22.24	261.12 ± 6.10 *ns*

CONTROL: negative control group receiving vehicle only. D-GalN/LPS: D-galactosamine 400 mg/kg with lipopolysaccharide 50 μg/kg; Units: ALT and AST μcat/l; CAT- μmol/ml; CD and TBARS- nmol/mg protein; GSH- μmol/mg protein; Values are mean ± S.E.M., n=8; *ns* non-significant value compared to the negative control group (CONTROL) $p>0.05$; *, **, *** value significant compared to CONTROL $p\leq0.05$*, $p\leq0.01$**, $p\leq0.001$***.

Fig. 1 further demonstrates changes in the gene expression of selected antioxidant enzymes as measured by the RT-PCR method. Glutathione peroxidase 1 (Gpx1) and superoxide dismutase 1 (SOD1) gene expressions were related to Gadph as the endogenous control, and measured in both D-GalN/LPS and the control groups. The increase of Gpx1 gene expression in the treated group is non significant ($p>0.05$); however, D-GalN/LPS treatment has caused a highly significant decrease of SOD1 gene expression in comparison to the untreated control group.

The extent of heme catabolism as shown in the Fig. 2a, illustrates significantly higher levels of bilirubin in plasma of D-GalN/LPS treated rats compared to that of the control group. The same trend is observed in the inducible HO-1 gene expression (Fig. 2b) relative to the Gapdh, where the seven fold increase in the D-GalN/LPS treated group is highly significant. In comparison to the untreated control animals, D-GalN/LPS treatment induced simultaneous statistically significant increase in both plasma NO_2^- levels (Fig. 3a) and NOS-2 expression relative to Gapdh as endogeneous control (Fig. 3b).

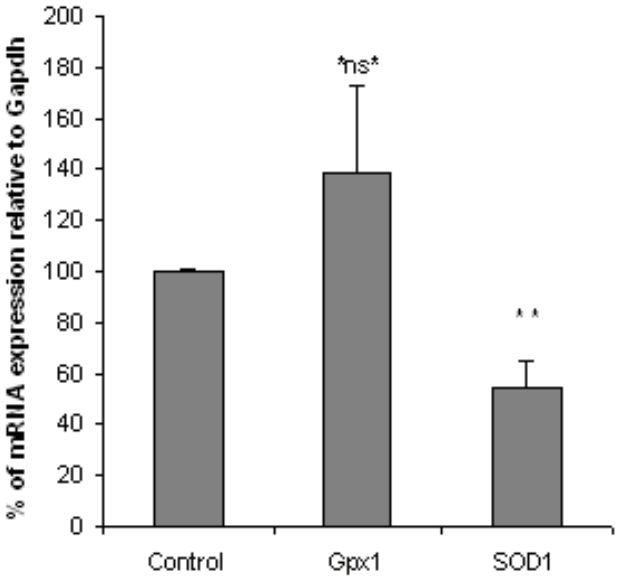

Fig. 1. Effect of lipopolysaccharide-induced hepatitis in D-galactosamine sensitized rats (D-GalN/LPS) on Gpx1 and SOD1 gene expressions relative to Gapdh as the endogenous control 24 h after injection. Control: vehicle only; D-GalN/LPS: D-galactosamine 400 mg/kg with lipopolysaccharide 50 μg/kg; Values are mean ± S.E.M., n=8; *ns* non-significant value compared to the negative control group (Control) $p>0.05$; ** value significant compared to Control $p\leq0.01$**.

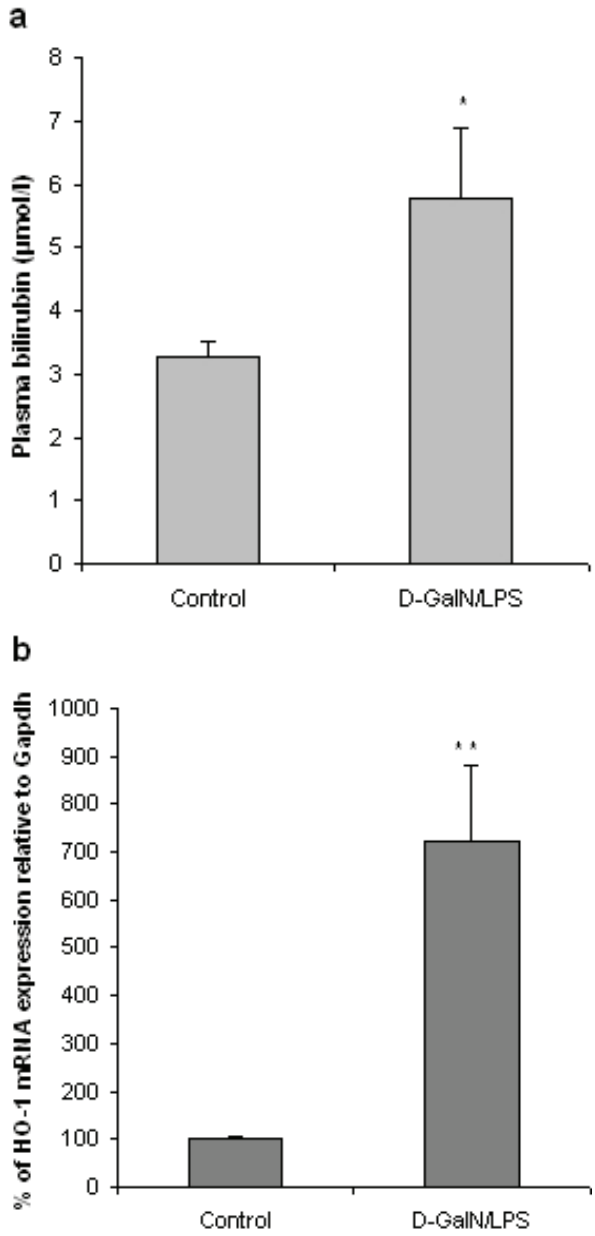

Fig. 2. Effect of lipopolysaccharide-induced hepatitis in D-galactosamine sensitized rats (D-GalN/LPS) on plasma bilirubin (**a**) and on HO-1 gene expression relative to Gapdh as the endogenous control (**b**) 24 h after injection. Control: vehicle only; D-GalN/LPS: D-galactosamine 400 mg/kg with lipopolysaccharide 50 µg/kg; Values are mean ± S.E.M., n=8; *,** value significant compared to Control p≤0.05*, p≤0.01**.

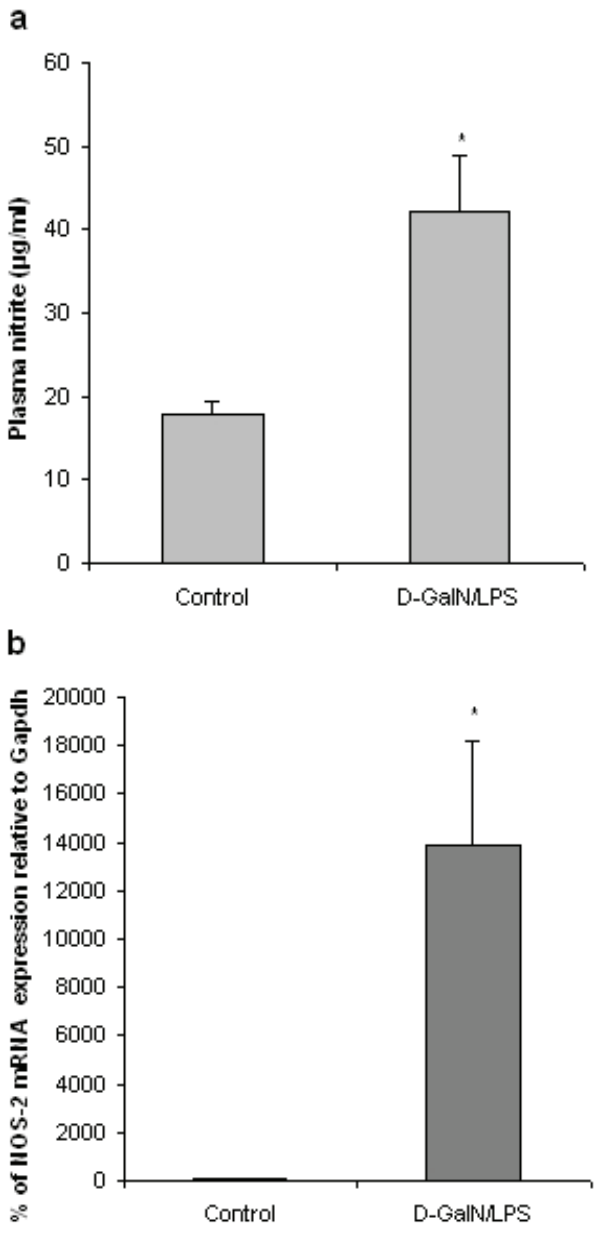

Fig. 3. Effect of lipopolysaccharide-induced hepatitis in D-galactosamine sensitized rats (D-GalN/LPS) on plasma NO_2^- (**a**) and on NOS-2 gene expression relative to Gapdh as the endogenous control (**b**) 24 h after injection. Control: vehicle only; D-GalN/LPS: D-galactosamine 400 mg/kg with lipopolysaccharide 50 µg/kg; Values are mean ± S.E.M., n=8; * value significant compared to Control p≤0.05*.

Effects of D-GalN/LPS treatment on apoptotic markers and morphological findings

Measurements of selected apoptotic parameters are illustrated in the Fig. 4. Caspase 3 activity, although slightly increased in the D-GalN/LPS group, is not significantly different from the control group (Fig. 4a): however, the expression of Casp 3 gene did show a significant increase. The same trend is also observed in the expressions of Bid and Bax genes, where the increase in the D-GalN/LPS treated group was statistically significant. Bax gene expression was more than two-fold and thus the highest of the three apoptotic genes that were measured.

The morphological evaluation has shown the well preserved cytological features of the control liver tissues. Specifically, the periphreal region of the central vein lobules consists of radially arranged cords of hepatocytes with more or less comparable cytological

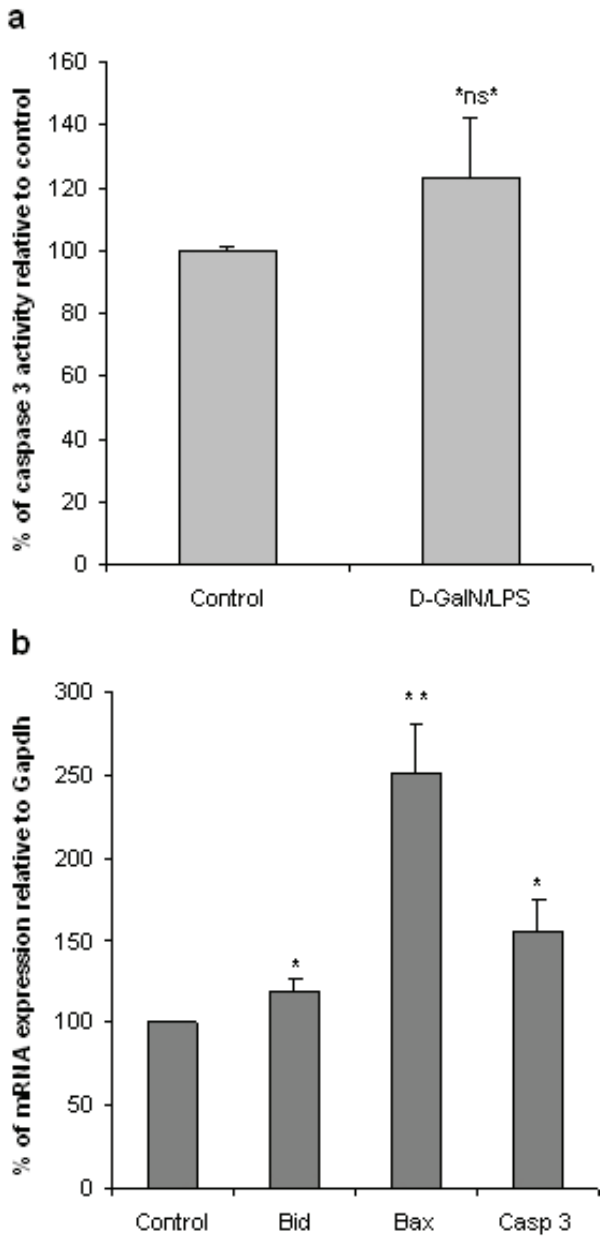

Fig. 4. Effect of lipopolysaccharide-induced hepatitis in D-galactosamine sensitized rats (D-GalN/LPS) on Caspase 3 activity (**a**) and on Bid, Bax and Casp 3 gene expressions relative to Gapdh as the endogenous control (**b**) 24 h after injection. Control: vehicle only; D-GalN/LPS: D-galactosamine 400 mg/kg with lipopolysaccharide 50 μg/kg; Values are mean ± S.E.M., n=8; *ns* non-significant value compared to the negative control group (Control) $p>0.05$; *,** value significant compared to Control $p\leq0.05$*, $p\leq0.01$**.

features, such as stainability of the cytoplasm and distribution of cell organelles (Fig. 5a). The administration of D-GalN/LPS has significantly affected morphological parameters of the rat livers. Even at the lower magnification (Fig. 5b) striking necrotic lesions can be observed in the peripheral and intermediate regions of the central vein lobules. Typical changes and aggregation of heterochromatin near the nuclear envelope confirm the occurence of apoptosis in some hepatocytes (Fig. 5c). At the peripheral region of some injured lobules transitional change of aponecrosis can be detected and the presence of pycnotic nuclei is clearly visible (Fig. 5d).

Discussion

Understanding the exact mechanism of xenobiotic hepatotoxicity is one of the major challenges hepatologists are faced with today. Recent advances in the studies of toxicogenomics have been useful in elucidating several different pathways of hepatotoxicity. Further research is needed to confirm these results as well to gain a mechanistic understanding of toxic changes that occur in the liver. As before mentioned, combination of D-GalN/LPS is a useful model for hepatotoxicity research that resembles fulminant hepatic failure. In this study, the administration of D-GalN/LPS significantly increased the levels of ALT and AST, which are indicative of failing liver function and are a cardinal feature in the FHF. The impairment of biliary function has been seen by the raised levels of bilirubin in the D-GalN/LPS treated animals. The present study revealed that the extent of lipid peroxidation in this model seems to be non significant, since the levels of conjugated dienes and the measured TBARS in plasma of D-GalN/LPS treated animals were not different from those of the control group.

Heme oxygenase-1 is the inducible isoform that is activated in response to cellular stress, playing a main role in degradation of heme into carbon monoxide, free iron and biliverdin. In turn, the enzyme biliverdin reductase converts biliverdin into bilirubin, a powerful antioxidant with cytoprotective capabilities that has been linked to increased heme oxygenase activity (Baranano *et al.* 2002, Clark *et al.* 2000). As well, the other heme degradation pathway products, biliverdin and carbon monoxide, play a protective role against oxidative stress which may explain the observed increase in HO-1 expression (Lehmann *et al.* 2010, Zhu *et al.* 2008). One of the ways lipopolysaccharide exerts its inflammatory action is by stimulation of production of pro-inflammatory cytokine TNF-α by the Kupffer cells. (Lichtman *et al.* 1994). The cooperative action of biliverdin/bilirubin and CO was reported to be responsible for the prolonged survival of mice in the D-GalN/LPS model of hepatotoxicity due to cytokine reduction, specifically TNF-α (Sass *et al.* 2004). This is relevant to the observed parallel increase in both HO-1 expression and bilirubin levels in this experiment.

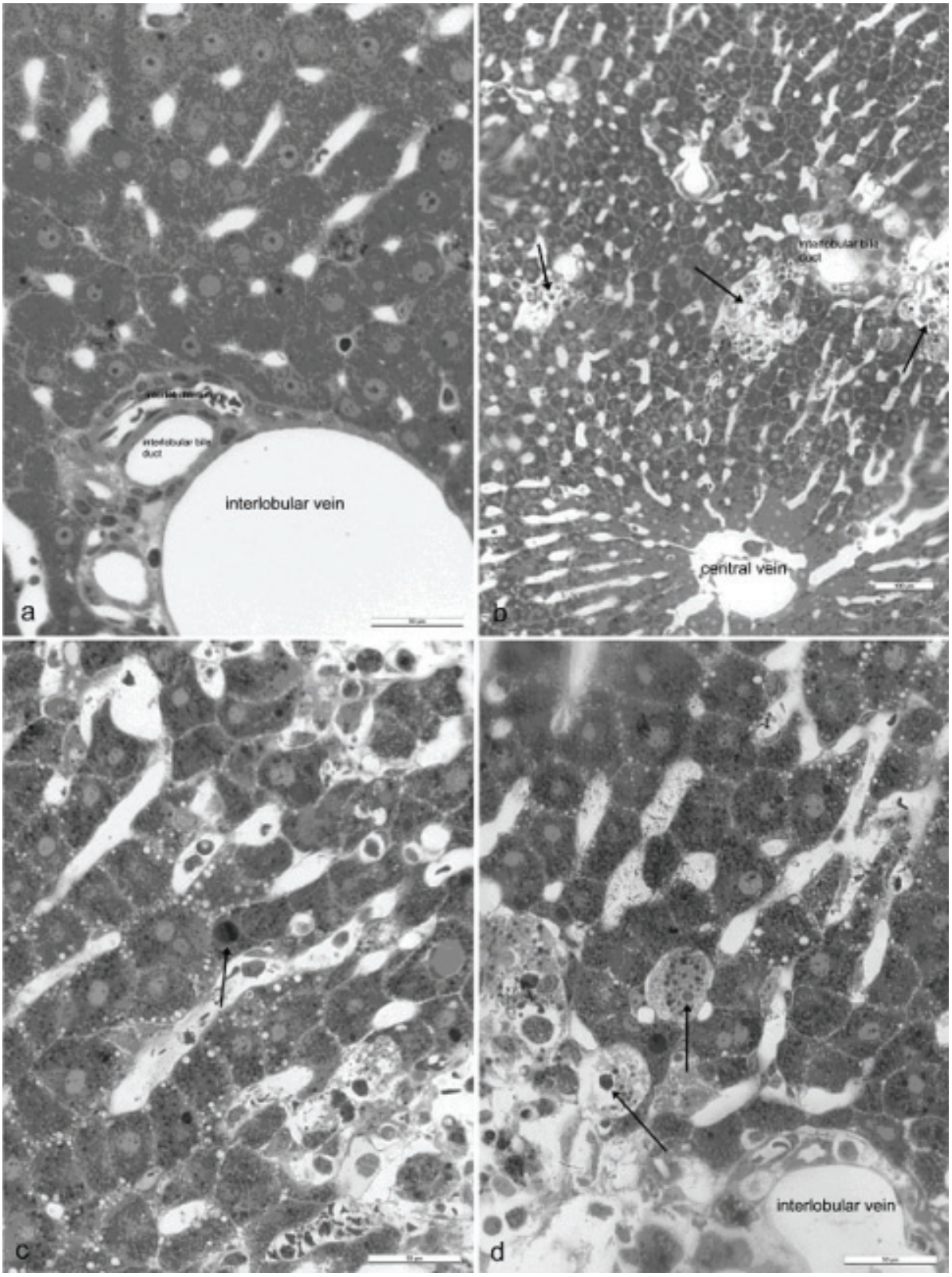

Fig. 5. Light microscopy morphological findings of rat liver of control and D-GalN/LPS treated samples: **a**) control hepatocyte liver-peripheral region of the central vein lobule; trabecular arrangement of polyhedral hepatocytes demonstrates well preserved cytological features; bar 50 μm. **b**) the effect of D-GalN/LPS treatment (low magnification) – striking necrotic lesions can be seen in the peripheral and intermediate (arrows) regions of a central vein lobule of the liver; many transparent pseudovacuoles are visible; bar 100 μm; **c**) the effect of D-GalN/LPS treatment (higher magnification) – increased number of neutral lipid droplets, increased distribution of dark granular accumulations in the cytoplasm and activated lysosomal apparatus of injured hepatocytes are seen; typical changes of demilunar apoptotic heterochromatin arrangement in the nucleus are indicated by an arrow; bar 50 μm; **d**) peripheral region of injured lobule – some transitional stages of aponecrosis can be detected; presence of pycnotic nuclei (arrows); marked disintegration of the cell cytoplasm; bar 50 μm; All samples prepared by semithin epon section, toluidine blue.

Oxidative stress causes an increase in production of nitric oxide, a molecule which plays a complex role in both oxidative stress and cell death responses. The activity of NO in this study was reflected in the measurement of its oxidation end product nitrite in plasma, which has been significantly increased in parallel with the gene expression of NOS-2 enzyme in the D-GalN/LPS group. One of the important influences of NOS-2 enzyme is that once it is induced by increased levels of TNF-α, it produces nitric oxide that in turn stimulates additional production of TNF-α resulting in inflammatory injury (Sass *et al.* 2001). Furthermore, nitric oxide is thought to play a dual role in apoptosis acting as both pro-apoptotic and anti-apoptotic mediator depending on various cellular conditions and cell types (Chung *et al.* 2001, Brune 2005). Some studies have shown that this inducible isoenzyme in certain cell types contributes to cell death by increasing caspase 3 activity due to increased cytokine levels such as TNF-α (Obara *et al.* 2010). Earlier studies on hepatocytes however have shown that nitric oxide exerts anti-apoptotic action through direct inhibition of caspase activity by S-nitrosylation, resulting in prevention of Bcl-2 cleaveage and cytochrome C release (Li *et al*. 1997, Kim *et al*. 1998). Although we have not been able to observe any significant change in Caspase 3 activity in D-GalN/LPS treated animals, there was a significant increase in Casp 3 and NOS-2 gene expressions. Furthermore, some studies have shown that the induced HO-1 enzyme exerts its cytoprotective action through inhibition of inflammatory NOS-2 induction, decrease in levels of cytokines and decreased Caspase 3 activity (Sass *et al.,* 2003, Wen *et al.* 2003). Therefore, the last reports support our present findings in so far as the relationship between HO-1 and billirubin from one hand and NOS-2 and Casp 3 gene expresion on the other hand.

It is well established that interdependence of members of Bcl-2 pro-apoptotic and antiapoptotic proteins plays a major role in apoptotic cell death, through their action on mitochondrial permeability pores, cytochrome C release and activation of caspases (Garcia-Saez *et al.* 2010, Chipuk and Green 2008). The increases in measured gene expressions of Bcl-2 pro-apoptotic members, Bid and Bax, as well as that of Casp 3 gene expression signify an early initiation of the apoptotic pathways. Furthermore, the morphological evaluation of D-GalN/LPS treated rats has shown the presence of pycnotic nuclei, which in those cells support a classification of running apoptotic process. In addition, the simultaneously marked disintegration of their cytoplasm shows a necrotic continuation, apparently, following process of apoptotic cell death. Typical changes and aggregation of heterochromatin near the nuclear envelope testify an occurence of apoptosis in some hepatocytes. These findings support the concept of the presence of apoptosis which was followed by necrotic changes, in other words aponecrotic cell death.

Reduced glutathione (GSH) is a powerful antioxidant that protects cells from oxidative injury by scavenging reactive oxygen/nitrogen species and a homeostatic decrease in the GSH pool can make cells more vulnerable to further damage by toxins (Ballatori *et al*. 2009). In this study, the GSH levels were similar between the control and D-GalN/LPS group, indicating that the GSH pool has remained intact. In addition to antioxidant action of GSH, the antioxidant enzymes SOD1, Gpx1 and catalase work together to counteract the oxidation of proteins, lipids and DNA, by removing ROS from the cell (Yuan and Kaplowitz 2008). Specifically, SOD reduces superoxide into hydrogen peroxide, which is further reduced to water by the action of catalase and glutathione peroxidase (Valdivia *et al.* 2009). It is noteworthy that within the present experimental conditions gene expression of SOD1 decreased significantly, while that of the Gpx1 remained unchanged. Catalase was significantly induced by D-GalN/LPS as was seen by significantly increased levels in plasma. It might be expected that under the present experimental conditions, the responses of these three parameters would be increased in parallel, however, under D-GalN/LPS toxicity the expected mutual relationship of these antioxidant enzymes was not seen. Differential response of these enzymes may be dependent on the dictating cellular needs in fight against increased levels of reactive oxygen species in induced oxidative stress states (Djordjevic *et al*. 2010).

In summary, D-GalN/LPS induced hepatotoxicity has resulted in a differential oxidative stress response as reflected by the alterations in expressions of certain oxidant/antioxidant genes, while the expression of others remained unchanged. Even though our findings were not able to confirm a direct relationship between the oxidative and apoptotic parameters that were tested, a parallel relationship between selected enzymes' gene expressions and their respective biochemical markers was seen. Thus, the real time PCR analysis of certain genes, which according to the present conditions is extremely sensitive, combined with conventional biochemical markers and morphology is potentially a very useful tool in

understanding various steps involved in D-GalN/LPS induced fulminant hepatic injury.

Conflict of Interest

There is no conflict of interest.

Acknowledgements

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The involvement of heme oxygenase 1 but not nitric oxide synthase 2 in a hepatoprotective action of quercetin in lipopolysaccharide-induced hepatotoxicity of D-galactosamine sensitized rats

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ABSTRACT

The objective of this study was to evaluate potential hepatoprotective capabilities of quercetin in relation to its modulation of the HO-1 and NOS-2 activities in an experimental model of fulminant liver failure. Liver insult was induced by *in vivo* administration of D-galactosamine (D-GalN, 400 mg/kg, *i.p.*) and lipopolysaccharide (LPS, 10 μg/kg, *i.p.*). The effects of quercetin (50 mg/kg, *i.p*) on D-GalN toxicity was evaluated by standard biochemical, RT-PCR and Western blot methods. Administration of D-GalN/LPS combination resulted in significantly higher plasma levels of aminotransferases, as well as increased mRNA and protein expressions of both HO-1 and NOS-2 enzymes. Quercetin exhibited cytoprotective effects on the liver, as evidenced by decreased aminotransferase plasma levels. Additionally, quercetin treatment in D-GalN/LPS treated rats significantly increased HO-1 mRNA and its protein expressions. On the contrary, quercetin did not exhibit any significant effects on the levels of nitrites, and NOS-2 mRNA and protein expressions in D-GalN/LPS treated rats. Quercetin when given alone did not have any significant changes on liver enzymes nor HO-1 and NOS-2 mRNA and protein expressions. It can be concluded that the quercetin's induction of HO-1 and its byproducts, without concomitant NOS-2 activity reduction, is among mechanisms contributing to the hepatoprotective effect in D-GalN/LPS hepatotoxicity.

1. Introduction

Throughout history it has been known that diets rich in fresh fruits, vegetables and grains have been associated with beneficial health effects. In the past years, polyphenols isolated from these natural food sources have received a great deal of the attention due to their various health benefits. One of these substances is quercetin, a member of flavonoid family, which exists as a glycoside and is commonly found in food sources such as apples, onions, green tea and wine. This flavonoid exhibits anti-inflammatory, antioxidant, anti-aging, anti-proliferative, among other favorable characteristics [1,2].

Small-scale cardiovascular studies testing the potential health benefits of quercetin were among the first to demonstrate its antioxidant potential in humans, where quercetin decreased blood low density lipoprotein levels and regulated blood pressure in hypertensive patients [3,4]. It is hypothesized that quercetin may exhibit its blood pressure lowering effect by enhancement of levels of the potent vasodilator, nitric oxide, by inducing endothelial nitric oxide synthase in spontaneous hypertensive rats [5]. Quercetin exerts its anti-inflammatory effect through inhibition of TNF-α and NF-κB production, which may further reduce oxidative stress markers [6]. Several studies, evaluating quercetin's effect on

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malignant cells, have revealed that this polyphenol increases expression of pro-apoptotic Bcl-2 proteins and it sensitizes malignant cell lines to a death ligand, both of which account for its favorable anti-proliferative properties [7,8]. Moreover, growing scientific evidence attributes oxidative stress and increased reactive oxygen species production as the main mediators of pathology of both acute and chronic liver injury caused by xenobiotics. In regard to the small number of studies done on liver, it is reported that quercetin exhibits its antioxidant properties by increasing the antioxidant status of the hepatocytes, decreasing the levels of pro-oxidant and inflammatory mediators, as well as altering gene expression of a number of antioxidant genes [9,10]. However the exact mechanism and hepatoprotective role of quercetin still remain to be further studied.

To date, the role of nitric oxide in liver damage remains controversial. It seems that this molecule plays a paradoxical effect in liver injury depending on the experimental conditions and current oxidative status in the cell [11]. Additionally, nitric oxide has been shown to influence the expression of heme oxygenase 1 (HO-1), an antioxidant enzyme that plays an important role in defense against oxidative stress [12–14]. In our previous work, we have addressed the potential cross talk between the HO-1 and nitric oxide synthase 2 (NOS-2) enzyme systems as targets of hepatoprotective agents [15,16]. We further aim to evolve our previous findings regarding the demonstrated hepatoprotective effects of natural products, resveratrol and curcumin, with definite roles of HO-1/NOS-2 hepatotoxicity/hepatoprotection. The present study, therefore, focuses on the evaluation of whether, and how, quercetin influences the activity of HO-1 and NOS-2 in the experimental model of hepatotoxicity, which resembles fulminant hepatic failure. Additionally, given the encouraging reported findings on the cytoprotective properties of quercetin, we would like to evaluate its potential hepatoprotective capabilities in relation to its modulation of the HO-1 and NOS-2 activities in rats.

2. Materials and methods

2.1. Chemicals

Lipopolysaccharide from *Escherichia coli* K-235 (LPS), D-galactosamine hydrochloride (D-GalN), quercetin, that is 2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4H-1-benzopyran-4-one with HPLC purity of >95%, (M. wt. 302.24), Tris–HCl, dimethyl sulfoxide, isopropyl alcohol, Tween 20, sodium dodecyl sulphate, ammonium persulfate, methanol, glycine, N,N,N′,N″-tetramethylethylenediamine, 2-mercaptoethanol, bromophenol blue, glycerol, N,N′-methylenebis (acrylamide), NaCl, KCl, Na_2HPO_4, KH_2PO_4, ammonium molybdate terahydrate, hydrogen peroxide, acrylamide, filter paper, nitrocellulose membrane, anti-mouse and anti-rabbit IgG (whole molecule) horse radish-peroxidase (HRP) conjugated secondary antibodies, rabbit primary NOS-2 antibody, mouse HO-1 primary antibody and beta-actin primary antibody were purchased from Sigma-Aldrich (Prague, Czech Republic). Non-fat dry milk was from Biotech A.S. (Prague, Czech Republic). NaCl 0.9% w/v i.v. infusion was from Bieffe Medital (Grosotto, Italy). Bio-Rad protein assay dye reagent was from Bio-Rad (Prague, Czech Republic).

2.2. Animals

Male Wistar rats (Velaz, Lysolaje, Czech Republic) of 300–400 g body weight were used in this study. They were given a standard granulated diet and water *ad libitum*. The animals were housed in standard environmental conditions: light (i.e. 12 h light and 12 h dark); temperature (22 ± 2 °C); relative humidity (50 ± 10%). The rats received humane care in accordance with the guidelines and approval by the Ethical Committee of the First Faculty of Medicine, Charles University in Prague, Czech Republic.

2.3. The study design

In the pilot experiments the effect of various doses of quercetin (50–500 μM) on tert-butylhydroperoxide induced toxicity in rat hepatocyte culture was evaluated. The results of these preliminary studies have indicated that 250 μM (57 mg/kg) of quercetin preserved hepatocyte viability and reduced ALT and AST leakage compared to the other doses that were tested (data not shown). The decision to use the dosage of 50 mg/kg in this experiment was also based on the studies of others dealing with quercetin's cytoprotective effects that include HO-1 induction and consequent NOS-2 reduction [17–19]. Thus we felt that this concentration complements our *in vitro* findings and is the most suitable one to test the aims of our study.

The rats were therefore divided randomly into four groups of six animals each and treated as follows:

- Group 1 — control rats received vehicle solution consisting of physiological solution and dimethyl sulfoxide (DMSO) intraperitoneally (i.p)
- Group 2 — rats were injected i.p. with a dose of 400 mg/kg D-GalN and 10 μg/kg LPS dissolved in physiological solution.
- Group 3 — rats were injected i.p. with a dose of 50 mg/kg of quercetin dissolved in DMSO.
- Group 4 — rats received a combination of 400 mg/kg D-GalN and 10 μg/kg LPS dissolved in appropriate vehicle solution, followed by a treatment dose of 50 mg/kg quercetin one hour later.

After 24 h, the animals were sacrificed and the blood samples were collected. In the next step, the livers were excised, preserved in liquid nitrogen for RT-PCR and Western blot studies.

2.4. Biochemical analyses

Determinations of plasma alanine aminotransferase (ALT), aspartate aminotransferase (AST) and total bilirubin plasma levels were carried out using Fluitest® GPT ALT, Fluitest® GOT AST and Fluitest® BIL-Total kits (Analyticon, Lichtenfels, Germany) respectively. Nitric oxide production was determined spectrophotometrically (540 nm) by measurement of one of its stable oxidation products, NO_2^-, in plasma using Griess reagent (1% sulfanilamide, 0.1% naphthylethylenediamine, 2.5% H_3PO_4). The medium nitrite levels were calculated by comparison with a sodium nitrite standard curve. The measurement of catalase in plasma was

performed according to the reaction between H_2O_2 and molybdenium ammonium as previously reported [20].

2.5. Real-time PCR gene expression measurements

Total RNA isolation was carried out according to the manufacturers' instructions of the Qiagen® RNeasy plus kit (Bio-Consult Laboratories, Prague, Czech Republic). Subsequently, the reverse transcription from total RNA to cDNA was carried out with universal GeneAmp® RNA PCR kit (Applied Biosystems, Prague, Czech Republic), using a murine leukemia virus (MuLv) as reverse trancriptase. Reverse transcription included the following phases: 10 min at 25 °C for reverse transcriptase enzyme activation, 30 min at 48 °C for PCR amplification and 5 min at 95 °C for denaturation.

The obtained total mRNA was reverse transcribed to cDNA with the help of ABI PRISM 7900, and TagMan® Gene Expression master mix (Applied Biosystems Prague, Czech Republic). Expressions of genes of interest were evaluated using real-time polymerase chain reaction (RT-PCR). The TagMan® Gene Expression Assays Kit (Applied Biosystems Prague, Czech Republic) was used. NOS-2 and HO-1 were used as genes of interest (target genes) and beta-actin gene as a control (housekeeping) gene, using the FAM colored primes and probes. Housekeeping gene-expression was stable and constant during the experiment and was used in comparison with target gene-expression. The conditions for thermal cycling for primer and probes optimization were as follows: 10 min at 90–95 °C for Taq polymerase activation, followed by 15 s at 95–99 °C for DNA denaturation and 1 min at 60 °C for annealing. The obtained Ct values were used in relative quantification of gene expression measurements relative to the endogenous gene control Ct measurements, and the relative gene expression was calculated using the ΔΔCt method [21].

2.6. Western blot analysis of proteins

Isolated liver samples were lysed with lysis buffer (1 M Tris HCl) and homogenized with an electric homogenizer. The samples were then centrifuged for 20 min at 12,000 rpm at 4 °C and supernatant was collected. The cell lysates were mixed (1:1) with sample buffer (b-mercaptoethanol, 10% SDS, glycerol, bromophenol blue, 0.5 M Tris HCl pH 6.8) and then heated for 5 min at 95 °C. Protein from the tissue samples (30 μg protein measured by Bradford method) were separated on 10% SDS-acrylamide gel and transferred to a nitrocellulose membrane by electrophoresis overnight at 4 °C. Membranes were blocked for 1 h with 5% non-fat milk in Tris-buffered solution, at room temperature. Membranes were then washed in a washing buffer (NaCl, KCl, Na_2HPO_4, KH_2PO_4, Tween, H_2O). They were then incubated with rabbit primary antibody against NOS-2 (1:1000), mouse HO-1 primary antibody (1:500) or mouse beta actin primary antibody (1:5000). After eluting the unconjugated primary antibody with the wash buffer, the membrane was then incubated with corresponding secondary antibody anti-rabbit IgG HRP conjugate (1:20,000) or anti-mouse IgG HRP conjugate (1:100,000). This step was followed by chemiluminescence labeling with Super Signal West Pico Chemiluminescent Substrate (GeneTiCA s.r.o. Prague, Czech Republic) for 2 min. Bands were detected with the use Molecular Imager VersaDoc™ MP 5000 System and analyzed by Quantity One 1-D Analysis Software (Bio-Rad, Prague, Czech Republic). Optical densities of NOS-2 and HO-1 bands were normalized by the corresponding loading control (ACTB) and then to the mean of the corresponding control group.

2.7. Statistical analysis

All experiments were performed in four groups of six rats with the reported results stated as ± standard error of mean (SEM). The statistical analysis was performed using one way ANOVA, followed with Tukey–Kramer comparison test. The p-values less than 0.05 were considered significant.

3. Results

3.1. Ameliorative effects of quercetin on D-GalN/LPS hepatotoxicity

The first question that we wanted to address was whether quercetin treatment (*i.p.*, 50 mg/kg) will have ameliorative effects on D-GalN (*i.p.*, 400 mg/kg) and LPS (*i.p.*, 10 μg/kg) model of hepatotoxicity. The results show that male Wistar rats treated with D-GalN and LPS developed acute hepatotoxicity within 24 h of the insult. This can be confirmed by a several-fold increase in the plasma levels of transaminases (AST and ALT) that are indicative of failing liver function. Quercetin alone had no influence on the plasma levels of these two liver enzymes. Interestingly, quercetin (Q) treatment in D-GalN/LPS rats resulted in significant lowering of the ALT (5.6-fold) and AST (5.9-fold) plasma levels, compared to the group receiving the hepatotoxic D-GalN/LPS combination. Thus, this data suggests a clear hepatoprotective effect as a result of quercetin treatment (Table 1).

Table 1
Effects of quercetin treatment in lipopolysaccharide-induced hepatitis in D-galactosamine sensitized rats (D-GalN/LPS) on levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and catalase. Control: vehicle only; Q: quercetin 50 mg/kg; D-GalN/LPS: D-galactosamine 400 mg/kg + lipopolysaccharide 10 μg/kg; D-GalN/LPS + Q: combination of D-galactosamine, lipopolysaccharide and quercetin treatment. The results are expressed as means ± SEM, n = 6.

	Control	Q	D-GalN/LPS	D-GalN/LPS + Q
ALT [U/l]	10.35 ± 0.11	29.08 ± 3.68	291.09 ± 35.53 [a]	51.59 ± 3.64 [a,b]
AST [U/l]	74.53 ± 4.91	60.51 ± 7.29	896.56 ± 135.64 [a]	151.85 ± 20.79 [a,b]
Catalase [μg/ml]	61.21 ± 4.88	49.52 ± 10.20	336.50 ± 14.92[a]	273.46 ± 33.24[a]

[a] Value significant compared to the control group (vehicle only), $p \leq 0.05$.
[b] Value significant compared to the respective positive control group (D-GalN/LPS), $p \leq 0.05$.

The plasma level measurements of the antioxidant molecule and product of heme degradation pathway, bilirubin, and antioxidant enzyme catalase were used as indicators of the oxidative status. In the groups treated with D-GalN/LPS, we observed significant 6.4-fold increase in bilirubin and 5.5-fold increase in catalase plasma levels. Quercetin treatment had no effect on the levels of these two antioxidants as can be seen by comparable values between D-GalN/LPS and D-GalN/LPS + Q groups (Table 1 and Fig. 1).

3.2. Effect of quercetin on HO-1, NOS-2 mRNA and protein expressions

The second question that we wanted to address in this study was to determine whether, and how, quercetin influences the activity of the potent antioxidant enzyme HO-1, as well as that of the enzyme NOS-2 in D-GalN/LPS model of hepatotoxicity. As postulated, both HO-1 mRNA and protein expressions were significantly increased (30.6-fold and 2-fold respectively, $p \leq 0.05$) in D-GalN/LPS group compared to control (Fig. 2). Conversely, quercetin alone did not exert any significant changes in HO-1 mRNA nor protein expressions, as the levels were comparable to those seen in the control group. However, when given to D-GalN/LPS treated rats, quercetin significantly ($p \leq 0.05$) increased the levels of HO-1 mRNA expression (by 2.3-fold) and protein expression (by 1.2-fold) in comparison to the group receiving hepatotoxic D-GalN/LPS combination (Fig. 2). These results clearly indicate the induction of HO-1 under hepatotoxic conditions, where quercetin further increased its activity under hepatotoxic, but not under physiological, conditions.

Furthermore, D-GalN/LPS challenge resulted in a significant 12.3-fold increase in NOS-2 mRNA and 2.4-fold increase in NOS-2 protein expressions in comparison to the control group ($p \leq 0.05$) (Fig. 3). Likewise, when compared to the control (vehicle only) group, addition of quercetin treatment to D-GalN/LPS treated rats resulted in significant 10-fold increase in NOS-2 mRNA and 2-fold increase in NOS-2 protein expressions ($p \leq 0.05$). Interestingly, however, there was no significant difference in NOS-2 mRNA and protein expressions between the D-GalN/LPS and D-GalN/LPS + Q groups, despite the notice of slight decrease in these levels upon addition of quercetin (Fig. 3). Moreover, quercetin did not show to have any influence on NOS-2 mRNA and protein expressions when given under normal physiological conditions (Fig. 3). Additionally, the levels of nitrites measured in plasma closely correlated to both NOS-2 mRNA and protein expression in the tested groups as was expected (Fig. 4).

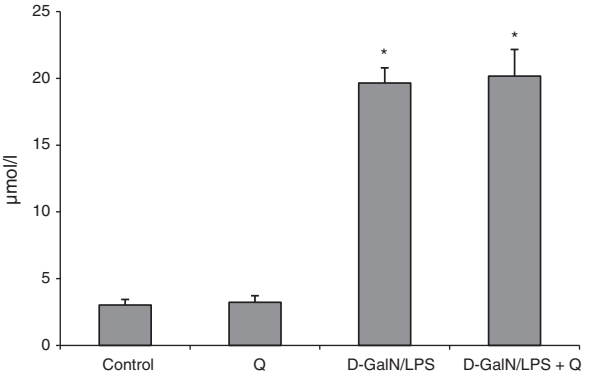

Fig 1. Effects of quercetin treatment in lipopolysaccharide-induced hepatitis in D-galactosamine sensitized rats (D-GalN/LPS) on plasma bilirubin levels. Control: vehicle only; Q: quercetin 50 mg/kg; D-GalN/LPS: D-galactosamine 400 mg/kg + lipopolysaccharide 10 µg/kg; D-GalN/LPS + Q: combination of D-galactosamine, lipopolysaccharide and quercetin treatment. The results are expressed as means ± SEM, n = 6. * Indicates significant values ($p \leq 0.05$) compared to the control group (vehicle only). The results are expressed as means ± SEM, n = 6.

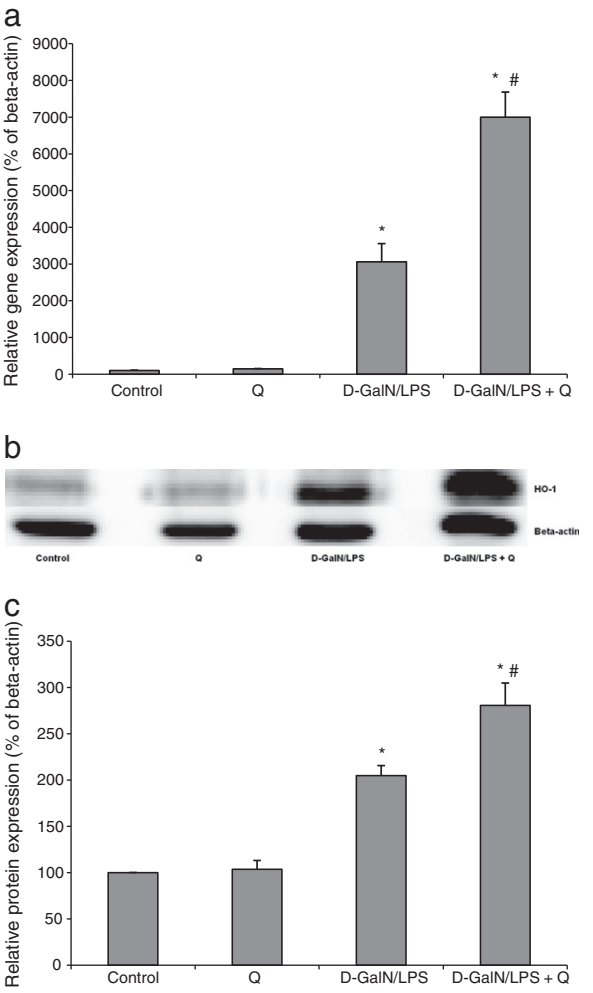

Fig. 2. Increase in HO-1 gene and protein expressions by quercetin treatment in lipopolysaccharide-induced hepatitis in D-galactosamine sensitized (D-GalN/LPS) rats. Gene expression of HO-1(a). The results are expressed as relative percentage of HO-1 gene expression against beta-actin as the endogenous control. Protein bands (b) and optical density (c) of HO-1 protein expression. The results are expressed as relative percentage of optical densities of HO-1 against beta-actin as the reference; Control: vehicle only; Q: quercetin 50 mg/kg; D-GalN/LPS: D-galactosamine 400 mg/kg + lipopolysaccharide 10 µg/kg; D-GalN/LPS + Q: combination of D-galactosamine, lipopolysaccharide and quercetin treatment. * Indicates significant values ($p \leq 0.05$) compared to the control group (vehicle only); # Indicates significant values ($p \leq 0.05$) compared to the respective positive control group (D-GalN/LPS). The results are expressed as means ± SEM, n = 6.

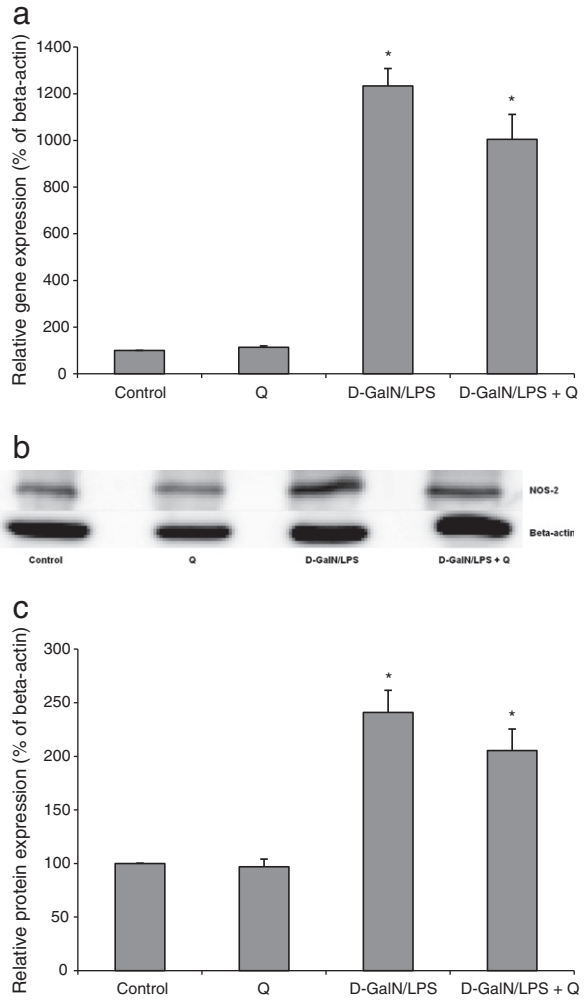

Fig. 3. Effects of quercetin treatment on NOS-2 on gene and protein expressions in lipopolysaccharide-induced hepatitis in D-galactosamine sensitized (D-GalN/LPS) rats. Gene expression of NOS-2 (a). The results are expressed as relative percentage of NOS-2 gene expression against beta-actin as the endogenous control. Protein bands (b) and optical density (c) of NOS-2 protein expression. The results are expressed as relative percentage of optical densities of HO-1 against beta-actin as the reference; Control: vehicle only; Q: quercetin 50 mg/kg; D-GalN/LPS: D-galactosamine 400 mg/kg + lipopolysaccharide 10 μg/kg; D-GalN/LPS + Q: combination of D-galactosamine, lipopolysaccharide and quercetin treatment. * Indicates significant values ($p \leq 0.05$) compared to the control group (vehicle only). The results are expressed as means ± SEM, $n = 6$.

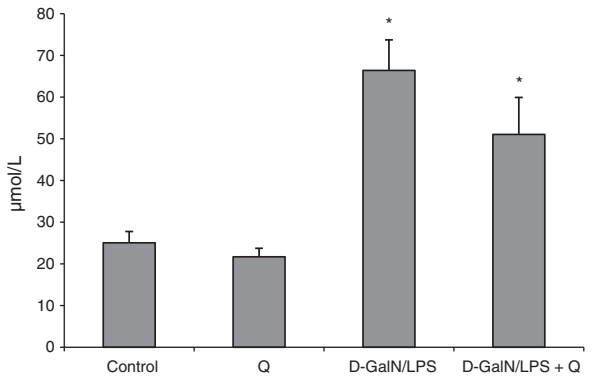

Fig. 4. Effects of quercetin treatment in lipopolysaccharide-induced hepatitis in D-galactosamine sensitized rats (D-GalN/LPS) on plasma nitrite levels. Control: vehicle only; Q: quercetin 50 mg/kg; D-GalN/LPS: D-galactosamine 400 mg/kg + lipopolysaccharide 10 μg/kg; D-GalN/LPS + Q: combination of D-galactosamine, lipopolysaccharide and quercetin treatment. * Indicates significant values ($p \leq 0.05$) compared to the control group (vehicle only). The results are expressed as means ± SEM, $n = 6$.

4. Discussion

Quercetin, a widely distributed dietary flavonoid has been shown to exert several hepatoprotective properties including its strong antioxidant potential in ameliorating alcoholic liver disease, anti-HBV activity, downregulation of expression of inflammatory mediators in cirrhotic rats and induction of cell death in hepatoma cell lines [22–27]. Despite the known aforementioned hepatoprotective properties of quercetin, many questions remain to be answered in relation to its cytoprotection involving induction of HO-1 and NOS-2 enzymes. In our previous studies on dietary polyphenols, resveratrol and curcumin, we have indicated that these agents may exert their hepatoprotective properties by induction of HO-1 and concomitant reduction of NOS-2 activities. This means that there is a kind of crosstalk between the two protein molecules including other cascade of effects which led to cytoprotection [15,16]. Thus the main goal of the current study was to assess potential hepatoprotective effects of quercetin in regard to its modulation of these two inducible enzymes that play important roles in oxidative stress.

In reference to the various mechanisms of hepatocellular damage of differing etiologies, oxidative stress is recognized as the most prominent and the most important mechanism resulting in a physiological misbalance of redox status of the cell favoring reactive oxidative species production and ensuing cellular injury [28,29]. Combination of D-GalN/LPS is a valuable hepatotoxic model that resembles clinical hepatitis, where oxidative stress plays a major role [30]. In short, D-galactosamine inhibits protein synthesis by depleting uridine triphosphate pool, resulting in early generation of reactive oxygen species and apoptosis [31–33]. On the other hand, lipopolysaccharide increases the release of pro-inflammatory cytokines (mainly TNF-α), induces the release of reactive nitrogen species and inflammatory prostaglandins due to the activation of NOS-1 and cycloxygenase 2 (COX-2) respectively [34–36]. In the present study administration of D-GalN/LPS caused an elevation of plasma aminotransferase (ALT, AST) levels that demonstrate failing liver function, as well it resulted in an increase in the catalase levels indicative of increased physiological antioxidant status of the cell. Quercetin treatment effectively improved D-GalN/LPS induced liver damage by lowering ALT and AST levels. However, the plasma level of catalase was not altered by quercetin treatment, indicating that increased antioxidant status of the cell was maintained in order to combat oxidative stress induced by D-GalN/LPS. For instance, the presence of catalase in the mitochondria of hepatocytes has shown to have important implications in the prevention of ROS generation and consequent activation of pro-apoptotic pathways induced by TNF-α [37].

Furthermore, the pathophysiological conditions of the liver involving oxidative stress initiate upregulation of HO-1 and increase in products of heme degradation pathway [13,38]. Among these products, biliverdin/bilirubin and CO are the key mediators of HO-1 mediated cytoprotection for the reason that they help restore intracellular homeostatic balance under oxidative stress conditions and suppressing inflammation through downregulation of pro-inflammatory mediators [38–41]. In agreement with our previous findings, administration of D-GalN/LPS toxicity resulted in higher levels of HO-1 gene and protein expressions, as well as a significant increase in bilirubin [30]. The augmented levels of bilirubin are of importance since this potent antioxidant ameliorates oxidative stress by scavenging peroxyl radicals, consequently preventing oxidation of fatty acids and proteins. Moreover, quercetin treatment resulted in a further increase of HO-1 gene and protein activities in D-GalN/LPS + Q rats, which is in accordance to other studies demonstrating hepatoprotective effect of quercetin by induction of HO-1 [38]. However, this increase in HO-1 activity was not paralleled by a simultaneous increase in bilirubin plasma levels. This could be explained by a postulated fine tune in bilirubin production, as the beneficial effects of this potent antioxidant is bypassed at higher serum levels resulting in cytotoxicity that targets the central nervous system [42].

As for the second enzyme of interest, NOS-2, its gene and protein expressions and the nitrite levels were significantly increased by D-GalN/LPS treatment, which can be attributed to the reported LPS-induced NO production [34,43]. Interestingly, in contrast to our previous experience with dietary polyphenols and those of others [15,16,44,45], quercetin did not have a significant reducing effect on NOS-2 activity or nitrate levels in D-GalN/LPS treated rats. It has been reported that quercetin mediates its cytoprotective actions by suppressing NO production due to induction of HO-1 [44–46]. However, considering the results of this study it could be postulated that there seems to be an alternative mechanism of HO-1 induction protection that is not related directly to NOS-2 activity reduction in this model of hepatotoxicity among other possible mechanisms. One possible explanation is that the products of HO-1 pathway, bilirubin and CO are mainly responsible for the quercetin mediated cytoprotection in this model given their aforementioned cytoprotective actions.

Furthermore, the mechanisms of HO-1 cytoprotection have been attributed in some models to be a result of upregulation of anti-inflammatory mediators such as IL-10 and IL-13, and inhibition of production of pro-inflammatory cytokines such as TNF- α [47,48]. Through inhibition of COX-2 activity, the IL-10 is responsible for the decrease in production of inflammatory prostaglandin E_2 that contributes to much of the damage due to LPS insult [49]. However, until now not much is known whether there exists a potential cross-talk between these two enzymes. On the other hand, the relationship between COX-2 and NOS-2 is such that peroxynitrite radicals resulting from increased NOS activity are able to inhibit the activation of COX-2 by tyrosine nitration [36]. Thus, high NOS-2 activity could contribute to reduction of pro-inflammatory damage caused by COX-2.

Another presumptions is that quercetin was extensively metabolized into its metabolite quercetin-3′-sulfate, which has been not been shown to possess NOS-2 and NO lowering properties [45,50]. Furthermore, it is known that quercetin, resveratrol, curcumin and related natural and synthetic compounds are SIRT1 activators. The latter is a NAD+ dependent deacetylase responsible for multiple beneficial effects [51]. SIRT1 activation could be achieved by several pathways through stimulation and/or inhibition of mediators, thus a direct effect on NOS-2/NO level due to quercetin treatment was not demonstrated. Moreover, an important finding is that quercetin administration to healthy rats without the presence of a toxicant did not produce any significant changes on the tested parameters. It could be assumed, therefore, that there exist different regulation mechanisms which are responsible for the differences seen under physiological versus pathological conditions.

In our previous findings dealing with dietary hepatoprotective substances we have found that the dosage of 100 mg/kg curcumin and 2.3 mg/kg of resveratrol has ameliorated D-GalN/LPS induced hepatotoxicity [15,16]. In this study, 50 mg/kg was shown to be cytoprotective in D-GalN/LPS model, thus being more potent than curcumin, but less potent than resveratrol.

In conclusion, the results of the present study indicate that quercetin's significant induction of HO-1 alone, without concomitant NOS-2 activity reduction, might be sufficient in combating cellular damage induced by D-GalN/LPS toxicity which resembles fulminant hepatitis. However, we must remain cautious in regard to many studies demonstrating various cytoprotective effects of quercetin, as this dietary polyphenol can also be toxic in some conditions. Such is the example of its potent inhibiting effect on topoisomerase II that may cause double-strand DNA lesions at topoisomerase binding sites including the MLL gene, which can lead to the development of secondary leukemias [52,53]. Nonetheless, given the wide availability of dietary quercetin as well as its cytoprotective properties in various disease models, the importance of this flavonoid in amelioration of hepatic diseases should be further studied.

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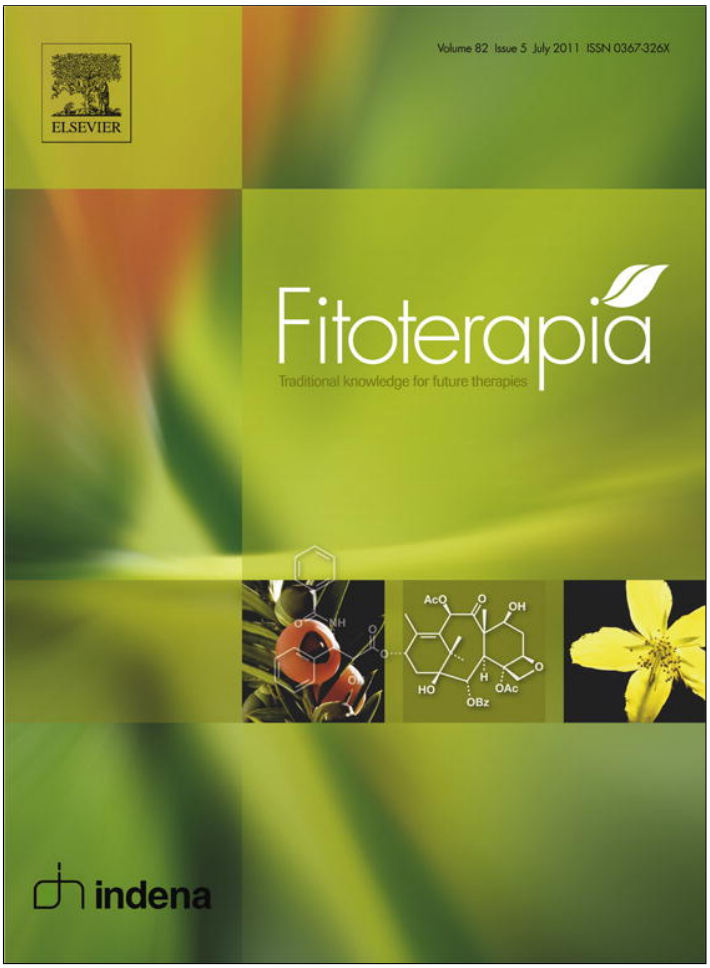

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Hepatoprotective effect of curcumin in lipopolysaccharide/D-galactosamine model of liver injury in rats: Relationship to HO-1/CO antioxidant system

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ABSTRACT

This work studied a relationship between HO-1/CO system and lipid peroxidation with consequent effects on liver functions and NOS-2. We focused on curcumin pretreatment in rat toxic model of D-galactosamine and lipopolysaccharide. Hepatocyte viability, lipid peroxidation, antioxidant status, ALT and AST were evaluated. HO-1 and NOS-2 expressions and respective enzyme activity were determined. Curcumin caused decreases in ALT and AST levels as well as in lipid peroxidation. Furthermore, curcumin pretreatment increased liver HO-1 (2.4-fold, $p = 0.001$), but reduced NOS-2 (4.1-fold, $p = 0.01$) expressions. In conclusion, the tuning of CO/NO pathways is important in shedding light on curcumin's cytoprotective effects in this model.

1. Introduction

Curcumin is a yellow pigment from rhizomatous herbaceous plant turmeric (*Curcuma longa*). This low-molecular-weight polyphenol exhibits reported anti-inflammatory [1] and antioxidant [2] properties. It is generally regarded as the most active constituent of the rhizomes and comprises 2–8% of most turmeric preparations. It has long been used as the yellow spice in Indian food and as a herbal medicine for the treatment of inflammatory diseases [3]. Several reports are dealing with curcumin as a potential cytoprotective agent [4–11]. Due to its antioxidant and anti-inflammatory properties, curcumin is considered as an hepatoprotective and anti-fibrotic compound [12,13]. Curcumin is also known as an inducer of enzyme heme oxygenase-1 by several mechanisms [14].

Heme oxygenase-1 (HO-1), an inducible enzyme in heme catabolic pathway, has emerged as an important mediator of antiinflammatory, antioxidant and cytoprotective actions. HO-1 and its metabolic products, CO and bilirubin, are potential drug targets for amelioration of liver insults [15]. Molecular targets of CO are heme proteins, including among several others nitric oxide synthases (NOSs). Nitric oxide produced in high amounts by NOS-2 greatly contributes to the pro-oxidative status of the cells and can also stimulate HO-1 [16]. Various dose combinations of LPS and DG were used to produce sub lethal liver failure which simulate clinical situations in viral, drug or alcohol-induced, immune-induced or under ischemia reperfusion hepatitis [17–22]. While LPS causes cytokine release and increase in reactive oxygen/nitrogen species, D-GalN inhibits protein synthesis due to depletion of the uridine triphosphate pool in addition to its well established pro-oxidative effect [23].

The aim of this study was therefore to evaluate whether curcumin, as an antioxidant and cytoprotective substance, exerts its inducing effects on HO-1 under normal physiological conditions as well as in response to stress conditions in

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the present liver failure model. The specific goal of the work was directed to study the mutual relationship between HO-1/CO system and lipid peroxidation with consequent effects on liver functions and inducible NOS-2/NO system.

2. Materials and methods

2.1. Materials

Lipopolysaccharide from *Escherichia coli* K-235 (LPS), D-galactosamine hydrochloride (DG) FW 179,17, curcumin (CUR) FW 368.38 - purity ≥ 80,1% curcumin, 16% other curcuminoids (demethoxycurcumin$^+$ bisdemethoxycurcumin) and the rest 3,9% is not defined, TRIS–HCl, EDTA sodium, sucrose, trichloracetic acid (TCA), ammonium molybdate tetrahydrate, hydrogen peroxide, heptane, isopropyl alcohol, potassium phosphate, nicotinamide adenine dinucleotide phosphate (NADPH), hemin, bovine serum albumin (BSA), sodium phosphate Na_3PO_4, anhydrous magnesium perchlorate and sulphosalicylic acid were obtained from Sigma-Aldrich (Prague, Czech Republic). Carbon monoxide as calibration gas for gas chromatography was purchased from Linde (Prague, Czech Republic). Bio-Rad protein assay dye reagent concentrate was obtained from Bio-Rad Company (Prague, Czech Republic). A reagent kit Cayman Chemical Company (An Arbor, MI) for NO_2^-/NO_3^- measurement. The universal kit GeneAmp® RNA PCR using murine leukemia virus (MuLv) reverse transcriptase (RT) and TagMan® Gene Expression Assays Kit for heme oxygenase-1, nitric oxide synthase-2, beta-2-microglobulin including probes, primers, $MgCl_2$, dNTP mixture, 10x PCR buffer and DNA-polymerase were obtained from Applied Biosystems (Prague, Czech Republic). Quiagen RNeasy Plus Mini Kit for total RNA isolation was obtained from Bio-Consult Laboratories (Prague, Czech Republic).

2.2. Animals and drug treatments

Male Wistar rats of 200–300 g body weight (Velaz-Lysolaje, Czech Republic) were used throughout the present study and were allowed water and standard granulated diet *ad libitum*. All rats received humane care in accordance with the general guidelines of the First Faculty of Medicine, Charles University in Prague. The study protocol was approved by the Faculty Ethical Committee.

2.2.1. Drug treatments

We used a dose of 400 mg/kg DG *i.p.* followed immediately by 10 μg/kg LPS *i.p.* At the beginning of our experiment, two models using two different concentrations of LPS, 10 μg/kg and 50 μg/kg, were used. Only the first one (10 μg/kg) has been chosen for our further studies, because it produced reversible fulminant hepatic failure which was suitable for the present study. According to several reported preclinical studies [24,25], we used an adequate dose of CUR 100 mg/kg *i.p.* 1 h before the hepatic injury by DG + LPS. 24 h later animals were sacrificed by decapitation and blood samples were collected. Plasma was immediately isolated by centrifugation at 805 ×*g* for 10 min and used for assessment of ALT, AST, total bilirubin, catalase and nitric oxide (NO) as NO_2^-. Meanwhile, liver samples were snap frozen in liquid nitrogen and stored at −80 °C for RT PCR and biochemical studies as explained further. In addition, liver homogenates were used for conjugated dienes, total carbon monoxide (CO) and HO-activity measurements.

2.3. Determination of plasma alanine aminotransferase (ALT), aspartate aminotransferase (AST), total bilirubin and NO_2^-

The hepatocyte membrane integrity was assessed from plasma ALT and AST concentrations. Determination of plasma alanine aminotransferase (ALT) was carried out using Fluitest® GPT ALT kit by Analyticon (Germany). The principle of this test is to catalyze a reaction with pyruvate as one of the end products. Pyruvate reacts in the next reaction that involves oxidation of NADH to NAD. The rate of decrease of NADH is measured photometrically and is directly proportional to the rate of formation of pyruvate, which is indicative of ALT activity.

Fluitest® GOT AST kit by Analyticon (Germany) was used in determination of aspartate aminotransferase (AST) plasma levels. Briefly, AST is an enzyme that catalyzes conversion of 2-oxoglutarate into oxalacetate, which further reacts with NADH. The rate of decrease of NADH is measured photometrically and is directly proportional to the rate of formation of oxaloacetate, which in turn is indicative of AST activity.

Total bilirubin in plasma was measured using Fluitest® BIL-Total kit (Czech Republic). This method involves photometric measurement of azobilirubin dye that is produced when bilirubin reacts with sulfanilic acid.

Assessment of plasma NO_2^- was carried out using a colorimetric kit of Cayman Chemical Company (USA) and a microplate reader according to manufacturer's instructions. Briefly, this method involves a conversion of nitrate (NO_3^-) to nitrite (NO_2^-) by nitrate reductase. This is followed by the addition of the Griess reagent (1% sulfanilamide, 0.1% naphtylethylendiamine, 2.5% H_3PO_4) that allows for a simple spectrophotometrical measurement (at 540 nm) of NO oxidation product NO_2^- without the inclusion of nitrate reductase. The NO_2^- levels were calculated by comparison with a $NaNO_2$ standard curve.

2.4. HO-activity and total tissue carbon monoxide measurement

Liver HO activity and tissue CO content were measured as previously described [26]. Briefly, for HO activity measurement, liver homogenates were incubated with methemalbumin (1.5 mM heme/0.15 mM albumin) and NADPH at 37 °C in CO-free septum-sealed vials for 15 min and reaction was terminated with the addition of 60% sulphosalicylic acid. For liver CO determination, liver homogenates were incubated in CO-free septum-sealed vials containing 60% (w/v) sulphosalicylic acid on ice. The amount of CO generated by the reaction and released into the vial headspace was quantitated by gas chromatography with a reduction gas analyzer (Trace Analytical, Menlo Park, CA, USA). HO activity and liver CO content were calculated as pmolCO/hr/mg protein and pmolCO/mg protein, respectively.

2.5. Assay of enzymatic antioxidant (catalase) and lipid peroxidation (conjugated dienes) markers

The catalase assay in liver tissue or plasma was performed spectrophotometrically based on the reaction between H_2O_2 and molybdenium ammonium as previously reported [27]. The results were expressed in μg/ml. Conjugated dienes (CD)

Table 1
Effect of CUR pretreatment in lipopolysaccharide-induced hepatitis in D-galactosamine sensitized rats *on plasma alanine aminotransferase (ALT), aspartate aminotransferase (AST) and bilirubin*; CONTROL — vehicle only; CUR curcumin 100 mg/kg; DG + LPS — D-galactosamine 400 mg/kg + lipopolysaccharide 10 μg/kg after 24 hour incubation; CUR + DG + LPS — curcumin 1-hour pretreatment before D-galactosamine and lipopolysaccharide toxic insult; means ± SEM, n = 7.

	CONTROL	DG + LPS	CUR	CUR + DG + LPS
ALT [μcat/l	27.77 ± 3.02	247.31 ± 54.79 ***	35.89 ± 4.79	113.05 ± 31.92 #
AST [μcat/l]	56.80 ± 4.48	127.87 ± 10.63 ***	57.90 ± 9.79	63.70 ± 9.39 ###
Bilirubin [μmol/l]	3,6748 ± 1,7752	19,3777 ± 3,4768 ***	4,8779 ± 0,8157	36,8939 ± 2,9646 ###

*** Value significant compared to respective group with vehicle only (CONTROL) $p<0.001$.
# Value significant to respective positive control group (DG + LPS) $p<0.05$.
Value significant to respective positive control group (DG + LPS) $p<0.001$.

as lipid peroxidation marker was assayed as described earlier [28]. The results were expressed in nmol/mg protein.

2.6. Expression of inducible genes for HO-1 and NOS-2-real time PCR

Twenty-four hours following drug administration, the liver samples were obtained to be used for total RNA isolation according to the manufacturers' instructions of the Qiagen® RNeasy plus kit. Following total RNA isolation, the reverse transcription from total RNA to cDNA was processed by universal kit GeneAmp® RNA using a murine leukemia virus (MuLv) reverse transcriptase (RT). Real-time PCR was performed using Taqman Gene Expression Assay Kit (Applied Biosystems, USA). Overall three genes were evaluated — HO-1, NOS-2 genes as target genes and beta-2-microglobulin as a control (endogenous or house-keeping) gene. House-keeping gene-expression was stable and constant during the experiment and was used in comparison with target gene-expression. Real-time quantitative PCR analysis was performed by use of ABI PRISM 7900 machine.

2.7. Statistical analysis

The data were expressed as mean ± SEM. All experiments performed in groups of rats resulted in reported means of 7 animals. There were four independent groups together labeled as: CONTROL (negative control group with saline treatment only), CUR (curcumin *per se*), DG + LPS (D-galactosamine and lipopolysaccharide fixed toxic combination; positive control group) and CUR + DG + LPS (group with toxic combination pretreated by curcumin). In each case, there was one independent measurement of each parameter from each rat (24 h after drug application). Data passed normality test, that's why the statistical significance of difference of mean scores was determined using one-way Analysis of Variance (ANOVA) with *post hoc* Bonferroni multiple comparisons test for comparing plasma, tissue and/or genetic alterations between treated (positive control) and intact (negative control) animals.

P-value less than 0.05 was considered statistically significant.

3. Results

3.1. Effect of curcumin pretreatment on liver functions

Significant increases in serum aminotransferases ALT, AST and plasma bilirubin (Table 1) levels were observed in DG + LPS animals reflecting severe hepatocellular injury in this group of animals as compared to rats with saline treatment (negative controls). CUR *per se* had no effect on these parameters. Importantly, pretreatment with CUR resulted in significant drop of ALT and AST in CUR + DG + LPS rats ($p<0.05$), while bilirubin levels further increased (Table 1).

3.2. Effect of curcumin pretreatment on HO activity, HO-1 gene expression and total carbon monoxide tissue content

Discrepancy between increasing plasma bilirubin levels and decreasing aminotransferase activities prompted us to measure the rate of bilirubin production in the liver of experimental animals. DG + LPS toxic combination resulted in significant 5-fold increase in HO-1 mRNA gene expression ($p=0.05$, Fig. 1). Furthermore, curcumin pretreatment was associated with almost 12-fold increase in HO-1 mRNA in CUR + DG + LPS group compared to vehicle-treated controls ($p<0.001$, Fig. 1). The similar increasing trend has been observed for total HO activity (Fig. 2) indicating increased bilirubin production in curcumin pretreated animals with toxic liver injury.

Surprisingly, carbon monoxide, another product of HO reaction, significantly increased in the liver of DG + LPS group (2.5-fold, $p<0.01$), but not in CUR + DG + LPS animals (Fig. 3). Interestingly, curcumin administration to animals without liver injury (CUR group) had no significant effect on

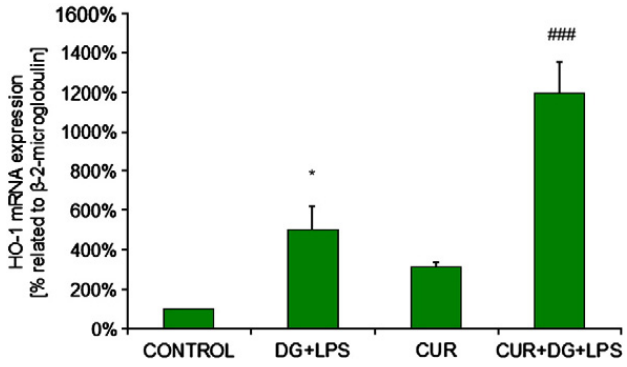

Fig. 1. *Effect of CUR* pretreatment in lipopolysaccharide-induced hepatitis in D-galactosamine sensitized rats *on HO-1 gene expression* related to beta-2-microglobulin as the endogenous control; CONTROL — vehicle only; CUR curcumin 100 mg/kg; DG + LPS — D-galactosamine 400 mg/kg + lipopolysaccharide 10 μg/kg after 24 hour incubation; CUR + DG + LPS — curcumin 1-hour pretreatment before D-galactosamine and lipopolysaccharide toxic insult; * value significant compared to negative control group (CONTROL) $p\leq0.05$, ### value significant to positive control group (DG + LPS) $p\leq0.001$; Means ± SEM, n = 7.

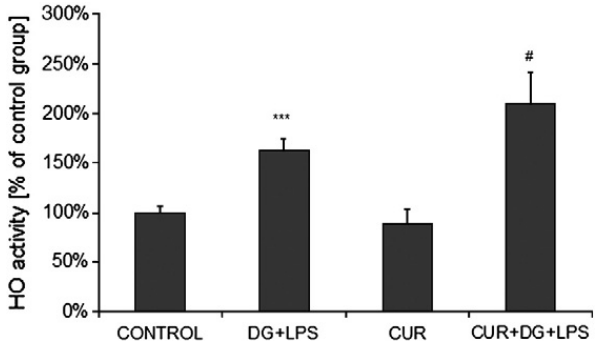

Fig. 2. Effect of CUR pretreatment in lipopolysaccharide-induced hepatitis in D-galactosamine sensitized rats *on total HO activity*; CONTROL — vehicle only; CUR curcumin 100 mg/kg; DG + LPS — D-galactosamine 400 mg/kg + lipopolysaccharide 10 μg/kg after 24 hour incubation; CUR + DG + LPS — curcumin 1-hour pretreatment before D-galactosamine and lipopolysaccharide toxic insult; *** value significant compared to group with vehicle only (CONTROL) $p<0.001$; # value significant to positive control group (DG + LPS) $p<0.05$; Means ± SEM, $n=7$.

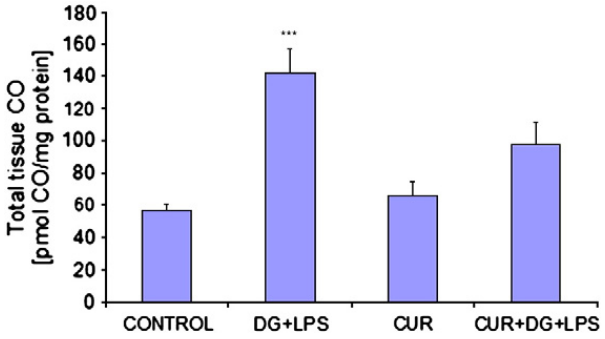

Fig. 3. *Effect of CUR* pretreatment in lipopolysaccharide-induced hepatitis in D-galactosamine sensitized rats *on total tissue carbon monoxide (CO)*; CONTROL — vehicle only; CUR curcumin 100 mg/kg; DG + LPS — D-galactosamine 400 mg/kg + lipopolysaccharide 10 μg/kg after 24 hour incubation; CUR + DG + LPS — curcumin 1-hour pretreatment before D-galactosamine and lipopolysaccharide toxic insult; *** value significant compared to group with vehicle only (CONTROL) $p<0.001$; Means ± SEM, $n=7$.

either HO gene expression/activity or CO/bilirubin levels (Figs. 1–3, Table 1).

Since CO is not only the product of heme catabolic pathway, but also one of the products of lipid peroxidation, we decided to measure the extent of lipid peroxidation and oxidative stress markers.

3.3. Effect of curcumin pretreatment on lipid peroxidation, oxidative stress markers and NOS-2/NO system

The extent of lipid peroxidation as evidenced by conjugated diene formation significantly increased in the livers of DG + LPS animals (3.5-fold, $p<0.001$ compared to controls). Curcumin pretreatment reduced the conjugated dienes levels to 1.6-fold of controls in CUR + DG + LPS ($p<0.01$, Table 2).

Interestingly, plasma catalase activity as a marker of antioxidant status was 2.4-fold elevated compared to controls in DG + LPS group and this level further increased to 3-fold following CUR pretreatment (CUR + DG + LPS group) clearly replicating plasma bilirubin concentration (Table 2).

Moreover, it was found that DG + LPS injection produced significantly more liver tissue NO_2^- as compared to control animals (Fig. 4). CUR was able to reduce DG + LPS-induced increase in NO_2^- production at statistically relevant level ($p<0.05$). However, single CUR injection did not affect NO_2^- production in liver of experimental animals. In accordance with these results, DG + LPS *per se* increased NOS-2 mRNA expression (240-fold) in the liver homogenates and CUR pretreatment highly significantly reduced this increase ($p<0.01$), as shown in Fig. 5.

4. Discussion

This work presents considerable hepatoprotective effect of curcumin in DG + LPS liver toxicity model mediated *via* modulation of HO/NOS systems.

Curcumin's cytoprotective, anti-inflammatory, antitumor, and antioxidant properties are based on several mechanisms. The reported ones are mainly based on the suppression of pro-inflammatory mediators like cyclooxygenase-2 (COX-2), lipooxygenase (LOX), inducible nitric oxide synthase (iNOS or NOS-2), nuclear factor kappa B (NF-κB) [29], tumor necrosis factor alpha (TNF-α) and others [30]. Another group of cellular constituents that can be affected by curcumin is the family of heat shock proteins. It was reported that induction of HSP 70 has cytoprotective effects [31], inhibition of HSP 90 results in the enhancement of anti-proliferative and pro-apoptotic activities [32] and induction of HSP 32 (= heme oxygenase-1) is associated with protection against oxidative stress [33].

The toxic combination of DG + LPS resembles clinical hepatitis [34–38]. In this model, we demonstrated ameliorative effects of curcumin pretreatment associated with regulation of HO-1. The liver protection was clearly demonstrated by the significant decrease in serum aminotransferases (ALT, AST) after CUR pretreatment in CUR-DG-LPS rats.

Table 2
Effect of CUR pretreatment in lipopolysaccharide-induced hepatitis in D-galactosamine sensitized rats *on catalase in plasma (CAT) and conjugated dienes in liver homogenate (CD)*; CONTROL — vehicle only; CUR curcumin 100 mg/kg; DG + LPS — D-galactosamine 400 mg/kg + lipopolysaccharide 10 μg/kg after 24 hour incubation; CUR + DG + LPS — curcumin 1-hour pretreatment before D-galactosamine and lipopolysaccharide toxic insult; means ± SEM, $n=7$.

	CONTROL	DG + LPS	CUR	CUR + DG + LPS
CAT [μg/ml]	55.43 ± 6.45	132.93 ± 10.20 ***	41.22 ± 3.23	164.35 ± 9.11 #
CD [nmol/mg/protein]	2.19 ± 0.37	7.76 ± 1.49 ***	1.77 ± 0.42	3.43 ± 0.60 ##

*** Value significant compared to respective group with vehicle only (CONTROL) $p<0.001$.
# Value significant to respective positive control group (DG + LPS) $p<0.05$.
Value significant to respective positive control group (DG + LPS) $p<0.01$.

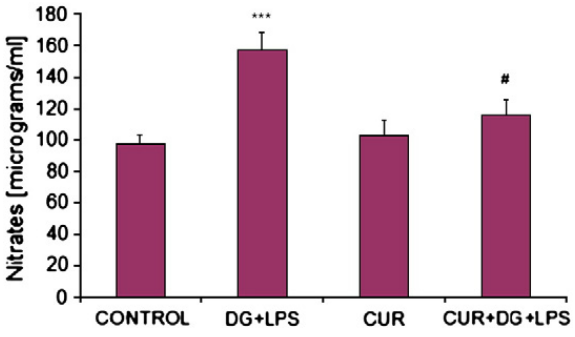

Fig. 4. *Effect of CUR* pretreatment in lipopolysaccharide-induced hepatitis in D-galactosamine sensitized rats *on cellular nitrates* (NO_2^-); CONTROL — vehicle only; CUR curcumin 100 mg/kg; DG + LPS — D-galactosamine 400 mg/kg + lipopolysaccharide 10 μg/kg after 24 hour incubation; CUR + DG + LPS — curcumin 1-hour pretreatment before D-galactosamine and lipopolysaccharide toxic insult; *** value significant compared to group with vehicle only (CONTROL) $p<0.001$, # value significant to positive control group (DG + LPS) $p<0.05$; Means ± SEM, n = 7.

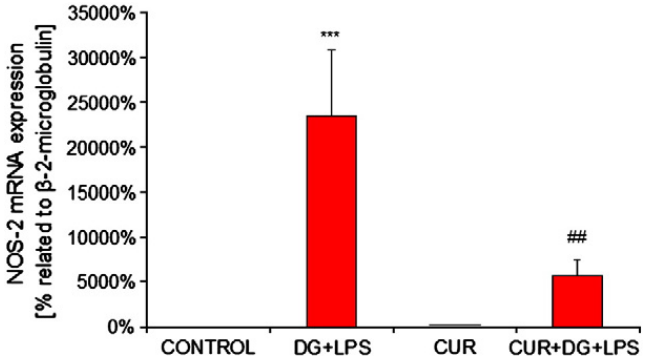

Fig. 5. *Effect of CUR* pretreatment in lipopolysaccharide-induced hepatitis in D-galactosamine sensitized rats *on NOS-2 gene expression* related to beta-2-microglobulin as the endogenous control; CONTROL — vehicle only; CUR curcumin 100 mg/kg; DG + LPS — D-galactosamine 400 mg/kg + lipopolysaccharide 10 μg/kg after 24 hour incubation; CUR + DG + LPS — curcumin 1-hour pretreatment before D-galactosamine and lipopolysaccharide toxic insult; *** value significant compared to negative control group (CONTROL) $p \leq 0.001$, ## value significant to positive control group (DG + LPS) $p \leq 0.01$; Means ± SEM, n = 7.

Interestingly, plasma bilirubin levels further increased in this group of animals. We demonstrate that this increase is not attributed to the severity of liver injury, but rather to the increased bilirubin production represented by concomitant elevation of HO activity and expression. This observation is of significant importance as bilirubin has been reported to possess strong antioxidant properties and to protect liver from oxidative stress-mediated injury [39,40]. Given its hydrophobic properties, unconjugated bilirubin has been shown to protect organism primarily against lipid peroxidation [41]. However, similar effect has been described for water-soluble conjugated bilirubin or albumin-bound bilirubin *in vitro* [42] and *in vivo* [43].

In this study, DG + LPS administration resulted in dramatic increase in lipid peroxidation as measured by conjugated dienes and nitrate production and NOS-2 mRNA expression in the liver and/or plasma of experimental animals. Curcumin pretreatment caused significant drop in all these parameters. Furthermore, increased catalase activity in plasma of curcumin pretreated rats reflects the increased antioxidant status of experimental animals under current pathophysiological conditions. Our results are in accordance with published data showing antioxidant and anti-inflammatory effects of curcumin in various models of organ injury [44–46].

Furthermore, our finding of HO-1/NOS system modulation is reflected in *in vitro* studies on RAW264.7 macrophages suggesting that inhibitory effect of curcumin on NO production is mediated via HO-1 up-regulation inactivating nuclear factor (NF)-kappa B [47].

Moreover, the decrease of lipid peroxidation after curcumin pretreatment might explain slightly lower level of liver CO after curcumin pretreatment despite higher HO activity. It has been published that CO is not only the product of heme catabolic pathway catalyzed by heme oxygenase, but also one of the products of lipid peroxidation [43,48]. Thus, lipid peroxidation might significantly influence final tissue pool of CO.

Importantly, single application of curcumin without concomitant oxidative stress and inflammatory liver injury had no effect either on HO-1 activity and expression or on NOS-2/NO system and oxidative status *in vivo*. We hypothesize that different regulation mechanisms might be involved under physiological and/or pathophysiological conditions as suggested in previous *in vitro* studies [14,49].

We conclude that this hepatoprotective effect of curcumin in LPS + DG model of liver injury is associated with significant up-regulation of HO-1 and/or its antioxidant and cytoprotective product bilirubin. This effect is followed by down-regulation of NOS-2 expression and consequent NO production and lower accumulation of lipid peroxidation products in liver and/or plasma of experimental animals.

Acknowledgments

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Resveratrol and related compounds as antioxidants with an allosteric mechanism of action in epigenetic drug targets

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Short title: Resveratrol and related compounds in epigenetic drug targets

Summary

The present review is intended to focus on naturally occurring cytoprotective agents such as resveratrol (trans-3,4',5-trihydroxystilbene) and other related compounds, probably with similar molecular mechanisms of action and high capacity to find applications in medical fields. Several physiological aspects have been ascribed to resveratrol and similar compounds. Resveratrol, among others, has been recently described as a silent information regulator T1 (SIRT1) activator that increases AMP-activated protein kinase (AMPK) phosphorylation and reduces the oxidative damage biomarkers during aging in laboratory settings. The reports on resveratrol and other SIRT1 activators from various sources are encouraging. The pharmacological strategies for modulation of sirtuins by small molecules through allosteric mechanisms should gain a greater momentum including human research. Resveratrol and resveratrol-like molecules seem to fulfill the requirement of a new horizon in drug research since these molecules cover a growing research means as antioxidants with allosteric mechanism in epigenetic drug targets. However, one should keep in mind the challenges of extrapolation of basic research into clinical results. Overall, the issue of sirtuins in biology and disease provides an insight on therapeutic potentials of sirtuin-based therapeutics and demonstrates the high complexity of drug-targeting these modalities for human applications.

Keywords: Resveratrol; SIRT1 activators; epigenetic drug target.

Background

Oxidative injury is well known to be associated both with the process of organism aging and with several chronic diseases that include among others, diabetes, atherosclerosis, age-related macular degeneration, cataract and Alzeheimer'disease. Moreover, oxidative stress that can be induced by toxins and environmental factors, leads to the accumulation of reactive oxygen/nitrogen species (ROS/RNS), further causing misbalance in pro-oxidant/antioxidant steady state. Protein crosslinking, lipid peroxidation, mitochondrial dysfunction and induction of cell death pathways are among the proposed mechanisms of cellular damage due to this misbalance (Jaeschke 2011; Jaeschke *et al.* 2012; Yeum *et al.* 2010). Foods, such as vegetables and fruits, rich in antioxidants are correlated with reduced risk of several chronic diseases. However, a recent meta-analysis of randomized trials, that included subjects treated with high doses of some antioxidants (e.g. beta-carotene, α-tocopherol), demonstrated various health problems (Christen *et al.* 2008; Dauchet *et al.* 2006; Dherani *et al.* 2008). As expected, it is likely that physiological doses of antioxidants combination, that might be included in adequate fruit and vegetable diet, are required to establish an effective antioxidant network *in vivo*.

Most of the basic science literature contains enormous numbers of molecules that promise to ameliorate almost any disease ranging from curing cancer to slowing the aging process itself. On the other hand most of these compounds were not sufficiently evaluated in humans. The present article is not intended to overview antioxidants of various sources but rather to focus on few naturally occurring cytoprotective agents such as resveratrol and curcumin. These polyphenolic compounds found in plant have a high potential to find applications in medical fields. Resveratrol (trans-3,5,4'-trihydroxystilbene, Fig. 1) has been

detected in fruits and some flowering plants, however, its major dietary sources include grapes, wine, peanuts, and peanut products.

Curcumin (Fig. 1) belongs also to a class of natural phenols called curcuminoids that are isolated from the Indian plant *Curcuma longa*. This phytochemical is highly consumed worldwide as a food colouring agent, food additive and it is a main component of the popular yellow spice turmeric. For many years, this substance has been largely used as a healing agent in much of the Indian and Chinese traditional medicine. Recently, however, curcumin has attracted attention of researchers due to its speculated anti-cancerous, anti-oxidant and anti-inflammatory properties (Aggarwal *et al.* 2007; Darvesh *et al.* 2012). Currently this drug is undergoing clinical trials for various cancers and neurodegenerative diseases, particularly Alzheimer's disease (Hatcher *et al.* 2008)**.** Its application in the treatment of Alzheimer's diseases is based on its ability to inhibit myeloid plaque formation; as well it has been shown to improve cognitive abilities of the elderly in small scale populations (Ng *et al.* 2006; Sikora *et al.* 2010).

Quercetin (Fig. 1) is another polyphenol isolated from natural sources including fruits (apples, cranberries), vegetables (onion, broccoli), tea leaves (*Camellia sinensis*), and grains (buckwheat). By itself, quercetin is the aglycone portion of several other flavonoid glycosides, including rutin and quercetrin, where it is bound to sugars such as rhamnose and rutinose. These polyphenols are also responsible for several health benefits of consuming fresh fruits and vegetables and are attractive focus of the current investigations. Several studies have shown quercetin to possess anti-inflammatory, anti-oxidant and anti-cancerous properties involving several different mechanisms (Chirumbolo 2010)**.** Cardiovascular studies have demonstrated that quercetin is a potent antioxidant that prevents depletion of a potent vasodilator, nitric oxide (NO), by scavenging free O_2^- , and thus lowering hypertension. Furthermore, in small-scale epidemiological studies done on hypertensive patients, quercetin

improved blood pressure and decreased oxidized LDL concentrations (Edwards *et al.* 2007; Egert *et al.* 2009; Galleano *et al.* 2010). As well, a small number of studies on hepatocytes have demonstrated quercetin's antioxidant potential where it increased antioxidant capacity of the hepatocytes, decreased pro-oxidant and inflammatory mediators and modulated expression of several antioxidant genes (Bharrhan *et al.* 2012; Ghosh *et al.* 2011; Jo *et al.* 2008; Weng *et al.* 2011; Zhao *et al.* 2011). Thus, given these encouraging findings of quercetin as a potent antioxidant, it is equally important to test this substance in its potential to ameliorate other diseases.

As for the focus of this review article, resveratrol represents an example to highlight the enormous difficulties in understanding general pharmacological profiles, determining side effects, and, ultimately, establishing mechanisms of action for a natural compound that belongs to the present topic (Smoliga *et al.* 2012). Resveratrol has multiple biological and pharmacological properties including antioxidant and anti-inflammatory effects. These properties were observed from classical studies reporting the beneficial effects of red wine which contains resveratrol in preventing cardiovascular disease, a phenomenon known as "French Paradox". This paradox refers to the fact that, in that geographical region, they have relatively low incidence of heart disease, despite the consumption of cheese and food high in fat. Although, this beneficial effect is mainly due to alcohol, resveratrol may add additional benefit. More recently, scientists have been exploring whether resveratrol could extend lifespan as well. The intention is that resveratrol or a chemically related compound might mimic the effects of a restricted diet, which has been shown to extend the life of mice (Baur 2010). A low level of caloric intake appears to be linked with activation of the class of proteins known as sirtuins that regulate metabolism, and are activated by natural substances such as resveratrol, which is very effective, experimentally, in this respect. Recent studies in this research area gave a cautious impression in that results involving *in vitro* or *in vivo*

studies often do not translate into drugs that work in humans. The experiments conducted so far have been based on a wide range of resveratrol dosage that would be impractical for human application. We have the relatively recent example of GlaxoSmithKline's concluded phase IIa study of SRT501 (a resveratrol pharmaceutical formulation in relatively high dose of resveratrol – 5 grams of SRT501 in advanced multiple myeloma). It was explained by the manufacturers that SRT501 offers minimal efficacy while having a potential to indirectly exacerbate a renal complication common in the patient population with multiple myeloma. Therefore researchers are hoping to find a way to concentrate the effect into a safe dose within an effective therapeutic range (Smoliga *et al.* 2012).

Additionally, the protective role of resveratrol and other polyphenols against a number of hepatic injuries (e.g. cholestasis) due to oxidative damage of primary rat hepatocytes was reported by several authors including our own *in vivo* and *in vitro* results (Ara *et al.* 2005; Cerný *et al.* 2009; Cerný *et al.* 2011; Farghali *et al.* 1994a; Farghali *et al.* 2009; Hebbar *et al.* 2005; Lekić *et al.* 2011; Kutinova-Canova *et al.* 2012; Wu *et al.* 2005; Yang *et al.* 2005). In addition, intraperitoneal administration of resveratrol in rats with ligated bile ducts maintained antioxidant defenses and reduced liver oxidative damage and ductular proliferation. Also, other naturally occurring substances of plant origin have been claimed to possess hepatoprotective actions and these include curcumin, tetrahydrocurcumin, catechin, quercetin and beta-carotene (Auger *et al.* 2005; Manda and Bhatia 2003; Pari and Murugan 2004; Raza *et al.* 2003).

Several other physiological aspects have been ascribed to resveratrol and polyphenolic compounds. Among these effects is the ability of resveratrol to attenuate obesity-associated peripheral and central inflammation and to improve memory deficit in mice fed high-fat diet (Jeon *et al.* 2012). Obesity-induced diabetes was shown to be associated with chronic inflammation and is considered a risk factor for neurodegeneration. It was hypothesized that

an AMP-activated protein kinase (AMPK) activator, resveratrol, which is known to exert a potent anti-inflammatory effects, would attenuate peripheral and central inflammation and improve memory deficit in mice fed a high-fat diet (HFD). Resveratrol treatment reduced hepatic steatosis, macrophage infiltration, and insulin resistance in HFD-fed mice. In the hippocampus of HFD-fed mice, the protein levels of pro-inflammatory tumor necrosis factor-alpha (TNF-α) and ionized calcium binding adaptor molecule 1 (Iba1) expression were reduced by resveratrol treatment. Choline acetyltransferase was increased, and the phosphorylation of tau protein was decreased in the hippocampus of HFD-fed mice upon resveratrol treatment. The authors found that resveratrol significantly improved memory deficit in HFD-fed mice (Jeon *et al.* 2012). These findings indicate that resveratrol reverses obesity-related peripheral and central inflammation and metabolic derangements and improves memory deficit in HFD-fed diabetic mice.

Resveratrol has been found to interact with multiple molecular targets, many of them associated with inflammation and immunity, thus its potential use in therapy of immune-mediated diseases was also reported (Švajger and Jeras 2012). Indeed, it has been shown to act directly on central players of both innate and adaptive immunity, such as macrophages, lymphocytes, and dendritic cells. Generally, resveratrol has been identified as a phytoalexin, antioxidant, cyclooxygenase (COX) inhibitor, peroxisome proliferator-activated receptor-alpha (PPAR-α) activator, endothelial nitric oxide synthase (eNOS) inducer, silent mating type information regulation 2 homolog 1 (SIRT1) activator belonging to a superfamily known as sirtuins whose name stems after their homology to the *Saccharomyces cerevisiae* gene silent information regulation-2 (Sir2).

Recent reviews on resveratrol and other SIRT1 activators from various sources are encouraging (Nakata *et al.* 2012; Villalba *et al.* 2012). This is related to developing strategies to protect against diet-induced metabolic imbalance, which is necessary in order to fight

against current obesity. It was suggested that the hypothalamus is a target for developing novel drugs that suppress SIRT1 degradation, as a strategy for treating metabolic syndrome. Deciphering the basic mechanism of sirtuin activators is essential to develop certain strategies to alter sirtuin activity. This is true regardless of the apparent controversy of whether *in vitro* activation of SIRT1 is direct or not, depending on the experimental design, and whether sirtuins may play a major role in longevity. The numerous studies on their positive effects against age-related diseases, obesity and other metabolic disorders are still valid, promising to positively influence the development of treatments to improve human health (Villalba *et al.* 2012).

In fact, resveratrol and similar compounds are attractive molecules that represent potential epigenetic targets in drug discovery with allosteric mechanism as will be discussed in the next sections.

Resveratrol and epigenetic drug target

Before discussing resveratrol as an epigenetic drug target, it is reasonable to shortly shed light on this topic, in so far as drug effect is concerned, in order to understand how some drugs may act. Epigenetics, at the molecular level, involves the dynamic regulation of covalent modifications to the histone proteins and DNA that influence gene expression and silencing, apoptosis, maintenance of stem cell pluripotency, X-chromosome inactivation and genomic imprinting without affecting DNA sequence (Sippl and Jung 2009). Therefore, epigenetics is considered as the conduit from genotype to phenotype. The epigenetic techniques emphasize the histone code and examine the utility of small molecule modulators of enzymes that modify histones and DNA. The dynamic remodeling of chromatin is essential to most DNA-based nuclear processes and it comes as no surprise that epigenetic changes are

implicated not only in normal development but also in various diseases. The large set of structural knowledge already obtained on epigenetic targets pave the way for drug design studies to act on major biological processes such as development, aging, diseases and cancer.

Among the basic knowledge gained on catalytic domains of the main histone modifying enzymes are histone deacetylases. Histone deacetylases (HDACs) catalyze the removal of acetyl groups from epsilon-N-acetylated lysine in a nucleosomal context, ensuring the reversibility of histone acetylation. Histone deacetylation is often associated with transcriptional repression and gene silencing, since it promotes chromatin of higher order structures and the recruitment of silencers. Among this superfamily is a HDACs class III which includes NAD^+-dependent deacetylases known as sirtuins (silent information regulator 2-related proteins). In the next section, we will discuss how resveratrol alters sirtuins in a specific mechanism that involves the concept of allosteric modulation.

Resveratrol as an allosteric modulator of the regulatory target SIRT1

Allosteric modulation of a receptor or a regulatory protein results from the binding of allosteric modulators to a "regulatory site" which is different from that of the endogenous ligand (an "active site") and enhances or inhibits the effects of the endogenous ligand. Under normal circumstances, allosteric modulators act by causing a conformational change in a receptor molecule, which results in a change in the binding affinity of the endogenous ligand. In this way, an allosteric ligand modulates the receptor's activation by its primary (orthosteric) ligand, and could act like a dimmer switch in an electrical circuit, adjusting the intensity of the response. A classical example is the $GABA_A$ receptor which has two active sites, one at which the neurotransmitter gamma-aminobutyric acid (GABA) binds, as well as the

benzodiazepine and general anesthetic agent regulatory binding sites. These regulatory sites can each produce positive allosteric modulation, potentiating the activity of GABA.

As a concept, allosterism was developed more than 50 years ago by Monod and coworkers to provide a framework for interpreting experimental studies on the regulation of protein function (Monod *et al.* 1965; Peracchi and Mozzarelli 2011). Basically, binding of a ligand at an allosteric site affects the function at a distant site exploiting protein flexibility and reshaping protein energy landscape. Both monomeric and oligomeric proteins can be allosteric. In the past decades, the behavior of allosteric systems has been analyzed in many investigations while general theoretical models and variations thereof have been steadily proposed to interpret the experimental data.

Allostery has been established as a fundamental mechanism of regulation in all organisms, governing a variety of processes that range from metabolic control to receptor function and from ligand transport to cell motility. A number of studies have shed light on how evolutionary pressures have favored and molded the development of allosteric features in specific macromolecular systems. The widespread occurrence of allostery has been recently exploited for the development and design of allosteric drugs that bind to allosteric sites leading to gain of function or loss of function. For example, small molecule activators of SIRT1 have been developed as therapeutics for the treatment of type 2 diabetes (Milne *et al.* 2007). Similarly to resveratrol, these compounds bind to the SIRT1 enzyme-peptide substrate complex at an allosteric site amino-terminal to the catalytic domain and increase the affinity for acetylated substrates. In the next section we will describe how resveratrol acts as an allosteric modulator of the regulatory protein SIRT1.

Molecular mechanism aspects of resveratrol actions

Several thousands of articles exist that deal with the biology and pharmacology of resveratrol, including many recent reports dealing with the molecular mechanisms of resveratrol´s cytoprotection (Suchankova *et al.* 2009; Wong *et al.* 2009). The vast interest in resveratrol which inspired enormous number of studies has led to the identification of multiple molecular targets of resveratrol (Athar *et al.* 2009; Pervaiz and Holme 2009; Smoliga *et al.* 2011; Smoliga and Rundell 2011).

Several potential beneficial effects of resveratrol could be attributed to its general effects as antioxidant (Aftab *et al.* 2010), anti-inflammatory (Bereswill *et al.* 2010), alteration of drug metabolizing enzymes (Chow *et al.* 2010), inhibition of cyclooxygenases (Wendeburg *et al.* 2009), and importantly specific effects on proteins and/or signaling cascades as SIRT1 and AMPK (Fullerton *et al.* 2010; Xiong *et al.* 2011) that are summarized in an inclusive table by Smoliga et al. (Smoliga *et al.* 2012). Hereby, we shed light on the most studied molecular mechanisms of resveratrol action, specifically with SIRT1, which controls a number of other regulatory factors associated with metabolism and inflammation as depicted in Fig. 2.

Resveratrol, as a SIRT1 activator, increased AMPK phosphorylation and reduce oxidative damage biomarkers during aging in F 2 hybrid mice (Wong *et al.* 2009). In addition, recent reports indicate intricate relationships between resveratrol, nuclear factors, autacoids and cytoprotection in various cells, tissues or organs. For instance in one study, resveratrol suppressed lipopolysaccharide (LPS)-induced nuclear translocation and activation of nuclear factor kappa B (NF-κB) in C6 microglia demonstrating an inhibiting effect of resveratrol on pro-inflammatory responses in microglia (Kim *et al.* 2007). Similar finding about the protective effect of resveratrol as an inhibitor of NF-κB-mediated vascular cell adhesion molecule (VCAM-1) induction was reported (Carluccio *et al.* 2007). Recently, NF-κB was suggested as a target for drug therapy in liver diseases where resveratrol was among several

agents that inhibits the aforesaid transcription factor. The fact that NF-κB has been associated with the induction of pro-inflammatory gene-expression makes research on agents which inhibit NF-κB an interesting current topic. Even other findings on experimental animals demonstrate that treatment with resveratrol can reduce structural airway remodeling changes and hyperreactivity which has important implications for the development of new therapeutic approaches to asthma (Royce *et al.* 2011). However, NF-κB has been considered as an anti-inflammatory factor in certain situations and thorough understanding of the function of the diverse NF-κB factors is needed to examine its relation with resveratrol or similar drugs with cytoprotective effects.

In our studies, we have investigated effects of resveratrol pretreatment on the enhancing action of D-Galactosamine (D-GalN) on LPS-induced liver failure in rats (Farghali *et al.* 2009) and in immobilized perfused hepatocytes as a short term bioreactor model with a chemical prooxidant (Cerný *et al.* 2009; Farghali *et al.* 1994b). Liver function was assessed together with plasma nitrite as a measure of NO, estimation of nonenzymatic and enzymatic antioxidants was performed in plasma and liver homogenate and morphological examinations were performed using light and electron microscopy. Observations related to pharmacological increases of inducible nitric oxide synthase (NOS-2) / NO and inducible heme oxygenase (HO-1) / carbon monoxide (CO) in fulminant hepatic failure and modulation by resveratrol were followed up by real-time reverse transcription PCR (RT-PCR) in liver tissue. In the last study we found that reduction in NO production, down-regulation of NOS-2 expression, modification of oxidative stress parameters and modulation of HO-1 are among the mechanisms responsible for the cytoprotective effect of resveratrol in the LPS/D-GalN liver toxicity and *tert*-butylhyroperoxide-induced hepatocyte toxicity models. This led to the overall improvement in hepatotoxic markers and morphology after the hepatic insult by resveratrol pretreatment.

From these results and due to the cardinal importance of sirtuins in the mode of action of resveratrol through AMPK activity, the next section will focus on this intricate inter-relationship.

What could be the pharmacologic benefits from SIRT1 activators?

Sirtuins constitute a family of highly conserved nicotinamide adenine dinucleotide (NAD^+)-dependent enzymes that deacetylate histones and residues of acetylated lysine. A common feature of the activity of sirtuins is their dependence on intracellular ratio of NAD^+ and its reduced form NADH. Sirtuins are reported to act as sensors that detect cellular energy availability leading to metabolic benefits (Chaudhary and Pfluger 2009), as calorie restriction extends lifespan in organisms ranging from yeast to mammals (Lin *et al.* 2004). It is shown that the mammalian Sir2 orthologue, SIRT1 (sirtuin 1), activates a critical component of calorie restriction in mammals; that is, fat mobilization in white adipocytes (Picard *et al.* 2004).

Recent studies suggest a key role for the mammalian SIRT1 in adequate cellular response to metabolic stress such as nutrient deprivation or overload and that SIRT1 and its activators play role in protection from the detrimental effects of metabolic stressors (Chaudhary and Pfluger 2009; Feige *et al.* 2008). A key role in the regulation of adipogenesis is played by the nuclear receptor PPAR-γ (peroxisome proliferator-activated receptor-gamma) (Spiegelman 1998). Indeed, upon food withdrawal SIRT1 protein binds to and represses genes controlled by the fat regulator PPAR-γ, including genes mediating fat storage. SIRT1 represses PPAR-γ by docking with its cofactors NCoR (nuclear receptor co-repressor) and SMRT (silencing mediator of retinoid and thyroid hormone receptors). Mobilization of fatty acids from white adipocytes upon fasting is compromised in SIRT1 (+/-) mice. Repression of

PPAR-γ by SIRT1 is also evident in 3T3-L1 adipocytes, where over-expression of SIRT1 attenuates adipogenesis, and RNA interference of SIRT1 enhances it. In differentiated fat cells, up-regulation of SIRT1 triggers lipolysis and loss of fat (Picard *et al.* 2004). As reduction in fat is sufficient to extend murine lifespan, the above-mentioned results provide a possible molecular pathway connecting calorie restriction to life extension in mammals.

In a study using liver-specific SIRT1-knockout mice, Chen *et al.* (2008) challenge the assumption that calorie restriction always activates SIRT1 in all tissue types by demonstrating that SIRT1 activity is reduced in the liver during calorie restriction, yet activated when mice are fed a high calorie diet. The study determines that liver-specific SIRT1-knockout mice have at least some protection, compared with wild-type mice, from accumulating fat while on a high-calorie diet. In contrast, while under calorie restriction, the liver-specific knockout mice have the same phenotype as wild-type mice. These observations suggest that hepatic SIRT1 may be inactivated during calorie restriction in normal mice and activated while on a high-calorie diet, opposite to what occurs in the muscle and the white adipose tissue that can be explained by different redox status and NAD/NADH ratio in the liver from other tissues under study conditions. It might raise the interesting possibility that SIRT1 inhibitors specifically targeted to the liver may be of benefit in treating obesity. Indeed, SIRT1 acts as cellular energy sensor that links metabolic stressors with an adequate cellular response. In the liver, SIRT1 activates PGC-1α (PPAR-γ coactivator-1α) during fasting to induce gluconeogenesis (Rodgers *et al.* 2005; Yang *et al.* 2007; Yeung *et al.* 2004).

Recent evidence demonstrates that there are similarities between AMPK (adenosine 5´-monophosphate-activated protein kinase) and SIRT1. They have similar effects on diverse processes such as cellular fuel metabolism, inflammation, and mitochondrial function. These similarities are due to the fact that AMPK and SIRT1 have regulatory influence on each other and share many common target molecules (Ruderman *et al.* 2010). These findings and the

concurrent demonstration by many laboratories of common activators, actions, and target molecules of SIRT1 and AMPK led to an examination of a possible linkage between SIRT1 and the primary upstream AMPK kinase, LKB1 (liver kinase B1). For example, the evidence for a SIRT1/LKB1/AMPK signaling mechanism was reported by Hou *et al.* (2008) who demonstrated that the ability of polyphenols (resveratrol, SI 17834) to activate AMPK in cultured HepG2 cells and mouse liver *in vivo* required the presence of both SIRT1 and LKB1. Likewise, in studies carried out predominantly in HepG2 cells, Suchankova et al. (Suchankova *et al.* 2009) noted that incubation with 25 vs. 5 mM glucose (6 h) or the SIRT1 inhibitor nicotinamide (10 mM, 2 h) down-regulated the activity of both AMPK and SIRT1 (indicated by increased PGC-1α acetylation), whereas incubation with pyruvate and the SIRT1 activator quercetin increased both of their activities. Similar effects of pyruvate and glucose on SIRT1 had been described previously in primary hepatocytes, suggesting that they are not unique to HepG2 cells (Rodgers *et al.* 2005)

Another important target for SIRT1 is NF-κB, a regulator of many processes, including cell cycle, apoptosis, and inflammation. SIRT1 down-regulates NF-κB-mediated pro-inflammatory effects by deacetylating the RelA/p65 subunit of NF-κB (Yang *et al.* 2007; Yeung *et al.* 2004). It is well-documented that chronic overfeeding, by increasing circulating fatty acids, might lead to inflammation, insulin resistance and injury in the liver (Mollica *et al.* 2011). SIRT1-overexpressing transgenic mice have decreased hepatic NF-κB activity, which protects from high-fat diet/lipid-induced hepatic inflammation, glucose intolerance, and nonalcoholic fatty liver disease (NAFLD) (Pfluger *et al.* 2008). Another study by Lee *et al.* (2009) finds that SIRT1-mediated attenuation of NF-κB signaling prevents cytokine-induced pancreatic β-cell damage.

The SIRT1 network is elegantly reviewed very recently by Kazantsev and Outeiro (2012), who discussed studies on human SIRT1, which illuminate functional relationships of

gene–protein interactions, controlling major metabolic pathways and the possibility of rational design of SIRT1 effectors. Overall, the issue of "sirtuins in biology and disease" provides an insight on therapeutic potentials of sirtuin-based therapeutics and demonstrates the high complexity of drug-targeting these modalities for human applications.

Do we have small molecules as sirtuin modulators?

Milne *et al.* (2007) identified novel small-molecule SIRT1 activators with 1000-fold higher pharmacologic potency compared with the structurally unrelated polyphenolic SIRT1 activator, resveratrol. Obese rodent models treated with these SIRT1 activators show improved whole-body glucose homeostasis and insulin sensitivity in liver, skeletal muscle, and adipose tissue, as well as an increased mitochondrial capacity in skeletal muscle. A very potent SIRT1 agonist, SRT1720 (Fig. 1), further increases endurance and enhances oxidative metabolism in muscle, liver, and brown adipose tissue, which might contribute to the observed protection from diet-induced obesity (Feige *et al.* 2008). Such increased metabolic performance might be induced by concerted deacetylation of the SIRT1 substrates PGC-1α, p53, FOXO-1 (forkhead box-containing protein-1) and FOX (forkhead box) proteins (a family of transcription factors that play important roles in regulating the expression of genes involved in cell growth, proliferation, differentiation, and longevity). SIRT1-mediated, indirect activation of AMPK and phosphorylation of acetyl-CoA carboxylase (ACC) might also contribute to the observed protection from NAFLD in SRT1720-treated mice fed a high-fat diet. Interesting reports on small-molecule SIRT1 activators also suggest promising new therapeutic approaches to treat metabolic diseases such as type 2 diabetes or NAFLD (Milne *et al.* 2007). Resveratrol, that is the most widely studied activator of SIRT1, as a small polyphenol improves insulin sensitivity and vascular function, boosts endurance, inhibits

tumor formation, and ameliorates the early mortality associated with obesity in mice. Most of these effects are consistent with the modulation of SIRT1 targets, such as PGC-1α and NF-κB. Moreover, it also activates AMPK, inhibit cyclooxygenases, and influence a variety of other enzymes (Baur 2010). The novel activator, SRT1720, as well as various methods to manipulate NAD+ metabolism, are emerging as alternative methods to increase SIRT1 activity, and in many cases recapitulate effects of resveratrol.

The role of sirtuins in modulating redox stressor and the links between sirtuins and their oxidative/redox environment and reviewing the control mechanisms that are regulated by the activity of sirtuin deacetylase proteins was reported recently (Webster *et al.* 2012). It is expected that pharmacological strategies for modulation of sirtuins by small molecules through allosteric mechanisms will gain a greater momentum including human research as was suggested in earlier reports (Dai *et al.* 2010; Sauve 2009).

Conclusion

Although allosteric drug sites and epigenetic targets in drug discovery have been studied for several years, more studies are needed in this area. Antioxidants, which are casually used together with the synthesis of new resveratrol-like molecules, are good drug models to follow up the various molecular events responsible for potential beneficial cytoprotective effects. Indeed, resveratrol and resveratrol-like molecules seem to open new horizons in drug research since these molecules cover a growing research area on antioxidant compounds with allosteric mechanism in epigenetic drug targets. However, one should keep in mind the challenges of extrapolation of basic research into clinical results which is well understandable.

In fact, all data give a strong justification for further experimental and clinical studies. Moreover, in spite of the copious research articles on resveratrol, this small structure entity gives further impetus to clinical pharmaceutical chemists to do the necessary computational biological chemistry and bioinformatics to synthesize more potent molecules, which could reproduce the required pharmacological and clinical effects. We need to investigate all about effects of resveratrol and similar compounds in various models *in vitro* and *in vivo* and then reassemble the results and perform, whenever possible, human studies that are very much lacking.

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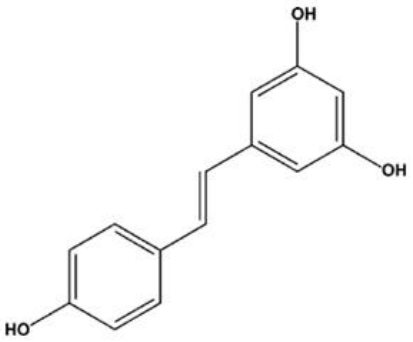
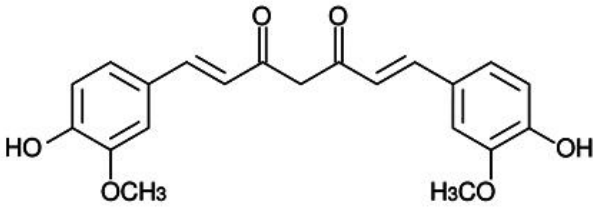
Figure 1. Sirtuin activators of natural (resveratrol, curcumin and quercetin) and synthetic (SIRT1720) origin.



Resveratrol



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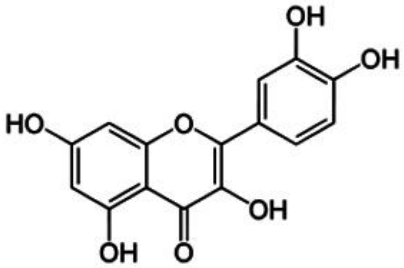
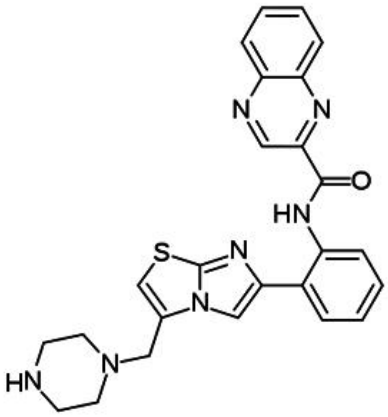


Quercetin



SRT1720

Figure 2. Proposed molecular mechanisms by which SIRT1 and AMPK activate each other and control other regulatory factors associated with metabolism and inflammation as they are described in the text (→ activation, ⊥ inhibiton). ACC, acetyl-CoA carboxylase; AMPK, AMP-activated protein kinase; eNOS (NOS-1), endothelial nitric oxide synthase; FOXOs, forkhead box-containing proteins; HO-1, inducible heme oxygenase; LKB1, liver kinase B1; NAD^+, nicotinamide adenine dinucleotide; NOS-2, inducible nitric oxide synthase; PPAR- γ, peroxisome proliferator-activated receptor-γ; PCG-1α, PPAR- γ coactivator-1α; NF-κB, nuclear factor kappa-B; SIRT1, silent information regulator T1; VCAM-1, vascular cell adhesion molecule-1.