A Pre-clinical investigation on hepatotoxicity and osteoporosis induced by amphetamine derivative (methamphetamine and 3, 4-methylenedioximethamphetamine) with associated metabolic pathways using rat

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Declaration

Not any portion of this work referred to this thesis paper has been submitted for any degree or qualification of the University of Dhaka or any other University or educational institution.

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List of abbreviations

ALT	Alanine transaminase
AST	Aspartate transaminase
ALP	Alkaline phosphatase
ATP	Adenosine triphosphate
ADP	Adenosine diphosphate
AP-1	Activator protein-1
ALD	Alcoholic liver disease
AFMU	5-acetylamino-6-formylamino-3-methyluracil
cAMP	Cyclic adenosine monophosphate
AOPP	Advanced oxidation protein product
ATPase	Adenosine triphosphatase
nACh	Nicotinic acetylcholine
BMD	Bone mineral density
BAFF	B cell activating factor
CYPs	Cytochrome P450s
COX-2	Cyclooxygenase-2
CXCR4	CXC chemokine receptor type 4
CCl_4	Carbon tetra chloride
CCL2	Chemokine (C-C motif) ligand 2
CLD	Chronic liver disease
CGRP	Calcitonin gene-related peptide
COMT	Catecholamine-O-methyl transferase
CREB	cAMP response element-binding protein
CAT	Catalase
Cu-ZnSOD	Copper-zinc superoxide dismutase
DNA	Deoxyribonucleic acid
DAMPs	Damage-associated molecular patterns
DCs	Dendritic cells
DEXA	Dual-energy X-ray absorptiometry
DTNB	5,5-dithiobis-2-nitrobenzoic acid

EI	Electron impact ionization
ECM	Extra cellular matrix
EROD	Ethoxyresorufin -O-deethylase
EC-SOD	Extracellular superoxide dismutase
FasL	Fas ligand
GSSG	Glutathione disulfide
GSH	Reduced glutathione
GGT	Gamma-glutamyl transferase
G-6-P	Glucose 6-phosphate
G-6-PDH	Glucose-6-phosphate dehydrogenase
GC-MS	Gas chromatography-mass spectrometry
HHMA	3,4-dihydroximethamphetamine
HMMA	4-hydroxy 3-methoxymethamphetamine
HMPA	(4-hydroxy-3-methoxyphenyl) acetone
5-HT	Serotonin
HIF1-α	Hypoxia-inducible factor-1α
HSCs	Hepatic stellate cells
HCV	Hepatitis C virus
HIV	Human immunodeficiency virus
4-HNE	4-hydroxynonenal
H_2O_2	Hydrogen per oxide
γ-IFN	γ-interferon
IP	Intraperitoneal
IP-10	Interferon gamma-induced protein 10
ICAM-1	Intercellular adhesion molecule-1
IgE	Immunoglobulin E
ILC2	Innate lymphoid cell2
IL	Interleukin
K_2HPO_4	Dipotassium phosphate
KC	Kupffer cell
LOD	Limit of detection

LOQ   Limit of quantification

LT   Leukotrienes

LSECs   Liver sinusoidal endothelial cells

LPS   Lipopolysaccharide

MCP-1   Monocyte chemoattractant protein-1

MMPs   Matrix metalloproteinases

MROD   Methoxyresorufin o-demethylase

Mn-SOD   Manganese-dependent superoxide

MDA   Malondialdehyde

MDA   3, 4-methylenedioxiamphetamine

Meth   Methamphetamine

MDMA   3, 4-methylenedioximethamphetamine

NADPH   Nicotinamide adenine dinucleotide phosphate

NF-κB   Nuclear factor kappa B

NFAT   Nuclear factor of activated T cells

Nrf2   Nuclear factor -E_2 related factor-2

NMR   Nuclear magnetic resonance

NEP   Norephedrine

NOD   Nucleotide-binding oligomerization-domain

NLRs   NOD like receptors

NO·   Nitric oxide radical

NOSs   Nitric oxide synthases

iNOS   Inducible nitric oxide synthases

nNOS   Neuronal nitric oxide synthases

eNOS   Endothelial nitric oxide synthases

NAFLD   Nonalcoholic fatty liver disease

NOX2   Nicotinamide adenine dinucleotide phosphate oxidase 2

NK   Natural killer

NKT   Natural killer T

NGO   Non government organization

PAMPs   Pathogen-associated molecular patterns

PB   Phenobarbital

PROD   Pentoxyresorufin-o-dealkylase

PBS   Phosphate buffer solution

PKA   Protein kinase

Prx   Peroxiredoxins

PRDX2   Peroxiredoxin-2

PGs   Prostaglandins

PDGF   Platelet-derived growth factor

PQCT   Peripheral quantitative computed tomography

RANTES   Regulated on activation, normal Tcell expressed and secreted

r^2 Regression co-efficient

RANKL   Receptor activator of nuclear factor kappa-B ligand

RNA   Ribonucleic acid

ROS   Reactive oxygen species

RNS   Reactive nitrogen species

STAT3   Signal transducer and activator of transcription 3

SOD   Superoxide dismutase

SEM   Standard error of mean

TBA   Thiobarbituric acid

TCA   Trichloroacetic acid

TRX   Thioredoxin

TNF-α   Tumor necrosis factor-α

TNFSF3   TNF superfamily member 3

TXA_2 Thromboxane A_2

TRAIL   TNF-related apoptosis-inducing ligand

TGF-β   Transforming growth factor beta

TLRs   Toll-like receptors

TIMPs   Tissue inhibitor metalloproteinases

TBARS   Thiobarbituric acid related substances

sTNFR   Soluble TNF receptors

TMJ   Temporomandibular joint disorder

TNFR   Tumor necrosis factor receptor

VMAT-2   Vesicular monoamine transporter-2

VCAM-1   Vascular cell adhesion molecule-1

VHD   Valvular heart disease

Abstract

Abuse of amphetamine derivative as methamphetamine (Meth, Yaba) or 3, 4-methylenedioximethamphetamine (MDMA, Ecstasy) is on a raising trend globally and Bangladesh is not an exception of this. The long term effects of abusing these drugs include risk of developing permanent brain damage with various psychological, cognitive and behavioral effects that may manifest as depression, anxiety, memory loss and various neuropsychotic disorders. Many studies, survey and case reports have described the clinical manifestations associated with the abuse of these drugs included mental disorder, cardiac disorder, jaundice, tooth decay, etc. But yet surprisingly little is known about the toxic effect of these drugs on vital organs like liver and bone. The purpose of this study was to look insight and reveal the effects of Yaba and Ecstasy on liver and bone using rat model.

The present study was conducted to determine the toxic effects of amphetamine derivatives using two representative tablets, Yaba and Ecstasy, where Yaba was subjected to measure liver and bone toxicity and Ecstasy to monitor the cytochrome P450 mediated metabolic pathways *in vivo*. Along with methamphetamine Yaba also contain a significant amount of caffeine. So the toxic action of Yaba might be due to the contribution of its both components. To clarify this, a separate study was also carried out with caffeine to determine its effect on liver and bone *in vivo.* The *in vivo* study performed with Yaba tablet used Long Evans rats for eight weeks to investigate liver and bone toxicities. ALT, AST, ALP level of plasma; AOPP, MDA, NO, Catalase, Glutathione and SOD level of plasma and tissue were measured. Histopathological study of rat liver was done with H&E and Picro Sirius Red staining. Rat tibias were tested by digital X-ray. Study with caffeine was performed following the same schedule as Yaba. The third experiment carried out with Ecstasy (3, 4-methylenedioximethamphetamine, MDMA) used Sprague Dawley rats, pretreated with phenobarbital (PB) or β-naphthoflavone (BNF), to monitor the metabolic pathways of MDMA in liver by measuring urine drug metabolites concentration and liver CYP isozymes content.

Liver inflammation was observed in Yaba treated rat liver with significantly elevated plasma level of ALT, AST, and ALP. Plasma and liver tissue level of MDA and NO were significantly increased. AOPP, Catalase, GSH, SOD activity in plasma and liver found unchanged as

compared to control. In caffeine treated rat, liver transaminases, ALT, AST, ALP; oxidation end product, AOPP, MDA and oxidative stress indicator, NO increased significantly, Catalase & GSH remained unchanged but SOD decreased significantly as compared to control. Histology of liver tissue showed invasion of inflammatory cells and progressive deposition of collagen fibre in both Yaba and caffeine treated group. Digital X-ray of rat tibia treated with Yaba showed radiolucency not significant as compared to control. In urine analysis of MDMA treated rats, end product of N-demethylation, 3, 4-methylenedioxiamphetamine (MDA) concentration was significantly decreased in PB and BNF treated rats and end product of O-methylation, 4-hydroxy 3-methoxy methamphetamine (HMMA) concentration was significantly increased in PB and BNF treated rats as compared to control. Hepatic enzyme assays showed increased activities of CYP1A1(600-fold), CYP1A2(4-fold), CYP2B(4-fold) and CYP3A(2-fold) related enzymes after 24 hours of MDMA administration in the inducer pretreated rats as compared to control. In PB treated rat urine MDMA concentration decreased significantly. The total percent dose recoveries of MDMA and three metabolites in urine samples was less than 35% of the administered dose.

The overall results suggested that methamphetamine tablet Yaba produced inflammation and fibrogenesis in liver. Caffeine, the second ingredient of Yaba showed similar liver toxicity in histochemical study. However, the extent of biochemical changes, notably higher in caffeine treated group than those of Yaba, suggested that methamphetamine in combination with caffeine might follow dissimilar pathways for liver toxicity to that followed by caffeine separately. The effect of Yaba on rat bone turnover was inconclusive. Metabolic pathway study results of 3,4-methylenedioximethamphetamine (MDMA) suggested that PB and BNF induced CYP isozymes might have inhibitory effects on N-demethylation of MDMA to MDA in rats. HHMA, the precursor of HMMA, increased through the process of O-demethylenation of MDMA by induced CYP isozymes. Decreased urinary concentration of MDMA in PB pretreated rats indicated its increased metabolism. The truly low percent recoveries of MDA, HMMA and HMPA in inducer pretreated rats suggested that other major pathways of MDMA metabolism might exist which were activated through inducer pretreatment.

Chapter One

Introduction

1.1. Drug abuse and health hazards: Bangladesh context

Drug abuse is a direct threat to the economy and social aspects of a country. In Bangladesh, it is a national crisis growing every day. People of different age group, ranging from 18 to 30, of different professions and of different social status are involved in this dreadful habit. There are millions of drug-addicted people in Bangladesh.

In Bangladesh, mostly abused recreational drugs are Heroin, Phensidyl, Pethidine, Ganja, Chorosh, Bhang, Seduxene, Opium etc (Shazzad et al., 2013). Recently a large portion of addicted population has become dependent on amphetamine and its derivatives. Methamphetamine (Meth, Yaba) has become a popular drug of abuse among Bangladeshi drug addicts. Over the past six years Yaba has reached almost to the apex of illicit drug list in this country with its increased abuse by 77 times where as other drugs like phensidyl and heroin market has decreased. Especially Yaba tablet has got increased popularity because of its availability, low cost, ease of administration and rapid action (within 5-6 minutes). However, the repeated and chronic use of Yaba is associated with undesirable and dangerous psychological and physiological effects as well. 3, 4-methylenedioximethamphetamine (MDMA, Ecstasy), another illicit drug abused by Bangladeshi addicts, have been shown by different studies to exert toxic effects on brain, heart, kidney, liver and skeletal system. Oxidative stress produced by this drug considered as causative factor for their toxicity. Parent drug or its oxidative metabolites are thought to provoke oxidative stress leading to different organ damage.

Myanmar is a regular supplier of a major part of the illicit drugs reported to be smuggled into Bangladesh. Apart from Myanmar, the drug is also smuggled into this country from some other regions including India, Nepal and Thailand b y air and land routes (Karim, 2016). Being produced in unauthentic manufacturing areas and without following any standard composition of raw materials Yaba has been spread throughout the Bangladeshi illegal drug market.

In past, methamphetamine was therapeutically used as nasal decongestant and anti-asthmatic drug. Especially at the time of Hitler this drug was given to his soldiers in World War-II to have energy and increased stamina (McPhe et al., 2010). In different dosage forms, methamphetamine can be administered through three routes- oral, inhalation or parenteral. According to literature, Yaba tablet usually composed of the synthetic drug 'methamphetamine' in combination with

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'caffeine' and/or 'heroin' in varying amount. Besides oral application, Yaba tablets also found to smoke by melting the whole tablet or crushed powder by heat. People use methamphetamine in the desire of getting euphoria, decreased appetite, insomnia, increased smartness, more energy, good feelings, increased self-reliance etc. Interview with Yaba abusers revealed that a regular user needs 8-10 tablets daily on an average and each tablet costs about Taka 600-1000. The worst thing of Yaba abuse includes rapid development of tolerance and progressive increase in amount of drug demand (Fattah, 2012; Richards, 2015; Sheridan et al., 2006). Increased tolerance or dependence developed from frequent use of MDMA is evident for its addiction by few pre-clinical or clinical data. However, some unreliable reports suggest that demand to achieve the powerful psychoactive effects same as the first day of use, provokes people to increase the amount of MDMA intake (Steele et al., 1994). Brain exhausted of serotonin by high doses of MDMA said to be associated with its getting tolerance. Evidences suggested that low or even moderate dose of MDMA, insufficient to produce serotonin depletion, only associated with persistent behavioral dysfunction rather development of dependency.

Recreational drug abuse eventually limits the normal activities of life through malfunctioning different vital organs including heart, kidney, brain, liver and bone as well. In a short time of abuse started, tolerance developed from most abused drugs and results in increasing dose. Common features associated with drug abuse include lack of patience, sleep disturbance, confusion, head-ache, feeling dizziness during interval of taking drugs, hallucination, purturbance, lethargy, sexual abnormality, violence, abolished humanity and judgment, indiscipline in personal life etc. Caffeine increases respiratory rate and exert broncho-dilatation effect. On GIT, caffeine exerts stimulating effect on gastric acid and pepsin secretion and sometimes reported to increase acid reflux by weakening lower esophageal sphincter (Robertson et al., 1978; Pincomb et al., 1985; Williams et al., 1985; Davis et al., 1988; Prineas et al., 1980; Sutherland et al., 1985; Benowitz et al., 1985; Smits et al., 1985; Murat et al., 1981; Gong et al., 1986; Benowitz, 1990).

The rapid increase rate of drug addicts reported in recent epidemiological surveys carried on the three divisions of Bangladesh reflected that this country is going to turn to a potential venue of drug abuse very soon. Increased criminal activities like hijacking, extortion etc. to collect money for purchasing drugs destroys social security and peace. Worldwide drug abuse has recognized as

a burning issue of public health. Besides the activities of law enforcement agencies and other NGOs to stop the trafficking and abuse of these drugs, health care department holds the responsibility to figure out possible health hazards occurred from these abused drugs. Information about toxic effects of drugs from different pre-clinical or clinical investigations would be supportive for making awareness among common people about the destructive sites of drug abuse. Immediate stoppage of illicit drug transportation into and within this country is essential to save our population and country from this deadly game (http://www.assignmentpoint.com/science/medical/current-status-in-bangladesh).

Deteriorative effects of amphetamine derivatives and caffeine abuse on human health particularly on vital organs including the proposed mechanisms are discussed in this chapter.

1.2. Effect of amphetamine derivatives and caffeine abuse on brain

Over the past decade drug addiction has been considered as a brain disease. Structural and functional changes of the brain due to repeated use of addictive drug can persist after even stopping them. When brain becomes adapted with different changed chemical and physiological conditions, addiction takes place (Leshner, 1997). Addiction, started with progressive increase of drug intake, manifested as a circuit of malfunctioning of brain with compulsion of taking it which ends with loss of control on taking it (Koob et al., 1997).

Drugs that are abused for achieving pleasant sensation mostly act by acting on neurotransmitter. Many of those increase dopamine release and cause persistent nerve stimulation which gives energetic and euphoric effects. This chronic and uncontrolled stimulation causes damage to nerve terminals.

 Several mechanism of neurotoxicity of abused drugs has been suggested as:

(i) Decrease in tryptophan hydroxylase activity by acute methamphetamine administration at higher dose

(ii) Damage of dopaminergic nerve terminals through increasing glutamate neurotransmitter which is mediated by α7 nicotinic acetylcholine (nACh) receptors. Elevated concentrations of extracellular glutamate, released as a result of neural injury, are toxic to neurons (Purves, 2001)

(iii) Decreased activity of vesicular monoamine transporter-2 (VMAT-2) and resultant dopamine deficiency which reflects damage to dopamine axon terminal (Little et al., 2003)

(iv) Reduced dopamine transporter function contributing to reduced dopamine reuptake;

(v) Neurodegeneration due to apoptosis induced by endoplasmic reticulum and mitochondria generated oxygen based radicals (Hotchkiss et al., 1980).

(vi) Effect on the serotonin system in the similar way as dopamine system (Krasnova et al., 2009) by stimulant drugs.

Significant health hazards are associated with both short term and long term use of methamphetamine. The mental disturbance observed in short term use include acute paranoia, anxiety, confusion etc. Prolonged use of methamphetamine develops depression, social isolation, mood disturbances and psychomotor dysfunction (Darke et al., 2008; Homer et al., 2008; Scott et al., 2007). Different studies suggested that methamphetamine might cause brain degeneration after long term use by damaging dopamine and serotonin axons. Activation of microglia, hypertrophic white matter, loss of gray matter in different brain areas are other associated features of neuronal apoptosis. Nerve terminal degeneration has been proposed to be mediated by oxidative stress, hyperthermia, neuroinflammatory responses, mitochondrial dysfunction, endoplasmic reticulum stress etc. (Krasnova et al., 2009).

Studies with methamphetamine neurotoxicity proposed that this drug causes excitotoxicity to neuron. One mechanism explained that methamphetamine binds with dopamine transporter, enters into presynaptic nerve terminal and force dopamine to be released from vesicles. Thus increases dopamine and serotonin concentration in synaptic cleft. Another mechanism included that methamphetamine decreases dopamine reuptake by reducing activity of dopamine transporter and thus causes persistent nerve stimulation which ends in nerve damage. Free radical mediated apoptosis of nerve cell also suggested as the pathway of methamphetamine induced neurotoxicity (Northrop et al., 2011).

3, 4-methylenedioximethamphetamine (MDMA), the ring substituted amphetamine derivative, has been reported to increase the release of both serotonin (5-HT) and dopamine neurotransmitter from nerve endings in the brain of animal under experiment. Rat experiment with long term use of MDMA revealed increased locomotor activity and other behavioral symptoms related to serotonin activation where as similar study on mice showed selective damage of dopamine nerve ending. As mechanism of neurotoxicity by MDMA, different studies

suggested the formation of highly reactive free radicals probably generated from the further breakdown of MDMA metabolites (Green et al., 2003). Increased levels of reactive species e.g. reactive oxygen and nitrogen species may be developed inside body from many sources other than abusive drugs such as exposure to heavy metals which is potentially toxic environmental contaminant; smoking, UV irradiation etc (Song et al., 2010). It is well established from animal studies that persistent stimulation and release of neurotransmitter by high doses of the drug can eventually develop persistent deficiency of neurotransmitter in brain (Baumann et al., 2009).

Disruption of mitochondrial function by psychostimulants has been proven by many studies characterized by a rapid and transient decrease in striatal ATP stores (Burrows et al., 2000). Byoung et al. suggested that oxidative-modifications of various mitochondrial proteins, leads to mitochondrial dysfunction and ultimate cell damage. Increased release of reactive oxygen species from mitochondrial respiratory chain mediates the oxidative changes which may be associated with neurodegenerative diseases (Burrows et al., 2006).

Caffeine (1, 3, 7-trimethylxanthine) exerts dose dependent effect on nervous system, heart and metabolic process. Maximum 250 mg of caffeine may be administered to get bodily positive effects like feelings of alertness, decreased fatigue, and eased flow of thought etc. Caffeine intake more than 250 mg and up to 500 mg result in adverse effects like restlessness, nervousness, insomnia, and tremors (Yew et al., 2014). Renata et al. suggested that chronic coffee and caffeine ingestion protected the endogenous antioxidant system in rat brain and reduced lipid peroxidation of brain membrane; thus prevented age related cognitive function turn down. Caffeine mainly acts by antagonizing adenosine receptors (A_1 & A_2) non selectively and reverse the action of adenosine. On central nervous system, caffeine induces low blood flow by vasoconstriction and releases catecholamines from presynaptic neurone by opposing action of adenosine (Abreu et al., 2011; Fredholm, 1985; Mathew et al., 1985).

1.3. Effect of amphetamine derivatives and caffeine abuse on heart

Amphetamine and its derivatives, reported as cardiotoxic drugs, manifest their toxic effects by increased heart rate, hypertension, cardiac myopathy, cardiac necrosis or myocardial infarction, cardiac arrhythmia or cardiac failure etc. Left ventricular hypertrophy mediated by oxidative

stress from methamphetamine abuse was described by a separate study (Lord et al., 2010). These drugs commonly induce hyperthermia (Jacobs, 2006).

Cardiotoxicity induced by stimulants usually commenced with excessive activity of catecholamines. Persistent release of dopamine by methamphetamine abuse increases heart muscle contraction and heart rate. Additionally, catecholamine toxicity produces fibrous tissue and increases the size of heart muscle cells. Study with acute or chronic administration of methamphetamine suggested its cardiotoxicity induction. Acute cardiotoxicity of methamphetamine marked with acute myocardial infarction and sudden cardiac death whereas its chronic cardiotoxicity associated with development of coronary artery disease and cardiac myopathy (Kaye et al., 2005). However, several clinical consequences resulted in cardiomyopathy with degraded cardiac mitochondria, extreme myofibril contraction and lost myofilament. Methamphetamine treated rats, diagnosed with myocytic degeneration and necrosis in heart, developed myocardial damage with sign of myocytolysis, contraction bands, atrophied myocytes, and spotty fibrosis (Song-Yue et al., 1996).

A relationship between MDMA induced increase in serotonin release and cardiotoxicity has been established. MDA (3, 4-methylenedioxiamphetamine), the N-demethylated metabolite of MDMA, reported by several review papers as a potent agonist of 5-HT2B receptors present in cardiac valve. However, the chronic MDMA abusers are at high risk of suffering from valvular heart disease (VHD) (Baumann et al., 2009). A different study suggested HHMA (3, 4-dihydroximethamphetamine), the O-demethylenated metabolite of MDMA, as a significant contributor to cardiovascular disease (Schindler et al., 2014). Hypertension and consequent heart failure frequently observed by MDMA and other related drugs abusers were evident to mediate through increased release of brain regulated noradrenaline hormone. The devastating cardiovascular toxicity signs associated to increased noradrenaline secretion include ruptured blood vessels, internal hemorrhage and tachycardia with a progressive cardiac workload.

On cardiovascular system, caffeine, by a moderate intake, induces peripheral vasoconstriction, slight increased blood pressure, reduced heat rate, systemic release of catecholamine and renin, a balanced change in cardiac output and contractility. Chronic coffee consumption is found to be associated with increased level of serum LDL cholesterol and cardiac arrhythmia. In contrast,

high dose of caffeine decreases blood pressure by vasodilatation and increases systemic catecholamine extremely.

1.4. Effect of amphetamine derivatives and caffeine abuse on kidney and thermoregulatory mechanism of the body

Being the major site of excretion, kidneys frequently victimized by drugs or their toxic biotransformed products either directly or indirectly. Amphetamine derivative especially methamphetamine reported to induce rhabdomyolysis with flow of massive toxins through kidney causing it to shut down. Another mechanism of kidney failure by the stimulant drug described as increased heart rate and contraction of blood vessel which cut off blood flow to the kidney (www.narconon.org/drug-abuse/stimulants/toxic-organs). Renal damage and peroxidative injury observed in rat kidney after acute and sub-acute administration of methamphetamine in immunohistochemical study in a separate investigation. Blood test revealed significant increase in creatinine and creatinine phosphokinase and decrease in K, Ca & P suggesting renal tubule damage by repeated administration of methamphetamine (Tokunaga et al., 2006)

Effect of MDMA on thermoregulatory mechanism of the body results in hyperpyrexia through the stimulation of serotonin and dopamine receptor systems which constructs pathogenesis by two ways: hyponatremia and inactivation of antioxidant enzymes. Increased thirst and extreme water intake due to copious sweating of restless and energetic drug abusers creates hyponatraemia which may develop hyponatraemic encephalopathy. Cellular damage by MDMA through increased lipid peroxidation is aggravated from decreased catalase and glutathione peroxidase activity in hyperthermic condition induced by MDMA as well. In another study, uncoupling of skeletal muscle mitochondria by MDMA in vivo was reported as underlying reason for increased thermogenesis which is supported by the elevated ambient temperature of the crowded rave parties. MDMA mediated increased noradrenaline release may be connected with the increased uncoupling event. Moreover, marked elevation of creatinine phosphokinase is also associated manifestation of hyperhermia. Hypovolumetric hypotension mediated acute tubular necrosis together with myoglobinuria, if persistent with excessive sweaing and hyperthermia, promote nephrotoxicity. In this connection, disseminated intravascular coagulation is suggested to give rise to acute kidney injury. Other mechanisms of MDMA induced nephrotoxicity suggested by different studies include proximal tubular cell damage by the

oxidative product of MDMA metabolites; obstruction of filtration by urinary bladder neck dysfunction; accelerated or malignant hypertension; water retention by the kidneys due to improper release of antidiuretic hormone from pituitary gland (Kalant, 2001; Shankaran et al., 1999; Bronstein et al., 1995; Michael et al., 2003; Carvalho et al., 2002; Rusyniak et al., 2005; Mallick et al., 1997; Gong et al., 1997; Hall et al., 2006; Fahal et al., 1992; Hall et al., 1996; Ninkovic et al., 2008; Kreth et al., 2000; Bryden et al., 1995; Woodrow et al., 1995; Satchell et al., 1994; Holden et al., 1996).

Glomerular filtration rate and tubular reabsorption, increased by caffeine creates hyponatraemia. Contribution of caffeine on increased body temperature is not supported by any strong evidence.

1.5. Effect of amphetamine derivatives and caffeine abuse on skeletal system

Bone remodeling oriented by the co-ordinate activities of osteoblast, osteocyte, osteoclast and bone lining cells continues good bone quality. The quantity of mineralized bone and bone per unit volume, known as bone mass and bone density respectively, represent bone quality. Inherited factors and environmental influences such as pathology, nutrition and drug use influence bone remodeling. However, exact reason of drug induced disruption of bone remodeling still requires thorough investigation. Few evidences suggested that drug induced bone defect might resulted from inhibition of growth of trabecular bone by increasing urinary excretion of calcium (in case of caffeine) or direct toxicity to osteoblast cells (alcohol). Drug induced bone toxicity frequently manifested as: osteomyelitis or bone infection- generated usually from intravenous drug abuse using dirty needles and contaminated drugs; osteoporosis- caused by the malnutrition seen in long-term stimulant users; osteopenia or reduced bone density- created usually from opiate addiction; temporomandibular joint disorder (TMJ), dental damage and decay- associated with stimulant abuse probably due to habitual teeth-grinding during stimulation and stress. Malnutrition among drug abusers causes poor joint health and arthritis. The poor postural habit of abusers also affects their spinal health causing weakening of spine's supporting muscles and increasing risk of spinal injury (http://www.drugrehabfl.net/effects-of-alcohol-abuse-on-skeletal-system).

Several studies suggested methamphetamine abuse to exert negative effect on bone. Bone disease induced by methamphetamine abuse may be of different types including osteomyelitis or bone infection, osteoporosis, osteopenia or reduced bone density, temporomandibular joint disorder, dental damage and decay etc. The strength of the calcaneus bone determined by ultrasound bone densitometer and broadband ultrasound attenuation found significantly lower among methamphetamine abusers as compared to control (Katsuragawa, 1999). In a separate study, methamphetamine decreased bone density in lumbar spine among its abusers (Kim et al., 2009). Low levels of Vitamin K associated with low rates of bone turnover increased risk of osteoporosis. However, methamphetamine toxicity in mice expressed in different ways as in human abusers. Researchers found increased osteoblast and decreased osteoclast activity in mice after long term treatment with methamphetamine resulted in increased bone formation (Tomita et al., 2015).

According to Dr. Vivek Shetty and colleagues at the University of California, Los Angeles, about 41% of methamphetamine abusers suffer from increased dental disease (www.drugabuse.gov, 2010). Meth causes dry mouth which is associated with hyposalivation. Methamphetamine induced mouth dryness results in tooth decay by reducing the protective action of saliva. Being a sympathomimetic amine, methamphetamine acts on α- and β-adrenergic receptors and stimulates the receptors in the vasculature of the salivary glands; vasoconstriction takes place which reduces salivary flow (www.researchgate.net / publication/ 7445408, 2005). The risk of caries and demineralization increases due to reduction of salivary flow resulted from hyposalivation. The pathology resulting from action of the drug on saliva production and abnormal dehydration of mouth are called xerostomia.

Methamphetamine users usually consume large quantities of carbonated sugary soft drinks. Furthermore, lack of personal and oral hygiene of long-term methamphetamine abusers associated with many behavioral side effects. The acidic composition of methamphetamine and the drug's capacity for increasing motor activity, such as excessive chewing, tooth grinding and clenching are additional risk factors to contribute to the destruction of teeth and oral health. These risk factors related with methamphetamine users predispose to extensive caries, giving rise a condition termed as "meth mouth" (Shaner, 2002; McGrath et al., 2005; Wynn, 1997). Besides

meth mouth, methamphetamine has been known to cause premature osteoporosis, clinically manifested by brittle teeth and bones vulnerable to break easily. A separate study suggested that methamphetamine induced adverse effects is dose-dependent and influenced bone metabolism through provoking osteoporosis in mice which was characterized by increased bone forming marker and decreased bone resorption marker in 5 and 10mg/kg methamphetamine dose group respectively (Tomita et al., 2014).

Yadav et al. (2013) suggested a link between chronic liver disease and a complicated bone disease, osteoporosis, diagnosed based on the bone mineral density (BMD) and assessment is done using dual-energy X-ray absorptiometry (DEXA) scan. Low bone mineral density in osteoporosis proven to be connected with various factors included vitamin D, calcium, insulin growth factor-1, activated receptor of nuclear factor-κB ligand (RANKL), bilirubin, fibronectin, leptin, proinflammatory cytokines, and genetic polymorphisms. Low production of active vitamin D in chronic liver disease lowers calcium absorption which in turn leads to bone diseases like rickets or osteomalacia. Cholestatic liver disease, where bile production and flow is impaired, lowers vitamin D level and cause bone disease. However, low vitamin D level also observed in non-cholestatic liver disease.

As a part of normal function, liver produces the active form of circulating vitamin D (25-OH vitamin D) which helps in calcium absorption (Nair S, 2010). Long term liver malfunction due to drug induced toxicity may be indirectly contribute to low calcium absorption and hence low bone quality. Moreover, vitamin D insufficiency, associated with secondary hyperparathyroidism, accelerates bone loss by increasing bone turnover. Chronic impaired bone mineralization because of chronic vitamin D scarcity leads to a pathologic condition, through accumulation of osteoid, known as osteomalacia. Two mechanisms usually work for bone loss- increased bone turnover and uneven bone remodeling. Bone remodeling process may be imbalanced by decreased bone formation or increased bone resorption or from both. Some researchers suggested chronic liver disease responsible for defective bone remodeling mediated by increased bone resorption while others showed decreased bone formation even in non osteoporotic condition.

Bone turnover can be diagnosed by two biochemical markers: bone resorption markers and bone formation markers. Collagen type 1 (procollagen propeptides), osteocalcin and alkaline

phosphatase isozyme generated from bone are clinically considered as the principal markers of bone formation. Abundance of alkaline phosphatase in chronic liver disease makes its accurate measurement difficult as a marker for bone formation and hence considered as weak identification marker. To assess bone resorption, the best way to measure the urinary concentration of deoxypyridinoline, type 1 collagen cross linked N-telopeptide and pyridinoline. These results are usually compared with the creatinine value of urine. Hydroxyproline concentration in urine is another marker less frequently used for bone resorption identification (Collier et al., 2002; Crosbie et al., 1999; Gallego-Rojo et al., 1998).

Considering this light of information, we have investigated the effect of Yaba tablet on bone quality of rat tibias after its long-term administration. In present study we have taken digital X-ray of rat tibias of Yaba treated and control rats to compare the radio lucency or radio opacity of bones.

Investigation on rats treated with caffeine revealed that coffee or caffeine intake inhibit calcium metabolism and results in higher levels of calcium in the urine and plasma as compared to bone, decreased bone mineral density and bone volume. Moreover, caffeine has negative effects on osteoblast function and bone matrix formation and thus bone repairing process is delayed. Caffeine suppresses calcium absorption from intestine to some extent. Thus people, in habit of caffeine intake and lack of calcium in diet, are highly at risk of developing osteoporosis. A separate study reported that high dose caffeine intake inhibits the secretion of parathyroid hormone (PTH) through the mechanism of decreasing intracellular cAmp. PTH contributes directly to the survival and differentiation of osteoblast cell and hence increased population of osteoblasts useful for the treatment of osteoporosis and bone formation. A correlation between high coffee consumption and low PTH serum levels has been observed in men which was opposite in a separate study with young women. However, this relationship requires further detailed investigation.

1.6. Effect of amphetamine derivatives and caffeine abuse on liver

Clinical evidences suggested that majority of patients in Bangladesh appeared in the department

of hepatology are diagnosed with liver diseases. In 80% of cases, death occurred due to liver disease is from acute hepatic failure (Khan, 2010; Rahman et al., 2014; Alam et al., 2009). The risk factors for liver damage detected as chronic and heavy alcohol consumption, viral hepatitis or chronic intake of liver toxic medicine. Drug induced hepatotoxicity comprises about 10% of all types of acute hepatitis (Zimmerman, 2000). Liver may be damaged by both hepatocellular and extracellular mechanisms. However, the exact pathway is not still clear. Some proposed mechanisms for drug induced hepatotoxicity included: hepatocyte damage through rupture of the membrane; transport protein disruption; bile flow blockade and resultant cholestasis; T cells activation and stimulation of many-sided immune responses by cytokines when drug binds with the P-450 enzyme and act as immunogen ; apoptotic pathways activation by the tumor necrosis factor-alpha receptor of Fas; mitochondrial disruption and decreased ATP production; injury to the bile duct epithelium by toxic metabolites(Yew et al., 2014).

Liver, the vital organ of vertebrates, perform numerous functions of which metabolism comprises the major part. Four most important highly specialized cells and vessels constitute liver. Majority of liver tissues composed of parenchymal cells known as hepatocytes which are the main site of metabolic activities such as various biochemical reactions regarding synthesis and breakdown of essential biomolecules, necessary for normal body activities. Hepatocytes work in separate groups to perform multiple functions included repairing of the injured liver by rapid regeneration and biotransformation of the harmful entities. Rest of the cells of liver tissue or non parenchimal cells are kupffer cells, stellate cells and liver sinusoidal cells which function as defender from harmful bacteria and antigens, store vitamin A and control the passage of nutrients and important molecules from blood vessel into liver. Hepatic stellate cells additionally contribute to liver regeneration and formation of fibrosis and tumor as well (Remmer, 1970).

In spite of other sites, liver is considered as the main site of drug metabolism. The liver's position between drug absorption site and systemic passage and moreover its metabolic role favors the drugs or toxic metabolites to attack it very easily as compared to other target organs (Russmann et al., 2009). Especially for oral tablet, due to passage of drug through portal circulation, liver is at higher risk of attack by toxic drug. Moreover, the same probability raises from its biotransformed toxic metabolites. Liver, by itself, produces the necessary enzymes for metabolism or biotransformation of nutrients or xenobiotics making those suitable for used by

body, stored or eliminated as whatever needed. Types of various chemical reactions associated with drug metabolism are grouped into phase I and phase II reactions included oxidation, reduction, hydrolysis, hydration, conjugation, condensation, or isomerization. Many drugs undergo metabolism by both or anyone of the phases. Phase I reactions, known as nonsynthetic type, involve formation, modification or cleavage of functional groups where as synthesis of new conjugated molecules with endogenous glucuronic acid, sulfate or glycine takes place in Phase II reactions. In brief, through the metabolic reactions take place in liver, water soluble or more polar compounds are produced in the aim of terminating them in dissolved form by urine or in bile through feces (Jennifer, 2014).

A group of enzymes, collectively known as mixed-function oxidase or monooxygenase, included cytochrome P450, cytochrome b5, and NADPH-cytochrome P450 reductase involved in drug-metabolism. Liver microsomes contain abundant cytochrome P450s (CYPs), a microsomal superfamily of isoenzymes, which exert their inductive or inhibitory effects to influence drug or xenobiotic metabolism in different ways (Sheweita, 2000). In phase I metabolism, cytochrome P-450 catalyses the oxidation of drug with the help of molecular oxygen. In this cycle, a flavoprotein NADPH–CYP450 reductase transfers electron from NADPH to cytochrome P-450. Through this process reactive oxygen species, known as superoxide ions, are produced. Among more than 50 enzymes of cytochrome P450 superfamily only 6 are reported to be involved in maximum drug metabolism of which CYP3A4 and CYP2D6 enzymes considered as the most significant participants (Lynch et al., 2007).

Synthesis of CYP isozymes in liver can be increased or inhibited by many drugs which have greater importance in drug interactions. The risk of this influence may be enhancement of one drug toxicity by another drug or reduction of the therapeutic effect. Phase II metabolism conjugates follow different routes to eliminate from body. Glucuronides are excreted through bile and urine equally where as glycine conjugates predominantly excreted in urinary pathway but in minor amount through bile. Sulfate conjugates mainly follow urinary route.

Hepatic efficacy of metabolism by cytochrome P-450s is age dependent. This capacity gradually reduces with the increase of age because of reduced hepatic blood flow. Hence, aged persons

experience prolonged half-life and variable effects of the same drug as compared to the younger persons. Unlikely, phase II conjugation processes are independent of aging. However, glucuronide formation rate is slow in neonates making them vulnerable to toxicity by phase I generated reactive metabolites (Jennifer, 2014). Genetic polymorphism in drug metabolizing enzymes is a significant factor behind the variable response of patients to common drugs manifested as very fast or very slow rate of drug metabolism (Lynch et al., 2007). Very rapid metabolism of a drug is associated with persistent lack of therapeutic level of drug in blood and tissue of the patient where as too slow rate of metabolism gives rise to prolonged high concentration of drugs that produce toxic effects even from usual dose. Besides genetic factors, some contemporaneous disorders like chronic liver disease, advanced heart failure or drug interactions affect drug metabolism rates in individuals.

Large amount of lipid soluble drug aggravates synthesis of cytochrome-P450 by hepatocytes which result in more rapid oxidation of drug and less conjugation. Competitive binding of overloaded drugs for non specific CYPs takes place giving rise to slow metabolism and resultant longer drug action. Phase I drug metabolism by CYP system sometimes generates active and toxic metabolites which might be injurious to hepatocytic cells (Yamamoto et al., 1998).

Possible ways of drug mediated liver toxicity may be summarized as-

- Toxic substances production through bioactivation of drugs by the enzyme system of liver normally
- Idiosyncratic or nonpredictable type drug induced liver injury manifested as hepatitis, cholestasis, or combination of both.
- Age, sex, chronic liver disease, alcohol ingestion or genetic predisposition considered as the host factors to support drug induced liver injury. Usually adults are more susceptible than children and women are at higher risk than men to drug induced liver toxicity
- Lipophilic drugs promote liver injury more depending on the dose of medication and its extent of metabolism in liver.
- Activation of several pathways of hepatocytic death included apoptosis, necrosis or necroptosis by many drugs initiates liver injury.

- Liver injury through inflammation is initiated and propagated by the parent drug and their active metabolites with the assistance of neighboring immune cells. The ultimate fate of this injury is hepatocytic death.

Drug produces toxicity by either directly or through its active metabolites. Metabolism study of methamphetamine in human and rat showed 4-hydroxymethamphetamine, the C-hydroxylated metabolite, as the main metabolite excreted in urine. Minor metabolites in human included hippuric acid, norephedrine, 4-hydroxyamphetamine, 4-hydroxy-norephedrine and benzylmethylketone where as in rat 4-hydroxynorephedrine, amphetamine, 4-hydroxyamphetamine and benzoic acid (Caldwell et al., 1972). A separate study reported that cytochromes P450 conduct C-hydroxylation and N-hydroxylation of methamphetamine followed by N-demethylation where the constitutive form of CYP showed more catalytic action on C-hydroxylation as compared to phenobarbital and 3-methylcholanthrene-inducible forms (Baba et al., 1988).

No tissue damage experienced from direct insertion of MDMA into brain or heart has been proven the dependence of MDMA on its biotransformation in the liver to exert its toxic action. Superoxide dismutase and other antioxidant enzymes, calcium-binding protein and metabolism involved proteins present in cytosol undergo structural alteration by MDMA generated oxidative stress and carry out hepatocyte damage through promoting apoptosis. Moreover, MDMA mediated phosphorylation of antiapoptic Bcl-2 protein by activating several kinases suggested accountable for the stimulation of tissue apoptosis in vitro (Upreti et al., 2011).

It has been reported that HHMA, the catecholamine intermediate and metabolite of MDMA may play vital role in neurotoxicity by forming thioether adducts in human (Segura et al., 2001; Perfetti et al., 2009). Hence plasma and urine concentration of HHMA is an important measurement to establish its existence in MDMA metabolism and possible contribution to the neurotoxicity produced from MDMA abuse by people.

The racemic mixtures of MDMA in its tablet form may be responsible for its variable pharmochological effects (acute and chronic). Selective metabolic rate of the enantiomers of MDMA possibly connected with its rapid and slow action. The (*S*)-enantiomer, metabolized faster than the (*R*)-enantiomer is reported to be consistent with the acute psychomotor activity and subjective effect of individuals exposed to it, whereas the much slower metabolism of (*R)-*

enantiomer corresponds to mood and cognitive effects commences from the following day of MDMA use (Pizarro et al., 2007).

Urine analysis of MDMA treated human revealed the exixtence of three forms of the drug: the unchanged parent drug, its phase I metabolites and phase II metabolites. MDMA is metabolized by two ways. According to the proposed mechanism, MDMA may be at first O-demethylenated, then methylated and in the next, conjugated into glucuronide or sulphate. Another way included first N-dealkylation, then deamination, oxidation and finally conjugation with glycine. Involvement of CYP2D6 isozyme and catecholamine-O-methyl transferase (COMT) was studied on O-demethylenation and O-methylation pathways respectively. The metabolites of the mentioned pathways are 3, 4-dihydroximethamphetame (HHMA) and 4-hydroxy-3-methoxymethamphetamine (HMMA) respectively. In human urine, sulfate conjugates of MDMA appeared more as compared to glucuronides. Research on MDMA metabolism suggested HHMA as the major metabolite with high plasma concentrations almost same as the parent compound (Segura et al., 2001). MDA, metabolite produced through MDMA dealkylation and α-methyldopa, produced from MDA form glutathione adduct and thus lower cellular glutathione (GSH) level. An in vitro study with freshly isolated rat hepatocytes resulted in α-methyldopa mediated profound cell death. MDMA induced hepatotoxic activity deteriorates more with the increase of temperature (Schwaninger et al., 2011; Hall et al., 2006; Carvalho et al., 2004; Carvalhoá et al., 2001).

Caffeine undergoes N-demethylation and/or ring oxidation through its biotransformation process and metabolites produced are theophylline, paraxanthine, theobromine and 1, 3, 7-trimethyluric acid in man. Further decomposition of these compounds takes place to give rise dimethylated uric acids, monomethylxanthines and monomethyluric acids. A separate study suggested that CYP1A2 isozyme catalyzes the demethylation process of caffeine which is further reportedly acetylated to 5-acetylamino-6-formylamino-3-methyluracil (AFMU) by the polymorphic acetyl transferase (Huang et al., 2002; Tassinari et al., 1991; Wink et al., 1996; Lacerda et al., 2010; Heaney, 2002; Lu et al., 2013; Jilka et al., 2007; Landin-Wilhelmsen et al., 1995; Paik et al., 2010; Tang-Liu et al., 1983; Butler et al., 1992).

Common markers of liver toxicity include abnormal liver enzyme level in blood, increased level of lipid and protein oxidation products, decreased level of antioxidant enzymes, increased

ammonia level etc. Several pathological conditions like chronic hepatitis, alcoholism, acetaminophen overdose and other toxic liver conditions can give rise to impaired liver function. Toxic ammonia then cannot be removed from the body rather it concentrate in blood which is clinically known as hyperammonemia. Study showed that increased blood level of ammonia causes nerve cells to become overexcited and engulf the brain's defenses (www.urmc.rochester.edu/news/story/3969, 2013). The magnitude of increase of liver transaminases (ALT, AST) and alkaline phosphatase (ALP) levels represent the type of liver damage (Leise et al., 2014). Elevation of ALP is the significant marker among the serious liver injury cases usually attended in clinical trials (Maddur et al., 2011). Other clinical outcomes of liver injury include allergic hepatitis, toxic hepatitis, chronic active toxic hepatitis, toxic cirrhosis, and liver vascular disorders etc.

From hepatic blood flow, through a transmembrane protein, drug enters into hepatocytes where it interacts with one of a number of enzymes, the most commonly with CYP2C9, 2C19, 2D6, and 3A4. Patient's nutritional state and genetic polymorphism influences these enzyme activities and thus varying individual risk for toxicity. Transport of conjugated metabolites from the hepatocyte by membrane proteins also affected by genetic polymorphism as well leading to some patients having an increased risk for toxicity. The unconjugated unstable metabolites damage lipids, proteins, or even DNA through the process of oxidation. Cellular death and reabsorption of dead cell by surrounding cells takes place. In response to cell damage, the inflammatory process is activated with the activation of Kupffer cells, killer cells, B-cells, and other T-cells and production of inflammatory cytokines. Drugs or active metabolites, transported or diffused into the mitochondria or the nucleus damage DNA, leading to mutagenicity and ultimately hepatic cancers (DiPiro et al., 9th Edition).

1.7. Possible pathways by which amphetamine derivatives and caffeine induce liver injury
Hepatocytic response to toxic insult depends on the co-existing factors. A good combination of age, sex, genetic, hormone, cellular energy, local oxygen supply, environmental factors and previous liver disease contribute to expression of cell death mediators (Pessayre et al., 1999). Generally, hepatocytes activate the defense mechanisms through hypertrophic endoplasmic reticulum, induced glutathione (GSH), and heat shock and acute phase proteins. The two major

pathways are there for drug induced death of hepatocytes: necrosis or/and apoptosis (Grattagliano et al., 2009). Hepatotoxicity by toxin generally follows a sequence like reactive metabolite formation, depletion of antioxidants and alkylation of protein. A separate study described one pathway of liver cell death where reactive oxygen and nitrogen species (ROS & RNS) causes mitochondrial dysfunction through mitochondrial membrane permeability transition and leads to membrane potential collapse, decreased ATP production, release of nucleases which ultimately activates death caspases (Jaeschke et al., 2006).

1.7.1. Cellular apoptosis

Apoptosis, the ATP dependent programmed cell death, initiated with the activation of caspase cascade and involves some death ligands and receptors. Drug induced hepatocytic apoptosis activation follows two pathways: intrinsic or mitochondrial pathway and extrinsic or death receptor pathway. Drug or its metabolites produce intracellular signals for intrinsic pathway activation (Scaffidi et al., 1998). Apoptic proteins target the mitochondria and cause its swelling by increasing membrane permeability. As a result, mitochondrial protein leak out into cytosol and binds with apoptosis inhibiting protein and blocks its function; thus allows apoptosis to proceed. Cytochrome C, released from mitochondria, serves regulatory function for apoptosis. In extrinsic way, pro-apoptic ligands bind with cell surface death receptors. Then the receptors cluster and form death-inducing signaling complex. For example, TNF-α acts as extrinsic mediator of apoptosis by binding with TNFR1 and TNFR2 receptors on cell surface (Ramzi et al., ISBN 0-7216-7335-X). Cytokines then binding with cell surface death receptors promotes a cluster of death domains and interleukin (IL)-1β converting enzyme activation which activates caspases and nucleases. However, hepatocytes show resistance to TNF-α-induced cytotoxicity in normal condition because some other surface receptors are stimulated to stimulate the synthesis of anti-apoptic molecules and enzymes (Hatano et al., 2001).

1.7.2. Cellular necrosis

The caspase-independent cell death induced by death receptors, referred to as "necrotic cell death" (Morgan et al., 2007), simply represents a more severe cellular response than apoptosis at higher concentration of injurious stimuli (Parola et al., 2001). Necrosis is a traumatic cell death. High amount of stress, generated from activated Kupffer cells and endothelial cells, causes

severe change in hepatic cellular homeostasis resulting in increased mitochondrial membrane permeability, loss of membrane potential, inhibition of Ca^{2+}-dependent ATPase, decreased ATP synthesis, cellular energy depletion, decreased capability to sequester Ca^{2+}, oxidation of actin, microfilament breakage, and membrane bleb formation (Vendemiale et al., 1996). Abnormal control of cell volume promotes hepatocyte necrosis. Increased cellular osmotic load causes cell swelling. The increase of cytosolic Ca^{2+} worsen the osmotic stress and results in plasma membrane rupture (Barros et al., 2001)

Loss of cell membrane potential as a result of cellular energy depletion, membrane protein damage and impairment of ATP dependent ion channel or pumps provokes hepatocytic necrosis. Malfunctioning of ion pumps of plasma membrane allows opening of some death channels through which cations enter and cause cytoplasm swelling and membrane rupture (Nishimura et al., 2001). Cell produces ATP from oxidative phosphorylation of ADP to ATP in mitochondrial matrix and from glycolysis in cytosol. Inhibition of mitochondrial respiration results in decreased ATP production causing decreased cellular energy which ultimately promotes necrosis.

Certain stimuli like increased intracellular Ca^{+2} ion, inorganic phosphate, basic pH or reactive oxygen species etc open mitochondrial transition pores of inner membrane and reduces transmembrane potential. Loss of protein gradient immediately shut down ATP synthesis through oxidative phosphorylation (Haworth et al., 1979). The persistent opening of transition pores leads to cell necrosis (Marzo et al., 1998). Intracellular Ca^{+2} plays important role in many cell signaling including necrosis. Some Ca^{+2} -dependent proteases calpains are activated when intracellular Ca^{+2} level is increased from influx of this ion through plasma membrane or released from endoplasmic reticulum. The activated proteases cleave the Na/ Ca^{+2}exchanger. The cells become unable to exclude Ca^{+2} and thus its increased concentration sustained (Bano et al., 2005). The high level of cytosolic Ca^{+2} triggers mitocholdrial Ca^{+2} overload leading to mitochondrial permeability transition, ATP depletion and also Ca^{+2} dependent protease activation. Concentration of Ca^{+2} more than 1μM leads to cell necrosis (McConkey et al., 1996). Cytoskeletal proteins, membrane proteins, adhesion molecules, iontransporters, kinases, phosphatases, and phospholipases are the substrates for proteases (Rami, 2003). Calpain leads to

release of lyso-somal enzymes by lysosomal membrane permeability (LMP) and causes necrotic cell death (Yamashima, 2004).

1.8. Drug induced liver injury and consequent inflammation

Inflammation, a kind of protective immune response of body towards the hazardous stimulants like pathogens, irritants or even dead cells, initially sensed by macrophage or mast cells and mediated by cytokines or chemokines and continued on affected tissue or organ (Medzhitov, 2010). The components of this protective immune response include blood vessels, mast cells , tumor necrosis factor (TNF-α), tryptase, chemokines, reactive oxygen species (ROS), histamine, bradykinin, prostaglandin and leukotrines, interleukin-1. The inflammatory process, strictly regulated by the body, included stoppage of harmful stimuli, clearance of damaged tissues and repairing of injury. The extent of this response determines its positive or negative effect on body. Extremely low level of inflammation can't stop tissue damage where as chronic inflammation frequently becomes the cause of disease itself.

Inflammatory response may be acute or chronic. Body initially protects harmful stimuli or injury by activating its inherent immunity regarded as acute inflammation. This short acting protective response usually not damaging to the host tissue. However, the second type long lasting chronic inflammation, harmful for host cell, may predispose various chronic illnesses, including cancer (Lin et al., 2007). During inflammation, mast cells and leukocytes lead to a 'respiratory burst' after recruited to the site of damage because of increased uptake of oxygen. This results in an increased release and accumulation of ROS at the site of damaged area (Hussain et al., 2003). The soluble mediators produced by inflammatory cells, such as metabolites of arachidonic acid, cytokines and chemokines, further recruit inflammatory cells to the injury site to produce more reactive species. These main mediators activate signal transduction cascades and cause changes in transcription factors like nuclear factor kappa B (NF-κB), signal transducer and activator of transcription 3 (STAT3), hypoxia-inducible factor-1α (HIF1-α), activator protein-1 (AP-1), nuclear factor of activated T cells (NFAT) and NF-E2 related factor-2 (Nrf2), which mediates cellular stress responses immediately. Induction of cyclooxygenase-2 (COX-2), inducible nitric oxide synthase (iNOS), abnormal expression of inflammatory cytokines e.g. tumor necrosis factor (TNF), interleukin-1 (IL-1), IL-6 and chemokines e.g. IL-8; CXC chemokine receptor 4

(CXCR4), along with altered expression of specific micro RNAs, have thought to play significant role in inflammation induced by oxidative stress (Hussain et al., 2007).

Ceasation of stimuli ultimately stops acute inflammation where as persistent stimulation gives rise to activation of chronic inflammatory pathway. In relation to inflammatory response, the resident Kupffer cells and other non-parenchymal cells get activated through binding of surface receptors with either pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs) and mediates the pathogen and host cell damage respectively (Ramzi S C, ISBN 0-7216-7335-X). The clinical outcomes of inflammatory response may be fever, anaphylactic shock, fibrosis or autoimmune diseases etc.

Liver disease of any type (inflammation or fibrosis) is primarily signal by abnormal liver enzyme levels or alteration in bile flow. The enzymes are grouped into two types as indicator of two sources of liver diseases: damage of hepatocytes and damage of billiary system or cholestasis. Usually abnormality in liver transaninases i.e. AST and ALT levels are clinically considered as indicator of hepatocellular major liver disease where as abnormal alkaline phosphatases (ALP) and γ-glutamyl transpeptidase (GGT) levels are for cholestatic major liver disease. However, mixed type of biochemical abnormality might be observed in certain cases. Increase in serum aminotransferase levels is common pathological finding in any type of liver damage, whether acute or chronic (Giannini et al., 2005).

1.8.1. Cell components involved in inflammatory process

1.8.1.1. Mast cells

Mast cells undergo degranulation to release histamine and other mediators to injurious stimuli as early phase response until inflammatory cells migrate from the circulation as late-phase response. According to the traditional understanding, when appropriate ligand binds with mast cell surface IgE receptors, exocytosis of the granules triggered with the immediate onset of vasodilatation, vascular leakage, smooth muscle contraction, and irritant nerve receptor stimulation. T-lymphocytes secret IL-4 when allergen fragments are displayed to it by antigen-presenting cells. Activated B lymphocytes are then matured into plasma cells and produce IgE antibodies specific to allergen. Binding of allergen molecules with mast cell and circulating

basophil surface activates enzyme cascades in the cell membrane including tyrosine kinase, protein kinase C, phospholipase C, phospholipase A2. Calcium ion influx results in the release of histamine and newly synthesized chemical mediators eg. leukotrienes, prostaglandins. The released mediators were thought to be degraded rapidly and not related with inflammation.

Recent observations suggested that mast cells participate in inflammatory process affecting different organs, including the heart, joints, lungs, and skin. Being stimulated by the stimulants like anaphylatoxins, immunoglobulin-free light chains, super antigens, neuropeptides, and cytokines, growth factors, and hormones etc mast calls release specific mediators including interleukin-6 and vascular endothelial growth factor, platelet-activating factor; several proteases; neuropeptides and, most importantly, the cytokines without degranulation. Interleukins and cytokines promote the expression of adhesion molecules and recruit the inflammatory cells and thus control the duration and intensity of the immune response. In the late-phase response, these inflammatory cells release additional chemical mediators and lead to chronic inflammation and tissue damage (Theoharides et al., 2007).

1.8.1.2. Hepatic stellate cells (HSCs)

Hepatic stellate cells (HSCs), located in the space of Disse, comprises 5%–8% of the cells in the liver. In normal and healthy individuals, HSCs remain dormant but get activated upon the stimulation from dead or injured hepatocytes and immune cells if liver suffer from injury by toxins. Activated HSCs, differentiated into myofibroblasts, produce a temporary scar of extra cellular matrix (ECM) to protect the liver from further injury. Besides protecting liver, HSCs additionally serve for liver regeneration by releasing cytokines and growth factor (Blaner et al., 2009).

Activated hepatic stellate cells express a combination of matrix metalloproteinases (MMPs) to degrade fibrillar and non-fibrillar collagens and elastin and their specific tissue inhibitors (TIMPs) as well which inhibits MMP's action to favour scar deposition. This dual contribution of HSCs appears depending on the periods of liver injury. At the initial stage of liver injury, HSCs temporarily express metalloproteinases and promotes matrix-degradation while in the later stages activated HSCs release a mixture of MMPs that perform different function including

degradion of normal liver matrix instead of fibrillar collagens, accumulated in fibrotic liver (Benyon et al., 2001).

HSCs promotes fibrogenesis through interaction with hepatocytes, hepatic macrophages, lymphocytes, and (liver sinusoidal endothelial cells) LSECs (Duffield et al., 2005). However, the fibrogenic process is terminated by interaction of natural killer (NK) cells with activated HSCs resulting in HSC death (Radaeva et al., 2006). In rodent and human liver, HSCs, highly sensitive to proinflammatory mediators like cytokines and lipopolysaccharide (LPS), stimulates the proinflammatory signaling pathways of nuclear factor kappa B (NF-kB) and activator protein 1 (AP-1) and subsequently produces the mediators of inflammation.

However, in vivo experiment has been shown that the extent of contribution of hepatic stellate cells in overall inflammatory process is not too high. Their participation orients mainly through the receiving of inflammatory signals, regulating the inflammatory pathways and ensuring the survival of activated HSCs. It is evident that livers at cirrhotic condition, may exhibit a lower degree of inflammation controlled by HSCs which suggests the significance of myofibroblasts in regulation of inflammation within the fibrotic liver than other inflammatory cells.

The involvement of hepatic stellate cell throughout the overall inflammatory process can be summarized as- (i) HSCs contribute to both fibrogenesis and fibrosis regression by throwing specific chemotactic signals and interacting with specific inflammatory cell types which results in activation and destruction of HSCs respectively. (iii) HSCs connect gut and liver through their high level of Toll-like receptors (TLRs) before activation and fibrosis (Seki et al., 2007). (iv) HSCs migrate towards the site of injury after receiving specific chemotactic signals.

1.8.1.3. Hepatocytes

Chronic liver inflammation and fibrosis from viral infection, steatosis, alcohol or drug induced toxicity is manifested by hepatocyte death because most hepatotoxic agents target hepatocytes (Higuchi et al., 2003). After injury, hepatocytes release fibrogenic mediators as well as ROS to induce inflammatory cells for recruiting white blood cells. Damaged hepatocytes undergoing apoptosis stimulates the liver myofibroblasts for fibrogenic actions (Canbay et al., 2004). Cell debris of apoptotic hepatocytes has been suggested to provoke further inflammation by

stimulating macrophages to secret proinflammatory and fibrogenic cytokines which activates HSC directly (Canbay et al., 2003). Moreover, hepatocytes have been reported to promote fibrogenesis directly or indirectly by releasing damage-associated molecular patterns (DAMPs) during dying (Luedde et al., 2014). Several studies suggested that hepatocytes suffering from various stress might release inflammatory cytokine, such as interleukin (IL), which ultimately take part in the promotion of fibrogenesis (McHedlidze et al., 2013).

1.8.1.4. Macrophages and dendritic cells

Liver resident macrophages, named as Kupffer cells, play significant role in liver injury by initiating and driving the inflammatory response. Proinflammatory cytokines and chemokines including tumor necrosis factor (TNF), interleukin-6 (IL-6), IL-1beta, or monocyte-chemoattractant protein-1, secreted by macrophages, activates the non-parenchymal endothelial or hepatic stellate cells. The usual mechanism of hepatocytic cell death by these mediators includes activation of caspase cascades. In contrast, activation of nuclear factor kappa B (NF-κB) pathway protects liver cell. The infiltration of inflammatory Ly6c+ (Gr1+) monocyte subset, precursors of tissue macrophages, takes place after ligation of chemokine receptor CCR2 with ligand MCP-1/CCL2 (Zimmermann et al., 2010). Macrophages from bone marrow joined with the liver-resident macrophages (KCs) in injured liver. Attenuation of fibrosis from depleted macrophage proves its importance in fibrogenic response (Duffield et al., 2005). Although the mechanisms as well as mediators of HSC activation by macrophages still not completely revealed, it has been suggested that macrophages promote the survival of activated HSCs and maintain their high concentration (Pradere et al., 2013). In addition, macrophages secret chemokine ligands to promote HSC migration towards injury (Tacke et al., 2014). Besides fibrogenetic activity, macrophages contribute to resolve fibrosis with increased matrix metalloproteinase expression which results in enhanced ECM degradation (Duffield et al., 2005). In addition, expression of TNF-related apoptosis-inducing ligand by macrophages may contribute to HSCs killing (Pellicoro et al., 2014). Dendritic cells (DCs), another immune cell, are closely related to macrophages which have been reported to resolve liver fibrosis by expressing matrix metalloproteinase -9 (Jiao et al., 2012).

1.8.1.5. Cholangiocytes

The epithelial cells of bile duct, cholangiocytes (Tietz et al., 2006), possess abundant inflammatory cytokines and chemokines. Additionally, due to high sensitivity to Toll like receptors, cholangiocytes can participate in liver inflammation and fibrosis. In cholangiopathies, interaction between cholangiocytes and T lymphocytes (Syal et al., 2012) results in activation of myofibroblasts surrounding bile ducts which ultimately expanded due to the duct proliferation. In cholestatic liver fibrosis, HSCs plays the main role to produce ECM (Mederacke et al., 2013). However, information about the interactions of cholangiocytes with HSCs and involvement of chemokines or other proinflammatory mediators in this interaction still not clear. On the other hand, several studies have reported about interaction between cholangiocytes and portal fibroblasts through chemokine ligands (Kruglov et al., 2006). Evidences suggested that cholangiocyte provides specific signals to promote myofibroblasts recruitment in cholestatic liver disease which ultimately provide fibrotic response to cholangiocarcinoma (Rizvi et al., 2013). Overall, the interaction of cholangiocytes with multiple cell types in cholestatic liver disease contributes to its involvement in chronic inflammation and fibrosis.

1.8.1.6. Liver sinusoidal endothelial cells (LSECs)

During the migration from blood vessels into tissue, LSECs interact with leukocytes and form functional units. Hepatic Stellate cells bind with LSEs in normal as well as injured liver. In normal condition of liver, LSECs bound with HSCs, actively suppress HSC activation where as in injury condition, LSECs loss their ability to suppress HSC activity (Deleve et al., 2008). Researchers suggested that LSECs play the key role to decide to regenerate or to promote HSC activation and fibrosis (Ding et al., 2014). However, the exact mechanism is not still clear. LSECs, source of multiple inflammatory mediators, are highly sensitive to Toll like receptors (TLR) ligands. Several data suggests that LSECs combines with HSCs to make a functional unit which promotes ultimately fibrogenesis by interacting with multiple other cell types in chronic inflammation.

1.8.1.7. Natural killer (NK) cells and natural killer T (NKT) cells

The unique lymphocytes NK and NKT cells, abundant in liver, play vital roles in innate immune system when body suffers from infection and tumors. In fibrotic liver, NK cells act as antifibrotic

agent (Radaeva et al., 2006) by interferon-gamma (IFN-c) cytokine and by TRAIL and Fas ligand (FasL) as killer of activated HSCs (Jeong et al., 2011). Clinical evidences on patients suggested that fibrosis attenuated with the increased action of NK cells against HSCs (Kramer et al., 2012). Increased transforming growth factor beta (TGF-β) level in fibrotic liver restrain the antifibrotic function of NK cells (Jeong et al., 2011).

NKT cells, having characteristics of both NK and T-cell, regulate innate and adaptive immunity by producing interferon-gamma and interleukin-4 cytokines. Although invariant NKT cells work to kill HSCs, in a separate study with CCl4-induced liver fibrosis activated invariant NKT cells found to augment fibrosis (Pellicoro et al., 2014). In chronic liver injury, lymphocytes and HSCs commonly undergo functional interactions which results in HSC activation and liver fibrosis (Novobrantseva et al., 2005).

Different study suggested that NK and NKT cells destroy hepatocytes by releasing proinflammatory cytokines and accelerate liver injury. The impact of hepatocytes killing by NK cells is inhibition of liver regeneration. NK cells play significant role in liver fibrosis regulation more than NKT cells because of tolerance of liver to NKT cells. However, still controversy exists on the role of NK cells and NKT cells on liver precursor cell proliferation and liver regeneration (Gao et al., 2009).

1.8.1.8. Neutrophils

Neutrophils are activated to terminate the invading organisms. However, persistant activation of neutrophils may destroy host liver tissue resulting in additional liver damage. Neutrophils found to be recruited by the action of Toll like receptor2 observed in CCl4 induced liver inflammation. Study carried on mice suggested that neutrophils play no direct role on liver fibrogenesis (Moles et al., 2014).

1.8.1.9. Platelets

Hypercoagulability, observed in chronic liver disease, generates from decreased number of platelets and their function. This pathologic condition most likely caused by decreased levels of protein C, proteinS, antithrombin, 2-macroglobulin, and heparin cofactor II and increased levels

of factor VIII and von Willebrand factor. This hypercoagulability might contribute to micro and macro thrombi formation in the liver. In this connection, platelets release a growth factor named as platelet-derived growth factor (PDGF) and contribute to activation of profibrotic coagulation cascades (Lewis, 2006). However, platelets have been reported to exert antifibrotic effects by several studies (Pessayre et al., 1988). Such as, liver fibrosis exacerbated in thrombocytopenic mice (Takahashi et al., 2013). Proper regulation of matrix degradation can reverse fibrosis and even cirrhosis. In other word, fibrosis progresses when fibril-forming or interstitial scar matrix exceeds the matrix degradation process (Friedman, 2008)

1.8.1.10. Innate lymphoid cells

Innate lymphoid cell2 (ILC2), newly discovered cell, produces interleukin-5 (IL-5), interleukin-9 (IL-9), and interleukin-13 (IL-13). In liver fibrosis, hepatic ILC2 contribute to HSC activation after being expanded by hepatocyte secreted interleukin-33(IL-33) (McHedlidze et al., 2013). In the lungs, ILC2 has also been reported to exert profibrotic role (Hams et al., 2014). Other subsets of ILC have not been yet clearly informed for their contribution to hepatic fibrosis.

1.8.2. Chemical mediators of inflammatory process

1.8.2.1. The nuclear factor NF-kB

The nuclear factor NF-kappa B pathway, typically considered as pro-inflammatory pathway, get activated by IL-1(interlukin-1) and TNF-α (Tumor necrosis factor) and stimulates the expression of pro-inflammatory genes like cytokines, chemokines, and adhesion molecules (Lawrence, 2009). Two different pathways are there for NF-kB activation: 'canonical' pathway-triggered by microbial product, TNF-α and IL-1, results in activation of RelA- or cRel-containing complexes, play an important role in chronic inflammatory diseases and 'alternative' pathway- activated by TNF-family cytokines-lymphotoxin β (TNFSF3); CD40 ligand (CD40L and TNFSF5), B cell activating factor (BAFF and TNFSF13B) and receptor activator of NF-κB ligand (RANKL and TNFSF11) - but not TNFα; results in activation of RelB/p52 complexes (Karin et al., 2000).

During inflammation, many pro- and anti- inflammatory mediators are produced. Balance between these two determines the disease progression. The role of NF-kB has been proven to

induce both pro- and anti- inflammatory gene expression by different studies (Lawrence, 2009). The controlled regulation such as activation or inhibition of this pathway can inhibit or induce apoptosis. Evidences suggested that physiological NF-kB activation prevents hepatocyte apoptosis and is therefore protective and pathological overactivation of NF-kB in hepatocytes or inflammatory cells increases production of proinflammatory, NF-kB-regulated cytokines, such as TNF-α,IL-1β,and IL-6 and promotes liver inflammation (Seki et al., 2015).

Neutrophil apoptosis is mediated by pro-apoptic role of NF-kB during acute inflammation and thus limited inflammatory and immune response. The NF-kB has found to inhibit pathogen induced macrophage apoptosis and thus increased protection against infection and reduced pathogen regulated inflammation during infection (Park et al., 2005). In contrast, prolonged macrophage activity represents the pro-inflammatory action of NF-kB.

1.8.2.2. Prostaglandin

Prostaglandin, generated from arachidonic acid, mediates the promotion and resolution of inflammatory process (Ricciotti et al., 2011). In inflamed tissue, prostaglandin production increases rapidly before the action of leukocyte and immune cells. Four types of bio activated prostaglandins are produced by the enzymatic action of cyclooxygenase-1(COX-1) and cyclooxygenase-2(COX-2): prostaglandin E_2, prostacyclin I_2, prostaglandin D_2, and prostaglandin $F_{2\alpha}$. Macrophages produce PGE_2 and thromboxane A_2 (TXA_2) at the site of inflammation (Tilley et al., 2001). PGE_2 has particular roles in all the phenomena of inflammation like redness, swelling and pain. PGE_2-mediated augmentation of arterial dilatation and increased micro vascular permeability increases blood flow to the inflamed site and causes redness and edema. PGE_2 act on peripheral sensory neurons and produces pain (Funk, 2001). After formation, PGE_2 cross plasma membrane through diffusion or ATP-dependent protein-4 and acts locally by binding with any of 4 receptors: EP1, EP2, EP3 and EP4.

Gene expression for a particular receptor in relevant tissue determines the pro- and anti-inflammatory action of PGE_2. PGE_2 can regulate the function of many cells including macrophages, dendritic cells (DCs) and T and B lymphocytes when binds with receptors. PGE_2 exerts its pro-inflammatory effect by regulating cytokine expression profile of DCs (Egan et al.,

2004).Anti -inflammatory actions of prostaglandins (PGs) are seen typically in allergic or immune inflammation such as action on neutrophils, monocytes, and natural killer cells and are usually balanced by pro- inflammatory actions of other PGs (Matsuoka et al., 2000). Prostaglandins, other than PGE_2, are also involved in inflammatory process including edema and pain by different receptor bonded pathways and likewise exert anti-inflammatory action in different settings. Arachidonic acid produces a collection of leukotrienes (LT) through the 5-lipoxygenase pathway.

1.8.2.3. Histamine

The amino acid histidine, decarboxylated to histamine in the Golgi apparatus of mast cells and basophils, stored in secretory granules in complexes with heparin, protein, or both (Uvnas et al., 1970). When specific allergen binds with IgE antibodies of mast cells and basophils, histamine is released through degranulation reaction. Histamine can participate in both allergic and anaphylactic reaction by immunologic (IgE-mediated) and nonimmunologic mechanisms respectively (Pearce, 1990). Smooth muscle cells, neurons, glandular cells (endocrine and exocrine), blood cells, and cells of the immune system are influenced by histamine (β-Imidazolylethylamine) effect (Pearce, 1991).

Besides immediate hypersensitivity reactions, histamine can perform anti-inflammatory activity through H_2 receptor. Inhibition of human neutrophil lysosomal enzyme release, inhibition of IgE-mediated histamine release from peripheral leukocytes, and activation of suppressor T-lymphocytes are example of its anti-inflammatory action (Metcalfe et al., 1981).

1.8.2.4. Cytokine and chemokine

Many multifunctional cytokines, present in exocytosis of mast cells, play important roles in late-phase inflammatory response. These include IL-1, -2, -4, -5, -6 and TNF-α (Bradding et al., 1996). IL-1 functions to grow T-helper cells and B cells with its proliferation whereas IL-2 influences the proliferation of T lymphocytes and activation of B lymphocytes. IL-4 helps to differentiate B lymphocytes into plasma cells to secret IgE. With TNF-α, IL-4 regulates the expression of both high- and low-affinity IgE receptor on antigen presenting cells. IL-5 activates B lymphocytes and helps in the differentiation of eosinophils, and IL-6 causes B lymphocytes to

increase the synthesis and secretion of immunoglobulins. Chemokines such as IL-8, macrophage inflammatory protein-1α, macrophage chemoattractant protein-1, -2, and -3, and RANTES etc are released to attract macrophages and circulating leukocytes (Luster, 1998). Additionally, these molecules induce histamine release from basophils and mast cells and activation of eosinophils (Alam et al., 1992).

1.8.2.5. Tumor necrosis factor-α (TNF-α)

TNF-α has been identified as a major controller of inflammatory response (Bradley, 2008). TNF, produced by activated macrophages and T lymphocytes, exerts its effects by binding with specific TNF receptors on cell surfaces. TNF receptors, known as transmembrane receptors, extend to the outside cell through the cell membrane from inside the cell. The lock and key binding takes place between TNF and protruding part of the receptors. These cell surface TNF receptors may be released as free-floating molecules, known as soluble TNF receptors (sTNFR).

TNF can bind with receptors whether still attached to cells or in soluble form. TNF known to activate the inflammatory response when bound to cell surface receptor. Action on vascular endothelium and interactions with endothelial leukocyte explain the proinflammatory function of TNF. In contrast, when TNF binds to floating receptors, it is found to inhibit the inflammatory cascade. Normally, the body strictly maintains the balance between the amounts of TNF produced the quantity of cell-surface bound and soluble TNF receptors (www.pauljanssenaward.com).

TNFα converting enzyme controls the pro- or anti-inflammatory activities of two different forms of TNF which depends on the cell type where it acts (e.g. macrophage or target endothelial cell) (Black et al., 1997). Elevation of serum and tissue TNF levels indicates inflammatory and infectious conditions (Robak et al., 1998) as well as the severity of disease (Kwiatkowski et al., 1990). Although TNF mainly released from monocytes or macrophages in inflammatory disease, it is the common mediator released from other cells such as mast cells, T and B lymphocytes, fibroblasts, endothelial cells, natural killer (NK) cells, neutrophils, smooth and cardiac muscle cells and osteoclasts.

Endothelial cells bound with TNF create a distinct temporal, spatial and anatomical pattern (Messadi et al., 1987) for leukocytes combining with different adhesion molecules like E-selectin, intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) to promote inflammation (Munro et al., 1989). When chemokines (including IL-8, MCP-1 and IP-10) (Rollins et al., 1990) merge with these responses, different populations of leukocytes, not dependent of antigen recognition, are recruited. Classical features of inflammation appear when TNF acts on endothelial cells locally. Extracellular production of vasodilatory PGI2 increased with resultant vasodilatation upon expression of cyclo-oxygenase 2 by TNF induction (Mark et al., 2001).

1.8.3. Involvement of drug generated reactive species or free radicals in tissue necrosis and inflammation

Among all of the established mediators for tissue necrosis as well as inflammation, reactive oxygen and nitrogen species are of greater importance. The body generates these unstable reactive species or free radicals to kill microorganisms but their presence lead to tissue injury in specific situations. Mitochondria produce free radicals as by product during energy consumption. During breathing, we make some free radicals but many factors of our lifestyle and environment can also produce them such as

- Too much calories, sugars and/or refined carbohydrates or charbroiled foods intake
- Too much or too little exercise
- Excessive alcohol consumption and exposure to tobacco smoke
- Exposure to fungal toxins, air pollutants, ionizing radiation
- Poor function of liver and gut to detoxify toxins or allergens
- Chronic infections
- Excessive stress and lack of sleep (www.wallerwellness.com)

Free radicals, the highly reactive atoms or molecules having unpaired electrons, are unstable. Biological system contains two types of free radicals included oxygen based radicals and nitrogen based radicals. Superoxide ion, hydroxyl radicals, peroxyl radicals and some non-radicals, such as hydrogen peroxide, hypochlorous acid and ozone, known as reactive oxygen

species (ROS), are generated from oxygen metabolism. Nitrogen based radicals and non-radicals includes nitrogen dioxide, nitric oxide radicals and peroxynitrite which are produced from nitric oxide and superoxide via inducible nitric oxide synthase (iNOS) and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, respectively .

Liver produces reactive oxygen species (ROS) in the endoplasmic reticulum and mitochondria via the action of CYP enzymes as a part of its normal activity. Unused radicals are converted into non reactive species by body's own mechanism. Repeated intake of abused drugs produces rushes of reactive species that causes damage to hepatic cell membrane and organelles. Free radicals and their related reactants are variably capable of inducing toxicity. The oxygen-based hydroxyl radical and the nitrogen-based peroxynitrite anion are found to be more reactive and harmful for bodily tissue. ROS and reactive nitrogen species (RNS) initiate lipid peroxidation, DNA strand break and arbitrarily oxidize the molecules of biological membranes and tissues and hence tissue injury. However, the body can remove ROS and RNS to a certain limit under physiological conditions which attenuates the chance of tissue injury by these reactive species.

Our body continuously producing reactive oxygen species (ROS) from inhaled atmospheric molecular oxygen through different aerobic cycle. However, the resultant excessive amount of ROS is highly toxic to cells. Imbalance between the production and the elimination of reactive oxygen and nitrogen species as well as decreased production of antioxidants generate oxidative and nitrosative stress, respectively. Nevertheless, oxidative stress plays positive role in certain specific physiological conditions. The cellular functions requiring ROS include activation of signal transduction pathways, killing of invaded microorganisms and mediating necessary gene expression for cell growth or death promotion. Some practical example may be presented as- strengthening of biological defense mechanisms during appropriate physical exercise and ischemia or inducing apoptosis to prepare the birth canal for delivery etc (Finkel et al., 2000; Apel et al., 2004; McCord, 2000; Cichoz-Lach et al., 2014; Mittler, 2002; Lee et al., 2012). However, other than such particular situations, in most cases, large levels of ROS and oxidative stress will induce cellular and tissue injury through necrotic and/or apoptotic cell death.

Studies showed that ROS can alter mitochondrial permeability and transition potential. All of these conformational changes lead to the release of pro-apoptotic factors (*e.g.*, cytochrome C) from mitochondria to cytosol which activates caspase-3 cascade among cells. Increased redox

state triggers decreased ATP synthesis, reduced mitochondrial protein synthesis, alteration of the oxidative phosphorylation system and damage to mitochondrial DNA (Wang et al., 2013; Wang et al., 2013; Sinha et al., 2013). Proteins affected by oxidative stress may be reversible or irreversible. Cysteine usually can reverse the alteration and restore the function of a protein and thus can protect a cell from irreversible damage. Unlikely, lysine and arginine undergo irreversible modifications caused by ROS, resulting in a permanent loss of functions and may contribute to accumulation of damaged proteins into cytoplasm after degrading them (Lu, 2008).

Investigations proposed that cellular proteins, lipids and DNA are mostly victims of oxidative attack. Various degenerative diseases, like diabetes, cancer, cardiovascular disorders or neurodegenerative diseases have been proven to be result of cytotoxicity of ROS (Apostolova et al., 2011). The mentioned diseases are undoubtedly the consequences of chronic oxidative stress. However, acute insult by high levels of ROS may also cause serious damage to vital organs, like liver damage during ischemia/reperfusion.

Superoxide, hydroxyl radicals, hydrogen peroxide, 4-hydroxy-2,3-nonenal and 4-hydroxy-2,3-alkenals (aldehyde generated), the reactive species, are released by activated phagocytes, macrophages, hepatocytes, inflammatory cells and stellate cells (Parola et al., 2001). Ethanol, polyunsaturated fatty acids and iron may enhance reactive oxygen species production. Cytochrome P450 produces reactive oxygen species (ROS) during the metabolic processes. Cytochrome P450 2E1 induction has been considered as classic pathway of ROS generation in hepatocytes, especially in alcoholic or non-alcoholic liver diseases (Castillo et al., 1992). ROS, generated from either mitochondrial or non-mitochondrial sources, has been suggested to induce death receptors to initiate necrotic cell death (Fiers et al., 1999). Various mechanisms for ROS mediated cell death included malfunctioning of cellular proteins, lipids, or nucleic acids through their oxidative damage or initiating cell death processes by activating various signaling cascades (Tietz et al., 2006). ROS directly reacts with the catalytic sites of phosphatases and affect signaling pathways. Based on several recent data, ROS, capable of inactivating phosphatases, have dual specificity (den Hertog et al., 2005). For example, tyrosine phosphatases have been proposed to be oxidized by ROS at their catalytic cysteine site and inactivated (Nakashima et al., 2002) which may be reversible or irreversible and depends on the oxidation state of the catalytic cysteine (Groen et al., 2005).

Over production of ROS frequently associated with inflammation. Chronic inflammation commences when acute inflammatory response and tissue destruction continued. Pathogenesis of chronic inflammation is mediated by immune cells like macrophages, neutrophils and eosinophils which perform their actions either directly or by producing inflammatory cytokines. Moreover, chronic inflammation is found to be seriously associated with a wide variety of diseases that develop with age such as cardiovascular disease, autoimmune diseases and diabetes. Chronic inflammatory process creates an imbalance between oxidative stress and cellular antioxidant capacity. Free radicals during chronic inflammation inactivate cell membrane fatty acids and proteins permanently. Additionally, free radicals, in worst case, can execute mutation and DNA damage leading to the predisposing factor for cancer (Khansari et al., 2009).

The imbalance between oxidants and antioxidants due to either excess production of reactive species or impaired activity of antioxidant systems creates the situation referred to as oxidative stress. This oxidative stress exerts damaging effect on important biomolecules and cells, which affect potentially on the whole organism (Khansari, 2010).Chronic inflammatory diseases mostly whether initiated by oxidative stress generated inflammation or inflammation generated oxidative stress- has not yet been identified. Oxidized peroxiredoxin-2 (PRDX2), a ubiquitous redox-active intracellular enzyme, induced by inflammatory stimuli, released in extracellular space and triggers the production and release of TNF-α by macrophages. The central regulation of immunity involves a process where glutathione (GSH) make oxidative couple with the cysteine residue of PRDX2 and initiate its release. The PRDX2 substrate thioredoxin (TRX), released along with PRDX2 from macrophages, modifies the redox status of cell surface receptors making those enable to induce inflammatory responses (Salzano et al., 2014).

ROS, produced from normal cellular metabolism, play significant role for signaling pathways stimulation in plant and animal cells depending on changes of intra- and extracellular conditions and in defense response against environmental pathogens as well (Jabs, 1999). Studies have found that NADPH oxidases, belong to a large family of enzymes and expressed in different isoforms, produce ROS and are tissue specific. Phagocytic neutrophils and macrophages utilize the isoform of NADPH oxidase, Nox2 to produce large quantities of superoxide for host defense. The non-phagocytic cell like fibroblasts, vascular smooth muscle cells, cardiac myocytes, and endothelial cells have been identified to utilize other family members of NADPH oxidase

(Morgan et al., 2007). However, there are some other enzymes which can also produce trace amount of ROS names as xanthine oxidase, lipoxygenases, aldehyde oxidase, myeloperoxidases, cyclooxygenases, heme oxygenase, monoamine oxidases, cytochrome P450-based enzymes etc. (Morgan et al., 2008). During inflammatory response, ROS may directly react with nuclear DNA, RNA, and lipids by oxidation, nitration and halogenation or activate the signaling pathways. Aerobic organisms produce greater energy with the help of the mitochondrial respiratory chain as compared to anaerobic organisms. However, in aerobic respiration, electron is continuously leaked to O_2 during mitochondrial ATP synthesis and hence gives rise to superoxide anion (O_2^-). Approximately 1–5% of total consumed oxygen in aerobic metabolism is converted into this ROS.

H_2O_2, another ROS, produced either from dismuted superoxide anion or molecular oxygen impulsively, is less reactive among all other ROS. However, H_2O_2 can contribute to many types of cellular injury after disperse throughout the mitochondria and across the cell membranes. Hydroxyl radical ($\cdot$OH), the most injurious and very unstable ROS in the mammalian cells, mainly produced in the presence of transition metals e.g. Fe, Cu, Co, or Ni in their reduced form in vivo. Fenton reaction, the representative reaction of $\cdot$OH production, where Fe^{2+} reacts with H_2O_2. DNA damage by $\cdot$OH radical generates 8-hydroxyguanosine (8-OHG) which is hydrolyzed into 8-hydroxydeoxyguanosine (8-OHdG), the most widely used fingerprint of radical attack towards DNA (Mates et al., 2000)

$NO^{\cdot}$, a short-lived free radical, generated from L-arginine, normally produced to fight against pathogens with the enzymatic action of nitric oxide synthase (NOS). The three types of NOSs included inducible NOS (iNOS), neuronal NOS (nNOS) and endothelial NOS (eNOS). Maximum amount of $NO^{\cdot}$ is synthesized by iNOS, usually after insult by immunological or inflammatory stimuli (120). The two others- nNOS and eNOS produce small amount of $NO^{\cdot}$ which function as a neurotransmittor and vasodilator, respectively. iNOS has been suggested to produce injurious amounts of RNS in inflammation where as eNOS and nNOS produce such amounts under physiological conditions which is considered beneficial. Several cytokines like γ-interferon (γ-IFN), TNF-α, IL-1, and lipopolysaccharide (LPS) induce iNOS. Another situation described for nitric oxide ($NO^{\cdot}$) production is through mitochondrial respiratory chain in hypoxic conditions. This reactive nitric oxide induces excessive lipid peroxidation and generates two

other reactive species- malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE). This reactive species also targets proteins and DNA for oxidation and induces cell death or increases the risk of mutagenesis by modifying these molecules (Moncada et al., 2012; Lowenstein et al., 2004; Xie et al., 1994; Poyton et al., 2009; Hussain et al., 2003; Schraufstatter et al., 1988).

The undesirable and dangerous event of ROS activity consists of their redox reaction with cellular components. Amino acids, such as tyrosine, tryptophan, histidine and, particularly, cysteine becomes the prey of these redox consequences. Proteins containing these specific amino acids are thus more vulnerable to oxidation by ROS. Structural modification of protein by ROS alters its function. Oxidized proteins are considered as unneeded or damaged and are degraded by proteolytic attack of proteasomes (Cesaratto et al., 2004). Liver cells are frequently attacked by ROS. Initially ROS induce liver injury through oxidation of parenchymal cell materials. Liver parenchymal cells can produce ROS which regulates liver fatty acid oxidation gene expression. Moreover, oxidative stress induce Kupffer cells, hepatic stellate cells and endothelial cells to produce a variety of cytokines like TNF-α , interferon-γ, interleukin-β(IL) etc which might increase inflammation and apoptosis. Lipid peroxidation caused by oxidative stress triggers the proliferation and collagen synthesis of hepatic stellate cells (Sanchez-Valle et al., 2012; Sakaguchi et al., 2011; Wu et al., 2009).

In spite of a complex antioxidant system working continuously to relieve oxidative stress, reactive species, produced in excess from oxygen and nitrogen, may still lead to oxidative damage to tissue and plays a significant role in liver diseases like, alcoholic liver disease and non-alcoholic steatohepatitis and other chronic and degenerative disorders through a set of pathological mechanism (Li et al., 2015; Li et al., 2014; Feng et al., 2011; Singal et al., 2011). Alcohol, drugs, environmental pollutants and irradiation are the examples of risk factors that may induce oxidative stress in liver. Different research suggested that the pathological factors, free radicals and immune responses have a complicated connection with each other to cause inflammation (Medina et al., 2005).

Lipid peroxidation of cell membrane is a vital destructive action of ROS. The reactive aldehyde produced during this process activates pro-inflammatory and pro-fibrotic process. As drugs

frequently target liver to exert toxicity as it is their main site of metabolism, increased level of radical species, specifically ROS and RNS are considered as an indicator of hepatotoxic potential and an early event of drugs hepatotoxicity (Videla, 2009). Many studies suggested that group of drugs including anti-inflammation drugs, anti-analgesic drugs, anti-cancer drugs and antidepressants could induce oxidative stress such as increase of cellular oxidants and lipid peroxidation, depletion of antioxidants in the liver (Linares et al., 2009; Mladenovic et al., 2009; Samarghandian et al., 2014; Pieniazek et al., 2013; Nguyen et al., 2015; Zlatkovic et al., 2014; Stine et al., 2015; Shuhendler et al., 2014; Karabulut et al., 2010). Old mitochondria can be a major source of ROS due to defective autophagy (Zhang et al., 2007). ROS produced by damaged mitochondria may perturb the signal transduction adaptor function of p62-controlling pathways and thus promote tumor development (Mathew et al., 2009).

1.9. Body's protective system against the reactive species

To attenuate free radicals and reactive metabolites, an anti oxidant system works for cell protective purpose included superoxide dismutases, glutathione peroxidases, thioredoxin reductase and peroxiredoxins enzymes, catalase as well as non-enzymatic antioxidants (Morgan et al., 2008). Tocopherol, vitamin E, beta-carotene, ascorbate and glutathione (GSH) are included in nonenzymatic group (Edeas et al., 2010; Majima et al., 2011; Mao et al., 2011). Members of this antioxidant system eventually lose their capability due to continuous engulfment of ROS and hence ROS concentration in body rises.

A variety of DNA repair enzymes exist to protect cell death from DNA damage besides various types of antioxidants exist to specifically and efficiently protect cells from radicals. The endogenous and exogenous and enzymatic and non-enzymatic antioxidants comprise the whole protective system.

The first characterized antioxidant enzymes, superoxide dismutase which dismutate two O_2^- anions to H_2O_2 and molecular oxygen (McCord et al., 1969). In human cells, three different types of SODs have been characterized included copper-zinc SOD (Cu-ZnSOD), Mn-SOD, and extracellular-SOD (EC-SOD). The manganese-superoxide dismutase (Mn-SOD), employed to degrade or dismutase superoxide anion into H_2O_2 and water, protects the cell components from

the harmful effect of this free radical (Karihtala et al., 2007). Another antioxidant, Catalase acts to detoxify H_2O_2 to water. Peroxiredoxins, another group of enzymes, reduce alkyl hydroperoxides and H_2O_2 to the corresponding alcohol or water (Reuter et al., 2010).

Cells produced endogenous antioxidant Glutathione (GSH) is a tripeptide which helps to protect cells from free radicals and peroxides. GSH can protect DNA damage by protecting ROS and electrophilic chemicals. GSH can also directly make conjugate with toxins through phase II metabolism to detoxify those and subsequently eliminate these chemicals from the cell. (Pompella et al., 2003; Valko et al., 2007; Calvert et al., 1998). Glutathione peroxidase (GPx), another group of enzymes, uses GSH as substrate to reduce hydroperoxides, including lipid hydroperoxides. Glutathione disulfide (GSSG), the oxidized form of GSH, again reduced by the specific enzyme glutathione reductase.

Cumulative evidence suggests that methamphetamine is able to cause oxidative stress by affecting the balance between ROS production and enzymatic and non-enzymatic antioxidant systems (Reiner et al., 2009).

1.10. Outcomes of inflammatory reactions

After effect of inflammatory reactions can be expressed by two ways-

Physiological outcomes: host defense against infection, tissue repair response, adaptation to stress and restoration of a homeostatic state

Pathological consequences: autoimmunity, inflammatory tissue damage and sepsis, fibrosis, metaplasia and / or tumor growth, development of diseases of homeostasis and / or auto inflammatory diseases (Medzhitov, 2008)

Injured tissue or infection stimulates acute inflammatory response where plasma and leukocytes exudates locally to the extravascular tissues at the site of infection or injury in a coordinated way (Majno et al., 2004). Toll-like receptors (TLRs) and NOD (nucleotide-binding oligomerization-domain protein)-like receptors (NLRs), the surface receptors of innate immune system, bound with mediators to initiate acute inflammatory response (Barton, 2008). Initially macrophages and mast cells, resident to affected tissue, responds to the inflammatory stimulation with the

production of inflammatory mediators (eg.chemokines, cytokines, vasoactive amines, eicosanoids and products of proteolytic cascades). The mechanism by which only neutrophils but not erythrocytes are allowed for extravasation included activation of endothelium of blood vessels after ligation of endothelial-cell selectins at the endothelial surface and in the extravascular spaces with integrins and chemokine receptors on leukocytes (Pober et al. 2007).

Neutrophils become activated after reaching the affected tissue site by two ways-either from direct contact with pathogens or by the actions of cytokines secreted from tissue-resident cells. The neutrophils secret the toxic contents of their granules included reactive oxygen species (ROS) and reactive nitrogen species, proteinase 3, cathepsin G to kill the invading agents (Nathan, 2006). During this secretion and killing, these highly potent effectors can not discriminate between microbial tissue and host tissue. So, host tissue is equally damaged with invading targets (Nathan, 2002). Successful elimination of infectious agents by acute inflammatory response follows the next step as resolution of inflammatory reaction and repairing of damaged organ, principally performed by tissue-resident and recruited macrophages. To bring the transition from inflammation to resolution, the pro-inflammatory prostaglandin, acted as lipid mediators, are converted into anti-inflammatory lipoxins. Lipoxins promote the recruitment of monocytes after ceasing the recruitment of neutrophils. Monocytes clear the dead cells by phagocytosis and start tissue remodeling. Another class of lipid mediator, resolvins and protectins with transforming growth factor-β and growth factors produced by macrophages, contribute their significant role in the resolution of inflammation as well as the initiation of tissue repair (Serhan et al., 2005).

In case of failure of pathogen elimination by the acute inflammatory response, the inflammatory process persists and appears with new characteristics. The neutrophil infiltrate is replaced with macrophages and T cells. Further failure of this combined effect commences a chronic inflammatory state with the formation of granulomas and tertiary lymphoid tissues (Drayton et al., 2006). Different types of effector T cells create different characteristics of inflammatory state. Apart from persistent pathogens, chronic inflammation may also result from other reasons of tissue damage like autoimmune responses (owing to the persistence of self antigens) or unbreakable foreign bodies. If the macrophages fail to engulf and destroy pathogens or foreign

bodies, then granulomas are formed in which the invaders are entrapped by the layers of macrophages, as a final step to protect the host (Barton, 2008).

After clearance of dead tissues by macrophages, tissue healing or tissue repair started which refers to the body's replacement of destroyed tissue by living tissue (Walter et al., 1987). Damaged organ healed by two ways namely- regeneration and repair. The two processes involve replacement with two types of tissue. In regeneration, wound is healed by same type of cells formed by the proliferation of surrounding undamaged specialized cells. The repair process involved replacement of lost tissue by granulation tissue which in turn matured to form scar tissue.

Chronic liver disease (CLD) continued with persistent cell death and inflammation eventually promotes the development of fibrosis. The wound-healing response in which a range of cell types and mediators work to encapsulate injury is referred to as scarring or fibrosis. Hepatic diseases, irrespective of type, when result in cellular death is recovered with this kind of response. Chronic viral hepatitis, nonalcoholic fatty liver disease (NAFLD), alcoholic liver disease (ALD), cholestatic and autoimmune liver disease are examples of clinical grounds in which patients are frequently diagnosed to develop liver fibrosis. Fibrogenesis, a multi-cellular wound-healing response, in which a group of cells work sequentially included hepatic stellate cells (HSCs), bone marrow (BM)-derived cells, hepatocytes, Kupffer cells (KCs), cholangiocytes, liver sinusoidal endothelial cells (LSECs), and infiltrating immune cells (Duffield et al., 2005). Myofibroblasts derived from hepatic stellate cells (HSCs) contribute to fibrosis by releasing approximately 90% of extracellular matrix (ECM) and stimulating inflammatory signaling pathways to recruit bone marrow (BM)-derived cells through cellular interactions (Mederacke et al., 2013). During the process of fibrosis, HSCs interacts closely with liver-resident cells. Classically cell death is the predisposing factor, which subsequently triggers inflammatory and fibrogenic signaling cascades activation. Moreover, cell death biomarkers with other factors contribute to the development of fibrosis.

Acute inflammatory responses exert some beneficiary effects which help to restore liver function and shape after acute attack such as- engulf of dead cell debris by immune cells, providing mechanical stability by fibrogenesis and parallel activation of liver regeneration process by

inflammatory signals. However, the prolonged hepatic cell death and sustained underlying disease or destructive stimuli make these wound-healing responses unable to adapt the continuous damage. As a result, chronic inflammation is triggered and liver fibrosis progressively developed. Increased cell death-inducing cytokines and ligands from the tumor necrosis factor (TNF) receptor (TNFR) family by invaded leukocytes intensify liver injury. However, cell death components render the process to continue repeatedly by further triggering the leukocyte infiltration and inflammation (Luedde et al., 2014). Although cell death and inflammation in the liver are nearly indivisible, it is still unclear at which degree of cell death during inflammation fibrosis is promoted.

Significant fibrosis accumulated with nonstop signal thrown by chronic tissue injury resulting from infection, drugs, metabolic disorders, or immune attack. Infrequently, fibrosis is observed to progress within weeks to month's period in some chronic diseases such as drug injury, hepatitis C, (Schluger et al., 1996) or human immunodeficiency virus (HIV)/HCV co-infection etc. (Bonnard et al., 2007). Fibrin, deposited during inflammation, is partially removed by the fibrinolitic enzyme in the process of damage repairing by fibrosis. The gap, gradually substituted with granulation tissue, eventually filled with the converted scar tissue. The more the volume of damaged tissue, the more the degree and concentration of scar formation. However, some clinical evidences have shown the development of some fibrosis normally in the absence of significant tissue destruction in chronic inflammation (Li et al., 2007). In liver fibrosis, the matrix, composed of transcriptionally and posttranscriptionally regulated Collagen type I produces fibril (Tsukada et al., 2006).

Evidences have shown that liver resident stellate cells are not the only source responsible for all fibrogenic cell production and fibrosis in case of liver injury. Rather, portal fibroblasts, circulating fibrocytes, bone marrow, and transition of epithelial–mesenchymal cell also involved in fibrogenic cell supply and contribute to fibrosis. Of course, all sources don't equally contribute to disease progression. The stellate cells act as host of cytokines and growth factors when activated by initiating stimuli. Similarly, these signals provoke the stellate cells to enhance proliferation, contractility, fibrogenesis, matrix degradation, and proinflammatory signaling to generate scar (Wells et al., 2004).

Liver fibrosis generated as a result of progressive accumulation and decreased remodeling of the extracellular matrix. Chronic inflammation of liver cell eventually gives rise to liver fibrosis which is characterized by proliferation of hepatic stellate cell (HSC) and differentiation to myofibroblast-like cells which secrets extracellular matrix (ECM) and collagen. The HSC releases excess amount of ECM components and disrupts the normal architecture of the liver (Elpek, 2014). It has been observed that oxidative stress often activate the synthesis and release of fibrogenic factors, alters hepatic functions and promotes fibrogenesis.

Drug can induce both acute and chronic types of hepatobiliary diseases. However, clinically these are presented as acute icteric hepatitis or cholestatic liver disease. Acute icteric hepatitis is more severe and often shows10% mortality rate; not dependent on the causative drug (Kaplowitz, 2001; Zimmerman, 2nd ed; 1999) and menifestated by markedly elevated serum transaminase levels but minimal increase in alkaline phosphatase level. Cholestatic disease or cholestatic hepatitis is not usually life threatening and characterized by mild increases in alanine amino transferase (ALT) level, jaundice, pruritus, and marked increases in alkaline phosphatase level. Intermediate to marked increase in ALT and alkaline phosphatase levels resemble atypical hepatitis or granulomatus hepatitis (Kaplowitz, 2004)

1.11. Rationale of study

1.11.1. Experiment with methamphetamine tablet Yaba to investigate liver inflammation and consequent fibrogenesis in rat

The neurotoxic effect of methamphetamine has been almost established by different studies. In few studies, liver damage is reported as remarkable symptom observed from long term abuse of amphetamine derivatives (Cui et al., 2009). But relevant information and data on the toxic effect of these drugs on liver is not available. The aim of our study was to evaluate the hepatotoxic potential of methamphetamine in rats using its tablet form Yaba as a representative of amphetamine derivatives. We estimated liver marker enzymes (ALT,AST) and alkaline phosphatase (ALP) level in rat plasma; antioxidant enzyme level such as catalase (CAT), glutathione (GSH), superoxide dismutase (SOD) and oxidation end products like advanced oxidation protein product (AOPP), lipid peroxidation product, malondialdehyde (MDA) and

level of nitric oxide (NO) in both plasma and liver tissue after long term administration of Yaba on rats. Histopathologic study of rat liver tissue was performed to monitor inflammation, fibrosis and iron deposition from all groups.

1.11.2. Experiment with caffeine to investigate liver inflammation and consequent fibrogenesis in rat

The recreational drugs abused by Bangladeshi people mostly contain caffeine as adjunct stimulant. Yaba, being the second positioned illicit drug contains larger amount of caffeine with the main active methamphetamine. The fatal dose of caffeine for adults is more than 10g. It is safe to keep average daily intake of caffeine below 500 mg (Yew et al., 2014). According to the standard formula, Yaba is composed of 25-35 mg of methamphetamine and 45-65 mg of caffeine. The source and composition of the supplied Yaba tablets for our study were unknown. For that reason, the tablets were subjected to chemical analysis. Yaba tablets used in our study were analyzed by HPLC and UV-Visible spectroscopy for its caffeine content and found 65mg (app.) caffeine per tablet having average weight approximately 90 mg.

From different investigations, a Yaba abuser on an average found to take 10-12 tablets daily due to being tolerant to the lower dose which starts within a short time of the first tablet intake. The total content of caffeine in daily intake through Yaba exceeds the safety range for daily use of this drug. Considering this, we have studied the effect of high dose caffeine on rat liver in vivo. As both caffeine and methamphetamine are established CNS stimulant drugs (Nehlig et al., 1992), we expected their synergistic effect when used jointly in Yaba tablets. To prove whether the evidences of liver toxicity observed from chronic use of Yaba were from methamphetamine only or the combined effect of methamphetamine and caffeine; we have conducted a separate study by administering pure caffeine in rats.

To investigate liver toxicity by caffeine we have estimated liver marker enzymes (ALT, AST) and alkaline phosphatases (ALP) level in rat plasma; antioxidant enzyme activity such as catalase CAT), glutathione (GSH), superoxide dismutase (SOD) and oxidation end products like advanced oxidation protein product (AOPP), lipid peroxidation product, malondialdehyde (MDA) and level of nitric oxide (NO) in both plasma and liver tissue after long term

administration of caffeine on rats. Histopathologic study of rat liver tissue was performed for inflammation and fibrosis from all groups.

1.11.3. Experiment with 3, 4-methylenedioximethamphetamine (MDMA) to investigate the involvement of cytochrome P450 (CYP) isozymes on metabolism of amphetamine derivatives in rat

Identification of CYP isozymes participated in metabolic pathways of xenobiotics has been made by different ways mostly through in vitro studies using fresh liver microsome. In our study, an in *vivo* investigation was made on metabolic pathways of MDMA considering the involvement of cytochrome P450 (CYP) isozymes. We have used CYP isozyme induction with phenobarbital (PB) or β-naphthoflavone (BNF) separately before MDMA administration in the aim of observing correlation between the induced CYP isozymes and the increased or decreased metabolites by detecting their urinary concentration. We have targeted three metabolites of MDMA as MDA, HMMA and (4-hydroxy-3methoxyphenyl)acetone (HMPA) and four CYP isozymes as CYP3A, CYP2B1, CYP 1A1and CYP1A2 for their involment in N-demethylation and O-demethylenation and O-methylation pathways of MDMA metabolism.

1.11.4. Experiment with methamphetamine tablet Yaba to investigate its effect on bone in rat

Drug abuse can affect a person both physically and mentally. People mostly aware of drugs toxic effects on different vital organs like brain, liver, heart, kidney etc. Usually less attention is given to the drug's effect on skeletal system. Methamphetamine is familiar for its CNS stimulant effect. Several mechanism of action of its toxicity on CNS has been studied. Its effect on liver, heart, kidney is almost known. But sufficient information regarding the effect of methamphetamine on the skeletal system and related mechanisms are still unknown. To our knowledge, bone quality study of methamphetamine abusers yet unexplored and can be a treasure for scientific investigations. As bone metabolism is controlled by nervous regulation, we suspected the ill effects of neurotoxic methamphetamine on skeletal system.

Chapter Two

Materials and methods

2.1. Investigation on liver inflammation and consequent fibrogenesis in rat with Yaba treatment

2.1.1. Chemicals and reagents

Yaba tablets used in present study were received from the Narcotics Control Dept. of Dhaka Metropolitan Police, Bangladesh. Thiobarbituric acid (TBA), glutathione in reduced form (GSH) and trichloroacetic acid were purchased from Sigma Chemical Company (USA) and J.I. Baker (USA) respectively. Alanine aminotransferase (ALT), aspartate amino transferase (AST) and alkaline phosphatase assay kits were obtained from DCI diagnostics (Budapest, Hungary), 50,50-dithiobis-2-nitrobenzoate (Ellman's reagent) and sodium hydroxide purchased from sigma (USA) and Merck (Germany) respectively. All other chemicals and reagents used in present study were of analytical grade.

2.1.2. Animal study and specimen collection

To determine hepatotoxicity of methamphetamine from long term administration, we have used male Long Evans rats. The period of drug treatment was eight weeks (56 days). Rats, of 10-12 weeks old, having body weight 180g - 200g were taken. We made three groups with six rats in each. Rats were supplied from the production area of animal house of Pharmaceutical Sciences Department of NSU. Yaba tablets were suspended in distilled water and administered into rats through oral route. Animals of low dose group received Yaba tablet suspension equivalent to 5mg of methamphetamine/kg of body weight once daily; Rats of high dose group, were treated with Yaba tablet suspension equivalent to 10 mg of methamphetamine/kg of body weight once daily. Rats of control group received only distilled water. We kept the rats in ordinary cages. Animals had free access to water and standard laboratory feed. They were kept with a 12 h light / dark cycles. The room temperature of animal house was $25\pm3°C$. We used protocol for the present study approved by The Ethical Committee of the Department of Pharmaceutical Sciences, NSU, Bangladesh for animal care and experimentation. We have monitored body weight, water and food intake of all animals and recorded every day.

We weighed, anaesthetized and sacrificed rats of three groups on the last day of eight weeks. Collected blood samples were centrifuged to separate plasma from it and stored at -20°C until used. Liver, kidney, heart, spleen were also collected and weighed immediately. Halves of the organs were stored at -20°C until used for biochemical tests and halves were processed for histological study according to the established method.

2.1.3. Assessment of liver enzymes

Rat plasma of three groups was assayed for liver transaminases (ALT and AST) and another isozyme, alkaline phosphatases (ALP). Chemical analysis was conducted by following the standard protocols of manufacturer provided with DCI diagnostics kits (Hungary).

2.1.4. Assessment of oxidative stress markers and antioxidant enzyme activity

Oxidative stress markers and antioxidant enzyme activity were measured in rat blood plasma and liver tissue. Liver tissue was homogenized in 10 times volume of ice cold phosphate buffer having pH 7.4 and centrifuged at 12,000X g for 30 min at 4°C. The supernatant was collected and used for the determination of protein and enzyme contents.

2.1.4.1. Estimation of malondialdehyde (MDA)

Following a colorimetric method, we measured malondialdehyde (MDA), one of the products of lipid peroxidation in plasma and liver tissue extract using thiobarbituric acid (TBA) as per method described by Niehaus and Samuelsson (Niehaus et al., 1968). The procedure briefly described as treatment of 0.1 ml of tissue extract or plasma in Tris-HCl buffer (pH 7.5) with 2ml of TBA-TCA-HCl reagent mixture taking equal amount of each (thiobarbituric acid 0.37%, 0.25 N HCl and 15% TCA). The final mixture was heated in hot water bath for 15 min and then cooled. The clear supernatant was taken and absorbance measured against reference blank at 535nm.

2.1.4.2. Assay of nitric oxide (NO)

Nitric oxide, in the form of nitrate, was measured following the method described by Tracey et

al.(1995). In present study, Griess-Illosvoy reagent was modified by replacing 5% 1-napthylamine with naphthyl ethylene diamine dihydrochloride (0.1% w/v). The reaction mixture containing tissue extract or plasma (2ml) and phosphate buffer saline (0.5ml) was incubated at 25°C for 150 min. The mixture color changed into pink due to formation of a pink colored chromophore. Using corresponding blank solutions, the absorbance of these solutions was measured at 540 nm. A standard curve, expressed as nmol/ml, was prepared to measure plasma and liver tissue content of nitric oxide.

2.1.4.3. Measurement of advanced oxidation protein products (AOPP)

The methods described by Witko-Sarsat et al.(1996) and Tiwari et al. (2014) were followed with slight modification to detect AOPP level . In brief, 2 ml mixture was made by diluting plasma or tissue extract with PBS in 1:5 ratio; then 0.1 ml of 1.16 M potassium iodide was added to each tube; after 2 min, 0.2ml acetic acid was added to it. The absorption of the reaction mixture was measured immediately at 340 nm using a blank containing 2ml of PBS, 0.1ml of potassium iodide and 0.2 ml of acetic acid. AOPP concentrations were expressed as nmol/L as chloramine-T equivalents where the absorbance of chloramine–T was linear within the range of 0 to 100 nmol/L at 340 nm.

2.1.4.4. Estimation of catalase (CAT) activity

The method described by Khan et al. (2012) was used to test catalase activity with some modifications. The 3 ml reaction mixture used for CAT activity contained: 0.1 ml of plasma or liver tissue enzyme extract, 2.5 ml of 50 mmol phosphate buffer (pH 5.0) and 0.4 ml of 5.9 mmol hydrogen peroxide. 1 min later changes in absorbance of the reaction mixture were determined at 240 nm. An absorbance change of 0.01 as units/min corresponds to one unit of CAT activity.

2.1.4.5. Measurement of glutathione (GSH) in reduced form

Reduced form of glutathione in plasma and liver was estimated by the method of Mitchell et al. (1973). 1.0 ml of (4%) sulfosalicylic acid was added to 1 ml of 10% tissue homogenate. Then the precipitated mixtures were kept for one hour at 4ºC. After 1 hour, mixtures were centrifuged at

1200 × g for 20 min at 4ºC. 0.1 ml filtered aliquot, 2.7 ml phosphate buffer (0.1 M, pH 7.4) and 0.2 ml of 100mM DTNB (5, 5-dithiobis-2-nitrobenzoic acid) were mixed to make 3 ml assay mixture. A yellow color of the mixture developed which was immediately subjected to take absorbance at 412 nm using Smart SpecTM plus Spectrophotometer. The calculated result was expressed in ng/mg protein.

2.1.4.6. Estimation of superoxide dismutase (SOD) activity

SOD was assayed in tissue homogenates by using previously described method (Tripathi et al., 2010). Sufficient amount of tissue extract mixed with PBS to make the volume 2.94 ml. Then 0.06 ml of 15 mM epinephrine was added to the mixture to start the reaction. Taking an interval of 15 sec, change in absorbance of reaction mixture was recorded for one min at 480 nm. Blank prepared with all the ingredients, except enzyme preparation used simultaneously for taking absorption. Auto-oxidation of epinephrine present in sample solution usually reduced to 50% with the action of one unit enzyme.

2.1.5. Histopathological observation

Liver tissues of all groups were prepared for histology study according to standard procedure. In short, tissues were fixed in neutral buffered formalin, treated with ethanol and xylene, and then embedded in paraffin. Using microtom, tissue paraffin blocks were sectioned at 5µm. Tissue sections were then stained with Hematoxylin and Eosin, Picro Sirius Red and Prussian blue separately for microscopic observation of the inflammatory cell invasion, deposition of collagen fibre and iron respectively in liver. Stained tissue sections were examined under light microscope at 40X magnifications.

2.1.6. Statistical analysis

The experimental results were evaluated by using the student's *t* test in Graph Pad Prism Software. The values are expressed as mean±standard error of mean (SEM). In all cases, statistical significance was considered $p < 0.5$.

2.2. Investigation on liver inflammation and consequent fibrogenesis in rat with caffeine treatment

2.2.1. Chemicals and reagents

Pure caffeine of pharmaceutical grade used in our study was kindly supplied by Pharmadesh Laboratories Limited. Alanine aminotransferase (ALT), aspartate amino transferase (AST) and alkaline phosphatases (ALP) assay kits were obtained from DCI diagnostics (Budapest, Hungary), Thiobarbituric acid (TBA) was purchased from Sigma Chemical Company(USA),50,50-dithiobis-2-nitrobenzoate (Ellman's reagent) from sigma (USA), glutathione (GSH) in reduced form and trichloroacetic acid were purchased from J.I. Baker (USA) and sodium hydroxide from Merck (Germany). All other chemicals and reagents used were of analytical grade.

2.2.2. Animal experiment

Ten to twelve weeks old, 18 male Long Evans rats (180-200g) were obtained from animal production unit of Animal House in the Department of Pharmaceutical Sciences, North South University, Dhaka, Bangladesh. The animals were kept in ordinary cages at room temperature of $25\pm3°C$ with 12 h dark / light cycles. They had free access to standard laboratory feed and water. The present study protocol was approved by the Ethical Committee of the Department of Pharmaceutical Sciences, North South University for animal care and experimentation. To study the toxic effect of caffeine on liver, all animals were equally divided into three groups (6 rats in each). Animals of group 1, considered as low dose group, were treated with 6mg caffeine / kg of body weight intragastrically, once daily. Rats of group 2, considered as high dose group, were treated with 12 mg caffeine / kg of body weight intragastrically, once daily. Group 3 considered as control group where rats received only distilled water. Rats of all groups were treated for eight weeks. Body weight, water and food intake of animals were monitored and recorded daily.

After 56 days, all the animals were weighed and sacrificed. Blood sample collected and plasma separated from it and stored at -20°C until used. Some major organs like liver, kidney, heart, spleen were also collected and weight immediately. Half of the organs were stored at

-20°C until used for biochemical tests and halves were processed for histological study.

2.2.3. Assessment of liver enzymes

Rat plasma of three groups was assayed for liver transaminases, ALT and AST and another isozyme, alkaline phosphatases (ALP). Chemical analysis was conducted by following the standard protocols of manufacturer provided with DCI diagnostics kits (Hungary)

2.2.4. Assessment of oxidative stress markers and antioxidant enzyme activity

Oxidative stress markers and antioxidant enzyme activity were measured in both blood plasma and liver tissue. Liver tissue was homogenized in 10 times volume of ice cold phosphate buffer having pH 7.4 and centrifuged at 12,000X g for 30 min at 4°C. The supernatant was collected and used for the determination of protein and enzymatic studies.

2.2.4.1. Estimation of malondialdehyde (MDA)

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2.2.4.2. Assay of nitric oxide (NO)

Nitric oxide, in the form of nitrate, was measured following the method described by Tracey et al.(1995). In present study, Griess-Illosvoy reagent was modified by replacing 5% 1-napthylamine with naphthyl ethylene diamine dihydrochloride (0.1% w/v). The reaction mixture containing tissue extract or plasma (2ml) and phosphate buffer saline (0.5ml) was incubated at 25°C for 150 min. The mixture color changed into pink due to formation of a pink colored

chromophore. Using corresponding blank solutions, the absorbance of these solutions was measured at 540 nm. A standard curve, expressed as nmol/ml, was prepared to measure plasma and liver tissue content of nitric oxide.

2.2.4.3. Measurement of advanced oxidation protein products (AOPP)

The methods described by Witko-Sarsat et al.(1996) and Tiwari et al.(2014) were followed with slight modification to detect AOPP level. In brief, 2 ml mixture was made by diluting plasma or tissue extract with PBS in 1:5 ratio; then 0.1 ml of 1.16 M potassium iodide was added to each tube; after 2 min, 0.2ml acetic acid was added to it. The absorption of the reaction mixture was measured immediately at 340 nm using a blank containing 2ml of PBS, 0.1ml of potassium iodide and 0.2 ml of acetic acid. AOPP concentrations were expressed as nmol/L as chloramine–T equivalents where the absorbance of chloramine–T was linear within the range of 0 to 100 nmol/L at 340 nm.

2.2.4.4. Estimation of catalase (CAT) activity

The method described by Khan et al. (2012) was used to test catalase activity with some modifications. The 3 ml reaction mixture used for CAT activity contained: 0.1 ml of plasma or liver tissue enzyme extract, 2.5 ml of 50 mmol phosphate buffer (pH 5.0) and 0.4 ml of 5.9 mmol hydrogen peroxide. 1 min later changes in absorbance of the reaction mixture were determined at 240 nm. An absorbance change of 0.01 as units/min corresponds to one unit of CAT activity.

2.2.4.5. Assay of glutathione (GSH) in reduced form

Reduced glutathione in liver was estimated by the method of Mitchell JR et al. (1973). 1.0 ml of (4%) sulfosalicylic acid is added to 1 ml of 10% tissue homogenate. Then the precipitated mixtures were kept for one hour at 4ºC. After 1 hour, mixtures were centrifuged at $1200 \times g$ for 20 min at 4ºC. 0.1 ml filtered aliquot, 2.7 ml phosphate buffer (0.1 M, pH 7.4) and 0.2 ml of 100mM DTNB (5,5-dithiobis-2-nitrobenzoic acid) were mixed to make 3 ml assay mixture. A yellow color of the mixture developed which is immediately subjected to take absorbance at 412nm using Smart SpecTM plus Spectrophotometer. The calculated result was expressed in ng/mg protein.

2.2.4.6. Estimation of superoxide dismutase (SOD) activity

SOD was assayed in plasma and tissue homogenates by using previously described method (Tripathi et al., 2010). Sufficient amount of tissue extract mixed with PBS to make the volume 2.94 ml. Then 0.06 ml of 15 mM epinephrine is added to the mixture to start the reaction. Taking an interval of 15 sec, change in absorbance of reaction mixture was recorded for one min at 480 nm. Blank prepared with all the ingredients, except enzyme preparation used simultaneously for taking absorption. Auto-oxidation of epinephrine present in sample solution will be reduced to 50% with the action of one unit enzyme.

2.2.5. Histopathological observation

Liver tissues of all groups were prepared for histology study according to standard procedure. In short, tissues were fixed neutral buffered formalin, treated with ethanol and xylene, then embedded in paraffin. Using microtom, tissue paraffin blocks were sectioned at 5µm. Tissue sections were then stained with Hematoxylin and Eosin and Picro Sirius Red separately for microscopic observation of the inflammatory cell invasion and deposition of collagen fibre respectively in liver. Stained tissue sections were examined under light microscope at 40X magnifications.

2.2.6. Statistical analysis

The experimental results were evaluated by using the student's *t* test in Graph Pad Prism Software. The values were expressed as mean±standard error of mean (SEM). In all cases, statistical significance was considered $p < 0.5$.

2.3. Investigation on the involvement of cytochrome P450 (CYP) isozymes on metabolism of amphetamine derivatives in rat with 3, 4-methylenedioximethamphetamine (MDMA) treatment

2.3.1. Chemicals and reagents

Clandestine tablets, supplied from Osaka Police Station, were subjected to extraction for MDMA and MDA. Purification done until the analytical grade obtained which was confirmed by nuclear magnetic resonance (NMR) spectroscopy. BNF, 7-pentoxyresorufin, ethoxyresorufin, norephedrine (NEP) hydrochloride, PB sodium, methoxyresorufin and β-glucuronidase (*Escherichia coli* type VII-A, lyophilized) purched from Sigma Aldrich (St. Louis, MO, USA). NADPH, glucose-6-phosphate (G-6-P) and G-6-P dehydrogenase were purchased from Oriental Yeast (Tokyo, Japan). HMMA was prepared according to the previously published method (Shima et al., 2007). HMPA and Heptafluorobutyric anhydride (HFBA) were purchased from Wako (Osaka, Japan). All other reagents used in present study were of the commercially available purest grade.

2.3.2. Treatment of animals

Male Sprague-Dawley rats, selected for MDMA metabolic study, were supplied by Japan Laboratory Animals (Tokyo, Japan). The animals' body weight was ranging from 180 to 200 g.

2.3.2.1. Phenobarbital pretreatment

Rats (n=4) were administered phenobarbital (PB) dissolved in saline as 80mg (calculated as free base) / kg body weight once daily through intra peritoneal route for 3 consecutive days. Control rats (n=4) were administered 0.9% of NaCl (saline) solution for those 3 days. On the 4th day, all the rats (both control and PB treated) were administered MDMA i.p. as 10 mg (calculated as free base) / kg body weight. The rats were then individually kept in metabolic cages in a room with controlled temperature and 12 hr light / dark cycle. Animals of all groups had free access to food

and water during the study period. Urine sample was collected at 12th & 24th h after MDMA injection. The samples were stored in plastic tubes with screw cap at -30°C until analysed.

2.3.2.2. β-naphthoflavone pretreatment

Rats (n=4) were administered β-naphthoflavone (BNF), dissolved in corn oil as 80mg (calculated as free base) / kg body weight, once daily by intra peritoneal injection for 3 consequtive days. Control rats (n=4) were administered corn oil (vehicle) for those 3 days. On the 4th day, all the rats (both control and BNF treated) were administered MDMA i.p. as 10 mg (calculated as free base). / Kg body weight. Rats were kept and urine samples were collected & stored following the same protocol as PB pretreatment.

The present animal experiment protocol was approved by the Animal Experiment Committee of Showa University. After collection of 24 hr urine, the rats were anaesthesized and killed by decapitation. The livers were removed immediately for the preparation of microsomes.

2.3.3. Instrumentation

The TRACE GC 2000 SERIES - GCQTM / POLARIS MS system was used to determine MDMA, MDA, HMMA and HMPA quantitatively.

2.3.3.1. GC conditions

Gas chromatograph was composed of a RTX-5 MS capillary column (30 m X 0.25 mm i.d, 0.25µm film thickness, 5% phenyl 95% methyl polysiloxane) inside the oven which is directly connected to the ion source of the mass spectrometer. Helium gas, as carrier, passed through the column with a persistant a flow rate of 1.0ml/min.The injection was processed using the splitless mode and the temperature of the injector was maintained at 150° C for 1.0 min following injection. The oven temperature was held at 80°C for I min after sample injected and then increased linearly at a rate 12°C /min upto 250°C, then at a rate 50°C/min upto 300°C. This burning temperature held for 3 minutes to clear the column from sample residue.

2.3.3.2. MS Conditions

Ion fragments of metabolites and internal standard were detected by Mass detector with electron impact ionization (EI) mode at 70eV. The temperature set for ion source was 250°C and for transfer line 275°C. Before conducting all analysis, the machine was tuned and report recorded. To recognize the fragmentation pattern of each sample component some ions were selected as: for MDMA m/z 162(target ion, t), 210, 254; for MDA m/z 135,162(t), 375; for HMMA m/z 210,254(t), 360; for HMPA m/z 305,333(t), 376 and for NEP m/z 240(t), 330, 303. Results were achieved by the Thermo Fisher Scientific Xcalibur data system comparing the sample values with standard curve.

2.3.4. Preparation of enzyme solutions and enzymatic hydrolysis of rat urine specimen

The conjugated MDMA and three of its metabolites excreted through rat urine was hydrolysed. We considered only the glucuronide conjugates. An enzyme solution of β-Glucuronidase, type VII-A (2000 Units/ 0.5 ml), prepared in 0.075M potassium phosphate (pH=6.8) buffer was added to each sample solution for hydrolysis. We hydrolyzed 0.1ml of urine specimen collected upto12 hr of MDMA administration and 0.5 ml for 13-24 hr collected sample suspecting very low metabolite concentration in urine specimen of 13-24 hr collection. 0.5 ml of the prepared buffered enzyme solution was added to each sample solution. Deionized water was added to each test tube to make the volume 1.5 ml and then incubated for 16 hrs at 37°C in a water bath with shaker. Gentle flow of nitrogen gas was passed into each sample tube for one min before incubation (Tam et al., 1990). Tight closure of cap was maintained in each step of analysis.

2.3.5. Extraction of MDMA and its metabolites from rat urine

Nor ephedrine, NEP was used in this method as internal standard (IS) to identify any shift in band position in GC-MS data. 0.1 ml (1μg) solution of NEP was added to all enzyme hydrolyzed samples. We followed the method provided by Terada et al. with slight modification (Terada et al., 1983) as reconstitution of the residue inside the tube with ethyl acetate and derivatization with HFBA (Katagi et al., 2005). Sufficient amount of deionized water added to each test tube to

make the total volume 2ml. Then to each test tube 0.2ml of Na_2CO_3 (20% solution), 0.75 gm of NaCl crystal and 6 ml of ethyl acetate were added. The sample was shaken properly for 10 minutes. The mixture was centrifuged at 3,000 rpm for 5 minutes. 5 ml of the organic layer was transferred to 10 ml conical-bottomed centrifuged tube with a screw cap. 20µl of Acetic acid was added into it. The extract was placed under a gentle stream of nitrogen at 40°C for evaporation. After drying, 0.2 ml of ethyl acetate & 0.1 ml of HFBA was added to the residue in each test tube for making derivative. The content was mixed by vortex mixture in tightly capped condition for 5 seconds and then incubated for 1 hour using temperature 60°C. The derivative synthesized in test tube solution again evaporated under nitrogen flow at 45°C. The mixture was not allowed to dry completely to prevent the decomposition of derivatives and approximately 10-20 µl concentrate was kept instead of drying. 0.3 ml of ethyl acetate was added to each test tube for reconstitution, vortex mixed for 5 seconds and made the preparation ready for analysis by gas chromatography- mass spectrometry (GC-MS).

2.3.6. Working standard solution preparation

Separately 1mg/ml stock solutions of MDA, MDMA, HMMA, HMPA & NEP were prepared by dissolving an appropriate amount of the standards in water. Working solution of both analytes and internal standard were made separately at concentration of 10 µg / ml by dilution of stock solution with water.All the working solutions were freshly prepared for each analysis.

2.3.7. Preparation of standard curve

Standard samples were prepared by adding 18µl, 90µl and 180µl(from 10µg/ml solution) of each of four analytes (MDA,MDMA,HMMA,HMPA) and following the same process for extraction and derivatization as described previously. Then 2 µl of each sample was injected into GC-MS for analysis under the same condition as for sample. Standard curves were constructed by plotting the ratios of the peak areas of the analyte to the internal standard (NEP) versus concentrations of the analyte. Routinely, nine standard samples of three different concentrations (0.09, 0.45 and 0.9µg/ml) were analysed along with samples from inducer pre-treated rats dosed with MDMA.

2.3.8. Validation of assay method

The linearity of assay method was checked for all analytes. The limit of detection (LOD) and the limit of quantification (LOQ) were measured for all analytes. For each analyte, the computer-generated regression of the calibration curve, r^2 (regression co-efficient) value was considered.

2.3.9. Recovery study of method validation

Recoveries of MDMA, MDA, HMMA and HMPA from urine were determined at the concentration of 0.45µg/ml. The spiked urine samples were processed as described in the assay procedure except that the addition of internal standard NEP. A reference standard solution was made in triplicate using water in place of drug-free urine and without NEP. Another exception is that ethyl acetate of 8ml in lieu of 6ml was added in reference standard solution to obtain maximum extraction. The peak area obtained from the spiked urine samples (n=3) were compared to those obtained from the reference standards. Though the standards were in salt form we couldn`t add them directly in to organic layer without hydrolysis in aqueous solution. So, extraction step also required to transfer the reference standard into organic layer from aqueous layer.

2.3.10. Rat liver microsome preparation and their enzyme assay

The rat livers, removed from the animals, were perfused with ice-cold 0.9% NaCl solution and homogenized with four volumes of ice cold 1.15% KCl solution immediately. The homogenates were centrifuged 20 min at 9000g. The resultant supernatant fractions collected and further ultracentrifuged for 60 min at 105000g. The obtained microsomal pellet was dispersed in 0.1M sodium potassium phosphate buffer (pH 7.4) containing 20% glycerol. The microsomal protein was assayed following the method provided by Lowry et al. (1951). According to the previously described method by Omura and Sato, the total cytochrome P450 content was determined (Omura et al., 1964).

CYP 3A isozyme dependent testosterone 6β-hydroxylation activity was determined by using the method of van der Hoeven (1984). CYP 2B1 dependent pentoxyresorufin-O-dealkylase activity,

CYP 1A1 dependent ethoxyresorufin-O-deethylase activity and CYP 1A2 dependent methoxyresorufin-O- demethylase activity were studied according to the method described by Burk and Mayer (1974). Slight modification of the method was done. The reaction mixture consisted of $2\mu M$ substrate, 5mM $MgCl_2$, 50 µg of microsomal protein, NADPH generating system (5mM G-6-P, 0.6mM NADP and 1U G-6-PDH) and 100mM K_2HPO_4 buffer (pH 7.5). Considering excitation and emission maxima at 530 nm and 585 nm respectively, resorufin formation was fluorimetrically identified. The fluorescence emitted by the produced resorufin in reaction mixture of microsome was compared with the fluorescence of known amount of resorufin and the activities of corresponding CYP isozymes were calculated.

2.4. Investigation on the effect of methamphetamine tablet Yaba on bone in rat

2.4.1. Chemicals and reagents

Yaba tablets, used in our study, were kindly supplied by the Narcotics Control Dept. of Dhaka Metropolitan Police, Bangladesh. Alkaline phosphatase assay kit was obtained from DCI diagnostics (Budapest, Hungary). All other chemicals and reagents used were of the purest analytical grade. The digital X-ray taken by the logistics of Lab Aid Limited, Mirpur, Dhaka, Bangladesh.

2.4.2. Animal experiment

18 male Long Evans rats (180-200g), age of ten to twelve weeks, were obtained from animal production unit of animal house in the Department of Pharmaceutical Sciences, North South University, Dhaka, Bangladesh. The animals were kept in ordinary cages at room temperature of $25 \pm 3°C$ with a 12 h day/ night cycles. The animals were supplied standard laboratory feed and water. The Ethical Committee of the Department of Pharmaceutical Sciences, North South University for animal care and experimentation approved the present study protocol. Animals were equally divided into three groups (6 rats in each). Rats of group 1, considered as low dose group, were treated with Yaba tablet powder suspended in distilled water equivalent to 5mg of methamphetamine/kg of body weight intragastrically, once daily. Rats of group 2, considered as high dose group, were treated with Yaba tablet powder suspended in distilled water equivalent to 10 mg of methamphetamine/kg of body weight intragastrically, once daily. Group 3 considered as control group where rats received only distilled water. Rats of all groups were treated for eight weeks. Body weight, water and food intake of animals were monitored and recorded daily.

After 56 days, all the animals were weighed and sacrificed. Blood sample collected and plasma separated from it and stored at -20°C until used. Livers also collected and homogenized and stored at -20°C. Rat tibias were collected; flash removed and kept in neutral buffer formalin at 4°C for72 hrs. After then bones were cleaned properly and made free from flesh and stored in 10% phosphate buffer until tested.

2.4.3. Assessment of parameters associated with bone metabolism

2.4.3.1. Measurement of alkaline phosphatase (ALP)

Alkaline phosphatase (ALP), considered as bone forming marker, was measured in rat plasma by using DCI diagnostics kits (Hungary) according to the manufacturer's protocol.

2.4.3.2. Assay of nitric oxide (NO)

NO was determined in the form of nitrate according to the method described by Tracey et al. (1995). In this study, we have modified the Griess-Illosvoy reagent and used naphthyl ethylene diamine dihydrochloride (0.1% w/v) instead of 5% 1-napthylamine. 3 ml of reaction mixture contained 2ml of liver homogenates and 0.5 ml of phosphate buffer saline and incubated at 25°C for 150 min. A pink colored chromophore was appeared. We took the absorbance of these solutions at 540 nm using the corresponding blank solutions. A standard curve was used to measure NO level and results were expressed in nmol/ml.

2.4.4. Digital X-ray of rat tibias

Rat tibias of 5mg and 10 mg dose groups and control group were tested for radiolucency or radiopacity by digital X-ray.

Chapter Three

Results

3.1. Investigation with methamphetamine tablet Yaba for liver inflammation and consequent fibrogenesis in rat

3.1.1 Physical & behavioral changes observed in rat during Yaba treatment

During the period of experiment, rats of both drug treated groups were found restless with palpitation and increased thirst. 30% of drug treated rat of high dose group had skin lesion in different parts of the body. Drug treated rats of low and high dose group rapidly got aged look with loosening of skin. Body temperature of rats in low dose and high dose groups increased by 7-8°C and persisted for 1h app. after Yaba administration where as body temperature remain unchanged in control rats.
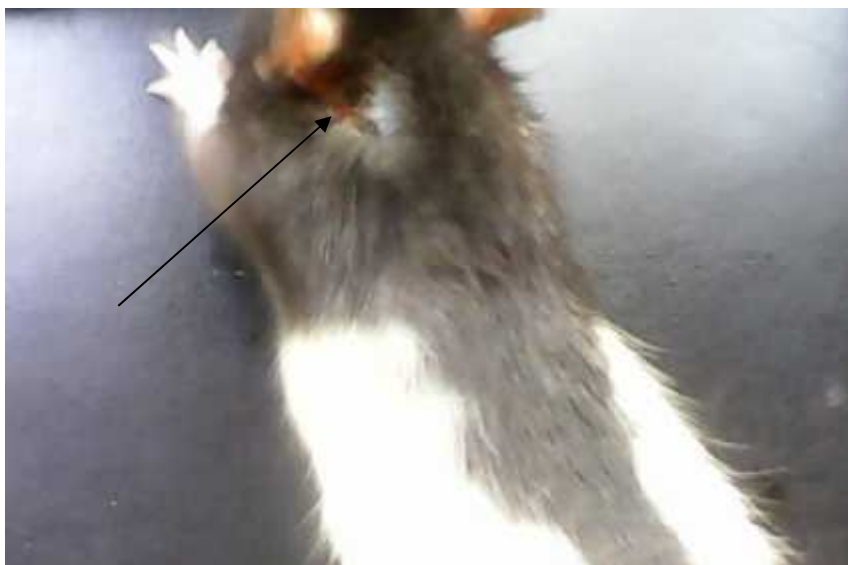

Figure 1: Skin lesion of rat during Yaba treatment

3.1.2. Effects of Yaba on body weight, food and water intake in rat

During the experimental period, daily record of body weight, food and water intake of rats of all groups showed that body weight of drug treated rats of both dose groups increased in a similar rate as control. Food intake was same in rats of all groups. Increased water intake observed in rats of drug treated groups as compared to control but that was not statistically significant.
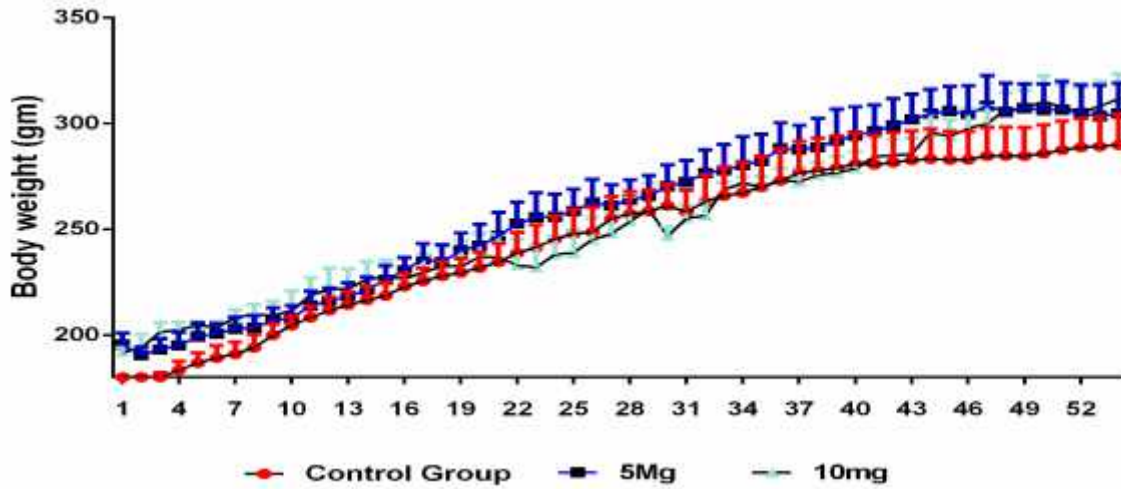
Figure 2: Daily body weight record of rat during Yaba treatment in liver toxicity study
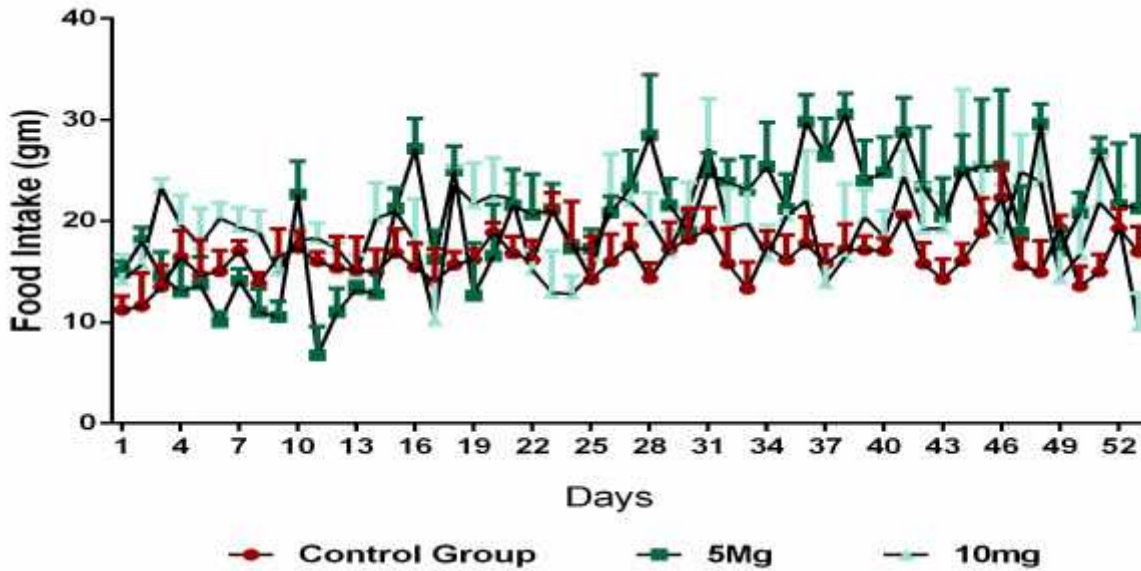

Figure 3: Daily food intake record of rat during Yaba treatment in liver toxicity study
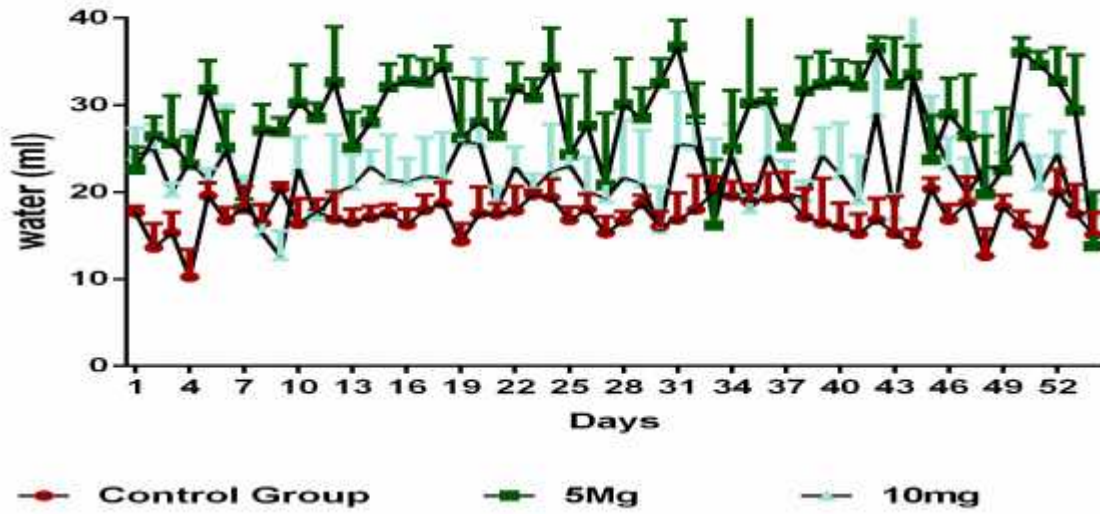
Figure 4: Daily water intake record of rat during Yaba treatment in liver toxicity study

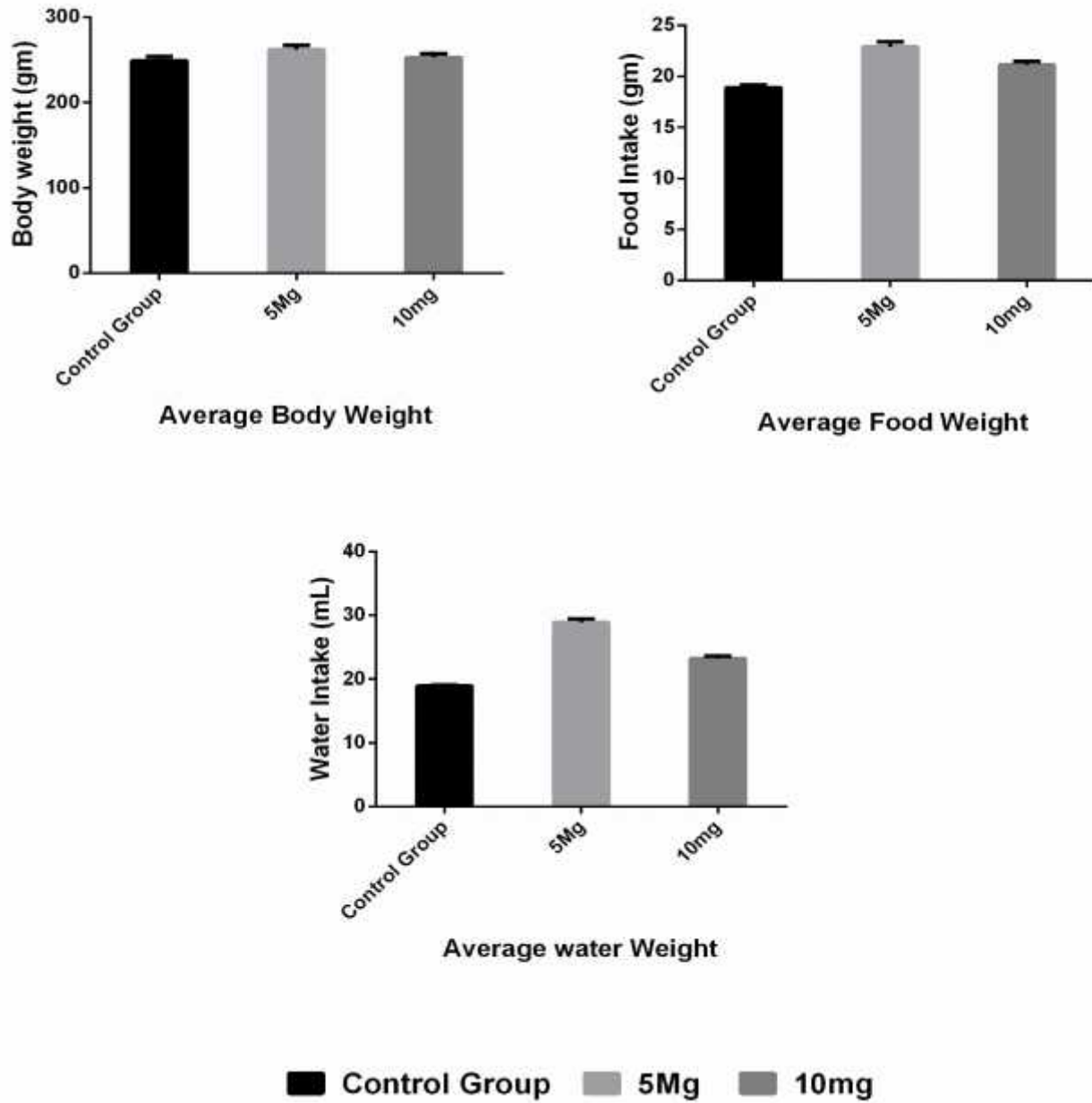Figure 5: Abscess in liver of rat after Yaba treatment

Figure 6: Average body weight, food and water intake of rat during Yaba treatment

in liver toxicity study

3.1.3. Effects of Yaba on organ weight in rat

Different vital organs included liver, heart, kidney and spleen showed no significant change in weight as compared to control values. 30% of drug treated rat had abscess in liver.
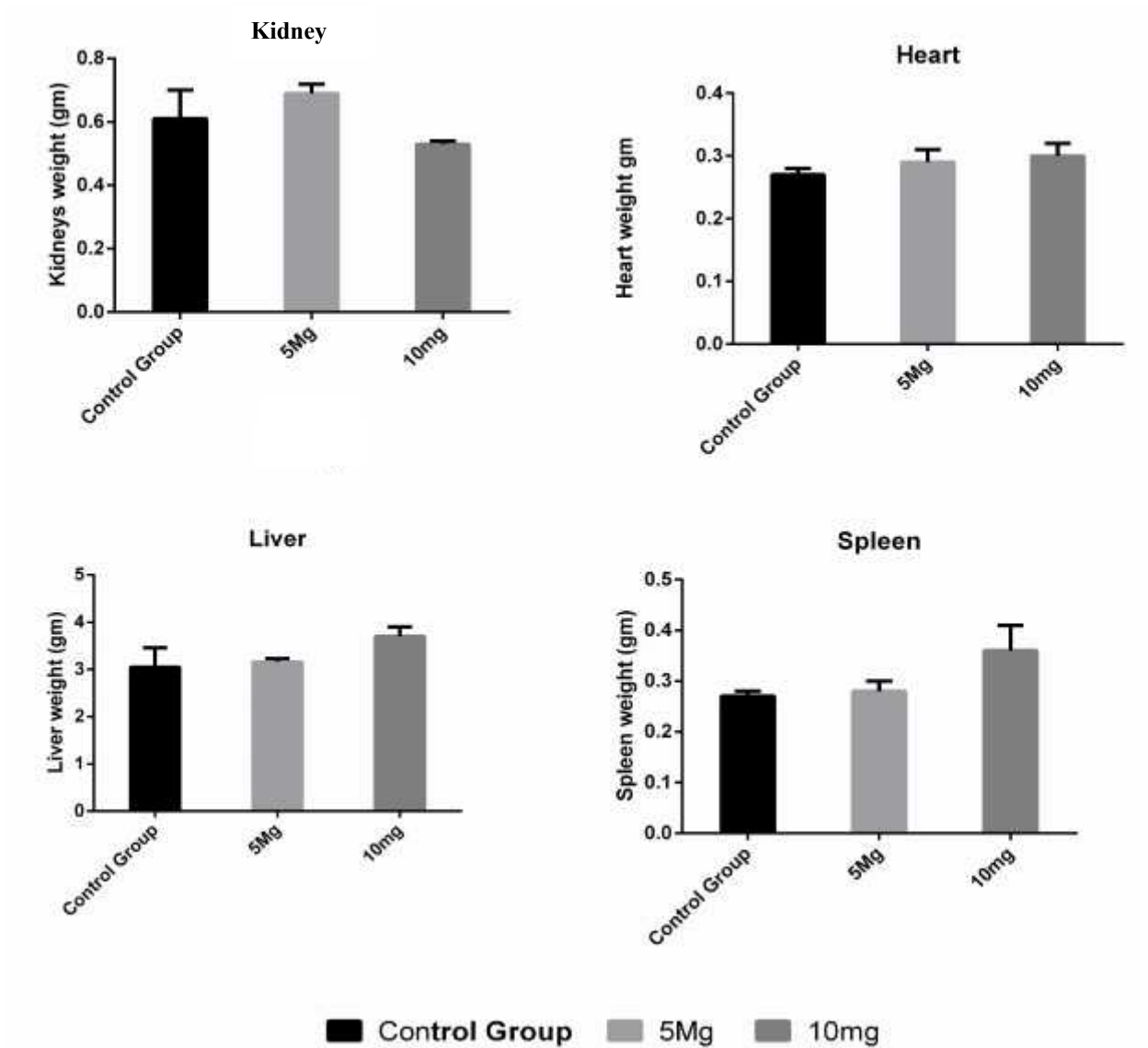

Figure 7: Comparison of different organ weight of Yaba treated rat with control

3.1.4. Effects of Yaba on liver marker enzymes in rat

Yaba treatment induced transaminase ALT to increase significantly to 87.86±14.00 in high dose group from 31.58±2.87 U/L(p< 0.01, N= 5-6). Another liver marker enzyme AST increased significantly to 47.37±6.19 in high dose group from 22.97±1.82 U/L(p< 0.01, n=5-6). Alkaline phosphatase (ALP) increased significantly to 165.32±10.14 in low dose group from 79.81±7.60 U/L(p< 0.01, n=5-6). The increase in liver transaminases, ALT and AST, increased by 2.8-fold and 2-fold respectively by high dose of Yaba and ALP by 2-fold by low dose of Yaba as compared to control value. Change in ALT and AST concentrations in low dose groups and ALP concentration in high dose group was not statistically significant.
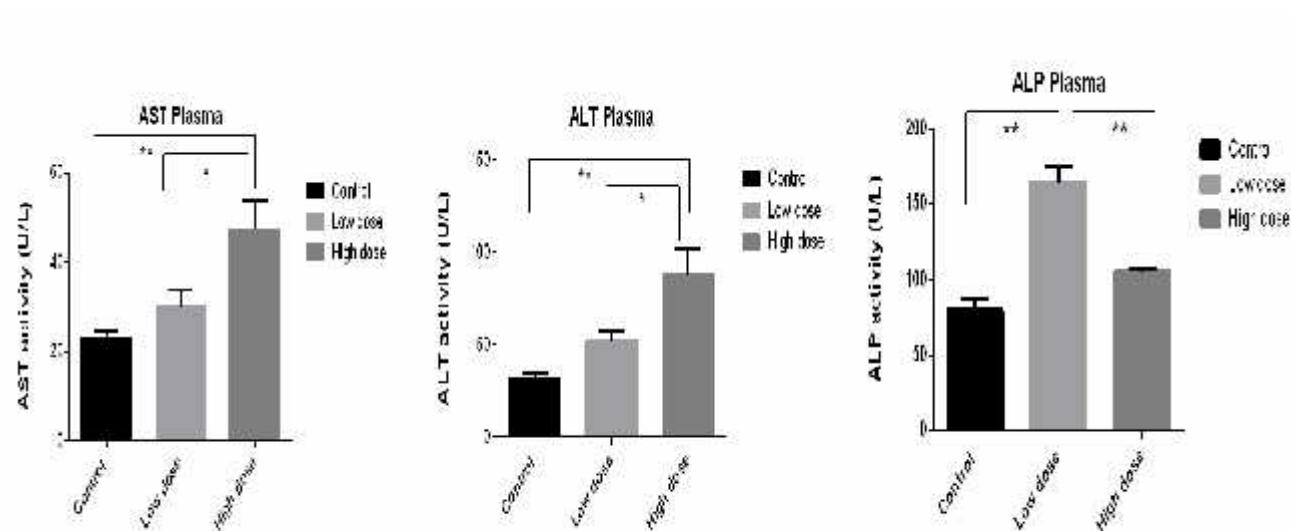

Figure 8: Effect of Yaba on liver transaminases and alkaline phosphatases in rat

3.1.5. Effects of Yaba on oxidative stress in rat

The plasma and liver tissue content of MDA, NO and AOPP were considered as markers of oxidative stress. Yaba treatment induced high concentration of lipid peroxidation product, MDA, which significantly increased to 50.58±2.06 nmol/ml in high dose group from 36.49±2.3 nmol/ml (p < 0.05, n=5-6) in plasma and to 181.54±11.96 nmol/ml in high dose group from 129.92±15.61 nmol/ml (p < 0.01, n=5-6) in liver homogenate. Yaba in high dose increased MDA

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by 1.4-fold both in rat plasma and liver tissue when compared with control value. However, low dose of Yaba couldn't induce significant change in MDA concentration in plasma and liver tissue. In drug treated rat liver tissue, nitrate level increased significantly in high dose group to 20.55 ± 1.01 nmol/ml from 17.43 ± 1.21 nmol/ml ($p < 0.05$, n=5-6) which was 1.2-fold increase as compared to control value. In rat plasma, NO level was not significantly changed in any dose group as compared to control. According to the figure11, no significant change in the value of AOPP level in plasma and liver tissue of drug treated groups found when compared with control.
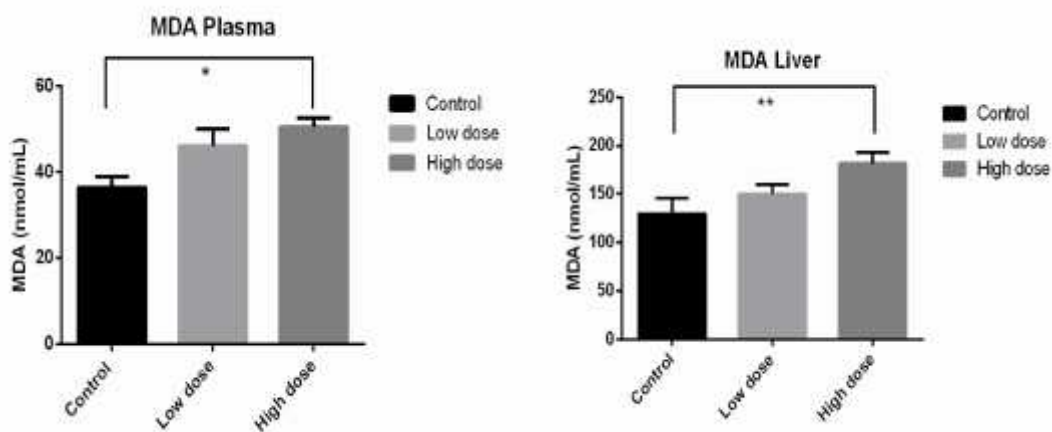
Figure 9: Effect of Yaba on lipid peroxidation in plasma and liver in rat
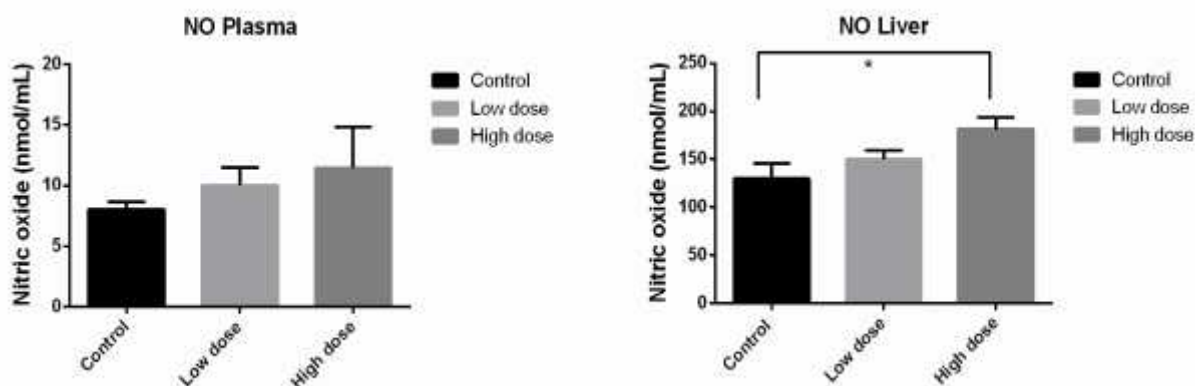
Figure 10: Effect of Yaba on nitric oxide level in plasma and liver in rat
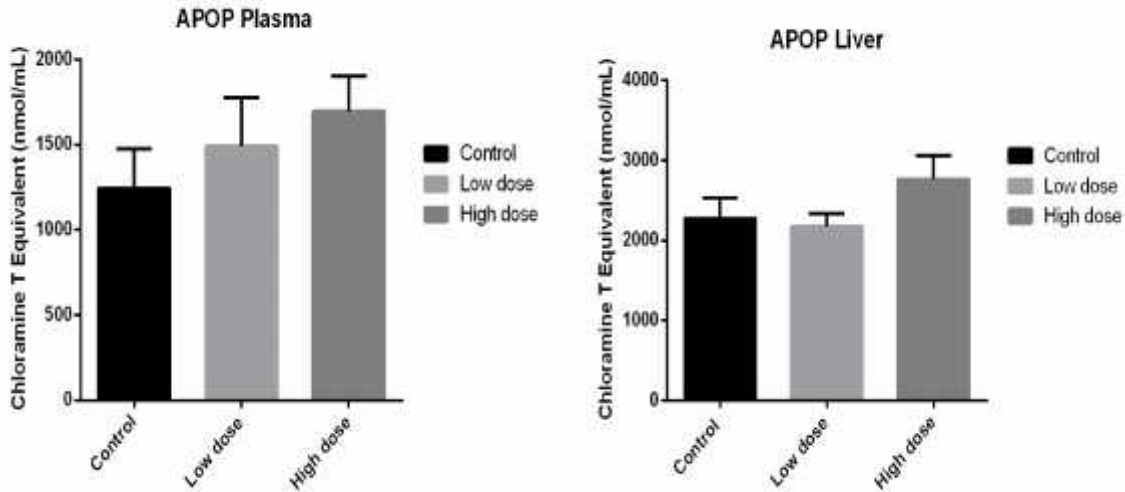
Figure 11: Effect of Yaba on advanced oxidation protein products level in plasma

and liver in rat

3.1.6. Effects of Yaba on antioxidant enzyme system in rat

Activity level of antioxidant enzymes such as GSH, CAT and SOD found almost unchanged in plasma or liver tissue of drug treated rats when compared with control values.

Figure12: Effect of Yaba on glutathione activity in plasma and liver in rat

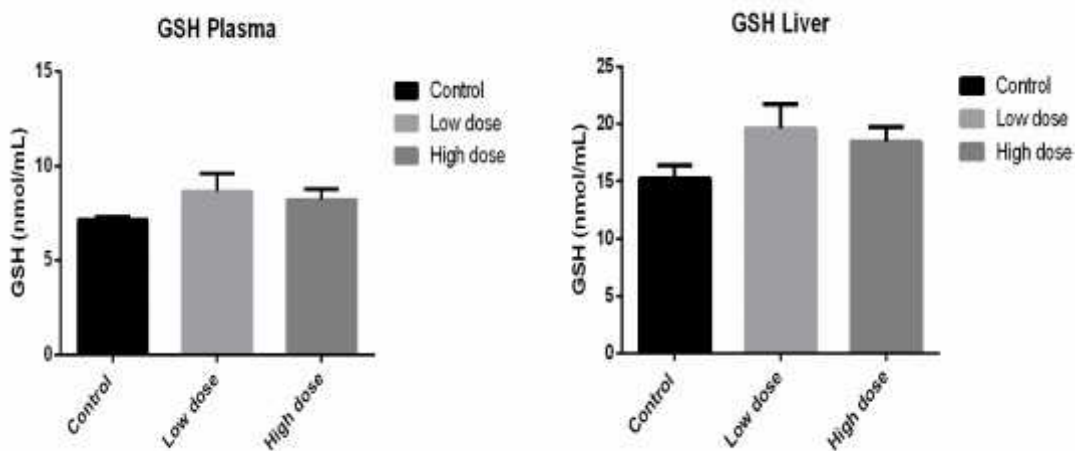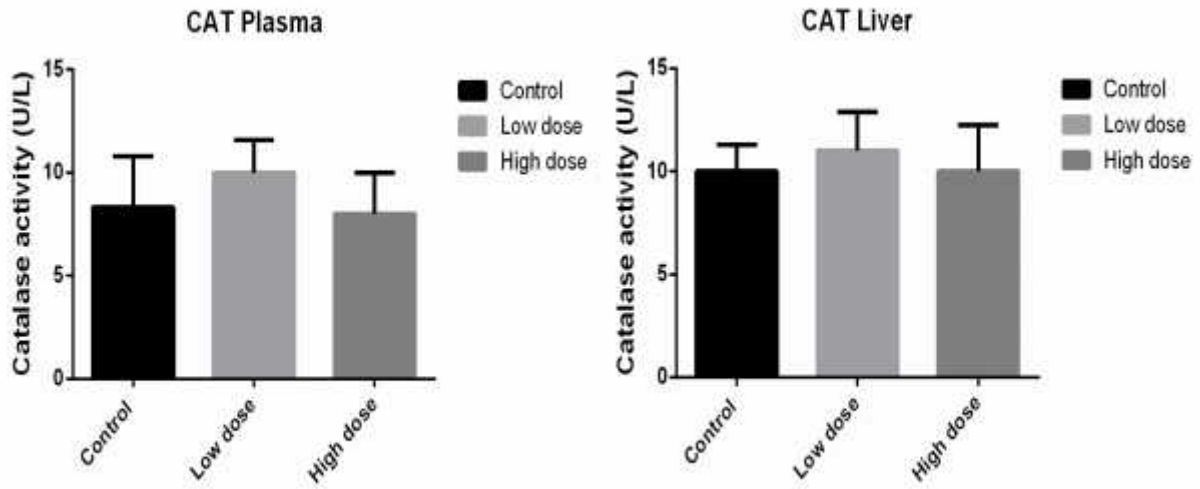Figure 13: Effect of Yaba on catalase activity in plasma and liver in rat
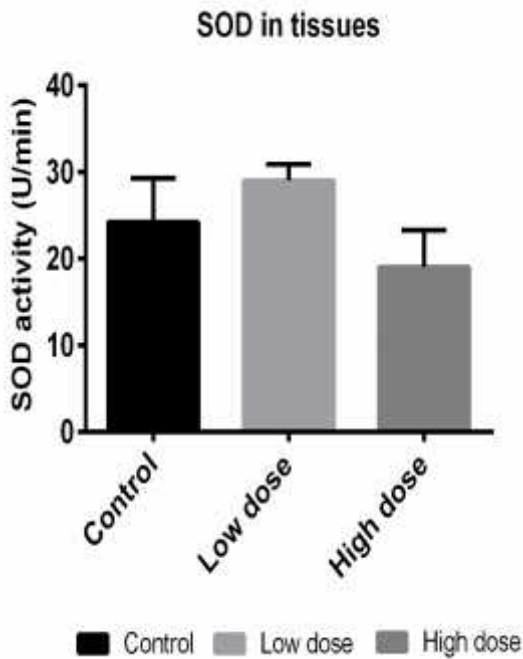
Figure 14: Effect of Yaba on superoxide dismutase activity in liver in rat

3.1.7. Histological changes after Yaba treatment in rat liver

Inflammation was seen in stained tissue section of drug treated rat liver. Massive invasion of inflammatory cells was found in the centrilobular part of liver section stained with H &E in low dose Yaba treated rat which progressively increased in high dose treated rat (Figure15). Liver fibrosis was evaluated histologically by visualizing the red color of collagen fibres deposition using Sirius Red stain in both dose groups. The collagen fibres were heavily deposited around portal tracts and central veins in Yaba treated group and extended from central vein to portal tract resulting in the formation of pseudolobules which was not seen in control rats. Extent of collagen fibre deposition was huge in high dose group than that of low dose group (Figure16).

Iron deposition was not seen in liver section of Yaba treated rats by Prussian blue staining (Figure17).

| Control | Low dose | High dose |
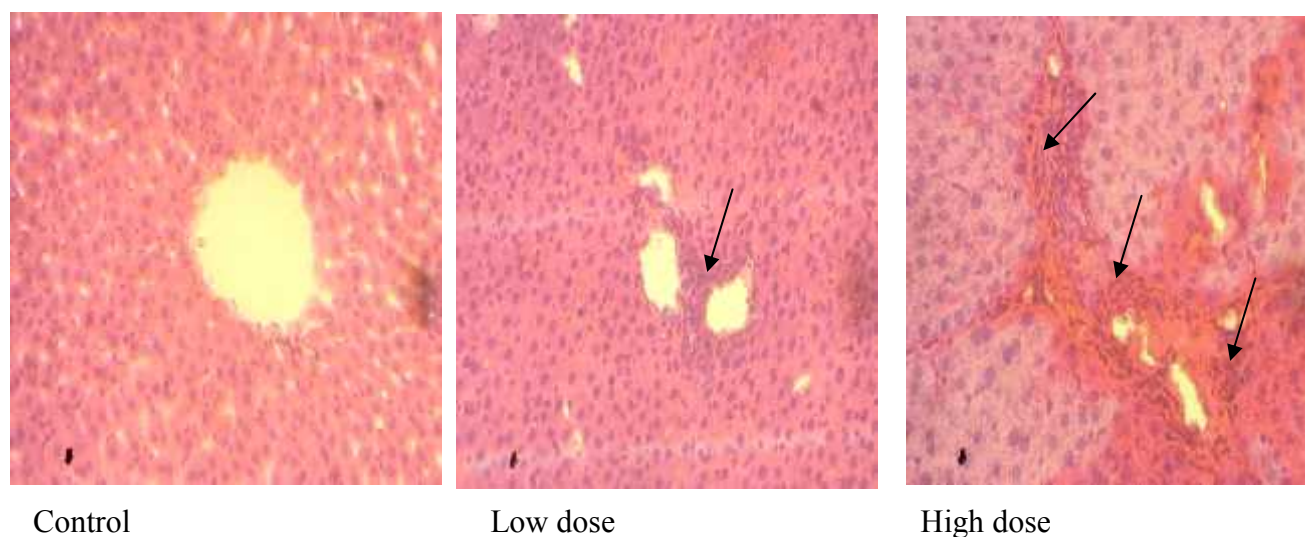
Figure 15: Invasion of inflammatory cells in the centilobular part of rat liver after Yaba treatment
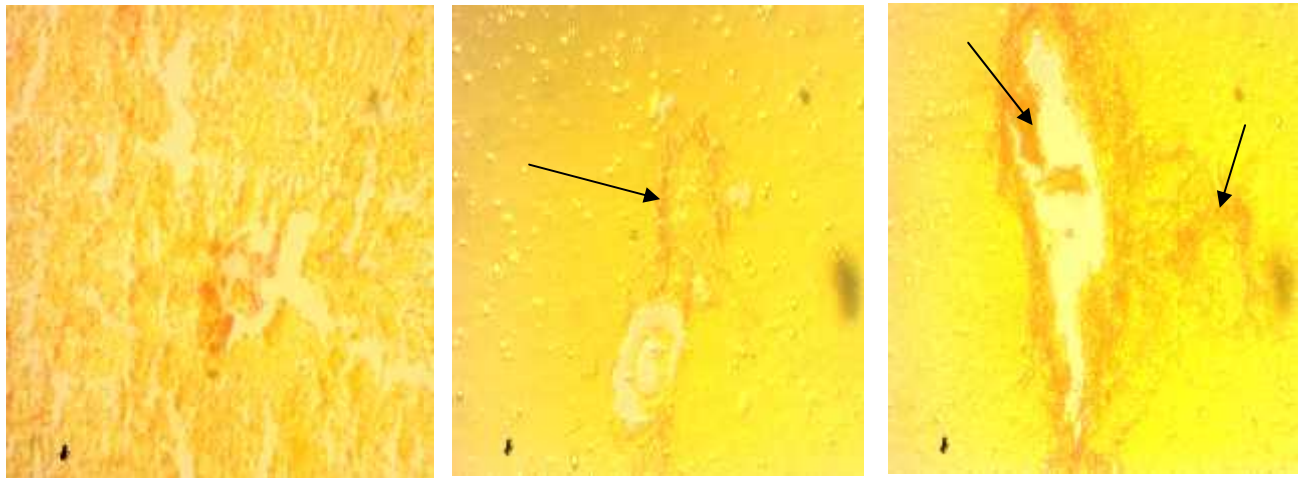
| Control | Low dose | High dose |

Figure 16: Deposition of collagen fibre in rat liver after Yaba treatment
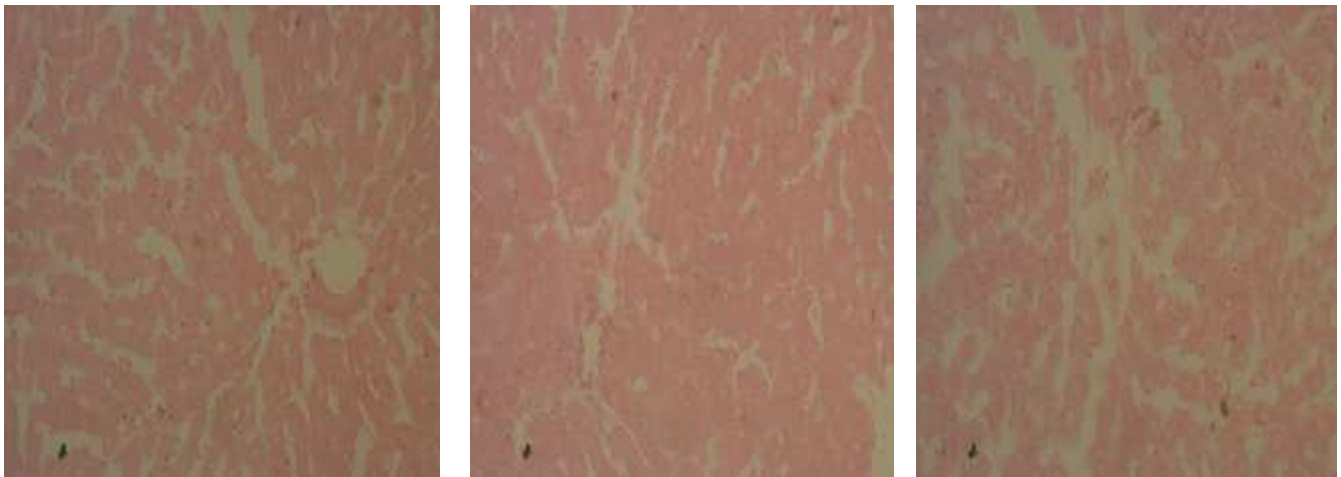
| Control | Low dose | High dose |

Figure 17: Iron deposition observation in rat liver after Yaba treatment

3.2. Investigation with caffeine for liver inflammation and consequent fibrogenesis in rat

3.2.1. Physical & behavioral changes observed in rat during caffeine treatment

During the period of experiment, rats of both dose treated groups were found restless with palpitation. No lesion observed on skin. Body temperature was normal after caffeine administration.

3.2.2. Effects of caffeine on body weight, food and water intake in rat

During the experimental period, daily body weight, food and water intake of rats of all groups were recorded. Body weight of rats increased equally in drug treated and control groups. Food and water intake was same for all groups.
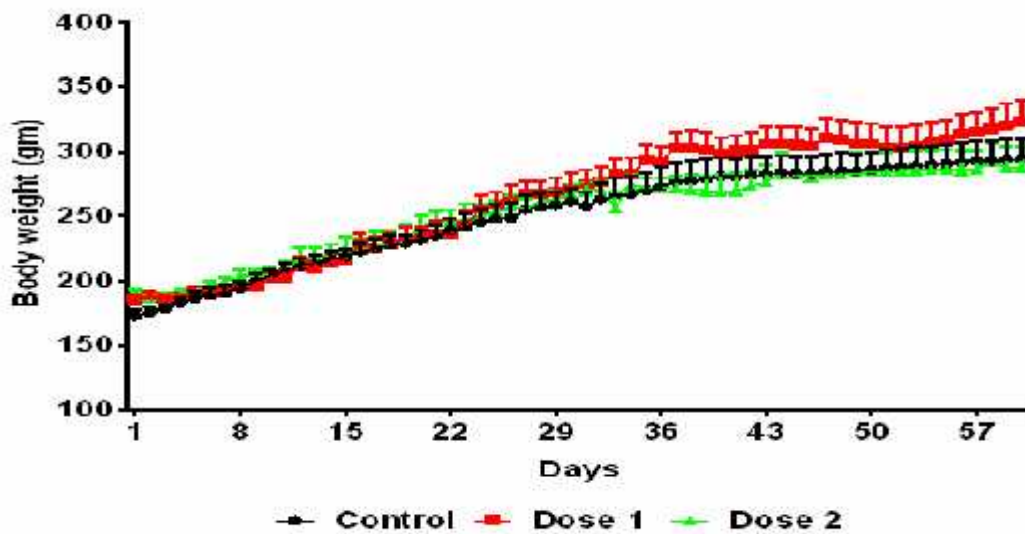
Figure 18: Daily body weight record of rat during caffeine treatment
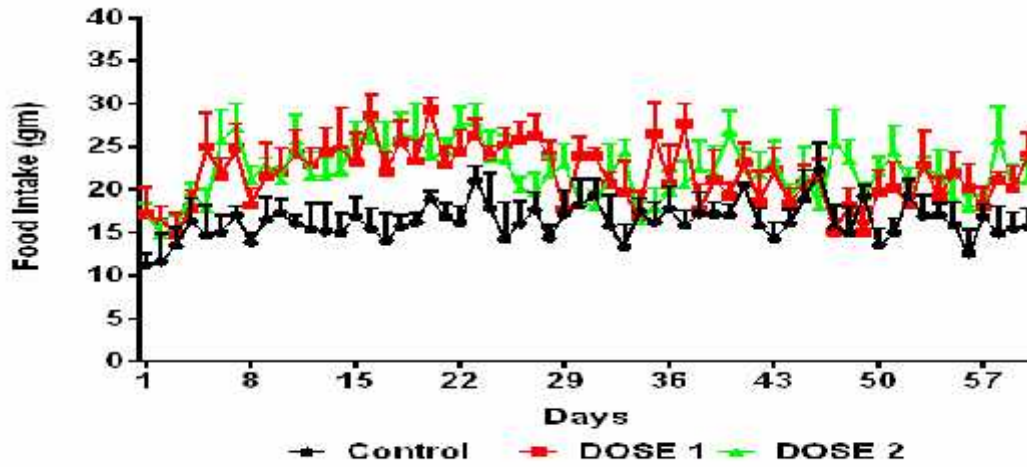
Figure 19: Daily food intake record of rat during caffeine treatment
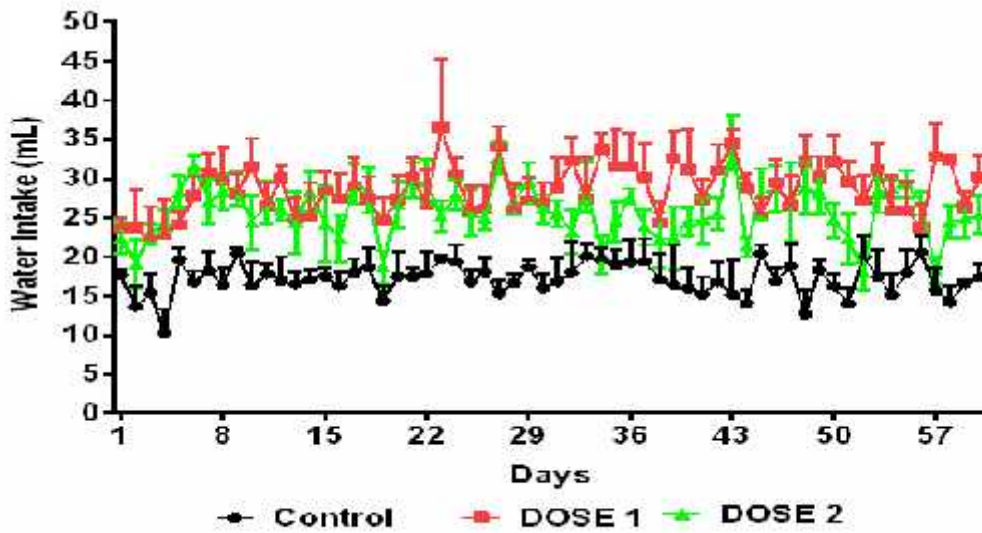
Figure 20: Daily water intake record of rat during caffeine treatment

3.2.3. Effects of caffeine on organ weight in rat

Liver weight of caffeine treated rats increased significantly from that of control in high dose group (P < 0.05, n = 6). Other vital organs such as heart, kidney and spleen showed no significant change in weight as compared to control values.
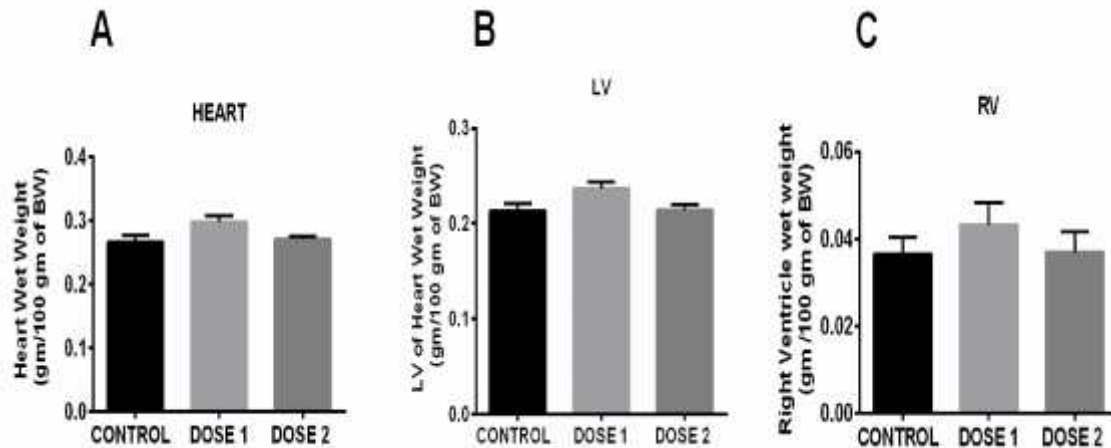
Figure 21: Comparison of weights of whole heart, left ventricle and right ventricle of caffeine treated rat with control group
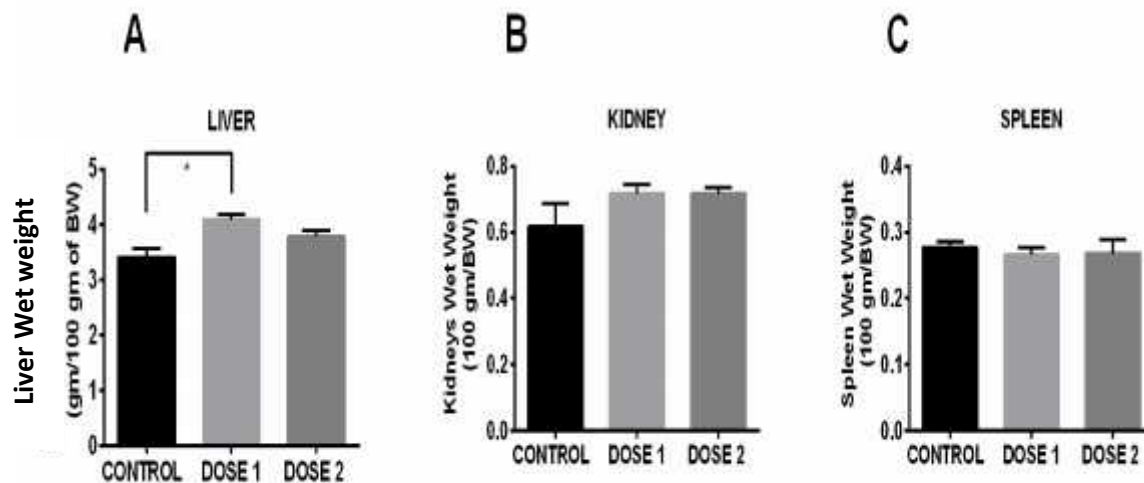
Figure 22: Comparison of liver, kidney and spleen weights of caffeine treated rat with control group

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3.2.4. Effects of caffeine on liver marker enzymes in rat

By caffeine treatment, transaminase ALT increased significantly to 71.78 ± 8.22 in low dose group and to 73.21 ± 5.33 in high dose group from 43.07 ± 4.45 U/L ($p < 0.01$, n= 6 in both groups). Another liver marker enzyme AST increased significantly to 41.63 ± 3.46 in low dose group and to 47.37 ± 4.85 in high dose group from 22.97 ± 2.87 U/L($p < 0.01$, n=6 in both dose groups). Alkaline phosphatase (ALP) increased significantly to 51.10 ± 3.85 in low dose group and to 48.75 ± 3.88 in high dose group from 30.88 ± 4.59 U/L($p < 0.01$, n=6 in both groups). The liver transaminases, ALT increased 1.7-fold in both dose groups; AST increased 1.8-2.0 fold in low dose and high dose group, respectively; ALP increased1.6-1.7 fold in low dose and high dose group respectively as compared to control value.
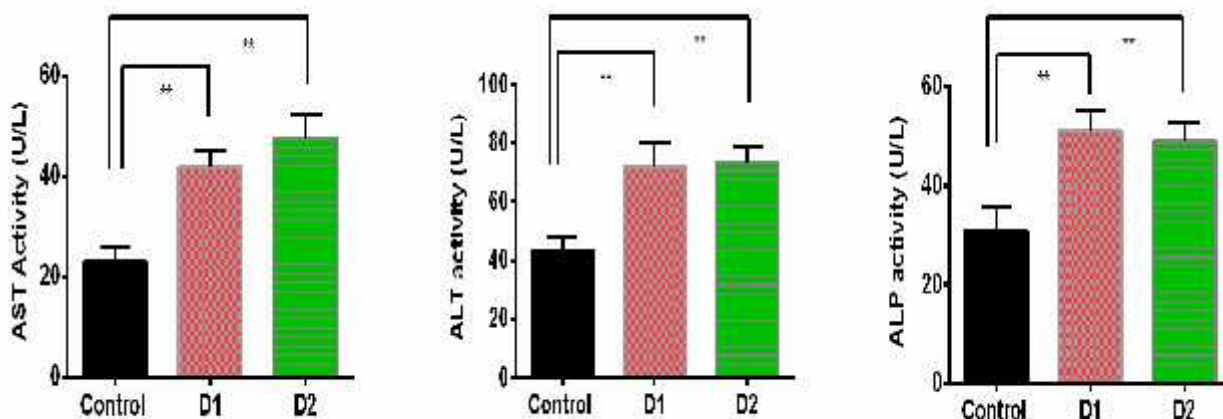
Figure 23: Effect of caffeine on liver transaminases and alkaline phosphatases in rat

3.2.5. Effects of caffeine on oxidative stress in rat

In our study, we have evaluated the MDA, nitric oxide and AOPP content in rat plasma and liver homogenates as markers of oxidative stress. The level of lipid peroxidation product, MDA in caffeine treated rats significantly increased from 97.72 ± 7.30 to 169.79 ± 11.09 by low dose and to 146.85 ± 14.08 nmol/ml by high dose ($p < 0.01$, n=6) in plasma. In liver homogenate, this value increased from 130.82 ± 10.89 to 179.08 ± 6.76 by low dose and to 194.46 ± 5.85 nmol/ml by high dose ($p < 0.01$, n=6). Caffeine increased MDA 1.5-1.7 fold by high and low dose respectively in rat plasma and 1.4-1.5 fold by low and high dose respectively in liver. NO,

measured as nitrate, increased significantly from 14.1 ± 0.91 to 17.24 ± 0.34 nmol/ml (p<0.05, n=6) by low dose in plasma and in liver tissue, from 12.63 ± 0.48 to 29.05 ± 2.01 by low dose and to 35.57 ± 4.08 nmol/ml (p<0.05, n=6) by high dose when compared with control values. Caffeine induced increase in NO level 1.2-fold by low dose in plasma and in liver 2.0-2.3 fold by low and high dose respectively as compared to control value.
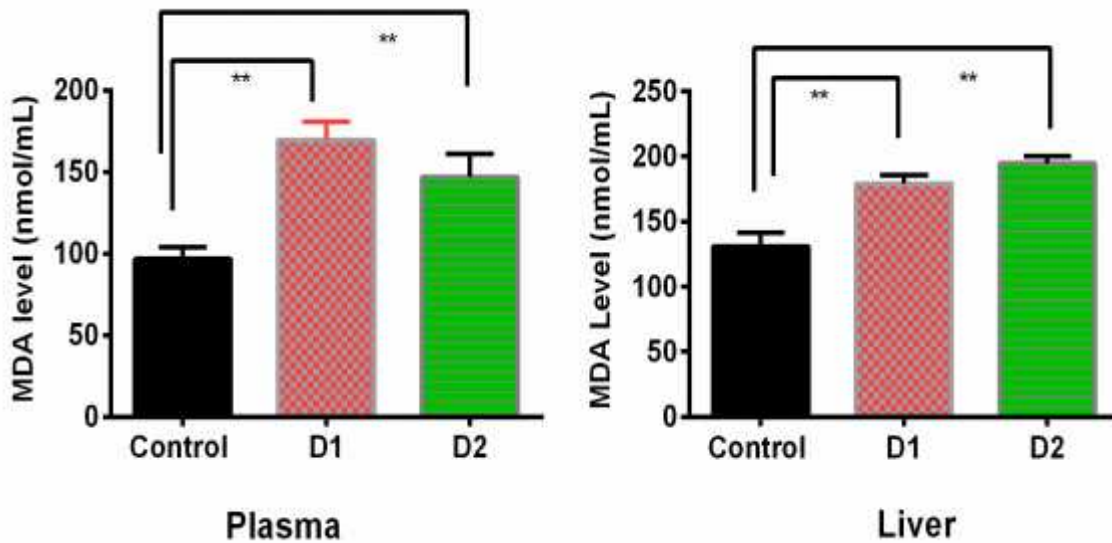
Figure 24: Effect of caffeine on lipid peroxidation in plasma and liver in rat
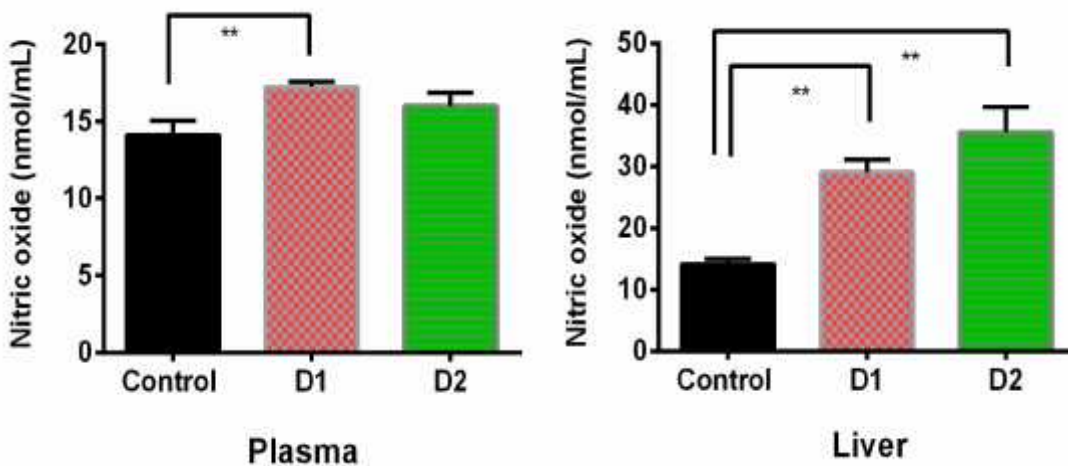
Figure 25: Effect of caffeine on nitric oxide level in plasma and liver in rat

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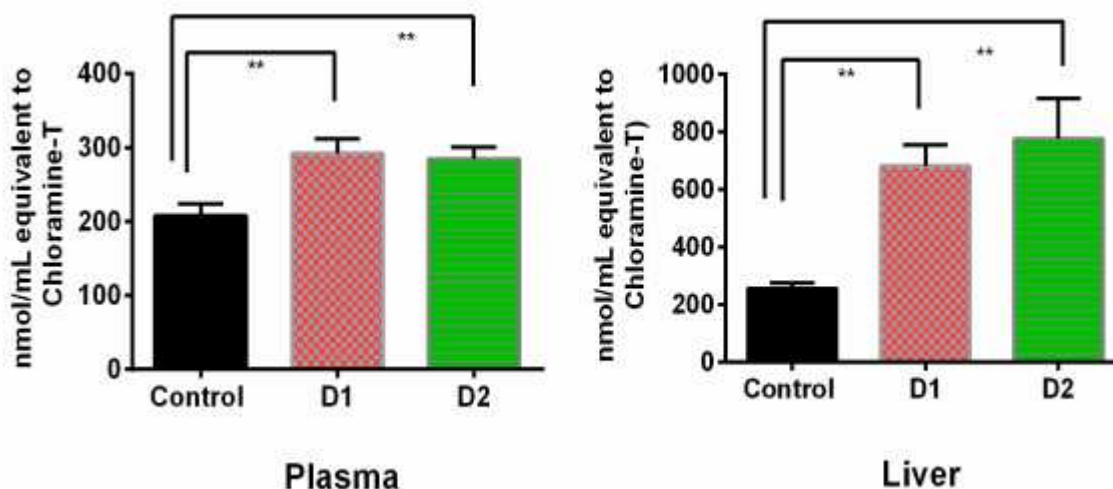
Figure 26: Effect of caffeine on advanced oxidation protein product level in plasma

and liver in rat

Caffeine treatment induced significant increase in AOPP concentration to 292.62 ± 19.03 and to 285.08 ± 15.40 from 207.70 ± 16.05 nmol/ml equivalent to chloramines-T ($p<0.01$, n=6) in plasma by low and high dose respectively. In liver it increased to 682.06 ± 72.34 and to 776.51 ± 139.36 from 256.11 ± 18.44 nmol/ml equivalent to chloramines-T ($p<0.01$, n=6).by low and high dose respectively. AOPP increased 1.3-1.4 fold by high and low dose respectively in plasma where as 2.6-3.0 fold by high and low dose respectively in liver as compared to control.

3.2.6. Effects of caffeine on antioxidant enzyme system in rat

Caffeine treatment significantly reduced superoxide dismutase activity by low dose from 20.83 ± 3.52 to 10.00 ± 1.83 in plasma; from 154.17 ± 17.77 to 83.33 ± 8.82 by low dose and to 94.17 ± 6.38 by high dose in liver. SOD activity reduced 2-fold by both dose groups in plasma and liver. Activities of other antioxidant enzymes such as GSH and CAT found unchanged in plasma and liver tissue in both dose groups when compared to control values.
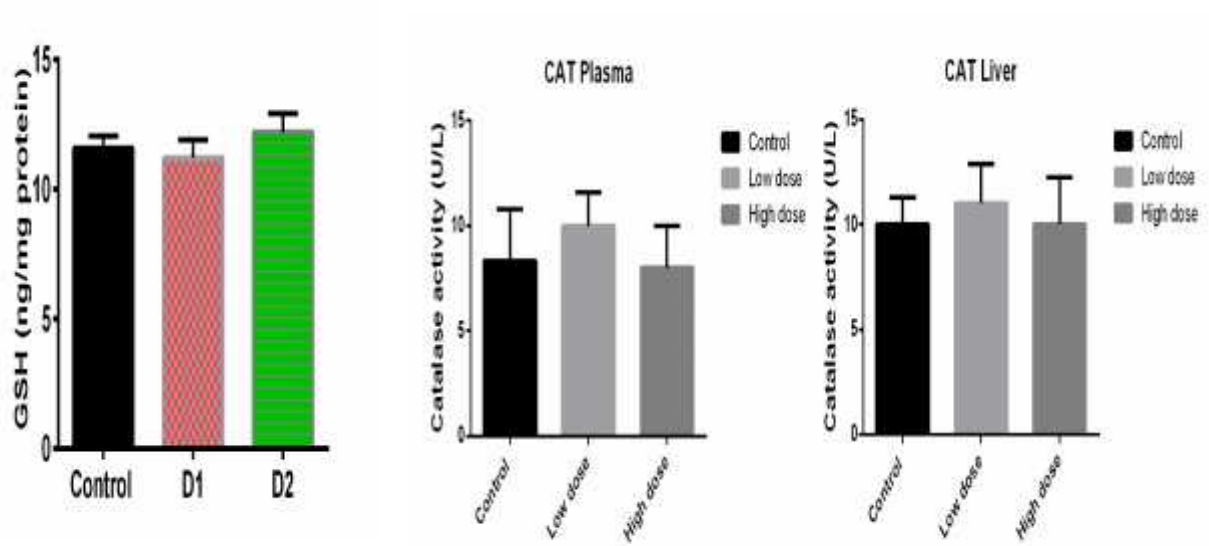
Figure 27: Effect of caffeine on glutathione and catalase activities in plasma and liver in rat
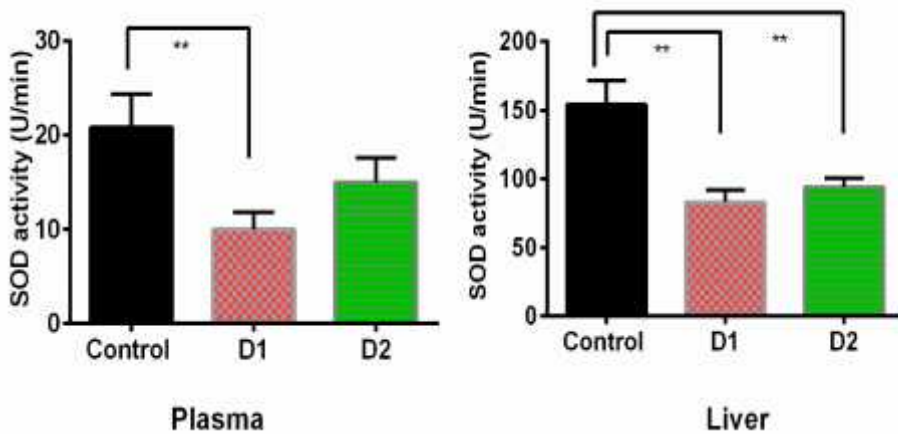
Figure 28: Effect of caffeine on superoxide dismutase activity in plasma and liver in rat

3.2.7. Histological changes after caffeine treatment in rat liver

Inflammation in drug treated rat liver was seen in stained tissue section. Massive invasion of inflammatory cells was found in the centrilobular part of liver section stained with H &E in low

dose group which progressively increased in high dose group (Figure29).

| Control | Low dose | High dose |
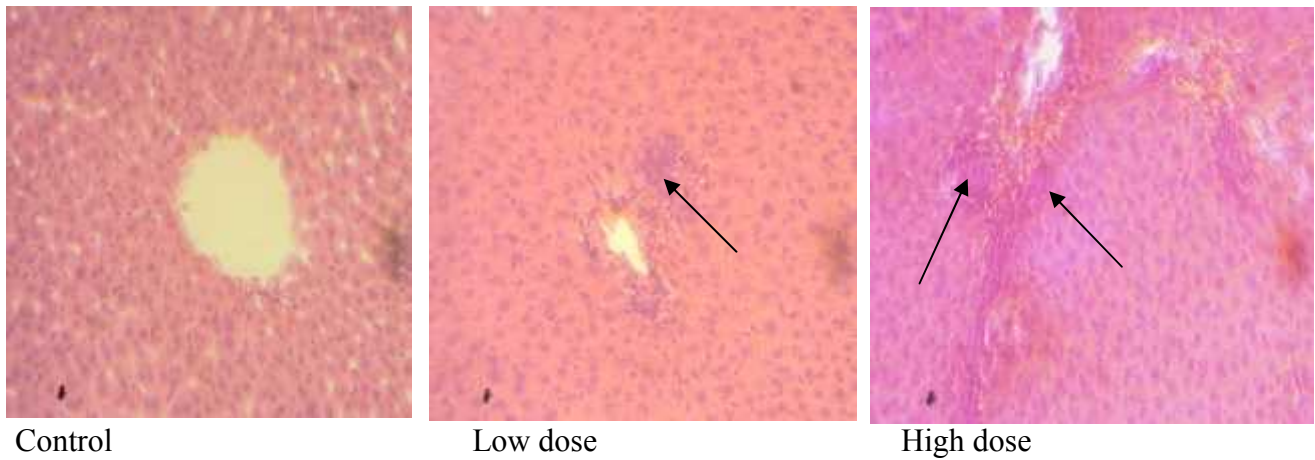
Figure 29: Invasion of inflammatory cells in the centilobular part of rat liver after caffeine

treatment

| Control | Low dose | High dose |
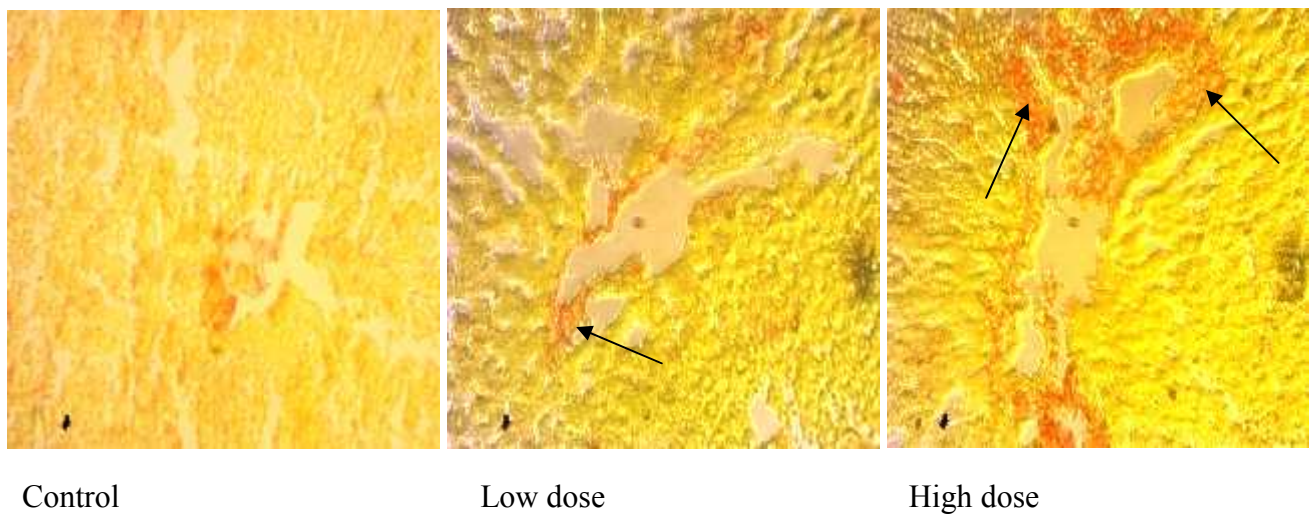
Figure 30: Deposition of collagen fibre in rat liver after caffeine treatment

Liver fibrosis was evaluated histologically by visualizing the red color of collagen fibres deposition using Sirius Red stain in both dose groups. The collagen fibres were heavily deposited around portal tracts and central veins in caffeine treated group and extended from central vein to portal tract resulting in the formation of pseudolobules which was not seen in control rats. Extent of collagen fibre deposition was enormous in high dose group than that of low dose group (Figure30).

3.3. Investigation with 3, 4-methylenedioximethamphetamine (MDMA) for the involvement of cytochrome P450 (CYP) isozymes on metabolism of amphetamine derivatives in rat

3.3.1. Validation of assay method by GC-MS

The method was linear from 0.045 to 0.9 µg / ml. for all analytes. The limit of detection (LOD) was 0.02 µg/ml and the limit of quantification (LOQ) was 0.09 for all analytes. For each analyte, the computer-generated regression line of the calibration curve gave an r^2 (regression co-efficient) value which are shown in the figure 31.
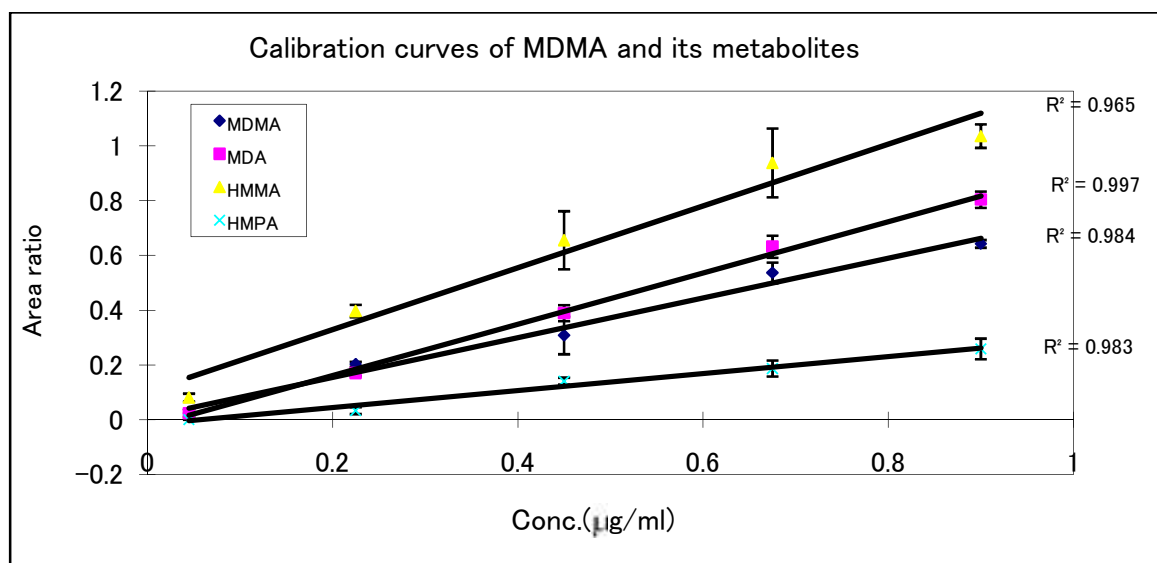
Figure 31: Calibration curves of MDMA, MDA, HMMA and HMPA extracted from

spiked rat urine

Table 1

Recovery study for determination of MDMA, MDA, HMMA and HMPA in spiked rat urine.

Target conc. (µg/ml)	Component name	Mean measured Conc.(µg/ml)	Mean % recovery
0.45	MDMA	0.401	89.1
	MDA	0.455	101.1
	HMMA	0.457	101.5
	HMPA	0.403	89.6

* RSD= relative standard deviation (n=3)

3.3.2. Effects of cytochrome P450 inducers on metabolism of 3,4-methylenedioximethamphetamine (MDMA) *in vivo*

The results calculated for MDMA and its three metabolites concentration based on GC-MS data were expressed as percent dose recovery(%) comparing with the initial dose (10mg / kg BW) of MDMA in Table2.

From phenobarbital pretreated rat urine total percent recovery of MDMA and its three of other metabolites (MDA, HMMA and HMPA) was 34.86% where as from that of control group was 42.62% after 24 hrs of MDMA administration. Intact parent drug (MDMA) found 11.33% and 17.93% from PB treated and control group respectively. MDA was excreted as 6.65%from PB treated group and 11.7% from control. HMMA was recovered from PB treated and control group as 15.5% and 11.63% respectively. HMPA recovery was similar in case of both groups as 1.35%.

Rats pretreated with β-naphthoflavone excreted through urine total 17.49% of MDMA and its three other metabolites after 24 hrs of MDMA administration and control rats excreted 28.16%. Intact MDMA assayed from both treated and control group were 2.83% and 13.23% respectively. MDA found 2.3% from BNF group and 7.6% from control group. HMMA

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recovered as 11.45% and 6.53% from both group respectively. HMPA recovery was 0.91% and 0.8% from BNF and control group respectively.

Pretreatment with phenobarbital decreased MDA synthesis and increased HMMA synthesis significantly as compared to untreated rats during first twelve hour of MDMA administration. In the next twelve hours, significant decrease observed in the concentration of both MDA and HMMA in the PB-pretreated rat urine when compared with the control values. HMPA synthesis was low and remains unchanged after PB treatment. Concentration of the parent molecule, MDMA was significantly decreased in urine samples in phenobarbital induced group as compared to control.

Table 2

Percent dose recoveries of MDMA and its metabolites calculated from their concentrations in rat urine after phenobarbital or β-naphthoflavone pretreatment

Sampling time after MDMA administration (h)	Pretreatment with	Dose recoveries (%)[a]			
		MDMA	MDA	HMMA	HMPA
0-12	Phenobarbital	10.5±1.0*	5.56±0.2*	13.6±0.6*	0.65±0.02
	Vehicle only	16.3±2.5	9.88±1.7	8.62±1.2	0.50±0.1
13-24	Phenobarbital	0.86±0.1*	1.09±0.1*	1.90±0.2*	0.70±0.1
	Vehicle only	1.63±0.3	1.82±0.4	3.01±0.1	0.86±0.1
0-12	β-naphthoflavone	1.61±1.7	2.18±0.2*	9.17±1.6*	0.74±0.1
	Vehicle only	12.2±2.3	7.39±0.7	4.78±0.5	0.67±0.1
13-24	β-naphthoflavone	1.22±0.3	0.12±0.01	2.28±0.3	0.17±0.02
	Vehicle only	1.03±0.3	0.21±0.1	1.75±0.2	0.13±0.02

MDMA, 3,4-methylenedioximethamphetamine; MDA, 3,4-methylenedioxiamphetamine;HMMA,4-hydroxy-3-methoxymethamphetamine; HMPA, (4-hydroxy-3-methoxyphenyl) acetone

*$P<0.05$ vs control (vehicle only); statistical comparison made by the unpaired Student's t-test and the nonparametric test (Wilcoxon or Mann-Whitney)

[a]Percentages of the administered dose of MDMA. Data given as mean±standard deviation (n=4)

Table 3

Effects of phenobarbital or β-naphthoflavone pretreatment on some enzyme activities being related to cytochrome P450 isozymes in rat liver

Enzyme activity		Pretreatment with	Measured activity (pmol/min/mg protein)[a]
Testosterone β-hydroxylase	CYP3A dependent	Phenobarbital	3.90±0.3***
		Vehicle only	1.85±0.2
Pentoxyresorufin O-dealkylase	CYP2B1 dependent	Phenobarbital	545±67.4***
		Vehicle only	136±44.2
Ethoxyresorufin O-deethylase	CYP1A1 dependent	β-naphthoflavone	1260±99.0**
		Vehicle only	231±32.0
Methoxyresorufin O-demethylase	CYP1A2 dependent	β-naphthoflavone	405±59.7**
		Vehicle only	62.3±6.7

[a] Mean± standard deviation (n=4)   **P<0.01; ***P<0.001 vs control (vehicle only)

Pretreatment with BNF decreased MDA synthesis and increased HMMA synthesis significantly as compared to untreated rats during 0-12 hour of MDMA administration. In the next twelve hours, no significant change observed in the concentration of both MDA and HMMA in the BNF-pretreated rat urine when compared with the control values. HMPA synthesis was low and remains unchanged after BNF treatment. Concentration of the parent molecule, MDMA remain unchanged in urine samples collected from β-naphthoflavone induced group as compared to control.

3.3.3. Effects of inducer pretreatment on microsomal cytochrome P450 content in rat liver

Total CYP contents of rat liver microsome significantly increased from 1.05± 0.06 to 1.85± 0.06 nmol/mg protein (P< 0.001, n=4) after phenobarbital pretreatment where as after treatment with β-naphthoflavone, CYP content significantly increased from 0.92± 0.05 to 1.34± 0.04 nmol/mg protein (P< 0.001, n=4). PB and BNF pretreatment induced about 1.8-fold and 1.4-fold increase in P450 contents of rat liver respectively as compared to control.

3.3.4. Effects of inducer pretreatment on cytochrome P450 related enzymes in rat liver

Testosterone 6β-hydroxylation and pentoxyresorufin O-dealkylation reactions increased significantly by two-fold and four-fold respectively as compared to control by phenobarbital pretreatment where as ethoxyresorufin O-deethylation and methoxyresorufin O-demethylation reactions were significantly increased by five- fold and six-fold respectively after β-naphthoflavone pretreatment. This result indicated increased synyhesis of CYP 3A and CYP 2B isozymes by PB and CYP1A1 and CYP 1A2 isozymes by BNF in rat liver.
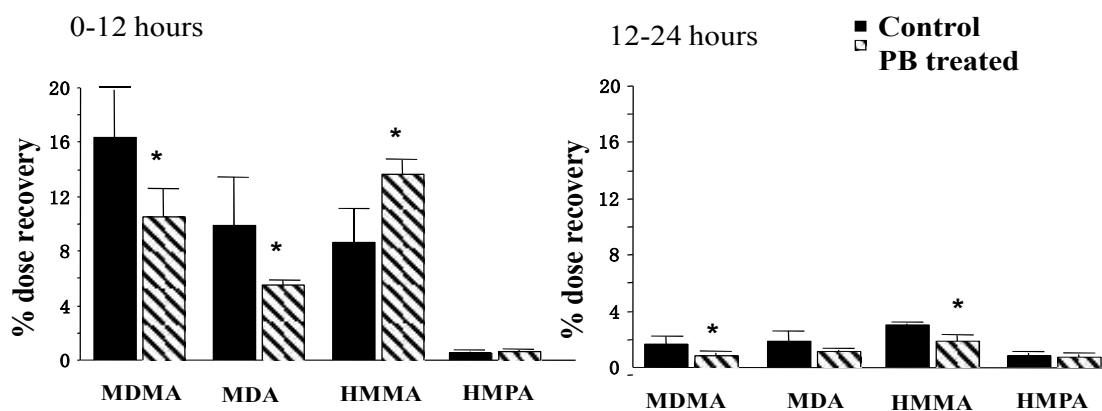
Figure 32: GC-MS analysis of MDMA and its metabolites extracted from PB-pretreated rat urine.

0-12 hours   12-24hours   □ **control**
                                                 □ **BNF treated**
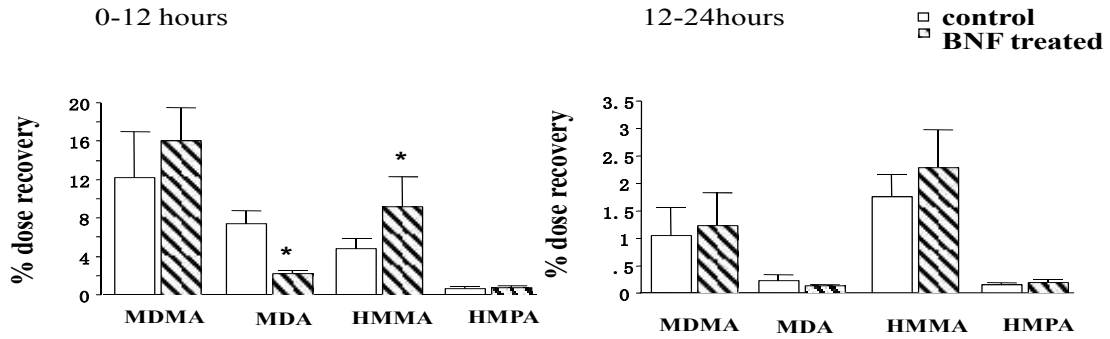
Figure 33: GC-MS analysis of MDMA and its metabolites extracted
from BNF-pretreated rat urine.
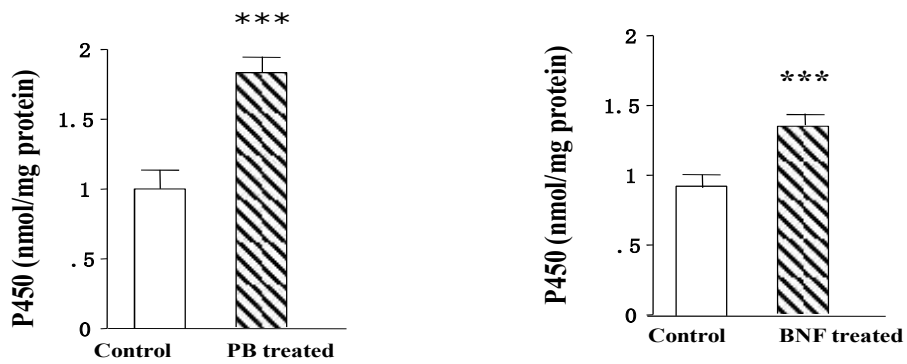
Figure 34: Total cytochrome P450 content of liver microsome increased in
rat livers after PB or BNF treatment.

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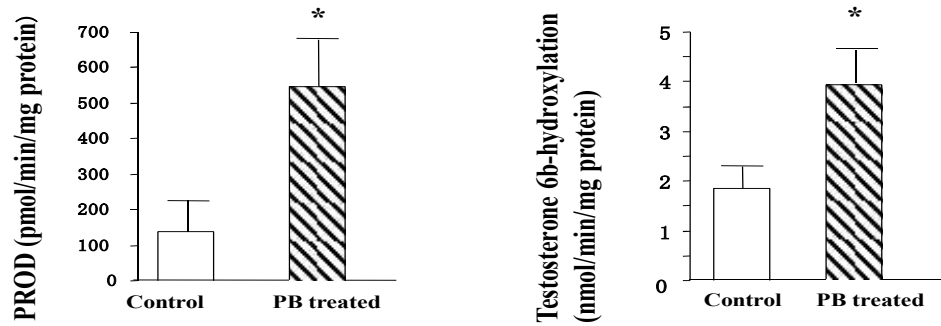
Figure 35:The pentoxyresorufin-O-dealkylase (PROD) activity (CYP2B dependent activity) and testosterone 6-beta-hydroxylase activity (CYP3A dependent activity) of rat liver increased after phenobarbital treatment
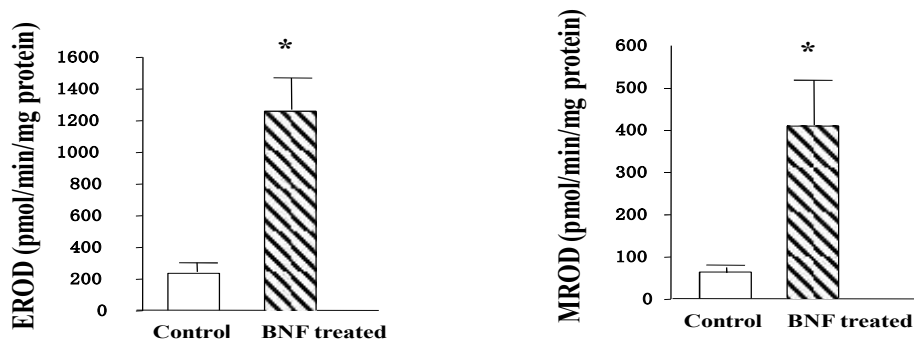
Figure 36: Ethoxyresorufin-O-deethylase (EROD) activity (CYP1A1 dependent activity) and methoxyresorufin-O-demethylase (MROD) activity (CYP1A2 dependent activity) of rat liver increased after BNF treatment

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3.4. Investigation with methamphetamine tablet Yaba for its effect on bone in rat

3.4.1. Body weight, food and water intake of rat during experiment

During the experiment period, daily body weight, food and water intake of rats of all groups were recorded. Increase of body weight, food and water intake of drug treated rats of both dose groups were same as that of control rats (Figure 37).
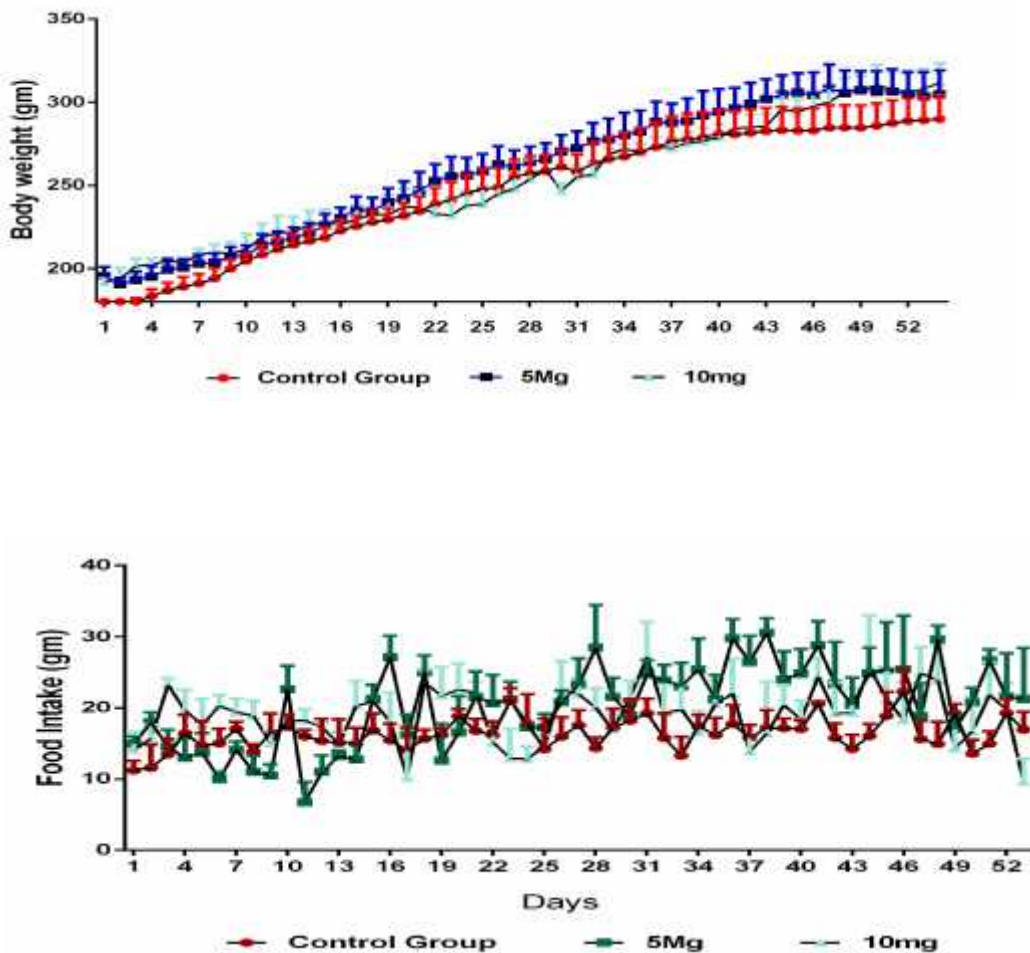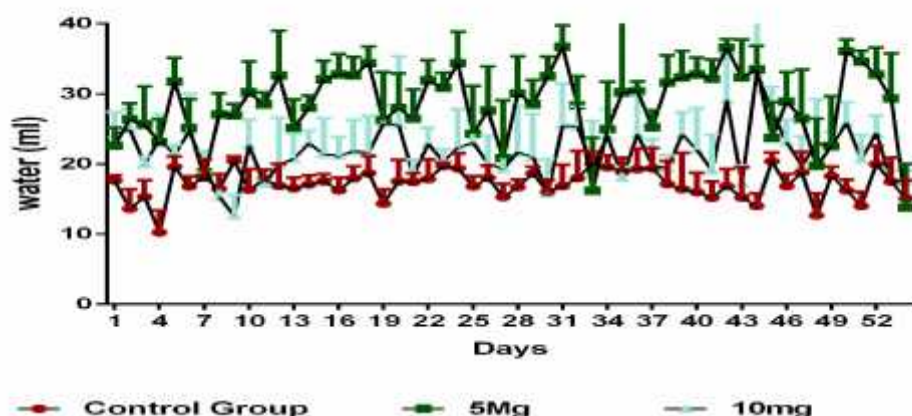
Figure 37: Body weight, food and water intake record of rat during Yaba treatment

 in bone toxicity study

3.4.2. Effect of Yaba tablet on plasma ALP in rat

Alkaline phosphatase (ALP) increased significantly to 165.32 ± 10.14 in low dose group from 79.81 ± 7.60 U/L($p< 0.01$, n=5-6). ALP increased by 2-fold by low dose of Yaba as compared to control value. High dose of Yaba showed decrease in ALP level in rat plasma but was not statistically significant.
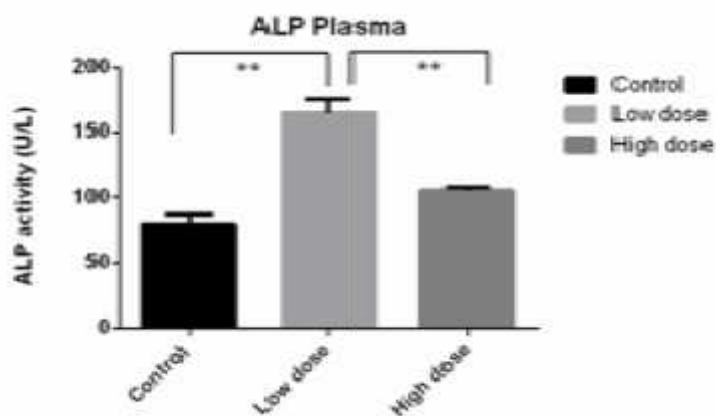
Figure 38: Change in alkaline phosphatases level after Yaba treatment in bone toxicity study

3.4.3. Effect of Yaba tablet on NO level in rat

In drug treated rat liver tissue, nitric oxide level increased significantly in high dose group from 17.43±1.21 to 20.55±1.01 nmol/ml (p<0.05, n=5-6). NO increased by 1.2-fold as compared to control value in liver
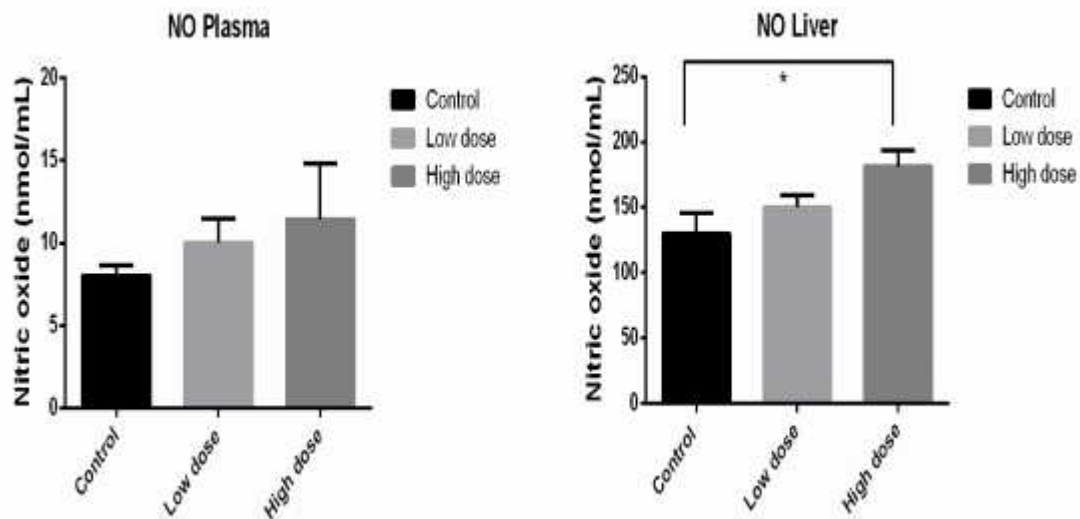
Figure 39: Change in nitric oxide level after Yaba treatment in bone toxicity study

3.4.4. Effect of Yaba on bone density in rat

Digital X-ray result showed that bones of both dose groups were more radiopaque (white part on bone) as compared to control representing more bone mass. However, this finding can be more clarified by measuring BMD by PQCT (peripheral quantitative computed tomography) or DEXA.
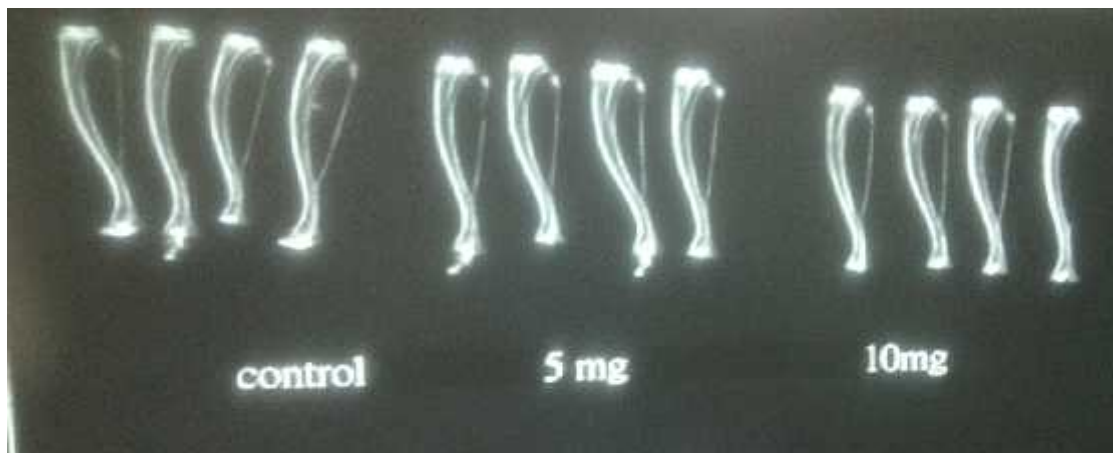
Figure 40: Digital X-ray image of rat tibia

Chapter Four

Discussion

4.1. Effect of methamphetamine tablet Yaba on liver inflammation and consequent fibrogenesis in rat

Based on length of use, acute or chronic- any form of liver disease might result from toxic illicit drugs. Drug mediated acute injury to the liver has been proposed as the action of cytochrome P450 isozymes, which breaks down drugs into electrophiles or free radicals. Resultant reactive metabolites then covalently bonded with protein or unsaturated fatty acids to induce lipid peroxidation (Kaplowitz et al., 1986).

Yaba, composed of methamphetamine 30% and caffeine70% (according to the literature), rapidly develops strong addiction in its abusers due to repeated use even within two or three days and results in undesirable and dangerous psychological and physiological effects. Liver disease in methamphetamine abusers is evident besides its toxic effects on other vital organs of the body. To evaluate chronic liver toxicity of methamphetamine, we have administered Yaba tablet orally to rats for long term and extensively investigated end products of several pathways signifying hepatcytic cell damage. Two doses of drug considered as low and high dose were applied to animal in order to observe the dose dependence of effects. Our investigation revealed that chronic administration of Yaba in rats induced oxidative stress, inflammation and fibrogenesis in liver.

In present study, significant elevation in liver enzymes (ALT, AST and alkaline phosphatase) took place in plasma as compared to control indicated liver abnormality after long term Yaba treatment. Several in vitro studies have been reported on significant increase in liver enzymes and ROS formation in rats which were subjected to meth- induced hepatotoxicity using isolated rat hepatocytes. The cytotoxicity produced by methamphetamine is proposed as mediated by oxidative stress (Mashayekhi et al., 2014; Eskandari et al., 2014).

During hepatic cell necrosis and membrane damage, the liver marker enzymes are released into circulation and hence their concentration increased in plasma and serum. High level of AST indicates liver damage in viral hepatitis as well as cardiac infarction where as ALT is more

specific to the liver and thus a better parameter for detecting liver injury. In hepatobiliary tract disease and bone abnormality, alkaline phosphatases level increased (Giannini et al., 2005).

Increased production of ROS and resultant oxidative stress plays a vital role in the development of liver damage. The reactive species stimulates inflammatory responses through the activation of pro-inflammatory mediators. The episodes of inflammation eventually stimulate pro-fibrogenetic mediators to initiate hepatic fibrogenesis (Galli et al., 2005; Ghiassi-Nejad et al., 2008).

Our investigation results revealed that Yaba treatment inflamed the production of thiobarbituric acid related substances (TBARS), the lipid peroxidation product, as compared to control group significantly which indicates profound oxidative damage in liver cell membrane. The antioxidant enzymes such as superoxide dismutase, glutathione peroxidase and catalase work collectively in human cells to protect from toxic effect of reactive oxygen species. Therefore, when body suffers from oxidative stress and pathology arises, the bodily defense system promotes the expression of these enzymes as protective measure (MatÉs et al., 1999).

By several studies it is proven that increase in ROS production ultimately lowers these enzymes levels (Koruk et al., 2004). In contrast to this information, antioxidant enzymes GSH, CAT, SOD were not depleted significantly in spite of evidence of increased lipid peroxidation after Yaba treatment. However, the mechanism is not still clear. Nitric oxide (NO), a mediator of systemic vasodilatation, has been reported to be increased in liver cirrhosis. Moreover, clinical studies showed that serum nitrite level in cirrhotic patients were significantly increased in comparison to that of control (Mohammed et al., 2003). In our study, NO level of rat liver, increased significantly by high dose of Yaba, directed to its contribution in liver cell damage.

During the experimental period, body temperature of rats increased after Yaba administration by 6-8°C and persisted for one hour approximately. Laura et al. described that methamphetamine induced persistent liver damage through hyperthermia which was characterized by increased plasma aspartate and alanine aminotransferase as well as ammonia (Halpin et al., 2013). Hyperthermia considered as destructive pro-oxidant factor. At high temperature glutathione peroxidase loses its activity. Another amphetamine derivative, MDMA has been suggested

primarily as to induce lipid peroxidation of cell membrane by producing reactive species and secondarily to reduce antioxidant enzyme activity through increasing body temperature (Carvalho et al., 2002). Hyperthermia caused by amphetamine derivative is thought to be mediated by activation of dopamine and serotonin receptor systems which are effectors for bodily thermal regulation and activator of pathways related to generation and conservation of heat (Shankaran et al., 1999; Bronstein et al., 1995). Additionally, hyponatremia caused by profuse sweating during uncontrolled physical activity of the abusers and interference of the drug on the thermoregulatory system of body proposed to be reason for drugs ill effect on renal system even after drinking plenty of water (Kalant, 2001).

Histopathological assessment in liver tissues of Yaba treated rats revealed typical hepatotoxic effects same as described previously in the literature (Nabeshima et al., 2006). Inflammatory cells have been accumulated in the necrotized region and along the bile ducts and blood vessels in liver of drug treated rats.

Inflammatory response involves a number of inflammatory cells. Identification test of those inflammatory cells were not performed in present study. In the development of liver fibrosis, contribution of infiltrating immune cells has recently been proven by studies besides the liver-resident Kupffer cells. Previous reports on mice suggested that in acute and chronic drug induced liver injury monocytes infiltrates at the site of inflammation. During inflammatory response, monocytic macro phages release their own cytokines which gives rise to chronic inflammation. Besides, hepatic stellate cells are activated, proliferated and differentiated into myofibroblasts which turn out collagen (Karlmark et al., 2009; Imamura et al., 2005). In our study, collagen fibre deposition varied in two different doses treated rat liver observed in Sirius Red staining. Progressive deposition of collagen fibre supported the inflammatory cell damage of rat liver treated with Yaba. Possibility of iron-induced oxidative stress was not proven from Prussian blue staining of Yaba treated rat liver tissue.

Based on the investigation results of present study, conclusion may be drawn in the way that evidences for inflammation and consequent fibrotic effect on rat liver were visible from long term Yaba treatment. The possible mechanism of damage may be due to increased lipid

peroxidation of hepatocytes by increased ROS and RNS which is followed by inflammatory response leading to deposition of collagen fibre by activated hepatic stellate cells. In spite of progressive fibrogenesis in drug treated rat liver observed in histologic study, the unchanged activity of antioxidant enzymes detected might suggest the specific stage and level of liver toxicity during the experimental eight weeks by Yaba tablet which require further investigation.

4.2. Effect of caffeine on liver inflammation and consequent fibrogenesis in rat

Regular coffee intake has been reported as attenuator of liver fibrosis progression in several studies. Sung Gon and Dae Won described caffeine as to inhibit hepatic stellate cells (HSCs) adhesion and activation and increased HSC apoptosis (Shim et al., 2013). Another study suggested that regular coffee intake lowers plasma liver transaminase level, inhibits the cAMP/PKA/CREB signal pathway through adenosine A2A receptors in HSC (Wang et al., 2015). However, caffeine had shown variable stimulating effect on acetaminophen induced hepatotoxicity mediated by microsomal CYP3A subfamily (Jaw et al., 1993).We suspected the synergistic effect of caffeine on methamphetamine induced hepatotoxicity when these two are used in combination in Yaba tablet.

In present study, we have performed investigations to figure out the effect of caffeine on liver. The biochemical test results indicated the contribution of caffeine on ROS induced liver cell damage through lipid peroxidation or protein oxidation. Other effects such as increased nitric oxide and decreased activity of antioxidant enzymes suggest the stressed condition with reactive oxygen or nitrogen species produced by caffeine. In our study, the high dose of caffeine (12mg / kg of BW) administered in rat once daily was equivalent to amount of caffeine taken by chronic Yaba abusers (adult of 60kg BW) daily through tablet (10-12 tablets app.).

High and low doses of caffeine induced elevation of serum ALT, AST and ALP levels as compared to control. The magnitude of aminotransferase alteration defines the severity of liver disease as "mild" (elevation less than 5 times of normal) "moderate" (elevation 5−10 times of normal) or "marked" (elevation more than 10 times of normal). Besides liver, alkaline phosphatases level indicates bone diseases even though this enzyme may also be released from other tissue damage included the placenta, kidneys, intestines or leukocytes. In drug induced

liver damage, alcoholic liver disease and non-alcoholic fatty liver disease, viral hepatitis (hepatitis B and C) and hemochromatosis, liver transaminases (ALT,AST) exhibit mild elevation (Green et al., 2002; Pratt et al., 2000; Gopal et al., 2000; Robert et al., 2011; Fishman, 1990). In present study, we found the magnitude of ALT and AST elevation in caffeine-treated rat as representing mild liver disease when compared with control values.

Drug toxicity can play significant role by triggering inflammatory response and activating innate immune cells by release of damage –associated molecular pattern. Resident kupffer cell, neutrophils, monocytes are activated in the liver after toxic induction of drug. ROS produced by the phagocytes mediates killing of target cells (e.g. the invaded organisms). In absence of bacteria, especially when injury is drug induced, hepatocytes and other liver cells becomes target of ROS; hence the damaged host cells initiate chronic inflammation. However, the nature of oxidative stress specifies the target and damages particular cellular component. The mechanism of ROS induced cell killing involves mitochondrial dysfunction leading to oncotic cell necrosis rather apoptosis.

Chronic release of cell contents amplifies the inflammatory injury. Jaeschke et al. described that inflammatory ROS cause cell death in sufficient concentration where as insufficient ROS triggers the resistance against future inflammatory oxidative stress by inducing transcription of antioxidant genes and thus promoting tissue repairing process (Jaeschke, 2011). Among ROS, superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2) mostly capable of damaging biochemical molecules including nucleic acids and aminoacids. However, the most damaging effect is the induction of lipid peroxidation. The cell membrane, composed of poly-unsaturated fatty acids, is a primary target for reactive oxygen attack leading to cell membrane damage (Ghiassi-Nejad et al., 2008). Our study revealed that caffeine administration resulted in a significant reduction of SOD in plasma and liver by both doses variably which was accompanied with a significant increase in lipid peroxidation product TBARS as compared to the control group. This finding suggested the elevated production of superoxide anion by caffeine treatment in rats.

Increased level of oxidized protein is an indicator of increased oxidative stress. Oxidized protein product equivalent to chloramines-T increased significantly in plasma and liver after caffeine

treatment indicated increased ROS production by both doses of the drug. Catalase and glutathione, which protect body from oxidative harm by reactive oxygen species, showed unchanged activity in caffeine treated groups when compared with control value. Nitric oxide (NO), another component of reactive species, takes part in lipid peroxidation. Role of NO in apoptic cell death of neuronal cells was described by Taotao et al. The same paper suggested that NO- induced apoptosis is partly mediated by ROS. In our study, caffeine induced significant increase in NO level in rat plasma and liver as compared to control indicating hepatic cellular apoptosis or necrosis.

In response to liver inflammation, the pro-fibrotic mediators are stimulated and initiate liver fibrogenesis (Cui et al., 2003; Galli et al., 2005). Sign of liver inflammation, due to significantly increased inflammatory mediators, detected by biochemical tests, was supported by the histopathological assessment of liver tissue. Typical hepatotoxic signs appeared in histopathological study of caffeine treated rat liver tissue as described in previous literature of Nabeshima et al., 2006 and Jin et al., 2013. Inflammatory cells were accumulated in the necrotized region and along the bile ducts and blood vessels in liver of drug treated rats.

However, we could not identify the inflammatory cell type in present study. Research suggested that immune cell infiltration coupled with liver-resident kupffer cells, contribute significantly to the development of liver fibrosis. Investigation on mice with drug induced liver injury revealed the involvement of monocytes in both acute and chronic inflammation (Karlmark et al., 2009). At the initial stage of fibrosis, macrophages derived from monocytes release several cytokines which initiates chronic inflammation through the activation of hepatic stellate cells. The activated hepatic stellate cells, proliferated and trans-differentiated into myofibroblasts start to produce collagen (Imamura et al., 2005). In our study, dose dependent collagen fibre deposition was seen in Sirius Red staining of rat liver. Progressive deposition of collagen fibre supported the sustained inflammatory cell damage of rat liver, treated with caffeine.

In present study, inflammation and consequent fibrogenetic effect on liver by caffeine was uncovered. The possible mechanism might be increased lipid peroxidation of hepatocytes and protein oxidation by increased ROS (especially $O_2.$) or RNS (especially nitric oxide); consequent

chronic inflammation stimulated by damaged cells and progressive collagen fibre deposition by myofibroblasts. Additionally histochemical finding proved the existence of inflammation and fibrogenesis in liver. However, in contrast with inflammatory response, antioxidant enzyme catalase and glutathione activity remain unchanged. Another enzyme superoxide dismutase activity significantly decreased suggesting abundance of superoxide ion as compared to other reactive species. Further study on other molecular mechanisms might be conducted to clarify the process of fibrogenesis mediated by caffeine.

4.3. Involvement of cytochrome P450 (CYP) isozymes on metabolism of 3, 4-methylenedioximethamphetamine (MDMA) in rat

Phenobarbital and β-naphthoflavone have been proven as good inducer of a number of CYP isozymes. Research papers suggested that in rats, PB activates the synthesis of CYP2A1, CYP2B1, CYP2B2, CYP2B12, CYP2C6, CYP2C7, CYP2D1, CYP3A1 and CYP 3A2 isozymes and BNF activates the synthesis of CYP1A1, CYP1A2 and CYP2A1 isozymes (Soucek et al., 1992; Kumagai et al., 1994). On the basis of this information, we studied the in vivo metabolism of MDMA by detecting three of its metabolites in urine of rats separately pretreated with PB or BNF; the detected compounds were MDMA, MDA, HMMA and HMPA. In relation to this study, we also determined total P450 contents of liver microsome to make a connection between metabolite synthesis and enzyme involvement. To confirm the success of inducers on CYP induction, we examined the enzymatic activities related to CYP isozymes such as CYP1A1, CYP1A2, CYP2B and CYP3A with the liver microsome of PB-pretreated or BNF-pretreated rats and compared the results with that of control rats. Results obtained from the urine analysis of pretreated and control rats showed that MDA, the N-demethylated product of MDMA, was decreased and the HMMA, the methylated product of MDMA metabolite HHMA, was increased in both PB-and BNF -pretreated rats as compared with the control values (Table 2).

In consistent with GC-MS assay, hepatic enzyme study revealed that the applied inducers increased the synthesis of CYP1A, CYP2B and CYP3A isozymes in rats (Table 3). It is suggested that the O-demethylenation product of MDMA is HHMA which is further O-methylated to HMMA. The urinary HMMA increase, therefore, indicated the action of increased

CYP isozymes induced by either PB or BNF on O-demethylenation pathway of MDMA and O-methylation of HHMA as well. In contrast to rat study, the enzyme catechol-O-methyltransferase (COMT) has been reported to regulate the O-methylation of HHMA to HMMA in human (Maurer et al., 2000). However, to our knowledge, rats have not been well studied with this enzymatic pathway yet. CYP1A2 was reported as the major catalyst in N-demethylation pathway of MDMA (de la Torre et al., 2004).The decreased urinary concentration of MDA, considered as the N-demethylation product of MDMA, after pretreatment with either PB or BNF (Table 2) suggested the inversely proportional relation of MDA production with six-fold increased CYP1A2 activity in liver microsome (Table 3). This can be concluded in the way that the PB- or BNF-activated CYP isozymes have inhibited the N-demethylation of MDMA to MDA in vivo in rats; however, the exact mechanism not yet clear.

From the urine analysis of PB treated rat, we observed that MDMA concentration decreased significantly as compared to control. This indicated the increased metabolism of MDMA after PB treatment. However, the total percent dose recoveries of three detected metabolites in urine samples from PB-pretreated and BNF pretreated rats was too low. This situation suggested that one or more other major pathways might be there for MDMA metabolism apart from N-demethylation and O-demethylenation followed by O-methylation in rats which requires further investigation.

4.4. Effect of methamphetamine tablet Yaba on bone in rat

Elevated ALP represents signature of the skeletal system disorders mediated by osteoblast hyperactivity and bone remodeling as well (Tietz, 1999). Alkaline Phosphatases, a group of enzymes abundant in the liver and bone. Liver resident ALP known as isoenzyme ALP-1 while those in bone named as isoenzyme ALP-2. Small amounts produced from intestinal cell lining

namely isoenzyme ALP-3. The placenta and the proximal convoluted tubules of kidney generated some ALP as well. Measurement of ALP in the blood corresponds to the total amount of alkaline phosphatases released from these tissues into the blood. Serum alkaline phosphatases can be increased due to rapid growth of bone since it is produced by bone-forming cells called osteoblasts. In our study, significantly elevated serum ALP observed by low dose of Yaba might

be indicative of increased osteoblast activity by this drug. However, test for serum billirubin content and gamma-glutamyl transferase (GGT) might clarify this possibility as GGT is absent in bone (Aragon et al., 2010; Chou et al., 2013; Hartwell et al., 2015; Oh et al., 2011; Ozer et al., 2008; Plebani et al., 2007; Woreta et al., 2014). Unchanged ALP level in high dose group suggests dose dependent action of the drug.

Bone remodeling or bone metabolism is regulated by the coordinated contribution of osteoclast and osteoblast cells. Loss of this coupling might give rise to several skeletal pathologies characterized by increased or decreased osteoclast and osteoblast activities (Rucci, 2008). Bone remodeling process is regulated by the action of nervous system. Inputs from the central and peripheral nervous system feed into the already complex regulatory machinery controlling bone remodeling. Florent et al. has proposed the participation of a number of "osteo-neuromediators" in bone metabolic process. The mediator and receptors are norepinephrine and the beta 2-adrenergic receptor, neuropeptide Y and the Y1 and Y2 receptors, endocannabinoids and the CB1 and CB2 receptors, as well as dopamine, serotonin and their receptors and transporters respectively. Calcitonin gene-related peptide (CGRP) also found to involve in the above mentioned process. Three types of nitric oxide synthases, connected variably with bone, comprise endothelial NOS (eNOS), expressed in bone normally; inducible NOS (iNOS), respond to inflammatory stimuli and neuronal NOS (nNOS), study found as potential negative regulator of bone remodeling (Elefteriou, 2008; van'T Hof et al., 2001). Research suggested eNOS as to regulate osteoblast activity. However, Nitric oxide synthases (NOSs) synthesize the metastable free radical nitric oxide (NO).

In our study, Yaba induced significant increase in NO level in liver tissue by high dose. This finding suggested the possibility of increasing NOSs by Yaba treatment and thus influencing bone remodeling. In the X-ray result, tibias of Yaba treated groups showed comparatively more radio opacity as compared to control as a sign of increased bone mass whereas effect of low dose group was prominent which was consistent with the increased plasma ALP (a product of osteoblast cell) concentration of this group. In present study, bone histology for osteoblast and osteoclast cells was not performed. Histological quantification of these two cells is warranted to give a clear picture about their role on bone metabolism after long term treatment with Yaba.

Chapter Five

Conclusion

We have conducted our research by choosing the crazy drug of abuse of present time 'Yaba' and 'Ecstasy', the two representative recreational drugs of amphetamine derivative. We have given emphasis on the health hazards that abusers of Bangladesh might experience from the use of these drugs sold in our local underground markets. Lack of availability of pure methamphetamine reference standard, present research could not include the assessment of methamphetamine content in supplied Yaba tablets and a separate study using pure methamphetamine in order to compare the results with that of Yaba treatment where caffeine is included as adjunct principle in these tablets. Treatment duration and doses were selected by keeping similarities with drug abuse pattern by people.

A significant part of our research was study with caffeine which occupies the major portion of Yaba tablets and other recreational drugs as well. To our knowledge, caffeine as liver toxicity inducer has not yet attracted the attention of researchers and hence extensive investigation on caffeine induced liver toxicity at particular dose still not performed. However, caffeine, as a well known anti oxidant, reported to inhibit ROS induced lipid peroxidation (Devasagayam et al., 1996) and also to attenuate liver cirrhosis (Liu et al., 2015) by several studies where in most of those coffee was administered instead of pure caffeine and confusion arises about whether coffee or caffeine worked to protect liver. In our study, a lucrative finding was there regarding caffeine's effect on liver after long term use, at dose equivalent to the amount present in Yaba tablet.

Liver was selected as target organ of drug toxicity because of its major participation in drug metabolism and frequency of jaundice in many Yaba addicted people diagnosed in clinical practice as well. We have examined the blood and tissue for oxidative stress markers induced liver toxicity. To our knowledge, large number of clinical trials on human has been conducted to assess liver toxicity by drugs but data regarding the role of oxidative stress in the development of liver disease is inadequate.

For example, research on paracetamol induced liver toxicity revealed its manifestation by mitochondrial dysfunction and DNA damage in patients, supporting the partial participation of oxidative stress in producing toxicity. However, the exact measurement of oxidative stress in

order to correlate with disease status in patients is still needed (O'Riordan et al., 2011). Furthermore, most of the investigations on drug induced liver toxicity made on the basis of retrospective study. Very few clinical trials carried on hepatotoxicity study of illicit and abused drugs included inadequate patient population. Several in vitro studies conducted using human cell line to simulate the pathogenesis developed in particular organ by drugs (Stine et al., 2015). On the whole, sufficient clinical data using proper size and experimental model, representing similar as human pathophysiology, can guide properly for the establishment of appropriate treatment protocol against drug toxicity.

On the basis of different data and statistics, it is estimated that the number of addicts in Bangladesh is more than six million (Khan, 2013). Investigation on addicts in a treatment centre revealed that daily expenditure of an addict to purchase drug is $1.9-$3.1. Expenses for drug abuse, treatment cost, reduction in working efficiency, crimes and accidents by the abusers have put serious deteriorative effect on the economy and social life (Shemul, 2009). This is high time to bring social stability by standing besides the derailed and helpless people sunk in the black world of drug. However, we could not establish any standard diagnostic test and treatment protocol for these unfortunate people yet. Bangladesh government is constantly trying to stop drug trafficking by law enforcement department and collaborating with some NGOs. Rehabilitation centers throughout the country engaged to bring them out of the cruel grab of addiction. Besides all of these approaches, we are still badly in need of establishing a complete support system including frequent health awareness program with research publications on health threats of abused drugs, standard diagnosis set up and proper treatment facilities to attenuate the associated toxic effects.

5.1. Research outcomes at a glance

After different investigations in present study we have proposed the following pathways for oxidative stress mediated liver toxicity induced by amphetamine derivatives (Yaba, Ecstacy) and caffeine in rat:

Repeated administration of drugs with gradually increased dose

* | Involvement of Cytochrome P450

Metabolism in liver

*

Production of reactive hydroxy-metabolites   Increased production of ROS by CYP

Increased production of   *   Decreased activities of anti-
RNS (NO)   *   oxidant enzymes (CAT, GSH, SOD)

Nitrosative stress + Oxidative stress

Oxidation of hepatic   Lipid peroxidation of   DNA damage   Mitochondrial
cellular protein   hepatocytic cell   dysfunction
                             membrane

*   *

Alteration of protein   Hepatic cell damage ———— * ——→ Liver enzymes
structure & function   (ALT,AST,ALP)
                                                                 released in blood
                              *

                    Invasion of inflammatory
                       cells into liver

                              *

Damaged liver regenerated or replaced by scar tissue composed of collagen fibre
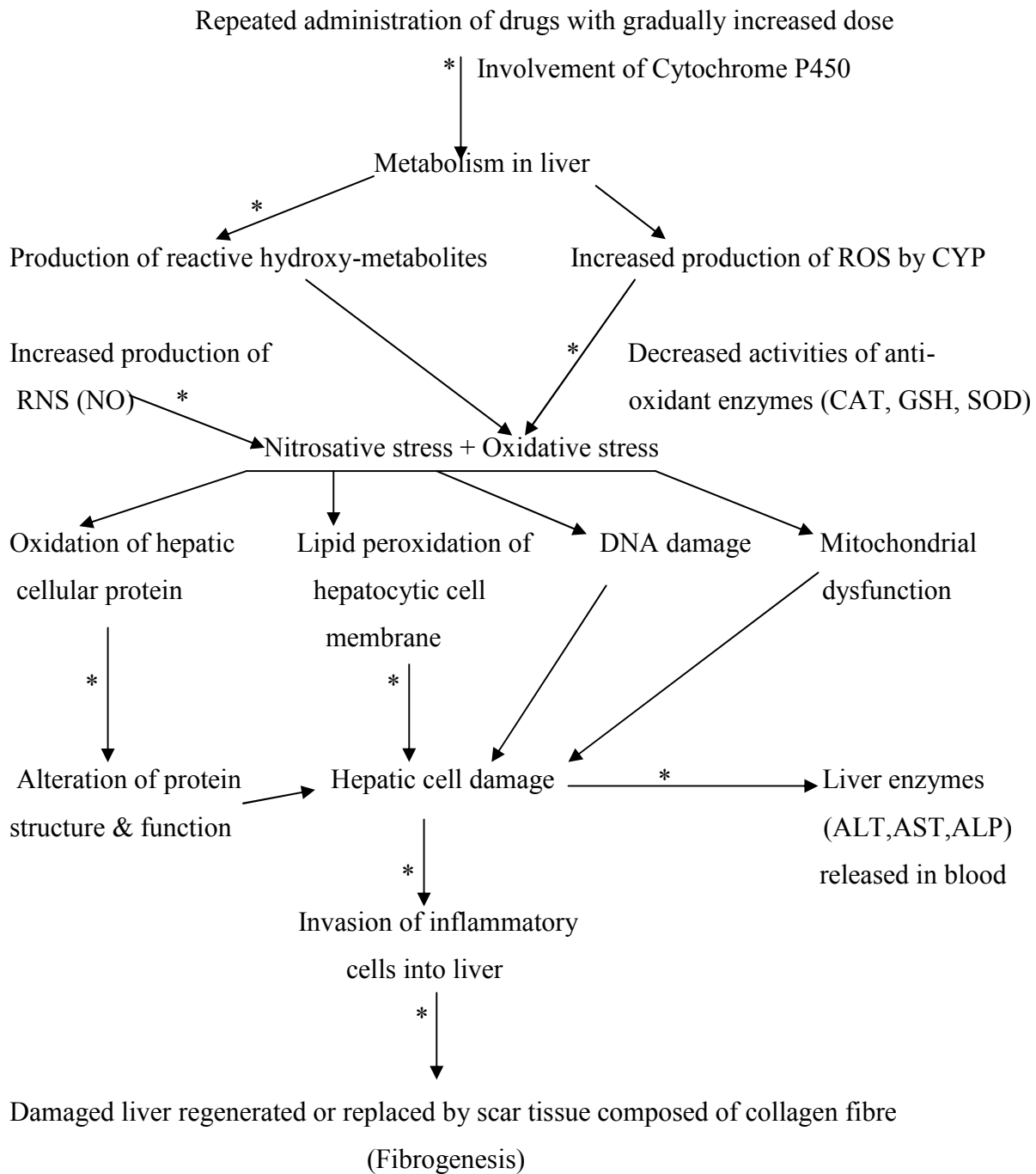   (Fibrogenesis)

Figure 41: Proposed pathways of amphetamine derivatives and caffeine induced liver
toxicity in rat.

* The pathways we investigated

To crosscheck the relevancy of biochemical findings representing disease status, we performed histology study of liver and observed directly the inflammatory and fibrotic status.

The biochemical test results of Yaba and caffeine treated group showed positive sign in favour of liver inflammation and fibrosis. We expected that there should be difference in test results values between Yaba and caffeine treated groups. However, caffeine resembled higher hepatotoxic potentiality indeed as compared to Yaba especially by some parameters we investigated, such as increased oxidation end products and decreased antioxidant enzyme level. If we consider the daily increasing recreational intake of caffeine through Yaba, the probability of liver health threat can easily be assumed especially with prolonged use.

5.2. Recommendations

These research findings can essentially be passed to common people through various social media or health related program or may be included in text books to disseminate the knowledge about the drawbacks of recreational drugs use. Government should play the vital role to assign this responsibility on respective department. We are hopeful about the awareness of people if they are provided the scientific information in a regular and effective way.

People are mostly concerned about the devastation and ultimate fate of liver cirrhosis. So, information on the recreational drug induced liver fibrosis leading to cirrhosis surely will alert them about this life threatening habit. Furthermore, disclosure of the information such as the stimulating function of Yaba is maximally done by the cheaper caffeine which is easily available in coffee, tea or chocolates etc. might reduce their excessive attraction for this illicit drug. Any institution, trustworthy to public, may take this responsibility with the circulation that Bangladeshi addicts are being cheated by purchasing cheap caffeine in the name of methamphetamine tablet, Yaba. Besides this, a scientific research unit should run continually with investigation on identifying molecular mechanisms of drug toxicity so as to develop targeted treatment to protect people from unexpected serious side effects.

Furthermore, we can discourage people to excessive intake (above the safety range) of caffeine through hot and cold drinks and alert them by circulating our research findings on caffeine study. Through our study, we have just initiated the investigation against the recreational drug, particularly Yaba, induced toxicity on liver. For future investigation we have the following recommendations:

- Extensive study on toxicity of other organs by this drug might bring under research protocol.

- Endogenous or exogenous molecules that can directly or indirectly restore membrane integrity may be searched to protect membrane permeability of amphetamine derivatives which is the identified mechanism of their toxicity.

- Cell damage by reactive species may be prevented by studying with traditional antioxidant agents, synthetic or natural, and importantly with particular dose.

- Identification of more enzymatic pathways of metabolism of these recreational drugs will open the scope to prevent toxic metabolites generation.

- The mechanistic pathways of amphetamine derivatives and caffeine on bone turn over should be monitored. Drug treated rat tibias can be experimented histologically for growth plate observation. Considering the participation of bone forming cell osteoblast and bone resorption cell osteoclast in bone metabolism, their number may be counted to understand the reason of bone quality change. However, in our study, the increased alkaline phosphatase level after Yaba treatment indicated increased osteoblast activity which has been reflected in X ray image.

- Bone mineral density and bone volume can be measured by using DEXA or PQCT.

- The bone degradation marker, deoxypyridinoline, in urine can be measured to get information about bone resorption. However, identification of overall effects of drugs on bone metabolism with molecular mechanism is required to prevent as well as treat drug abusers from bone related diseases including osteoporosis or bone cancer.

Besides all medical and scientific research supports, it is also equally necessary to activate familial, social and religious values among people to prevent drug addiction and associated crimes through frequent awareness program. Law enforcement agencies should be very strict and honest to stop these recreational drugs trafficking throughout the country.

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Permission letter for animal study with Yaba tablets from the Department of Narcotics Control, Government of the People's Republic of Bangladesh