EFFECTS OF BENZENE ON LIVER, KIDNEY AND LUNG CYP1A, CYP2B4, CYP2E1 AND CYP3A6 mRNA, PROTEIN LEVEL, AND DRUG METABOLIZING ENZYME ACTIVITIES AND TOXICITY IN DIABETIC RABBITS

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ABSTRACT

EFFECTS OF BENZENE ON LIVER, KIDNEY AND LUNG CYP1A, CYP2B4, CYP2E1 AND CYP3A6 mRNA, PROTEIN LEVEL, AND DRUG METABOLIZING ENZYME ACTIVITIES AND TOXICITY IN DIABETIC RABBITS

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The effects of diabetes on cytochrome P450 dependent drug metabolizing enzymes have not to be clarified yet. The most widely used animals in these studies have been rats, and information regarding the effects of diabetes on cytochrome P450 dependent procarcinogen/carcinogen metabolism in rabbits is limited. In the present study, we investigated, for the first time, the influence of benzene on liver, kidney and lung microsomal cytochrome P450 dependent drug metabolizing enzyme activities, protein and mRNA levels in diabetic and non-diabetic rabbits.

Male New Zealand rabbits were made diabetic by a single dose of alloxan treatment in this study. AST, ALT and LDH enzyme activities in the blood serum and lipid peroxidation in liver microsomes were found to increase in diabetic, benzene treated and benzene treated diabetic rabbits. Besides these, CYP2E1 dependent NDMA N-demethylase and *p*-nitrophenol hydroxylase activities and CYP2E1 protein level were found to increase in liver and kidney of diabetic and benzene-treated rabbits. The combined effects of benzene and diabetes on these activities and protein level were found to be additive. Although diabetes caused

induction of pulmonary CYP2E1 protein level and associated enzyme activities, benzene treatment of rabbits resulted in no change in enzyme activities and protein level in lung. The level of mRNA was investigated by Real-Time PCR. Accordingly, hepatic CYP2E1 mRNA level was increased 6.71-, 10.53- and 12.93-fold in diabetic, benzene treated and benzene treated diabetic rabbits with respect to the control animals. Similarly, renal CYP2E1 mRNA level was found in increase in these rabbits. In addition to CYP2E1, CYP3A6 associated enzyme activity, erythromycin N-demethylase, CYP3A6 protein and mRNA level were found to increase in diabetic rabbit liver and lung. Unlike diabetes, benzene treatment caused suppression of CYP3A6 protein and inhibition of associated enzyme activity in liver. There was no significant change in the erythromycin N-demethylase activity and CYP3A6 level of liver and lung as a result of benzene treatment of diabetic rabbits. Moreover, diabetes induced CYP1A2 protein and mRNA level and CYP1A associated enzyme activities in the rabbit liver. On the other hand, benzene caused statistically insignificant decreases in CYP1A dependent enzyme activities and CYP1A2 protein level in liver. CYP1A associated enzyme activities, CYP1A2 protein and mRNA levels were not changed in the liver of benzene treated diabetics.

The results of the present work indicate that both diabetes and benzene stimulate metabolic activation toxic chemicals metabolized by CYP2E1 such as NDMA and benzene by inducing CYP2E1 which results in the formation of increased amounts of reactive metabolites. Application of benzene to diabetic rabbits further elevates expression and activities of the CYP2E1. As a result of additive induction of the CYP2E1 in benzene treated diabetics, further increase the risk of hepatotoxicity produced by toxins may be observed when compared to the separate treatments. This may in turn further potentiate the risk of organ toxicity and mutagenesis in liver and kidney of these subjects. As in the case of CYP2E1, the risk of carcinogenesis due to induction of CYP1A may be increased in diabetic subjects. Moreover, in diabetic and benzene exposed subjects, alteration of drug clearance and clinical drug toxicity may be observed due to induction or suppression of CYP3A.

Key Words: Diabetes, Benzene, CYP1A, CYP2B4, CYP2E1, CYP3A6, Drug Metabolizing Enzyme Activities, Real-time PCR, Liver and Kidney Toxicity.

ÖZ

DİYABETLİ TAVŞANLARDA BENZEN'İN KARACİĞER, BÖBREK VE AKCİĞER CYP1A, CYP2B4, CYP2E1 VE CYP3A6 mRNA, PROTEİN SEVİYESİ VE İLAÇ METABOLİZE EDEN ENZİM AKTİVİTELERİ VE TOKSİSİTE ÜZERİNE ETKİLERİ

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Diyabetin sitokrom P450'ye bağlı ilaçları metabolize eden enzimler üzerine olan etkileri henüz belirlenmemiştir. Bu çalışmalarda en sık kullanılan hayvan sıçan olup, tavşanlarda diyabetin sitokrom P450'ye bağlı önkarsinojen/karsinojen metabolismasına olan etkileri göz önünde bulundurulduğunda bilgi sınırlıdır. Bu çalışmada, ilk defa, benzenin karaciğer, böbrek ve akciğer mikrozomal sitokrom P450'ye bağlı ilaç metabolize eden enzimlerin aktiviteleleri, protein seviyeleri ve mRNA seviyeleri üzerine olan etkileri diyabetik ve diyabetik olmayan tavşanlarda incelenmiştir.

Erkek Yeni Zellanda türü tavşanlar bu çalışmada tek doz alloksan verilmesiyle diyabet yapıldı. Kan serumundaki AST, ALT ve LDH enzim aktiviteleri ve karaciğer mikrozomlarındaki lipid peroksidasyonu diyabetik, benzen uygulanmış ve benzen uygulanmış diyabetik tavşanlarda arttığı bulunmuştur. Bunların yanında, CYP2E1'e bağlı NDMA N-demetilaz ve *p*-nitrofenol hidroksilaz aktiviteleri ve CYP2E1 protein seviyesinin diyabetik ve benzen uygulanmış tavşanların karaciğer ve böbreklerinde arttığı bulunmuştur. Bu aktiviteler ile protein seviyesi benzen ve diyabetin birleştirilmiş etkisinde eklenmiş olarak bulundu. Diyabet akciğer CYP2E1 protein seviyesi ve enzim aktivitelerinin indüksiyonuna

sebeb olmasına rağmen, benzen uygulaması akciğerlerde enzim aktiviteleri ve protein seviyesinde hiçbir değişikliğe yol açmamıştır. mRNA seviyesi Gerçek zamanlı PZR ile belirlenmiştir. Buna göre, hepatik CYP2E1 mRNA seviyesi diyabetik, benzen uygulanmış ve benzen uygulanmış diyabetik tavşanlarda kontrollere göre sırasıyla 6.71-, 10.53- ve 12.93-kat artmıştır. Benzer şekilde, renal CYP2E1 mRNA seviyesi tüm bu tavşanlarda artığı bulunmuştur. CYP2E1 yanı sıra, CYP3A6'ya bağlı eritromisin N-demetilaz aktivitesi, CYP3A6 protein ve mRNA seviyeleri diyabetik tavşan karaciğer ve akciğerlerinde artmıştır. Diyabetin tersine, benzen uygulaması karaciğerde CYP3A6 protein seviyesinin baskılanmasına ve bu proteine bağlı enzim aktivitesinin inhibisyonuna sebep olmuştur. Benzen uygulanmış diyabetik tavşanların karaciğer ve akciğer mikrozomlarındaki eritromisin N-demetilaz aktivitesi ve CYP3A6 seviyesinde anlamlı bir değişim yoktur. Bunların yanında, diyabet CYP1A2 protein ve mRNA seviyesi ve CYP1A'ya bağlı enzim aktivitelerini tavşan karaciğerinde indüklemiştir. Diğer yandan, benzen karaciğerde CYP1A'ya bağımlı enzim aktiviteleri ve CYP1A2 protein seviyesinin istatiksel olarak anlamlı olmayan düşüşene sebep olmuştur. CYP1A'ya bağlı enzim aktiviteleri, CYP1A2 protein ve mRNA seviyeleri benzen uygulanmış diyabetik tavşanların karaciğerlerinde değişmemiştir.

Bu çalışmanın sonuçları hem diyabet hem de benzen maruziyetinin, CYP2E1'i indükleyerek, CYP2E1 tarafından metabolize edilen NDMA ve benzen gibi toksik kimyasalların metabolik aktivasyonlarını arttırdığını göstermiştir. Bu artış, reaktif metabolitlerin yüksek miktarda oluşmasıyla sonuçlanır. Benzenin diyabetik tavşanlara uygulanması CYP2E1'in ekspresyonunu ve aktivitelerini daha da arttırmıştır. CYP2E1'in benzen uygulanmış diyabetiklerde eklemeli olarak artışı sonucunda, toksinler tarafından üretilen hepatoksisite riski ayrı ayrı uygulamalarla karşılaştırıldığında daha da artması gözlemlenebilir. Bunun sonucunda organ toksisitesi ve mutajenez riski bu kişilerin karaciğer ve böbreklerinde daha da artabilir. CYP2E1'de olduğu gibi, karsinojenez riski, CYP1A'ın diyabetlilerde indüklenmesinden dolayı artabilir. Aynı zamanda, diyabetli ve benzene maruz kalmış kişilerde, CYP3A'nın indüksiyonu ya da baskılanması sonucunda ilaç temizlenmesinde değişiklikler ve klinik ilaç toksisitesi görülebilir.

Anahtar kelimeler: Diyabet, Benzen, CYP1A, CYP2B4, CYP2E1, CYP3A6, İlaç Metabolize Eden Enzimlerin Aktiviteleri, Gerçek-Zamanlı PZR, Karaciğer ve Böbrek Toksisitesi.

Dedicated to my parents

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AHR	Aryl hydrocarbon receptor
APS	Ammonium per sulphate
ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
BHT	Butylated hydroxytoluene
BSA	Bovine serum albumine
BIS	N, N'-Methylene bisacryiamide
CAR	Constitutively active receptor
CYP	Cytochrome P450
ε-ACA	ε-Amino caproic acid
EDTA	Ethylenediamine tetra acetic acid
DM	Diabetes mellitus
EDHF	Endothelium-derived hyperpolarizing factor
FAD	Flavine adenine dinucleotide
GLUT2	Glucose transporter
GR	Glucocorticoid receptor
GSH	Glutathione reduced form
HEPES	N-2-hydroxyethyl piperazine-N'-2 ethane sulfonic acid
HNF1α	Hepatic nuclear factor alpha
IDDM	Insulin dependentent diabetus mellitus.
LDH	Lactate dehydrogenase
MAO	Monoamine Oxidase
MDA	Malondialdehyde
MFO	Mixed function oxidases
MPO	Myeloperoxidase
NADH	Nicotineamideadenine dinucleotide, reduced form

$NADP^+$	Nicotineamideadenine dinucleotide phosphate
NADPH	Nicotineamideadenine dinucleotide phosphate, reduced form
NDMA	N-nitrosodimethylamine
NIDDM	Non-insulin dependent diabetus mellitus
NNK	4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone
PAGE	Polyacrylamide gel electrophoresis
PAH	Polycyclic aromatic hydrocarbons
PMSF	Phenylmethylsulfonyl floride
PPAR	Proliferator activated receptor
PPBREM	Penobarbital-responsive enhancer module
PXR	Retinoid X receptor
RT-PCR	Reverse transcriptase polymerase chain reaction
SDS	Sodium dodecyl sulfate
STZ	Streptozotocin
TBA	Thiobarbituric acid
TBARS	Thiobarbituric acid reactive substances
TEMED	N-N-N'-N'-tetramethylenediamine
TRIS	Tris (hydroxymethyl) aminomethane
XRE	Xenobiotic response element

CHAPTER 1

INTRODUCTION

The human body is continuously exposed by an immense diversity of natural and man-made environmental insults such as environmental pollutants, food additives, industrial chemicals, drugs, herbicides and pesticides, which can be totally called as xenobiotics. The body recognises the xenobiotics as foreign, potentially detrimental to its survival, and its immediate response is to protect itself. However, as most of these chemicals are largely lipophilic, an essential requirement for them to traverse the body's lipoid membranes, they are very difficult to eliminate through excretion in the bile and urine. Consequently, in order to facilitate their excretion, the body has to metabolically convert them to readily excretable hydrophilic entities. A number of enzyme systems capable of metabolizing xenobiotics have been identified and extensively investigated. Undoubtedly, the most important enzyme system is the cytochrome P450-dependent mixed-function oxidases, a ubiquitous system of heme-thiolate enzymes encountered in almost every human organ, but with the highest concentration in the liver, which consequently functions as the centre of xenobiotic metabolism, being capable of catalysing both oxidation and reduction pathways. Also, this enzyme system is a crucial catalyst of the metabolism, both biosynthesis and degradation, of endogenous substrates including steroid and other hormones, eicosanoids and certain vitamins.

It is well established that relative concentrations and activities of cytochrome P450-dependent mixed-function oxidases are modulated by not only diverse array of chemical compounds but also disease state such as diabetes. Diabetes is a universal health problem estimated to affect more than 246 million people worldwide. By 2025, the figure is expected to rise to 380 million. It is the fourth leading cause of global death by disease (American Diabetes Association, 2004 and International Diabetes Federation, 2007). It is well known that diabetus mellitus affects the carbohydrate, fat, lipid and electrolyte metabolisms. Besides these effects, it affects the other metabolic events in the body. According to recent studies, cytochrome P450 dependent monooxygenases, also known as drug metabolizing enzymes, are modulated as a result of diabetes (Chen *et al*., 1996; Sakuma *et al*., 2001; Sindhu *et al*., 2006; Arınç *et al*., 2007). The outcome of the alterations in cytochrome P450 compositions and levels was that efficacy or the toxicity of drugs and carcinogens may either decrease or increase in diabetes subjects. This results in further increase the risk of adverse events such as higher risk of organ toxicity and carcinogenicity observed in diabetic patients.

1.1 Diabetus Mellitus

Diabetes mellitus (DM) is a generalized metabolic disorder characterized by certain abnormalities in carbohydrate, fat, electrolyte and protein metabolisms which ultimately lead to several acute and chronic complications. The basis of the abnormalities in carbohydrate, fat and protein metabolisms in diabetes is deficient action of insulin on target tissues due to inadequate insulin secretion or diminished tissue responses to insulin at one or more points in the complex pathways of insulin action. Besides hyperglycemia and glycosuria, there are other signs and symptoms such as excessive thirst, frequent urination, unexplained weight loss, blurred vision that may indicate diabetus mellitus.

High concentration of glucose or other biochemical abnormalities such as ketoacidosis due to defects in insulin, insulin action or both is associated with

several acute and chronic complications. Hyperglycemia, ketoacidosis and non-ketotic hyperosmolar syndrome are acute complications which are involved in the chronic or long-term complications. The major chronic complications of diabetes include retinopathy, nephropathy, neuropathy, angiopathy, gastroparesis and macrovascular complications. Patients with diabetes have an increased incidence of atherosclerotic cardiovascular, peripheral arterial and cerebrovascular diseases. Hypertension and abnormalities of lipoprotein metabolism are often found in people with diabetes. These complications have devastating consequences including visual impairment, blindness, renal failure, myocardial infarction, stroke, lower extremity amputation, and shortened life span. (American Diabetes Association, 2004; Wang *et al*., 2007). Chronic complications of diabetes develop over time. Therefore, early detection and identification of the risks of the development of diabetic complications may aid in the prevention and/or management of these complications and are important strategies to undertake to minimize the human suffering and costs associated with this disease.

Generally, there are two main forms of diabetes mellitus; Type 1 and Type 2 diabetes. Type 1 or Insulin Dependent Diabetes Mellitus (IDDM) was once called juvenile-onset diabetes because it usually appears in childhood or teens. But it is not limited to these patients. It results from a cellular-mediated autoimmune destruction of β-cells of pancreas. Patients with insulin dependent diabetes mellitus become dependent on administered insulin for survival because of the complete lack of endogenous insulin. Oral anti-diabetic agents such as sulphonyl urea group of drugs are not effective in treatment of IDDM (American Diabetes Association, 2004; Eikeles, 1983; Devlin, 1997).

The second one, Type 2 or Non-Insulin Dependent Diabetes Mellitus (NIDDM), is the most common type of diabetes (85-90% of total diagnosed cases of diabetes) and is also called maturity-onset diabetes mellitus, because it affects middle aged obese people (more than 80% of patients with NIDDM are overweight). Unlike the IDDM, non-insulin dependent diabetes mellitus is caused not only by insulin resistance but also by impaired β-cells function resulting in

relative insulin deficiency, i.e., although insulin level is close to or above the normal level, the target cells can not respond to insulin. The specific etiologies are not known in NIDDM, but hereditary factors, aging and obesity are important risk factors. Ketoacidosis is not common at the time of diagnosis of NIDDM because the pancreas can still secrete the minimal amount of insulin required for the suppression of the lipolysis. Most patients do not require insulin injection and oral anti-diabetic agents are used for treatment. But, weight control by exercise and good planned diets are enough to control the NIDDM (American Diabetes Association, 2004; Eikeles, 1983; Devlin, 1997).

1.1.1 Experimental Models of Diabetus Mellitus

In order to understand the etiology of diabetes mellitus and its complications, this disease is induced in different animals by different methods. Although experimentally induced diabetes mellitus is not exactly equivalent to clinical diabetes, almost all diabetic complications can be observed in experimental models of diabetes and it also permits to test the effectiveness of potential treatment such as drugs, toxins and diet. Development of diabetic animal models fall into two categories. The first one is temporary diabetes which mimics the type 2 diabetes. Hormone application such as adrenalin, adrenocorticol steroids, pituitary growth hormone and glucagon and extensive glucose administration are used for development of type 2 diabetes according to type of the species (Lazarus and Volk, 1962). Also, some genetic models such as leptin and leptin receptor deficient mice (C57BL/6J ob/ob and C57BL/KsJ db/db), yellow obese mice and zucker diabetic rats were used for studying type 2 diabetes (Wang *et al*., 2007).

The second category is the permanent diabetes which mimics type 1 diabetes. In order to induce permanent experimental diabetes, destruction of β-cells of pancreas must occur. This can be achieved by surgical removal of most or the entire pancreas, administration of chemicals which are selectively toxic to the β-cells (Lazarus and Volk, 1962). There are some genetic models (animals) of permanent diabetes such as non-obese diabetic mice and BB rats (Wang *et al*.,

2007). Generally, the induction of diabetes using chemicals is very convenient and simple to use when compared to others. Many chemicals and drugs are used for induction of experimental diabetes, widely. Among these chemicals alloxan and streptozotocin (STZ) are the most specific and convenient chemicals to induce diabetus mellitus. The cytotoxic action of both chemicals to β-cells of pancreas is mediated by reactive oxygen species; however, their generation was different in the case of alloxan and streptozotocin.

Alloxan, an uracil derivative (Figure 1.1), causes permanent diabetus mellitus in virtually all animals with the exception of the guinea pig and birds (Rerup, 1970). The action of alloxan is preceded by its rapid uptake by the β-cells. Rapid uptake of insulin-secreting cells has been proposed to be one of the important features of determining alloxan diabetogenicity. Another aspect concerns the formation of reactive oxygen species. Alloxan and the product of its reduction dialuric acid establish redox cycle with the formation of superoxide radicals. These radicals undergo dismutation to hydrogen peroxide. Thereafter, highly reactive hydroxyl radicals are formed by the Fenton reaction. One of the targets of the reactive oxygen species is DNA of the pancreatic islets. Its fragmentation takes place in β-cells exposed to alloxan. Moreover, it is proposed that increase in intracellular calcium is another reason of rapid destruction of the β-cells (Szkudelski, 2001).

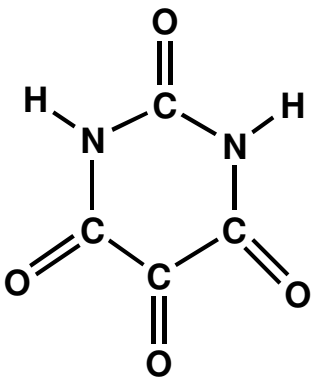


Figure 1.1 Chemical structure of Alloxan

The animals treated with alloxan exhibit triphasic blood glucose level as shown in Figure 1.2. There is an initial rise in the glycemic level during the first few hours after injection of diabetogen. This is followed by a profound fall in blood sugar between 12 to 24 hours due to β-cells necrosis with an increase in serum insulin level. Finally, secondary permanent hyperglycemic state is observed. The dose of alloxan depends on the type of organism and range of diabetes (Lazarus and Volk, 1962; Schenkman 1991).

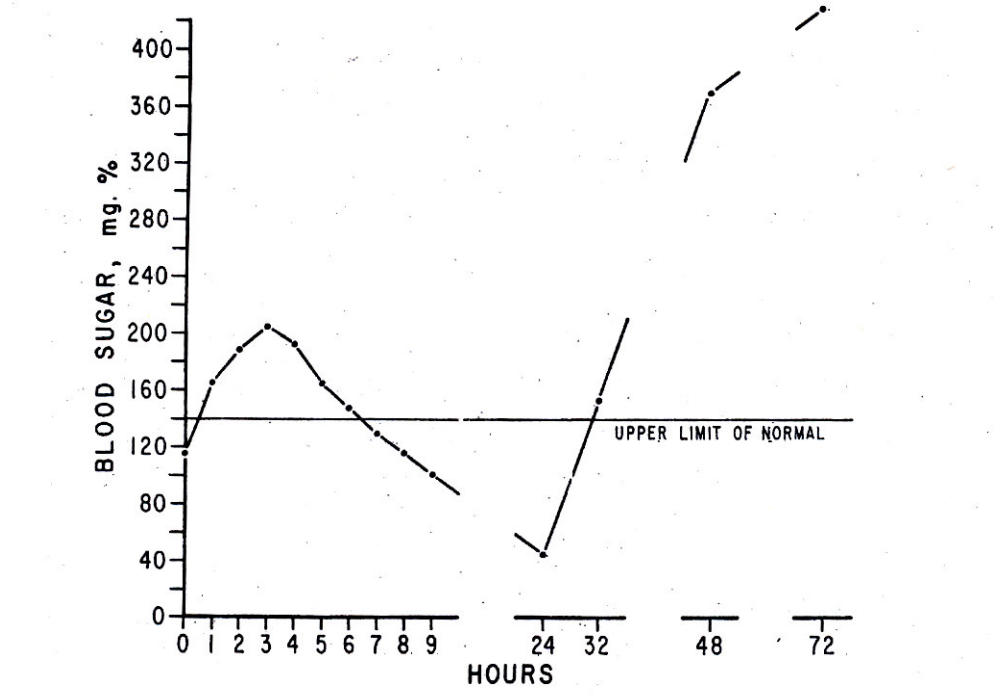


Figure 1.2 Effect of Alloxan on blood glucose level (taken from Lazarous and Volk 1962).

Streptozotocin, an antibiotic extracted from *Streptomyces achromogenes*, is another specific β-cells toxin used as an agent for generating experimental diabetus mellitus (Figure 1.3). As in the case of alloxan, animals show triphasic blood glucose levels after treatment of STZ (Figure 1.2). Streptozotocin enters the β-cells via a glucose transporter, GLUT2, and causes alklylation of DNA due to nitrosourea

moiety of STZ. The induction of DNA damage causes activation of nuclear poly (ADP-ribose) polymerase. Poly ADP-ribosylation leads to depletion of cellular NAD^+ and ATP. Enhanced ATP dephosphorylation after streptozotocin treatment supplies a substrate for xanthine oxidase resulting in the formation of superoxide radicals. As a result of superoxide anion generation, hydrogen peroxide and hydroxyl radicals are generated. These are involved in fragmentation of DNA. Furthermore, streptozotocin causes production of toxic amount of nitric oxide that inhibits aconitase activity and participates in DNA damage. As a result of streptozotocin action, β-cells undergo the destruction by necrosis. (Szkudelski, 2001).

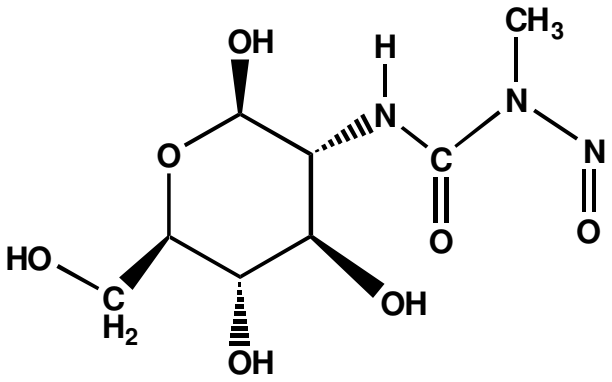


Figure 1.3 Chemical structure of Streptozotocin

1.1.2 Relation of Diabetes and Drug Metabolizing Enzymes

It is well known that diabetes mellitus affects the carbohydrate, fat and lipid metabolisms. Besides these effects, diabetes mellitus modulates the other metabolic events in the body. According to the recent studies, rat, mouse, hamster, rabbit and human cytochrome P450 dependent monooxygenases, also known as drug metabolizing enzymes, are modulated as a result of diabetes (Favreau and Schenkman, 1988; Chen *et al.,* 1996; Lucas *et al*., 1998; Sakuma *et al*., 2001**;** Arınç

et al., 2005, 2007). Induction of the diabetes in experimental animals caused increases, decreases, or no changes in hepatic monooxygeanase activity depending on the species and sex of the animals and duration of diabetes. These alterations have been explained, at least in part, by the altered pathophysiological conditions in diabetes such as decreased levels of pituitary growth hormone, thyroid hormone and testosterone, increased levels of ketone bodies and free fatty acid (Barnett *et al.,* 1988; Favreau and Schenkman, 1988; Kimura *et al.,* 1989; Yamazoe *et al.,* 1989; Barnett *et al.,* 1992). Due to alteration of several forms of cytochrome P450 dependent drug metabolizing enzymes, qualitative and quantitative changes may be observed in the drug metabolizing capacities in diabetes.

1.2 Cytochrome P450

The cytochrome P450 isoenzymes are a superfamily of haemoproteins that are the terminal oxidases of the mixed function oxidase system. These enzymes play critical roles in the bioactivation and detoxification of a wide variety of xenobiotic substances. Besides, they also have roles in the metabolism and synthesis of endogenous compounds. This group of protein has a unique absorbance spectrum that is obtained by adding reducing agent such as sodium dithionate to a microsomal preparation, followed by carbon monoxide (CO) gas bubbling. CO is bound to the reduced heme protein and produces an absorbance spectrum with a peak at 450 nm; thus name P450 for a pigment with an absorbance at 450 nm (Omura and Sato 1964). Cytochrome P450s are present in just about every phylum: in prokaryotes, unicellular eukaryotes, plants, invertebrates, insects, fish and mammals. While the prokaryotic enzyme is a soluble hemeprotein, that of higher organisms is membrane bound. In mammals, it is mainly located in the mitochondrial inner membrane as well as in the smooth endoplasmic reticulum membranes (Schenkman, 1991). It is well known that the cytochrome enzymes in humans and most other animals are involved in the activation or inactivation of xenobiotics such as drugs including antibiotics, carcinogens, organic solvents, dyes, pesticides, alcohols and environmental chemicals. These enzymes are important in the oxidative, peroxidative and reductive metabolism of endogenous physiological

compounds such as steroids, bile acids, fatty acids, prostaglandin, biogenic amines, fat soluble vitamins and retinoids (Lu and Levin, 1974; Arınç and Philpot, 1976; Nebert and Gonzalez, 1987; Gonzales, 1989; Porter and Coon, 1991; Chang and Kam, 1999; Hasler, 1999; Stoilov, 2001).

It is well established that cytochrome P450 moonooxygenase system functions as a multi-component electron transport system. Lu and Coon (1968), for the first time, demonstrated that liver microsomal cytochrome P450 dependent monooxygenase system has three components; cytochrome P450, FAD and FMN containing NADPH dependent cytochrome P450 reductase and lipid. All three components of cytochrome P450 dependent monooxygenase system (NADPH-cytochrome P450 reductase, cytochrome P450 and lipid) are required to reconstitute the full hydroxylation activity (Lu and Coon, 1968; Lu and Levin, 1974; Arınç and Philpot, 1976; Black and Coon, 1986; Adalı and Arınç, 1990; Arınç, 1993, 1995). In the microsomal systems, FAD and FMN containing flavoprotein, NADPH dependent cytochrome P450 reductase catalyzes electron transfer from NADPH to cytochrome P450. Lipid is involved in the transfer of electrons from NADPH-cytochrome P450 reductase to cytochrome P450 in the monooxgenase system.

The reaction catalyzed by P450s involves the oxidation of a substrate (R) where there is a heterocyclic ring, an aromatic ring, an alkane, or an alkene substituent, in the presence of atmospheric oxygen (O_2) and reduced cofactor ($NADPH^+$,H). This reaction can be expressed as:

$$NADPH+H+\mathbf{O}_2+RH \longrightarrow NADP^+ + H_2\mathbf{O} + R\text{-}\mathbf{O}H \qquad (1.1)$$

Only one atom of oxygen is incorporated into the substrate in this reaction, with the other atom being reduced to water; thus the cytochrome P450 dependent enzymes are generally classified as monooxygenases whose primary task is to add or expose a functional groups to both endogenous and exogenous compounds which will help in further excretion of these compounds (Lu and Coon, 1968; Lu and Levin, 1974; Arınç and Philpot, 1976; Black and Coon, 1986).

Cytochrome P450 dependent monooxygenases metabolize a variety of lipophilic compounds of endogenous and exogenous origin. These enzymes may catalyze simple hydroxylation of carbon atom of a methyl group, insertion of a hydroxyl group into a methylene carbon of an alkane, hydroxylation of aromatic ring to form a phenol or addition of oxygen atom across a double bond to form epoxide. In dealkylation reactions, the oxygen is inserted into the carbon-hydrogen bond, but resulting product is unstable and rearranges to primary alcohol, amine or sulphydryl compound. Oxidation of nitrogen, sulphur, and phosphorous atoms and dehalogenation reactions are also catalyzed by cytochrome P450 (Schenkman, 1991; Guengerich, 1993).

At present, cytochrome P450s are found in both eukaryote and prokaryote organisms including vertebrate and invertebrate, animals, plants, fungi, yeast, bacteria and archaea. Total 7703-cytochrome P450 sequence (2740 animals, 2675 plant, 1231 fungi, 813 bacteria, 226 protists and 18, archaea) has been reported as of September 2007 (http://drnelson.utmem.edu/CytochromeP450.html).

Due to presence of so many P450s in various organisms and many of the individual P450s catalyze multiple reactions, the usual method of naming enzymes which depend on spectral properties, electrophoretic mobility or their substrates is inadequate for this group of heme proteins. Therefore, a systemic nomenclature has been devised which is based on structural homology (amino acid sequence) (Nebert *et al.,* 1987). According to this system, the cytochrome P450 superfamily is categorized into respective families and subfamilies. P450 proteins exhibiting more than 40% amino acid sequence similarity are classified within the same family, while proteins exhibiting more than 55% sequence similarity are grouped into the same subfamily (Nebert and McKinnon, 1994; Nelson *et al.,* 1996). In naming of the individual cytochrome P450 enzymes, the abbreviation 'CYP' (Cyp for Drosophila and mouse) -meaning for CYtochrome P450-, is used as a preface to designate the protein under question is a cytochrome P450-dependent monooxygenase. Proteins belonging to the same family are designated with an Arabic numeral, and those belonging to the same subfamily with a capital letter.

Lastly, the individual isoform is shown by an Arabic numeral following the capital letter. For instance, the CYP2 family has several subfamilies such as CYP2B, CYP2C, CYP2D and CYP2E. The individual enzyme is denoted by a numeral, as in CYP2E1. The gene encoding the cytochrome P450 isoforms are similarly denoted, but with italics, like *CYP2E1* (Nebert *et al.,* 1991; Nelson *et al.,* 1993).

The completion of the human genome sequence enabled scientists to determine the human P450 genes. As of January 5, 2007, the human genome sequence contains 57 probable functional P450s in 18 families, though the substrates are not identified for all of them yet (http://drnelson.utmem.edu/CytochromeP450.html). Table 1.1 represents the human cytochrome P450 families and their functions. The enzymes in the families 1-4 are mostly active in the metabolism of xenobiotics -thus called xenobiotic metabolizing P450s-, whereas the families 5-51 have important endogenous functions. Some of the members of families 2 and 3 also metabolize endogenous compounds, such as steroid hormones and arachidonic acid (Gonzalez 1992, Capdevila *et al.,* 2000). The microsomal cytochrome P450 (CYP) family 4 monooxygenases are the major fatty acid ω-hydroxylases. In addition to endogenous substrates, recent evidence indicates that CYP4 monooxygenases can also metabolize xenobiotics, including therapeutic drugs (Hsu *et al*., 2007).

Table 1.1 Human cytochrome P450 families and their main functions (adapted from Hukkanen, 2000; Nebert and Russel, 2002 and http://drnelson.utmem.edu/ Cytochrome P450.html).

CYP Family	Main Functions
CYP1	Xenobiotic metabolism
CYP2	Xenobiotic metabolism Arachidonic acid metabolism
CYP3	Xenobiotic and steroid metabolism
CYP4	Fatty acid hydroxylation Xenobiotic metabolism
CYP5	Thromboxane synthesis
CYP7	Cholesterol 7α-hydroxylation
CYP8	Prostacyclin synthesis
CYP11	Cholesterol side-chain cleavage Steroid 11β-hydroxylation
CYP17	Steroid 17α-hydroxylation
CYP19	Androgen aromatization
CYP20	Developmental signaling
CYP21	Steroid 21-hydroxylation
CYP24	Steroid 24-hydroxylation
CYP26	Retinoic acid hydroxylation
CYP27	Steroid 27-hydroxylation
CYP39	24-hydroxycholesterol 7α-hydroxylation
CYP46	Cholesterol 24-hydroxylation
CYP51	Sterol biosynthesis

1.3 Modulation of Cytochrome P450 Dependent Drug Metabolizing Enzymes in Diabetus Mellitus.

As mentioned earlier, administration of the diabetogenic agents for induction of diabetes such as streptozotocin and alloxan results in modification of the cytochrome P450 dependent drug metabolizing enzymes in various species (Shimojo *et al.,* 1993; Chen *et al.,* 1996; Sakuma *et al*., 2001**;** Arınç *et al.*, 2007). Modulation in CYP450 activities are attributed to resultant pathophysiological changes in diabetes such as, increased levels of ketone bodies and free fatty acids, rather than to the chemical (alloxan and streptozotocin) per se (Chawalit *et al.,* 1982 and Favreau and Schenkman, 1988). The outcome of the alteration of CYP450 composition and levels was that the diabetic animals became more susceptible to the toxicity of chemicals such as carbon tetrachloride, thioacetamide, acetaminophen and other chemical toxins (Hanasona *et al*., 1975; Watkins *et al*., 1988; Wang *et al*., 2000a,b). Moreover, hepatic preparations from diabetic rats were significantly more effective in converting various promutagens to mutagenic species in the Ames mutagenicity assay due to CYP450 alterations (Ionnides *et al*., 1988; Flatt *et al*., 1989).

An increasing number of studies have suggested that diabetes can regulate the composition and levels of many CYP450s belonging to the CYP1, CYP2, CYP3 and CYP4 families in different species and in different organs. Moreover, marked species-related differences exist in the modulation of cytochrome P450 isozymes because of diabetes (Shimojo *et al*., 1993; Chen *et al.,* 1996; Sakuma *et al*., 2001 and Arınç *et al*., 2007). Alterations in the level and composition of cytochrome P450 dependent drug metabolizing enzymes in different species due to diabetes are given below in detail.

1.3.1 CYP1 Family

This family of microsomal cytochrome P450s comprises three members, CYP1A1, CYP1A2 and CYP1B1 in human and rodents such as rat and mouse

(Danielson, 2002 and http://drnelson.utmem.edu/CytochromeP450.html). There are two members of the CYP1A family (CYP1A1 and CYP1A2) showing greater than 70% amino acid sequence identity in humans, but they display very different patterns of tissue expression. CYP1A2 is expressed primarily in the liver. On the other hand, there are conflicting results on its low level expression in the extrahepatic tissues. In contrast, CYP1A1 is expressed primarily in extrahepatic tissues such as the lungs, lymphocytes and placenta while only low-level expression has been reported in liver tissue (Ding and Kaminsky, 2003; Shimada *et al*., 2003; see review Ioannides and Lewis, 2004; Bièche *et al*., 2007; Özkarslı *et al*., 2008). Besides, CYP1B1 is expressed at low levels in different organs such as brain, heart, lung, kidney, leukocytes, small intestine and thymus (Sutter *et al*., 1994; Hukkanen *et al*., 2002; Ding and Kaminsky, 2003; Shimada, 2006). CYP1A family is one of the most conserved families within the phylogenetic tree. Human CYP1A proteins share extensive structural similarity and display similar specificity to the orthologous rodent proteins. Moreover, DNA sequence analysis of the 5'- regulatory regions and comparison to the rodent and human CYP1A genes demonstrated that the human CYP1A1 and CYP1A2 genes were most similar to their rabbit orthologs (Strom *et al*., 1992).

CYP1A1 and CYP1A2 are substrate inducible cytochrome P450s that are responsible for the metabolism of both exogenous and endogenous substrates. Although there is an apparent selectivity for some compounds, both enzymes exhibit overlapping substrate specificities. The substrates of CYP1A subfamily are essentially lipophilic planar molecules, composed of fused rings and characterized by a small depth (Lewis *et al.*, 1987). CYP1A1 and CYP1A2 can cause metabolic activation of well known human carcinogens including polycyclic aromatic hydrocarbons (PAH) such as benzo(a)pyrene, aromatic and heterocyclic amines found in cigarette smoke and in charred foods (Adamson *et al*., 1996; Hümmerich *et al*., 2004; Kim and Guengerich, 2005; Ma and Lu, 2007). In addition to its role in the bioactivation of carcinogens, CYP1A2 also plays a role in the metabolism of numerous pharmaceuticals and other xenobitics such as caffeine, acetaminophen, antipyrine, melatonin, phenacetin, theophylline, imipramine and warfarin (Sharer

and Wrighton, 1996; Yamazaki and Shimida 1997; Facciolá *et al*., 2001; Walsky and Obach, 2004). CYP1A2 can metabolize the some endogenous substrates such as bilirubin and uroporphyrinogen (Sinclair *et al*., 1998, 2000; Zaccaro *et al*., 2001). Susbtrates, inducers, and inhibitors of the CYP1A2 are given in Table 1.2.

Table 1.2 Substrates, inducers and inhibitors of CYP1A2 (taken from Danielson, 2002)

Substrates		**Inhibitors**	**Inducers**
Amitriptyline	Phenacetin	Amiodarone	Methyl cholanthrene
Benzo(a)pyrene	Paracetamol	Cimetidine	Modafinil
Bilirubin	PAH	Fluoroquinolones	Nafcillin
Caffeine	Paracetamol	Fluvoxamine	α-naphthoflavone
Clomipramine	Lidocaine	Furafylline	Omeprazole
Cyclobenzaprine	Propafenone	Interferon	Tobacco
Fluvoxamine	Propranolol	Methoxsalen	TCDD
Flutamide	Riluzole	Mibefradil	
Estradiol	Ropivacaine	Ticlopidine	
Haloperidol	Triamterene		
Imipramine	Theophylline		
Mianserin	Uroporphyrinogen		
Mexiletine	Verapamil		
Naproxen	Warfarin		
Ondansetron	Zileuton		
	Zolmitriptan		

Regulation of induction of CYP1 family is the best studied and well characterized among the cytochrome P450 families because of its association with the etiology of several cancers that are thought to arise through the formation of adducts between DNA and the oxidized products of CYP1 catalyzed reactions. There are at least two types of regulatory DNA sequences found in the cell. The first one is designated as xenobiotic responsible elements (XRE) that works as an inducible enhancer in response to inducers. This cis-acting regulatory DNA element is important in terms of inductive response (Fujisava-Sehara *et al*., 1987; Whithlock *et al*., 1996). The other regulatory element is named as basal transcription element (BTE) that is involved in the constitutive expression of the gene (Yanagida *et al*., 1990; Fuji-Kuriyama *et al*., 1992). It has been shown that these regulatory elements exist in human CYP1A1 gene. XRE works via a trans acting regulatory factor which is found in the cytosol. It is identified and named as XRE-binding factor or Ah (aromatic hydrocarbon) receptor. Aryl hydrocarbon receptor (AHR) is a basic helix-loop-helix (bHLH) protein belonging to the Per-Arnt-Sim (PAS) family of transcription factors. It transcriptionally induces expression of human CYP1A1, CYP1A2, and CYP1B1 (Quattrochi *et al*., 1994; Tang *et al*., 1996; Whitlock, 1999), as well as several other genes, including some phase II metabolizing enzymes (Schmidt and Bradfield, 1996). AHR ligands include PAHs and TCDD (Whitlock, 1999). The unliganded AHR is maintained in cytoplasm in a complex containing chaperon proteins, such as a dimer of HSP90 (heat shock protein 90), ARA9 (also called AIP1 and XAP2) and p23. These other proteins are involved in the correct folding and stabilization of AHR (Gu *et al*., 2000). Upon ligand binding, AHR sheds the chaperon proteins and translocates to the nucleus, where it forms a heterodimer with the AHR nuclear translocator (ARNT) (Hoffman *et al*., 1991). This heterodimer binds to the xenobiotic response elements (XRE) of CYP genes activating transcription (Hankinson, 1994). Consequently, messenger RNA synthesis is increased, resulting in elevated protein level. ARNT also belongs to the bHLH/PAS family. A novel PAS protein called AHR repressor inhibits AHR signal transduction by competing with AHR for ARNT and also by binding to XRE. The AHR repressor is induced by AHR, thus forming a negative feedback loop for the regulation of AHR (Mimura *et al*., 1999; Gu *et al*., 2000).

CYP1A1/CYP1A2 dependent catalytic activities and protein levels were changed due to diabetes in different species. CYP1A1/1A2 associated hepatic ethoxyresorufin O-deethylase, methoxyresorufin O-deethylase and ethoxycoumarin O-deethylase activities was increased 2-4-fold in STZ induced diabetic male rats (Barnett *et al*., 1993; Clarke *et al*., 1996; Raza *et al.*, 1996; Engels *et al*., 1999; Raza *et al*., 2000 and Sheweita *et al*., 2002; Shankar *et al*., 2003b). The STZ treatment given to female rats produced a similar change to those seen in the diabetic male animals (Barnett *et al*., 1993). Consistently, expression of hepatic CYP1A1/1A2 protein was also increased in streptozotocin induced male and female diabetic rats (Barnett *et al*., 1992; Barnett *et al*., 1993; Raza *et al.*, 1996; Raza *et al*., 2000; Shankar *et al*., 2003b). Extrahepatic CYP1A1/CYP1A2 dependent activities were also determined in diabetic rats. Ethoxycoumarin O-deethylase activity was increased 1.9-, 2.0- and 1.32 fold in diabetic kidney, brain and testis, respectively (Raza *et al*., 2000).

Different laboratories have obtained different results about CYP1A protein expression following to diabetogen treatment in hamsters. The effects of acute and chronic streptozotocin diabetes on kidney and liver microsomal monooxygenases were studied by Chen *et al.,* in 1996 using hamsters 2 days and 6 weeks following treatment with diabetogen. Benzo(a)pyrene hydroxylation and ethoxycoumarin O-deethylation (P4501A1 associated) rates were similar to controls in the acute diabetic hamsters' kidney and liver. On the other hand, the chronic diabetes caused inhibition in benzo(a)pyrene and 7-ethoxycoumarin oxidation in kidney microsomes. The western-blot data showed that diabetes caused induction of the renal and hepatic protein cross reactive with P4501A1. The discrepancy between CYP1A1 associated enzyme activities and protein level was explained by that diabetes caused induction of catalytically different P450 such as CYP1A2 or antibody can recognize inactive apoprotein of CYP1A1. Unlike Chen *et al*., 1996, Takatori and co-workers found that CYP1A protein in hamster was suppressed due to STZ induced diabetes (Takatori *et al*., 2002). At one and six months after SZ-injection, the levels of CYP1A protein of STZ-injected hamsters were lower than those of age-matched control hamsters (2-fold and 1.5-fold, respectively). Diabetes

caused 1.2 fold suppression of CYP1A protein level at 3 months after STZ injection but this was not found statistically significant (Takatori *et al*., 2002).

In 2001, Sakuma *et al*., examined the effect of diabetes on mouse hepatic and renal cytochrome P4501A2. Immunochemical and catalytic analyses (CYP1A2 dependent methoxyresorufin O-deethylase activity in mouse) demonstrated that the CYP1A2 was not changed in the streptozotocin induced diabetic male and female mouse. In fact; methoxyresorufin O-deethylase (MROD) activity is not dependent solely to CYP1A2. It has been shown to be catalyzed by CYP1A1 and CYP1A2 in mammals (Burke et al., 1994). Moreover, in the reconstituted system, the purified *L. saliens* liver CYP1A1 showed both EROD and MROD activity (Şen and Arınç, 2000). On the other hand, CYP1A protein in mouse liver was suppressed about 2-fold due to STZ induced diabetes (Shankar *et al*., 2003b). These results indicate that species related difference in CYP1A was present in diabetic animals. No data is available about the effect of diabetes on CYP1A dependent enzyme activities, protein level and mRNA level in rabbit up to now.

Induction of CYP1A protein in rats and hamsters appear to be mediated by the hyperketonaemia that accompanies diabetes (Barnett *et al*., 1992). When animals treated with triacylglycerols that induce hyperketonaemia, similar changes in the CYP1A was observed as in STZ-induced diabetes (Barnett *et al*., 1988 and Barnett *et al*., 1990a). The role of ketones in induction of CYP1A activities and protein levels was also supported that treatment of rats with acetone enhanced both activities and levels of these proteins (Barnett *et al*., 1992).

1.3.2 CYP2 Family

This is the largest mammalian cytochrome P450 family, comprising a number of distinct subfamilies including CYP2A, CYP2B, CYP2C, CYP2D and CYP2E which, unlike the CYP1 subfamilies, exhibit markedly different substrate specificity, and are under different regulatory control. This family of microsomal cytochrome P450s is classified into 13 subfamilies that consist of 16 functional

genes in humans (http://drnelson.utmem.edu/CytochromeP450.html). CYP2 family members oxidize numerous compounds of clinical and toxicological significance, including pharmaceuticals, pro-carcinogens, and tobacco smoke constituents.

Cytochrome P4502A subfamily is a complex family which has a highly similar gene sequence in different species but their regulation and substrate specificities are diverse (Ioannides and Lewis 2004; Wang *et al*., 2003 referred by Su and Ding, 2004; http://drnelson.utmem.edu/CytochromeP450.html). There are at least three functional proteins (CYP2A6, CYP2A7, and CYP2A13) in human and two functional CYP2A proteins (CYP2A10 and CYP2A11) in rabbit (http://drnelson.utmem.edu/CytochromeP450.html). Rabbit CYP2A10 and CYP2A11 are expressed abundantly in olfactory and respiratory nasal mucosa and have relatively low levels in liver (Ding *et al*., 1994). The predicted amino acid sequence of these two proteins differ only in eight positions, and are over 80% identical to the sequence of CYP2A3 (Rat), CYP2A4, CYP2A5 (Mouse), CYP2A6 and CYP2A7 (Human) (Ding *et al*., 1994). On the other hand, human CYP2A6 is expressed dominantly in the liver, and at much lower levels, in tissues of the respiratory tract, including nasal mucosa, trachea, and lung, and in the skin (Janmohamed *et al*., 2001; Saeki *et al*., 2002). In contrast, CYP2A13 is expressed mainly in the respiratory tract (Koskela *et al*., 1999; Su *et al*., 2000). CYP2A7 appears to be liver-specific (Koskela *et al*., 1999; Su *et al*., 2000). Gender and tissue specific expressions of CYP2A proteins were observed in different species despite their similar gene sequences (see review Su and Ding, 2004).

Collectively, the CYP2A enzymes metabolize numerous xenobiotic compounds such as coumarin, aflatoxin B1, *N*-nitrosodiethylamine, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), nicotine, cotinine, 1,3-butadiene, hexamethylphosphoramide, acetaminophen, and 2,6-dichlorobenzonitrile, as well as endogenous compounds, including testosterone, progesterone, and other steroid hormones (see reviews, Ding and Dahl, 2003; Ding and Kaminsky, 2003; Fernandez-Salguero and Gonzalez, 1995; Honkakoski and Negishi, 1997). Human CYP2A6 is responsible for the systemic clearance of

nicotine, and thus may influence the level of cigarette consumption in addicted smokers (Tyndale and Sellers, 2002), whereas CYP2As in extrahepatic tissues may play important roles in target-tissue metabolic activation of xenobiotics (Ding and Kaminsky, 2003). Variations in the expression of the *CYP2A* genes probably alter the risks of chemical toxicity for numerous toxicants that are CYP2A substrates.

Cytochrome P4502B subfamily has received a great deal of attention in recent years because of its capacity to metabolize testosterone, androstedione and several important pharmaceutical agents, and because of its inducibility by phenobarbital (Guengerich *et al.*, 1982; Fukui *et al.*, 1992; Ryan *et al.*, 1993). CYP2B has been studied, cloned and sequenced in many different mammalian species, and 17 different proteins have been reported as of January 2003 (http://drnelson.utmem.edu/ CytochromeP450.html). There are at least two different CYP2B proteins present in rabbits (CYP2B4, CYP2B5). The highly related rabbit cytochromes CYP2B4 and CYP2B5 differ in only 12 amino acid positions, but only CYP2B5 has an activity toward progesterone (He *et al*., 1998). In rabbit, P4502B4 (LM2 or P450 2) is a major constitutive P450 in lung, but is present in very small amounts in liver (Serabjit-Singh *et al.*, 1979; Parandoosh *et al.*, 1987). Treatment with Phenobarbital increases the level of CYP2B4 many-fold in liver, but does not change CYP2B4 amounts in lung (Serabjit-Singh *et al.*, 1983, Arınç, 1993). On the other hand, only one CYP2B (CYP2B6) appears to be poorly expressed in human liver where it constitutes 1% of the human hepatic P450 complement (Gonzalez and Gelboin, 1994).

There is a substantial structural diversity in CYP2B substrates, although many tend to be lipophilic, of intermediate molecular weight with respect to those of CYP2E (low molecular weight) and CYP3A (high molecular weight), and are generally characterized by non-planar molecular geometries often comprising two aromatic rings, flanking a central tetrahedral carbon atom, which consequently adopts a V-shape or 'butterfly-wing' conformation (Lewis and Lake, 1997; Lewis *et. al.*, 1998). A representative list of CYP2B substrates, inducers and inhibitors is shown in Table 1.3. There is a diversity in both structural class and position of

metabolism. As mentioned before, the CYP2B subfamily is also involved in the metabolism of endogenous steroids, such as testosterone and androstenedione (Lewis and Lake, 1997).

Table 1.3 Substrates, inducers and inhibitors of CYP2B (adapted from Danielson, 2002)

Substrates	Inhibitors	Inducers
2,2'-Dichlorobiphenyl 7-Pentoxyresorufin Androstedione Benzphetamine Bupropion Chloramphenicol Cocaine Cyclophosphamide DDT Febrazole Hexobarbital Ifosphamide Nicotine Phenobarbital Phenylbutazone Promethazine *p*-Iodotoluene Sertraline Tamoxifen Testesterone	Allylisopropylacetamide Guanidinium ion Metyrapone n-Octylamine Proadifen (SKF-525A) Secobarbital	Phenobarbital Phenytoin Rifampin

Phenobarbital is the classic archetype of a large number of structurally diverse compounds inducing numerous xenobiotic-metabolizing enzymes as well as affecting various other cellular processes. The xenobiotic-metabolizing genes induced by phenobarbital include CYPs in the subfamilies 2A, 2B, 2C and 3A. The most effectively induced genes are members of the CYP2B family (Denison and Whitlock 1995), CYP2B6 in humans (Chang *et al.,* 1997). Phenobarbital induction

of CYP2B6 is mediated by constitutively active receptor (CAR, also called constitutive androstane receptor) even though phenobarbital is not a ligand of CAR (Honkakoski and Negishi 1998; Moore *et al.,* 2000a). The exact mechanism of phenobarbital induction is still unclear, but it is suggest that phenobarbital not only facilitates the translocation of CAR to the nucleus, but also activates CAR in the nucleus. These steps are dependent on phosphorylation, since translocation and activation are inhibited by protein phosphatase (PP) and CaM kinase (CK) inhibitors, respectively (Negishi, 2000). This model is supported by the finding that, in mouse primary hepatocytes, CAR is located in the cytoplasm and is only translocated to the nucleus after inducer treatment (Kawamoto *et al.,* 1999). Thus, the regulation of CAR function would be dependent not only on the repression and derepression of constitutive activity, but also on the nuclear translocation and activation of CAR (Honkakoski and Negishi 2000; Tzameli *et al.,* 2000). Recently, CAR-null mice were produced showing no induction of CYP2B by phenobarbital (Wei *et al.,* 2000). CAR is down-regulated by the inflammatory cytokine interleukin-6, which could explain the repression of CYPs by inflammatory mediators (Abdel-Razzak *et al.,* 1993; Muntane-Relat *et al.*, 1995).

CAR acts differently than the more traditional receptors: CAR is constitutively active without ligand. CAR requires the heterodimerization partner, retinoid X receptor (RXR), to enable binding to DNA. CAR/RXR heterodimers bind to a conserved 51-base pair element called phenobarbital-responsive enhancer module (PBREM) in the 5'-flanking region of the *CYP2B* genes and to the ER6 element (everted repeat with a 6 bp spacer) of the *CYP3A4* gene (Honkakoski *et al.,* 1998a; Sueyoshi *et al.,* 1999). PBREM has been shown to mediate the induction by phenobarbital and phenobarbital-like inducers (Honkakoski *et al.,* 1998b). As mentioned above, phenobarbital is not a CAR ligand (Moore *et al.,* 2000a). But, deactivators or inverse agonists, such as testosterone metabolites, androstanol and androstenol, bind to human CAR and so its constitutive activity can be inhibited by superphysiological concentrations of the inhibitors (Forman *et al*., 1998). These androstanes inhibit the interaction of CAR with the steroid receptor coactivator 1 (SRC-1), suggesting that "deactivation" is mediated by direct binding to the

orphan receptor. (Forman *et al.,* 1998; Moore *et al.,* 2000a). As a result, CYP2B6 transcription is suppressed. This suppression is overcome by agonist binding to CAR, which abolishes the inhibitory inverse agonists from CAR leading to the induction of CYP2B6 (Waxman 1999).

CYP2C is a large subfamily, composed of at least four in humans (CYP2C8, CYP2C9, CYP2C18 and CYP2C19) which are more than 80% identical at the amino acid level. CYP2C9 is the main CYP2C in human liver, followed by CYP2C8 and CYP2C19 (Edwards *et al.,* 1998). The CYP2C18 protein is not expressed in liver (Richardson *et al.,* 1997). Low levels of CYP2C mRNAs and proteins have also been detected in small intestine and other extra-hepatic tissues (Klose et al., 1999). At least nine CYP2C proteins were identified in different tissues of rabbit (CYP2C1, CYP2C2, CYP2C3, CYP2C4, CYP2C5, CYP2C14, CYP2C15, CYP2C16 and CYP2C30) (http://drnelson.utmem.edu /CytochromeP450 html). Rabbit CYP2C amino acid sequences can be divided into two subgroups. The first group, CYP2C4, CYP2C5, CYP2C15 and CYP2C16 exhibit a minimum of 90% of homology and contain 487 amino acids. On the other hand, the other CYP2C group, CYP2C1, CYP2C2, CYP2C3 and CYP2C14 contains 490 amino acids and amino acid sequence similarity, relative to the CYP2C14, ranges from 65% to 83%. (Henderson and Wolf, 1992). CYP2C1 is only present in liver after phenobarbital treatment, whereas CYP2C2 is both constituvely expressed and inducible by phenobarbital in liver and kidney (Leighton and Kemper, 1984 referred by Henderson and Wolf, 1992). On the other hand, CYP2C3 is present in untreated animals, but no induction was seen following the phenobarbital treatment. Neither CYP2C1 and CYP2C2 nor CYP2C3 is detected in lung, either in control or phenobarbital treated rabbits (Leighton and Kemper, 1984 referred by Henderson and Wolf, 1992). CYP2C16 is found in lung and testis with phenobarbital inducibility. CYP2C4 is found only in rabbit liver and is phenobarbital inducible (Hassett and Omiecinski, 1990 referred by Henderson and Wolf, 1992). On the other hand, CYP2C5 is found in liver but not inducible by phenobarbital. Moreover, it is absent in kidney (Finlayson et al., 1987 referred by Henderson and Wolf, 1992).

The proteins encoded by *CYP2C* genes account for approximately 20% of the total liver cytochrome P450 content in humans (Ioannides and Lewis, 2004). These proteins are responsible for the metabolism of approximately 20% of all clinically administered drugs including anti-coagulants (warfarin), anti-diabetic agents (tolbutamide), barbiturate sedatives (hexobarbital, ibuprofen), nonsteroidal anti-inflammatory drugs, anti-convulsants (phenytoin and trimethadone) and proton pump inhibitors (omeprazole), benzodiazepines (diazepam), anti-depressants and the anti-malarial drug (proguanil), as well as many endogenous substrates such as arachidonic acid (Leemann *et al*., 1993; Carlile *et al*., 1999; Tréluyer *et al*., 2000; DeLozier *et al*., 2004; Soga *et al*., 2004; Klotz, 2006). Recently, it was postulated that CYP2C8 has important physiological functions in the production of endothelium-derived hyperpolarizing factor (EDHF) (Fisslthaler *et al.,* 1999). Human CYP2C subfamily plays a very minor role in the bioactivation of chemical carcinogens (Shimada *et al*., 1989; Shou *et al*., 1996).

CYP2C mRNA and protein are induced in primary hepatocytes by phenobarbital and rifampicin (Chang *et al.,* 1997; Gerbal-Chaloin *et al*., 2001). Rifampicin and barbiturates can induce CYP2C proteins and related activities *in vivo* (Zilly *et al.,*. 1977, Perrot *et al.,* 1989, Treluyer *et al.,* 1997). Recent results indicate that PXR, CAR and Glucocorticoid receptor (GR) mediate CYP2C8 and CYP2C9 induction in human hepatocytes (Pascussi *et al.,* 2000; Gerbal-Chaloin *et al*., 2001). As a result of binding of PXR/RXR, CAR/RXR heterodimers and GR/GR homodimers to different promoter response elements on DNA, CYP2C8 and CYP2C9 transcription is increased, resulting in elevated protein level. In contrast to the other CYP2C messengers, CYP2C18 mRNA was not inducible in any of the cultures tested. Furthermore, CYP2C18 protein was not detected in the microsomes prepared either from human liver tissue or cultured hepatocytes. These data suggest that *CYP2C18* is not significantly expressed at the protein level in the liver and is regulated differently from the other members of this subfamily (Gerbal-Chaloin *et al*., 2001).

The CYP2D subfamily consists of a single functional member in human (CYP2D6) and two members in rabbit (CYP2D23 and CYP2D24). CYP2D6 is mainly expressed in liver but its expression was found at the level of mRNA by reverse-transcriptase PCR in a number of extrahepatic tissues such as small intestine, brain and heart (Pelkonen and Raunio 1997; Zanger *et al*., 2004). Although, CYP2D6 constitutes about 2% of total hepatic CYP450s (Shimada *et al.,* 1994, Imaoka *et al.*, 1996), it is involved in oxidative metabolism of more than 70 different pharmaceuticals. CYP2D23 and CYP2D24 are functionally expressed in rabbits, and have different organ distributions and metabolic properties. The open-reading frames of rabbit CYP2D23 and CYP2D24 cDNAs encode proteins that are each composed of 500 amino acids. The amino acid sequence identity of CYP2D23 with CYP2D24 is 91.6%, and the homology of these two isozymes with other known mammalian CYPs in the CYP2D subfamily range from 64.9 to 79.8%. Using RT-PCR, the distribution of these two isozymes was determined in nine major organs, including brain tissue sections. CYP2D23 mRNA was abundantly expressed in the liver and small intestine, but only slightly in the brain sections, whereas CYP2D24 mRNA was expressed in the liver, small intestine, and stomach (Yamamoto *et al*., 1998).

CYP2D6 has been one of the most investigated of the drug-metabolizing cytochrome P450s, primarily because of a well known polymorphism in the gene and the number of its drug substrates (Legrand-Andréoletti *et al*., 1998; Dorado *et al*., 2007 and Ingelman-Sundberg *et al.,* 2007). The CYP2D6 enzyme supports the oxidative metabolism of more than 70 different pharmaceuticals including β-adrenergic blocking agents (labetalol, timolol), anti-arrhythmics (flecainide, mexiletine), anti-psychotics (clorpromazine, haloperidol), anti-depressants (paroxetine, venlafaxine, fluoxetine, prozac, trazadone) calcium antagonist (perhexiline), MAO-inhibitors (amiflamine), anti-histamines (promethazine), anti-diabetic (phenformine), vasodilatators (cinnarizine) and narcotic analgesics (codeine,fentanyl, meperidine) (Buchert and Woosley 1992; Otani and Aoshima, 2000; Zanger *et al*., 2004; Micallef *et al*., 2006; Stamer and Stüber, 2007 and

Volotinen *et al*., 2007). No major role in the bioactivation of chemicals is associated with this subfamily.

Cytochrome P4502E1, ethanol inducible form of P450, is also known as P450 LM3a, P450j, P450ac or P450alc. It was first purified in rabbits (Koop *et al.,* 1982) and later in rats and humans (Ryan *et al.,* 1985; Patten *et al.,* 1986; Wrighton *et al.,* 1986). The rat and human liver CYP2E1 cDNAs have been isolated and sequenced (Song *et al.,* 1986). The deduced amino acid sequence of rat and human 2E1 both contain 493 amino acids with calculated molecular weights of 56 634 Da and 56 916 Da, respectively. CYP2E1 is one of the most conserved forms in the CYP2 family and the orthologues share the same substrate specificity. Human CYP2E1 shares 75% nucleotide and 78% amino acid similarities to rat CYP2E1. Amino acid alignment revealed that CYP2E1 was 48% similar to CYP2B1 and CYP2B2 and similar to CYP2C6 and CYP2C7, but had lower similarities to other P450s (Umeno *et al.,* 1988; Gonzales, 1989; Yang *et al.,* 1990).

Unlike other mammals, the CYP2E sub-family in rabbit liver is found to contain two genes, *CYP2E1* and *CYP2E2*. CYP2E2 expression begins at birth and is abundant in one- to three- week old rabbits and its expression diminishes after three weeks. CYP2E1 is not present at birth, it appears during the fourth week and reaches to a high level at two months and stays at the high level at least until the rabbits are six months old. Only CYP2E1 is expressed in rabbit kidney (Bonfils *et al*., 1991; Ding *et al*., 1991; Rich and Boobis, 1997).

CYP2E1 is mainly found in liver, but significant amounts are also found in extrahepatic tissues including lung, kidney, brain, endothelium of large blood vessels, heart, bone marrow and nasopharyngeal tissues (Ding *et al.,* 1986; Ingelman-Sundberg *et al.,* 1993). In liver, the enzyme is not homogenously distributed: the expression of enzyme, both constitutively and after induction, is restricted to the centrilobular region of the liver and, in particular, to the three to four layers of hepatocytes proximal to the central vein (Ingelman-Sundberg et *al.*,

1988). Thus, hepatic damage caused by CYP2E1 activity is mainly observed in these regions of the liver.

A number of different chemicals of diverse structures have been found to be selectively metabolized by CYP2E1. Besides ethanol, CYP2E1 metabolizes some other endogenous compounds, but most of its substrates are exogenous, including industrial solvents, procarcinogens and a few pharmaceutical drugs. It is difficult to find a common structure among all these substrates, but it appears that small (Molecular weight < 100) and hydrophobic compounds in general provide efficient targets for CYP2E1-dependent catalysis (Ingelman-Sundberg *et al.,* 1993). Examples for the substrates, inducers and inhibitors of CYP2E1 are listed in Table 1.4.

CYP2E1 can metabolize many endogenous compounds such as acetone, acetate and long chain fatty acids (arachidonic acid, lauric acid, oleic acid and linoleic acids) (Lieber, 1999; Klaz and Ammon, 1998), suggesting that it has a physiological importance (Table 1.4). Acetone and ketone bodies are metabolized by P4502E1 to acetal and methylglyoxal (Koop and Casazza, 1985) which can be used for synthesis of glucose. It has been estimated that, under physiological conditions, most of the acetone is metabolized via this oxidative pathway (Landau and Brunengraber, 1987).

Table 1.4 Substrates, inducers and inhibitors of CYP2E1 (Adapted from Tanaka *et al.*, 2000 and Bolt *et al.*, 2003).

Substrates		
Endogenous	**Exogenous**	
	Therapeutic drugs/ anaesthetic gases	**Solvents and other chemicals**
Ethanol Acetone Acetoacetate Acetol Acetaldeyde Fatty acids Glycerol	Acetaminophen Chlorzoxazone Dapsone Enflurane Sevoflurane, Isoflurane Halothane Disulfiram Isoniazid *p*-nitrophenol Phenacetin Trimetmethadione Acetylsalicylic acid Thioacetamide Felbamate Fluoxetine	Aniline Acrylonitrile, Methacrylonitrile Alcohols, ethers, alkanes Acetone Benzene (and derivatives) Styrene Chloroform Carbontetrachloride Pyrazole Phenol Pyridine Acrylamide Nitrosamines (e.g. NDMA) Ethyl carbamate Vinyl carbamate Vinyl chloride Diethylether Hexane Butadiene Ethylene dichloride Methyl chloride Methylene chloride 1,1,1-trichloropropanol 1,2-dichloropropanol

Inducers	**Inhibitors**
Ethanol Acetone Benzene Isoniazid Pyrazole Pyridine Starvation Diabetes	Diallylsulfide, Diallylsulfone Chlormethiazole Diethyldithiocarbamate Isothiocyanates 4-methyl-pyrazole Disulfiram

As it can be seen on Table 1.4, an important portion of CYP2E1 exogenous substrates involves industrial chemicals and procarcinogens/carcinogens. Among them are butadiene (Melnick and Kohn, 1995), small molecular weight hydrocarbons such as benzene and styrene (Guengerich, 1995), certain chloroalkanes and chloroalkenes like chloroform, tetrachloromethane, trichloroethane and vinyl chloride (Guengerich *et al.,* 1991; Gonzalez and Gelboin, 1994), heterocyclic compounds like pyridine (Arınç *et al*., 2000a,b) and nitrosamines including NDMA (Garro *et al.,* 1981; Arınç *et al.*, 2007). CYP2E1 activates these chemicals into more toxic or carcinogenic forms and therefore receives a great deal of attention in terms of occupational liver-diseases and cancer. In addition, several halogenated anaesthetics (e.g. halothane, sevoflurane and isoflurane) and drugs such as acetaminophen, chlorzoxazone, trimetmethadione and acetylsalicylic acid (aspirin) are also mainly catalyzed by CYP2E1 (Wu and Cederbaum, 2001; Tanaka *et al.*, 2003, Gonzales, 2005 and Khemawoot *et al*., 2007). Cytochrome P450 chemical inhibitors are widely used to define the role of individual cytochrome P450 isozymes in metabolic processes. There are several selective inhibitors for CYP2E1 for this purpose. 4-methylpyrazole, disulfram, diethyl-dithiocarbamate, phenylethyl-isothiocyanite, diallysulfide and its metabolites inhibit CYP2E1 activity *in vivo* and *in vitro* (Bourie *et al.,* 1996; Nissbrandt *et al.,* 2001; Hazai *et al.,* 2002).

Besides ethanol, CYP2E1 is also induced by acetone, isopropanol, pyridine, benzene and some pathophysiological conditions such as starvation, diabetes, obesity, high fat feeding (Koop and Casazza, 1985; Hong *et al.,* 1987; Song *et al.,* 1987; Kim *et al.,* 1990; Raucy *et al.,* 1990; Arınç *et al.,* 1991; Yoo *et al.,* 1991; Arınç *et al.,* 2000a,b and Arınç *et al.,* 2007). It has received a great deal of attention in recent years because of its vital role in the activation of many low molecular carcinogens such as nitrosoamines, pyridine, benzene and other toxic agents (Yang *et al*., 1990; Arınç *et al*., 1991; Yamazaki *et al.*, 1992; Arınç *et al.,* 2000). In addition, cytochrome P4502E1 induction due to diabetes and obesity or chemicals given above and in Table 1.4 has been found to potentiate hepatotoxicity of certain

compounds such as thioacetamide, chloroform, CCl_4 and bromobenzene (Hanasono *et al.,* 1975; El-Hawari and Plaa, 1983; Watkins *et al.,* 1988; Wang *et al.,* 2000).

Another important and peculiar feature of CYP2E1 is its capability to reduce molecular oxygen, resulting in the formation of H_2O_2 and $O_2^{\bullet-}$ radicals (Gorsky *et al.,* 1984; Elkstrom and Ingelman-Sundberg, 1989; Persson *et al.,* 1990). It is known that, unlike most other P450s, CYP2E1 contains a proportion of the hemoprotein that is present naturally in the high-spin state even in the absence of a substrate (Guengerich and Johnson, 1997), and this feature of CYP2E1 would let it be reduced by an electron provided by NADPH-cytochrome P450 reductase (in the absence of a substrate), resulting in the generation of reactive oxygen species (Kukielka and Cederbaum, 1994; Wu and Cederbaum, 1999; Cederbaum *et al.*, 2001).

It appears that oxygen radicals generated by CYP2E1 have the capability to initiate membranous lipid peroxidation. Thus, CYP2E1 turned out to be the most efficient isozyme in the initiation of NADPH-dependent lipid peroxidation. Its importance is verified with studies showing almost complete inhibition of NADPH-dependent lipid peroxidation in microsomes using CYP2E1 antibodies (Elkstrom and Ingelman-Sundberg, 1989). So, it is clear that CYP2E1 plays a key role in the pathogenesis of liver injury.

Membranous lipids are not the sole target of free radical attack. The increased microsomal generation of reactive oxygen derivatives could also contribute to ethanol toxicity through radical-mediated inactivation of metabolic enzymes (Dicker and Cederbaum, 1988), including CYP2E1 itself (Koop and Tierney, 1990).

As mentioned above, most CYP2E1 substrates and some pathophysiological conditions induce CYP2E1. The regulation of CYP2E1 expression is complex, involving transcriptional, post-transcriptional, and post-translational events with polymorphism playing a role (Song, 1995). Transcriptional regulation seems to have a minor role, in contrast to many other xenobiotic-metabolizing P450 forms. A summary of the modes of CYP2E1 regulation is given in Figure 1.4.

Transcriptional activation of CYP2E1 was demonstrated in rats right after birth (Song *et al.,* 1986) which is accompanied by demethylation of cytosine residues located within the 5'-flanking region of the gene (Umeno *et al.,* 1988 and Lieber, 1997).

It is reported that hepatic nuclear factor-1α (HNF1α) -a homodomain-containing transcription factor, that is expressed in liver, kidney, intestine, stomach and pancreas (Lee *et al.,* 1998; Blumenfeld *et al.,* 1991), can bind to *CYP2E1* gene between –113 bp to –7 bp in 5'-flanking region, and probably controls the transcription of this gene (Liu and Gonzalez, 1995). In a recent study, it is established that *CYP2E1* gene is found out to be expressed at markedly lower levels in the livers of HNF1α-deficient mice, with a corresponding decrease in the protein level, verifying that HNF1α may act as a positive regulator of *CYP2E1* gene (Cheung *et al.,* 2003). However, more recent studies have revealed that control of *Cyp2E1* expression in mice is not solely dependent on Hnf1α expression. (Sekine *et al*., 2006). These results show that the role of this transcription factor in the regulation of *CYP2E1* gene needs further investigation. Transcriptional activation of *CYP2E1* gene is also seen after extensive starvation (Albano *et al.,* 1993), and at high ethanol concentrations (Badger *et al.,* 1993; Ronis *et al.,* 1993). Acute ethanol treatment does not influence the CYP2E1 mRNA levels, indicating that no specific ethanol responsive element is present within the *CYP2E1* gene (Ingelman-Sundberg *et al.,* 1993).

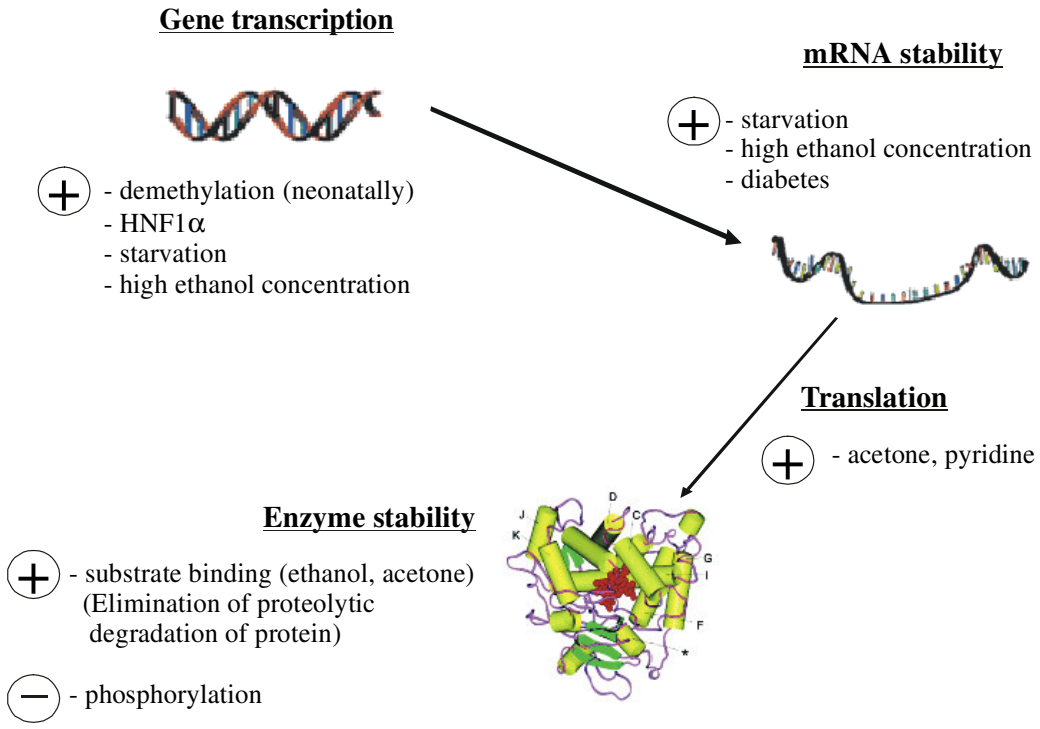


Figure 1.4 Mechanisms of regulation of CYP2E1 expression (adapted from Ingelman-Sundberg *et al.,* 1993).

One of the most profound examples of post-transcriptional regulation is seen with the CYP2E1 enzyme. Administration of small organic compounds such as acetone, pyrazole, and ethanol to rats caused a rapid induction of CYP2E1 protein without affecting the levels of CYP2E1 mRNA (Khani *et al.,* 1987; Song *et al.*, 1989). Furthermore, in a HepG2 cell line, which stably and constitutively expresses the coding sequences of human CYP2E1, the addition of ethanol (2-100 mM) for 2 days resulted in an increased CYP2E1 content without any raise in mRNA levels (Carrocio *et al.,* 1994). These results indicate a post-transcriptional regulation of CYP2E1 at either the translational level or by stabilization of the protein against degradation. The stabilization of enzyme was proposed to be achieved by binding of substrates to CYP2E1 as revealed by in vivo labelling of proteins with 14C-sodium

bicarbonate in rats treated with acetone (Song *et al*., 1989). In untreated animals, CYP2E1 exhibits biphasic turnover with endoplasmic reticulum proteolytic pathway (with half-life of 7 hours) and lysosomal proteolytic pathway (with half-life of 37 hours). When rats were administered acetone in the drinking water, endoplasmic reticulum proteolytic pathway was lost, thus accounting for the increase in microsomal content of CYP2E1. This data has suggested that the protein is stabilized by the presence of the substrate as a result of loss of the rapid turnover component. (Song *et al*., 1989; Eliasson *et al.,* 1992).

Controversially, other studies on hamsters (Kubota *et al.,* 1988), rats (Diehl *et al.,* 1991; Kim and Novak, 1994) and humans (Takashi *et al.,* 1993), showed that administration of low molecular weight substrates of CYP2E1 like ethanol results with an increase in CYP2E1 mRNA levels in liver. According to these results, it appears that dose of inducer triggers different modes of induction such that CYP2E1 induction with ethanol occurs in two steps: 1) post-translational mechanism at low ethanol concentrations, and 2) an additional transcriptional one at high ethanol levels (Ronis *et al.,* 1993; Badger *et al.,* 1993).

In spontaneously diabetic rats or in rats made diabetic by treatment with streptozotocin or alloxan, CYP2E1 protein was induced up to 9-fold above the level seen in untreated rats (Raza *et al*., 2000; Wang *et al*., 2000a; Shankar *et al*., 2003b Sindhu *et al*., 2006). This increase was accompanied by up to a 10-fold increase in CYP2E1 mRNA in the absence of an increase in transcription of the *CYP2E1* gene (Song *et al.,* 1986). Nuclear run-on transcription analysis showed that this mRNA increase is not due to transcriptional activation, but is due to specific stabilization of the P4502E1 mRNA (Song *et al.,* 1986). The stabilization of the CYP2E1 mRNA that is reversed by insulin (Woodcroft *et al*., 2002) is less well understood. Recent studies revealed the presence of a 16-nucleotide sequence in the 5' region of the CYP2E1 mRNA that is responsible for insulin-mediated destabilization of the mRNA (Truong *et al*., 2005). Fasting also caused an increase in CYP2E1 mRNA and protein (Hong *et al.,* 1987), suggesting that the rate of mRNA degradation may have been retarded under these conditions (Hong *et al.,* 1987; Song *et al.,* 1987).

Several studies have also shown that fasting and diabetes result in elevated levels of ketone bodies (Miller and Yang, 1984), which are also the substrates of CYP2E1, so they are thought to be involved in the induction response either directly, or indirectly (Bellward *et al.,* 1988).

The regulation mechanisms of CYP2E1 by benzene have not been clarified yet. Benzene was found to stabilize CYP2E1 by inhibiting protein degradation like acetone (Koop and Tierney, 1990). But, recently, it is found that benzene increases the content of CYP2E1 mRNA in both liver and peripheral lymphocytes detected by an ad hoc RT-PCR strategy. The levels of CYP2E1 mRNA in liver and lymphocytes were well correlated to each other in both the acute and subacute treatment of benzene (Gonzalez-Jasso *et al*, 2003). However, whether, the observed mRNA increase resulting from benzene is transcriptional or post-transcriptional remains to be elucidated.

Except CYP2G and CYP2T subfamilies, remaing six CYP2 subfamilies (CYP2F, CYP2J, CYP2R, CYP2S, CYP2U and CYP2W) are expressed to functional gene products in humans (http://drnelson.utmem.edu/Cytochrome P450.html). Aside from CYP2F, however, little is known about the role of the enzymes encoded by these CYP2 genes in xenobiotic metabolism (Lanza and Yost, 2001; Baldwin *et al*., 2005).

The effect of chemically induced diabetes on many isozymes of CYP2 family was studied in rat, mice, hamster and rabbits. In male rats, hepatic CYP2A1 dependent enzyme activity (testosterone 7α-hydroxylation) and expression were increased in the chemically induced diabetes (Yamazoe *et al*., 1989 and Shimojo *et al*., 1993). On the other hand, male specific form of CYP2A, CYP2A2, was decreased in STZ-induced diabetic rats (Shimojo *et al*., 1993; Thummel and Schenkman, 1990). Moreover, CYP2B1 protein level and its associated enzyme activity, *O*-pentoxyresorufin dealkylase, were induced in STZ-diabetic rats (Barnett *et al*., 1994; Clarke *et al.*, 1996; Yamazoe *et al.,* 1989). Furthermore, hepatic CYP2C11 and CYP2C13 (male-specific isozymes) and their enzyme activities were

suppressed in male STZ-induced diabetic rats (Favreau and Schenkman, 1988; Thummel and Schenkman, 1990; Yamazoe *et al*., 1989; Shimojo *et al.,* 1993). In contrast, female-specific isoform, CYP2C12, was unchanged or increased in the liver of STZ-induced diabetic rats (Shimojo *et al*., 1993; Thummel and Schenkman, 1990). Hepatic CYP2C6 and CYP2C7 are increased in STZ-induced diabetic rats (Shimojo *et al*., 1993 and Shimoho, 1994).

The most prominent effect of diabetes is the induction of CYP2E1. Hepatic CYP2E1 is induced 2- to 8-fold in STZ-induced diabetic rats (Thummel and Schenkman, 1990; Shimojo *et al*., 1993; Barnett *et al*., 1993; Raza *et al*., 1996 and 2000; Wang *et al*., 2000a). Consistent with the induced protein level, increased enzyme catalytic activities of CYP2E1, aniline hydroxylation, *p*-nitrophenol hydroxylation, *N*-dimethynitrosamine *N*-demethylation, were increased 2 to 4 fold due to diabetes (Barnett *et al*., 1994; Dong *et al*., 1988; Raza *et al*., 1996, 2000; Wang *et al.*, 2000a; Sheweita *et al*., 2002). Besides CYP2E1 protein level and enzyme activities, mRNA levels of CYP2E1 were increased 4 to 7 fold in both STZ and alloxan-induced diabetic rats (Dong *et al*., 1988).

The effect of STZ induced diabetes on the CYP2 isozymes in mice was also determined. Recent studies have shown that mouse CYP2A1 proteins or their enzyme activities were either significantly lower or unchanged in the STZ-induced diabetic mice compared to the non-diabetic counterparts (Sakuma *et al*., 2001; Shankar *et al*., 2003b). The catalytic activity (pentoxyresorufin o-dealkylase) protein level and mRNA level of CYP2B was induced by streptozotocin induced diabetes (Sakuma *et al*., 2001). Western blot analysis of hepatic CYP2C and CYP2D related proteins indicate that these proteins were not changed as a result of diabetes in mice. Unlike the rats, CYP2E1 level in the liver and kidney of the diabetic mice was not changed due to diabetes while slight enhancement was observed in its associated enzyme activity, aniline 4-hydroxylase (Sakuma *et al*., 2001). Also, Shankar *et al.* (2003b) showed that mouse CYP2E1 proteins and their enzyme activities were significantly decreased in the STZ-induced diabetic mice compared to the non-diabetic counterparts.

The effects of acute and chronic streptozotocin diabetes on hamster's kidney and liver CYP2 isozymes were studied by Chen *et al.,* in 1996. They found that acute diabetes caused 2-fold increases of aniline 4-hydroxylase and N-nitrosodimethylamine demethylation activities in hamster liver and kidney microsomes (P4502E1 associated enzymes). In contrast, the acute diabetic condition caused 66% and 48% decrease of renal and hepatic pentoxyresorofin-*O*-dealkylation activity (P4502B1 associated), respectively. Also immunoblotting of microsomal proteins showed that diabetes induced proteins immunorelated to P4502E1 in kidney and liver. In diabetic conditions, the level of P4502B-immunorelated protein(s) was decreased (Chen *et al.,* 1996 and Takatori *et al*., 2002). The level of CYP2C11 expression was slightly depressed in one month and six month diabetes duration, although almost equivalent to the control hamsters in three month diabetes duration. The level of CYP2C6 was significantly lower than those of age matched control hamsters in 1 month and 6 month diabetes duration (Takatori *et al*., 2002).

As described above, although the effect of diabetes mellitus on rat, hamster and mice cytochrome P450 and monooxgenase activities have been studied extensively (Barnett *et al*., 1990a,b; Shimojo *et al*., 1993; Yang and Hong, 1995; Chen *et al*., 1996; Wang *et al*., 2000; Sakuma *et al*., 2001), no data has been available regarding the influence of diabetes on rabbit cytochrome P450 and enzyme activities until recently (Arınç *et al*., 2005, 2007). These current studies have showed that the level of CYP2B4 and associated enzyme benzphetamine N-demethylase activity have not been changed in diabetic rabbit liver, lung and kidney. On the other hand, the data obtained from immunoblot analysis and enzymatic activity studies clearly demonstrated that diabetes induced CYP2E1 and associated enzyme activities not only in rabbit liver but also in kidney and lung (Arınç *et al*., 2005, 2007).

A large number of studies have demonstrated that diabetes modifies a number of drug metabolizing enzymes in liver and kidney and marked species-related differences exist in the modulation of members of CYP2 family.

1.3.3 CYP3 Family

CYP3 is the most abundant CYP family in human liver. It consists of a single subfamily (CYP3A) within which there are four functional genes (*CYP3A4, CYP3A5, CYP3A7, CYP3A43*) in humans. They share more than 71% amino acid sequence identity with each other. It was demonstrated that five different CYP 3A isoforms was constitutively expressed in rat, whereas the rabbit expresses the CYP 3A6, the only CYP 3A isoform identified in the rabbit so far (Guengerich, 1997; Chirulli *et al*., 2005; http://drnelson.utmem.edu/CytochromeP450.html). The rabbit isoform CYP3A6 and the human isoform CYP3A4 have similar P450 predominance and substrate specificity (Weber *et al*., 2001).

The CYP3A subfamily is considered to have the greatest overall impact on human pharmacotherapy because it is the most abundantly expressed CYPs in the human liver and small intestine and possesses metabolic activity toward an extremely broad spectrum of xenobiotic substrates; CYP3A4 (CYP3A6 in rabbit) is known to metabolize more than 50% of all known therapeutic drugs, as well as many endogenous compounds in humans. Not surprisingly, it is involved in many clinically relevant drug-drug interactions. In all, cytochrome CYP3A4 is known to metabolize more than 120 different drugs include macrolide antibiotics (erythromycin), anti-arrhythmics (quinidine), sedatives (midazolam), immune system modulators (cyclosporine), HIV-directed antiviral agents (ritonavir and saquinavir), prokinetic agents (cisapride), anti-histamines (astemizole), calcium channel blockers (nifedipine and verapamil), HMG CoA reductase inhibitors (lovastatin), opioid analgesic, stimulants and non-benzodiazepine hypnotics (zolpidem) (Martin and Krum, 2003; Arayne *et al*., 2005; van Herwaarden *et al*., 2005; Sica, 2006; Sugimoto *et al*., 2006; de Wildt *et al.,* 2007; Klotz, 2007). Also,

CYP3A4 is involved in the metabolism of endogenous steroidal substrates including testosterone, progesterone, and androstenedione (Yamazaki and Shimada, 1997; Wang *et al*., 2000). A representative list of CYP3A substrates, inducers and inhibitors is shown in Table 1.5.

Table 1.5 Substrates, inducers and inhibitors of CYP3A (adapted from Danielson, 2002)

Substrates		**Inhibitors**	**Inducers**
Alfentanyl	Lovastatin	Amiodarone	Barbiturates
Alprazolam	Methadone	Azithromycin	Carbamazepine
Amlodipine	Midazolam	Cimetidine	Efavirenz
Androstenedione	Nelfinavir	Ciprofloxacin	Glucocorticoids
Astemizole	Nifedipine	Clarithromycin	Modafinil
Atorvastatin	Nisoldipine	Delaviridine	Nevirapine
Azithromycin	Nitrendipine	Diltiazem	Phenobarbital
Buspirone	Odanestron (In Part)	Erythromycin	Phenytoin
Caffeine	Pimozide	Fluconazole	Pioglitazone
Cerivastatin	Progesterone	Fluvoxamine	Rifampin
Chlorpheniramine	Propranolol	Gestodene	St. John's Wort
Cisapride	Quinidine	Grapefruit Juice	Troglitazone
Clarithromycin	Quinine	Indinavir	
Cocaine	Ritonavir	Interleukin-10	
Codeine	Salmeterol	Itraconazole	
Cyclosporine	Saquinavir	Ketoconazole	
Dapsone	Sildenafil	Mibefradil	
Dextromethorphan	Simvastatin	Mifepristone	
Diazepam	Tacrolimus (FK506)	Nefazodone	
Diltiazem	Tamoxifen	Nelfinavir	
Erythromycin	Taxol	Norfloxacin	
Estradiol	Terfenadine	Norfluoxetine	
Felodipine	Testosterone	Ritonavir	
Fentanyl	Trazodone	Saquinavir	
Finasteride	Triazolam		
Haloperidol	Verapamil		
Hydrocortisone	Vincristine		
Indinavir	Zaleplon		
Lidocaine	Zolpidem		

Changes in CYP3A proteins were monitored using diagnostic enzyme activities (testosterone 2β- and 6β-hydroxylation) and immunologically utilizing specific polyclonal antibodies in rats. According to these studies, CYP3A expression and its associated enzyme activities were significantly increased in STZ-induced diabetic rats (Barnett *et al*., 1990b, 1993 and 1994; Shimojo *et al*., 1993; Raza *et al*., 1996). On the other hand, Thummel and Schenkman reported that CYP3A protein level was decreased in male rats and increased in female rats (Thummel and Schenkman 1990).

Moreover, hepatic CYP3A dependent catalytic activity, erythromycin *N*-demethylase, was significantly higher in the streptozotocin-induced diabetic mouse of both sexes than in the respective control group In contrast to the microsomal enzyme activity, there was no clear increase in CYP3A (CYP3A11) related protein bands in diabetic mouse. Moreover, when mouse CYP3A subfamily mRNA levels (3A11, 13, 16, and 41) were analysed, only CYP3A11 showed induction in the livers of the streptozotocin-induced diabetic ddY, whereas the other three isoforms were not altered. These results showed that each CYP3A isoform has a different sensitivity to diabetic complicatios and these CYP3A isoforms are not controlled by uniform regulatory mechanisms (Sakuma et al., 2001). The apparent discrepancy between apoprotein and mRNA levels may be attributable to the other unaltered CYP3A related proteins (Sakuma *et al*., 2001). In another study, Shankar and coworkers found that CYP3A related protein was decreased about 3-fold in diabetic mouse (Shankar *et al*., 2003b).

As mentioned above, different laboratories have reported either decreased or increased CYP3A protein expression following STZ treatment in rats and mouse. The conflicting data on diabetic regulation of CYP3A proteins might be due to different specificities of the antibodies used for the Western blot assays, coupled with differential regulation of CYP3A family members or different duration of the diabetes in animals (Cheng and Morgan, 2001).

Futhermore, CYP3A protein in hamster was suppressed about 1.5-fold in STZ induced diabetes (Takatori *et al*., 2002). Although the effect of diabetes on CYP3A protein level and associated enzyme activities were extensively studied in rat, mouse and hamster, there was no available data on rabbit CYP3A protein up to now.

1.3.4 CYP4 Family

The CYP4 family consists of 18 subfamilies (CYP4A-CYP4T), which encode constitutive and inducible isozymes expressed in mammals, insects and fish. (http://drnelson.utmem.edu/CytochromeP450.html; Simpson, 1997). In humans, CYP4 family consists of five subfamilies within which there are 12 functional genes. These family members are involved in the fatty acid ω-hydroxylation and eicosanoids metabolism. In addition to endogenous substrates, recent evidence indicates that CYP4 monooxygenases (CYP4B) can also metabolize xenobiotics, including valproic acid, 3-methylindole, 4-ipomeanol, 3-methoxy-4-aminoazobenzene, and numerous aromatic amines and other therapeutic drugs (Baer and Rettie, 2006; Hsu *et al*., 2007). Because of their role in metabolism of endogenous substrates, it is not surprising that members of CYP4 family are constitutively expressed in liver and other tissues such as kidney, lung and intestine.

The CYP4A genes, the most extensively studied members of the CYP4 family, are expressed constitutively in mammalian liver and kidney and their expression is induced by a class of chemicals known as peroxisome proliferators, which includes the hypolipidemic drug, clofibrate. Induction of CYP4A expression by clofibrate is due to transcriptional activation, mediated possibly via a peroxisome proliferator activated receptor (PPAR) (Simpson, 1997). There are at least two functional proteins (CYP4A11 and CYP4A22) in human and four functional CYP4A proteins (CYP4A4, CYP4A5, CYP4A6 and CYP4A7) in rabbit (http://drnelson.utmem.edu/CytochromeP450.html). They share 74-90% sequence homology with each other and also human CYP4A11 shares more than 80% amino acid similarities to rabbit CYP4A4, CYP4A5, CYP4A6, CYP4A7 (Okita and Okita,

2001). The CYP4A subfamily is capable of hydroxylating the terminal ω-carbon and, to a lesser extent, the (ω-1) position of saturated and unsaturated fatty acids such as arachidonic and lauric acids as well as the enzymes active in the ω-hydroxylation of various prostaglandins (Sharma *et al.,* 1989; Okita and Okita, 2001).

Studies with diabetic rats showed that CYP4A expression and/or activities are induced STZ-induced diabetic rats. Shimojo and co-workers reported that CYP4A2 and 4A3 protein levels and associated CYP4A catalyzed lauric acid *ω*- and *ω*-1-hydroxylations were induced 2 to 4-fold n STZ induced diabetic rats. Unlike CYP4A2 and 4A3, CYP4A1 was not changed in the liver of diabetic rats (Shimojo *et al*., 1993). Barnett and co-workers reported that diabetes enhanced the hydroxylation of lauric acid in both male and female rats. Western blot studies showed that increases in CYP4A protein were only in male rats (Barnett *et al*., 1990b and 1993). Moreover, Barnett *et al*., (1994) showed that hepatic CYP4A1 protein and lauric acid hydroxylase activity peaked at four weeks following the treatment of STZ in rats, and declined at 12 weeks of diabetes to levels similar to those in the non-diabetic rats. Furtermore, hepatic mRNA levels of CYP4A1, 4A2, and 4A3 were induced three- to eightfold in the STZ-induced diabetic rats (Kroetz *et al*., 1998). Also, CYP4A1 and 4A2 inductions in extrahepatic tissues such as kidney and/or brain of STZ-induced diabetic rats have also been shown (Clarke *et al*., 1996; Raza *et al*., 1996; Engels *et al*., 1999).

In the mouse model of STZ-induced diabetes, protein levels and mRNA of two isoforms of CYP4A, CYP4A10 and CYP4A14 were increased in both sexes (Sakuma *et al*., 2001). Moreover, hamster CYP4A protein was increased in STZ-induced diabetes (Takatori *et al*., 2002). But, no data is available on the effect of diabetes on rabbit CYP4A protein level and activities.

The effects of type 1 diabetes on the modification of cytochrome P-450 isozymes in rat, mice, hamster and rabbit are as shown in Table 1.6.

Table 1.6 Modification of hepatic cytochrome P-450 by diabetes in rat, mouse, hamster and rabbit

CYP450	RAT		MOUSE		HAMSTER		RABBIT	
	CHANGE	**REFERENCES**	**CHANGE**	**REFERENCES**	**CHANGE**	**REFERENCES**	**CHANGE**	**REFERENCES**
CYP1A	~ 2-fold ↑	Yamazoe *et al*., 1989 Barnett *et al*., 1990a Raza *et al*., 1996 Engels *et al*., 1999 Shankar *et al*., 2003b Sindhu *et al*., 2006	~ 2-fold ↓ **N.C.**	Shankar *et al*., 2003b Sakuma *et al*., 2001	~ 4-fold ↑ ~ 2-fold ↓	Chen *et al*., 1996 Takatori *et al*., 2002		
CYP1B	~ 5-fold ↑	Sindhu *et al*., 2006						
CYP2A	**2A1** 2-4 fold **↑ F** **2A2**~4-fold **↓ M**	Yamazoe *et al*., 1989 Thummel and Schenkman, 1990 Shimojo *et al*., 1993	**N.C.**	Sakuma *et al*., 2001				
CYP2B	~9-fold ↑	Barnett *et al*., 1990a Raza *et al*., 1996 Sindhu *et al*., 2006	↑	Sakuma *et al*., 2001	~ 1.5-2-fold ↓	Chen *et al*., 1996 Takatori *et al*., 2002	**N.C.**	Arınç *et al*., 2005, 2007
CYP2C	**2C6** ~2-fold **↑ F** **2C7** ~2-fold **↑ F** **2C11** 4-9 fold **↓ M**	Yamazoe *et al*., 1989 Donahue and Morgan, 1990 Thummel and Schenkman, 1990 Donahue *et al*., 1991 Shimojo *et al*., 1993 Sindhu *et al*., 2006	**N.C.**	Sakuma *et al*., 2001	**2C6** ~ 2-fold **↓ M** **2C11 ↓ / N.C M**	Takatori *et al*., 2002		

Table 1.6 Modification of hepatic cytochrome P-450 by diabetes in rat, mouse, hamster and rabbit (Continued)

CYP2C	**2C12** ↑ **/N.C**. **F** **2C13** ~4 fold ↓ **M**							
CYP2E1	~3-9-fold ↑	Yamazoe *et al*., 1989 Barnett *et al*., 1993 Shimojo *et al*., 1993 Clarke *et al*., 1996 Raza *et al*., 1996 Engels *et al*., 1999 Wang etal., 2000 Shankar *et al*., 2003b Sindhu *et al*., 2006	**N.C**. ~3-fold ↓	Sakuma *et al*., 2001 Shankar *et al*., 2003b	~ 2-fold ↑	Chen *et al*., 1996 Takatori *et al*., 2002	~ 2-fold ↑	Arınç *et al*., 2005, 2007
CYP3A	~2-fold ↑ ~2-fold ↓ **N.C.**	Barnett *et al*., 1990b Thummeland Schenkman, 1990 Barnett *et al*., 1993 Shimojo *et al*., 1993 Raza *et al*., 1996 Shankar *et al*., 2003b	~ 2-fold ↓ **N.C.**	Shankar *et al*., 2003b Sakuma *et al*., 2001	~ 1.5-fold ↓	Takatori *et al*., 2002		
CYP4A	**4A1**~2-fold ↑ **4A2**~3-fold ↑ **4A3**~2-fold ↑	Barnett *et al*., 1990b Barnett *et al*., 1993 Shimojo *et al*., 1993 Raza *et al*., 1996 Kroetz *et al*., 1998	↑	Sakuma *et al*., 2001	~ 1.3-fold ↑	Takatori *et al*., 2002		

F: Female dominat form and **M**: Male dominat form **N.C**. No change

1.4 Modulation of Toxicity in Diabetus Mellitus.

It is well established that induction of the experimental diabetes by STZ or alloxan in rodents results in alteration of activities and/or expressions of cytochrome P450 dependent drug-metabolizing enzymes. Since the vast majority of chemical carcinogens are metabolized by the Mixed Function Oxidase system, it is reasonable to assume that their metabolism will also be changed by diabetes. Indeed, STZ administration to rats for induction of diabetes enhances the metabolism and hepatotoxicity of some well-known human carcinogens such as carbon tetrachloride, thioacetamide, bromobenzene, 1, 1, 2-trichloroethane, nitrosoamines and chloroform. (Hanasono *et al*., 1975; Lorr *et al*., 1984; Watkins *et al*., 1988; Aniya *et al*., 1989; Wang *et al*., 2000a). In contrast to the enhanced hepatotoxicity in type 1 diabetic rats, diabetes in mice unexpectedly protects animals from normally lethal hepatotoxic chemicals such as acetaminophen, carbon tetrachloride, thioacetamide, bromobenzene (Shankar *et al*., 2003a,b,c and Sawant *et al*., 2006a).

Potentiation of metabolic activation of NDMA in diabetic rabbit liver, kidney and lung microsomes was shown by an increase of NDMA N-demethylase activity in parallel to significant CYP2E1 induction in all three tissues (Arınç *et al*., 2007). NDMA requires a metabolic activation to elicit its carcinogenic effects. It is well established that NDMA is converted to its carcinogenic form after it is metabolized by CYP2E1 associated NDMA N-demethylase. Therefore, mutagenicity and carcinogenicity of NDMA are dependent on the activity of NDMA N-demethylase (Yang and Hong, 1995; Liteplo and Meek 2001). Also of concern is the increased potential carcinogenic risk to diabetic patients posed by long term continuous exposure to relatively low level of nitrosoamines from dietary sources including a wide variety of food products and alcoholic beverages, water, soil, industrial sites and environment and tobacco smoke. Thus, we can say that diabetes mellitus stimulates the metabolic activation of *N*-nitrosodimethylamine by inducing CYP2E1 dependent NDMA N-demethylase in liver, kidney and lung,

which results in the formation of increased levels of highly reactive methylated agents from nitrosamines. Thus, it is expected that, in diabetic subjects, the risk of nitrosamine induced carcinogenesis may be greater in liver, kidney and lung (Arınç *et al*., 2007).

Application of another inducer of the CYP2E1 such as benzene, isoniazid or pyridine to the diabetic rabbits may further elevate expression and activities of the CYP2E1. As a result of further or additive induction of the CYP2E1 by two treatments (diabetes and chemical inducer of the CYP2E1), an increased risk of hepatotoxicity produced by toxins which are activated by CYP2E1 may be observed when compared to the separate treatments. In this regard, we claimed that benzene application to the diabetic rabbits may further increase the risk of CYP2E1 induced chemical carcinogenesis such as benzene or nitrosamine by formation of more reactive methylated agents from these compounds.

1.5 Benzene

Benzene, also known as benzol, is a colorless liquid with a sweet smell. Benzene is chemically characterized by six carbon atoms linked in a planar symmetrical hexagon (equal C-C bond lengths) with each carbon atom attached to a hydrogen atom (Figure 1.5). It has a relatively low boiling point (80.1^{o}C) and a high vapour pressure (13.3 kPa), which causes it to evaporate rapidly at room temperature (Merck index, 2006). It is a good solvent for many organic and inorganic compounds, but its use as a solvent has decreased in many countries due to the concern over carcinogenic effects. Benzene is found in air, water, and soil as a result of both human activities and natural processes. Today, benzene is made mostly from petroleum products. Various industries use benzene to make other chemicals, such as ethylbenzene (used in the synthesis of styrene), styrene (for Styrofoam and other plastics), cumene (for various resins), nitrobenzene (used in the synthesis of aniline) and cyclohexane (for nylon and synthetic fibers). Benzene is also used for the manufacturing of some types of rubbers, lubricants, dyes,

detergents, drugs, and pesticides. Because of its wide use, benzene ranks in the top 20 in production volume for chemicals produced in the United States. Natural sources of benzene, which include volcanoes and forest fires, also contribute to the presence of benzene in the environment. Benzene is also a part of crude oil and gasoline and cigarette smoke. The general population is exposed to benzene primarily by tobacco smoke (by both active and passive smoking) and by inhaling contaminated air (motor vehicle exhaust gases). Exposure to benzene can also result from ingestion of contaminated food or water.

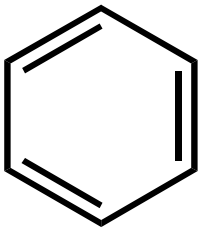


Figure 1.5 Molecular formula of benzene

Acute exposure to high concentrations of benzene by both inhalation and ingestion results in neurological and gastrointestinal toxicity and may sensitize the myocardium to endogenous catecholamines. On the other hand, chronic exposure to benzene causes hematotoxicity, including aplastic anemia, pancytopenia, anaemia, chromosomal abberations, thrombocytopenia and leukopenia (Aksoy *et al*., 1971; Aksoy *et al*., 1974; Aksoy, 1985; Aksoy, 1989; Aksoy, 1991).

Benzene is characterized as a known human carcinogen (Category A) for all routes of exposure based upon convincing human evidence as well as supporting evidence from animal studies. (U.S. EPA, 1985, 1998; ATSDR, 1997). Both epidemiologic studies and case studies have showed that there is a causal association between exposure to benzene and acute myelogenous leukemia, acute lymphocytic leukemia, acute erythroleukemic leukemia, acute myelomonocytic leukemia, acute promyelocytic leukemia, acute undifferentiated leukemia, chronic myelogenous leukemia, chronic lymphocytic leukemia, hairy cell leukemia,

Hodgkin's lymphoma, Non-Hodgkin's lymphoma, lymphosarcoma, multiple myeloma and reticulum cell sarcoma in humans. Besides leukemia and lymphosarcoma, benzene also causes cancers of the lung, liver, stomach, esophagus, nasopharnyx, and intestine (see the review by Mehlman, 2002). Other neoplastic conditions that are associated with an increased risk in humans are hematologic neoplasms, blood disorders such as aplastic anemia (Aksoy *et al*., 1984; Hayes *et al*., 1997). These human data are supported by animal studies (Maltoni *et al*., 1983, 1988, 1989). These long-term bioassays demonstrated that benzene caused tumors in Sprague-Dawley rats, Wistar rats, Swiss mice, and RF/J mice including cancers of the zymbal gland, oral cavity, lung, skin, nasal cavity, forestomach, Harderian gland, mammary gland, ovary, and uterus, lymphoma, hemolymphoreticular neoplasia and all types of leukemias (Cronkite *et al*., 1989; Huff *et al*., 1989; Maltoni *et al*., 1983, 1989).

1.5.1 Benzene Metabolism

The mechanisms by which exposure to benzene and its metabolites exerts its toxic and carcinogenic effects remain elusive. Animal studies showed that benzene itself was unlikely to be the actual toxicant. In order to exert its toxic effects, it must be metabolized by cytochrome P450 system. Benzene has not been found to produce mutagenicity in short-term *in vitro* tests such as Ames Salmonella test, yeast mutagenicity test, unscheduled DNA synthesis and 6-thioguanine resistance test in Chinese hamster V79 cells (Dean, 1985). The Ames test is a biological assay to assess the mutagenic potential of chemical compounds. The bacterium used in the test is a strain of *Salmonella typhimurium* that carries a defective gene making it unable to synthesize the amino acid histidine from the ingredients in culture. When the chemical is given to bacteria, some of them undergo mutations if it is mutagenic. Following a particular type of mutation, the bacteria can grow without histidine. On the other hand, many chemicals such as benzo(a)pyrene are not mutagenic in their native forms, but they are converted into mutagenic intermediates by metabolism in the liver. Since bacteria do not have the same metabolic capabilities as mammals, some Ames test protocols utilize extracts of rat

liver enzymes with the standard induction procedures (Aroclor 1254 or a combination of phenobarbital (PB) and beta-naphthoflavone) in order to promote metabolic conversion of the test chemical. These chemicals induce CYP1A1, 1A2 and CYP2B. Benzene has been classified as a non-mutagen in the Ames test (Dean 1985), possibly because of the inadequacy of the S9 microsomal activation system. Because Aroclor 1254 or phenobarbital or beta-naphthoflavone do not induce CYP2E1 (Burke *et al*., 1994), it has been suggested that this may account for the negative finding with benzene in the Ames test. If S9 fraction is prepared from rats pretreated with pyrazole, ethanol or other inducers of CYP2E1, positive Ames test result will be obtained about benzene mutagenecity. Moreover, inhibition studies by toluene, a competitive inhibitor of benzene, resulted in a decrease in benzene metabolism and a reduction in benzene toxicity (Andrews *et al*., 1977; Medinsky *et al*., 1994). Furthermore, decreasing the hepatic metabolism of benzene by partial hepatectomy reduced benzene toxicity (Sammett *et al*., 1979). Besides these, CYP2E1 dependent metabolism is a prerequisite for the cytotoxic and genotoxic effects of benzene as shown in CYP2E1 knockout mice (Powley and Carlson, 2001). These evidences are quite strong showing that metabolism of benzene must be required for its toxicity. Due to its carcinogenic and toxic effects, elucidation of the metabolic pathway for benzene biotransformation is essential for a full understanding of the mechanism of toxicity and carcinogenecity.

Metabolism of benzene is extremely complex and not yet fully understood. It occurs primarily in the liver. In addition to hepatic metabolism, it appears that secondary metabolism of benzene metabolites in bone marrow contributes to toxicity. There are several toxification pathways and detoxification pathways of benzene metabolism. The first step in benzene metabolism is the formation of benzene oxide, an epoxide, by cytochrome P-450 dependent mixed function oxidases (Post and Snyder, 1983). There are at least two isoforms of CYP450 involved in this step. One of them is CYP2B (CYP2B1 in rat). It was characterized as a high-Km isoform for benzene oxidation (Km for CYP2B is 10 mM). The second CYP450 isoform involved in benzene oxidation is CYP2E1. Km value for the CYP2E1 was 10-fold lower than that of CYP2B (Km for CYP2E1 is 1 mM)

(Post and Snyder, 1983). Induction of the CYP2E1 by ethanol, isopropanol and acetone increased benzene metabolism in rat and rabbit liver microsomes and rat hepatocytes (Johansson and Ingelman-Sundberg, 1988; Schrenk *et al*., 1992). Also, inhibition of CYP2E1 by diethyldithiocarbamic acid caused a decrease in the metabolism of benzene by 96% in liver microsomes and 54% in lung microsomes from rat (Chaney and Carlson 1995). Studies with CYP2E1 knockout mice was shown that benzene metabolism was decreased in knockout mice. Furthermore, genotoxicity or cytotoxicity were not detected in bone marrow, spleen, thymus, or blood of CYP2E1 knockout mice but were detected in the wild-type controls following a benzene exposure (Valentine *et al*., 1996). All these studies suggest that CYP2E1 is the cytochrome P450 isozyme primarily responsible for metabolizing benzene although CYP2B may be involved at high substrate concentrations.

The resulting benzene oxide establishes equilibrium with its oxepin or may rearrange non-enzymatically to form phenol. Cytochrome P4502E1 mediated metabolism of phenol (major metabolite) leads to the formation of hydroquinone and catechol (secondary metabolites) and the subsequent formation of 1,2,4-benzenetriol. The hydroxylated aromatic benzene metabolites are further metabolized to sulfate or glucuronic acid conjugates as well as phenol. Moreover, hydroquinone and catechol are further metabolized by myeloperoxidase in bone marrow to form reactive *p*-benzoquinone and *o*-benzoquinone (Eastmond *et al*., 1987; Sadler *et al*., 1988). Benzene oxide is also a substrate for epoxide hydrolase, which adds water across the epoxide to yield benzene dihydrodiol (1,2,-dihydroxy-3,5-cyclohexadiene), which, in turn, can be oxidized by dihydrodiol dehydrogenase to yield catechol (Henderson *et al*., 1989). An alternative pathway involves the conjugation of benzene oxide with glutathione as the first step in a pathway that ultimately results in the formation of phenyl mercapturic acid (Henderson *et al*., 1989, Schafer *et al*., 1993). Another alternative pathway is a ring opening either at the benzene oxide or oxepin stage with the resulting formation of muconaldehyde through a NADPH mediated process. An indicator for muconaldehyde formation sould be the appearance of trans, trans-muconic acid in the urine (Goldstein *et al*., 1981; Latriano *et al*., 1986). Metabolic pathways of benzene are given in Figure 1.6.

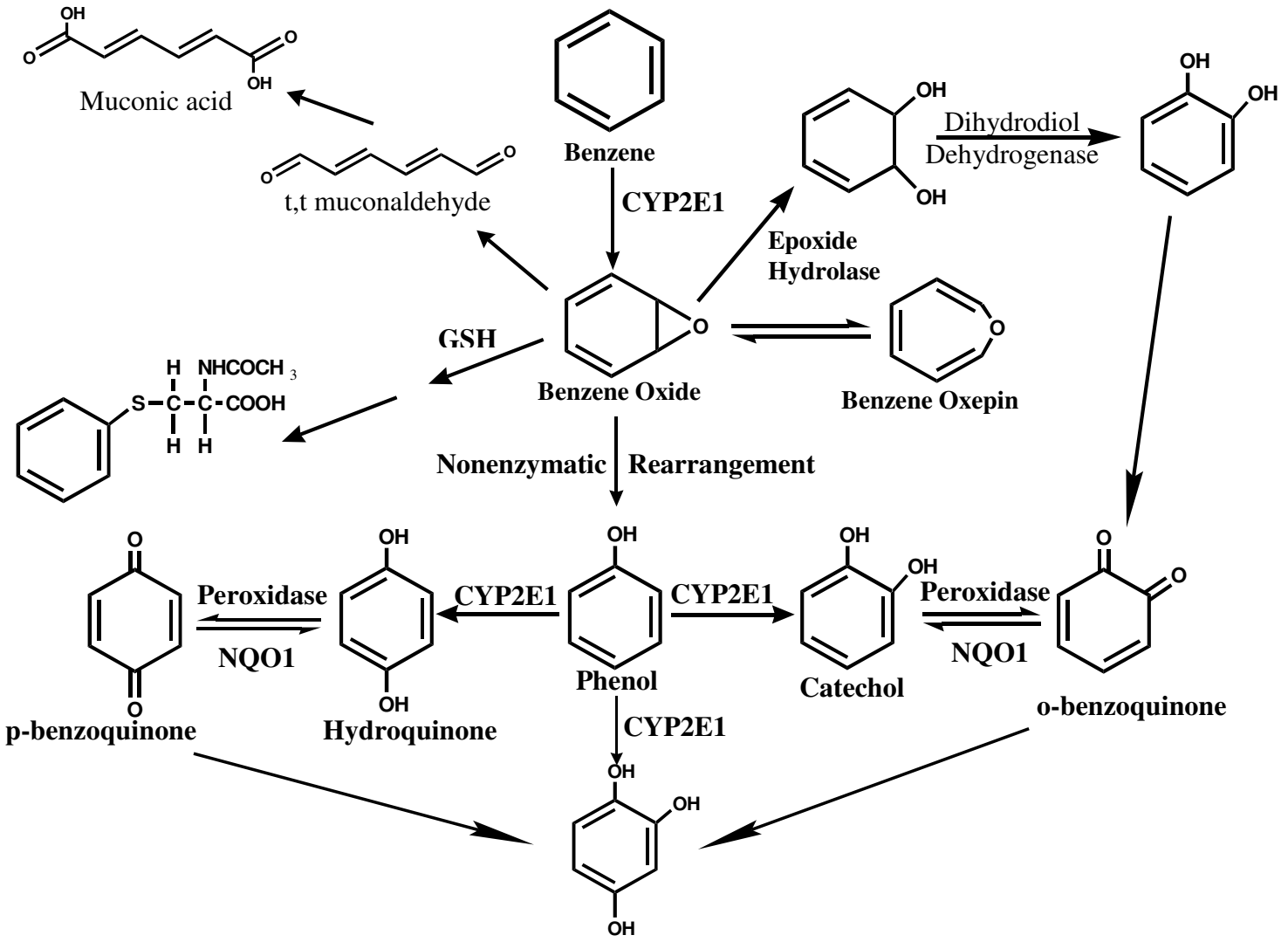


Figure 1.6 Metabolic pathways of benzene adopted from Daiker et al., 1996, Ross, 1996 and Snyder and Hedli, 1996.

1.5.2 Benzene Toxicity

Although the actual molecular mechanism of action has not yet been fully elucidated, many authors described adducts formed by the covalent binding of benzene reactive metabolites with proteins, DNA and RNA. Oxidative stress produced by glutathione-depleting metabolites of benzene and damage of oxygen radicals on nucleic acids and proteins seems to be another mechanism of toxicity (Eastmond *et al*., 1987; Parke, 1989; Yardley-Jones *et al*., 1991; Snyder *et al*., 1993).

The search for one or more mechanisms of toxicity began with the hypothesis that toxicity was related to the production of one or more of the following metabolites: phenol, hydroquinone (and by extension, *p*-benzoquinone), catechol (and by extension, *o*-benzoquinone), 1,2,4-benzenetriol, and muconaldehyde in liver that is followed by their transport to the bone marrow and other target organs. The target organs for benzene carcinogenicity in rodents are rich in enzymes that may confer tissue sensitivity to benzene, as is human bone marrow (Low *et al*., 1989, 1995). The bone marrow, Zymbal gland, and Harderian gland all contain peroxidases, which can activate phenols to toxic quinones and free radicals. The peroxidases reported to be present in bone marrow include myeloperoxidase (MPO) (Bainton *et al*., 1971), prostaglandin synthase (Gaido and Weirda, 1987), and eosinophil peroxidase; of these, MPO is known to be present in bone marrow in high concentrations, and such a peroxidase can readily bioactivate benzene- derived phenolics to reactive quinones and free radicals in situ in bone marrow (Bhat *et al*., 1988). Sulfatases, which remove conjugated sulfate and thus reform free phenols, are also present at high levels in these target organs. Phenolic metabolites of benzene can be activated by peroxidases to reactive quinone derivatives (Subrahmanyam *et al*., 1991). Peroxidase activation of hydroquinone is known to result in covalent binding to protein (Subrahmanyam *et al*., 1989; Ganousis *et al*., 1992) and the formation of DNA adducts, as detected by ^{32}P-post-labeling (Lévay *et al*., 1993), in both murine and human bone marrow in vitro. 3'-OH-1,N^2-benzetheno-2'-deoxyguanosine, 3'-OH-3,N^4-benzetheno-2'-Deoxycytidine

and 3'-OH-1,N^6-benzetheno-2'-deoxy- adenosine are the major deoxyribonucleoside adducts of *p*-benzoquinone (Pongracz *et al*.,1990 and Lévay *et al*. 1993). The synergistic interactions between phenolic metabolites exacerbate benzene toxicity (Chen and Eastmond 1995; Eastmond *et al*. 1987; Subrahmanyam *et al*., 1990). This mechanism of multi-metabolite genotoxicity is an unique aspect of benzene that distinguishes it from other chemicals in terms of the mechanism of its toxicity and carcinogenicity. It was shown that phenol and hydroquinone, when administered together, produced bone marrow toxicity in mice that was very similar to that produced by benzene, whereas alone they did not (Eastmond *et al*., 1987). Phenol enhanced the conversion of hydroquinone into 1,4-benzoquinone catalyzed in vitro by myeloperoxidase (Smith *et al*., 1989) and that catechol would also stimulate the peroxidase-dependent activation of hydroquinone (Subrahmanyam *et al*.,1991). Catechol and hydroquinone also produced a synergistic genotoxic effect in human lymphocytes (Robertson *et al*., 1991). The electrophilic 1,4-benzoquinone thus formed is able to bind to cellular proteins and DNA. Although in vitro studies have established that reactive metabolites of benzene covalently bind to DNA, in vivo evidence of covalent binding has been more difficult to demonstrate because of low covalent binding index of benzene. In vivo studies using rats and mice, demonstrated covalent binding to DNA of several animal tissues including liver, bone marrow, spleen, kidney, stomach, and lung (Arfellini *et al*., 1985; Mazullo *et al*., 1989; Reddy *et al*., 1989). Conflicted results were obtained for the identification of adduct formation in vivo using the [^{32}P] postlabeling method (Hedli, 1994 and Reddy *et al*., 1990, 1994 referred by Synder, 2002). Benzene treatment by gavage caused three faint spots in the post-labeling assay in the Zymbal gland of rats (Reddy *et al*., 1994 referred by Synder, 2002). On the other hand, Hedli (1994), also using the post labeling assay, failed to detect DNA adducts in the bone marrow of male Sprague Dawley rats treated with benzene intraperitoneally (Hedli, 1994 referred by Synder, 2002). These problems may be related to the low covalent binding index of benzene, the complex nature of the bone marrow, and difficulties in establishing both an optimal treatment regimen and an animal model system that accurately reflects all the toxic responses to benzene observed in humans (Synder and Hedli, 1996).

As a results of binding of reactive metabolites to DNA and proteins, DNA strand breaks (Lee and Garner, 1991), topoisomerase II inhibition (Chen and Eastmond 1995), and damage to the mitotic spindle, histone proteins and other DNA associated proteins (Irons, 1985) can be observed. This leads to mitotic recombination (Celi and Akbaş 2005), chromosome translocations and aneuploidy (the loss and gain of whole chromosomes) (Chung and Kim, 2002 and Zhang *et al*., 2007). These genotoxic events will, in turn, cause leukemia and multiorgan carcinogenicity.

The potential for oxidative stress to contribute to benzene toxicity is closely tied to specific benzene metabolites. It was suggested that reactive oxygen species could be generated as a result of auto-oxidation of 2-OH-5-glutathionyl *p*-benzoquinone formed by glutathione conjugation with either 2-OH-*p*-benzoquinone or 2,3-epoxy-*p*-benzoquinone, each of which is a potential metabolite of benzene (Brunmark and Cedenas, 1988). Moreover, phenolic metabolites of benzene (hydroquinone, *p*-benzoquinone and 1,2,4-benzenetriol) could modify the catalase, superoxide dismutase (SOD), nitric oxide, sulfhydryl level, hydrogen peroxide and superoxide production in different manner (Rao and Synder, 1995). All of these cause oxidation of protein, lipid and DNA and single strand break. These oxidation events will, in turn, cause tissue necrosis and carcinogenesis.

It is well established that metabolic activation and detoxification of benzene and its phenolic metabolites appeared to be important steps in benzene hematotoxicity. Most of the enzymes involved in benzene metabolism such as CYP2E1, NQO1, Glutathione S-Tranfereseases (GST) and Myeloperoxidases (MPO) are known to vary substantially among individuals and among ethnic groups (Ada *et al*., 2004: Ross, 2005; Ulusoy *et al*., 2007a; Zhamg *et al*., 2007). Therefore, the deficiency or altered activity of enzymes involved in benzene metabolism would significantly affect susceptibility to benzene toxicity. Recent studies showed that the combined effect of polymorphisms in *NQO1, CYP2E1,* and *GSTT1* genes and enviromental factors such as cigarette smoking and alcohol consumption might contribute to benzene poisoning (Wan *et al*., 2006; Ulusoy *et al*., 2007b).

1.5.3 Effects of Benzene Treatment on Cytochrome P450 Dependent Drug Metabolizing Enzymes

Benzene has been found to stimulate its own metabolism, thereby increasing the rate of toxic metabolite formation. Pretreatment of mice, rats, and rabbits subcutaneously with benzene increased benzene metabolism by increasing CYP2E1 concentrations (Gonasun *et al*., 1973; Arinc *et al*. 1991; Gut *et al*., 1993; Wang *et al*., 1996).

The effects of pretreatment with benzene on hepatic and pulmonary microsomal CYP450 enzymes were studied in male rats by Pyykkö *et al*., in 1987. In this study, male Sprague Dawley rats were treated with single dose of 5 mmol/kg b.w. benzene. P450 contents, cytochrome b5 levels and NADPH-cytochrome P450 reductase activities were not significantly changed by benzene treatment. Moreover, CYP1A dependent aryl hydrocarbon hydroxylase and 7-ethoxycoumarin O-deethylase activities were not changed in liver and lung by benzene treatment. Slight increase in 7-erthoxyresorufin O-deethylase activity was observed in liver and lung microsomes but, this increase was not found to be statistically significant.

In 1993, Gut *et al*., demonstrated that benzene administration (4 mg/l, 20 h per day for 4 days in a dynamic inhalation apparatus) to rats elevated hepatic CYP2E1 level approximately 5-fold as compared to controls. This was supported by the substantial 2-fold increase in aniline 4-hydroxylase activity. Moreover, in vitro CYP2E1 related ^{14}C benzene oxidation (0.35 mM, low Km) was also induced about 5-fold in benzene treated rats microsomes. Besides CYP2E1 and its associated enzyme activities, CYP2B1 protein level was increased in benzene treated rats. On the other hand, CYP1A1/1A2, CYP2A1 and CYP3A1 protein levels was supressed 1.5-, 2- and 1.5-fold as a result of benzene treatment, respectively.

The effects of hydrocarbon treatment including benzene on CYP450s were examined in 1993 by Backes and co-workers. Rats were treated with daily i.p. injections of 10mmol/kg b.w. benzene for 3 days. P450 content, cytochrome b5

level and NADPH-cytochrome P450 reductase activity was not changed by benzene treatment. Moreover, p-nitroanisole demethylation and benzphetamine N-demethylation were not changed. Aniline 4-hydroxylase and NDMA N-demethylase activities were not changed in benzene treated rat liver, also. Although benzene treatment produced a smaller inductive response in CYP2B1/2 protein level, CYP2E1 did not appear to be induced by benzene treatment. CYP1A1/2 protein level and associated 7-ethoxyresorufin O-deethylase activity were increased 2- and 4-fold by benzene treatment, respectively. On the other hand, CYP2C11 protein level was suppressed approximately 3-fold in benzene treated rat liver.

The effects of benzene exposure (4000 ppm for 6 hour) on cytochrome P450 isozymes and their associated enzyme activities were examined by Wang and co-workers in 1996. Benzene treatment caused significant induction of CYP2E1 (1.31-fold) and CYP2B1/2 level. This can be observed from the results of western blot with anti-CYP2E1 and anti-CYP2B1/2, and also from the increased inhitable toluene side-chain oxidase activities by antibodies at low and high substrate concentrations. These activities can be attributed to CYP2E1 and CYP2B1/2, respectively. At the same time, CYP2E1 associated NDMA N-demethylase activity and CYP2B associated 7-pentoxyresorufin O-dependentylase activity were increased abot 1.4- and 5-fold due to benzene treatment. Moreover, benzene also induced CYP2A1 and CYP4A1 protein level about 1.4- and 1.3-fold, respectively. CYP3A1 and CYP1A protein levels were not changed as a result of benzene treatment. There was no agreement with report in which CYP2A1 and CYP3A1 were suppressed (Gut *et al*., 1993). This was explained by different exposure protocols being applied in these two studies (Wang et al., 1996). Furthermore, toluene metabolism inhibition studies with CYP2C11 antibodies and a specific inhibitor, cimetidine, showed that benzene inhibited the level of CYP2C11 in liver microsomes. On the other hand, western blot and inhibition studies with specific inhibitors showed that CYP2C13 and CYP1A1 levels were not changed as a result of benzene treatment. In this study, there was determined that benzene metabolism was increased in the rats exposed to benzene by measuring benzene metabolites, phenol and hydroquinone.

The effects of benzene on the components of mixed function oxidase system and cytochrome P450 dependent drug metabolizing enzymes were determined in rabbit liver, kidney and lung (Arınç *et al*., 1988, 1991). Rabbits were treated with benzene (880mg/kg/day), s.c. for 3 consecutive days in these studies. Benzene treatment caused 3.8-and 5.7-fold increases in aniline 4-hydroxylation rates of liver and kidney microsomes, respectively. Similarly, benzene markedly enhanced hydroxylation rates of p-nitrophenol by liver and kidney by 7.2- and 4.2-fold, respectively. Benzene treatment did not alter CYP2E1 dependent activities in lung microsomes. Moreover, benzphetamine N-demethylase, associated with P4502B4, was not altered significantly in liver and lung microsomes (Arınç *et al*., 1988). Cytochrome b5 content was not changed in liver and kidney microsomes by benzene treatment. Although the total cytochrome P-450 contents of liver and kidney microsomes were not altered significantly by the benzene treatment, in the case of liver microsomes, formation of a new cytochrome P-450 with an apparent Mr of 51.400 was observed on SDS-PAGE. On the other hand, in the kidney microsomes, the intensity of the bands corresponding to approximate Mr of 50.000 and 51.400 was markedly increased (Arınç *et al*., 1991).

1.6 Aim of This Study

It is well established that cytochrome P450 dependent drug metabolizing enzyme activities and expression can be profoundly altered in disease states. The levels of affected cytochrome P450 enzymes are depressed/induced by diseases, causing potential and documented impairment of drug clearance and clinical drug toxicity. The susceptibility of patients with altered cytochrome P450 enzymes to the adverse effects of chemicals including environmental pollutants, toxins, carcinogens and other xenobiotics is also a very important concern. In addition, analysis of cytochrome P450 isozymes in patients is helpful in elucidating the impaired metabolism of some endogenous substrates catalyzed by the cytochrome P450, such as steroid hormones and fatty acids. Diabetes is very important disease that affects millions of people worldwide every day. Both metabolic and hormonal disturbances

following insulin deficiency in diabetics are responsible for changes of cytochrome P450 dependent enzymes.

Induction of diabetes by diabetogens enhances the metabolism and hepatotoxicity of some well-known human carcinogens such as carbon tetrachloride, thioacetamide, bromobenzene, 1, 1, 2-trichloroethane, nitrosoamines and chloroform in rats. (Hanasono *et al*., 1975a,b; Lorr *et al*., 1984; Watkins *et al*., 1988; Aniya *et al*., 1989 and Wang *et al*., 2000a). Unlike the diabetic rats, diabetes in mice protects animals from normally lethal hepatotoxic chemicals such as acetaminophen, carbon tetrachloride, thioacetamide, bromobenzene (Shankar *et al*., 2003 a, b,c and Sawant *et al*., 2006a). Although rabbits are widely used for the cytochrome P450 dependent drug metabolism studies, there has been only one available study about the effect of diabetes on hepatotoxicity of chemicals in rabbits (Arınç *et al*., 2007).

Benzene was selected in this study. Because, despite intensive studies over several decades, the mechanisms underlying benzene-induced hematotoxicity and carcinogenicity are still not fully understood. Benzene is a preferential substrate of one particular cytochrome P450 family, namely cytochrome P4502E1 (CYP2E1), which also metabolizes alcohol and aniline. CYP2E1 can be induced by these substrates and is associated with the generation of hydroxyl radicals, probably via futile cycling of the cytochrome (Chepiga *et al*., 1990; Parke 1989; Snyder *et al*., 1993). Therefore, exposure to chemicals or pathophysiological situations that stimulate the activity of this enzyme system prior to exposure to benzene could increase the rate of benzene metabolism. This may further increase the risk of chemical carcinogenesis by formation of more reactive agents from benzene.

Classic carcinogens, such as benzo[a]pyrene, aromatic amines, and aflatoxin, are thought to be activated to a single, ultimately carcinogenic metabolite. Typically, these metabolites are highly electrophilic and bind strongly to DNA in a covalent fashion. Covalent binding of this type is readily measured as bound radioactivity. Classic carcinogens and their metabolites are also highly mutagenic in

the Ames Salmonella test producing point mutations and small deletions. On the other hand, benzene presents the exact opposite scenario. It does not form a single highly electrophilic metabolite. Its epoxide, benzene oxide, is highly unstable and rapidly rearranges to the major metabolite phenol. Alternatively, it is metabolized via epoxide hydrolase and dihydrodiol dehydrogenase to catechol (Smith, 1996). None of the prominent metabolites of benzene are "hard" electrophiles and thus little binding to DNA is detected as bound radioactivity when radiolabeled benzene is administered to experimental animals (Lutz, 1986). As decribed previously, benzene and the majority of its metabolites are also not mutagenic in the Ames Salmonella test (Dean 1985). However, they produce chromosomal damage both in vitro and in vivo (Wolman 1977; Dean 1985; Yager *et al*., 1990). Primary benzene metabolites include phenol, hydroquinone, catechol, and *trans-trans* muconic acid (Ross, 2000). The synergistic interactions between these phenolic metabolites exacerbate benzene toxicity (Eastmond *et al*., 1987; Subrahmanyam *et al*., 1990; Chen and Eastmond 1995; Smith, 1996). This mechanism of multi-metabolite genotoxicity is another unique aspect of benzene that distinguishes it from other chemicals in terms of the mechanism of its toxicity and carcinogenicity. Moreover, benzene can be characterized further in terms of its multi-site carcinogenicity including the hemopoietic system, Zymbal gland, Harderian gland, preputial gland, mammary gland, ovary, liver and lung (Huff *et al*., 1989; Maltoni *et al*., 1989).

Effects of diabetes on cytochrome P450 (CYP) dependent drug metabolizing enzymes have not been clarified yet. Rats, mice and hamsters have been widely used animal in these studies. Rabbits are widely used for the cytochrome P450 dependent drug metabolism studies. However information regarding the effect of diabetes on cytochrome P450 dependent drug metabolizing enzyme activities is limited in rabbits (Arınç *et al*., 2005, 2007). In order to understand species and tissue specific metabolism of drugs and bioactivation and detoxification of toxic compounds in diabetic animals, it is necessary to carry out comparative studies.

As can be seen Thalidomide Episode, before marketing the drug in late 1950s, toxicity studies were carried out only with rat. From 1956 to 1962, thousands of children were born with birth defects. Thalidomide was accepted as the teratogenic agent. Therefore, it was withdrawn from commercial sale in late 1961. After withdrawn, thalidomide's teratogenic effect has been tested in 10 strains of rats, 15 strains of mice, 11 breeds of rabbit, 2 breeds of dogs, 3 strains of hamsters, 8 species of primates and other such varied species as cats, armadillos, guinea pigs, swine and ferrets. Eventually, only after the administrating of thalidomide to certain species of rabbit (New Zealand White) and primates showed teratogenic toxicity which was similar to those of humans (Hawkins, 1983). Now, it is accepted that toxicity studies of the drugs have to be carried out in at least two species. The first animal to be used is the rat the second animals is the rabbit. Thus, in P450-depentent drug metabolism studies, it is necessary to carry out the experiments in more than one type animal and do comparative studies. Although rabbits are used in P450 dependent drug metabolism studies, it has not been widely used in drug metabolism studies in diabetes (Arınç *et al*., 2005, 2007).

In this regards, the aims of this study were;

1. to determine the influence of benzene on liver, kidney and lung microsomal cytochrome P450 dependent drug metabolizing enzyme activities, protein levels and mRNA levels in diabetic and control rabbits.

2. to analyze the existence of tissue specificity in the induction of rabbit P450 isozymes by benzene in diabetic and control rabbits.

3. to examine changes in mRNA and protein levels of certain P450 isozymes in the liver, kidney and lung of the alloxan induced diabetic rabbits and control rabbits.

4. to investigated whether species related differences exist in diabetic rabbit in terms of Cytochrome P450 isozyme expression and their associated drug metabolizing enzyme activities.

5. to evaluate the changes in antioxidant status of the rabbits due to diabetes, and benzene in liver.

In order to achieve these goals, first of all, experimental diabetes was induced in rabbit by single dose of alloxan. Then, benzene was applied to non-diabetic and diabetic rabbits. Cytochrome P450 dependent enzyme activities and protein levels were investigated in all groups. Besides, mRNA level of cytochrome P450 isozymes were determined by using Real-time PCR detection system. Some antioxidant parameters such as GST izozymes and catalase enzyme activities were also investigated in the liver of control, diabetic, benzene treated and benzene treated diabetic animals.

CHAPTER 2

MATERIAL AND METHODS

2.1 Materials

Benzene (01782), Cupper sulphate ($CuSO_4$; 02790), ethylene diamine tetra acetic acid disodium salt (EDTA; 08421), glacial acetic acid (0056), glycerol (04093), magnesium chloride ($MgCl_2$; 05833), methanol (02500), *p*-nitrophenol (106798), potassium chloride (KCl 104935), potassium dihydrojen phosphate (KH_2PO_4; 04871), di-potassium hydrojen phosphate (K_2HPO_4; 05101), sodium carbonate (06398), sodium hydroxide (06462), were the products of E. Merck, Darmstadt, Germany.

Acetic acid (glacial) (27225), acetylacetone (33005), acrylamide (A8887), alloxan monohydrate (A7413), ammonium acetate (A7672), ε-amino caproic acid (ε-ACA; A2504), amonium persulfate (APS; A 3678), borate (11607), bovine serum albumin (BSA; A7511), bromochloroindoylphosphate (BCIP; B8503), caffeine (C0750), chloroform (C2432), N'-N'-dimethylformamide (F7508), erythromycin (E-0774), ethanol (32221), ethidium bromide (E-7637), ethoxyresorufin (E3763), D-glucose–6-phosphate dehydrogenase type IX (G8878), glutathione reduced form (GSH; G4251), N-2-hydroxyethylpiperazine-N-2,ethane sulfonic acid (HEPES; H3375), β-mercaptoethanol (M6250), N,N'-methylene bisacrylamide (BIS; M7256), N-nitrosodimethylamine (NDMA; N3632), nitrotetrazolium blue (NBT; N6876), phenazine methosulfate (P9625), phenol

(P4557), phenylmethane sulfonyl flouride (PMSF; P7626), polyxyethylene sorbitan monolaurate (Tween 20; P1376), potassium chloride (13424), sodium chloride (13423), sodium potassium tartarate (Rochell salt; S2377), 2-amino-2(hydroxymethyl)-1,3-propandiol (Tris; T1378), anti-rabbit IgG-ALP conjugate (A3687) were purchased from Sigma Chemical Company, Saint Louis, Missouri, USA.

2, 4 Dinitrophenyl hydrazine, formaldehyde and sodium dodecyl sulphate (SDS) were obtained from Fluka A.G., Switzerland. N, N, N', N'-tetramethylene diamine (TEMED; 161-0801) were purchased from Bio-Rad Laboratories, Richmond, California, USA.

Agarose (A2114), diethylpyrocarbonate (DEPC), D-glucose-6-phosphate monosodium salt (A3789), glycine (A4554), guanidinium thiocyanate (GTC), β-nicotinamide adenine dinucleotide phosphate ($NADP^+$; A1394), were purchased from Applichem, Darmstadt, Germany. Benzphetamine-HCl was kindly provided by Dr. J. F. Stiver of UpJohn Co., USA.

Polyclonal anti-rabbit CYP2E1 was obtained from Oxford Biomedical Research, MI, USA. Polyclonal anti-rat CYP1A2 was purchased from Chemicon International, Temecula, CA, USA and polyclonal anti-human CYP3A4 was obtained from BD Biosciences, San Jose, CA, USA. Polyclonal antibodies against to sheep lung cytochrome P4502B4 homologue was obtained from Dr. O. Adalı.

RNeasy kit was obtained from Qiagen, Hilden, Germany. Moloney-Murine Leukemia Virus Reverse Transcriptase, dNTP mix (#R0191), Gene Ruler^{TM} 50 bp DNA Ladder (#SM0371) were purchased from MBI Fermentas, USA. Primer pairs of CYP2E1, CYP1A2, CYP3A6, GAPDH and oligo dT were purchased from Alpha DNA, Montreal, Quebec, Canada.

All the other chemicals used in this study were of analytical grade and were obtained from commercial sources at the highest grade of purity.

2.2 Animals and Treatments

2.5 months old Adult male New Zealand white rabbits, initially weighing 1.5-2.2 kg, were purchased from Lameli Husbandary, Ankara. The rabbits were caged separately and maintained at 23-25 ^{o}C on a 12 h light/12h dark cycle with free access to water and commercial chow for 7 days before the beginning of the treatments. The procedures involving animals and their care were carried out in accordance with the Declaration of Helsinki.

Experimental diabetus mellitus was induced by injecting a single dose of alloxan (125 mg/kg, freshly dissolved in saline solution) into lateral ear vein after overnight fasting. Because alloxan injection is irritating and painful, rabbits were sedated with intramuscular 50 mg of ketamine just before the injection of alloxan. The age-matched control rabbits received an equal volume of saline. Blood glucose concentrations of both control and alloxan-treated rabbits were monitored on a weekly basis. Five weeks after alloxan injection, animals having blood glucose levels more than 200 mg/dl were considered as diabetic and used for present study. Then, control and diabetic rabbits were randomly selected and divided into four groups of 7-9 animals in each. The first group consisted of control animals. The second group was the diabetic control. The third group was benzene control group which was injected three dose of benzene at a dose of 750 mg/kg body weight, subcutaneously, on day 1, day 5 and day 7. The fourth group was benzene treated diabetic animals which were received same dose protocol of benzene (750 mg/kg body weight, subcutaneously, day 1, day 5 and day 7). Schematic representation of the treatments was represented in Figure 2.1. All of the animals were sacrified 20h after last treatment by decapitation.

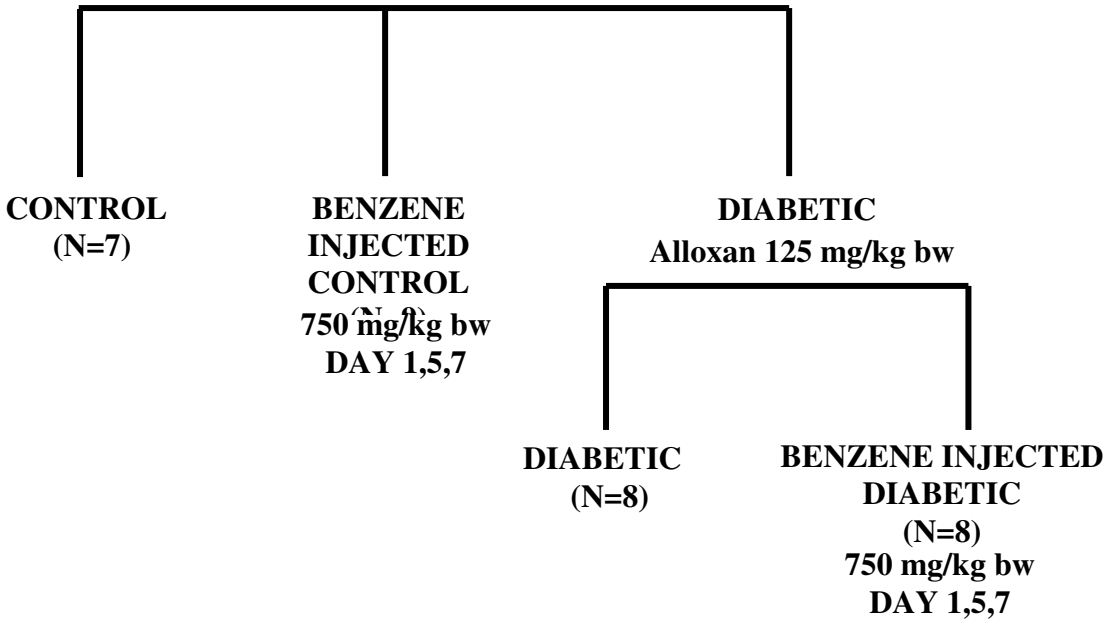


Figure 2.1 Schematic representation of the experimental design.

2.3 Methods

2.3.1 Preparation of Rabbit Liver Microsomes

Rabbit liver microsomes were prepared as described by the method of Arınç *et al*., (1991) except that homogenization was carried in 1.15% KCl solution containing 2 mM EDTA, 0.25 mM ε-ACA, 0.1 mM PMSF. The livers each weighing about 40-80 g were removed immediately after killing the animals by decapitation. Gall bladders were removed from the livers to prevent release of its contents that are known to be inhibitory to monooxygenase activity. All subsequent steps were carried out at 0-4^{0}C. After removal of the connective and fatty tissues, livers were washed with cold distilled water and then with 1.15% KCl solution sevaral times to remove the excess blood. After blotting the tissues by the help of a filter paper, tissues were weighed and chopped with scissors. The resulting minced tissues were homogenized in 1.15% KCl solution containing 2 mM EDTA pH 7.2, 0.25 mM ε-ACA and 0.1 mM PMSF at a volume of equal to 3 times of weigth of

liver by using Potter-Elvehjem glass homogenizer coupled with a motor (Black and Decker, V850, multispeed drill)-driven teflon pestle at 2 400 rpm. Fifteen passes were made for the homegenization of liver tissue.

The resulting liver homogenate was centrifuged at 10 031 rpm (10 800 xg) (Sigma 3K30 Centrifuge Osterode am Harz, Germany) with 12156 rotor for 25 minutes to remove cell debris, nuclei and mitochondria. The supernatant fraction containing endoplasmic reticulum and soluble fraction of the cell was filtered through double layers of cheese cloth in a Buchner funnel. The microsomes were sedimented from the supernatant by centrifugation at 45 000 rpm (145 215 xg) for 50 minutes using T1270 rotor or at 40 000 rpm (115 632 xg) for 60 minutes using A-841 rotor in Sorvall Combi Plus Ultracentrifuge (Sorvall, Du Pont Company, Newton, Connecticut, USA). The supernatant fraction (cytosol) was taken and shocked with liquid nitrogen and then stored at -80 ^{0}C until use in studies related with cytosolic enzymes. The firmly packed microsomal pellet was suspended in 1.15% KCl solution containing 2 mM EDTA and was resedimented by ultracentrifugation at 145 215 xg for 50 minutes or at 115 632 xg for 60 minutes by using Sorvall Combi Plus Ultracentrifuge to remove excess hemoglobin. Then the washed microsomal pellet was resuspended in 25% glycerol containing 1 mM EDTA at a volume of 0.5 ml for each gram of liver tissue. In order to obtain a homogenous microsomal suspension, resuspended microsomes were homogenized manually using the glass-teflon homogenizer.

The microsomal suspensions containing approximatelly 25-45 mg of microsomal protein per milliliter were then gassed with nitrogen in eppendorf tubes and stored at -80 ^{0}C for enzymatic assays.

2.3.2 Preparation of Rabbit Kidney and Lung Microsomes

Rabbit kidney and lung microsomes were prepared essentially with the same procedure used for the preparation of rabbit liver microsomes with some modifications.

The kidneys and lungs were removed immediatelly after killing the animals and placed on crushed ice. All subsequent steps were carried out at 0-4^{0}C. After removal of the connective and fatty tissues, organs were washed with cold distilled water and then with 1.15% KCl solution sevaral times to remove the excess blood. Then the tissues were drained by a filter paper, minced with scissors to get a homogenous kidney and lung samples. The resulting minced tissues were homogenized in 1.15% KCl solution containing 2 mM EDTA pH 7.2, 0.25 mM ε-ACA and 0.1 mM PMSF at a volume of equal to 2.2 times of weigth of kidney and 2.5 times of weigth of lung by using Potter-Elvehjem glass homogenizer coupled with a motor (Black and Decker, V850, multispeed drill)-driven teflon pestle at 2 400 rpm. Fifteen passess were used for the homogenization of both kidney and lung tissues.

The homogenate was centrifuged at 10 031 rpm (10 800 xg) (Sigma 3K30 Centrifuge Osterode am Harz, Germany) with 12156 rotor for 25 minutes to remove cell debris, nuclei and mitochondria. The supernatant fraction containing endoplasmic reticulum and other soluble fraction of the cell was filtered through double layers of cheese cloth. The microsomes were sedimented from the supernatant by centrifugation at 45 000 rpm (145 215 xg) for 50 minutes using T1270 rotor or at 40 000 rpm (115 632 xg) for 60 minutes using A-841 rotor in Sorvall-Combi ultracentrifuge (Du Pont Company, Newton, Connecticut, USA). The supernatant fraction (cytosol) was taken and shocked with liquid nitrojen and then stored at -80 ^{0}C in order to use in studies related with cytosolic enzymes. The firmly packed microsomal pellet was suspended in 1.15% KCl solution containing 2 mM EDTA and resedimented by ultracentrifugation at 145 215 xg for 50 minutes or at 115 632 xg for 60 minutes by using Sorvall Combi Plus Ultracentrifuge to remove excess hemoglobin. Then the washed microsomal pellet was resuspended in 25% glycerol containing 1 mM EDTA at a volume of 0.3 ml for each gram of kidney and lung tissues. Resuspended microsomes were homogenized manually using the glass-teflon homogenizer to obtain homogenous suspension.

The microsomal suspensions containing approximatelly 25-50 mg of microsomal protein per milliliter for kidney and 8-25 mg of microsomal protein per milliliter for lung were then gassed with nitrogen in eppendorf tubes and stored at -80 ^{0}C for enzymatic assays.

2.3.3 Determination of Serum Glucose Concentration

In this study, glucose concentration in the serum of control and diabetic rabbits were determined according to the enzymatic method by using ABX Glucose PAP 100 kit (ABX Diagnostics, 34187 Montpellier, France) in Cobes Mira Plus cc autoanalyzer (Roche Corporation, Switzerland) in the Health Center of Middle East Technical University.

In this method, glucose and oxygen react in the presence of glucose oxidase to form gluconic acid and hydrogen peroxide. Hydrogen peroxide generated by the oxidation is decomposed by peroxidase in the presence of 4-amino-antipyrine and phenol to yield a quinoneimine dye. The absorbance of this dye measured at 505 nm is proportional to the glucose concentration. The reactions are given below in Figure 2.2.

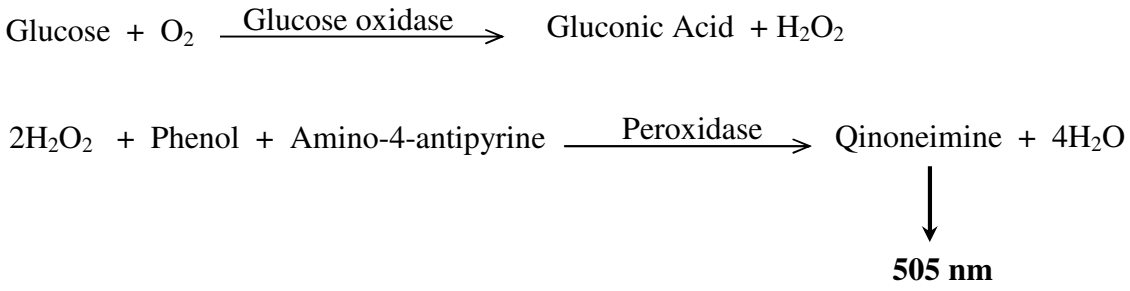


Figure 2.2 Reactions of glucose determination.

2.3.4 Determination of Aspartate Aminotransferase (AST) Activity

The transaminases constitute a group of enzymes which catalyze the interconversion of amino acids and α-ketoacids by transfer of amino groups. The α-ketoglutarate/L-glutamate couple serves as the amino group acceptor and donor in pair in all amino transfer reactions. Animal cells contain a variety of amino transferases. Aspartate Aminotransferase (AST) is found in practically every tissue of the body, including red blood cells. It is particularly high concentration in cardiac muscle and liver, intermediate in skeletal muscle and in much lower concentrations in others.

In this work, the AST enzyme activity of blood serum was determined according to the method of Reitman and Frankel (1957). The method involves the direct combination of oxalate with 2, 4-dinitrophenyl hydrazine (DNPH) and the measurement of the color in alkaline solution. The AST catalyzed reaction and measurement of AST activity are given in Figure 2.3 and 2.4. Although the ultraviolet (UV) procedure is the reference method, the colorimetric method eliminates the need of specific instruments and lends itself more readily to multiple analysis, while giving results which compare favorably with the ultraviolet technic.

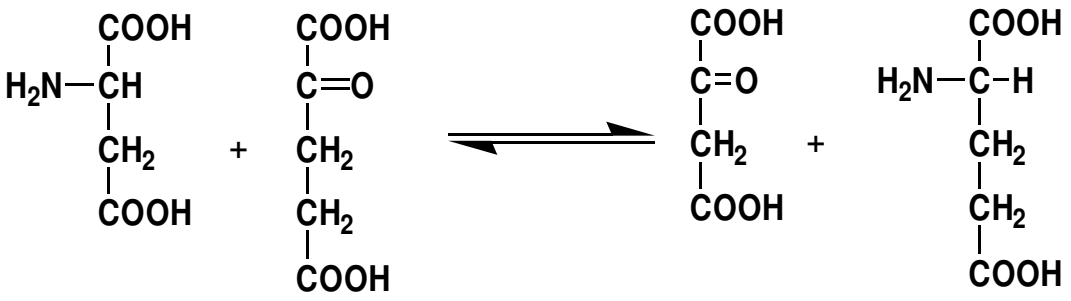
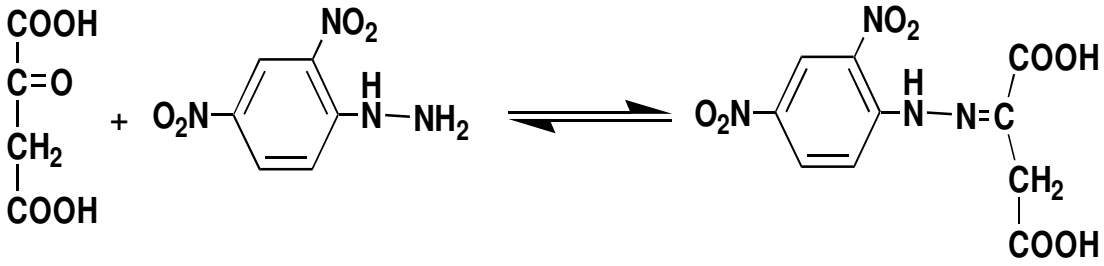


Figure 2.3 The reaction catalysed by AST



Oxaloacetate | 2, 4, Dinitrophenyl hydrazine (DNPH) | Oxalate-DNP hydrazone (Highly colored)

Figure 2.4 The reaction used for the determination of AST activity

The incubation mixture contained 1 ml aspartic acid and α-ketoglutaric acid as a substrate and 0.2 ml serum. Substrate solution was prepared by the addition of 40 ml of 1 N NaOH to the mixture of 0.0584 g of α-ketoglutarate and 5.32 g D-L-aspartate. The pH of the solution was adjusted to 7.4±0.1 by adding 1N NaOH dropwise, with stirring. Then the solution was diluted to 200 ml with 100 mM phosphate buffer, pH 7.4.

All the tubes were mixed and only the test tubes were incubated in a water bath at 37 °C for 1 hour. After the incubation, the test tubes were removed from the water bath and 1 ml of color reagent which was prepared by dissolving 0.039 g DNPH in 200 ml of 1N HCl was added to all tubes including blanks and standards. Then, all tubes were incubated at room temperature for 20 minutes.

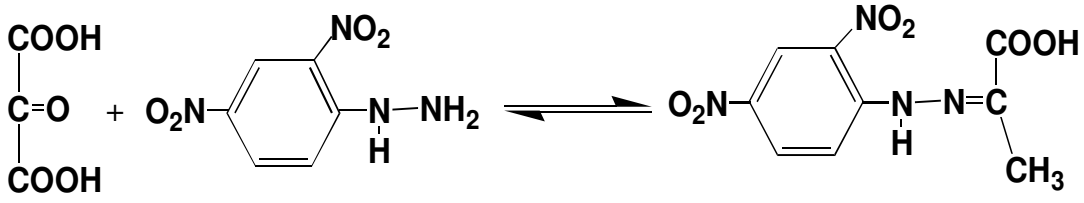
Colorimetric method assigns transaminase unit values to several concentration of the pyruvate, an intermediate standard. Pyruvate standard was prepared freshly by dissolving 20 mg pure Na-pyruvate in 100 ml phosphate buffer, pH 7.4. Four different concentrations of pyruvate standards (0.1 ml, 0.2 ml, 0.3 ml and 0.4 ml pyruvate standard solution were added to tubes and all tubes were completed to 1 ml with substrate solution) which refer to 24, 61, 114, 190 AST

units were used for the construction of standard curve. The enzyme activities of samples were calculated using this linear standard curve.

Finally, 10 ml of 0.4 N NaOH was added to all tubes and they were allowed to stand for at least 5 minutes. Then, the blank was set to 0.25 absorbance in spectrophotometer at 505 nm. The absorbances of the tubes developing brown color were read. A standard curve was constructed by using the absorbances and AST units of the standards. Blank was plotted at zero concentration and 0.25 absorbance directly. The AST units of the samples were calculated by using this calibration curve. One transaminase unit is defined, in terms of the UV procedure, is the amount of enzyme activity in 1 ml of serum that will lower the absorbance by 0.001 in 1 minute under the described conditions of that method (Reitman and Frankel, 1957).

2.3.5 Determination of Alanine Aminotransferase (ALT) Activity

Glutamate pyruvate transaminase is similar to AST and was determined by the same method (Reitman and Frankel, 1957) except that substrate contained DL-alanine instead of aspartic acid and the product measured was pyruvic acid rather than oxalacetic acid. The measurement of ALT activity is given in Figure 2.5



Pyruvate

2, 4, Dinitrophenyl hydrazine (DNPH)

Pyruvate-DNP hydrazone (Highly colored)

Figure 2.5 The reaction used for the determination of ALT activity

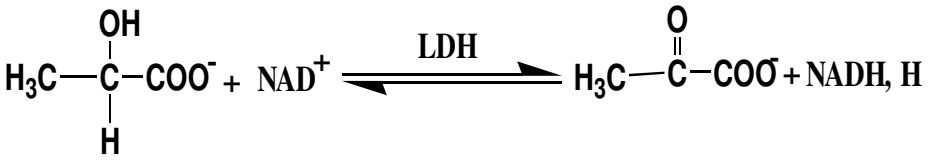
The incubation mixture contained 1 ml alanine and α-ketoglutaric acid as a substrate and 0.2 ml serum. Substrate solution was prepared by the addition of 20 ml of dH_2O to the mixture of 0.0584 g of α-ketoglutarate and 3.56 g D-L-Alanine. The pH of the solution was adjusted to 7.4±0.1 by adding 1N NaOH dropwise, with stirring. Then the solution was diluted to 200 ml with 100 mM phosphate buffer, pH 7.4.

Pyruvate standard was prepared as described in AST activity. Three different concentrations of pyruvate (0.1 ml, 0.2 ml, and 0.3 ml pyruvate standard solution was added to tubes and all tubes were completed to 1 ml with substrate solution) which refer to 28, 57, 97 ALT units were used for the construction of standard curve. The enzyme activities of samples were calculated using this linear standard curve.

All the tubes were mixed and only the test tubes were incubated in a water bath at 37 °C for 30 minutes. After the incubation, the test tubes were removed from the water bath and 1ml of color reagent was added to all tubes including blanks and standards. Then, all tubes were incubated at room temperature for 20 minutes.Finally, 10 ml of 0.4 N NaOH was added to all tubes and they were allowed to stand for at least 5 minutes. Then, the blank was set to 0.25 absorbance in spectrophotometer at 505 nm. The absorbances of the tubes developing brown color were read. A standard curve was constructed by using the absorbances and ALT units of the standards. Blank was plotted at zero concentration and 0.25 absorbance directly. The ALT units of the samples were calculated by using this calibration curve.

2.3.6 Determination of Lactate Dehydrogenase (LDH) Activity

Oxidation-reduction reactions are among the most fundamental in the living organisms. LDH represents an enzyme that catalyses such a reaction shown in Figure 2.6.



Lactate Pruvate

Figure 2.6 LDH reaction

The enzyme is distributed in all human tissues, but is present in high concentrations in liver, cardiac and skeletal muscles, red blood cells and other tissues. In this work, LDH activity determination of blood serum samples was carried out using the spectrophotometric method of Wroblewski and La Due (1955) who adapted the classical assay of Kubowitz and Ott (1943) to serum. The activity of LDH was measured by monitoring the rate at which the substrate, pyruvate is reduced to lactate. The reduction is coupled with the oxidation of NADH, which is followed spectrophotometrically in terms of reduced absorbance at 340 nm.

The reaction mixture was contained 2.85 ml of 0.1M phosphate buffer, pH 7.5, 0.05 ml serum and 0.2 mg NADH. All the tubes were mixed and left in 25 °C for 20 minutes. After the exact period of 20 minutes, 0.1 ml of 22.7 mM Na-pyruvate in 0.1 M phosphate buffer, pH 7.5 was added to the tubes. They were mixed well and the content of tubes was transferred to a cuvette of 1cm lightpath. Then the absorbance was read at 340 nm in each 30-second intervals for 3 minutes against water as reference.

The LDH activities of serums were determined by selecting a period where the decrease in absorbance is linear with time and ≤ OD340 /min was calculated for this period. Then the following formula was used for the calculation of enzyme activity in terms of units/ml.

Serum LDH activity (units/ml) =[OD_{340} / min x TCF**]/[0.001x0.05x1(cm)] (2.1)

where:

0.001=OD_{340} equivalent to 1 unit of LDH activity in a 3 ml volume with 1 cm light path at 25 °C

0.05 =serum volume in cuvette

TCF =Temperature correction factor (1 at 25 °C)

Therefore, if a 1 cm lightpath cuvette is used, the above equation reduces to;

Serum LDH activity (units/ml) =OD_{340} x 20 000 xTCF

2.3.7 Protein Determination

The protein concentrations of microsomes and cytosols were determined by the method of Lowry *et al*. (1951). Crystalline bovine serum albumin was used as a standard. Before the preparation of the reaction tubes, initial dilution was done for microsomes and cytosols. In addition to initial dilutions, dilution within tube was carried out by taking 0.1 to 0.5 ml of initially diluted samples into reaction tubes and completed it to final volume of 0.5 ml with distilled water. After that, 2.5 ml of Lowry alkaline copper reagent (prepared by 2% copper sulphate, 2% sodium potassium tartarate and 0.1 N NaOH containing 20% sodium carbonate in a ratio of 1:1:100 in the written order) was added to each tube. All tubes were mixed and let stand for 10 minutes at room temperature for copper reaction in alkaline medium. Then 2 N folin reageant was diluted 1:1 ratio by distilled water and 0.25 ml of diluted reagent was added to the each tube and mixed within 8 seconds by vortex. The tubes were incubated for 30 minutes at room temperature. The intensity of resulting color was measured at 660 nm. Standard tubes with five different protein concentrations (20, 50, 100, 150, 200 μg BSA / ml) were prepared from crystalline bovine serum albumin and same steps were carried out for standard tubes. A standard curve was drawn by using intensity readings of standards and slope of this curve was used for the calculation of protein amounts in the sample.

2.3.8 Determination of Mixed Function Oxidase (MFO) Enzyme Activities

Mixed function oxidase activities of the microsomes were measured by using the following procedures at indicated substrate concentrations: N-nitrosodimethylamine (2.5 mM), Benzphetamine (1.5 mM), Erythromycin (1.0 mM) and Caffeine (30 μM) N-demethylase activities were determined by measuring the quantity of formaldehyde formed according to the method of Nash (1953), as modified by Cochin and Axelrod (1959). The hydroxylation of *p*-nitrophenol (0.25 mM) to 4-nitrocatechol (1,2-dihydroxy-4-nitrobenzene) was determined using the method of Reinke and Moyer (1985). Ethoxyresorufin (1.5 μM) O-dealkylase was measured using the fluorimetric method of Burke and Mayer (1974). The details of the procedures were given in the following sections.

2.3.8.1 Determination of N-Nitrosodimethylamine (NDMA) N-Demethylase Activity

NDMA N-demethylase activity of rabbit liver, kidney and lung microsomes was determined according to the method of Gorsky and Hollenberg (1989) and formaldehyde formed was measured by the method Nash (1953) as modified Cochin and Axelrod (1959). N-demethylase reaction is shown in Figure 2.7.

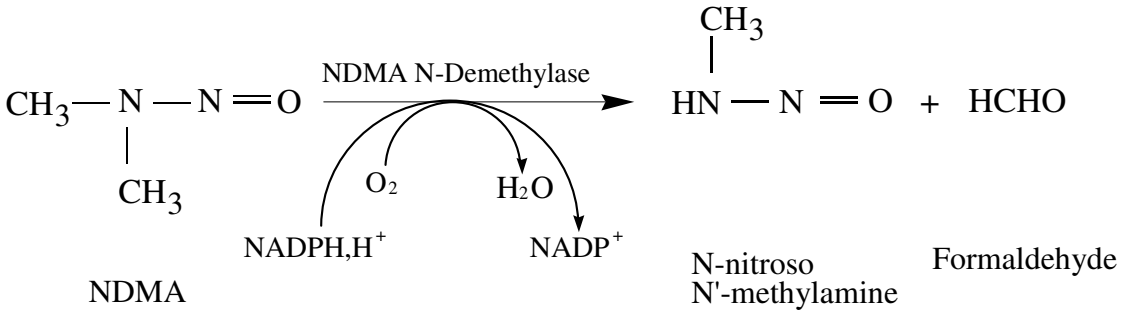


Figure 2.7 NDMA N-Demethylation reaction.

A 0.5 ml of incubation mixture given in Table 2.1 contained 100 mM HEPES buffer, pH 7.7, 2.5 mM NDMA, 0.75 mg microsomal protein for liver and 2 mg microsomal protein for kidney and 1 mg for lung and 0.5 mM NADPH generating system in final concentration.

NADPH generating system was composed of 2.5 mM $MgCl_2$, 14.6 mM HEPES pH 7.8, 2.5 mM Glucose-6-phosphate, 0.5 mM $NADPH^+$ and 0.5 units of glucose-6-phosphate dehydrogenase (Table 2.2). The test tube containing generating system then incubated at 37°C for 5 minutes. One unit of glucose 6-phosphate dehydrogenase reduces 1 μmol of $NADP^+$ per min at 25°C.

The enzyme reaction was initiated by the addition of 0.075 ml NADPH generating system to incubation mixture and to zero times blank to which 0.5 ml of 0.75 N perchloric acid was added just before the cofactor. The reaction was carried out at 37 ^{0}C for 20 minutes under the air with constant moderate shaking in a water bath.

The enzyme reaction was stopped by the addition of 0.5 ml 0.75 N perchloric acid at the end of 20 minutes. Then the contents of the tubes were transferred to the eppendorf centrifuge tubes and denatured proteins were removed by the centrifugation at 13 000 xg for 25 minutes using Thermo Scientific Microlite RF refrigerated microcentrifuge (Thermo Scientific, Milford, Massachusetts, USA) in order to remove denatured proteins.

Finally, 0.5 ml aliquots of supernatant were taken and were mixed with freshly prepared 0.375 ml of Nash reagent (prepared by the addition of 0.4 ml acetylacetone, just before use, to 100 ml solution containing 30.8 g ammonium acetate and 0.6 ml of glacial acetic acid). The mixture was incubated for 10 minutes at 50 ^{0}C in a water bath and the intensity of yellow color developed was measured at 412 nm using Schimadzu UV-1240 spectrophotometer (Schimadzu Co., Analytical Instruments Division, Kyoto, Japan).

A 0.5 mM freshly prepared formaldehyde solution was used as a standard. The tubes containing standards at four concentrations (12.5, 25, 50, 100 nmoles) with other incubation constituents were run under the same conditions as for reaction tubes. A standard formaldehyde calibration curve was constructed and the slope of this curve was used for calculation of enzyme activity.

Table 2.1 The constituents of the incubation mixture for determination of NDMA N-Demethylase activity of rabbit liver, kidney and lung microsomes.

Constituents	**Stock solution**	**Volume to be added (ml)**	**Final concentration in 0.5 ml incubation mixture**
HEPES buffer pH 7.7	400 mM	0.125	100 mM
NDMA	25 mM	0.050	2.5 mM
Microsomal protein	---------	Depending on the protein concentration of microsomes.	0.75 mg for liver 2 mg for kidney 1 mg for lung in 0.5 ml
NADPH generating System *		0.075	
Distilled water		to 0.5 ml	

* NADPH generating system was prepared as described in Table 2.2.

Table 2.2 Preparation of NADPH generating system.

Constituents	Stock Solutions	Volume to be taken (ml)	Final concentration in 1 ml reaction mixture
Glucose-6-phospate	100 mM	0.025	2.5 mM
$MgCl_2$	100 mM	0.025	2.5 mM
HEPES, pH:7.8	200 mM	0.073	14.6 mM
$NADP^+$	20 mM	0.025	0.5 mM
Glucose-6-phosphate dehydrogenase	1750 U/ml	0.00029	0.5 Units
Distilled water		to 0.150	

2.3.8.2 Determination of p-Nitrophenol Hydroxylase Activity

Hydroxylation of *p*-Nitrophenol is associated with CYP2E1 isozyme of cytochrome P450 enzyme family (Arınç *et al*. 2005). The reaction of *p*-nitrophenol hydroxylation is given in Figure 2.8.

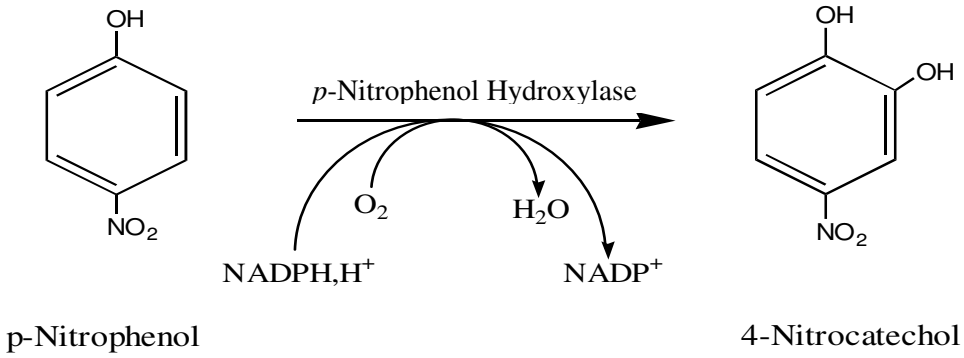


Figure 2.8 *p*-Nitrophenol Hydroxylation reaction.

The hydroxylation of *p*-nitrophenol to 4-nitrocatechol (1,2-dihydroxy-4-nitrobenzene) was determined using the method of Reinke and Moyer (1985). The assay conditions that were optimized for lung microsomal *p*-nitrophenol hydroxylase by Arınç and Aydoğmuş (1990) were also used for the determination of rabbit liver, kidney and lung microsomal *p*-nitrophenol hydroxylase activity.

The reaction medium given in Table 2.3 contained 100 mM Tris-HCl buffer, pH 6.8, 0.25 mM p-nitrophenol, 1.5 mg, 4 mg and 2 mg of microsomal protein for liver, kidney and lung, respectively and 0.5 mM NADPH generating system in a final volume of 1.0 ml.

p-Nitrophenol hydroxylation reaction was started by the addition of 0.15 ml NADPH generating system and carried out for 10 minutes at 37 ^{0}C with moderate shaking in a water bath. To zero time blank tubes, 0.5 ml of 0.6 N perchloric acid was added just before addition of NADPH generating system. The enzyme reaction was terminated by the addition of 0.5 ml 0.6 N perchloric acid. The contents of mixture transferred to eppendorf tubes and were centrifuged 13 000 xg for 25 minutes for removal of denatured proteins using Thermo Scientific Microlite RF refrigerated microcentrifuge (Thermo Scientific, Milford, Massachusetts, USA).

After centrifugation, 1.0 ml aliquot of the supernatant was taken and mixed with 0.1 ml of 10 N NaOH for complete ionization of 4-nitrocatechol. Then 4-nitrocatechol formed as a result of *p*-nitrophenol hydroxylase activity of microsomal enzymes was determined spectrally at 546 nm (Schimadzu UV-1240 spectrophotometer, Schimadzu Co., Analytical Instruments Division, Kyoto, Japan). The enzyme activity was determined by using extinction coefficient of 9.53 mM^{-1} cm^{-1} (Koop, 1986).

Table 2.3 The constituents of incubation mixture for the determination of *p*-Nitrophenol Hydroxylase activity of rabbit liver, kidney and lung microsomes.

Constituents	**Stock solution**	**Volume to be added (ml)**	**Final concentration in 1 ml incubation mixture**
Tris buffer pH 6.8	400 mM	0.250	100 mM
p-Nitrophenol	2.5 mM	0.100	0.25 mM
Microsomal protein	---------	Depending on the protein concentration of microsomes.	1.5 mg for liver 4 mg for kidney 2 mg for lung in 1ml
NADPH generating system *		0.150	0.5 mM
Distilled water		to 1 ml	

* NADPH generating system was prepared as described in Table 2.2.

2.3.8.3 Determination of Benzphetamine is N-demethylase Activity

Benzphetamine is a specific substrate for the phenobarbital inducible forms of CYP isozymes. N-demethylation of benzphetamine is found to be associated with cytochrome P450 2B isozymes (Adalı and Arınç, 1990) Benzphetamine is N-demethylated by mixed function oxidases in the presence of molecular oxygen and NADPH. At the end of the reaction norbenzphetamin, $NADP^+$, water and formaldehyde are produced (Figure 2.9).

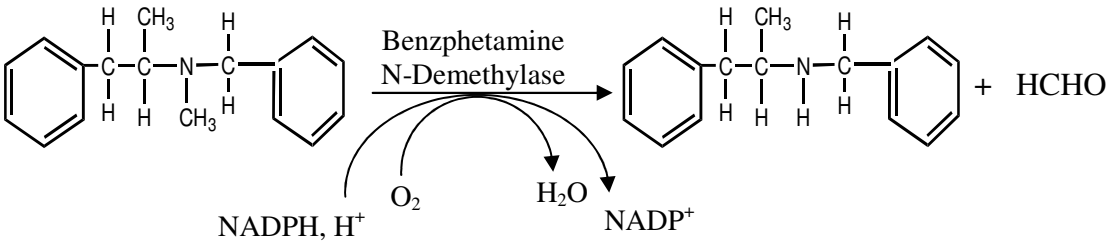


Figure 2.9 Benzphetamine N-Demethylation reaction

Benzphetamine N-demethylase activity of rabbit liver, kidney and lung microsomes was determined colorimetrically by measuring the amount of formaldehyde formed according to the method of Nash (1953) as modified by Cochin and Axelrod (1959). The typical assay conditions for benzphetaime N-demetylation, shown in Table 2.4, contained 100 mM HEPES buffer pH 7.7, 1.5 mM benzphetamine, 0.5 mg microsomal protein for rabbit liver and lung and 1.5 mg protein for kidney and finally 0.5 mM NADPH generating system in 0.5 ml final volume.

Benzphetamine N-demethylation reaction were initiated by the addition of 0.075 ml of NADPH generating system to incubation mixture and to zero time blanks to which 0.5 ml of 0.75 N perchloric acid was added just before the additon of the cofactor. Then, the reaction was carried out at 37 ^{0} C for 10 minutes with constant moderate shaking in a shaking water bath. After 10 minutes, the enzyme reaction was finalized by the addition of 0.5 ml 0.75 N percloric acid solution. Then, the denatured proteins were transferred to the eppendorf centrifuge tubes, and centrifuged at 13 000 xg at microcentrifuge (Thermo Microlite R. F., Milford, North America) for 25 minutes at 4 ^{0} C.

Table 2.4 The constituents of incubation mixture for the determination of Benzphetamine N-Demethylase activity of rabbit liver, kidney and lung microsomes

Constituents	**Stock solution**	**Volume to be added (ml)**	**Final concentration in 0.5 ml incubation mixture**
HEPES buffer pH 7.7	400 mM	0.125	100 mM
Benzphetamine	7.5 mM	0.100	1.5 mM
Microsomal protein	---------	Depending on the protein concentration of microsomes.	0.5 mg for liver 1.5 mg for kidney 0.5 mg for lung in 0.5 ml
NADPH generating System *		0.075	0.5 mM
Distilled water		to 0.5 ml	

* NADPH generating system was prepared as described in Table 2.2.

After centrifugation, 0.5 ml aliquots were mixed with 0.375 ml Nash reagent (prepared by the addition of 0.4 ml acetylacetone, just before use, to 100 ml solution containing 30.8 g ammonium acetate and 0.6 ml of glacial acetic acid) again in eppendorf tubes and the mixture was incubated for 10 minutes at 50 ^{0} C in a water bath. Then, the formaldehyde amount was determined by measuring the absorbance at 412 nm using Schimadzu UV-1240 spectrophotometer (Schimadzu Co., Analytical Instruments Division, Kyoto, Japan).

A 0.5 mM freshly prepared formaldehyde solution was used as a standard. The tubes containing standards at four concentrations (12.5, 25, 50, 100 nmoles) with other incubation constituents were run under the same conditions as for reaction tubes. A standard formaldehyde calibration curve was constructed and the slope of this curve was used for calculation of enzyme activity.

2.3.8.4 Determination of Erythromycin N-demethylase Activity

N-demethylation of Erythromycin is associated with CYP3A isozyme of cytochrome P450 enzyme family (Combalbert *et. al.*, 1989; Wang *et. al.*, 1997). Erythromycin is N-demethylated by P450 dependent mixed function oxidases in the presence of molecular oxygen and formaldehyde is produced (Figure 2.10).

Erythromycin N-demethylase activity of rabbit liver and lung microsomes were determined by measuring the amount of formaldehyde formed according to the method of Nash (1953) as modified by Cochin and Axelrod (1959). The typical assay mixture for erythromycin N-demethylation, shown in Table 2.5, contained 100 mM HEPES buffer pH 7.8, 1.0 mM erythromycin, 1.5 mg microsomal protein for liver and lung and finally 0.5 mM NADPH generating system in a final volume of 0.5 ml.

Erythromycin N-demethylation reaction was initiated by the addition of 0.075 ml of NADPH generating system to incubation mixture and to zero time blanks to which 0.5 ml of 0.75 N perchloric acid was added just before the additon of the cofactor. Then, the reaction was carried out at 37 ^{0} C for 15 minutes with constant moderate shaking in a shaking water bath. After 15 minutes, the enzyme reaction was finalized by the addition of 0.5 ml 0.75 N percloric acid solution. Then, the denatured proteins were transferred to the eppendorf centrifuge tubes, and centrifuged at 13 000 xg at microcentrifuge (Thermo Microlite R. F., Milford, North America) for 25 minutes at 4 ^{0} C.

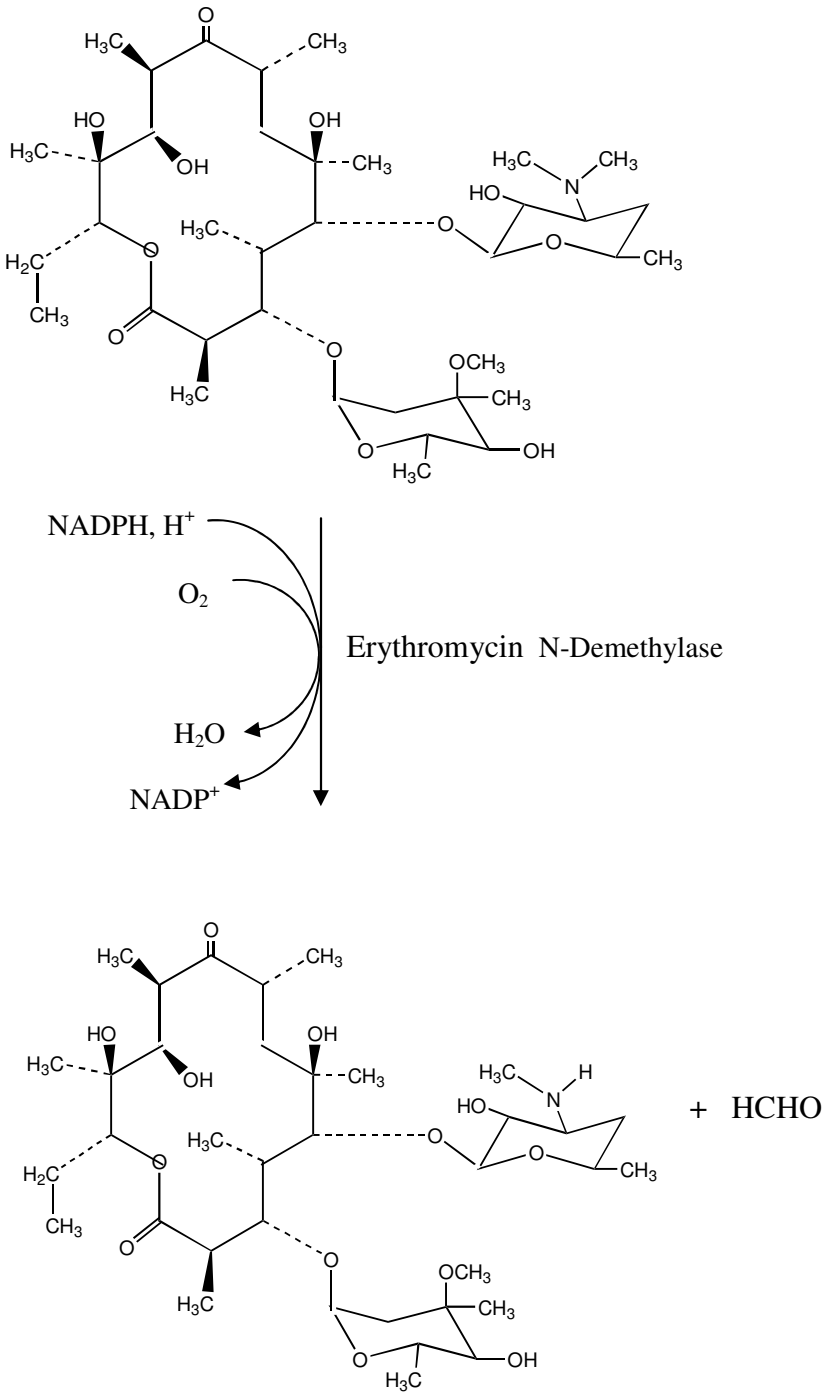


Figure 2.10 Erythromycin N-Demethylation reaction

Table 2.5 The constituents of incubation mixture for the determination of Erythromycin N-Demethylase activity of rabbit liver, kidney and lung microsomes

Constituents	**Stock solution**	**Volume to be added (ml)**	**Final concentration in 0.5 ml incubation mixture**
HEPES buffer pH 7.8	400 mM	0.125	100 mM
Erythromycin[&]	7.5 mM	0.010	1 mM
Microsomal protein	---------	Depending on the protein concentration of microsomes.	1.5 mg for liver 1.5 mg for lung in 0.5 ml
NADPH generating System *		0.075	0.5 mM
Distilled water		to 0.5 ml	

[&] Erythromycin was prepared by dissolving 73.4 mg of erythromycin in 50 % ethanol. The final concentration of ethanol 1 % reaction mixture

*NADPH generating system was prepared as described in Table 2.2.

After centrifugation, 0.5 ml aliquots were mixed with 0.375 ml Nash reagent (prepared by the addition of 0.4 ml acetylacetone, just before use, to 100 ml solution containing 30.8 g ammonium acetate and 0.6 ml of glacial acetic acid) again in eppendorf tubes and the mixture was incubated for 10 minutes at 50 ^{0} C in a water bath. Then, the formaldehyde amount was determined by measuring the absorbance at 412 nm using Schimadzu UV-1240 spectrophotometer (Schimadzu Co., Analytical Instruments Division, Kyoto, Japan).

A 0.5 mM freshly prepared formaldehyde solution was used as a standard. The tubes containing standards at four concentrations (12.5, 25, 50, 100 nmoles) with other incubation constituents were run under the same conditions as for reaction tubes. A standard formaldehyde calibration curve was constructed and the slope of this curve was used for calculation of enzyme activity.

2.3.8.5 Determination of 7-Ethoxyresorufin–O-deethylase Activity

Cytochrome P450 1A (CYP1A) associated 7-Ethoxyresorufin–O-deethylase (EROD) activity of rabbit liver microsomes was determined by the method of Burke and Mayer (1974) with some modifications. Figure 2.11 shows the dealkylation reaction of ethoxyresorufin catalyzed by monooxygenases in the presence of molecular oxygen and NADPH.

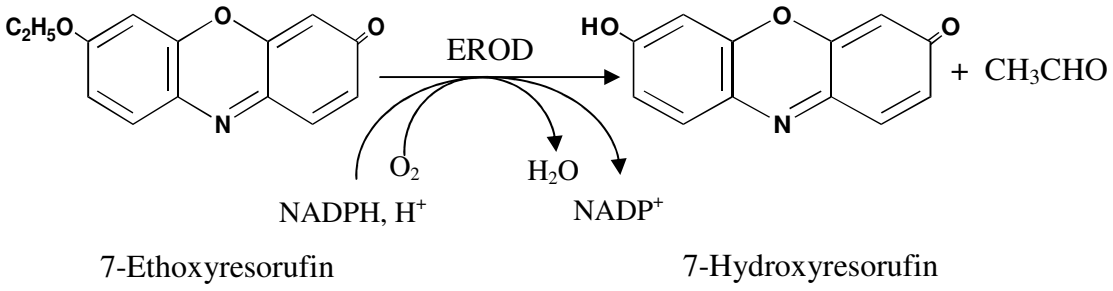


Figure 2.11. Ethoxyresorufin O-Deethylase reaction

A 0.5 mM stock substrate solution was first prepared by dissolving appropriate amount of 7-ethoxyresorufin in DMSO. Then 10 µM daily solution was prepared by diluting 1:50 with 0.2 M potassium phosphate buffer pH 7.8 containing 0.2 M NaCl. 1 mM stock resorufin standard was prepared by dissolving appropriate amount of resorufin in DMSO. Then 5 µM daily solution was prepared by diluting 1:200 with 0.2 M potassium phosphate buffer pH 7.8 containing 0.2 M NaCl. As it is shown in Table 2.6, a typical reaction mixture contained 0.1 M potassium phosphate buffer pH 7.8, 0.1 M NaCl, 2.4 mg BSA, 1.5 µM 7-ethoxyresorufin, 0.5

mg microsomal protein, 0.5 mM NADPH generating system (constituents of generating system the were given in Table 2.2) in a final volume of 2.0 ml in a fluorometer cuvette. The reaction was initiated by the addition of substrate and followed for three minutes in spectrofluorometer (Hitachi F2000, Hitachi ltd., Tokyo, Japan) at 535 nm (excitation) and 585 nm (emission) wavelengths. Finally a known amount of resorufin was added as an internal standard to the reaction mixture and the increase in fluorescence was recorded.

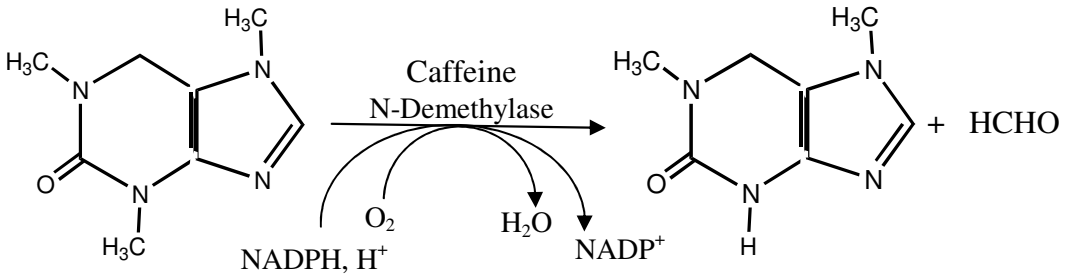
Table 2.6 The constituents of the reaction mixture for the determination of EROD activity in rabbit liver microsomes.

Constituents	**Stock solution**	**Volume to be added (ml)**	**Final concentration in 2 ml incubation mixture**
Potassium phosphate buffer, at pH 7.8 containing 0.4 M NaCl	400 mM	0.500	100 mM
Ethoxyresorufin	10 µM	0,300	1.5 µM
BSA	12 mg/ml	0.200	1.2 mg/ml
Microsomal protein	---------	Depending on the protein concentration of microsomes.	0.5 mg for liver in 2 ml
NADPH generating System *		0.300	0.5 mM
Distilled water		to 2 ml	

*NADPH generating system was prepared as described in Table 2.2.

2.3.8.6 Determination of Caffeine N-demethylase Activity

N-demethylation of Caffeine is associated with CYP1A2 isozyme of cytochrome P450 enzyme family in mammals. (Berthou *et al*., 1992). Figure 2.12 shows the N-demethylation reaction of caffeine catalyzed by CYP1A2 in the presence of molecular oxygen and NADPH.



Caffeine Paraxanthine Formaldehyde

Figure 2.12 Caffeine N-Demethylation reaction

Caffeine N-demethylase activity of rabbit liver microsomes was determined by measuring the amount of formaldehyde formed according to the method of Nash (1953) as modified by Cochin and Axelrod (1959). The typical assay mixture for caffeine N-demethylation, shown in Table 2.7, contained 100 mM HEPES buffer pH 7.5, 30 µM caffeine, 1.5 mg microsomal protein for liver and 0.5 mM NADPH generating system in a final volume of 0.5 ml.

Caffeine N-demethylation reaction was initiated by the addition of 0.075 ml of NADPH generating system to incubation mixture and to zero time blanks to which 0.5 ml of 0.75 N perchloric acid was added just before the additon of the cofactor. Then, the reaction was carried out at 37 ^{0} C for 15 minutes with constant moderate shaking in a shaking water bath. After 15 minutes, the enzyme reaction was finalized by the addition of 0.5 ml 0.75 N percloric acid solution. Then, the denatured proteins were transferred to the eppendorf centrifuge tubes, and centrifuged at 13 000 xg at microcentrifuge for 25 minutes at 4 ^{0} C.

Table 2.7 The constituents of incubation mixture for the determination of Caffeine N-Demethylase activity of rabbit liver, kidney and lung microsomes

Constituents	**Stock solution**	**Volume to be added (ml)**	**Final concentration in 0.5 ml incubation mixture**
HEPES buffer pH 7.5	400 mM	0.125	100 mM
Caffeine	1 mM	0.015	30 µM
Microsomal protein	---------	Depending on the protein concentration of microsomes.	1.5 mg for liver in 0.5 ml
NADPH generating System *		0.075	0.5 mM
Distilled water		to 0.5 ml	

*NADPH generating system was prepared as described in Table 2.2.

After centrifugation, 0.5 ml aliquots were mixed with 0.375 ml Nash reagent in eppendorf tubes and the mixture was incubated for 10 minutes at 50 ^{0} C in a water bath. Then, the formaldehyde amount was determined by measuring the absorbance at 412 nm using Schimadzu UV-1240 spectrophotometer (Schimadzu Co., Analytical Instruments Division, Kyoto, Japan).

A 0.5 mM freshly prepared formaldehyde solution was used as a standard. The tubes containing standards at four concentrations (12.5, 25, 50, 100 nmoles) with other incubation constituents were run under the same conditions as for

reaction tubes. A standard formaldehyde calibration curve was constructed and the slope of this curve was used for calculation of enzyme activity.

2.3.9 Determination of Glutathione S-Transferase Activity

2.3.9.1 Determination of Total Glutathione S-Transferase (GST) Activity

Total GST activity was measured according to the method of Habig *et. al.* (1974) with some modifications. 1-chloro-2, 4-dinitrobenzene (CDNB) is a common substrate for all isozymes of GST. CDNB was used as substrate for the determination of total GST activity in the presence of the cofactor reduced glutathione (GSH) by monitoring the increase in the absorbance at 340 nm due to the colored adducts formation.

The assay was performed in a 1 ml quartz cuvette. Into 1 ml quartz cuvette, 835 µl of 120 mM potassium phosphate buffer, pH 7.0, 65 µl of 20 mM GSH and 50 µl of 20 mMCDNB were added, and the reaction was started by the addition of 50 µl of 1/1000 diluted liver cytosols. Then thioether formation was followed for 2 minutes at 340 nm at Hitachi U-2800 Spectrophotometer (Hitachi High Technologies Corporation, Tokyo, Japan). Each time, blank readings (reaction with no enzyme) were subtracted from enzyme reaction. Then the enzyme activity was calculated as the amount of nmol thioether formed by 1 mg total protein containing cytosol in one minute using 9.6 $mM^{-1}.cm^{-1}$ as an extinction coefficient of thioether formed by GST.

2.3.9.2 Determination of GST Mu Isozyme Activity

GST Mu activity was measured according to the method of Habig *et. al.,* (1974) with some modifications. 1,2-dichloro-4-nitrobenzene (DCNB) was used as substrate for the determination of GST Mu isozyme activity in the presence of the cofactor reduced glutathione (GSH) by monitoring the thioether formation at 345 nm.

The assay was performed in a 1 ml quartz cuvette. Into 1 ml quartz cuvette, 835 µl of 120 mM potassium phosphate buffer, pH 7.0, 65 µl of 20 mM GSH and 50 µl of 20 mM DCNB were added, and the reaction was started by the addition of 50 µl of enzyme (no dilutions were performed for both liver). Then the activity increase was followed for 2 minutes at 345 nm at Hitachi U-2800 Spectrophotometer (Hitachi High Technologies Corporation, Tokyo, Japan). Each time, blank readings (reaction with no enzyme) were subtracted from enzyme reaction. Then the enzyme activity was calculated as the amount of nmol thioether formed by 1 mg total protein containing liver cytosol in one minute by using 8.5 mM^{-1}. cm^{-1} as an extinction coefficient of thioether formed by GST.

2.3.9.3 Determination of GST Pi Isozyme Activity

GST Pi activity was measured according to the method of Habig *et. al.* (1974) with some modifications. Etacyrinic acid was used as substrate for determination of GST pi isozyme activity in the presence of the cofactor reduced glutathione (GSH) is achived by monitoring the thioether formation at 270 nm.

The assay was performed in a 1 ml quartz cuvette. Into 1 ml quartz cuvette, 850 µl of 120 mM potassium phosphate buffer, pH 6.5, 50 µl of 6 mM GSH and 50 µl of 5 mM Etacyrinic acid were added, and the reaction was started by the addition of 50 µl of 5X diluted liver cytosol. Then the activity increase was followed for 2 minutes at 270 nm at Hitachi U-2800 Spectrophotometer (Hitachi High Technologies Corporation, Tokyo, Japan). Each time, blank readings (reaction with no enzyme) were subtracted from enzyme reaction. Then the enzyme activity was calculated as the amount of nmol thioether formed by 1 mg total protein containing liver cytosol in one minute by using 5.0 $mM^{-1}.cm^{-1}$ as an extinction coefficient of thioether formed by GST.

2.3.9.4 Determination of GST Theta Isozyme Activity

GST Theta activity was measured according to the method of Habig *et. al.* (1974) with some modifications. 1,2-epoxy-3-(*p*-nitrophenoxy)propane (EPNP) is a substrate for Theta isozyme of GST. Determination of GST theta isozyme activity in the presence of the cofactor reduced glutathione (GSH) is achived by monitoring the thioether formation at 360 nm.

The assay was performed in a 1 ml quartz cuvette. Into 1 ml quartz cuvette, 850 µl of 120 mM potassium phosphate buffer, pH 6.5, 50 µl of 100 mM GSH and 50 µl of 5 mM 1,2-epoxy-3-(*p*-nitrophenoxy)propane (EPNP) were added, and the reaction was started by the addition of 50 µl of liver cytosols (no dilutions were performed). Then the activity increase was followed for 2 minutes at 360 nm at Hitachi U-2800 Spectrophotometer (Hitachi High Technologies Corporation, Tokyo, Japan). Each time, blank readings (reaction with no enzyme) were subtracted from enzyme reaction. Then the enzyme activity was calculated as the amount of nmol thioether formed by 1 mg total protein containing liver cytosol in one minute by using 0.5 $mM^{-1}.cm^{-1}$ as an extinction coefficient of thioether formed by GST.

2.3.10 Determination of Catalase Activity

Catalase activities of cytosols were determined according to the method of Aebi (1964). In the UV range, H_2O_2 shows a continual increase in absorption with decreasing wavelength. The decomposition of H_2O_2 was followed directly by the decrease in absorbance at 240 nm. Catalase activity was calculated from the difference in the absorbance at 240 nm per unit time using an extinction coefficient of 0.0364 $mM^{-1}.cm^{-1}$

Before mixing the assay medium, enzyme solution (Liver Cytosol) was pretreated with 1% Triton X-100 for 10 minutes (ten fold dilution of cytosol; 1 part

sample, 9 parts Triton X-100), and then the mixture diluted further 200 or 300 fold to make a total dilution of 2000 or 3000 with phosphate buffer. After that 2 ml of diluted sample is added into quartz cuvettes and the reaction was started by the addition of 1 ml of 30 mM H_2O_2 and followed by the decrease in absorbance at 240 nm for about 1 min. The decrease in absorbance was recorded against a blank tube containing 1 ml of buffer instead of substrate H_2O_2. Catalse activity was calculated as;

$$\text{Activity} = (OD_{240}/0.00364)*\text{DF (2000 or 3000)}*\text{Tube dilution}(3/2) \qquad (2.2)$$

2.3.11 Determination of Lipid Peroxidation

The lipid peroxidation products were measured according to the method of Jain and Levine (1995), as described below. Malondialdehyde (MDA) which is an end product of lipid peroxidation reacts with thiobarbituric acid (TBA) and forms a colored complex having a maximum absorbance at 532 nm.

In assay medium, 0.2 ml of liver microsome was suspended in 0.8 ml of 0.018 M phosphate-buffered saline (pH 7.4) and 0.025 ml of butylated hydroxytoluene (BHT). 0.5 ml of 30% trichloroacetic acid was then added. The tubes were mixed and allowed to stand in ice for at least two hours. The tubes were centrifuged at 2000 *g* for 15 min. 1 ml of supernatant was then transferred to another tube to which 0.075 ml of 0.1 M EDTA and 0.25 ml of 1% TBA in 0.05 N NaOH was added. The tubes were mixed and kept in boiling water bath for 45 min. The samples were cooled to room temperature and the absorbances were measured at 532 nm and 600 nm. The absorbances of the samples at 600 nm were subtracted from 532 nm. This process was applied for the deduction of free MDA in the sample. MDA values in nmol were calculated with the extinction coefficient of MDA - TBA complex at 532 nm (ε_{532}= 1.56 x 10^5 L/mol.cm). TBA reactivity was expressed per mg of protein. TBARS test determines any compound that reacts with thiobarbituric acid, but the most abundant product is malondialdehyde. Therefore,

the test is named as thiobarbituric acid reactive substances (TBARS) test. Butylated hydroxytoluene (BHT) is used to prevent artificial increase of MDA during the experiment.

2.3.12 Western Blot Analysis – Protein Blotting

Liver, lung and kidney cytochrome P450 protein levels were determined as described by Towbin et al. (1979) with some modifications. Polyclonal anti-rabbit CYP2E1 (Oxford Biomedical Research, MI, USA), Polyclonal anti-rat CYP1A2 (Chemicon Internatinol, Temecula, CA, USA), Polyclonal anti-human CYP3A4 (BD Biosciences, San Jose, CA, USA), anti-sheep lung CYP2B was used as primary antibodies. Anti-sheep lung CYP2B antibody was obtained in our laboratory by injecting purified sheep lung CYP2B into female rabbits (Adalı and Arınç, 1992, Arınç et al., 1995; Arınç and Şen, 1999). Immunoblot analysis of purified sheep lung CYP2B and PB-induced rabbit liver and lung CYP2B4 using the antibodies raised against sheep lung CYP2B showed that these cytochromes were homologous (Adalı and Arınç, 1992; Arınç et al., 1995). Similar results were also obtained when purified sheep lung CYP2B and rabbit liver CYP2B4 were probed with anti-rabbit liver P4502B4 (Adalı and Arınç, 1990; Arınç, 1993).

First, microsomal proteins were separated by polyacrylamide gel electrophoresis in the presence of SDS, in a discontinuous buffer system as described by Laemmli (1970) by using 4% stacking gel and 8.5% separating gel. The separating and stacking gel solutions were prepared just before use as given in Table 2.8 in a given order.

Table 2.8 Components of separating and stacking gel solutions

Constituents	**Separating Gel (8.5%) (0.375 M Tris, pH 8.8)**	**Stacking Gel (4%) (0.125 M Tris, pH 6.8)**
Gel solution (ml)	8.5	1.3
Distilled water (ml)	13.55	6.1
Separating gel buffer (ml)	7.5	-
Stacking Gel buffer (ml)	-	2.5
10% SDS (ml)	0.3	0.1
Ammonium persulfate (ml)	0.15	0.05
Temed (ml)	0.015	0.01
Total volume	30	10

Vertical slab gel electrophoresis was carried out using Scie-plasV10-CDC vertical electrophoresis unit (Southam, England). Polyacrylamide slab gels were prepared using the gel sandwich. The gel sandwich was prepared between two glass plates leaving 1 mm space between plates and central gel running module was assembled. The module was placed in melted agar and both sides were sealed with agar to prevent a leakage of separating gel and stacking gel solution. First separating gel solution was transferred to the center of gel sandwich until the desired height of the solution in the sandwich was obtained. The top of the gel polymerizing solution was covered with a layer of isobutanol to ensure the formation of smooth gel surface. After polymerization of separating gel, the layer of alcohol was poured off completely. The stacking gel polymerization solution was prepared and poured into the center of gel sandwich over the separating gel until the sandwich was filled completely. After adding stacking gel, a 1.0 mm Teflon comb with 12 wells was inserted into stacking gel solution. After polymerization, Teflon comb was carefully removed without tearing the wells. Wells were filled with electrode running buffer (25 mM Tris, 192 mM glycine and 0.1% SDS) using

syringe with a fine needle to remove any air bubbles and unpolymerized chain particles. Then gel running module was filled with a necessary volume of electrode running buffer. Protein samples were diluted 1:3 (3 part sample and 1 part buffer) with 4× sample dilution buffer containing 0.25 M Tris-HCl, pH 6.8, 8% SDS, 40% glycerol, 20% β-mercaptoethanol, and 0.01% bromophenol blue and were boiled in a boiling water bath for 2 minutes. Each sample was applied to different wells by Hamilton syringe.

After application of the samples, gel running module were placed in the main buffer tank filled with an appreciate amount of electrode running buffer. The electrophoresis unit was connected to the power supply Bio-Rad model 2 (Bio-Rad Laboratories, Richmond, California, USA) and electrophoresis was run at 10mA and 100V in stacking gel and 20mA 200V in separating gel. When electrophoresis was completed, gel was removed for western blot. Gels were equilibrated in transfer buffer (25 mM Tris, 192 mM glycine and 20% methanol) for 15 minutes in order to adjust the final size of gel and remove the buffer salts and SDS which were used in the SDS-Polyacrylamide gel electrophoresis. Nitrocellulose membrane was cut 1 cm larger then the dimension of the gel and two pieces of filter paper (Whatman #1) were cut to a dimension a little bit larger than the membrane. Nitrocellulose membrane, two filter paper and fiber pads of the transfer sandwich were placed in transfer buffer and saturated with this solution. Later, the sandwich was put into the Bio-Rad Trans-Blot Cell and the cell was filled with cold transfer buffer. Voltage and current were set to 90V and 400 mA, respectively. Transfer process was carried out at cold room (4°C) for 90 minutes. At the end of this period, the membrane having the transferred protein on it, i.e. “blot” was obtained and taken from the cell and placed into a plastic dish in such a way that protein side facing up and washed with TBST (Tris Buffered Saline plus Tween 20: 20mM Tris-HCl, pH 7.4, 0.5 M NaCl, and 0.05 % Tween 20) for 10 minutes in order to remove the salts and buffers of transfer medium. Then the blot was incubated with blocking solution (5% Non-Fat Dry-Milk in TBST) for 40 minutes in order to fill the empty spaces between bound proteins by this way prevent the non-specific binding.

The blot was incubated with primary antibody for 2 hours. As primary antibody, Polyclonal anti-rabbit CYP2E1 (1:1.000 ratio), Polyclonal anti-rat CYP1A2 (1:2.000 ratio), Polyclonal anti-human CYP3A4 (1:500 ratio), anti-sheep lung CYP2B (1:1.000 ratio) were used. Then the blot was washed 3 times with 50 ml TBST for 5 minutes each. The washing steps are necessary to remove excess antibody from the membrane. The blot was then incubated with secondary antibody conjugated to marker enzyme-alkaline phosphatase (anti-rabbit IgG-ALP conjugate, 1:5.000 ratio) for 1 hour. The blot was washed three times with TBST for 5 minutes each to remove excess antibody. Since the excess antibody will give reaction with substrate solution nonspecifically the complete removal of the excess antibody between each washing steps are extremely important. Finally, blot was incubated with substrate solution given in Table 2.9 as described by Ey and Ashman (1986) to visualize the specifically bound antibodies. The final images were photographed by using computer based gel imaging instrument (Infinity 3000-CN-3000 darkroom) (Vilber Lourmat, Marne-la-Vallee Cedex 1, France) by using Infinity-Capt Version 12.9 software. Protein bands were quantified using Scion Image Version Beta 4.0.2 software.

Table 2.9 Preparation of substrate solution for immunodetection

Solution A: 2.67 ml of 1.5 M Tris-HCl, pH 8.8 4.0 ml of 1M NaCl 0.82 ml of 100 mM $MgCl_2$ 0.04 ml of 100 mM $ZnCl_2$ 0.096 ml of DEA 12.2 mg NBT Distilled water to 40 ml (pH of the solution was adjusted to 9.55 with saturated Tris before completing to final volume)
Solution B: 2 mg/ml phenazine methosulfate in distilled water
Solution C: 5.44 mg/0.136 ml N, N-dimethyl formamide
Finally NBT/BCIP substrate solution was prepared by mixing solution A with solution C and 0.268 ml of solution B.

2.3.13 Gene Expression Analysis by Real time PCR

Gene expression analysis plays an increasingly important role in many fields of toxicology. Precise quantification of mRNA expression by conventional methods, such as Northern blotting and ribonuclease protection assays, require large amounts of RNA, and are not always feasible when the transcripts of interest display low expression levels and/or sample size may be limiting. Conventional reverse transcription-polymerase chain reaction (RT-PCR) can overcome some of these limitations but requires elaborate and very time-consuming standardization protocols and post-PCR manipulations, making high-throughput analyses difficult. Over the past decade, several investigators have reported the development of real-time PCR quantification methods that have many advantages over conventional RT-PCR in terms of accuracy, sensitivity, dynamic range, high-throughput capacity,

and absence of post-PCR manipulation. In these approaches, sequencespecific fluorescence-labeled probes or primers (e.g. Taq-Man, Molecular Beacons, and Scorpions) and non-specific fluorescence dyes (Syber Greeen) have been considered as detection formats in many research applications and have greatly enhanced our ability to measure and accurately quantify low copy number transcripts in very small samples. One drawback to these methods is that relatively expensive probes, primers, or both are required for each amplicon under investigation.

Although the conventional PCR is a powerful tool in the detection and identification of target nucleic acids, it has its limitations in quantitative analysis. Introduction of the real-time PCR technique offers the ability to quantify the initial target nucleic acids in one reaction. The real-time PCR allows the absolute quantification of the initial target DNA by the measurement of the amplification products at each cycle throughout the PCR procedure. The monitoring of the accumulation of PCR products in real time has been made possible by labeling of primers, oligonucleotide probes or amplification products with fluorescent dyes. During PCR, the accumulation of PCR products is measured automatically after each cycle by a fluorimeter. The signal strength is correlated to the amount of amplification products present during each cycle, and increases as the amount of specific amplification products increases.

Real-Time chemistries allow for the detection of PCR amplification during the early phases of the reaction. Measuring the kinetics of the reaction in the early phases of PCR provides a distinct advantage over traditional PCR detection. Traditional methods use Agarose gels for detection of PCR amplification at the final phase or end-point of the PCR reaction. End-point detection is very time consuming. Results are based on size discrimination, which may not be very precise. The end point is variable from sample to sample. While gels may not be able to resolve these variabilities in yield, real-time PCR is sensitive enough to detect these changes. Agarose Gel resolution is very poor, about 10 fold, on the other hand, Real-Time PCR can detect as little as a two-fold change.

Syber Green I is a fluorescent dye that binds to the minor groove of double-stranded DNA (i.e., amplicons) and fluoresces when bound to DNA and excited by a light source. Syber Green I provide the simplest and most economical format for detecting and quantitating PCR products in real-time reactions. Syber Green I binds double-stranded DNA, and upon excitation emits light. Thus, as a PCR product accumulates, fluorescence increases. The advantages of Syber Green I are that it is inexpensive (when compared to Taq-Man, Molecular Beacons, and Scorpions), easy to use, and sensitive. The disadvantage is that Syber Green I will bind to any double-stranded DNA in the reaction, including primer-dimers and other non-specific reaction products, which results in an overestimation of the target concentration. For single PCR product reactions with well designed primers, Syber Green I can work extremely well, with spurious non-specific background only showing up in very late cycles. Syber Green I is the most economical choice for real-time PCR product detection. Since the dye binds to double-stranded DNA, there is no need to design a probe for any particular target being analyzed. However, detection by Syber Green I requires extensive optimization.

2.3.13.1 Total RNA Isolation from Rabbit Liver, Kidney and Lung

Prior to RNA isolation, all the plastic and glass equipments for RNA work were treated with distilled water containing 0.1% (v/v) diethylpyrocarbonate (DEPC) in order to inhibit RNAse activity. Excess DEPC was evaporated under hood for over night and autoclaved before use. Distilled water for preparing solutions was also treated with DEPC (0.1 % v/v) and then autoclaved

Total RNA from rabbit liver tissue was extracted according to modified and optimized Guanidum Thiocynate (GTC) method as described by Chomczynski and Sacchi, 1987. On the other hand, rabbit kidney and lung total RNA were isolated by using RNeasy kit (Qiagen, GmbH, D40727, Hilden, Germany) according to the manufacturer's instructions.

In the GTC method, Firstly, 75 mg liver tissue samples were powdered using a mortar and liquid nitrogen. Powdered tissues were transferred into DEPC treated microcentrifuge tubes and were dissolved in a solution of 596 µl of 4 M GTC and 4 µl of 0.1 M β-mercaptoethanol. Then, sequentially, 100 µl of 2 M sodium acetate pH 4.0, 1 ml of citrate saturated acid phenol and 200 µl of chloroform were added to tubes. After incubation 15 minutes in ice, tubes were centrifuged at 13000 rpm, 4ºC for 15 minutes. After centrifugation, the upper aqueous phase that was containing RNA was transferred to new microcentrifuge tube carefully. Then, two volume of 95% ethyl alcohol was added and the content was centrifuged at 13000 rpm, 4ºC for 15 minutes. After centrifugation for 15 minutes, the supernatant was discarded, and the pellet containing RNA was resuspended in 200 µl of 70% ethyl alcohol. The RNA was sedimented again by centrifugation at 13000 rpm, 4ºC for 10 minutes. Then, the supernatant was discarded and the pellet was air-dried at room temperature. After that step, pellet was dissolved in 40 µl of DEPC treated sterile distilled water by incubation at 60ºC for 10 minutes. The RNA samples were stored at -80 ^{0}C for further use.

For isolation of the RNA from rabbit kidney and lung tissues, RNeasy Mini Total RNA isolation kit was used according to the manufacturer's instructions. Previously frozen 30 mg kidney and lung tissues were placed in liquid nitrogen cooled mortar and was grinded with a pestle. Tissue powder in liquid nitrogen was poured out into RNAase-free liquid nitrogen cooled 2 ml micrrocentrifuge tubes. After evaporation of the liquid nitrogen without thawing the tissue powder, 600 µl Buffer RLT containing guanidium thiocyanate was added to tubes and was homogenized by passing the lysate at least 5 times through a 20-gauge needle fitted to an RNAase-free syringe. Then, lysate was centrifuged at full speed for 3 minutes. Supernatant was removed carefully by pipetting and was transferred into new microcentrifuge tube. After that, one volume of 70% ethanol was added to lysate and mixed by pipetting immediately. Then, up to 700 µl of this ethanol containing sample including any precipitate that may have formed was placed into RNeasy spin column in a 2 ml collection tubes. The content was centrifuged at 9 000 xg for

15 seconds. Flow-through was discarded and 700 µl Buffer RW1 containing ethanol was added to the RNeasy spin column. The content was again centrifuged at 9 000 xg for 15 seconds in order to wash the spin column membrane. After centrifugation, spin column from the collection tube was carefully removed and placed in new collection tube. 500 µl Buffer RPE was added to RNeasy spin column and the content was centrifuged at 9 000 xg for 15 seconds to wash the spin column membrane. Then, flow-through was discarded and 500 µl buffer RPE was added to RNeasy spin column. The column was centrifuged at 9 000 xg for 2 minutes. After that, RNeasy spin column was placed into 1.5 ml new collection tube and 40 µl RNAase-free water was added directly to the spin column. Spin column was centrifuged at 9 000 xg in order to elute RNA. Eluted RNA was stored at -80^0C for further use.

2.3.13.2 Determination of RNA Concentration

For the determination of the concentration of RNA in the sample, absorbance values at 260 nm and 280 nm were measured in quartz cuvettes using Schimadzu UV-1201 Spectrophotometer (Schimadzu Corporation, Analytical Instruments Division, Kyoto, Japan). As the RNA molecule gave maximum absorption at 260 nm, reading at this wavelength was used to calculate the concentration of nucleic acid in the sample. Based on the knowledge that an optical density of 1.0 corresponded to approximately 40 µg/ml for RNA, the concentration of RNA in the sample was calculated according to the formula:

$$\text{Concentration } (\mu g/mL) = OD_{260nm} \times 40\ (\mu g/ml) \times \text{Dilution Factor.} \qquad (2.3)$$

The ratio between OD values at 260 nm and 280 nm (OD_{260}/OD_{280} ratio) was used to estimate the purity of the nucleic acid. Pure RNA preparations gave the ratio of 2 while the higher or lower values showed either DNA or protein (or phenol) contaminations, respectively.

2.3.13.3 Qualification of RNA by Agarose Gel Electrophoresis

Intactness of 18S and 28S ribosomal rRNA bands was checked on 1 % (w/v) agarose gel by using Biogen horizontal agarose gel electrophoresis unit having a gel tray in 8 cm x 9 cm dimensions.

1% (w/v) agarose gel was prepared by adding 0.4 g of agarose to 40 ml 0.5X TBE (Tris-Borate-EDTA) buffer, pH 8.3. All of the grains of agarose were dissolved in microwave oven. The solution was cooled to approximately 60°C, but not directly on the bench top to prevent heterogenous cooling. When cooled enough, ethidium bromide was added from a stock solution of 10 mg/ml in water to a final concentration of 0.5 µg/ml and the solution was mixed throughly. Agarose gel solution was poured into electrophoresis tray in which comb was placed for well formation prior to solidification and any air bubbles-if present-, especially under or between the teeth of the comb were removed with the help of a pipette tip.

The gel tank was filled with approximately 300 ml of 0.5x TBE buffer. The comb was carefully removed from the gel and the gel in the plastic tray was mounted in the electrophoresis tank so that the slots of the gel faced towards the negative pole-cathode. 0.5x TBE buffer was added to the tank until it covered the gel to a depth of about 1 mm. Any air bubbles in the wells, if present, were removed with the help of a pipette tip.

5 µl of RNA sample was mixed with 1 µl of gel loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF, 15% Ficoll.) by sucking in and out of a micropipette and the mixture was slowly loaded into the slots of the gel. Electrophoresis was performed at 70V for 1 hour. The gel was observed under UV light and photographed.

2.3.13.4 cDNA Synthesis

Reverse transcription is the process by which RNA is used as a template to synthesize cDNA. For cDNA synthesis, 2.5-5 µg of total RNA isolated from rabbit liver, kidney and lung and 0.5 µg (1 µl from 500 µg/ml stock) of oligo (dT) primers (Alpha DNA, Montreal, Quebec. Canada), for poly (A) tail, were mixed. Volume of the RNA was variable due to different concentration. Volume was completed to 11 µl with RNAase-free water and mixture was incubated at 70 ºC for 5 mins. 4 µl of 5X reaction buffer (250mM Tris-HCl pH 8.3, 250 mM KCl, 20 MM $MgCl_2$ and 50 mM DDT) was added to be 1X in the reaction mixture. 2 µl of four dNTP (MBI Fermentas, USA) mix to make a final concentration of 1 mM was added and volume was completed to 19 µl with RNAase-free water. After incubation for 5 minutes at 37°C for annealing of oligo (dT), 0.5 µl (50 units) of Moloney-Murine Leukemia Virus Reverse Transcriptase (MBI Fermentas, USA) was added. Synthesis was performed by incubation for 60 minutes at 42°C and the reaction was stopped by heating to 70°C for 10 minutes. cDNA was stored at -20°C for further use.

2.3.13.5 Real Time PCR

Quantitative real-time PCR assays for rabbit Cytochrome P450 mRNA levels were performed using LightCycler® 1.5 Instrument (Roche Applied Science, Basel, Switzerland) according to manufacturer's instructions. Reactions were performed in 20 µl volumes using Light Cycler-FastStart DNA Master^{Plus} SYBR Green I (Roche Applied Science, Basel, Switzerland). Briefly, 5µl of cDNA was added to a total reaction volume of 20 µl consisting of 9 µl PCR-grade water, 2 µl gene-specific primers (Primer for CYP2E1, CYP1A2 and CYP3A6) and 4 µl Master mix containing FastStart Tag DNA polymerase, $MgCl_2$, SYBR Green I dye and dNTP mix. All components in the reaction mix of the Light Cycler-FastStart DNA Master^{Plus} SYBR Green I are optimized for almost all primer combinations by manufacturer. All samples were run in duplicates. PCR-grade water and a sample in which Reverse transcriptase was omitted during reverse transcription, were included

in every PCR-run as negative controls to confirm that there was no genomic DNA contamination in the cDNA samples.

Primer pairs purified by desalting were purchased from Alpha DNA (Alpha DNA, Montreal, Quebec. Canada). The oligo sequences used as forward and reverse primers for rabbit CYP1A2 and CYP3A6 mRNA were based on those reported in Yang *et al*., (2003) which were designed with the aid of PRIMER PREMIER Software program (Premier Biosoft International). The forward and reverse primers for rabbit CYP2E1 mRNA were previously reported (Peng and Coon, 1998). The sequnces of reverse and forward primers of housekeeping gene, Glyceraldehyde-3-phosphate Dehydrogenase (GAPDH) were based on Reno *et al*., 1998. The nucleotide-nucleotide Blast has confirmed that there was no match between the primers and the rabbit mRNA sequence except for the target genes. Change in concentration and annealing temperatures of primer pairs were tested to check whether an improvement in the efficiency of Real-time PCR was obtained or not. Primer sequences and real-time conditions were described in Table 2.10.

Table 2.10 Oligonucleotide primers sequences and amplification conditions.

Gene	Primer sequences	Annealing Temparture (^{o}C)	Product Length (bp)	T*m* (^{o}C)
CYP2E1	**F** 5'-CATCGGGAATCTTCTCCAGTTGG-3' **R** 5'-TGAAGGGTGTGCAGCCGATGACAA-3'	55	410	90.3
CYP1A2	**F** 5'-GAGCACTATGAGGACTTCG-3' **R** 5'-GTTGATTTGCCACTGGTTTAT-3'	57	441	91.7
CYP3A6	**F** 5'-CCCAATCAATTATCATTCTC-3' **R** 5'-ATTCATCAGGCTCAGTCC-3'	55	348	85.5
GAPDH	**F** 5'-TCACCATCTTCCAGGAGCGA-3' **R** 5'-CACAATGCCGAAGTGGTCGT-3'	54	293	89.6

F: Forward, **R**: Reverse **T*m***: Melting temperature of product

The following LightCycler run protocol was used: Preincubation program was at 95 °C for 15 min in order to activate Taq polymerase and to denature DNA, amplification and quantification program repeated 45 times (95 °C for 10 seconds, 54-57 °C annealing (depending on the gene) for 5 seconds and 72 °C extension for 10-15 seconds with a single fluorescence measurement), melting curve program (65–95 °C with a heating rate of 0.1 °C/s and continuous fluorescence measurement), and finally a cooling step to 40 °C for 30 seconds. Extension period at 72 °C varied with specific primers depending on the length of product (~1seconds/25 base pair). Melting curve analysis of the amplification product was performed at the end of each amplification reaction in order to confirm that a single PCR product was detected or not. Quantities of specific mRNAs in the sample were measured according to the corresponding gene specific relative standard curve derived from dilution series of a control animal cDNA and expression level of the target genes was measured relative to the housekeeping gene, glyceraldehydes-3-phosphate dehydrogenase. LightCycler quantification software (Version 3.5) was used to compare amplification in experimental samples during the log linear phase to the standard curve from the dilution series of a control cDNA. PCR protocol was given in Table 2.11.

2.4 Statistical Analysis

Statistical analyses were performed by using Minitab statistical software package and the SPSS statistical package for Windows. All results were expressed as means with their Standard Error of Means (SEM). Comparison between two groups was performed by Student's *t*-test and $p < 0.05$ was chosen as the level for significance. Statistical comparisons of between four groups were assessed by one-way analysis of variance (ANOVA). When *F* ratios were significant ($p<0.05$), one-way ANOVA was followed by Tukey's Post hoc test for comparisions of multiple group means.

Table 2.11 Real-Time PCR protocol for quantification of different CYP450 mRNAs

Programme Name	Segment	Target Temperature	Hold Time	Acquisition Mode
Preincubation	--	95 °C	15 min	none
Quantification 45 Cycle	Denaturation	95 °C	10 sec	none
	Annealing	Primer dependent (Table 2.9)	5 sec	none
	Extension	72 °C	~1seconds /25 base pair	single
Melting Curve	Denaturation	95 °C	0 sec	none
	Annealing	65 °C	15 sec	none
	Melting	95 °C (slope 0.1 °C/s)	0 sec	continuous
Cooling	--	40 °C	30 sec	none

CHAPTER 3

RESULTS

3.1 Induction of Experimental Diabetus Mellitus by Alloxan Treatment of Rabbits

Adult male New Zealand White rabbits were made diabetic by injecting a single dose of alloxan (125 mg/kg, dissolved in normal saline) into lateral ear vein. Since the blood glucose concentration is an important criteria for induction and level of diabetus mellitus, the fasting blood glucose levels of control and alloxan injected rabbits were measured as described in "Material and Methods".

The fasting blood glucose levels of control and diabetic animals are given in Table 3.1. Alloxan did not cause diabetes in all the rabbits used in this study. The rabbits having a blood glucose level below 200 mg/dl were not used during this work. As given in Table 3.1, blood glucose levels of the diabetic rabbits after 5 weeks of alloxan injection were found as 369, 539, 426, 351, 580, 756, 414, 600, 449, 462, 306, 371, 384, 576, 453, and 800 mg glucose/dl blood with an average value of 490 ± 35.6 mg/dl (mean ± SEM, N=16). The average fasting blood glucose level of these animals prior to the alloxan injection was calculated as 134.4 ± 2.03 mg/dl (mean ± SEM, N=16). As can be seen in Table 3.1, the fasting blood glucose levels of control rabbits were found to be 130, 132, 133, 132, 140, 130, 135, 130, 137, 127, 132, 135, 130, 125, 127 and 137 mg glucose/dl blood with an average value of 132 ± 1.0 mg /dl (mean ± SEM, N=16). The results showed that

the intravenous injection of single dose of alloxan (125 mg/kg body weight) induced the diabetus mellitus as determined by statistically significant 3.7-fold increase in the blood glucose level ($p<0.001$).

Table 3.1 Serum glucose concentrations of control and alloxan injected rabbits.

Glucose Concentration (mg/dl)		
Rabbit	**Control**	**Diabetic***
1	130	369
2	132	539
3	133	426
4	132	351
5	140	580
6	130	756
7	135	414
8	130	600
9	137	449
10	127	462
11	132	306
12	135	371
13	130	384
14	125	576
15	127	453
16	137	800
Average	132 ± 1.0	490 ± 35.6
Change (Fold)	**1.0**	**3.70 X ↑**

* Significantly different from the respective control value $p<0.001$

3.2 Effects of Diabetes, Benzene and Benzene Treatment of Diabetic Rabbits on Blood Serum Lactate Dehyrogenase (LDH), Aspartate Aminotransferase (AST) and Alanine Aminotransferase (ALT) Activities

Table 3.2 tabulates the effects of diabetes, benzene and benzene treatment of diabetic rabbits on LDH, AST and ALT enzyme activities of rabbit blood serum. LDH enzyme activities were found to be 5.5, 6.0, 3.5, 5.0, 2.5 and 3.0 unit/min/mg protein which resulted in an average value of 4.5 ± 0.6 unit/min/mg protein (N=7) for the blood serum of control rabbits and 8.5, 9.5, 9.0, 10.0, 6.5, 10.6 and 6.8 unit/min/mg protein which gave an average value of 8.7 ± 0.6 unit/min/mg protein (mean ± SEM, N=7) of the diabetic rabbits. Thus, induction of diabetes by 125 mg/kg body weight of alloxan caused statistically significant increase (1.93-fold, $p<0.005$) in LDH enzyme activity in rabbit blood serum. As seen in Table 3.2, LDH enzyme activities of the blood serum in response to benzene treatment were 10.5, 16.5, 6.0, 5.5, 16.5, 8.0 and 6.5 which resulted in an average value of 9.9 ± 1.8 unit/min/mg protein (mean ± SEM, N=7). Thus; upon benzene treatment of rabbits, LDH enzyme activity of blood serum increased significantly (2.20-fold, $p<0.05$) with respect to that of the control animals. The enzyme activities recorded for the benzene treated diabetic rabbits were 10.5, 7.5, 11.5, 10.5, 10.5, 11.0 and 8.5 unit/min/mg protein which gave an average of 10.0 ± 0.5 unit/min/mg protein (mean ± SEM, N=7). As can be observed, benzene injection to the diabetic rabbits caused 2.22-fold increase ($p<0.05$) in LDH activity in blood serum.

The effects of diabetes, benzene and benzene treatment of diabetic rabbits of them on AST and ALT enzyme activities of blood serum were also studied. The AST activities obtained from the blood serum of control rabbits were found to be, 0.83, 0.62, 0.52, 0.65, 0.58, 0.34 unit/min/mg protein which resulted in an average value of 0.59 ± 0.07 (mean ± SEM, N=6) unit/min/mg protein and 0.96, 1.12, 0.93, 0.67, 0.85, 0.92, 0.97 unit/min/mg protein which gave an average value of 0.92 ± 0.05 unit/min/mg protein (mean ± SEM, N=7) of the diabetic rabbits. As can be

Table 3.2 Blood serum LDH, AST and ALT enzyme activities in control, diabetic, benzene and benzene treated diabetic rabbits.

Rabbit	Lactate Dehydrogenase (LDH) Unit/min/mg protein				Aspartate Aminotransferase (AST) Unit/min/mg protein				Alanine Aminotransferase (ALT) Unit/min/mg protein			
	Control	Diabetes*	Benzene*	Benzene + Diabetes*	Control	Diabetes**	Benzene*	Benzene + Diabetes*	Control	Diabetes**	Benzene**	Benzene + Diabetes**
1	5.5	8.5	10.5	10.5	0.83	0.96	0.71	0.85	ND	0.34	0.33	0.34
2	6.0	9.5	16.5	7.5	0.62	1.12	0.72	1.05	0.18	0.47	0.31	0.40
3	6.0	9.0	6.0	11.5	0.52	0.93	1.11	0.65	0.24	0.44	0.47	0.33
4	3.5	10.0	5.5	10.5	0.65	0.67	0.71	0.88	0.30	N.D.	0.35	0.40
5	5.0	6.5	16.5	10.5	0.58	0.85	0.65	0.75	0.21	0.34	0.28	0.35
6	2.5	10.6	8	11.0	0.34	0.92	0.67	0.74	0.23	0.36	0.39	0.30
7	3.0	6.8	6.5	8.5	N.D.	0.97	0.68	0.66	0.21	0.39	N.D.	N.D.
8						N.D.	N.D.	1.01			N.D.	0.39
9							0.81				0.33	
Average	4.5±0.6	8.7±0.6	9.9±1.8	10±0.5	0.59±0.07	0.92±0.05	0.76±0.05	0.82±0.05	0.22±0.02	0.39±0.02	0.35±0.02	0.36±0.01
Change (Fold)	**1.0**	**1.93 X ↑**	**2.20 X ↑**	**2.22 X ↑**	**1.0**	**1.56 X ↑**	**1.29 X ↑**	**1.39 X ↑**	**1.0**	**1.77 X ↑**	**1.59 X ↑**	**1.64 X ↑**

*Significantly different from the respective control value $p<0.05$
**Significantly different from the respective control value $p<0.005$
N.D. Not Determined

observed, diabetes caused 1.56-fold increase in AST activities of blood serum, with respect to the control animals. AST enzyme activities of the serum in response to benzene treatment were 0.71, 0.72, 1.11, 0.71, 0.65, 0.67, 0.68, 0.81 unit/min/mg protein which resulted in an average value of 0.76 ± 0.05 unit/min/mg protein (mean ± SEM, N=8). Thus, upon benzene treatment of rabbits, AST enzyme activity of blood serum increased significantly (1.29-fold, $p<0.05$). Furthermore, in benzene treated diabetic rabbits, the AST enzyme activities of blood serum were found as 0.85, 1.05, 0.65, 0.88, 0.75, 0.66, 1.01 unit/min/mg protein. The average specific activity was calculated to be 0.82 ± 0.05 unit/min/mg protein. Therefore, with benzene treatment of diabetic rabbits, the AST enzyme activity was increased by 1.39-fold with respect to the control rabbits (Table 3.2).

The ALT enzyme activities of blood serum obtained from the control rabbits were noted as; 0.18, 0.24, 0.30, 0.21, 0.23, 0.21 unit/min/mg protein which gave an average value of 0.22 ± 0.02 unit/min/mg protein (mean ± SEM, N=6). The activities of the enzyme from the diabetic rabbits were found as; 0.34, 0.47, 0.44, 0.34, 0.36, 0.39 unit/min/mg protein which resulted in an average value of 0.39 ± 0.02 unit/min/mg protein (mean ± SD, N=6). Thus, alloxan injection of the animals, i.v. at a dose of 125 mg/kg body weight caused significant 1.77-fold increase with respect to control animals ($p<0.005$). Also, in benzene treated rabbits, the ALT enzyme activities of blood serum were found as 0.33, 0.31, 0.47, 0.35, 0.28, 0.39 and 0.33 unit/min/mg protein. The average specific activity was calculated to be 0.35 ± 0.02 unit/min/mg protein (mean ± SD, N=7). Therefore, with benzene treatment, the ALT enzyme activity was increased by 1.59-fold with respect to the control rabbits (Table 3.2). Moreover, The enzyme activities recorded for the benzene treated diabetic rabbits were 0.34, 0.40, 0.33, 0.40, 0.35, 0.30 and 0.39 unit/min/mg protein which gave an average of 0.36 ± 0.01 unit/min/mg protein (mean ± SEM, N=7). As can be observed, benzene injection to the diabetic rabbits caused 1.64-fold increase in ALT activity in blood serum ($p<0.005$) (Table 3.2).

3.3 Effects of Diabetus Mellitus, Benzene and Benzene Treatment of Diabetic Rabbits on Mixed Function Oxidase Enzyme Activities

The effects of diabetes, benzene and benzene treatment diabetic rabbits on hepatic, renal and pulmonary microsomal mixed function oxidase (MFO) activities was studied by measuring, NDMA N-demethylase, *p*-nitrophenol hydroxylase, benzphetamine N-demethylase, erythromycin N-demethylase, caffeine N-demethylase and ethoxyresorufin O-dealkylase activities. These MFO enzyme activities have been shown to be valuable criteria in characterizing the specific cytochrome P450 isoenzymes in mammals.

3.3.1 Effects of Diabetes, Benzene and Benzene Treatment of Diabetic Rabbits on NDMA N-Demethylase Activity of Rabbits

3.3.1.1 Liver NDMA N-Demethylase Activity

Microsomal NDMA N-demethylase activity in rabbit liver was determined using a protein concentration of 0.75 mg/0.5 ml for diabetic, benzene treated, benzene treated diabetic rabbits and control animals. As shown in Table 3.3, the specific enzyme activities of hepatic microsomes obtained from the diabetic rabbits were found to be 0.79, 0.84, 1.04, 0.73, 0.67, 0.67 0.64 and 0.86 nmol HCHO/min/mg protein which gave an average value of 0.78 ± 0.05 nmol HCHO/min/mg protein (mean ± SEM, N=8). The specific activities of the enzyme from the control animals were found to be 0.36, 0.43, 0.47, 0.38, 0.44, 0.31 and 0.37 nmol HCHO/min/mg protein which resulted in an average value of 0.39 ± 0.02 nmol HCHO/min/mg protein (mean ± SEM, N=7). Thus, induction of the diabetes by intravenous injection of alloxan (125 mg/kg body weight) enhanced NDMA N-demethylase activity of liver microsomes by 2.0- fold with respect to control animals ($p<0.001$). Moreover, N-demethylation of NDMA in benzene treated rabbits was found to be 1.47, 1.17, 1.70, 0.90, 1.22, 1.15, 0.94 and 1.27 which resulted in an average value of 1.23 ± 0.10 nmol HCHO/min/mg protein (mean ±

SEM, N=8). These results showed that benzene treatment (750 mg/kg body weight for 3 days) caused statistically significant 3.15-fold increase ($p<0.005$) in NDMA N-demethylation rates of rabbit liver microsomes. Finally, the specific activities of the enzyme from the benzene treated diabetic rabbits were found as 1.71, 2.19, 1.99, 2.14, 1.72, 1.70, 1.23 and 1.57 nmol HCHO/min/mg protein which resulted in an average value of 1.78 ± 0.11 nmol HCHO/min/mg protein (mean ± SEM, N=8). Thus, *in vivo* treatment of diabetic rabbits with benzene, s.c., at dose of 750 mg/kg body weight on days 1, 5 and 7 caused 4.56-fold increase in NDMA N-demethylase activites of liver microsomes with respect to controls. Moreover, the activity obtained from benzene treated diabetic rabbits was significantly different from respective diabetic and benzene values ($p<0.001$). The liver microsomal NDMA N-demethylase activities of control, diabetic, benzene treated and benzene treated diabetic rabbits were presented as relative percentages in Figure 3.1.

Table 3.3 Effects of diabetes, benzene and benzene treatment of diabetic rabbits on hepatic microsomal NDMA N-Demethylase activity.

RABBIT	NDMA N-Demethylase Activity (nmol HCHO/min/mg protein)			
	CONTROL	**DIABETES****	**BENZENE***	**BENZENE + DIABETES**,&**
1	0.36	0.79	N.D.	1.71
2	0.43	0.84	1.47	2.19
3	0.47	1.04	1.17	1.99
4	0.38	0.73	1.70	2.14
5	0.44	0.67	0.90	1.72
6	0.31	0.67	1.22	1.70
7	0.37	0.64	1.15	1.23
8		0.86	0.94	1.57
9			1.27	
AVERAGE	0.39 ± 0.02	0.78 ± 0.05	1.23 ± 0.10	1.78 ± 0.11
CHANGE (FOLD)	**1.0**	**2.0 X ↑**	**3.15 X ↑**	**4.56 X ↑**

*Significantly different from the respective control value $p<0.005$.

& Significantly different from the respective diabetes and benzene value $p<0.001$

**Significantly different from the respective control value $p<0.0001$.

N.D. Not Determined

Values given in Table 3.3 were the average of duplicate determinations.

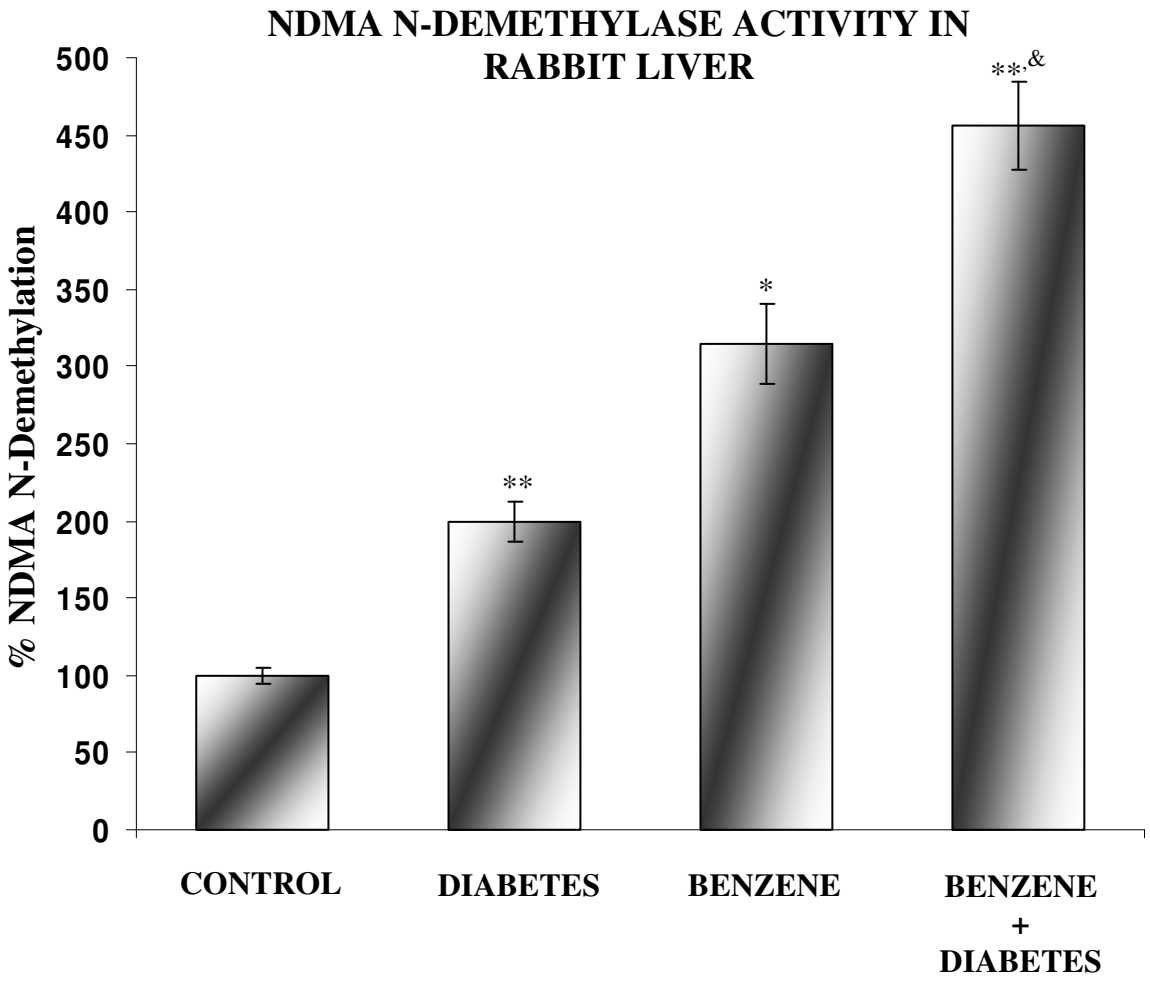


Figure 3.1 Effects of diabetes, benzene and benzene treatment of diabetic rabbits on hepatic CYP2E1 dependent NDMA N-demethylase activity. The value obtained from the control animals was taken to be 100%, and the value obtained from the diabetic, benzene and benzene treated diabetic group was expressed as a percentage of control.

*Significantly different from the respective control value $p<0.005$.

& Significantly different from the respective diabetes and benzene value $p<0.001$

**Significantly different from the respective control value $p<0.0001$.

3.3.1.2 Kidney NDMA N-Demethylase Activity

NDMA N-demethylase activity of rabbit kidney microsomes was determined in a reaction mixture containing 2 mg/0.5 ml microsomal protein. As shown in Table 3.4, the specific enzyme activities of renal microsomes of control rabbits were found to be 0.05, 0.05, 0.045, 0.045, 0.045 and 0.035 nmol HCHO/min/mg protein. The average NDMA N-demethylase activity of diabetic rabbits was calculated as 0.045 ± 0.002 nmol HCHO/min/mg protein (mean ± SEM, N=6). The specific activities of the enzyme from diabetic rabbits were found to be 0.055, 0.080, 0.080, 0.080, 0.065, 0.070 and 0.075 nmol HCHO/min/mg protein which resulted in an average value of 0.072 ± 0.004 nmol HCHO/min/mg protein (mean ± SEM, N=7). Thus, induction of the diabetes by injection of a single dose (125 mg/kg body weight) of alloxan resulted in 1.6-fold increase in NDMA N-demethylation rates of kidney microsomes. Moreover, as shown in Table 3.4, the specific NDMA N-demethylase activities were found as 0.100, 0.085, 0.085, 0.090, 0.090, 0.100, 0.090, 0.120 and 0,130 nmol HCHO/min/mg protein which resulted in an average value of 0.099 ± 0.005 nmol HCHO/min/mg protein (mean ± SEM, N=9) for the kidney microsomes obtained from the benzene treated rabbits, and 0.12, 0.11, 0.12, 0.13, 0.14, 0.15, 0.11 and 0.14 nmol HCHO/min/mg protein (average was 0.13 ± 0.005 nmol HCHO/min/mg protein, N=8) for the microsomes from the diabetic animals treated with benzene. Thus, benzene treatment of rabbits at a dose of 750 mg/kg body weight on days 1, 5 and 7 caused statistically significant 2.2-fold increase in NDMA N-demethylase activity in rabbit kidney microsomes ($p<0.0001$). Also, 2.89-fold increase was observed in diabetic animals treated with benzene ($p<0.0001$) when compared to control animals. This was also found significantly different from the respective diabetes and benzene values. The renal microsomal NDMA N-demethylase activities of control, diabetic, benzene treated and benzene treated diabetic rabbits were presented as relative percentages in Figure 3.2.

Table 3.4 Effects of diabetes, benzene and benzene treatment of diabetic rabbits on renal microsomal NDMA N-Demethylase activity.

RABBIT	NDMA N-Demethylase Activity (nmol HCHO/min/mg protein)			
	CONTROL	**DIABETES***	**BENZENE***	**BENZENE + DIABETES*,&**
1	0.050	0.055	0.100	0.12
2	0.050	0.080	0.085	0.11
3	0.045	0.080	0.085	0.12
4	0.045	0.080	0.090	0.13
5	0.045	0.065	0.090	0.14
6	0.035	0.070	0.100	0.15
7		0.075	0.090	0.11
8			0.120	0.14
9			0.130	
AVERAGE	0.045 ± 0.002	0.072 ± 0.004	0.099 ± 0.005	0.13 ± 0.005
CHANGE (FOLD)	**1.0**	**1.60 X ↑**	**2.20 X ↑**	**2.89 X ↑**

*Significantly different from the respective control value $p<0.0001$

& Significantly different from the respective diabetes and benzene value $p<0.0001$

Values given in Table 3.4 were the average of duplicate determinations.

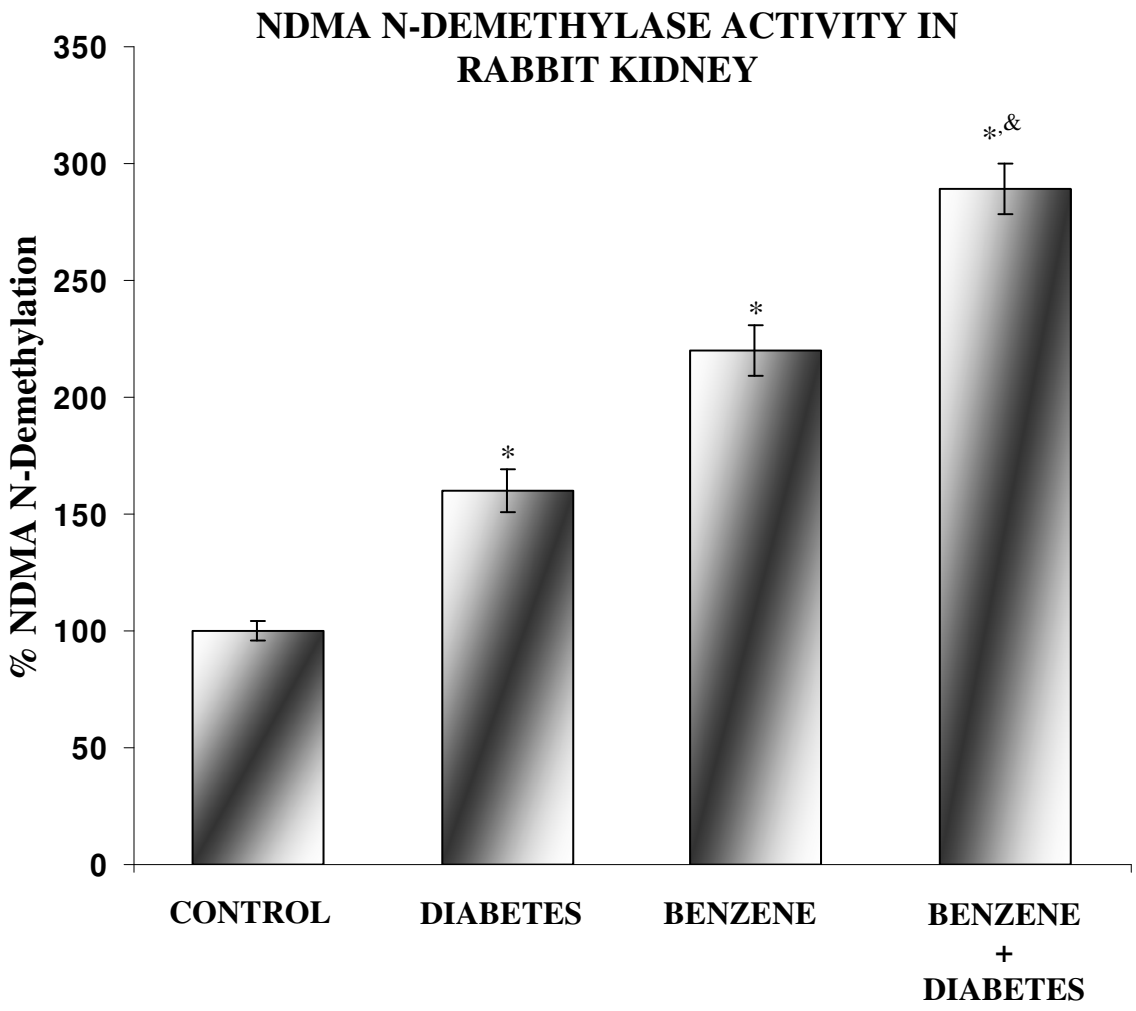


Figure 3.2 Effects of diabetes, benzene and benzene treatment of diabetic rabbits on renal CYP2E1 dependent NDMA N-demethylase activity. The value obtained from the control animals was taken to be 100%, and the value obtained from the diabetic, benzene and benzene treated diabetic group was expressed as a percentage of control.

*Significantly different from the respective control value $p<0.0001$

& Significantly different from the respective diabetes and benzene value $p<0.0001$

3.3.1.3 Lung NDMA N-Demethylase Activity

Microsomal NDMA N-demethylase activity of rabbit lung was determined in an incubation mixture containing 1 mg/0.5ml microsomal protein as described in Material and Methods. As seen in Table 3.5, the specific NDMA N-demethylase activities of pulmonary microsomes obtained from the control animals were found to be 0.19, 0.16, 0.13, 0.12 0.13, 0.16, and 0.18 nmol HCHO/min/mg protein which gave an average value of 0.15 ± 0.01 nmol HCHO/min/mg protein (mean ± SEM, N=7). The specific activities of the enzyme from the control rabbits were found as 0.28, 0.28, 0.22, 0.23, 0.29, 0.26, 0.21 and 0.26 nmol HCHO/min/mg protein. The average activity was calculated as 0.25 ± 0.01 nmol HCHO/min/mg protein (mean ± SEM, N=8). Induction of diabetes by a single dose of alloxan (125 mg/kg body weight) resulted in 1.66-fold increase in lung microsomal NDMA N-demethylase activity with respect to the microsomal enzyme obtained from the control animals. Moreover, the specific enzyme activities of hepatic microsomes obtained from the benzene treated animals were found to be 0.11, 0.15, 0.15, 0.18, 0.13, 0.13, 0.07, 0.17, 0.11 nmol HCHO/min/mg protein which gave an average value of 0.13 ± 0.01 nmol HCHO/min/mg protein (mean ± SEM, N=9). The observed decrease in the actvity was not found statistically significant in N-demethylation rates of NDMA of lung microsomes. Finally, the NDMA N-demethylase activities from the benzene treated diabetic rabbits were found as 0.22, 0.13, 0.19, 0.11, 0.16, 0.13, 0.16, 0.14 nmol HCHO/min/mg protein which resulted in an average value of 0.16 ± 0.01 nmol HCHO/min/mg protein (mean ± SEM, N=8). These results show that benzene treatment of diabetic rabbits caused no significant changes in N-demethylation rates of NDMA of lung microsomes. The pulmonary microsomal NDMA N-demethylase activities of control, diabetic, benzene treated and benzene treated diabetic rabbits were summarized as relative percentages in Figure 3.3.

Table 3.5 Effects of diabetes, benzene and benzene treatment of diabetic rabbits on Pulmonary microsomal NDMA N-Demethylase activity.

RABBIT	NDMA N-Demethylase Activity (nmol HCHO/min/mg protein)			
	CONTROL	DIABETES *, #	BENZENE	BENZENE + DIABETES
1	0.19	0.28	0.11	0.22
2	0.16	0.28	0.15	0.13
3	0.13	0.22	0.15	0.19
4	0.12	0.23	0.18	0.11
5	0.13	0.29	0.13	0.16
6	0.16	0.26	0.13	0.13
7	0.18	0.21	0.07	0.16
8		0.26	0.17	0.14
9			0.11	
AVERAGE	0.15 ± 0.01	0.25 ± 0.01	0.13 ± 0.01	0.16 ± 0.01
CHANGE (FOLD)	**1.0**	**1.66 X ↑**	**0.87 X ↓**	**1.07 X ↑**

*Significantly different from the respective control value $p<0.0001$

#Significantly different from the respective " benzene + diabetes" value $p<0.0001$

Values given in Table 3.5 were the average of duplicate determinations.

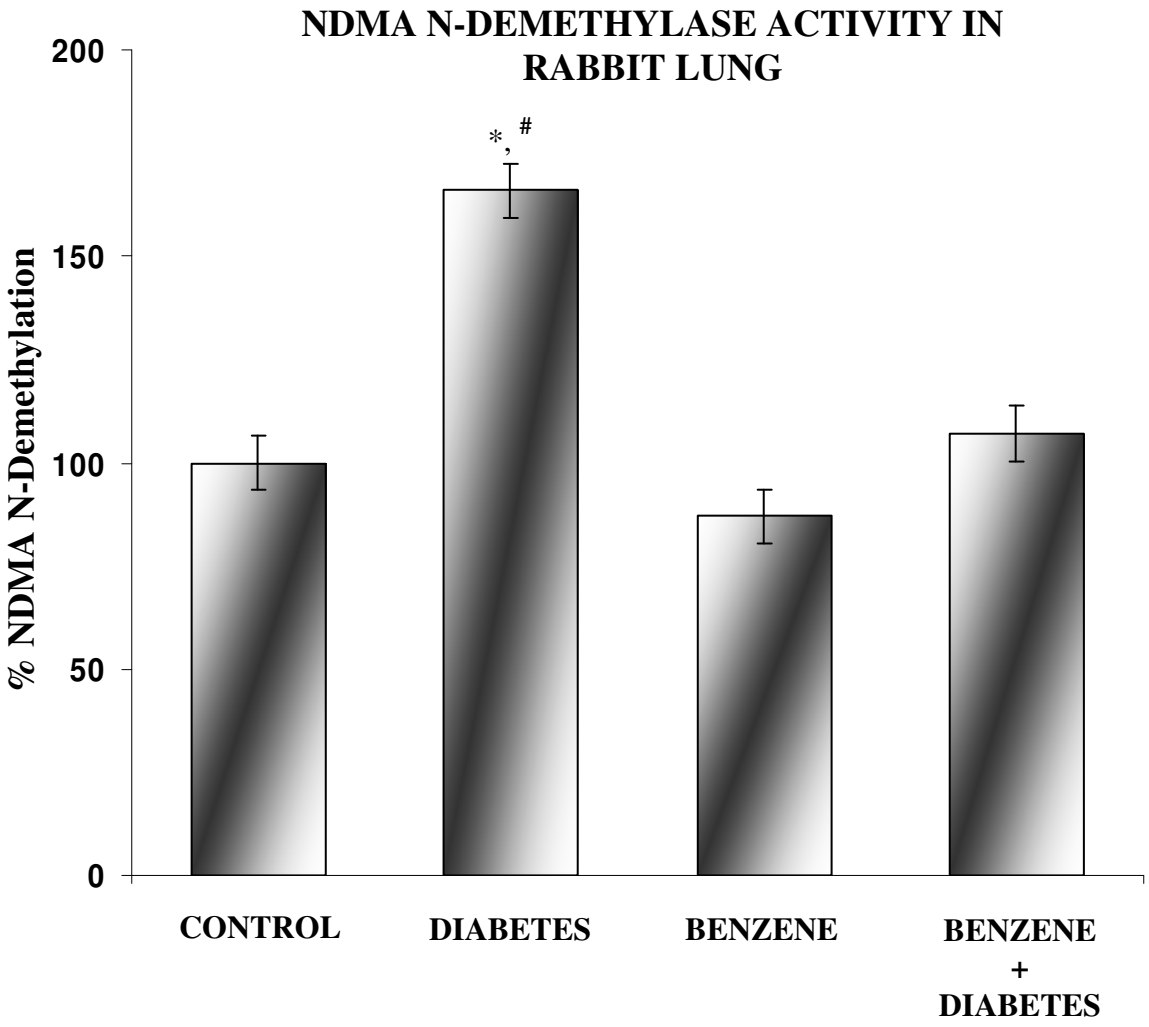


Figure 3.3 Effects of diabetes, benzene and benzene treatment of diabetic rabbits on pulmonary CYP2E1 dependent NDMA N-demethylase activity. The value obtained from the control animals was taken to be 100%, and the value obtained from the diabetic, benzene and benzene treated diabetic group was expressed as a percentage of control.

*Significantly different from the respective control value $p<0.0001$

#Significantly different from the respective "benzene + diabetes" value $p<0.0001$.

3.3.2 Effects of Diabetes, Benzene and Benzene Treatment of Diabetic Rabbits on p-Nitrophenol Hydroxylase Activity of Rabbits

3.3.2.1 Liver *p*-Nitrophenol Hydroxylase Activity

The hydroxylation of *p*-nitrophenol was determined by using 1.5 mg/1ml of hepatic microsomal protein from control, diabetic, benzene treated and benzene treated diabetic rabbits. As shown in Table 3.6, the specific *p*-nitrophenol hydroxylase activities were found as 0.26, 0.26, 0.28, 0.29, 0.25, 0.30 and 0.36 nmol product/min/mg protein which resulted in an average value of 0.30 ± 0.02 nmol product /min / mg protein (mean ± SEM, N=7) for liver microsomes obtained from the control rabbits, and 0.51, 0.55, 0.40, 0.55, 0.53, 0.43, 0.61 and 0.59 nmol product/min/mg protein for the diabetic rabbits which gave an average value of 0.52 ± 0.03 nmol product/min/mg protein (mean ± SEM, N=8). These results showed that induction of diabetus mellitus by alloxan (125 mg/ kg body weight) caused 1.73-fold increase in *p*-nitrophenol hydroxylation rates of rabbit liver microsomes with respect to control animals. Besides, the specific activities of benzene treated rabbits were found to be 0.62, 0.91, 0.77, 0.79, 0.54, 0.73, 0.70, 0.48, 0.84 nmol product/min/mg protein which resulted in average value of 0.71 ± 0.05 nmol product/min/mg protein (mean ± SEM, N=9). Thus, benzene treatment of rabbits caused statistically significant 2.37-fold increase in liver microsomes ($p<0.0001$). At the same time, hydroxlation rate of *p*-nitrophenol were found as 1.00, 1.15, 1.21, 1.20, 1.19, 1.20, 1.01, 1.27 nmol product/min/mg protein in diabetic rabbits treated with benzene. These activities were resulted in an average value of 1.15 ± 0.03 nmol product/min/mg protein (mean ± SEM, N=8). Thus, benzene treatment of diabetic rabbits caused 3.83-fold increase in *p*-nitrophenol hydroxylase activity in liver microsomes with respect to those of the control animals ($p<0.0001$). This was also found significantly different from the respective diabetes and benzene values ($p<0.001$). The hepatic microsomal *p*-nitrophenol hydroxylase activities of control, diabetic, benzene treated and benzene treated diabetic rabbits were summarized as relative percentages in Figure 3.4.

Table 3.6 Effects of diabetes, benzene and benzene treatment of diabetic rabbits on hepatic microsomal *p*-Nitrophenol Hydroxylase activity.

RABBIT	***p*-Nitrophenol Hydroxylase Activity (nmol product/min/mg protein)**			
	CONTROL	**DIABETES***	**BENZENE***	**BENZENE + DIABETES*,&**
1	0.26	0.51	0.62	1.00
2	0.26	0.55	0.91	1.15
3	0.28	0.40	0.77	1.21
4	0.29	0.55	0.79	1.20
5	0.25	0.53	0.54	1.19
6	0.30	0.43	0.73	1.20
7	0.36	0.61	0.70	1.01
8		0.59	0.48	1.27
9			0.84	
AVERAGE	0.30 ± 0.02	0.52 ± 0.03	0.71 ± 0.05	1.15 ± 0.03
CHANGE (FOLD)	**1.0**	**1.73 X ↑**	**2.37 X ↑**	**3.83 X ↑**

& Significantly different from the respective diabetes and benzene value $p<0.001$

*Significantly different from the respective control value $p<0.0001$

Values given in Table 3.6 were the average of duplicate determinations.

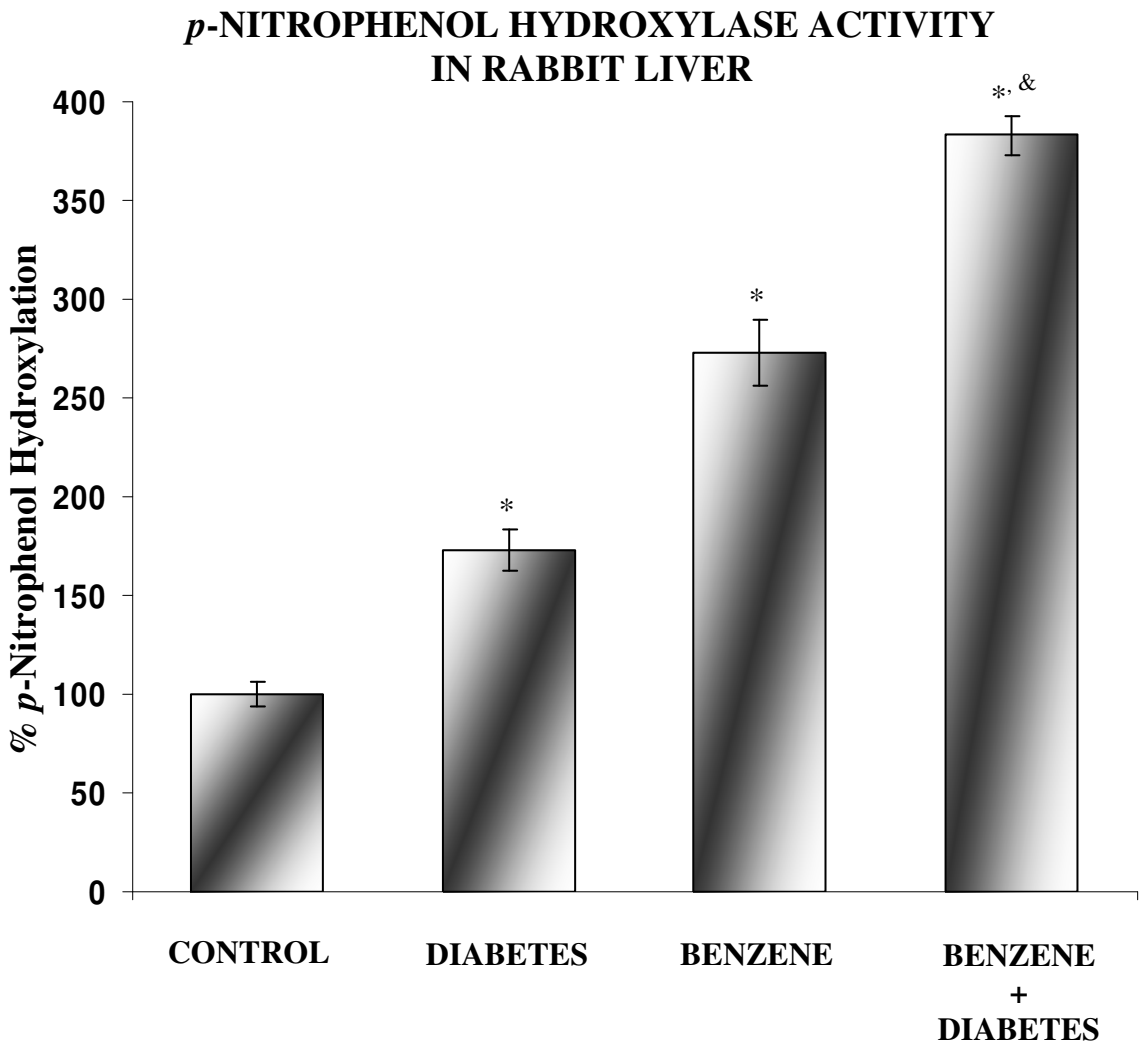


Figure 3.4 Effects of diabetes, benzene and benzene treatment of diabetic rabbits on hepatic CYP2E1 dependent *p*-nitrophenol hydroxylase activity. The value obtained from the control animals was taken to be 100%, and the value obtained from the diabetic, benzene and benzene treated diabetic group was expressed as a percentage of control.

[&] Significantly different from the respective diabetes and benzene value $p<0.001$

*Significantly different from the respective control value $p<0.0001$.

3.3.2.2 Kidney *p*-Nitrophenol Hydroxylase Activity

Microsomal *p*-nitrophenol hydroxylase activity in kidney was determined using 4 mg/ml microsomal protein concentration. As can be seen in Table 3.7, the specific activities of this enzyme from control rabbits were found to be 0.016, 0.016, 0.016, 0.018, 0.023, 0.014, 0.016 nmol product/min/mg protein which gave the average as 0.017 ± 0.001 nmol product/min/mg protein, (mean ± SEM, N=7). The enzyme activities of renal microsomes obtained from diabetic rabbits were found to be 0.064, 0.039, 0.050, 0.045, 0.032, 0.032, 0.030 and 0.037 nmol product/min/mg protein. The average activity was calculated as 0.041 ± 0.004 nmol product/min/mg protein (mean ± SEM, N=8). These results indicated that induction of the diabetes in rabbits by single dose of alloxan caused significant 2.41-fold increase in *p*-nitrophenol hydroxylase activity of kidney microsomes ($p<0.001$). Furthermore, *p*-nitrophenol hydroxylase enzyme activities of microsomes obtained from the benzene treated rabbits were noted as 0.058, 0.065, 0.047, 0.040, 0.042, 0.045, 0.059, 0.048 and 0.056 nmol product/min/mg protein which gave an average value of 0.051 ± 0.003 nmol product/min/mg protein. (mean ± SEM, N=9). Thus, *in vivo* treatment of benzene, s.c., at a dose of 750 mg/kg body weight on days 1, 5 and 7 caused 3.00-fold increase in *p*-nitrophenol hydroxylase activity in rabbit kidney microsomes ($p<0.0001$). Finally, *p*-nitrophenol hydroxylase activities from the benzene treated diabetic rabbits were found as 0.074, 0.079, 0.074, 0.10, 0.074, 0.079, 0.053, 0.082 nmol product/min/mg protein which resulted in an average value of 0.077 ± 0.005 nmol product/min/mg protein (mean ± SEM, N=8). Thus, it can be postulated that benzene treatment of diabetic rabbits resulted in statistically significant 4.53-fold increase in hydroxylation of *p*-nitrophenol of kidney microsomes with respect to the control animals ($p<0.0001$). Hydroxylation rate of *p*-nitrophenol in benzene treated diabetic rabbits was also significantly different from respective diabetes and benzene values ($p<0.0001$). The renal microsomal *p*-nitrophenol hydroxylase activities of control, diabetic, benzene treated and benzene treatment of diabetic rabbits were represented as relative percentages in Figure 3.5.

Table 3.7 Effects of diabetes, benzene and benzene treatment of diabetic rabbits on renal microsomal *p*-Nitrophenol Hydroxylase activity.

RABBIT	***p*-Nitrophenol Hydroxylase Activity (nmol product/min/mg protein)**			
	CONTROL	**DIABETES***	**BENZENE****	**BENZENE + DIABETES**,&**
1	0.016	0.064	0.058	0.074
2	0.016	0.039	0.065	0.079
3	0.016	0.050	0.047	0.074
4	0.018	0.045	0.040	0.10
5	0.023	0.032	0.042	0.074
6	0.014	0.032	0.045	0.079
7	0.016	0.030	0.059	0.053
8		0.037	0.048	0.082
9			0.056	
AVERAGE	0.017 ± 0.001	0.041 ± 0.004	0.051 ± 0.003	0.077 ± 0.003
CHANGE (FOLD)	**1.0**	**2.41 X ↑**	**3.00 X ↑**	**4.53 X ↑**

*Significantly different from the respective control value $p<0.001$.

**Significantly different from the respective control value $p<0.0001$.

& Significantly different from the respective diabetes and benzene value $p<0.0001$

Values given in Table 3.7 were the average of duplicate determinations.

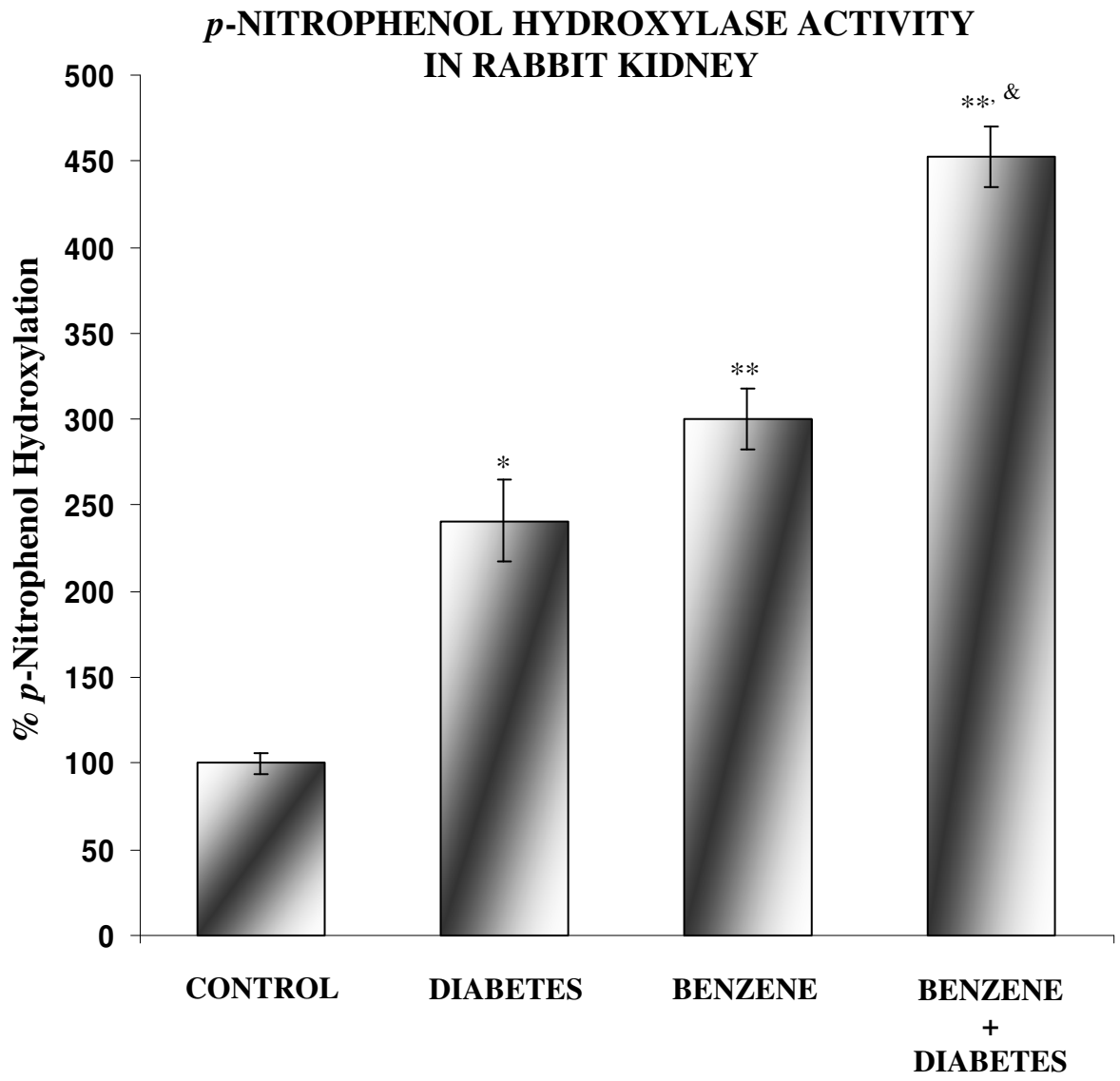


Figure 3.5 Effects of diabetes, benzene and benzene treatment of diabetic rabbits on renal CYP2E1 dependent *p*-nitrophenol hydroxylase activity. The value obtained from the control animals was taken to be 100%, and the value obtained from the diabetic, benzene and benzene treated diabetic group was expressed as a percentage of control.

*Significantly different from the respective control value $p<0.0001$.

**Significantly different from the respective control value $p<0.001$.

[&] Significantly different from the respective diabetes and benzene value $p<0.0001$

3.3.2.3 Lung p-Nitrophenol HydroxylaseActivity

The hydroxylation of *p*-nitrophenol to 4-nitrocatechol was determined in a reaction mixture containing 2 mg microsomal protein. As seen in Table 3.8, the specific activities of pulmonary microsomes obtained from the control animals were found to be 0.16, 0.12, 0.13, 0.15, 0.12, 0.13, 0.14 nmol product/min/mg protein which gave an average value of 0.14 ± 0.006 nmol product/min/mg protein (mean ± SEM, N=7). The specific activities of the enzyme from the diabetic rabbits were found as 0.21, 0.24, 0.19, 0.19, 0.22, 0.21, 0.19 and 0.26 nmol product/min/mg protein which resulted in an average value of 0.21 ± 0.009 nmol product/min/mg protein (mean ± SEM, N=8). Thus, induction of diabetus mellitus by single dose of alloxan caused 1.50-fold increase in *p*-nitrophenol hydroxylase activity of lung microsomes with respect to control animals ($p<0.0001$). Enzyme activities of hepatic microsomes obtained from the benzene treated animals (at a dose of 750 mg/kg body weight) were found to be 0.13, 0.10, 0.12, 0.15, 0.16, 0.14, 0.13, 0.11, 0.15, 0.14 nmol HCHO/min/mg protein which gave an average value of 0.13 ± 0.006 nmol HCHO/min/mg protein (mean ± SEM, N=9). These results indicate that benzene treatment of rabbits caused no significant change in hydroxylation rates of *p*-nitrophenol in lung microsomes with respect to control animals. Furthermore, the specific activities of the enzyme from the benzene treated diabetic rabbits were found as 0.14, 0.14, 0.16, 0.13, 0.15, 0.13, 0.13, 0.14 nmol product/min/mg protein which resulted in an average value of 0.14 ± 0.004 nmol product/min/mg protein (mean ± SEM, N=8). Thus, *in vivo* treatment of diabetic rabbits with benzene caused no change in *p*-nitrophenol hydroxylase activities of lung microsomes. The pulmonary microsomal *p*-nitrophenol hydroxylase activities of control, diabetic, benzene treated and benzene treated diabetic rabbits were represented as relative percentages in Figure 3.6.

Table 3.8 Effects of diabetes, benzene and benzene treatment of diabetic rabbits on pulmonary microsomal *p*-Nitrophenol Hydroxylase activity.

RABBIT	***p*-Nitrophenol Hydroxylase Activity (nmol product/min/mg protein)**			
	CONTROL	**DIABETES*,#**	**BENZENE**	**BENZENE + DIABETES**
1	0.16	0.21	0.10	0.14
2	0.12	0.24	0.12	0.14
3	0.13	0.19	0.15	0.16
4	0.15	0.19	0.16	0.13
5	0.12	0.22	0.14	0.15
6	0.13	0.21	0.13	0.13
7	0.14	0.19	0.11	0.13
8		0.26	0.15	0.14
9			0.14	
AVERAGE	0.14 ± 0.006	0.21 ± 0.009	0.13 ± 0.006	0.14 ± 0.004
CHANGE (FOLD)	**1.0**	**1.50 X ↑**	**0.93 X ↓**	**1.0**

*Significantly different from the respective control value $p<0.0001$.

#Significantly different from the respective "benzene + diabetes" value $p<0.0001$.

Values given in Table 3.8 were the average of duplicate determinations

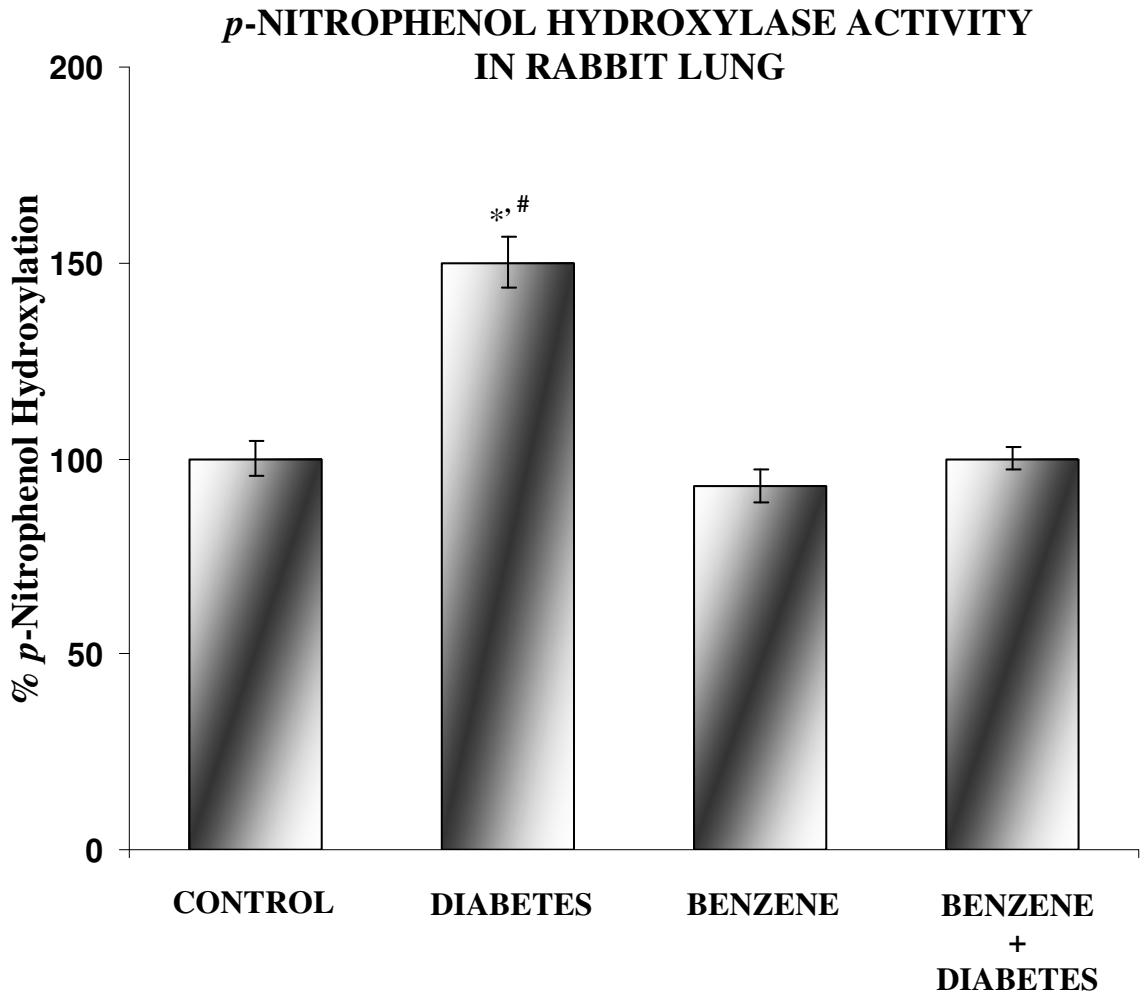


Figure 3.6 Effects of diabetes, benzene and benzene treatment of diabetic rabbits on pulmonary CYP2E1 dependent *p*-nitrophenol hydroxylase activity. The value obtained from the control animals was taken to be 100%, and the value obtained from the diabetic, benzene and benzene treated diabetic group was expressed as a percentage of control.

*Significantly different from the respective control value $p<0.0001$.

#Significantly different from the respective "benzene + diabetes" value $p<0.0001$.

3.3.3 Effects of Diabetes, Benzene and Benzene Treatment of Diabetic Rabbits on Benzphetamine N-Demethylase Activity of Rabbits

3.3.3.1 Liver Benzphetamine N-Demethylase Activity

Microsomal benzphetamine N-demethylase activity in rabbit liver was determined using a protein concentration of 0.5 mg/0.5 ml for diabetic, benzene treated, benzene treated diabetics and control animals. As shown in Table 3.9, the specific enzyme activities of hepatic microsomes obtained from the control animals were found to be 5.38, 5.22, 5.40, 5.11, 7.52, 4.76 and 5.39 nmol HCHO/min/mg protein which gave an average value of 5.54 ± 0.3 nmol HCHO/min/mg protein (N=7). The specific activities of the enzyme from the diabetic rabbits were found as 5.82, 6.77, 6.16, 6.40, 6.37, 5.73, 5.82 and 6.10 nmol HCHO/min/mg protein which resulted in an average value of 6.15 ± 0.1 nmol HCHO/min/mg protein (N=8). These results indicate that diabetes caused no significant change in N-demethylation rates of benzphetamine of liver microsomes. Moreover, enzyme activities of hepatic microsomes obtained from the benzene treated animals were found to be 5.54, 6.25, 5.62, 5.31, 5.53, 5.95, 6.67, 4.01, 6.60 nmol HCHO/min/mg protein which gave an average value of 5.72 ± 0.3 nmol HCHO/min/mg protein (mean ± SEM, N=9). These results show that benzene treatment of rabbits also caused no significant change in benzphetamine N-demethylase activity with respect to control animals. Finally, the specific activities of the enzyme from the benzene treated diabetic rabbits were found as 6.30, 7.15, 6.65, 6.48, 6.21, 6.64, 6.04, 6.67 nmol product/min/mg protein which resulted in an average value of 6.52 ± 0.1 nmol product/min/mg protein (mean ± SEM, N=8). Thus, *in vivo* treatment of diabetic rabbits with benzene caused no significant change in benzphetamine N-demethylase activities of liver microsomes.

Table 3.9 Effects of diabetes, benzene and benzene treatment of diabetic rabbits on hepatic microsomal Benzphetamine N-Demethylase activity.

RABBIT	Benzphetamine N-demethylase Activity (nmol HCHO/min/mg protein)			
	CONTROL	DIABETES	BENZENE	BENZENE + DIABETES
1	5.38	5.82	5.54	6.30
2	5.22	6.77	6.25	7.15
3	5.40	6.16	5.62	6.65
4	5.11	6.40	5.31	6.48
5	7.52	6.37	5.53	6.21
6	4.76	5.73	5.95	6.64
7	5.39	5.82	6.67	6.04
8		6.10	4.01	6.67
9			6.60	
AVERAGE	5.54 ± 0.3	6.15 ± 0.1	5.72 ± 0.3	6.52 ± 0.1
CHANGE (FOLD)	**1.0**	**1.11 X ↑**	**1.03 X ↑**	**1.18 X ↑**

Values given in Table 3.9 were the average of duplicate determinations

3.3.3.2 Kidney Benzphetamine N-Demethylase Activity

The N-demethylation of benzphetamine in rabbit kidney was determined in a reaction mixture containing 1.5 mg microsomal protein per 0.5 ml. As shown in Table 3.10, the specific benzphetamine N-demethylase activities were found as 0.80, 0.79, 0.44, 0.40, 0.43, 0.35 nmol HCHO/min/mg protein which resulted in an average value of 0.54 ± 0.08 nmol HCHO/min/mg protein (N=6) for the kidney microsomes obtained from the control rabbits, and 1.25, 0.49, 0.35, 0.36, 0.40, 0.60, 0.39 nmol HCHO/min/mg protein (average was 0.55 ± 0.12 nmol HCHO/min/mg protein, N=7) for the microsomes from the diabetic animals. Thus, single dose of alloxan treatment of rabbits at a dose of 125 mg/kg body weight caused no significant increase in benzphetamine N-demethylase activity in rabbit kidney microsomes with respect to age-matched control animals. Besides, enzyme activities of renal microsomes obtained from the benzene treated animals were found to be 0.56, 0.51, 0.43, 0.66, 0.65, 0.72, 0.37, 0.44 and 0.52 nmol HCHO/min/mg protein which gave an average value of 0.54 ± 0.04 nmol HCHO/min/mg protein (mean ± SEM, N=9). These results indicate that *in vivo* benzene treatment of rabbits caused no significant change in benzphetamine N-demethylase activity of renal microsomes, also. Finally, the specific benzphetamine N-demethylase activities of benzene treated diabetic rabbit microsomes were calculated to be 0.55, 0.53, 0.66, 0.76, 0.64, 0.52, 0.73, 0.56 nmol HCHO/min/mg protein which resulted in an average value of 0.62 ± 0.03 nmol HCHO/min/mg protein (mean ± SEM, N=8). Thus, it can be postulated that benzene treatment of diabetic rabbits resulted in no significant increase in benzphetamine N-demethylase activity of kidney microsomes.

Table 3.10 Effects of diabetes, benzene and benzene treatment of diabetic rabbits on renal microsomal Benzphetamine N-Demethylase activity.

RABBIT	Benzphetamine N-demethylase Activity (nmol HCHO/min/mg protein)			
	CONTROL	DIABETES	BENZENE	BENZENE + DIABETES
1	0.80	1.25	0.56	0.55
2	0.79	0.49	0.51	0.53
3	0.44	0.35	0.43	0.66
4	0.40	0.36	0.66	0.76
5	0.43	0.40	0.65	0.64
6	0.35	0.60	0.72	0.52
7	N.D.	0.39	0.37	0.73
8		N.D.	0.44	0.56
9			0.52	
AVERAGE	0.54 ± 0.08	0.55 ± 0.12	0.54 ± 0.04	0.62 ± 0.03
CHANGE (FOLD)	**1.0**	**1.02 X ↑**	**1.0**	**1.15 X ↑**

N.D. Not Determined

Values given in Table 3.10 were the average of duplicate determinations

3.3.3.3 Lung Benzphetamine N-Demethylase Activity

Activity of microsomal benzphetamine N-demethylase in rabbit lung was determined with an incubation mixture containing 0.5 mg/0.5ml microsomal protein. As seen in Table 3.11, the specific enzyme activities of pulmonary microsomes obtained from the control animals were found to be 11.32, 11.96, 11.88, 15.32, 11.44, 12.16, 10.78 nmol HCHO/min/mg protein (the average was 12.1 ± 0.6 nmol product/min/mg protein, N=7). The specific activities of the enzyme from the diabetic rabbits were found as 10.24, 15.12, 9.28, 9.56, 10.60, 14.28, 8.32, 13.36 (the average was 11.3 ± 0.9 nmol HCHO/min/mg protein, N=8). Injection of a single dose of alloxan at a dose of 125 mg/kg body weight did not alter the activity of lung microsomal benzphetamine N-demethylase activity with respect to the microsomal enzymes obtained from the control animals. At the same time, effect of benzene treatment on benzphetamine N-demethylase enzyme activity of lung microsomes was also studied and enzyme activities were found to be 13.24, 11.44, 10.36, 10.72, 13.44, 14.00, 9.96, 3.28, 6.64 nmol HCHO/min/mg protein (N=9). The average specific activity was calculated to be 10.3 ± 1.2 nmol HCHO /min/mg protein (mean ± SEM, N=9). Thus, upon benzene treatment of rabbits, benzphetamine N-demethylase activity of pulmonary microsomes did not change significantly. Furthermore, there was also no significant change in the enzyme activity of lung microsomes as a result of benzene treatment of diabetic rabbits. The enzyme activities of the lung microsomes were found to be 13.28, 13.02, 8.48, 9.64, 13.40, 12.96, 11.99, 14.02 nmol HCHO/min/mg protein and the average specific activity was calculated to be 12.1 ± 0.7 nmol HCHO /min/mg protein (mean ± SEM, N=8). The hepatic, renal and pulmonary microsomal benzphetamine N-demethylase activities of control, diabetic, benzene treated and benzene treated diabetic rabbits were given as relative percentages in Figure 3.7.

Table 3.11 Effects of diabetes, benzene and benzene treatment of diabetic rabbits on pulmonary microsomal Benzphetamine N-Demethylase activity.

RABBIT	Benzphetamine N-demethylase Activity (nmol HCHO/min/mg protein)			
	CONTROL	**DIABETES**	**BENZENE**	**BENZENE + DIABETES**
1	11.32	10.24	13.24	13.28
2	11.96	15.12	11.44	13.02
3	11.88	9.28	10.36	8.48
4	15.32	9.56	10.72	9.64
5	11.44	10.60	13.44	13.40
6	12.16	14.28	14.00	12.96
7	10.78	8.32	9.96	11.99
8		13.36	3.28	14.02
9			6.64	
AVERAGE	12.1 ± 0.6	11.3 ± 0.9	10.3 ± 1.2	12.1 ± 0.7
CHANGE (FOLD)	**1.0**	**0.93 X ↓**	**0.85 X ↓**	**1.0**

Values given in Table 3.11 were the average of duplicate determinations

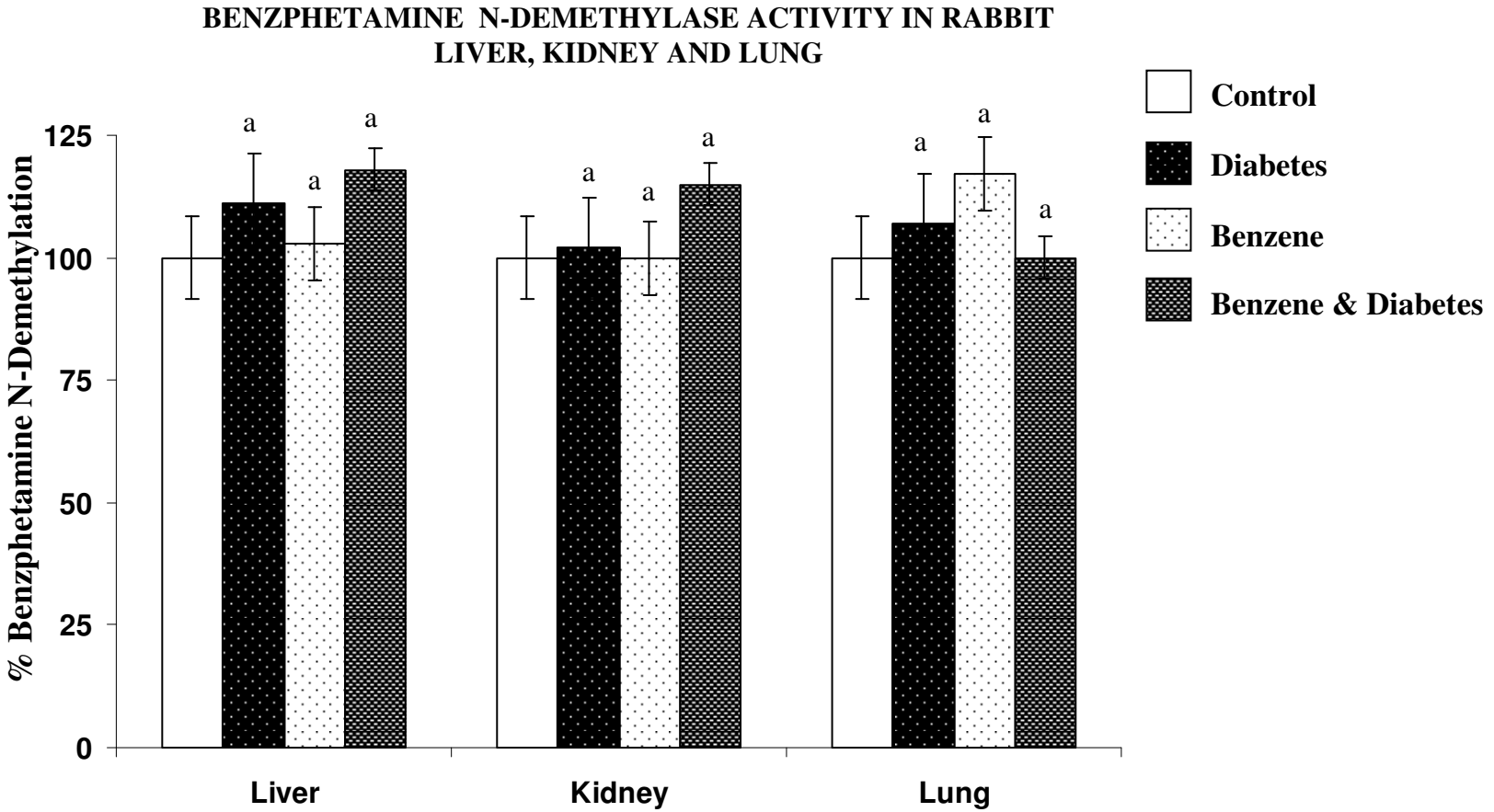


Figure 3.7 Effects of diabetes, benzene and benzene treatment of diabetic rabbits on hepatic, renal and pulmonary CYP2B4 dependent benzphetamine N-demethylase activity. The value obtained from the control animals was taken to be 100%, and the value obtained from the diabetic, benzene and benzene treated diabetic group was expressed as a percentage of control.

[a] Not significantly different from respective control value.

3.3.4 Effects of Diabetes, Benzene and Benzene Treatment of Diabetic Rabbits on Erythromycin N-Demethylase Activity of Rabbits

3.3.4.1 Liver Erythromycin N-Demethylase Activity

Hepatic microsomal N-demethylation of erythromycin was determined using a protein concentration of 1.5 mg/0.5 ml for diabetic, benzene treated, benzene treated diabetic rabbits and control animals as described in Material and Methods. As can be seen in Table 3.12, the specific enzyme activities of hepatic microsomes of the control rabbits were found to be 0.33, 0.28, 0.34, 0.24, 0.34, 0.45 nmol HCHO/ min/mg protein which gave an average value of 0.33 ± 0.03 nmol HCHO/min/mg protein(mean ± SEM, N=6). The specific activities of the enzyme from the diabetic rabbits were calculated to be 0.83, 0.58, 0.99, 0.47, 0.47, 0.31, 0.65 nmol HCHO/min/mg protein which resulted in an average value of 0.61 ± 0.09 nmol HCHO/min/mg protein (mean ± SEM, N=7). Thus, induction of diabetes by *in vivo* treatment with alloxan (i.v.) at a dose of 125 mg/kg body weight resulted in significant 1.85-fold increase in erythromycin N-demethylation rates of liver microsomes ($p<0.05$). Moreover, erythromycin N-demethylase enzyme activities of microsomes obtained from the benzene treated rabbits were noted as 0.19, 0.15, 0.14, 0.19, 0.17, 0.16, 0.22, 0.19, 0.2, and 0.9 nmol HCHO/min/mg protein which gave an average value of 0.18 ± 0.01 09 nmol HCHO/min/mg protein (mean ±SD, N=9). Thus, benzene treatment of the animals, s.c. at a dose of 750 mg/kg body weight caused significant reduction of the erythromycin N-demethylase activity in liver microsomes ($p<0.005$). Finally, the specific erythromycin N-demethylase activities of benzene treated diabetic rabbit microsomes were calculated to be 0.21, 0.37, 0.27, 0.24, 0.27, 0.55, 0.26, 0.23 nmol HCHO/min/mg protein which resulted in an average value of 0.30 ± 0.04 nmol HCHO/min/mg protein (mean ± SEM, N=8). Thus, it can be postulated that benzene treatment of diabetic rabbits resulted in no significant change in erythromycin N-demethylase activity of liver microsomes. The hepatic microsomal erythromycin N-demethylase activities of control, diabetic, benzene treated and benzene treated diabetic rabbits were summarized as relative percentages in Figure 3.8.

Table 3.12 Effects of diabetes, benzene and benzene treatment of diabetic rabbits on hepatic microsomal Erythromycin N-Demethylase activity.

RABBIT	Erythromycin N-demethylase Activity (nmol HCHO/min/mg protein)			
	CONTROL	DIABETES*,#	BENZENE**	BENZENE + DIABETES
1	0.33	0.83	0.19	0.21
2	0.28	0.58	0.15	0.37
3	0.34	0.99	0.14	0.27
4	0.24	0.47	0.19	0.24
5	0.34	0.47	0.17	0.27
6	0.45	0.31	0.16	0.55
7	N.D.	0.65	0.22	0.26
8		N.D.	0.19	0.23
9			0.20	
AVERAGE	0.33 ± 0.03	0.61 ± 0.09	0.18 ± 0.01	0.30 ± 0.04
CHANGE (FOLD)	**1.0**	**1.85 X ↑**	**0.55 X ↓**	**0.91 X ↓**

*Significantly different from the respective control value $p<0.05$.

#Significantly different from the respective "benzene + diabetes" value $p<0.05$

**Significantly different from the respective control value $p<0.005$.

N.D. Not Determined

Values given in Table 3.12 were the average of duplicate determinations

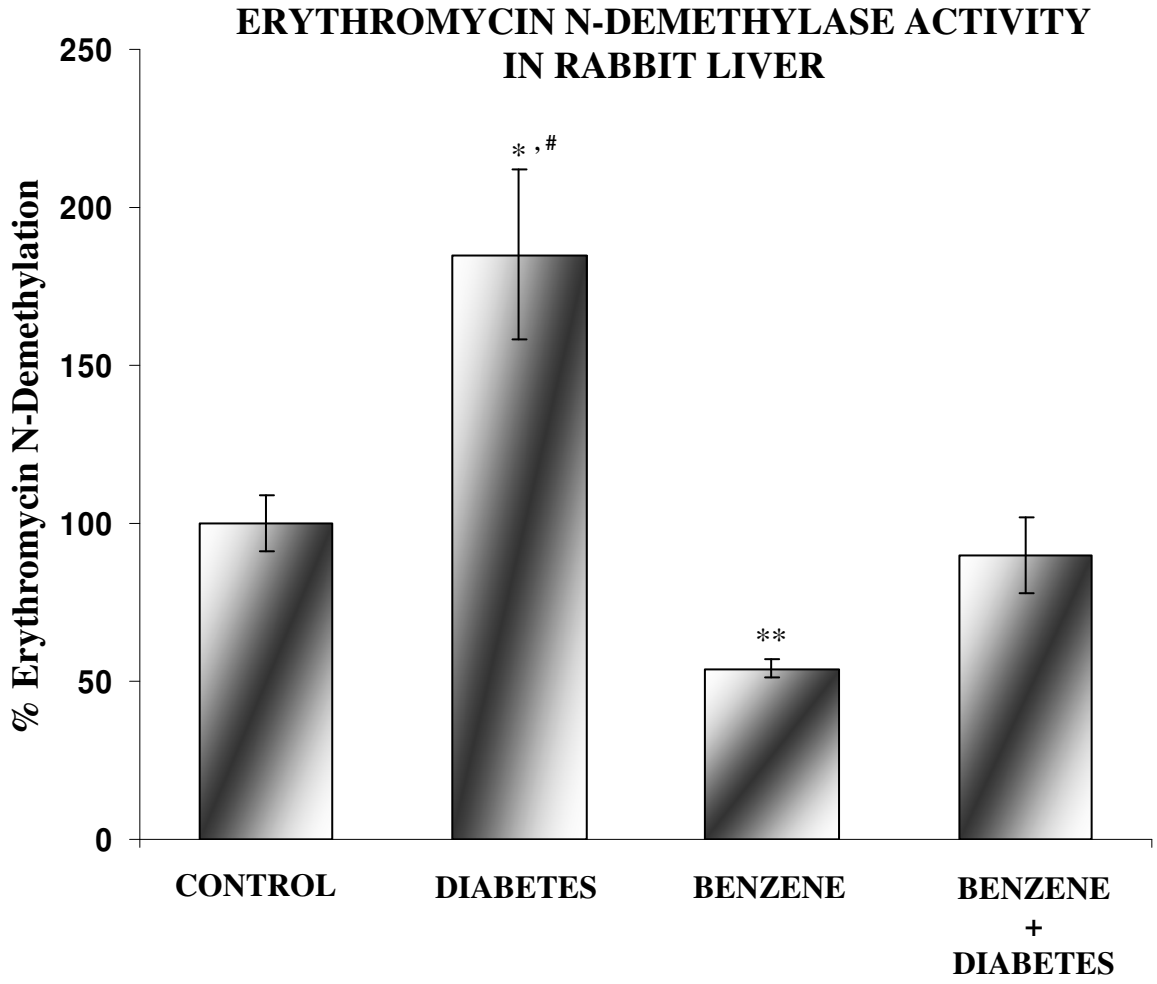


Figure 3.8 Effects of diabetes, benzene and benzene treatment of diabetic rabbits on hepatic CYP3A6 dependent erythromycin N-demethylase activity. The value obtained from the control animals was taken to be 100%, and the value obtained from the diabetic, benzene and benzene treated diabetic group was expressed as a percentage of control.

*Significantly different from the respective control value $p<0.05$.

#Significantly different from the respective "benzene + diabetes" value $p<0.05$

**Significantly different from the respective control value $p<0.005$.

3.3.4.2 Kidney Erythromycin N-Demethylase Activity

The N-demethylation of erythromycin was not detected in rabbit kidney.

3.3.4.3 Lung Erythromycin N-Demethylase Activity

The N-demethylation of erythromycin in rabbit lung was determined in a reaction mixture containing 1.5 mg microsomal protein per 0.5 ml. As shown in Table 3.13, erythromycin N-demethylase activities were found as 0.11, 0.14, 0.06, 0.07, 0.16, 0.12 nmol HCHO/min/mg protein which resulted in an average value of 0.11 ± 0.02 nmol HCHO/min/mg protein (N=6), for the lung microsomes obtained from the control rabbits. The activities of the enzyme from the diabetic rabbits were calculated to be 0.23, 0.13, 0.15, 0.13, 0.24, 0.20, 0.16 nmol HCHO/min/mg protein which resulted in an average value of 0.18 ± 0.02 nmol HCHO/min/mg protein (mean ± SEM, N=7). Thus, induction of diabetes by single dose of (125 mg/kg body weight) alloxan caused 1.64-fold increase in erythromycin N-demethylase activity in rabbit lung microsomes ($p<0.05$). Furthermore, effect of benzene treatment on erythromycin N-demethylase enzyme activity of lung microsomes was also studied and enzyme activities were found to be 0.08, 0.12, 0.08, 0.11, 0.15, 0.14, 0.09, 0.14 and 0.22 nmol HCHO/min/mg protein (N=9). The average specific activity was calculated to be 0.13 ± 0.02 nmol HCHO /min/mg protein (mean ± SEM, N=9). Thus, upon benzene treatment of rabbits, erythromycin N-demethylase activity of pulmonary microsomes did not change significantly. Finally, erythromycin N-demethylase activities of benzene treated diabetic rabbit microsomes were found to be 0.09, 0.15, 0.23, 0.12, 0.15, 0.23, 0.09, 0.09 nmol HCHO/min/mg protein which resulted in an average value of 0.14 ± 0.02 nmol HCHO/min/mg protein (mean ± SEM, N=8). Thus, it can be postulated that benzene treatment of diabetic rabbits resulted in 1.27-fold increase which was found statistically insignificant in erythromycin N-demethylase activity of lung microsomes with respect to control animals. Lung microsomal erythromycin N-demethylase activities of control, diabetic, benzene treated and benzene treated diabetic rabbits were summarized as relative percentages in Figure 3.9.

Table 3.13 Effects of diabetes, benzene and benzene treatment of diabetic rabbits on pulmonary microsomal Erythromycin N-Demethylase activity.

RABBIT	Erythromycin N-demethylase Activity (nmol HCHO/min/mg protein)			
	CONTROL	**DIABETES***	**BENZENE**	**BENZENE + DIABETES**
1	0.11	0.23	0.08	0.09
2	0.14	0.13	0.12	0.15
3	0.06	0.15	0.08	0.23
4	0.07	0.13	0.11	0.12
5	0.16	0.24	0.15	0.15
6	0.12	0.20	0.14	0.23
7	N.D.	0.16	0.09	0.09
8		N.D.	0.14	0.09
9			0.22	
AVERAGE	0.11 ± 0.02	0.18 ± 0.02	0.13 ± 0.02	0.14 ± 0.02
CHANGE (FOLD)	**1.0**	**1.64 X ↑**	**1.18 X ↑**	**1.27 X ↑**

*Significantly different from the respective control value $p<0.05$.

N.D. Not Determined

Values given in Table 3.13 were the average of duplicate determinations

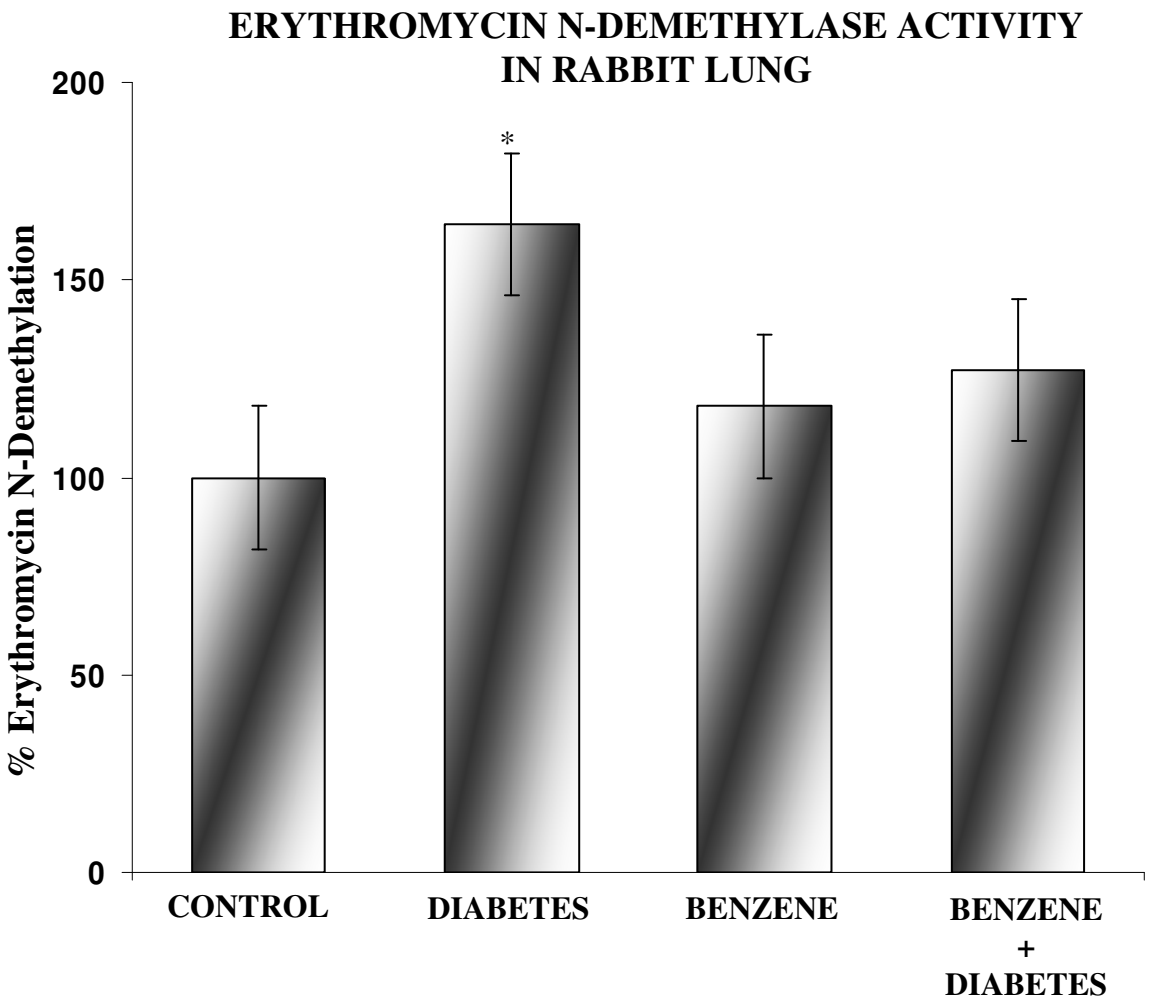


Figure 3.9 Effects of diabetes, benzene and benzene treatment of diabetic rabbit on pulmonary CYP3A6 dependent erythromycin N-demethylase activity. The value obtained from the control animals was taken to be 100%, and the value obtained from the diabetic, benzene and benzene treated diabetic group was expressed as a percentage of control.

*Significantly different from the respective control value $p<0.05$.

3.3.5 Effects of Diabetes, Benzene and Benzene Treatment of Diabetic Rabbits on Caffeine N-Demethylase Activity of Rabbits

3.3.5.1 Liver Caffeine N-Demethylase Activity

The N-demethylation of caffeine was determined by using 1.5 mg/0.5ml microsomal protein for control, diabetic, benzene treated and benzene treated diabetic rabbits. As can be seen in Table 3.14, the specific enzyme activities of hepatic microsomes of the control rabbits were found to be 0.05, 0.03, 0.03, 0.05, 0.09, 0.05 nmol HCHO/ min/mg protein which gave an average value of 0.050 ± 0.001 nmol HCHO/min/mg protein(mean ± SEM, N=6). The specific caffeine N-demethylase activities of the enzyme from the diabetic rabbits were calculated to be 0.13, 0.10, 0.07, 0.09, 0.09, 0.08, 0.09 nmol HCHO/min/mg protein which resulted in an average value of 0.092 ± 0.007 nmol HCHO/min/mg protein (mean ± SEM, N=7). Thus, *in vivo* treatment of rabbits with alloxan (i.v.) at a dose of 125 mg/kg body weight resulted in significant 1.84-fold increase in caffeine N-demethylation rates of liver microsomes with respect to age-matched control animals ($P<0.005$). Besides, the specific activities of benzene treated rabbits were found to be 0.05, 0.03, 0.03, 0.05, 0.03, 0.05, 0.04 and 0.04 nmol HCHO/min/mg protein which resulted in average value of 0.040 ± 0.003 nmol HCHO/min/mg protein (mean ± SEM, N=8). These results showed that benzene treatment caused slight decrease in caffeine N-methylation rates of rabbit liver microsomes with respect to control animals. However, this was not found statistically significant. Furthermore, there was also no significant change in the enzyme activity of liver microsomes as a result of benzene treatment of diabetic rabbits. The enzyme activities of liver microsomes were found to be 0.08, 0.05, 0.08, 0.05, 0.03, 0.05, 0.05, 0.03 nmol HCHO/min/mg protein and the average specific activity was calculated to be 0.053 ± 0.006 nmol HCHO /min/mg protein (mean ± SEM, N=8). Liver microsomal caffeine N-demethylase activities of control, diabetic, benzene treated and benzene treated diabetic rabbits were represented as relative percentages in Figure 3.10.

Table 3.14 Effects of diabetes, benzene and benzene treatment of diabetic rabbits on hepatic microsomal Caffeine N-Demethylase activity.

RABBIT	Caffeine N-demethylase Activity (nmol HCHO/min/mg protein)			
	CONTROL	**DIABETES***	**BENZENE**	**BENZENE + DIABETES**
1	0.05	0.13	0.05	0.08
2	0.03	0.10	0.03	0.05
3	0.03	0.07	0.03	0.08
4	0.05	0.09	0.05	0.05
5	N.D.	0.09	0.03	0.03
6	0.09	0.08	0.05	0.05
7	0.05	0.09	N.D.	0.05
8		N.D.	0.04	0.03
9			0.04	
AVERAGE	0.05 ± 0.001	0.092 ± 0.007	0.04 ± 0.003	0.053 ± 0.006
CHANGE (FOLD)	**1.0**	**1.84 X ↑**	**0.80 X ↓**	**1.06 X ↑**

*Significantly different from the respective control value $p<0.005$.

N.D. Not Determined

Values given in Table 3.14 were the average of duplicate determinations

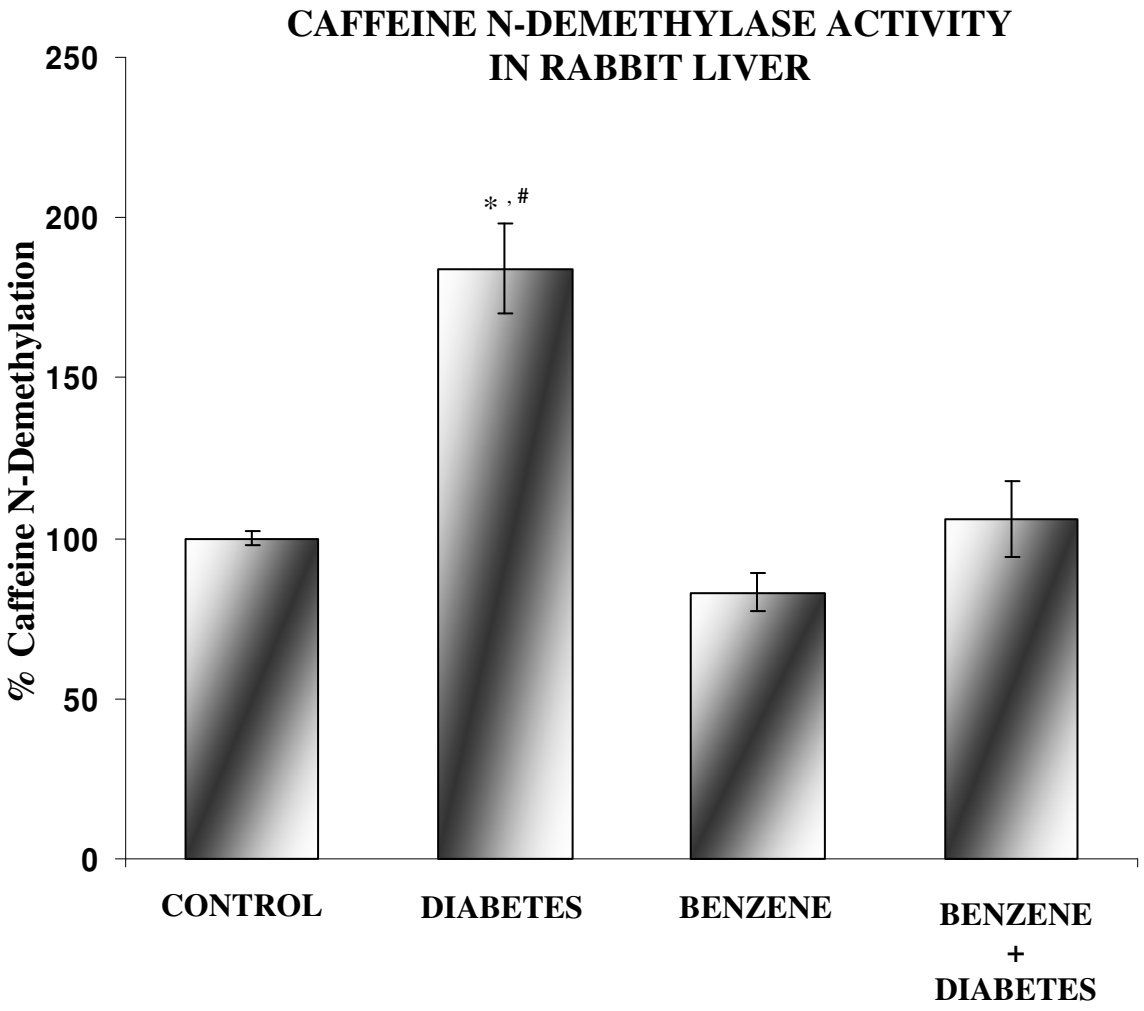


Figure 3.10 Effects of diabetes, benzene and benzene treatment of diabetic rabbit on hepatic CYP1A2 dependent caffeine N-demethylase activity. The value obtained from the control animals was taken to be 100%, and the value obtained from the diabetic, benzene and benzene treated diabetic group was expressed as a percentage of control.

*Significantly different from the respective control value $p<0.005$.

#Significantly different from the respective "benzene + diabetes" value $p<0.001$.

3.3.6 Effects of Diabetes, Benzene and Benzene Treatment of Diabetic Rabbits on 7-Ethoxyresorufin–O-deethylase Activity of Rabbits

3.3.6.1 Liver 7-Ethoxyresorufin–O-Deethylase Activity

EROD activities of P4501A from liver microsomes were determined by using 7-ethoxyresorufin O-deethylase reaction. 0.5 mg/ 2ml microsomal protein concentration from both treated and control animals were used for 7-ethoxyresorufin–O-deethylase reaction. The specific activities of hepatic 7-ethoxyresorufin–O-deethylase were found as 51.8, 43.9, 53.6, 22.5, 37.7, 34.6 pmol resorufin/min /mg protein which gave an average of 40.7 ± 4.8 pmol resorufin /min /mg protein (mean ± SEM, N=6) for control rabbits, and 50.0, 62.3, 33.3, 121.1, 46.7, 43.7, 69.9 pmol resorufin /min /mg protein which resulted in an average of 61.0 ± 11.0 pmol resorufin /min /mg protein (mean ± SEM, N=7) for diabetic rabbits as given in Table 3.15. Alloxan treatment of rabbits at a dose of 125 mg/kg body weight caused 1.5-fold increase in activity in rabbit liver microsomes ($p<0.05$). On the other hand, specific activities of the enzyme from the benzene treated rabbits were found as 27.9, 41.5, 13.1, 33.1, 37.5, 30.5, 35.1, 39.7 pmol resorufin/min/mg protein which resulted in an average value of 32.3 ± 3.2 pmol resorufin /min/mg protein (mean ± SEM, N=8). The observed decrease in O-deethylation rates of 7-ethoxyresorufin in liver microsomes was not found to be statistically significant in benzene treated rabbits with respect to control animals. Moreover, 7-ethoxyresorufin–O-deethylase enzyme activities of the benzene treated diabetic microsomes were found to be 42.8, 52.3, 26.4, 52.2, 37.8, 39.4, 45.6, 36.7 pmol resorufin/min/mg protein and the average specific activity was calculated to be 41.7 ± 3.0 pmol resorufin /min/mg protein (mean ± SEM, N=8). Therefore, there was also no significant change in the enzyme activity of liver microsomes as a result of benzene treatment of diabetic rabbits. Liver microsomal 7-ethoxyresorufin O-deethylase activities of control, diabetic, benzene treated and benzene treated diabetic rabbits were represented as relative percentages in Figure 3.11.

Table 3.15 Effects of diabetes, benzene and benzene treatment of diabetic rabbits on hepatic microsomal 7-Ethoxyresorufin–O-Deethylase activity.

RABBIT	7-Ethoxyresorufin–O-Deethylase Activity (pmol resorufin/min/mg protein)			
	CONTROL	DIABETES*	BENZENE	BENZENE + DIABETES
1	51.8	50.0	27.9	42.8
2	43.9	62.3	41.5	52.3
3	53.6	33.3	13.1	26.4
4	22.5	121.1	33.1	52.2
5	N.D.	46.7	37.5	37.8
6	37.7	43.7	30.5	39.4
7	34.6	69.9	35.1	45.6
8		N.D.	39.7	36.7
9			N.D.	
AVERAGE	40.7 ± 4.8	61.0 ± 11.0	32.3 ± 3.2	41.7 ± 3.0
CHANGE (FOLD)	**1.0**	**1.50 X ↑**	**0.79 X ↓**	**1.02X ↑**

*Significantly different from the respective control value $p<0.05$.

N.D. Not Determined

Values given in Table 3.14 were the average of duplicate determinations

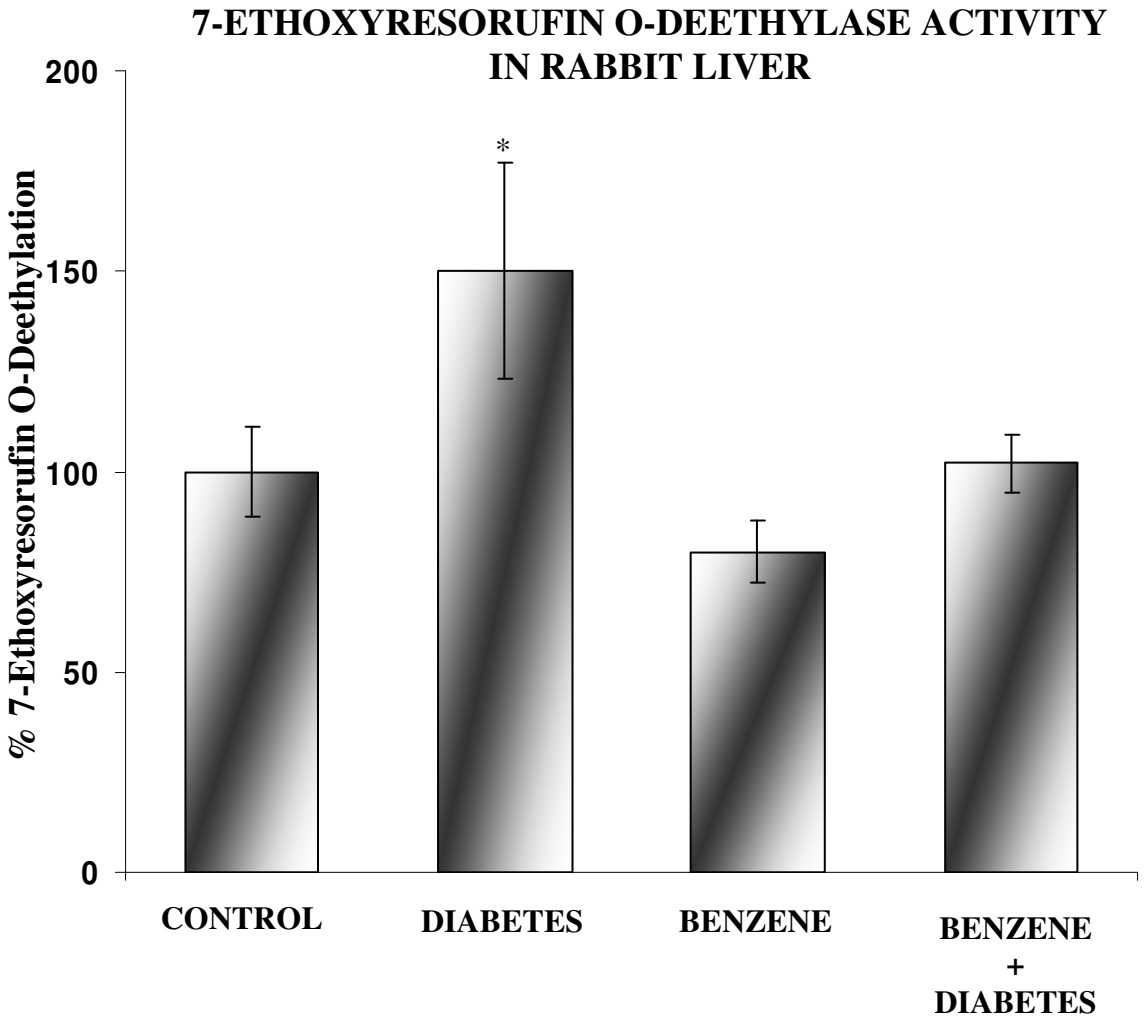


Figure 3.11 Effects of diabetes, benzene and benzene treatment of diabetic rabbits on hepatic CYP1A dependent 7-ethoxyresorufin O-deethylase activity. The value obtained from the control animals was taken to be 100%, and the value obtained from the diabetic, benzene and benzene treated diabetic group was expressed as a percentage of control.

*Significantly different from the respective control value $p<0.05$.

3.4 Effects of Diabetes, Benzene and Benzene Treatment of Diabetic Rabbits on CYP450 Protein Levels as Described by Western Blot Analysis.

Changes in the cytochrome P450 protein levels in hepatic, renal and pulmonary microsomes due to diabetes, benzene and benzene treatment of diabetic rabbits were determined by western blotting. For the analysis, different CYP450 antibodies and Alkaline phosphotase conjugated anti-rabbit IgG were used as primary and secondary andibodies, respectively. Protein bands were quantified as an arbitrary unit, relative peak area (RPA), by using Scion Image Version Beta 4.0.2 software.

3.4.1 Effects of Diabetes, Benzene and Benzene Treatment of Diabetic Rabbits on CYP2E1 Protein Level in Liver, Kidney and Lung.

In order to assess the effects of alloxan induced diabetes, benzene and both on the level of CYP2E1 protein in liver, kidney and lung microsomes, western blot analysis was carried out utilizing 1/1000 diluted polyclonal antibodies raised against rabbit CYP2E1 (Oxford Biomedical Research, MI, USA). Western blot analysis of liver CYP2E1 was illustrated in Figure 3.12. The result of densitometric scanning analysis of the protein bands for control animals (lane 1, 2, 3) was found to be 330 ± 13.3 RPA (mean ± SEM, N=3). The band intensities were increased to 875 ± 114 RPA (mean ± SEM, N=3) by injection of single dose of alloxan (lane 4, 5, 6). Moreover, the band intensities of benzene treated rabbit (lane 7, 8, 9) and benzene treated diabetic rabbits (lane 10, 11, 12) were calculated as 1270 ± 217 RPA (mean ± SEM, N=3) and 2541 ± 129 RPA (mean ± SEM, N=3) respectively. The results of densitometric analysis of the liver CYP2E1 shown in Figure 3.12 indicate that hepatic CYP2E1 expression was increased significantly 2.65-, 3.85-, and 7.70-fold in the diabetic, benzene treated and benzene treated diabetic rabbits with respect to control animals. Moreover, the expression of CYP2E1 in benzene treated diabetic rabbits was significantly different from respective diabetic and benzene values ($p<0.05$). The content of liver CYP2E1 protein in the benzene treated diabetic animals' appeared to be additive of the separate treatments.

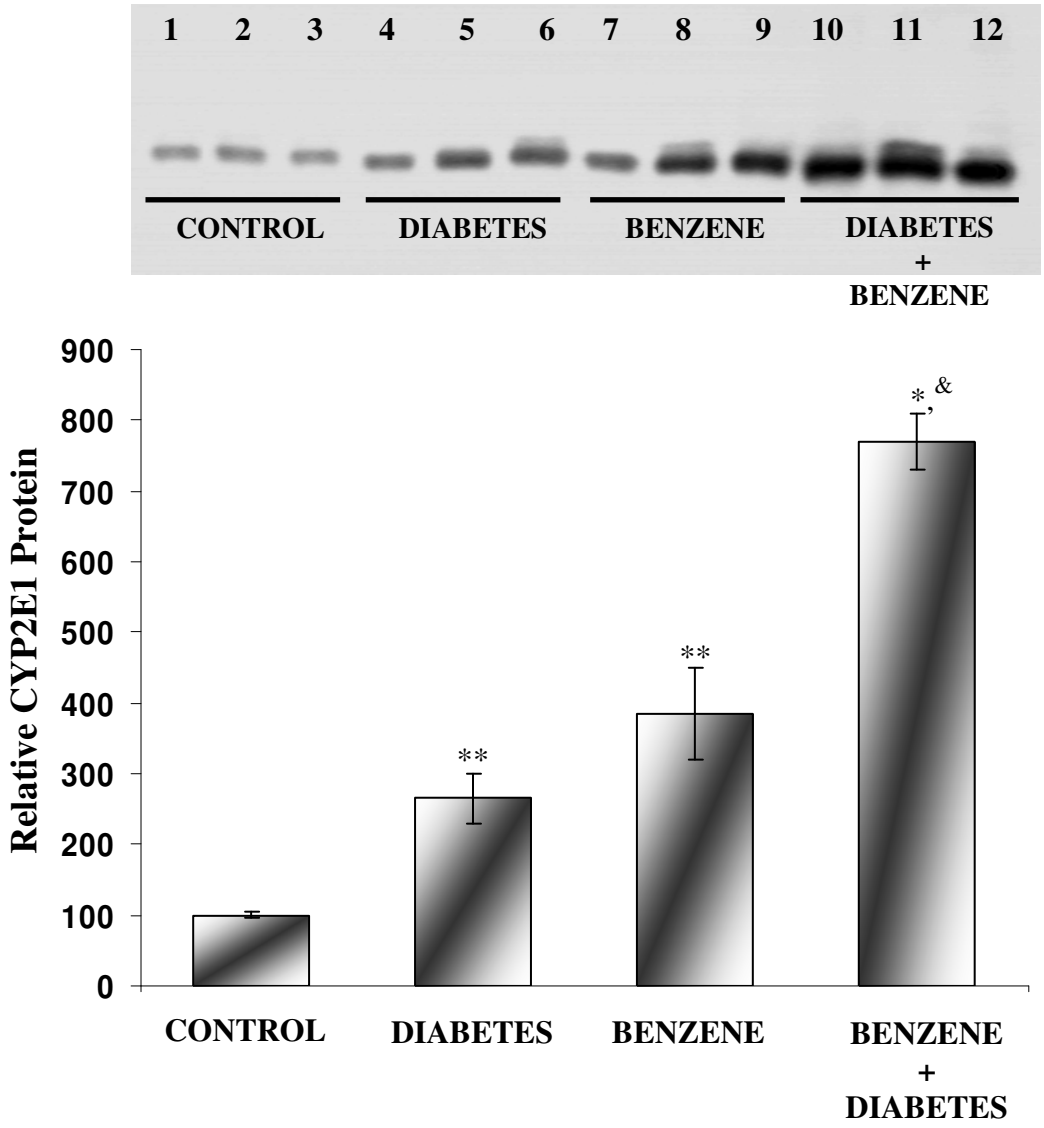


Figure 3.12 Effects of diabetes, benzene and benzene treatment of diabetic rabbits on hepatic CYP2E1 expression. Liver microsomal CYP2E1 expression from the control (lane 1-3), diabetic (lane 4-6), benzene (lane 7-9) and benzene treated diabetic rabbits (lane 10-12) (10 µg in each well) were detected by Western blot analysis and quantitated by densitometry. The value obtained from control animals were taken to be 100%, and the values obtained from the diabetic, benzene and benzene treated diabetic group were expressed as a percentage of control.

*Significantly different from the respective control value $p<0.005$.

**Significantly different from the respective control value $p<0.05$.

[&] Significantly different from the respective diabetes and benzene value $p<0.05$

Western blot analysis of kidney CYP2E1 was illustrated in Figure 3.13. Intensity of each band was quantified as Relative Peak Area (RPA) by Scion Image software. The densitometric analysis result for control microsomes (lane 1, 2, 3) was found to be 510 ± 152 RPA (mean ± SEM, N=3). The band intensities were calculated as 1270 ± 21.7 RPA (mean ± SEM, N=3) in diabetic rabbits (lane 4, 5, 6). Furthermore, band intensities of benzene treated rabbit (lane 7, 8, 9) and benzene treated diabetic rabbits (lane 10, 11, 12) were found to be 1425 ± 90.4 RPA (mean ± SEM, N=3) and 1760 ± 30.4 RPA (mean ± SEM, N=3), respectively. These results showed that CYP2E1 expression was increased significantly ($p<0.05$) 2.50-, 2.80-, and 3.45-fold in the diabetic, benzene treated and benzene treated diabetic rabbits' kidney, respectively (Figure 3.13). CYP2E1 protein level in benzene treated diabetic rabbits was significantly different from respective diabetic value ($p<0.001$).

Western blot analysis of lung CYP2E1 was illustrated in Figure 3.14. The band intensities of control animals (lane 1, 2, 3) was calculated to be 1121 ± 61 RPA (mean ± SEM, N=3). The band intensities were found to be 1982 ± 90.7 RPA (mean ± SEM, N=3) in diabetic rabbits (lane 4, 5, 6). Besides, band intensities of benzene treated rabbit (lane 7, 8, 9) and benzene treated diabetic rabbits (lane 10,11,12) were found to be 1119 ± 185 RPA (mean ± SEM, N=3) and 1175 ± 48.5 RPA (mean ± SEM, N=3), respectively. As can be seen in Figure 3.14, lung CYP2E1 protein level was increased 1.77-fold in diabetic rabbits with respect to control animals ($p<0.005$). On the other hand, lung CYP2E1 level was not changed in benzene and benzene treatment of diabetic rabbits.

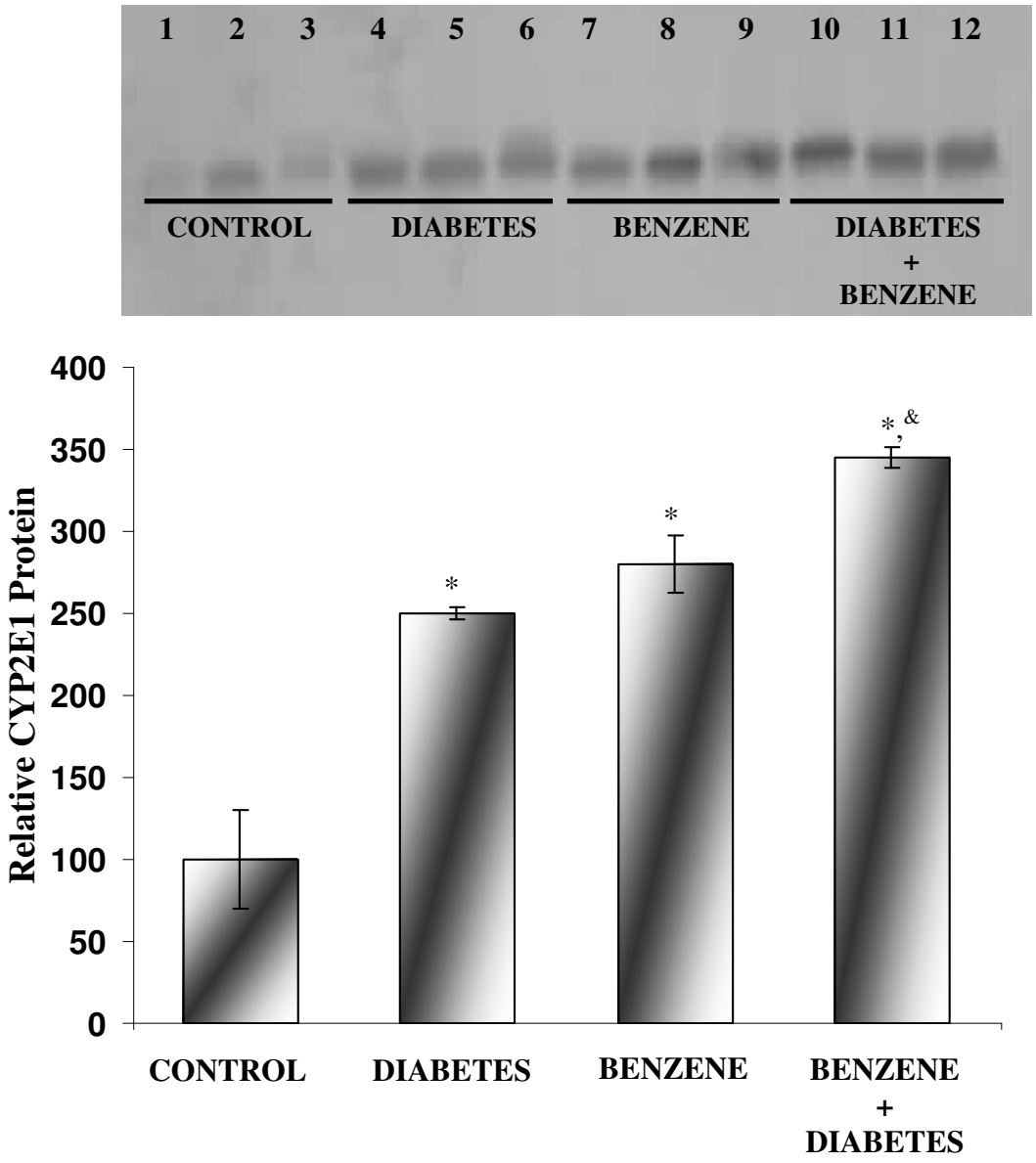


Figure 3.13 Effects of diabetes, benzene and benzene treatment diabetic rabbits on renal CYP2E1 expression. Kidney microsomal CYP2E1 expression from control (lane 1-3), diabetic (lane 4-6), benzene (lane 7-9) and benzene treated diabetic rabbits (lane 10-12) (20 μg in each well) were detected by Western blot analysis and quantitated by densitometry. The value obtained from the control animals were taken to be 100%, and the values obtained from the diabetic, benzene and benzene treated diabetic group were expressed as a percentage of control.

*Significantly different from the respective control value $p<0.05$.

& Significantly different from the respective diabetes and benzene value $p<0.05$

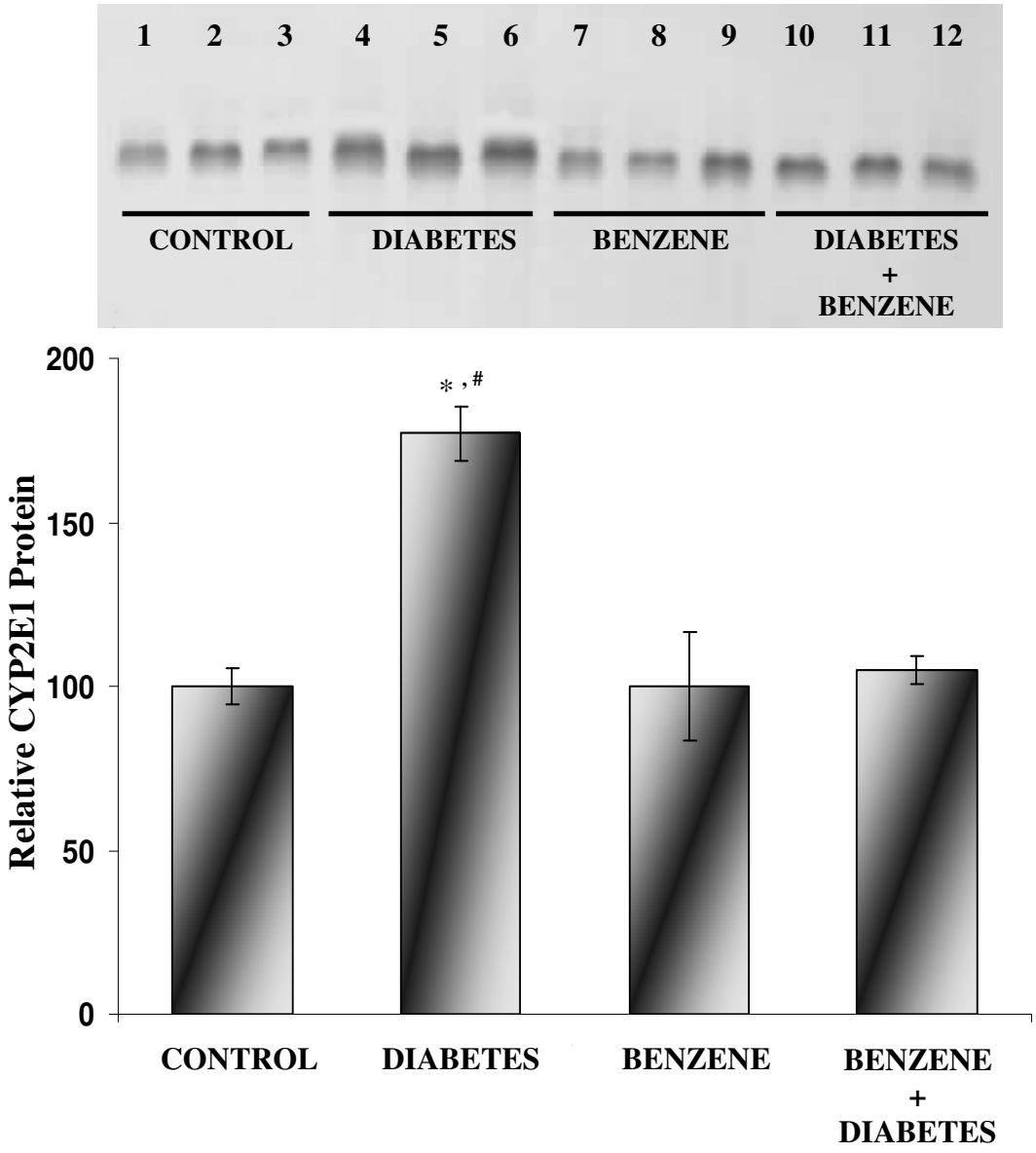


Figure 3.14 Effects of diabetes, benzene and benzene treatment of diabetic rabbits on pulmonary CYP2E1 expression. Lung microsomal CYP2E1 expression from control (lane 1-3), diabetic (lane 4-6), benzene (lane 7-9) and benzene treated diabetic rabbits (lane 10-12) (10 μg in each well) were detected by Western blot analysis and quantitated by densitometry. The value obtained from control animals were taken to be 100%, and the values obtained from the diabetic, benzene and benzene treated diabetic group were expressed as a percentage of control.

*Significantly different from the respective control value $p<0.005$.

#Significantly different from the respective "benzene + diabetes" value $p<0.005$.

3.4.2 Effects of Diabetes, Benzene and Benzene Treatment of Diabetic Rabbits on CYP3A6 Protein Level in Liver and Lung.

Effects of diabetes, benzene treatment and combination of both treatment on the level of rabbit liver and lung CYP3A6 protein was determined by using western blot analysis. 1/1000 diluted polyclonal antibodies raised against human CYP3A4 (an orthologue of rabbit CYP3A6) was utilized in this study. Western blot analysis of liver CYP3A6 was illustrated in Figure 3.15. The band intensities of control animals (lane 1, 2, 3) was found to be 285 ± 19.7 RPA (mean ± SEM, N=3). The band intensities were calculated as 428 ± 24.6 RPA (mean ± SEM, N=3) in diabetic rabbits (lane 4, 5, 6). Moreover, band intensities of benzene treated rabbit (lane 7, 8, 9) were found to be 190 ± 7.2 RPA (mean ± SEM, N=3). Finally, protein bands obtained from benzene treated diabetic rabbits (lane 10, 11, 12) were analysed and found to be 294 ± 32.7 RPA (mean ± SEM, N=3), respectively. As can be seen in Figure 3.15, liver CYP3A6 protein level was increased 1.50-fold in diabetic rabbits with respect to control animals ($p<0.05$). On the other hand, protein level level was decreased 1.50-fold in benzene treated animals ($p<0.05$). In benzene treated diabetic group, liver CYP3A6 level was not changed significantly (Figure 3.15).

Changes in CYP3A6 expression due to diabetes, benzene treatment and benzene treatment of diabetics were also determined in rabbit lung. Western blot analysis of lung CYP3A6 was illustrated in Figure 3.16. The band intensities of control animals (lane 1, 2, 3) was found to be 1577 ± 54.5 RPA (mean ± SEM, N=3). The band intensities were calculated as 2380 ± 90.7 RPA (mean ± SEM, N=3) in diabetic rabbits (lane 4, 5, 6). Moreover, band intensities of benzene treated rabbit (lane 7, 8, 9) and benzene treated diabetic rabbits (lane 10, 11, 12) were found to be 1496 ± 225 RPA (mean ± SEM, N=3) and 1950 ± 106 RPA (mean ± SEM, N=3) respectively. As shown in Figure 3.16, lung CYP3A6 protein level was increased 1.51-fold in diabetic rabbits with respect to control animals ($p<0.005$). On the other hand, protein level was not changed significantly in benzene treated and benzene treated diabetic rabbits.

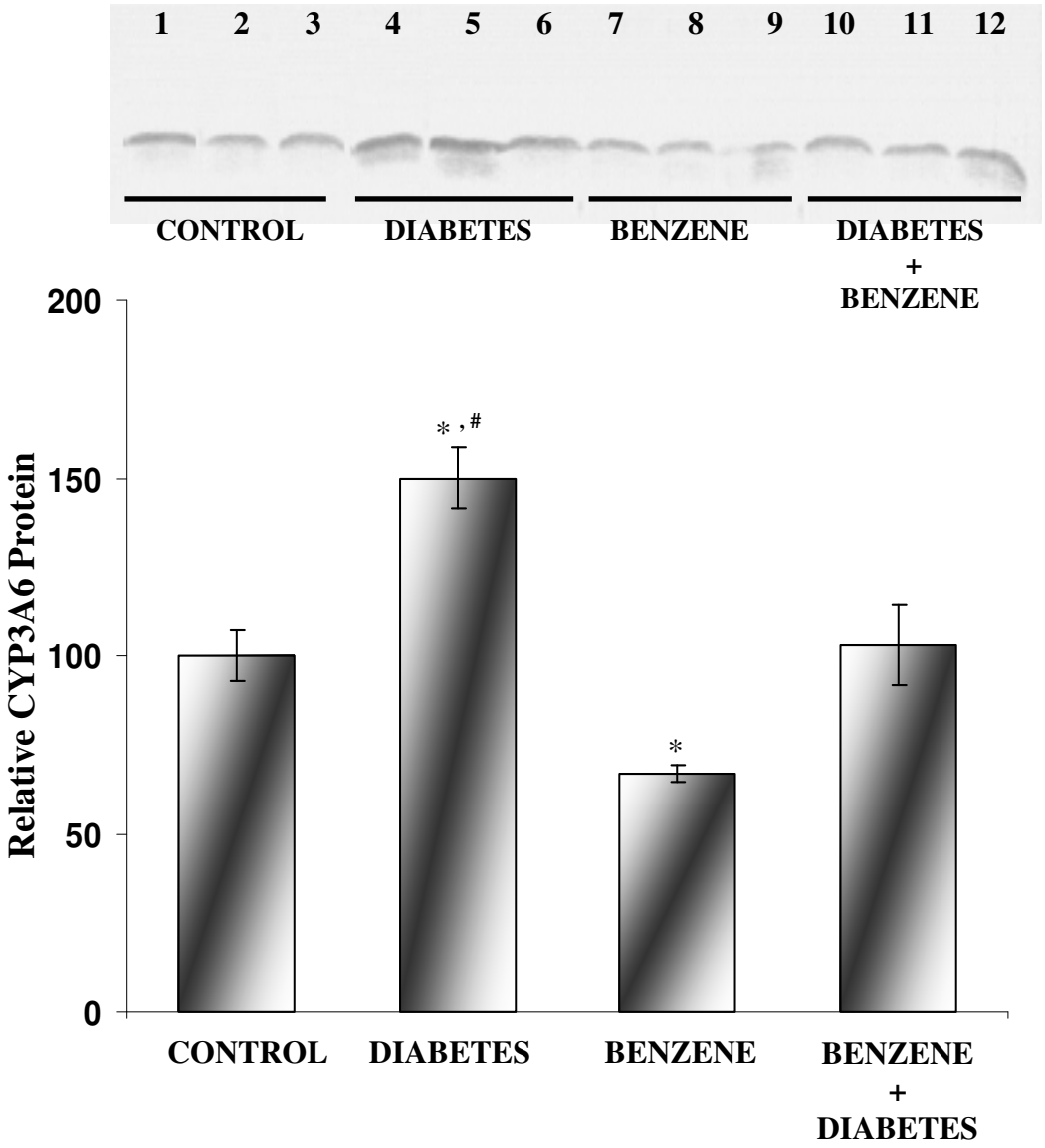


Figure 3.15 Effects of diabetes, benzene and benzene treatment of diabetic rabbits on hepatic CYP3A6 expression. Liver microsomal CYP3A6 expression from control (lane 1-3), diabetic (lane 4-6), benzene (lane 7-9) and benzene treated diabetic rabbits (lane 10-12) (30 μg in each well) were detected by Western blot analysis and quantitated by densitometry. The value obtained from control animals were taken to be 100%, and the values obtained from the diabetic, benzene and benzene treated diabetic group were expressed as a percentage of control.

*Significantly different from the respective control value $p<0.05$.

#Significantly different from the respective "benzene + diabetes" value $p<0.05$.

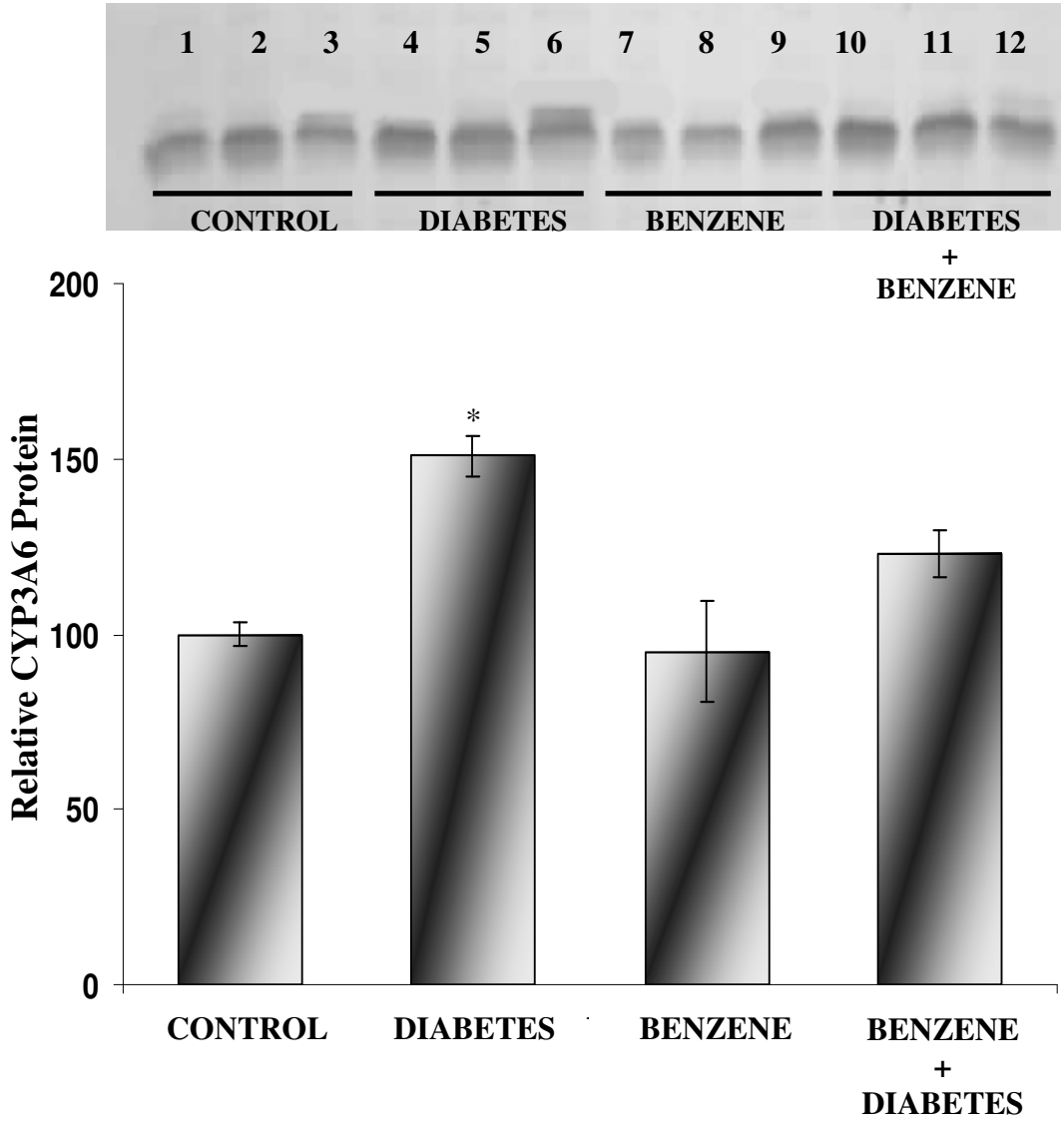


Figure 3.16 Effects of diabetes, benzene and benzene treatment of diabetic rabbits on pulmonary CYP3A6 expression. Lung microsomal CYP3A6 expression from control (lane 1-3), diabetic (lane 4-6), benzene (lane 7-9) and benzene treated diabetic rabbits (lane 10-12) (30 μg in each well) were detected by Western blot analysis and quantitated by densitometry. The value obtained from control animals were taken to be 100%, and the values obtained from the diabetic, benzene and benzene treated diabetic group were expressed as a percentage of control.

*Significantly different from the respective control value $p<0.005$.

3.4.3 Effects of Diabetes, Benzene and Benzene Treatment of Diabetic Rabbits on CYP1A2 Protein Level in Liver.

To determine the effects of alloxan induced diabetes, benzene and both treatment on the level of CYP1A2 protein in rabbit liver, western blot analysis was carried out utilizing 1/2000 diluted polyclonal antibodies raised against rat CYP1A2. Western blot analysis of liver CYP1A2 was illustrated in Figure 3.17. The result of densitometric scanning analysis of the protein bands for control animals (lane 1, 2, 3) was found to be 1839 ± 180 RPA (mean ± SEM, N=3). The band intensities were increased to 2583 ± 134 RPA (mean ± SEM, N=3) by injection of single dose of alloxan (lane 4, 5, 6). Moreover, the band intensities of benzene treated rabbit (lane 7, 8, 9) and benzene treated diabetic rabbits (lane 10, 11, 12) were calculated as 1333 ± 114 RPA (mean ± SEM, N=3) and 1801 ± 266 RPA (mean ± SEM, N=3), respectively. The results of densitometric analysis of the liver CYP1A2 shown in Figure 3.17 indicate that hepatic CYP1A2 expression was increased significantly 1.40-fold in the diabetic rabbits with respect to control animals ($p<0.05$). On the other hand, CYP1A2 protein expression decreased as a result of benzene treatment (1.37-fold) but this decrease was not found statistically significant. Also, protein level was not changed in benzene treated diabetic rabbits (Figure 3.17).

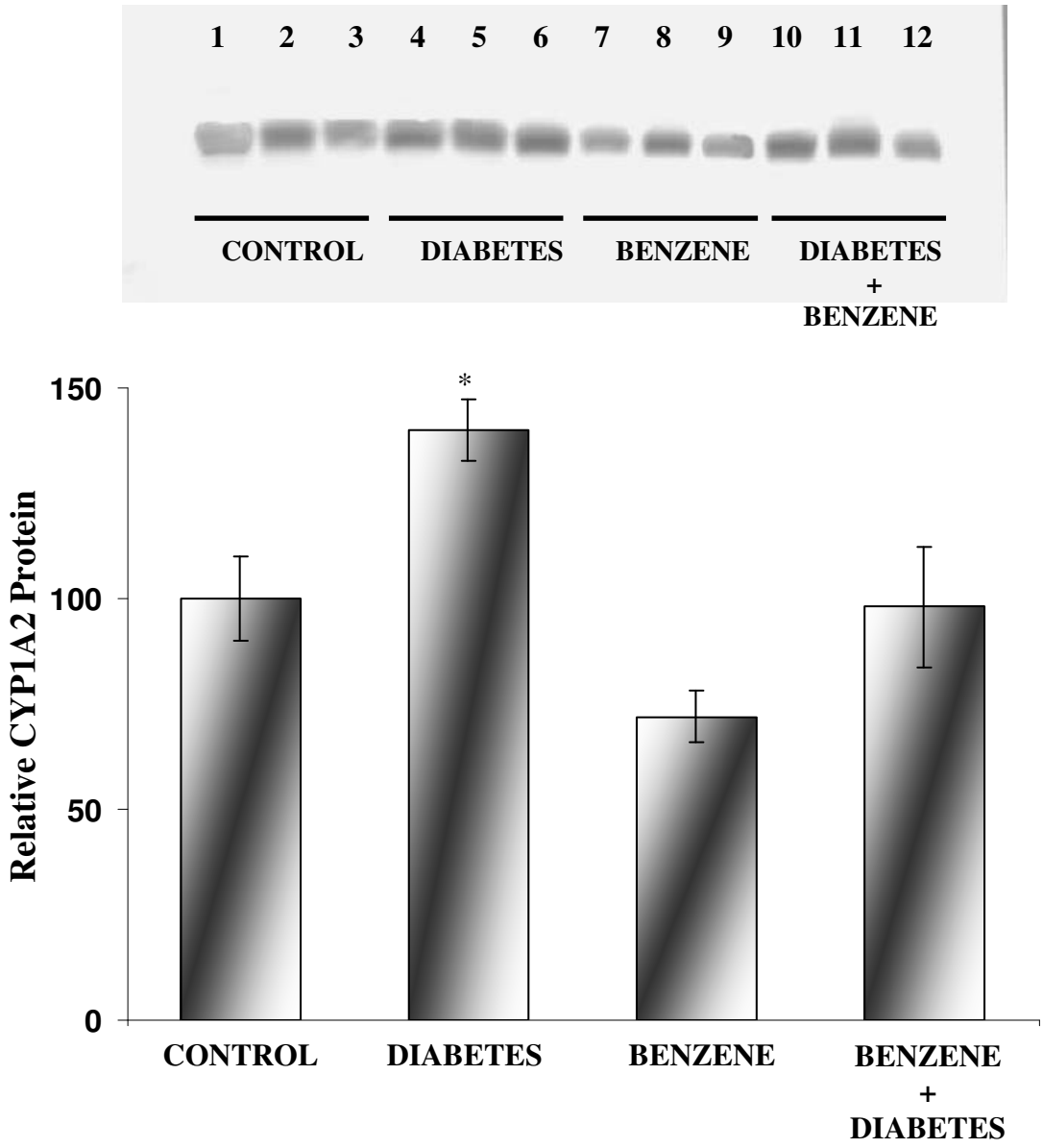


Figure 3.17 Effects of diabetes, benzene and benzene treatment of diabetic rabbits on hepatic CYP1A2 expression. Liver microsomal CYP1A2 expression from control (lane 1-3), diabetic (lane 4-6), benzene (lane 7-9) and benzene treated diabetic rabbits (lane 10-12) (25 μg in each well) were detected by Western blot analysis and quantitated by densitometry. The value obtained from control animals were taken to be 100%, and the values obtained from the diabetic, benzene and benzene treated diabetic group were expressed as a percentage of control.

*Significantly different from the respective control value $p<0.05$.

3.4.4 Effects of Diabetes, Benzene and Benzene Treatment of Diabetic Rabbits on CYP2B4 Protein Level in Liver Kidney and Lung.

To assess the effects of alloxan induced diabetes, benzene and benzene treatment of diabetic rabbits on the level of CYP2B4 protein in rabbit liver, kidney and lung, western blot analysis was performed utilizing anti-sheep lung CYP2B antibody. Western blot analysis of liver CYP2B4 was illustrated in Figure 3.18. The result of densitometric scanning analysis of the protein bands for control animals (lane 1, 2, 3) was found to be 1640 ± 301 RPA (mean ± SEM, N=3). The band intensities of diabetics (lane 4, 5, 6) were found to be 1370 ± 169 RPA (mean ± SEM, N=3). Moreover, the band intensities of benzene treated rabbit (lane 7,8,9) and benzene treated diabetic rabbits (lane 10,11,12) were calculated as 1306 ± 37 RPA (mean ± SEM, N=3) and 1278 ± 257 RPA (mean ± SEM, N=3), respectively. The results of densitometric analysis of the liver CYP2B4 shown in Figure 3.18 indicate that hepatic CYP2B4 expression was decreased slightly in the diabetic, benzene treated and benzene treated diabetic rabbits with respect to control animals, respectively. But these decreases were not found to be statistically significant (Figure 3.18).

Alterations in CYP2B4 expression due to diabetes, benzene treatment and benzene treatment of diabetics were also determined in rabbit kidney. Western blot analysis of kidney CYP2B4 was illustrated in Figure 3.19. The band intensities of control animals (lane 1, 2, 3) and diabetic animals (lane 4, 5, 6) was found to be 1142 ± 57 RPA (mean ± SEM, N=3) and 1268 ± 122 RPA (mean ± SEM, N=3), respectively. Moreover, band intensities of benzene treated rabbit (lane 7, 8, 9) and benzene treated diabetic rabbits (lane 10, 11, 12) were found to be 1480 ± 113 RPA (mean ± SEM, N=3) and 1473 ± 31 RPA (mean ± SEM, N=3) respectively. As shown in Figure 3.19, kidney CYP2B4 protein level was not changed significantly in diabetic, benzene treated and benzene treated diabetic rabbits.

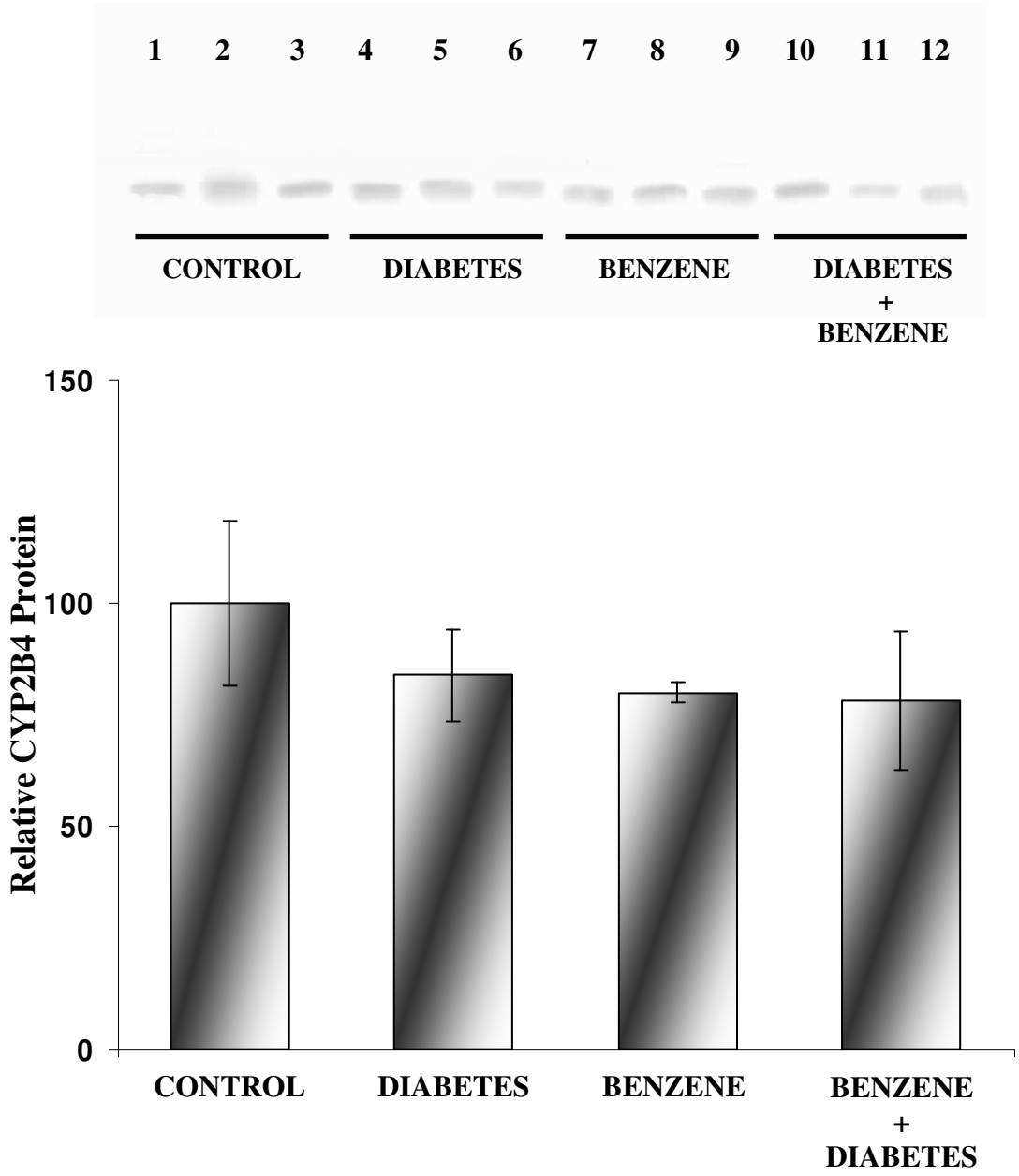


Figure 3.18 Effects of diabetes, benzene and benzene treatment of diabetic rabbits on hepatic CYP2B4 expression. Liver microsomal CYP2B4 expression from control (lane 1-3), diabetic (lane 4-6), benzene (lane 7-9) and benzene treated diabetic rabbits (lane 10-12) (20 μg in each well) were detected by Western blot analysis and quantitated by densitometry. The value obtained from control animals were taken to be 100%, and the values obtained from the diabetic, benzene and benzene treated diabetic group were expressed as a percentage of control.

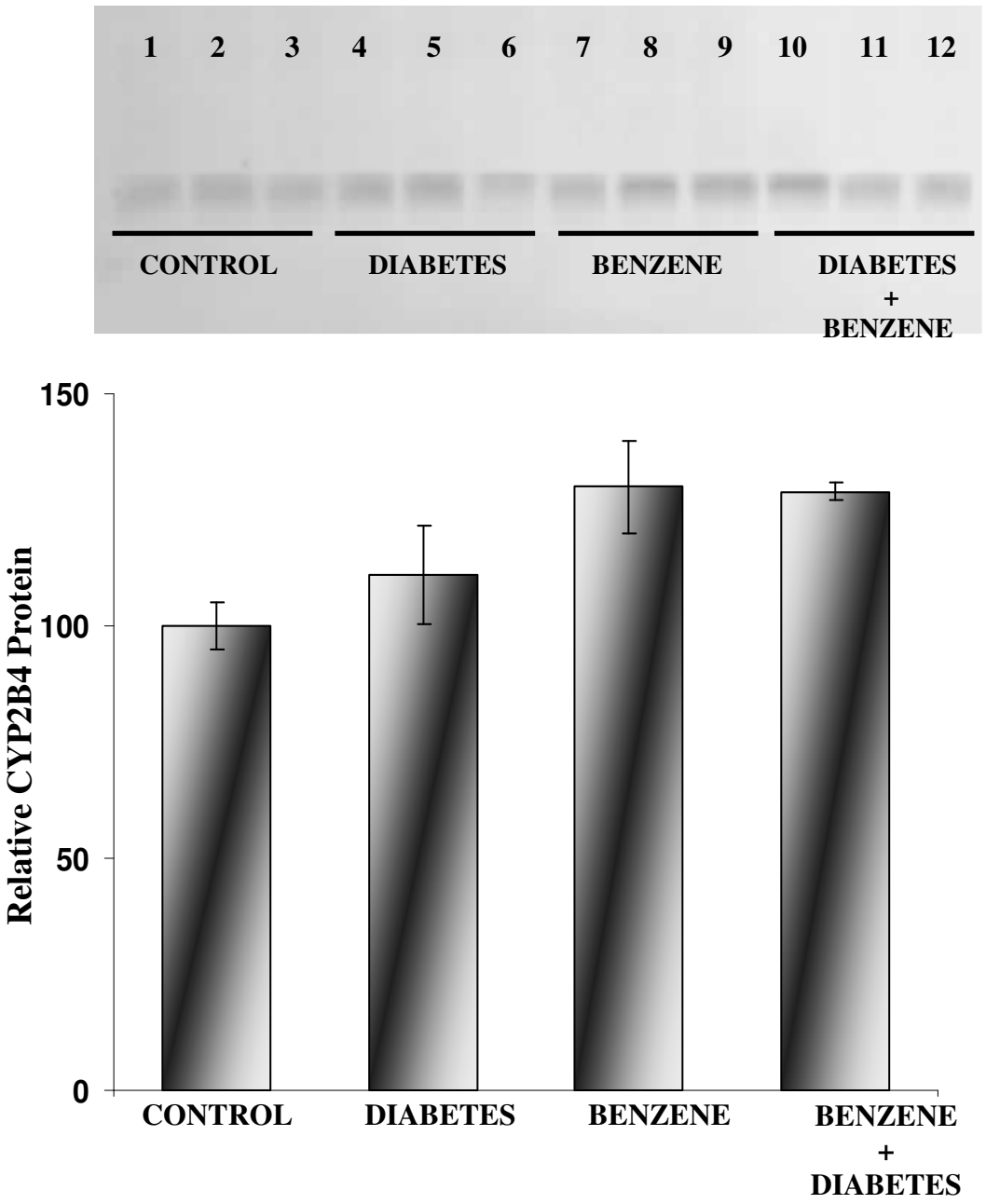


Figure 3.19 Effects of diabetes, benzene and benzene treatment of diabetic rabbits on renal CYP2B4 expression. Kidney microsomal CYP2B4 expression from control (lane 1-3), diabetic (lane 4-6), benzene (lane 7-9) and benzene treated diabetic rabbits (lane 10-12) (25 μg in each well) were detected by Western blot analysis and quantitated by densitometry. The value obtained from control animals were taken to be 100%, and the values obtained from the diabetic, benzene and benzene treated diabetic group were expressed as a percentage of control.

Western blot analysis of lung CYP2B4 was illustrated in Figure 3.20. The band intensities of control animals (lane 1, 2, 3) was calculated to be 748 ± 42 RPA (mean ± SEM, N=3). The band intensities were found to be 573 ± 74 RPA (mean ± SEM, N=3) in diabetic rabbits (lane 4, 5, 6). Besides, band intensities of benzene treated rabbit (lane 7, 8, 9) and benzene treated diabetic rabbits (lane 10, 11, 12) were found to be 650 ± 50 RPA (mean ± SEM, N=3) and 560 ± 30 RPA (mean ± SEM, N=3), respectively. As can be seen in Figure 3.20, lung CYP2B4 protein level was decreased in diabetic, benzene and benzene treatment of diabetic rabbits. But these decreases were not found to be statistically significant.

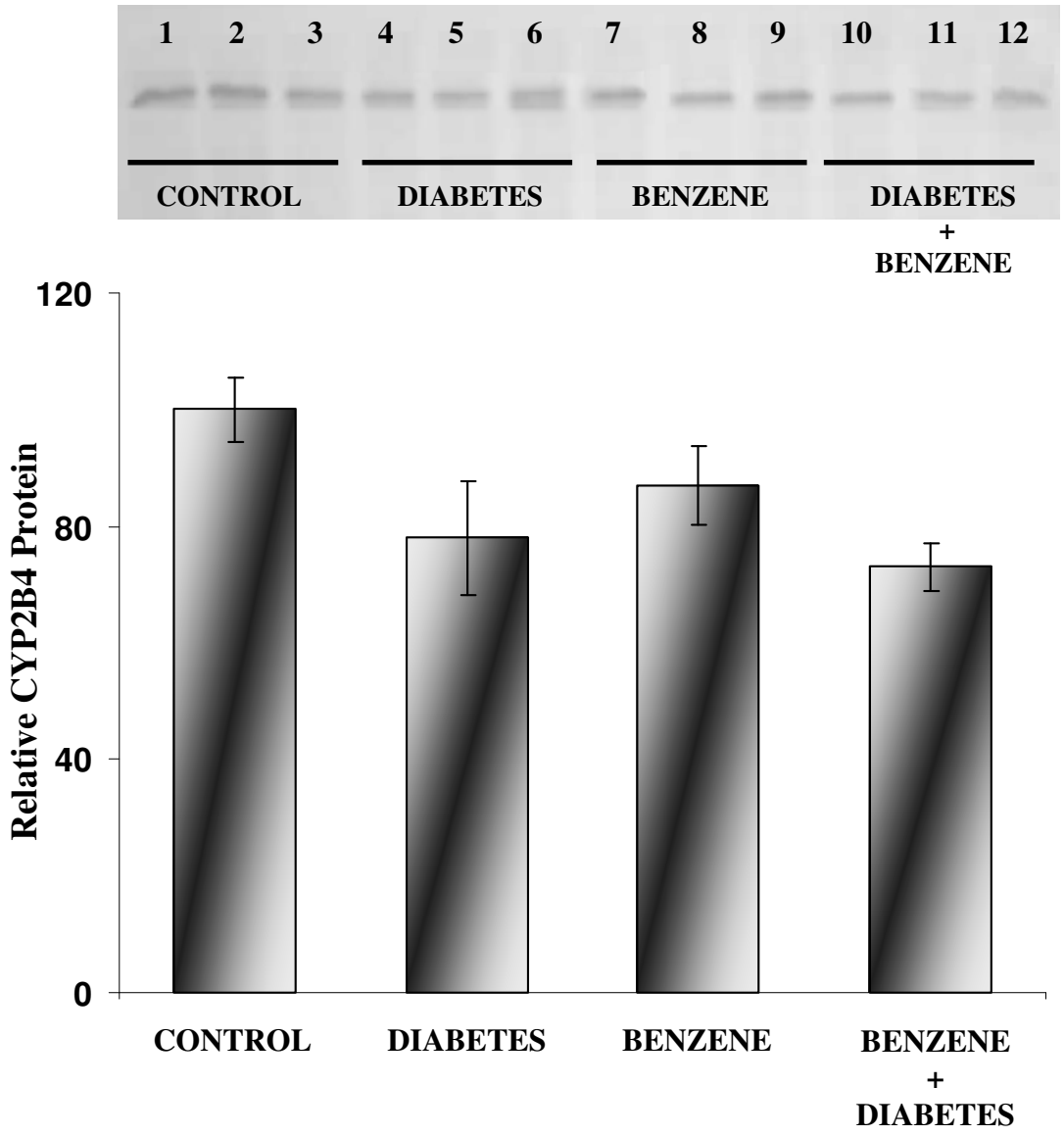


Figure 3.20 Effects of diabetes, benzene and benzene treatment of diabetic rabbits on pulmonary CYP2B4 expression. Lung microsomal CYP2B4 expression from control (lane 1-3), diabetic (lane 4-6), benzene (lane 7-9) and benzene treated diabetic rabbits (lane 10-12) (20 μg in each well) were detected by Western blot analysis and quantitated by densitometry. The value obtained from control animals were taken to be 100%, and the values obtained from the diabetic, benzene and benzene treated diabetic group were expressed as a percentage of control.

3.5 Effects of Diabetes, Benzene and Benzene Treatment of Diabetic Rabbits on CYP450 mRNA Levels in Liver, Kidney and Lung.

3.5.1 Total RNA Isolation from Rabbit Liver, Kidney and Lung.

Total cellular RNAs were isolated from liver, kidney and lung of rabbit as described in the Materials and Methods. RNA isolation was critical in this part of study as contamination of DNA interferes with the subsequent Real-Time PCR. Purity and quality of the isolated RNAs were assessed by $OD_{260/280}$ ratio and by agarose gel electrophoresis RNA samples having OD_{260}/OD_{280} ratio below 1.7 or above 2.0, were discarded and the isolation procedure was repeated for those samples. The isolation procedure yielded highly purified RNAs of sufficient quality to be used in Real-Time PCR studies. Figure 3.21 shows the representative agarose gel electrophoresis patterns of liver, lung and kidney total RNA samples. Majority of the RNA preparations had an $OD_{260/280}$ ratio of between 1.8 - 2.0 and produced intact and well-separated 28S and 18S RNA bands after gel electrophoresis.

3.5.2 Real-Time PCR

The effects of diabetes, benzene and benzene treatment of diabetic rabbits on gene expression analysis of CYP2E1, CYP1A2 and CYP3A6 were performed in rabbit liver, kidney and lung tissues by using real-time PCR with SYBR Green I detection chemistry. Real time-PCR quantification methods have many advantages over conventional RT-PCR in terms of accuracy, sensitivity, dynamic range, high throughout capacity and absence of post-PCR manipulation.

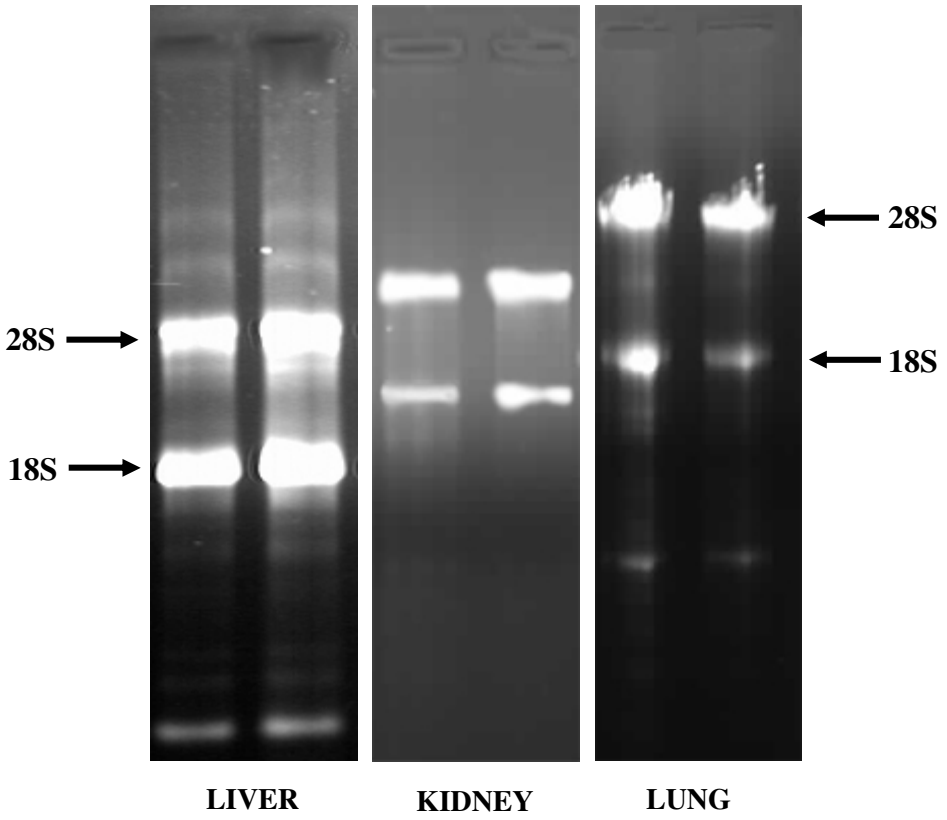


Figure 3.21 Agarose gel electrophoresis pattern of RNA isolated from rabbit liver, kidney and lung tissues. All lanes contain 5.0 µl of total RNA, with different concentrations.

3.5.2.1 Standard Curve of Dilution Series of Control cDNA

In this study, serial dilution series of each gene specific standard were run with samples in each experiment to minimize the inter-assay variability. The external standard curve is the linear regression line through the data points of crossing point (Threshold detection cycle) versus the logarithm of concentration of the standard samples. Given the crossing point-cycle number of any unknown, concentration can be calculated from the standard curve. The standards used in this study were serial dilutions of control cDNA with defined dilution steps (1, 1:10, 1:100, 1:1000 etc) representing arbitrary relative concentrations (10^7, 10^6, 10^5 etc). An example of the standard curve was given in Figure 3.22.

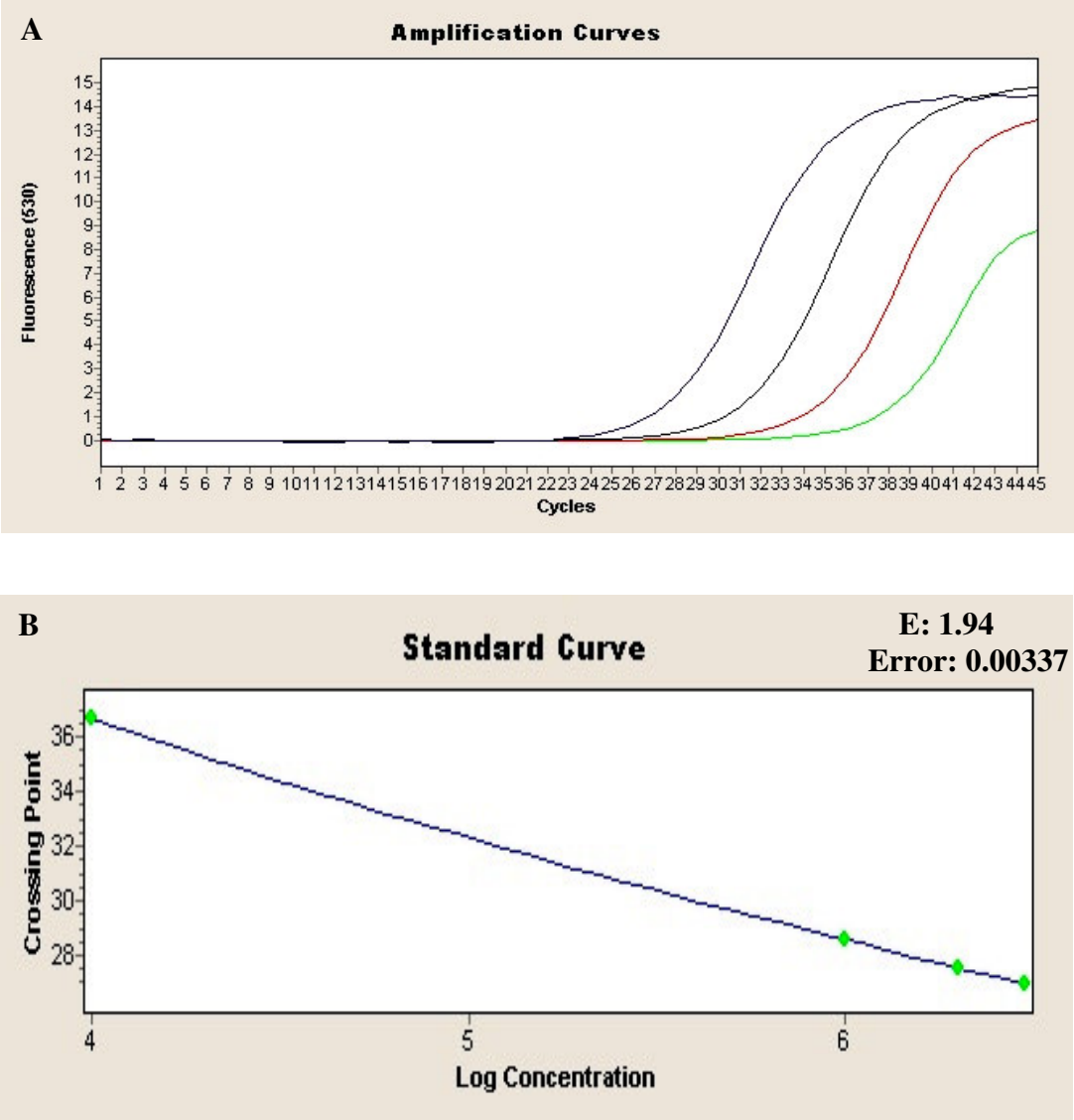


Figure 3.22 Amplification of the serial dilution series of each gene specific standards using the Roche Light Cycler 1.5 detection system. **A.** Amplification plot showing the changes in fluorescence of SYBR Green I dye versus cycle number of serial diluted cDNA (5 µl). **B.** Standard curve showing the crossing point of same diluted samples versus the log of the initial amount of cDNA. Efficiency of standard curve was found as 1.94 with an error of 0.003337

3.5.2.2 Effects of Diabetes, Benzene and Benzene Treatment of Diabetic Rabbits on CYP2E1 mRNA Level in Liver, Kidney and Lung.

Real Time-PCR results of liver CYP2E1 mRNA level were represented in Figure 3.23. The normalized liver CYP2E1 mRNA level (CYP2E1/GAPDH ratio) was found to be 0.28 ± 0.07 (mean ± SEM, N=6) for control animals. CYP2E1 mRNA amount was calculated as 1.88 ± 0.5 (mean ± SEM, N=7) for liver tissue of diabetic animals. Thus, induction of the diabetes by single dose of alloxan was caused significant 6.71-fold increase in CYP2E1 mRNA level with respect to controls ($p<0.05$). Moreover, CYP2E1 mRNA level for benzene treated and benzene treated diabetic rabbits were found as 2.95 ± 0.9 (mean ± SEM, N=8) and 3.62 ± 0.6 (mean ± SEM, N=8), respectively. Therefore, these results indicate that CYP2E1 mRNA level in benzene treated and benzene treated diabetic rabbit were increased 10.53- and 12.93-fold compared to control animals, respectively ($p<0.05$).

Changes in CYP2E1 mRNA level due to diabetes, benzene treatment and benzene treatment of diabetics were also determined in rabbit kidney. The result of Real-time PCR analysis of kidney CYP2E1 was illustrated in Figure 3.24. The normalized CYP2E1 mRNA level of control animals was found to be 1.10 ± 0.3 (mean ± SEM, N=5). Kidney CYP2E1 mRNA level of the diabetic animals was calculated as 2.90 ± 0.5 (mean ± SEM, N=5) in diabetic rabbits. Moreover, normalized kidney mRNA level (CYP2E1/GAPDH ratio) of benzene treated rabbit and benzene treated diabetic rabbits were found to be 3.20 ± 0.6 (mean ± SEM, N=5) and 4.30 ± 0.5 (mean ± SEM, N=5) respectively. As shown in Figure 3.24, relative CYP2E1 mRNA levels were increased 2.64-, 2.91 and 3.91-fold in diabetic, benzene treated and benzene treated diabetic rabbits with respect to control animals, respectively ($p<0.05$).

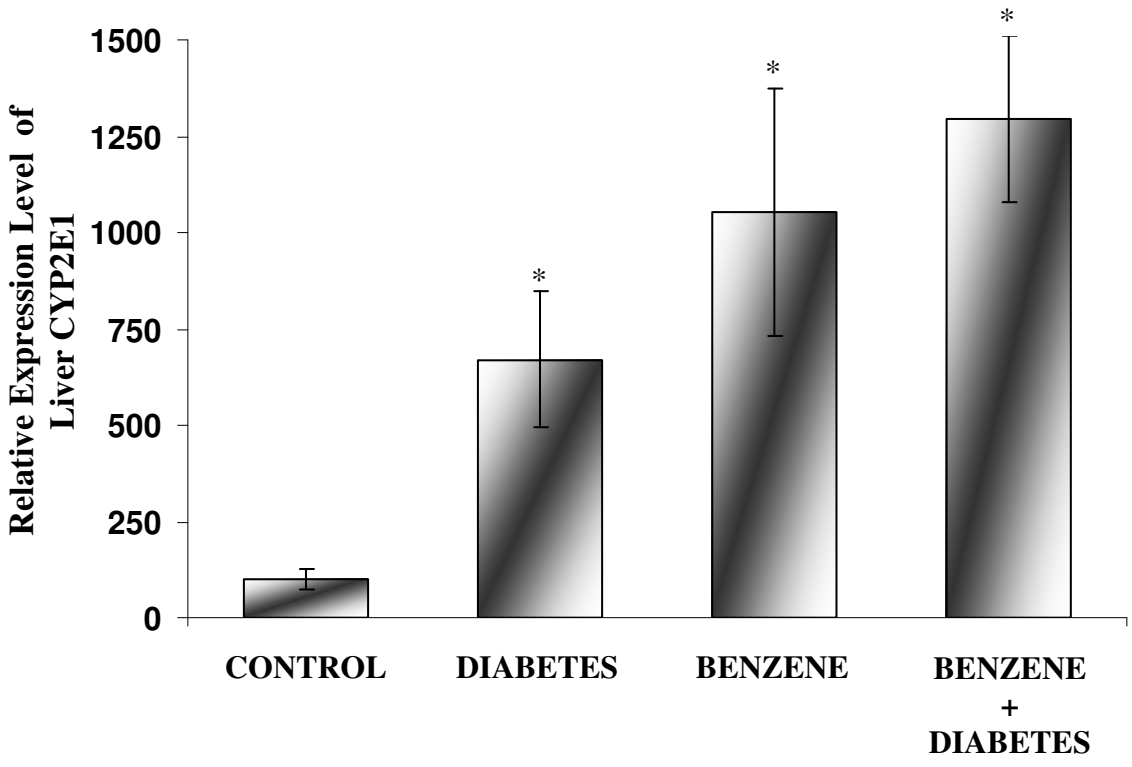


Figure 3.23 CYP2E1 gene expression of control, diabetic, benzene treated and benzene treated diabetic rabbit liver. Total RNA from rabbit liver tissues was isolated and 5 µg of RNA from each sample was used for cDNA synthesis. Real-time PCR for CYP2E1 and GAPDH was conducted by amplifying a 5µl aliquot of cDNA. Individual gene expression levels were normalized by using GAPDH. The value obtained from control animals was taken to be 100%, and the values obtained from the diabetic, benzene and benzene treated diabetic group were expressed as a percentage of control.

*Significantly different from the respective control value $p<0.05$

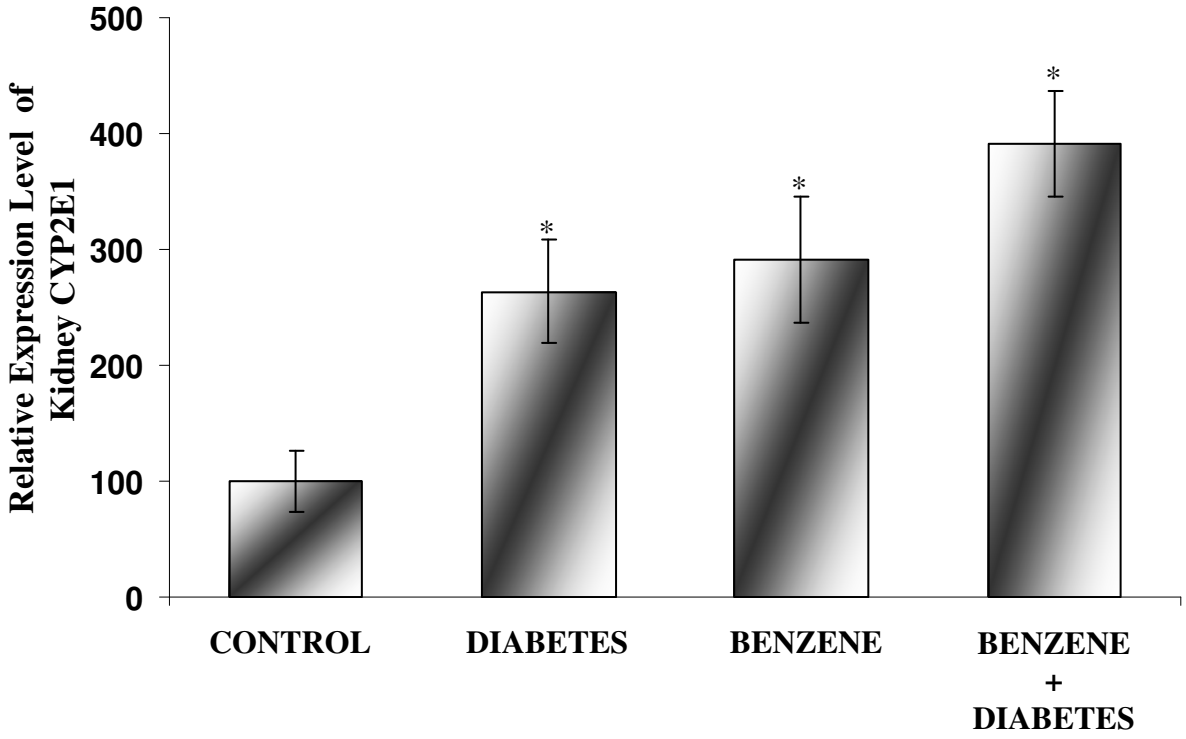


Figure 3.24 CYP2E1 gene expression of control, diabetic, benzene treated and benzene treated diabetic rabbit kidney. Total RNA from rabbit kidney tissues was isolated and 2.5 µg of RNA from each sample was used for cDNA synthesis. Real-time PCR for CYP2E1 and GAPDH was conducted by amplifying a 5µl aliquot of cDNA. Individual gene expression levels were normalized by using GAPDH. The value obtained from control animals was taken to be 100%, and the values obtained from the diabetic, benzene and benzene treated diabetic group were expressed as a percentage of control.

*Significantly different from the respective control value $p<0.05$.

3.5.2.3 Effects of Diabetes, Benzene and Benzene Treatment of Diabetic Rabbits on CYP1A2 mRNA Level in Liver.

To determine the effects of alloxan induced diabetes, benzene and benzene treatment of diabetic animals on CYP1A2 mRNA level in rabbit liver, Real-time PCR analysis was carried out utilizing SYBR Green I detection chemistry. The result of Real-time PCR analysis of liver CYP1A2 was illustrated in Figure 3.25. The normalized (CYP1A2/GAPDH ratio) CYP1A2 mRNA level for control animals was found to be 0.21 ± 0.04 (mean ± SEM, N=6). The liver mRNA level of CYP1A2 were increased to 0.38 ± 0.02 (mean ± SEM, N=7) by injection of animals with single dose of alloxan. Moreover, normalized CYP1A12 mRNA level for benzene treated rabbit and benzene treated diabetic rabbits were calculated as 0.29 ± 0.07 (mean ± SEM, N=6) and 0.22 ± 0.06 (mean ± SEM, N=8), respectively. The results of Real-time PCR analysis of the liver CYP1A2 shown in Figure 3.25 indicate that hepatic CYP1A2 mRNA level was increased significantly 1.81-fold in the diabetic rabbits with respect to control animals ($p<0.05$). On the other hand, CYP1A2 mRNA level was slightly (1.38-fold) increased as a result of benzene treatment, but this increase was not found statistically significant. Also, liver mRNA level was not changed in benzene treated diabetic rabbits as shown in Figure 3.25.

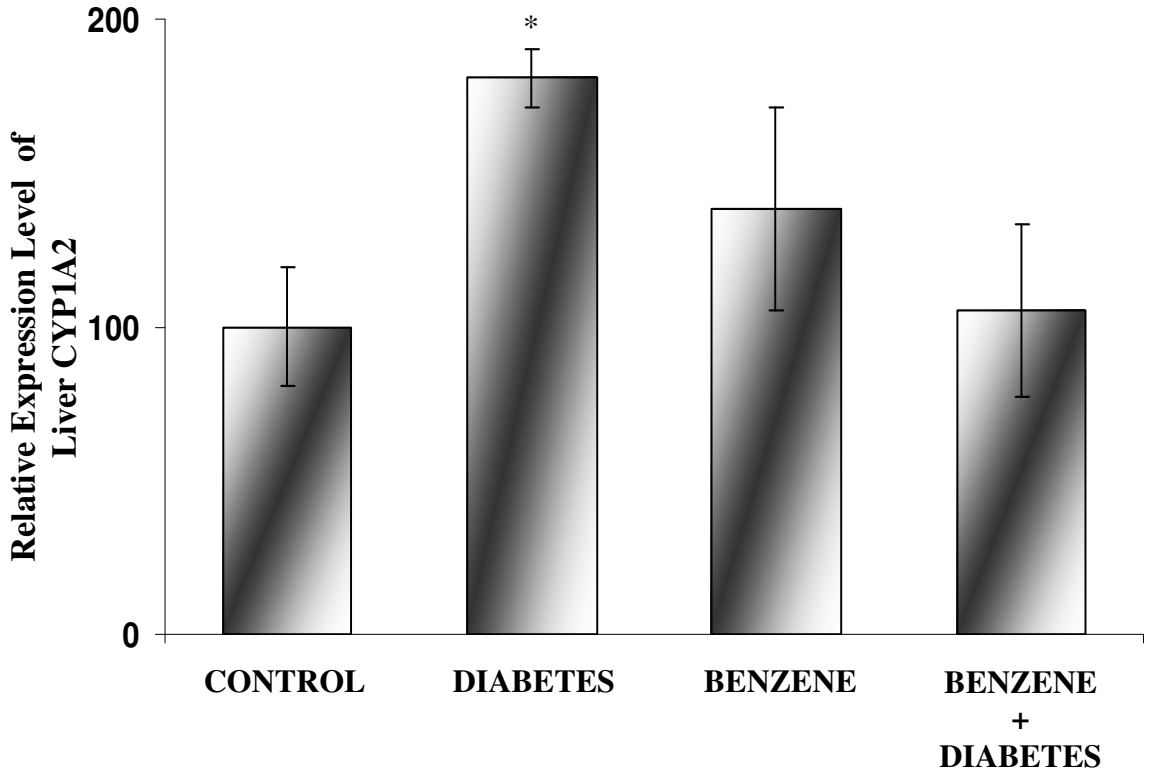


Figure 3.25 CYP1A2 gene expression of control, diabetic, benzene treated and benzene treated diabetic rabbit liver. Total RNA from rabbit liver tissues was isolated and 5 µg of RNA from each sample was used for cDNA synthesis. Real-time PCR for CYP1A2 and GAPDH was conducted by amplifying a 5µl aliquot of cDNA. Individual gene expression levels were normalized by using GAPDH. The value obtained from control animals was taken to be 100%, and the values obtained from the diabetic, benzene and benzene treated diabetic group were expressed as a percentage of control.

*Significantly different from the respective control value $p<0.05$.

3.5.2.4 Effects of Diabetes, Benzene and Benzene Treatment of Diabetic Rabbits on CYP3A6 mRNA Level in Liver.

The effects of diabetes, benzene and combined treatment of animals on the level of CYP3A6 mRNA level in rabbit liver were determined by Real-time PCR analysis. The results of Real-time PCR analysis of liver CYP3A6 was illustrated in Figure 3.26. The normalized (CYP3A6/GAPDH ratio) CYP3A6 mRNA level of control animals was found to be 5.60 ± 1.1 (mean ± SEM, N=6). CYP3A6 mRNA level was calculated as 75.90 ± 22.8 (mean ± SEM, N=7) in diabetic rabbits. Moreover, mRNA level of benzene treated rabbit and benzene treated diabetic rabbits were found to be 3.70 ± 0.7 (mean ± SEM, N=6) and 31.40 ± 8.4 (mean ± SEM, N=7), respectively. As shown in Figure 3.26, relative CYP3A6 mRNA level was increased 13.55 and 5.61-fold in diabetic and benzene treated diabetic rabbits compared to control animals, respectively ($p<0.05$). On the other hand, benzene treatment was caused 1.51-fold reduction of CYP3A6 mRNA level but this decrease was found statistically insignificant.

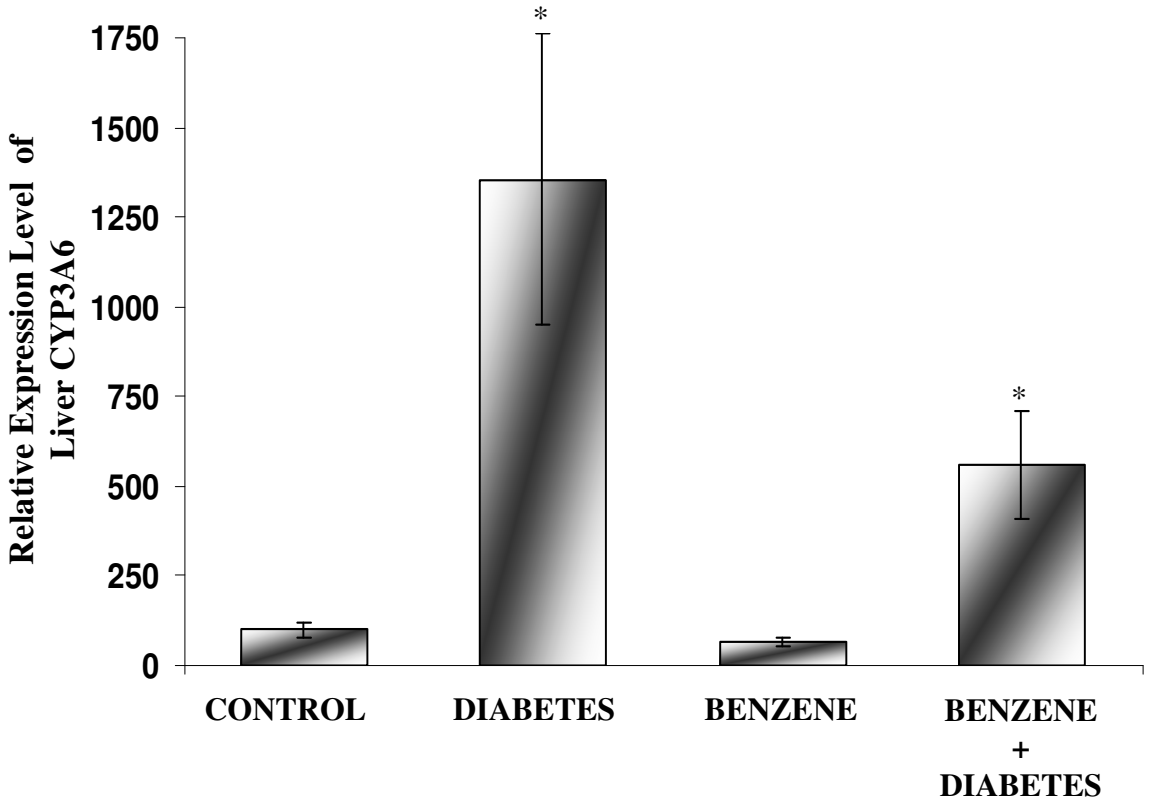


Figure 3.26 CYP3A6 gene expression of control, diabetic, benzene treated and benzene treated diabetic rabbit liver. Total RNA from rabbit liver tissues was isolated and 5 µg of RNA from each sample was used for cDNA synthesis. Real-time PCR for CYP3A6 and GAPDH was conducted by amplifying a 5µl aliquot of cDNA. Individual gene expression levels were normalized by using GAPDH. The value obtained from control animals was taken to be 100%, and the values obtained from the diabetic, benzene and benzene treated diabetic group were expressed as a percentage of control.

*Significantly different from the respective control value $p<0.05$.

3.5.3 Melting Curve Analysis of the Real-Time PCR Products.

The melting curve analysis is a technique used for determination of nature of of PCR products. After completing Real time-PCR, the resulting PCR product is slowly heated as described in methods. Each double-stranded DNA has its own specific melting temperature (Tm), which is defined as the temperature at which 50% of the DNA becomes single stranded. During a melt program, the fluorescence of the sample is monitored while the temperature is steadily increased to melt probes off the target strands.

Melting curve analysis is a simple, straightforward way to check Real time-PCR reactions for primer-dimer artefacts and contamination and to ensure reaction specificity and accurate quantification. In this study, melting curve analysis was performed in every PCR run in order to confirm that a single PCR product was detected or not. An example of the melting curve was given in Figure 3.27. As can be seen in the Figure, all reactions with cDNA template showed one sharp and fully overlapping melting peaks indicating the specificity of primer and no contamination. Single product was produced as a result of PCR. Arrow in Figure 3.27 indicates that there was no amplification of the product when water or No-RT control was used instead of cDNA.

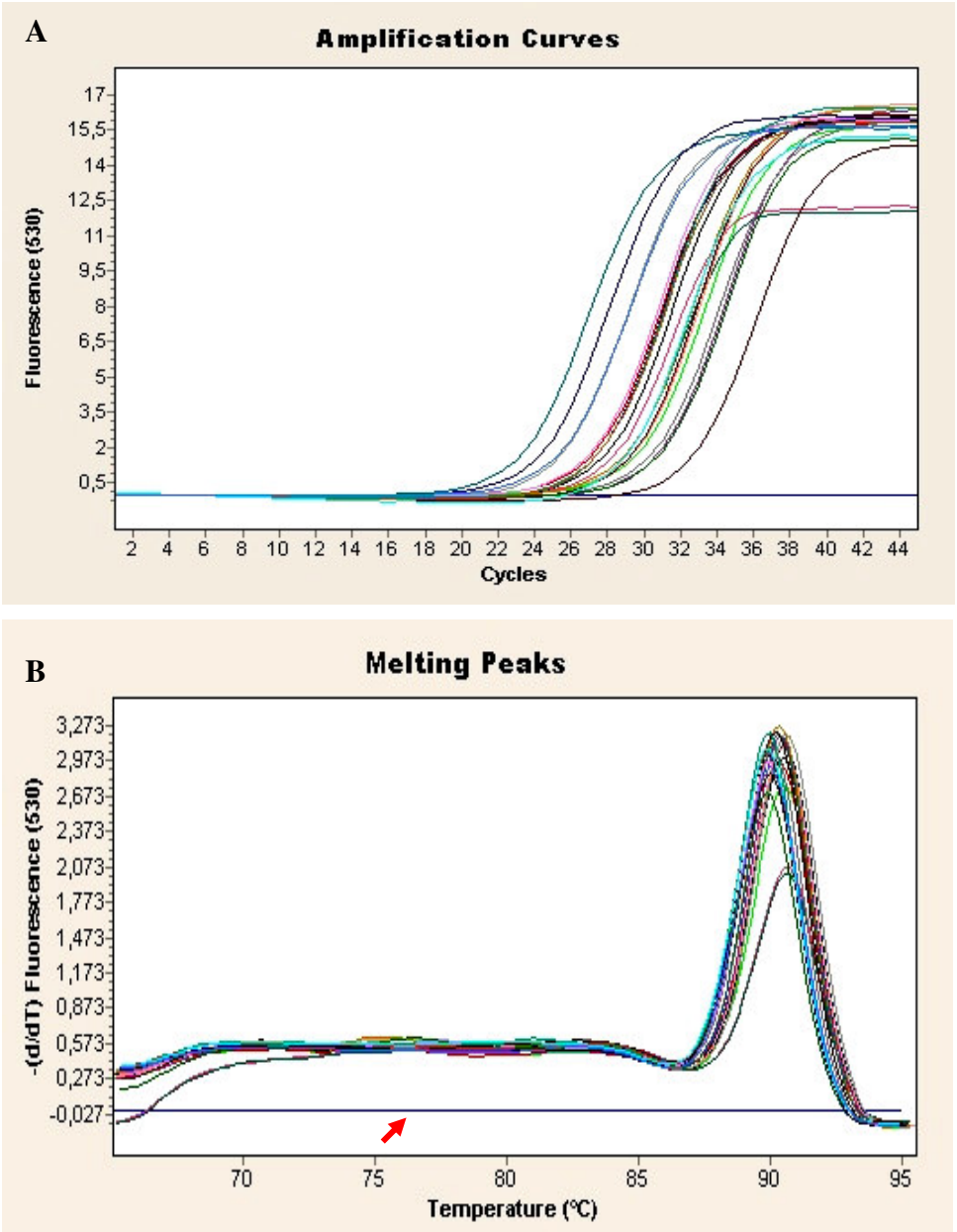


Figure 3.27 Amplification of the liver CYP2E1 mRNA by using Roche Light Cycler 1.5 detection system. **A.** Amplification plot showing the changes in fluorescence of SYBR Green I dye versus cycle number of control, diabetic, benzene treated and benzene treated diabetic rabbits' target gene (CYP2E1). **B.** Melting curve showing the fluorescence of SYBR Green I dye versus the temperature. One peak in melting analysis confirms that a single PCR product was detected.

3.6 Effects of Diabetes, Benzene and Benzene Treatment of Diabetic Rabbits on Total GST, Glutathione S-Transferase Isozymes, Catalase and Lipid Peroxidation.

Table 3.16 tabulates the effects of diabetes, benzene and both treatment on Total GST, Mu-GST, Pi-GST, Theta-GST, Catalase and Lipid Peroxidation. Total GST enzyme activities of rabbit liver cytosols were found be 3.5 ± 0.3 (n=6), 3.9 ± 0.3 (n=7), 4.1 ± 0.2 (n=8) and 3.8 ± 0.3 (n=7) μmol/min/mg protein (mean ± SEM) in control, diabetic, benzene treated and benzene treated diabetic rabbits, respectively.. Moreover, Mu-GST in control, diabetic, benzene treated and benzene treated diabetic rabbits were calculated as 4.3 ± 0.4 (n=6), 4.5± 0.4 (n=7), 4.3 ± 0.5 (n=7) and 4.3 ± 0.4 (n=7) μmol/min/mg protein (mean ± SEM), respectively. At the same time, Pi-GST activities of control, diabetic, benzene treated and benzene treated diabetic rabbit were found as; 3.5 ± 0.3 (n=6), 3.0 ± 0.2 (n=7), 2.8 ± 0.3 (n=7) and 3.2 ± 0.2 (n=7) nmol/min/mg protein, (mean ± SEM), respectively. Besides these, activities of Theta isozymes of GST were found to be 8.1 ± 2 (n=6), 10 ± 3 (n=7), 5.8 ± 1.8 (n=7) and 14.4 ± 4 (n=7) nmol/min/mg protein (mean ± SEM) in control, diabetic, benzene treated and benzene treated diabetic rabbits' cytosols, respectively. However, changes in activities in both Total GST and GST isozymes were not found to be statistically significant.

Catalase activities of the rabbit liver cytosols obtained from control, diabetic, benzene treated and benzene treated diabetic rabbit were found to be 37 ± 5 (n=6), 54 ± 5 (n=7), 52 ± 7 (n=7), 48 ± 4 (n=7) μmol/min/mg protein (mean ± SEM), respectively. These results shoved that induction of diabetes caused statistically significant 1.46-fold increase with respect to control animals activity ($p<0.05$). Although, , catalase enzyme activity of cytosols increased 1.40-fold upon benzene treatment of rabbits, this increase was not found to be statistically significant.

Table 3.16 Effects of diabetes, benzene and benzene treatment diabetic rabbits on liver cytosolic total GST, GST isozymes, catalase and lipid peroxidation

ENZYME ACTIVITY	RABBITS			
	Control	**Diabetes**	**Benzene**	**Benzene + Diabetes**
Total GST(CDNB) (µmol/min/mg prot)	3.5 ± 0.3 (n=6)	3.9 ± 0.3 (n=7)	4.1 ± 0.2 (n=8)	3.8 ± 0.3 (n=7)
GST Mu (EA) (nmol/min/mg prot)	4.3 ± 0.4 (n=6)	4.5± 0.4 (n=7)	4.3 ± 0.5 (n=7)	4.3 ± 0.4 (n=7)
GST Pi (DCNB) (µmol/min/mg prot)	3.5 ± 0.3 (n=6)	3.0 ± 0.2 (n=7)	2.8 ± 0.3 (n=7)	3.2 ± 0.2 (n=7)
GST Theta (EPNP) (nmol/min/mg prot)	8.1 ± 2 (n=6)	10 ± 3 (n=7)	5.8 ± 1.8 (n=7)	14.4 ± 4 (n=7)
Catalase (µmol/min/mg prot)	37 ± 5 (n=6)	54 ± 5** (n=7)	52 ± 7 (n=7)	48 ± 4 (n=7)
Lipid Peroxidation (nmol/mg prot)	0.04 ± 0.004 (n=6)	0.07 ± 0.005* (n=7)	0.06 ± 0.002** (n=7)	0.063 ± 0.005* (n=7)

*Significantly different from the respective control value $p<0.005$
**Significantly different from the respective control value $p<0.05$

Lipid peroxidation in liver microsomes of all rabbits was using TBARS test determined throughout this study. The amount of lipid peroxidation product in control, diabetic, benzene treated and benzene treated diabetic rabbits was found to be 0.04 ± 0.004 (n=6), 0.07 ± 0.005 (n=7), 0.06 ± 0.002 (n=7) and 0.063 ± 0.005 (n=7) nmol product/mg protein (mean ± SEM). These results showed that induction of diabetes by alloxan, benzene treatment and benzene treatment of diabetic rabbits caused statistically significant 1.75-, 1.50- and 1.58-fold increase in lipid peroxidation product in rabbit liver microsomes, respectively.

CHAPTER 4

DISCUSSION

It is well known that a diverse array of compounds influence to changes in the relative concentrations and/or activities of P450s. Moreover, expression of P450s is modulated not only by exposure to various chemicals, but also in some pathophysiological conditions such as diabetes, hypertension and obesity (Favreau and Schenkman, 1988; Raucy *et al*., 1990; Arınç *et al*., 2007). Over the past four decades, induction or suppression of MFO enzymes has been shown to significantly alter pharmacologic or toxicologic responses *in vivo* in laboratory animals and humans (Conney, 1967, 1982; Goldberg, 1980; Park and Breckenridge, 1981; Okey *et al.,* 1986). There are two primary practical concerns related to cytochrome P450 dependent MFO induction: (1) induction may alter the efficacy of therapeutic agents (2) induction may cause an undesirable imbalance between rates of "toxification" versus "detoxification" in diseased organisms and organisms exposed to drugs and environmental chemicals (Okey *et al.,* 1986; Okey, 1990; Schenkman *et al.,* 1989). Altered pharmacological responses *in vivo* generally are in proportion to the magnitude of increase in P450 dependent catalytic activities that can be measured *in vitro* on microsomes from the "induced" animals.

Diabetes is a universal health problem that is the fourth leading cause of global death by disease (American Diabetes Association, 2004). Despite the extensive research, the scientific community is still far away from understanding the mechanisms and irreversible changes caused by the disease on both structure and function of organs at the molecular level. In addition to the effects of carbohydrate,

fat, protein and electrolyte metabolisms due to insulin deficiency or diminished tissue response to insulin, diabetus mellitus affects the other metabolic events in the body. According to the recent studies, cytochrome P450 monooxygenases are altered as a result of diabetes (Favreau and Schenkman, 1988; Chen *et al.,* 1996; Sakuma *et al.,* 2001; Arınç *et al*., 2005). These studies indicate that several forms of cytochrome P450 are enhanced or suppressed as a result of diabetes due to metabolic and hormonal alterations (Schenkman *et al.,* 1989; Thummel and Schenkman, 1989).

Diabetic patients are treated with several drugs and, at the same time, they are exposed to a variety of toxic carcinogenic chemicals in the environment and in the diet. These drugs and other chemicals are oxidatively metabolized by cytochrome P450 dependent enzymes into more water soluble metabolites. It is well known that this system serves as a route of detoxification and, in contrast, also as a route of metabolic activation to yield reactive metabolites which initiate toxic and carcinogenic events. Multiple forms of P450 show different substrate specificities and reactivities toward these compounds (Lu and Levin, 1974; Arınç and Philpot, 1976; Arınç and Adalı, 1983; Watanabe, 1999). The balance between detoxification and bioactivation of a compound in a particular species or an organ is highly dependent on the relative amounts and/or activation of different forms of P450s that are expressed.

It is well established that vast majority of chemical carcinogens are metabolized by the Mixed Function Oxidase system. Therefore, it is reasonable to assume that their metabolism will also disrupted by diabetes. Induction of diabetes in rat enhances the metabolism and hepatotoxicity of some well-known human carcinogens such as carbon tetrachloride, thioacetamide, bromobenzene, 1, 1, 2-trichloroethane, nitrosoamines and chloroform. (Hanasono *et al*., 1975; Lorr *et al*., 1984; Watkins *et al*., 1988; Aniya *et al*., 1989; Wang *et al*., 2000a). On the other hand, diabetes in mice unexpectedly protects animals from normally lethal hepatotoxic chemicals such as acetaminophen, carbon tetrachloride, thioacetamide, bromobenzene (Shankar *et al*., 2003a,b,c; Sawant *et al*., 2006a). To our best

knowledge, there has been only one study on modulatory effects of diabetes on cytochrome P450 dependent procarcinogen/carcinogen metabolism and hepatoxicity in rabbits (Arınç *et al*., 2005, 2007). In order to understand species and tissue specific metabolism of drugs and bioactivation and/or detoxification of toxic compounds in diabetic animals, it is necessary to carry out comparative studies.

Application of another inducer of the CYP2E1 such as benzene to the diabetic rabbits may further elevate expression and activities of the CYP2E1. As a result of further or additive induction of the CYP2E1 by two treatments (diabetes and chemical inducer of the CYP2E1), an increased risk of hepatotoxicity produced by toxins which are activated by CYP2E1 may be observed when compared to the separate treatments. In this regard, we claimed that benzene application to the diabetic rabbits may further increase the risk of CYP2E1 induced chemical carcinogenesis such as benzene or nitrosamine by formation of more reactive methylated agents from these compounds.

Despite intensive studies over several decades, the mechanisms underlying benzene-induced hematotoxicity and carcinogenicity are still not fully understood. Classic carcinogens are thought to be activated to single, ultimate carcinogenic metabolites that are highly electrophilic and bind strongly to DNA in a covalent fashion. As a result, they damage to DNA, RNA or protein. On the other hand, for benzene, this scenario is opposite. It does not form a single highly electrophilic metabolite (Smith, 1996). None of the prominent metabolites of benzene are electrophiles (Lutz, 1986). However, they produce chromosomal damage both in vitro and in vivo (Wolman 1977; Dean 1985; Yager *et al*., 1990). Primary benzene metabolites show synergistic interactions to exacerbate benzene toxicity (Eastmond *et al*., 1987; Subrahmanyam *et al*., 1990; Chen and Eastmond 1995; Smith, 1996). This mechanism of multi-metabolite genotoxicity is another unique aspect of benzene that distinguishes it from other chemicals in terms of the mechanism of its toxicity and carcinogenicity.

Benzene is a preferential substrate of one particular cytochrome P450 family, namely CYP2E1 (Post and Snyder, 1983). Therefore, exposure to chemicals or pathophysiological situations that stimulate the activity of this enzyme system prior to exposure to benzene could increase the rate of benzene metabolism. This may further increase the risk of chemical carcinogenesis by formation of more reactive agents from benzene.

In this regard, this study was undertaken to determine the influence of benzene on liver, kidney and lung microsomal cytochrome P450 dependent drug metabolizing enzyme activities, protein levels and mRNA levels in diabetic and control rabbits and on the biomarkers used to measure chemical-induced toxicity including Lactate Dehyrogenase (LDH), Aspartate Aminotransferase (AST) and Alanine Aminotransferase (ALT) and on some oxidative stress markers in rabbit liver which have not been addressed in this context before.

In this study, diabetes was induced in rabbits experimentally by intravenous injection of 125 mg/kg alloxan. Induction of diabetus mellitus by a single dose of alloxan was confirmed by 3.7-fold increase in the blood glucose level. This condition may also indicate an alteration in carbohydrate metabolism due to diabetus mellitus. Creatinine and urea concentration of rabbit blood serum were increased in a statistically significant manner, 1.32- and 2-fold, respectively in diabetics rabbits with respect to control ones (Arslan, 2003; Arınç *et al*., 2007). The increase in blood serum urea concentration in diabetus mellitus was possibly due to increased rate of deamination of amino acids in liver as a result of tissue protein mobilization. This condition may reflect an alteration in protein metabolism due to diabetus mellitus.

Among all the P450s studied, P4502E1 is most active in catalyzing the NADPH dependent formation of H_2O_2 and $O_2^{\cdot-}$ *in vitro* (Ekstrom and Ingelman-Sundberg, 1989; Gorsky *et al.,* 1984; Persson *et al.,* 1990). Antibodies to P4502E1 almost completely inhibited the NADPH dependent lipid peroxidation in microsomes (Ekstrom and Ingelman-Sundberg, 1989). It was postulated that

induction of P4502E1 in the liver results in increased oxygen stress by generating higher levels of H_2O_2 and $O_2^{\cdot-}$. Thus, at the beginning of this study it has been speculated that some degree of membrane damage may occur as a result of lipid peroxidation which could result in enhancement of transaminases (AST and ALT) and LDH activities in serum. As given in Table 3.16, the amount of lipid peroxidation product in diabetic, benzene treated and benzene treated diabetic rabbits was increased 1.75-, 1.50- and 1.58-fold in liver microsomes, respectively. The integrity of the lipid membrane may be lost due to increase in lipid peroxidation. As a result, activities of LDH, AST and ALT in blood serum were increased in these rabbits with respect to controls. As shown in Table 3.2, LDH activity was increased about 2-fold in serum of diabetic, benzene treated and benzene treated diabetic rabbits. Moreover, AST activity was increased 1.59-, 1.29- and 1.39-fold in serum of diabetic, benzene treated and benzene treated diabetic rabbits. Similarly, ALT activity of diabetic, benzene treated and benzene treated diabetic rabbits was increased 1.77-, 1.59- and 1.64-fold with respect to controls.

It is well established that diabetes or benzene treatment modify oxidative stress markers such as catalase and reduced glutathione levels (Akkuş *et al*., 1996; Verma and Rana, 2004). Among these oxidative stress markers, the amount of thiobarbituric acid-reactive substances (TBARS) in tissues and serum estimates the extent of free radical damage and routinely employed by investigators. Several studies in humans and rodents have shown increased serum and tissue TBARS due to diabetes or benzene treatment (Akkuş *et al*., 1996; Sukhodub and Padalko, 1999; Rauscher *et al*., 2000, 2001; Verma and Rana, 2004). As a result of damage to lipid membrane, cytoplasmic components, especially cytoplasmic enzymes, pass to blood. Among these enzymes, LDH, AST and ALT alone or in combination are primarily recommended for the assessment of hepatocellular injury in rodents and non-rodents in non-clinical studies.

LDH found in the cytoplasmic portion of cells is distributed in all tissues, but it is present in high concentration in liver, cardiac and skeletal muscles, red blood cells and other tissues. In pathological states involving tissue necrosis and

neoplasia, leakage of enzyme from even a small amount of damaged tissue can significantly raise levels in various body fluids including blood serum. In this study, we observed such an increase in serum that shows us mild tissue damage due to diabetes and benzene treatment. Moreover, Aspartate Aminotransferase (AST) and Alanine Aminotransferase (ALT) are found practically in every tissue of the body, including red blood cells. They are in particularly high concentration in cardiac muscle and liver, intermediate in skeletal muscle and in much lower concentrations in others. AST and ALT activities were increased in blood serum of diabetic, benzene treated and benzene treated diabetic rabbits. This may also reflect the mild degree of tissue damage in liver as a result of these treatments.

As mentioned before, diverse array of compounds and some pathophysiological conditions influence the relative concentrations and/or activities of P450s. The most prominent effect of diabetes is the induction of CYP2E1. In consistent with previous studies in our laboratoy (Arınç *et al*., 2005, 2007), the data obtained from immunoblot analysis and enzymatic activity studies clearly demonstrated that diabetes induced CYP2E1 and associated enzyme activities (NDMA N-demethylase and *p*-nitrophenol hydroxylase) not only in rabbit liver but also in kidney and lung. As shown in Table 4.1 and Figure 4.1, enhancement of CYP2E1 dependent NDMA N-demethylase (2.0-fold) and *p*-nitrophenol hydroxylase (1.73-fold) activities were paralleled by a similar increase (2.65-fold) in hepatic CYP2E1 protein content of these animals, as assessed on immunoblots cross-reacted with anti-rabbit CYP2E1. Furthermore, CYP2E1 content of lung and kidney microsomes and its associated enzyme activities were increased significanly in diabetic animals (Table 4.1 and Figure 4.1). The activities of NDMA N-demethylase and *p*-nitrophenol hydroxylase and CYP2E1 protein level were increased 1.60-, 2.41- and 2.50-fold diabetic rabbit kidney, respectively. In addition to liver and kidney, CYP2E1 dependent enzyme activities, NDMA N-demethylase and *p*-nitrophenol hydroxylase, and CYP2E1 protein level were increased 1.66-, 1.50- and 1.77-fold in the lung of diabetic rabbits, respectively.

Table 4.1 Effects of diabetes, benzene and benzene treatment of diabetic rabbits on liver, kidney and lung CYP2E1 associated enzyme activities, NDMA N-demethylase and *p*-Nitrophenol hydroxylase, CYP2E1 protein and mRNA levels.

Tissue		NDMA N-Demethylase nmol/min/mg prot	Change (Fold)	*p*-Nitrophenol Hydroxylase nmol/min/mg prot	Change (Fold)	CYP2E1 Protein Level (RPA)	Change (Fold)	CYP2E1 mRNA Level (CYP2E1/GAPDH)	Change (Fold)
LIVER	**Control**	0.39 ± 0.02	**1.0**	0.30 ± 0.02	**1.0**	330 ± 13.3	**1.0**	0.28 ± 0.07	**1.0**
	Diabetes	0.78 ± 0.05[a]	**2.0 X ↑**	0.52 ± 0.03[a]	**1.73 X ↑**	875 ± 114[d]	**2.65 X ↑**	1.88 ± 0.5[d]	**6.71 X ↑**
	Benzene	1.23 ± 0.10[b]	**3.15 X ↑**	0.71 ± 0.05[a]	**2.37 X ↑**	1270 ± 217[d]	**3.85 X ↑**	2.95 ± 0.9[d]	**10.53 X ↑**
	Benzene+Diabetes	1.78 ± 0.11[a,f]	**4.56 X ↑**	1.15 ± 0.03[a,f]	**3.83 X ↑**	2541 ± 129[b,g]	**7.70 X ↑**	3.62 ± 0.6[d]	**12.93 X ↑**
KIDNEY	**Control**	0.045 ± 0.002	**1.0**	0.017 ± 0.001[c]	**1.0**	510 ± 152	**1.0**	1.10 ± 0.3	**1.0**
	Diabetes	0.072 ± 0.004[a]	**1.60 X ↑**	0.041 ± 0.004[a]	**2.41 X ↑**	1270 ± 21.7[d]	**2.50 X ↑**	2.90 ± 0.5[d]	**2.64 X ↑**
	Benzene	0.099 ± 0.005[a]	**2.20 X ↑**	0.051 ± 0.003[a,e]	**3.00 X ↑**	1425 ± 90.4[d]	**2.80 X ↑**	3.20 ± 0.6[d]	**2.91 X ↑**
	Benzene+Diabetes	0.13 ± 0.005[a,e]	**2.89 X ↑**	0.077 ± 0.003	**4.53 X ↑**	1760 ± 30.4[d,g]	**3.45 X ↑**	4.30 ± 0.5[d]	**3.91 X ↑**
LUNG	**Control**	0.15 ± 0.01	**1.0**	0.14 ± 0.006	**1.0**	1121 ± 61	**1.0**	ND	--
	Diabetes	0.25 ± 0.01[a,h]	**1.66 X ↑**	0.21 ± 0.009[a,h]	**1.50 X ↑**	1982 ± 90.7[b,j]	**1.77 X ↑**	ND	--
	Benzene	0.13 ± 0.01	**0.87 X ↓**	0.13 ± 0.006	**0.93 X ↓**	1119 ± 185	**1.0**	ND	--
	Benzene+Diabetes	0.16 ± 0.01	**1.07 X ↑**	0.14 ± 0.004	**1.0**	1175 ± 48.5	**1.05 X ↑**	ND	--

[a]Significantly different from the respective control value $p<0.0001$.
[b]Significantly different from the respective control value $p<0.005$.
[c]Significantly different from the respective control value $p<0.001$.
[d]Significantly different from the respective control value $p<0.05$.
[e]Significantly different from the respective diabetes and benzene value $p<0.0001$
[f]Significantly different from the respective diabetes and benzene value $p<0.001$
[g]Significantly different from the respective diabetes and benzene value $p<0.05$
[h]Significantly different from the respective "benzene + diabetes" value $p<0.0001$
[j]Significantly different from the respective "benzene + diabetes" value $p<0.005$
N.D. Not Determined

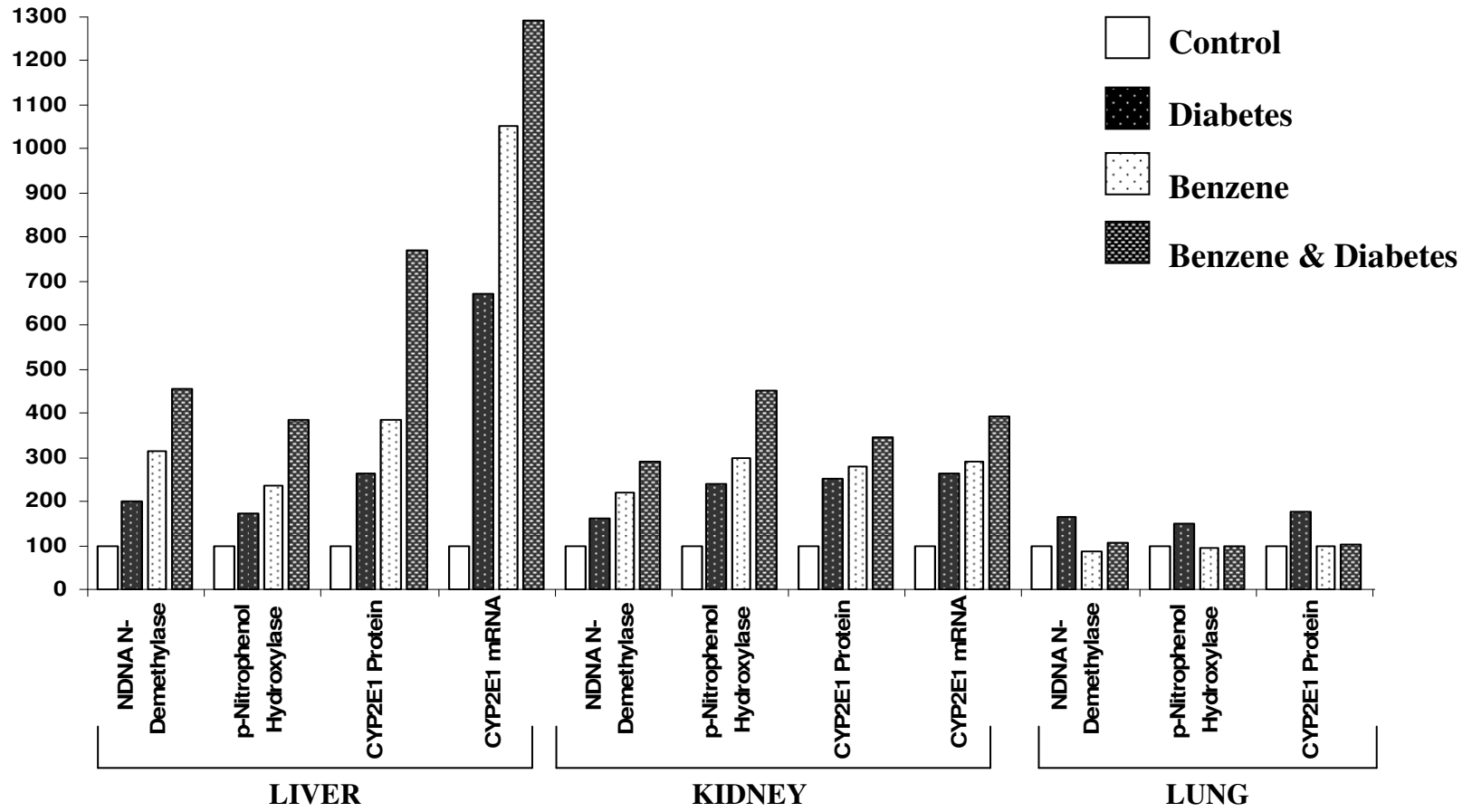


Figure 4.1 Effects of diabetes, benzene and benzene treatment of diabetic rabbits on hepatic, renal and pulmonary CYP2E1 dependent enzyme activities, CYP2E1 protein and mRNA levels. The value obtained from the control animals was taken to be 100%, and the value obtained from the diabetic, benzene and benzene treated diabetic group was expressed as a percentage of control.

Essentially similar results were obtained in our previous studies carried out in 2005 and 2007 (Arınç *et al*., 2005, 2007). These studies showed that induction of diabetes in rabbits by single dose of alloxan (100 mg/kg body weight) displayed elevated CYP2E1 protein level and its associated enzyme activities, aniline 4-hydroxylase, NDMA N-demethylase and *p*-nitrophenol hydroxylase not only in liver but also in kidney and lung. The results of the present study are in good correlation with those of Arınç *et al*., 2005 and 2007.

Extensive studies have implicated that possible three mechanisms: increased ketone bodies, impaired insulin secretion and suppressed growth hormone levels in diabetes are involved in induction of CYP2E1 in diabetes. These factors play a role in the induction of hepatic CYP2E1 in diabetes at transcriptional, post-transcriptional, and post-translational levels (Figure 4.2) (see the review Wang *et al*., 2007). Studies have indicated that the major factor responsible for the induction of rat CYP2E1 is elevated levels of ketone bodies. It has been well accepted that post-translational stabilization of CYP2E1 by high levels of ketone bodies in diabetes might result in induced CYP2E1 in diabetes (Abdelmegeed *et al*., 2005 and Shimojo, 1994 referred by Wang *et al*., 2007). In addition to elevated ketone bodies, decreased growth hormone secretion has been contributed to the induction of CYP2E1 in diabetes (Yamazoe *et al*., 1989; Barnett *et al*., 1993; Shimojo, 1994). However, conflicting results were also reported. It was showed that replacement of human growth hormone failed to significantly reverse the diabetes-induced hepatic CYP2E1; furthermore, CYP2E1 was induced in diabetic female rats without a reduction in growth hormone secretion (Thummel and Schenkman, 1990). Impaired insulin secretion may also involve in diabetes-associated increases in hepatic CYP2E1 which can be prevented by treatment with insulin (Favreau and Schenkman, 1988; Shimojo, 1994; Shimojo *et al.,* 1993). Regulation of CYP2E1 by insulin on both transcriptional and post-transcriptional levels has been studied in primary cultured rat hepatocytes. Insulin caused not only decrease the level of CYP2E1 mRNA precursor-heterogeneous nuclear mRNA, leading to decreased CYP2E1 gene transcription, but also decrease CYP2E1 mRNA half-life (Woodcroft *et al*., 2002).

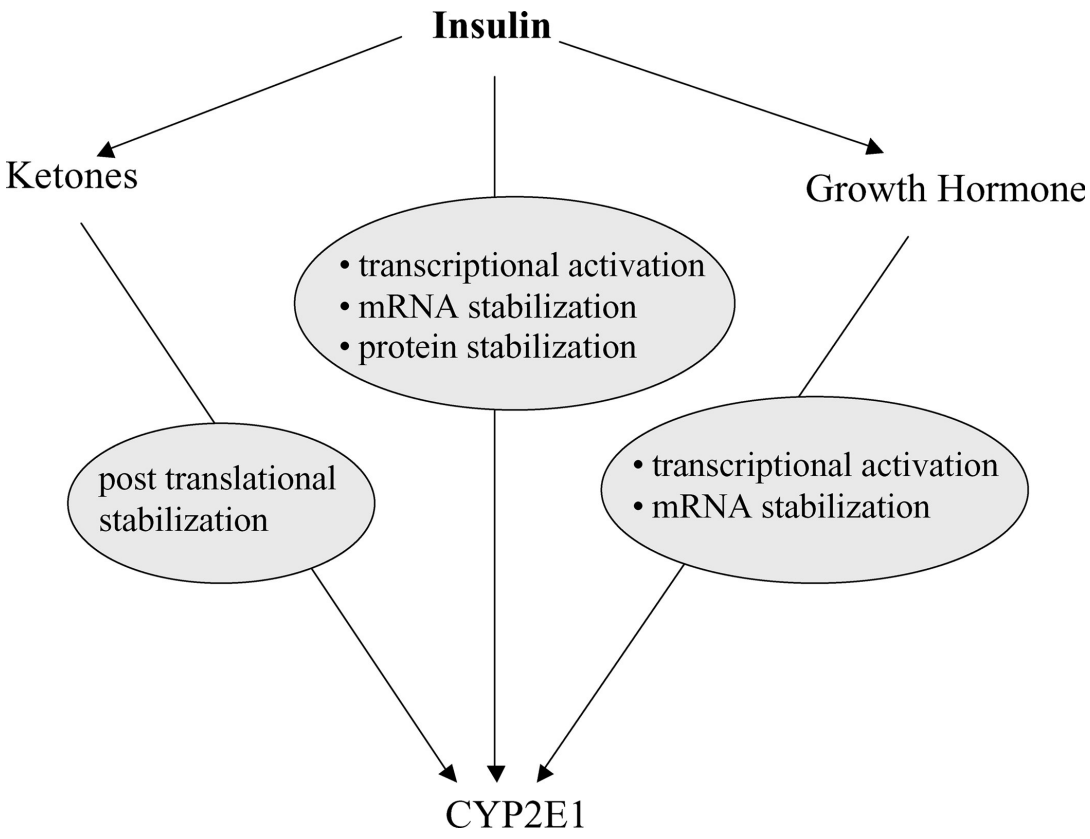


Figure 4.2 Proposed mechanisms involved in the induction of CYP2E1 in diabetes (Taken from Wang et al., 2007)

When the effects of diabetes mellitus on rat, hamster and mice cytochrome P450 and monooxgenase activities were studied, it was demonstrated that CYP2E1 and associated enzyme activity, aniline 4-hydroxylase, activity was found to be markedly increased in diabetic rat (Shimojo *et al*., 1993; Sakuma *et al*., 2001) and hamster (Chen *et al*., 1996) liver and kidney. In accordance with the results obtained with rat and hamster, diabetes increased CYP2E1 protein levels and associated enzyme activities in rabbit liver, kidney and lung. On the other hand, in contrast to the results obtained with rats, hamster and rabbits, CYP2E1 was not induced even decreased in diabetic ddY mouse liver and kidney but there was a statistically significant increase in aniline 4-hydroxylase activity of liver (Sakuma *et*

al., 2001). In the light of the data presented here and in previous works by other researchers, it is evident that a marked species-related differences exist in modulation of CYP2E1 level and associated enzyme activities in response to diabetes not only in liver but also in extrahepatic tissues such as kidney and lung. Many hormonal interactions and metabolic alterations play a significant role in modulation of CYP2E1 level in diabetic animals.

Benzene has been found to stimulate its own metabolism, thereby increasing the rate of toxic metabolite formation. The effect of benzene treatment on CYP2E1 protein level and its associated enzyme activities were determined in present study also. Benzene caused 3.15-, 2.37- and 3.85-fold increases in NDMA N-demethylase, p-nitrophenol hydroxylase activities and CYP2E1 protein level in liver microsomes, respectively (Table 4.1 and Figure 4.1). Benzene treatment also enhanced NDMA N-demethylase, p-nitrophenol hydroxylase activities and CYP2E1 protein level of kidney microsomes by 2.20-, 3.0- and 2.8-fold. In contrast to liver and kidney, benzene treatment caused no significant changes in CYP2E1 protein level and its associated enzyme activities of lung microsomes (Table 4.1 and Figure 4.1). In parallel to the results obtained in this study, although western blot analysis was not performed, the subcutaneous benzene treatment of rabbits induced CYP2E1 associated enzyme activities in the liver and kidney microsomes but not in lung (Arınç *et al*., 1988, 1991). Moreover, benzene treatment increased CYP2E1 and its associated enzyme activities in rats (Gut *et al*., 1993; Wang *et al*., 1996). However, benzene treatment did not influence CYP2E1 associated NDMA N-demethylase and aniline 4-hydroxylase activities and protein level in rat (Backes *et al*., 1993). The reason for these discrepancies in rat liver CYP2E1 may possibly be result of differences in dose, treatment period, or strain differences (Backes *et al*., 1993). These results showed that response of the rats to the benzene is similar as rabbits in terms of CYP2E1 induction

Benzene treatments of rabbits caused significant increases in NDMA N-demethylase, *p*-nitrophenol hydroxylase and CYP2E1 protein level in liver and kidney. On the other hand, unlike liver and kidney, lung NDMA N-demethylase, *p*-

nitrophenol hydroxylase and CYP2E1 protein level were not changed with benzene treatment. The results obtained in this study showed the existence of tissue specificity in the induction of rabbit CYP2E1 by benzene and diabetes. Moreover, another cytochrome P450 isozyme may be involved in benzene metabolism in rabbit lung. Studies with CYP2E1 knock-out mice and non-swiss albino (NSA) mice showed that CYP2F2 played as much of a role in benzene metabolism as CYP2E1 in lung (Powley and Carlson, 2000, 2001). Further experiments are needed to enlighten the involvement of CYP2F in benzene metabolism in rabbit lung. Furthermore, alloxan-induced diabetic condition in rabbits caused a significant increase in CYP2E1 associated enzyme activities and protein level in lung. In contrast to diabetes, benzene treatment of rabbits resulted in no change in CYP2E1 associated enzyme activities and protein level in lung. These results suggest that different factors may regulate induction of CYP2E1 in response to diabetes and benzene in rabbit lung.

During this study, benzene was given to the rabbits subcutaneously in high doses. In industry, repeated and rather low dose exposure of benzene is most common to workers, and benzene enters to the body via inhalation. Thus, it is expected that the lung is the first target organ to be affected. However, several reports of epidemiologic studies (Aksoy *et al*., 1974, Aksoy 1985, Kalf *et al*., 1987 referred by Arınç *et al*., 1991) and the results of low level benzene inhalation studies of mice over a 54-day period (Baarson *et al*., 1974; Dempster and Snyder 1986 referred by Arınç *et al*., 1991) did not show any toxic effect of benzene in the lung tissue. Benzene taken by any route, that is, inhalation, subcutaneous or intraperitoreal injection resulted in the hematotoxic and genotoxic effects (Gram et al., 1986, Kalf et al., 1987 referred by Arınç et al., 1991).

The combined effect of diabetes and benzene on CYP2E1 associated enzyme activity and protein level was also studied in this work. This is the most prominent part of this study. NDMA N-demethylase, p-nitrophenol hydroxylase activities and CYP2E1 protein level of liver microsomes increased by 4.56-, 3.83- and 7.70-fold in benzene treated diabetic rabbits, respectively (Table 4.1 and Figure

4.1). Similar trend was observed in kidney microsomes. Benzene treatment of diabetic rabbits caused 2.89-, 4.53- and 3.45-fold increases in NDMA N-demethylase, p-nitrophenol hydroxylase activities and CYP2E1 protein level in kidney microsomes, respectively. In contrast to liver and kidney, benzene treatment caused no significant changes in CYP2E1 protein level and its associated enzyme activities of lung microsomes. The content of CYP2E1 and associated catalytic activities in liver and kidney microsomes of benzene treated diabetic rabbits appeared to be additive of the diabetic and benzene group.

Cytochrome P4502E1 has received a great deal of attention in recent years because of its vital role in the activation of many toxic chemicals. Its possible role in the activation of xenobiotics to electrophilic, potentially mutagenic metabolites and in tumour development has been demonstrated in studies with benzene (Mehlman, 1991; Nakajima *et al.,* 1992), low molecular weight halogenated hydrocarbon species, like CCl4 (Guengerich *et al.,* 1991) and nitrosamines (Yoo *et al.,* 1988; Yang *et al.,* 1990; Yamazaki *et al.,* 1992).

Benzene is found in air, water, and soil as a result of both human activities and natural processes. Various industries use benzene to make other chemicals, such as ethylbenzene, styrene. Benzene is also used for the manufacturing of some types of rubbers, lubricants, dyes, detergents, drugs, and pesticides. The general population is exposed to benzene primarily by tobacco smoke (both active and passive smoking) and by inhaling contaminated air (motor vehicle exhaust gases). Exposure to benzene can also result from ingestion of contaminated food or water.

Benzene is an important class of multitarget environmental carcinogens known to cause cancer upon metabolic activation in humans and in animals. Benzene is activated to its carcinogenic forms by CYP2E1 (Figure1.6). Both epidemiologic studies and case studies showed that there is causal association between exposure to benzene and acute myelogenous leukemia, acute lymphocytic leukemia, acute erythroleukemic leukemia, acute myelomonocytic leukemia, acute promyelocytic leukemia, acute undifferentiated leukemia, chronic myelogenous

leukemia, chronic lymphocytic leukemia, hairy cell leukemia, Hodgkin's lymphoma, Non-Hodgkin's lymphoma, lymphosarcoma, multiple myeloma and reticulum cell sarcoma in human. Besides leukemia and lymphosarcoma, benzene also causes cancers of the lung, liver, stomach, esophagus, nasopharnyx, and intestine (see review Mehlman, 2002). These human data are supported by animal studies (Maltoni *et al*., 1983, 1988 and 1989). These long-term bioassays demonstrated that benzene caused tumors in Sprague-Dawley rats, Wistar rats, Swiss mice, and RF/J mice including cancers of the zymbal gland, oral cavity, lung, skin, nasal cavity, forestomach, Harderian gland, mammary gland, ovary, and uterus, lymphoma, hemolymphoreticular neoplasia and all types of leukemias (Cronkite *et al*., 1989; Huff *et al*., 1989 and Maltoni *et al*., 1983 and 1989).

As emphasized before, CYP2E1 is responsible for the bioactivation of an extensive array of drugs, solvents, and environmental carcinogens. Among those molecules, *N*-nitrosodimethylamine (NDMA) has a special interest due to its carcinogenic potential. NDMA requires a metabolic activation to elicit its carcinogenic effects. It is well established that NDMA is converted to its carcinogenic form after it is metabolized by CYP2E1 associated NDMA N-demethylase and that mutagenicity and carcinogenicity of NDMA are dependent on the activity of NDMA N-demethylase enzyme (Yang and Hong, 1995; Liteplo and Meek 2001).

p-Nitrophenol, an intermediate of benzene and nitrobenzene, is a food and environmental contaminant. The formation of catechols (4-nitrodihydroxy benzene) from *p*-nitrophenol by CYP2E1-associated *p*-nitrophenol hydroxylase has been implicated in the carcinogenic activity of these chemicals. The resulting product, 4-nitrocatechol, is oxidized to bioactive compounds, semiquinone and quinines that have ability to bind macromolecules such as DNA and proteins, which ultimately cause cellular necrosis, mutagenesis and malignant transformation (Billing, 1985; Kalf *et al*., 1987).

The results of the present work indicate that both diabetus mellitus and benzene exposure stimulate metabolic activation of *N*-nitrosodimethylamine, *p*-nitrophenol, benzene (and other toxic chemicals metabolized by CYP2E1) by inducing CYP2E1 which results in increased amounts of reactive metabolites formation. Application of benzene to the diabetic rabbits further elevates expression and activities of the CYP2E1. As a result of further or additive induction of the CYP2E1 by two situations (diabetes and benzene treatment), increased risk of hepatotocixity produced by toxins which are activated by CYP2E1 may be observed when compared to the separate treatments. This may in turn further potentiate the risk of organ toxicity, mutagenesis and malignant transformation in liver and kidney of these subjects.

The effects of diabetes, benzene, benzene treatment of diabetics on mRNA level of CYP450 isozymes was determined in this study by using real time PCR technique. In this technique, detection of PCR product occurs during the early phases of the reaction. Measuring the reaction kinetics in the early phases of PCR provide a distinct advantage over traditional PCR detection. At the same time, a relatively small amount of PCR product (DNA, cDNA or RNA) can be quantified in real time PCR. On the other hand, traditional methods use gel electrophoresis for the detection of PCR amplification in the final phase or at end-point of the PCR reaction. Endpoint detection is very time consuming and results may not be obtained for days. Results depend on size discrimination, which may not be very precise. This detection also is variable from sample to sample. Real Time PCR is based on the detection of the fluorescence produced by a reporter molecule which increases, as the reaction proceeds. This occurs due to the accumulation of the PCR product with each cycle of amplification. These fluorescent reporter molecules include dyes that bind to the double-stranded DNA (SYBR Green) or sequence specific probes (Molecular Beacons or TaqMan Probes). Real time PCR facilitates the monitoring of the reaction as it progresses. One can start with minimal amounts of nucleic acid and quantify the end product accurately. Moreover, there is no need for the post PCR processing which saves the resources and the time. Real time PCR

assays are now easy to perform, have high sensitivity, more specificity, and provide scope for automation.

The effect of diabetes, benzene and benzene treatment of diabetic rabbits on gene expression analysis of CYP2E1 was performed in rabbit liver and kidney tissues by using real-time PCR with SYBR Green I detection chemistry. As can be seen in Table 4.1 and Figure 4.1, liver CYP2E1 mRNA level in diabetic, benzene treated and benzene treated diabetic rabbit was increased 6.71-, 10.53- and 12.93-fold with respect to control animals, respectively ($p<0.05$). Moreover, relative kidney CYP2E1 mRNA level was increased 2.64-, 2.91 and 3.91-fold in diabetic, benzene treated and benzene treated rabbits with respect to control animals, respectively ($p<0.05$). In liver and kidney, mRNA level of CYP2E1 was increased in additive manner in benzene treated diabetic rabbits when compared to diabetes and benzene group, separately. This is in turn that more toxic agents may be formed when compared to separate treatments. Tissue necrosis and carcinogenicity may further increase in diabetic subjects that expose to benzene.

Hepatic mRNA level for CYP2E1 was determined by Northern blot analysis in diabetic rats. The mRNA level of CYP2E1 increased 4- to 10-fold, in alloxan induced and STZ induced diabetic rats (Song *et al*., 1986; Kim *et al*., 2005). Nuclear run-on transcription analysis showed that this mRNA increase is not due to transcriptional activation but is due to specific stabilization of the P4502E1 mRNA (Song *et al*., 1986). The stabilization of the CYP2E1 mRNA is reversed by insulin (Woodcroft *et al*., 2002). Moreover, it was observed an increase in CYP2E1 mRNA expression in peripheral mononuclear cells in both types of diabetes mellitus in human subjects which was determined by RT-PCR and Real-time PCR (Haufroid *et al*., 2003; Wang *et al*., 2003). These results are consistent with the observation that was obtained in our study. However, the observed mRNA increase resulting from diabetes in rabbits is transcriptional or post-transcriptional remains to be elucidated. Therefore, nuclear run-on transcription analysis will be performed in order to enlighten these possibilities.

The levels of CYP2E1 mRNA in rabbit liver and kidney were increased significantly due to benzene. Benzene was found to stabilize CYP2E1 by inhibiting protein degradation like acetone. (Koop and Tierney, 1990). Moreover, benzene increases the content of CYP2E1 mRNA in both liver and peripheral lymphocytes detected by an ad hoc RT-PCR strategy. The levels of CYP2E1 mRNA in liver and lymphocytes were well correlated to each other in both the acute and sub-acute treatment of benzene in rats (Gonzalez-Jasso *et al.*, 2003). Whether, however, the observed mRNA increase resulting from benzene is transcriptional or post-transcriptional remains to be elucidated in rats and rabbits. Moreover, Benzene treatment of diabetic rabbits was also increase the CYP2E1 mRNA level. But, this increase was additive when compared to the separate treatments. The regulation of the CYP2E1 in benzene treated diabetic rabbits is complex that post-transcriptional and post-translational events may play a role.

We have also examined whether diabetes, benzene and benzene treatment of diabetics stimulate microsomal CYP2B4 dependent drug metabolizing enzyme activity, benzphetamine N-demethylase and its protein level in rabbit liver, kidney and lung. Benzphetamine N-demethylase activity and CYP2B4 protein level were not altered in diabetic, benzene treated and benzene treated diabetic rabbit liver, kidney and lung when compared to those of control animals (Table 4.2).

When the effect of diabetes mellitus on rat, hamster and mice cytochrome P450 and monooxgenase activities were studied, it was demonstrated that CYP2B levels were suppressed in diabetic hamster liver and kidneys (Chen *et al*., 1996) while CYP2B10 and CYP2B1 levels were increased in diabetic mouse (Sakuma *et al.*, 2001) and rat livers (Barnett *et al*., 1990; Sakuma *et al*., 2001), respectively. In contrast to what was reported for rat, hamster and mouse, the results obtained in present study and recently published studies (Arınç *et al*., 2005, 2007) showed that the level of CYP2B4 and benzphetamine N-demethylase activity were not changed in diabetic rabbit liver, kidney and lung. All of these results show that species related differences exist in diabetic rabbit liver, kidney and lung with rat, mouse and hamster in terms of CYP2B4 expression and drug metabolizing enzyme activities.

Table 4.2 Effects of diabetes, benzene and benzene treatment of diabetic rabbits on liver, kidney and lung CYP2B4 associated enzyme activity, Benzphetamine N-demethylase and CYP2B4 protein level.

Tissue		Benzphetamine N-Demethylase nmol/min/mg prot	Change (Fold)	CYP2B4 Protein Level (RPA)	Change (Fold)
LIVER	**Control**	5.54 ± 0.3	**1.0**	1640 ± 301	**1.0**
	Diabetes	6.15 ± 0.1[a]	**1.11 X ↑**	1370 ± 169[a]	**0.84 X ↓**
	Benzene	5.72 ± 0.3[a]	**1.03 X ↑**	1306 ± 37[a]	**0.79 X ↓**
	Benzene+Diabetes	6.52 ± 0.1[a]	**1.18 X ↑**	1278 ± 257[a]	**0.78 X ↓**
KIDNEY	**Control**	0.54 ± 0.08	**1.0**	1142 ± 57	**1.0**
	Diabetes	0.55 ± 0.12[a]	**1.02 X ↑**	1268 ± 122[a]	**1.11 X ↑**
	Benzene	0.54 ± 0.04[a]	**1.0**	1480 ± 113[a]	**1.30 X ↑**
	Benzene+Diabetes	0.62 ± 0.03[a]	**1.15 X ↑**	1473 ± 31[a]	**1.29 X ↑**
LUNG	**Control**	12.1 ± 0.6	**1.0**	748 ± 42	**1.0**
	Diabetes	11.3 ± 0.9[a]	**0.93 X ↓**	573 ± 74[a]	**0.77 X ↓**
	Benzene	10.3 ± 1.2[a]	**0.85 X ↓**	650 ± 50[a]	**0.87 X ↓**
	Benzene+Diabetes	12.1 ± 0.7[a]	**1.0**	560 ± 30[a]	**0.75 X ↓**

[a]Not significantly different from respective control value

As mentioned above, benzene treatments of rabbits caused no significant change in benzphetamine N-demethylase and CYP2B4 protein level in liver, kidney and lung. Essentially similar results were obtained in previous studies carried out in our laboratory (Arınç *et al*., 1988, 1991). Although western blot studies were not performed in these studies, the subcutaneous benzene treatment of rabbits caused no change in CYP2B4 associated enzyme activity in rabbit liver, kidney and lung microsomes. In contrast to what was obtained in present study and previously published studies (Arınç *et al*., 1988, 1991), benzene taken by different route, that is, inhalation, and intraperitoreal injection resulted in induction of CYP2B protein level and its associated enzyme activities in rat liver (Backes *et al*., 1993; Gut *et al*., 1993; Wang *et al*., 1996). These results show that species related differences exist in inductive response of benzene in rat and rabbit in terms of CYP2B4 expression and associated drug metabolizing enzyme activity.

Another cytochrome P450 isozyme modulated by diabetes is CYP1A. Although the effect of diabetes mellitus on rat, hamster and mice CYP1A dependent monooxygenase system has been studied extensively (Favreau and Schenkman, 1988; Schenkman *et al*., 1989; Shimojo *et al*., 1993; Chen *et al*., 1996; Raza *et al*., 2000; Sakuma *et al*., 2001), no data is available concerning the influence of diabetes on rabbit CYP1A dependent drug metabolizing enzymes, CYP1A2 protein and mRNA levels. In this study, for the first time, CYP1A dependent drug metabolism was studied in alloxan induced diabetic rabbits.

There are two members of the CYP1A family (CYP1A1 and CYP1A2) showing highly amino acid sequence identity in mammals, but they display very different patterns of tissue expression. CYP1A2 is expressed primarily in the liver. On the other hand, there are conflicting results on its low level expression in the extrahepatic tissues. In contrast, CYP1A1 is expressed primarily in extrahepatic tissues such as the lungs, lymphocytes and placenta while only low-level expression has been reported in liver tissue (Şen and Arınç, 2000; Ding and Kaminsky, 2003; Shimada *et al*., 2003; see review Ioannides and Lewis, 2004; Bièche *et al*., 2007; Özkarslı *et al*., 2008).

The present study showed that diabetes induced CYP1A protein level and increased the associated enzyme activities in the rabbit liver. Both, 7-Ethoxyresorufin–O-deethylase (EROD) and caffeine N-demethylation activities are associated with CYP1A isozymes of cytochrome P450 enzyme family in mammals. The increase of CYP1A dependent EROD (1.50-fold) and caffeine N-demethylation (1.84-fold) activities were paralleled by a similar increase (1.40-fold) in hepatic CYP1A2 protein content of these animals, as assessed on immunoblots cross-reacted with CYP1A2 (Table 4.3). The present study demonstrated, for the first time, that diabetes induced CYP1A in rabbit liver.

Hepatic mRNA level of diabetic rabbits for CYP1A2 was determined by Real-time PCR analysis in this study for the first time. As shown in Table 4.3, hepatic CYP1A2 mRNA level was increased significantly 1.81-fold in the diabetic rabbits with respect to control animals. This result is consistent with the previous observation (Kim *et al*., 2005). mRNA level for CYP1A2 was determined by Northern blot analysis in alloxan and STZ induced diabetic rats. The hepatic mRNA level of CYP1A2 increased 3.4- and 4.2-fold, in alloxan and STZ-induced diabetic rats, respectively (Kim *et al*., 2005). Binding of the ligand to Aryl hydrocarbon receptor (AHR) causes increase in mRNA synthesis. It results in elevated CYP1A protein level.

CYP1A1/CYP1A2 dependent catalytic activities and protein levels were changed due to diabetes in different species. CYP1A1/1A2 associated hepatic ethoxyresorufin O-deethylase, methoxyresorufin O-deethylase and ethoxycoumarin O-deethylase activities and hepatic CYP1A1/1A2 protein level were increased in STZ induced diabetic male and female rats (Barnett *et al*., 1993; Clarke *et al*., 1996; Raza *et al.*, 1996; Engels *et al*., 1999; Raza *et al*., 2000; Sheweita *et al*., 2002; Shankar *et al*., 2003b). There are conflicting results on the effect of diabetes in hamster (Chen *et al*., 1996; Takatori *et al*., 2002). The western-blot data showed that diabetes caused induction of the renal and hepatic protein cross reactive with P4501A1. On the other hand, Takatori and co-workers found that CYP1A protein in hamster was suppressed due to STZ induced diabetes (Takatori *et al*., 2002).

Table 4.3 Effects of diabetes, benzene and benzene treatment of diabetic rabbits on liver CYP1A associated enzyme activities, Caffeine N-demethylase and Ethoxyresorufin O-deethylase (EROD), CYP1A2 protein and mRNA levels.

Tissue		Caffeine N-Demethylase nmol/min/mg prot	Change (Fold)	EROD nmol/min/mg prot	Change (Fold)	CYP1A2 Protein Level (RPA)	Change (Fold)	CYP1A2 mRNA Level (CYP1A2/GAPDH)	Change (Fold)
LIVER	**Control**	0.05 ± 0.001	**1.0**	40.7 ± 4.8	**1.0**	1839 ± 180	**1.0**	0.21 ± 0.04	**1.0**
	Diabetes	0.092 ± 0.007[a, c]	**1.84 X ↑**	61.0 ± 11.0[b]	**1.50 X ↑**	2583 ± 134 [b]	**1.40 X ↑**	0.38 ± 0.02 [b]	**1.81 X ↑**
	Benzene	0.04 ± 0.003	**0.80 X ↓**	32.3 ± 3.2	**0.79 X ↓**	1333 ± 114	**0.73 X ↓**	0.29 ± 0.07	**1.38 X ↑**
	Benzene+Diabetes	0.053 ± 0.006	**1.06 X ↑**	41.7 ± 3.0	**1.02 X ↑**	1801 ± 266	**0.98 X ↓**	0.22 ± 0.06	**1.05 X ↑**

[a]Significantly different from the respective control value $p<0.005$.
[b]Significantly different from the respective control value $p<0.05$.
[c]Significantly different from the respective "benzene + diabetes" value $p<0.001$

Immunochemical and catalytic analyses demonstrated that the CYP1A2 was not changed in the streptozotocin induced diabetic male and female mouse (Sakuma *et al*., 2001). On the other hand, CYP1A protein in mouse liver was suppressed about 2-fold due to STZ induced diabetes (Shankar *et al*., 2003b). In the light of the data presented here and in previous works by other researchers, it is evident that a marked species-related differences exist in modulation of CYP1A level and associated enzyme activities in response to diabetes in liver.

Induction of CYP1A protein appears to be mediated by the hyperketonaemia that accompanies diabetes (Barnett *et al*., 1992). When animals treated with triacylglycerols that induce hyperketonaemia, similar changes in the CYP1A was observed as in STZ-induced diabetes (Barnett *et al*., 1988; Barnett *et al*., 1990a). The role of ketones in induction of CYP1A activities and protein levels was also supported that treatment of rats with acetone enhanced both activities and levels of these proteins (Barnett *et al*., 1992). Thus hyperketonemia could contribute to the induction of CYP1A, but other factors such as insulin and glucagon have not been examined for this enzyme (Cheng and Morgan, 2001).

Among the all cytochrome P450 isoforms, CYP1A holds the priority due to its role in metabolism of carcinogens, mutagens and environmental pollutants. Two members of this family, CYP1A1 and CYP1A2 involve in the activation of potentially mutagenic chemicals such as benzo(a)pyrene, aromatic and heterocyclic amines found in cigarette smoke and in charred foods (Adamson *et al*., 1996; Hümmerich *et al*., 2004; Kim and Guengerich, 2005; Ma and Lu, 2007).

CYP1A mostly activates certain classes of PAH pro-carcinogens and other chemicals by forming oxygenated compounds. Oxygenation of benzo(a)pyrene by CYP1A1 in the presence of epoxide hydrolase results in the formation of the ultimate carcinogen, benzo(a)pyrene 7,8 dihydrodiol 9,10-epoxide (BPDE), which forms DNA-adducts. Greater CYP1A induction may result in high levels of activated carcinogens, and consequently to higher degree of persistent DNA-adduct formation or to an enhanced oxidative DNA damage. Induction of CYP1A1 has

been correlated with the developmentof PAH-associated cancers and other disorders in mammals (Nebert, 1989, Parke et al., 1991, Stegeman, 1995 referred by Arınç *et al*., 2000). Moreover, heterocyclic amines found in charred foods are substrates for CYP1A2, which exclusively and efficiently catalyses their conversion to genotoxic hydroxylamines (Gooderham *et al*., 1996; Ma and Lu, 2007). These active metabolites preferentially form DNA adducts with guanine bases, primarily at the C-8 position (Fradsen *et al*., 1992 and Turesky *et al*., 1992 referred by Gooderham *et al*., 1996). Epidemiological studies have releaved that high levels of CYP1A2 and comsumption of diets rich in well-done meat and fish is associated with increased relative risk of developing some types of cancer especially colorectal cancer (Gerhardsson et al., 1991 and Kadlubar et al., 1992 referred by Gooderham *et al*., 1996). This present work indicates that diabetus mellitus stimulate metabolic activation of benzo(a)pyrene, aromatic and heterocyclic amines by inducing CYP1A which results in increased amounts of reactive metabolites formation. This may in turn potentiate the risk of organ toxicity, mutagenesis and malignant transformation in liver of diabetic subjects.

The effect of benzene treatment on CYP1A protein level and its associated enzyme activities were also determined in present study. Benzene caused 1.26-, 1.20- and 1.37-fold decreases in ethoxyresorufin-O-deethylase (EROD), caffeine N-demethylation and CYP1A2 protein level in liver microsomes, respectively. But these decreases were not found to be statistically significant. Different laboratories have reported conflicting results on the effect of benzene to CYP1A protein and associated enzyme activities. CYP1A1/2 protein level and associated 7-ethoxyresorufin O-deethylase activity were not changed by benzene treatment in rat liver (Pykkkö *et al*., 1987; Wang *et al*., 1996). On the other hand, rat liver CYP1A1/1A2, protein level was suppressed 1.5-fold as a result of benzene treatment (Gut *et al*., 1993). In contrary to these studies, Backes and co workers found that CYP1A protein level was induced 2-fold by benzene treatment in rat liver (Backes *et al*., 1993). These differences in CYP1A response to benzene treatment may be explained by different application routes of benzene to rats.

Exposure to benzene was caused to lower the rat cytochrome P450 content *in vivo* and *in vitro* (Souĉek *et al*., 1994; Gut *et al*., 1996). Quinonic benzene metabolites (catechol, hydroquinone, and benzoquinone) were involved in CYP450 destruction in liver microsomes from rats pre-treated with various inducers (Souĉek *et al*., 1994) and in human liver microsomes (Souĉek, 1999). The main mechanism of CYP450 destruction is covalent binding of the oxidized quinone form to protein and heme moieties of CYP450 (Gut *et al*., 1993; Souĉek, 1999). The spectrophotometric evaluations of the total CYP content, assay of CYP marker activities, and electrophoresis with immunoblotting after incubation of microsomes with quinones revealed that CYP1A and CYP3A being the most sensitive isoforms *in vitro* in rat liver and human liver microsomes (Souĉek *et al*., 1994; Souĉek, 1999). The observed decreases in CYP1A protein level and associated enzyme activities in benzene treatment may be due to quinone mediated destruction of CYP1A protein. This remains to be elucidated. Therefore, further studies will be needed.

Unlike the diabetes or benzene treatment, there was no significant change in the CYP1A dependent enzyme activities, CYP1A2 protein level and mRNA level of liver microsomes as a result of benzene treatment of diabetic rabbits (Table 4.3). It can be proposed that induction of CYP1A due to diabetes may be relieved by benzene treatment causing no change in CYP1A in benzene treated diabetic rabbits.

In addititon to CYP2E1, CYP2B4 and CYP1A isozymes, the effects of diabetes, benzene treatment and combined treatment (diabetes and benzene) on CYP3A6 associated enzyme activity, erythromycin N-demethylase and protein level were determined in rabbit liver and lung. As shown in Table 4.4, induction of diabetes by single dose of alloxan (125 mg/kg body weight) caused increase in CYP3A6 associated enzyme activity (1.85-fold) and induction CYP3A6 protein level (1.50-fold) in rabbit liver, significantly ($p<0.05$). Similarly, erythromycin N-demethylase activity and CYP3A6 protein level were increased 1.64- and 1.51-fold in rabbit lung, respectively. To best of our knowledge, these results demonstrated, for the first time, that diabetes induced CYP3A6 in rabbit liver and lung (Table 4.4).

Table 4.4 Effects of diabetes, benzene and benzene treatment of diabetic rabbits on liver and lung CYP3A6 associated enzyme activity, Erythromycin N-demethylase, CYP3A6 protein and mRNA levels.

Tissue		Erythromycin N-demethylase nmol/min/mg prot	Change (Fold)	CYP3A6 Protein Level (RPA)	Change (Fold)	CYP3A6 mRNA Level (CYP3A6/GAPDH)	Change (Fold)
LIVER	Control	0.33 ± 0.03	**1.0**	285 ± 19.7	**1.0**	5.60 ± 1.1	**1.0**
	Diabetes	0.61 ± 0.09[b, c]	**1.85 X ↑**	428 ± 24.6[b, c]	**1.5 X ↑**	75.90 ± 22.8[b]	**13.55 X ↑**
	Benzene	0.18 ± 0.01[a]	**0.55 X ↓**	190 ± 7.2[b]	**0.67 X ↓**	3.70 ± 0.7	**0.66 X ↓**
	Benzene+Diabetes	0.30 ± 0.04	**0.91 X ↓**	294 ± 32.7	**1.03 X ↑**	31.40 ± 8.4[b]	**5.61 X ↑**
LUNG	Control	0.11 ± 0.02	**1.0**	1577 ± 54.5	**1.0**	ND	--
	Diabetes	0.18 ± 0.02[b]	**1.64 X ↑**	2380 ± 90.7[a]	**1.51 X ↑**	ND	--
	Benzene	0.13 ± 0.02	**1.18 X ↑**	1496 ± 225	**0.95 X ↓**	ND	--
	Benzene+Diabetes	0.14 ± 0.02	**1.27 X ↑**	1950 ± 106	**1.23 X ↑**	ND	--

[a]Significantly different from the respective control value $p<0.005$

[b]Significantly different from the respective control value $p<0.05$.

[c]Significantly different from the respective "benzene + diabetes" value $p<0.05$

N.D. Not Determined

The effect of diabetes on the level of CYP3A6 mRNA level in rabbit liver was determined by Real-time PCR analysis in this study also. As shown in Table 4.4, relative CYP3A6 mRNA level was increased 13.55-fold in diabetic with respect to control animals, respectively ($p<0.05$). Similar result was obtained in diabetic rats carried out in 2005 (Kim *et al*., 2005). The mRNA level of CYP3A23 increased 2.2 and 1.6-fold in STZ and alloxan induced diabetic rats, respectively. The results of this study and previous study (Kim *et al*., 2005) showed that CAR and PXR mediated trasnscriptional activation of the CYP3A protein in rats and rabbits cause increase in mRNA level of this protein in liver. This may in turn induction of CYP3A protein in rat and rabbit liver.

Changes in CYP3A proteins were monitored in diabetic rats, hamsters and mouse. CYP3A expression and its associated enzyme activities were significantly increased in STZ-induced diabetic rats (Barnett *et al*., 1990b, 1993, 1994; Shimojo *et al*., 1993; Raza *et al*., 1996). On the other hand, Thummel and Schenkman reported that CYP3A protein level was decreased in male rats and increased in female rats (Thummel and Schenkman 1990). On the other hand, CYP3A protein in hamster was suppressed about 1.5-fold in STZ induced diabetes (Takatori *et al*., 2002). Different laboratories have reported either decreased or increased CYP3A protein expression following STZ treatment in mouse (Sakuma *et al*., 2001; Shankar *et al*., 2003b). Hepatic CYP3A dependent catalytic activity, erythromycin *N*-demethylase, was significantly higher in the streptozotocin-induced diabetic mouse of both sexes than in the respective control group. In contrast to the microsomal enzyme activity, there was no clear increase in CYP3A11 related protein band in diabetic mouse. Moreover, when mouse CYP3A subfamily mRNA levels (3A11, 13, 16, and 41) were analysed, only CYP3A11 showed induction in the livers of the streptozotocin-induced diabetic ddY, whereas the other three isoforms were not altered (Sakuma *et al.,* 2001) In another study, Shankar and coworkers found that CYP3A related protein was decreased about 3-fold in diabetic mouse (Shankar *et al*., 2003b). In the light of the data presented here and in previous works by other researchers, it is evident that a marked species-related

differences exist in modulation of CYP3A level and associated enzyme activities in response to diabetes in liver.

The effect of benzene treatment on CYP3A6 protein level and its associated enzyme activity, erythromycin N-demethylase, were also determined in present study. Unlike diabetes, benzene treatment caused suppression of CYP3A6 protein (1.50-fold) and inhibition of its associated erythromycin N-demethylase activity (1.83-fold) in rabbit liver (Table 4.4). Besides enzyme activity and protein level, the effect of benzene on CYP3A6 mRNA level was determined in this study. Benzene treatment was caused 1.51-fold reduction of CYP3A6 mRNA level but this decrease was not found statistically significant (Table 4.4). On the other hand, upon benzene treatment of rabbits, erythromycin N-demethylase activity and CYP3A6 protein level of pulmonary microsomes did not change significantly (Table 4.4). Conflicting results were reported about the effect of benzene on CYP3A in rats (Gut *et al*., 1993; Wang *et al*., 1996). CYP3A1 protein level was not changed as a result of benzene treatment (Wang *et al*., 1996). There was no agreement with report in which CYP3A1 was suppressed (Gut *et al*., 1993). This was explained by different exposure protocols being applied in these two studies (Wang *et al*., 1996).

As mentioned above, exposure to benzene was caused to lower the cytochrome P450 content in vivo and in vitro (Souček *et al*., 1994; Gut *et al*., 1996; Souček, 1999). Quinonic benzene metabolites (catechol, hydroquinone, and benzoquinone) were involved in CYP450 destruction (Souček *et al*., 1994; Souček, 1999). The spectrophotometric evaluations of the total CYP content, assay of CYP marker activities, and electrophoresis with immunoblotting after incubation of microsomes with quinones revealed that CYP1A and CYP3A being the most sensitive isoforms in vitro (Souček, 1999). Our results and previous results (Wang *et al*., 1996) showed that CYP3A may be subjected to quinone mediated destruction in benzene treated rabbits and rats. Decreases in CYP3A protein level and associated enzyme activities due to benzene treatment remain to be elucidated. Therefore, further studies will be needed.

The CYP3A4 (CYP3A6 in rabbit) is known to be one of the most important enzyme in drug metabolism due to its impact on human pharmacotherapy. Because, it is the most abundantly expressed CYPs in the human liver and small intestine and possesses metabolic activity toward an extremely broad spectrum of xenobiotic substrates; CYP3A4 is known to metabolize more than 50% of all known therapeutic drugs, as well as many endogenous compounds in humans. Cytochrome CYP3A4 is known to metabolize more than 120 different drugs include macrolide antibiotics, anti-arrhythmics, sedatives, calcium channel blockers, HMG CoA reductase inhibitors and opioid analgesic (Martin and Krum, 2003; Arayne *et al*., 2005; van Herwaarden *et al*., 2005; Sica, 2006; Sugimoto *et al*., 2006; de Wildt *et al* 2007; Klotz, 2007). Therefore in diabetic and benzene exposed subjects, alteration of drug clearance and clinical drug toxicity may be observed due to induction or suppression of CYP3A6. The results of these analyses will provide insight into the prescription of drugs for diabetic and benzene exposed subjects.

It is well established that many drugs were prescribed for treatment and control of chronic complications of the diabetes. Troglitazone is an antidiabetic drug that cause severe and in some cases fatal hepatotoxicity in a number of diabetic patients. As a result of determination of hepatotoxic events related to this drug, it was withdrawn from the British and U.S. markets (Wang *et al*., 2007). It was shown that its metabolites bind covalently to microsomal protein and glutathione (GSH) following activation by cytochrome P450 isozymes (He et al., 2004). CYP3A4 and CYP2C8 are the major Cytochrome P450 isozymes involved in troglitazone metabolism. It is also inducer of the CYP3A4 (Li *et al*., 2002; Masubuschi, 2006). Induction of the CYP3A by not only diabetes but also by troglitazone may increase the hepatotoxicity of the drug and other members of the thiazolidinediones such as pioglitazone and rosiglitazone. Moreover, Acetaminophen, one of the most widely used analgesics, undergoing oxidative metabolism by CYP isozymes, CYP2E1, CYP1A2 and CYP3A (Raucy *et al*., 1989; Thummel *et al.*, 1993; Snawder *et al*., 1994). *N*-acetyl-*p*-benzoquinoneimine is the reactive product of cytochrome P450 dependent oxidative metabolism of acetaminophen. It is normally detoxified by conjugating with reduced GSH or binds

to hepatic macromolecules covalently (see in reviews Gonzalez, 2005 and David Josephy, 2006). Induction of the CYP3A by diabetes may cause formation of more reactive metabolites of acetaminophen by inducing CYP2E1, CYP1A and CYP3A. Thus, it is expected that, in diabetic subjects, the risk of acetaminophen induced hepatotoxicity and carcinogenecity may be greater in liver when compared to non-diabetics.

Unlike the diabetes and benzene treatment, there was no significant change in the CYP3A6 dependent enzyme activity and its protein level of liver and lung microsomes as a result of benzene treatment of diabetic rabbits. However, as shown in Table 4.4, benzene treatment of diabetic rabbits caused induction of the CYP3A6 mRNA level in liver (5.61-fold). It can be proposed that mRNA in these rabbits may not be translated as much as diabetic rabbit or induction of CYP3A6 by diabetes may be relieved by benzene treatment. As a result of benzene metabolism, quinones are formed and these reactive quinones destroy CYP3A6's heme or apoprotein structure. This causes no change in CYP3A6 in benzene treated diabetic rabbits.

The effects of diabetes, benzene and benzene treatment of diabetic rabbits on Glutathione S-Transferase isozymes and catalase were determined throughout this study, also. As can be seen in Table 3.16, there were no significant changes in activities both total GST and GST isozymes (Mu, Pi and Theta) in liver cytosols. On the other hand, catalase activity was increased 1.46-fold with respect to control animals activity ($p<0.05$). Upon benzene treatment of rabbits, catalase enzyme activity of liver cytosols was increased 1.40-fold; this increase was not found to be statistically significant. Conflicting results were obtained in the effect of diabetes on GST activity. These results showed that GST activities were reported both increased and decreased in alloxan- or STZinduced diabetic rats (See the rewiev, Wang *et al*., 2007). The results obtained in STZ induced diabetic mouse indicated that GST activities were increased (Rouer *et al*., 1981 and Mukherjee *et al*., 1998 referred by review Wang *et al*., 2007). The present study and these studies showed that there is species related differences in the regulation of the GST activities in diabetes.

Moreover, contradictory results were obtained about the effect of diabetes on catalase activity in rabbit, rat and mouse. These results showed that catalase activity decreased or increased due to diabetes in rabbits, rats and mouse (Maritim *et al*., 2002; Gumieniczek, 2005; Düzgüner and Kaya, 2007; Panda and Kar, 2007; Sepici Dinçel *et al*., 2007; Sadi *et al*., 2008).

CHAPTER 5

CONCLUSION

In summary, in the present study, we demonstrated, for the first time, that the influence of benzene on liver, kidney and lung microsomal cytochrome P450 dependent drug metabolizing enzyme activities, protein levels and mRNA levels in diabetic and control rabbits and on the biomarkers used to measure chemical-induced toxicity including Lactate Dehyrogenase (LDH), Aspartate Aminotransferase (AST) and Alanine Aminotransferase (ALT) and on some oxidative stress markers in rabbit liver were determined.

In this study, diabetes was induced in rabbits experimentally by intravenous injection of 125 mg/kg alloxan. Induction of diabetus mellitus by a single dose of alloxan was confirmed by 3.7-fold increase in the blood glucose level. Lipid peroxidation in diabetic, benzene treated and benzene treated diabetic rabbits was increased 1.75-, 1.50- and 1.58-fold in liver microsomes, respectively. Moreover, LDH activity was increased about 2-fold in serum of diabetic, benzene treated and benzene treated diabetic rabbits. Similarly, AST and ALT activities were increased significantly between 1.3- to 1.8-fold in serum of diabetic, benzene treated and benzene treated diabetic rabbits with respect to controls.

CYP2E1 dependent NDMA N-demethylase and *p*-nitrophenol hydroxylase activities were increase 2.0- and 1.73-fold in diabetic liver. These were paralleled by a similar increase (2.65-fold) in hepatic CYP2E1 protein content of these animals, as assessed on immunoblots cross-reacted with anti-rabbit CYP2E1. The

activities of NDMA N-demethylase and *p*-nitrophenol hydroxylase and CYP2E1 protein level were increased 1.60-, 2.41- and 2.50-fold diabetic rabbit kidney, respectively. In addition to liver and kidney, CYP2E1 dependent enzyme activities, NDMA N-demethylase and *p*-nitrophenol hydroxylase, and CYP2E1 protein level were increased 1.66-, 1.50- and 1.77-fold in the lung of diabetic rabbits, respectively. Moreover, benzene caused 3.15-, 2.37- and 3.85-fold increases in NDMA N-demethylase, p-nitrophenol hydroxylase activities and CYP2E1 protein level in liver microsomes, respectively. Benzene treatment also enhanced NDMA N-demethylase, p-nitrophenol hydroxylase activities and CYP2E1 protein level of kidney microsomes by 2.20-, 3.0- and 2.8-fold. In contrast to liver and kidney, benzene treatment caused no significant changes in CYP2E1 protein level and its associated enzyme activities of lung microsomes. The combined effect of diabetes and benzene on CYP2E1 associated enzyme activities and protein level was also studied in this work. This is the most prominent part of this study. NDMA N-demethylase, p-nitrophenol hydroxylase activities and CYP2E1 protein level of liver microsomes increased by 4.56-, 3.83- and 7.70-fold in benzene treated diabetic rabbits, respectively. Similar trend was observed in kidney microsomes. In contrast to liver and kidney, benzene treatment caused no significant changes in CYP2E1 protein level and its associated enzyme activities of lung microsomes. The content of liver and kidney CYP2E1 and catalytic activities in the benzene treated diabetic rabbits appeared to be additive of the diabetic and benzene group. The effects of diabetes, benzene, benzene treatment of diabetics on mRNA level of CYP450 isozymes were determined in this study by using real time PCR technique. Accordingly, liver CYP2E1 mRNA level in diabetic, benzene treated and benzene treated diabetic rabbit was increased 6.71-, 10.53- and 12.93-fold with respect to control animals, respectively ($p<0.05$). Moreover, relative kidney CYP2E1 mRNA level was increased 2.64-, 2.91 and 3.91-fold in diabetic, benzene treated and benzene treated rabbits with respect to control animals, respectively ($p<0.05$).

We also examined whether diabetes, benzene and benzene treatment of diabetics effect microsomal CYP2B4 dependent drug metabolizing enzyme activity, benzphetamine N-demethylase and its protein level in rabbit liver, kidney and lung.

Benzphetamine N-demethylase activity and CYP2B4 protein level were not altered in diabetic, benzene treated and benzene treated diabetic rabbit liver, kidney and lung when compared to those of control animals.

The present study showed that diabetes induced CYP1A protein level and increased the associated enzyme activities in the rabbit liver. The increase of CYP1A dependent EROD (1.50-fold) and caffeine N-demethylation (1.84-fold) activities were paralleled by a similar increase (1.40-fold) in hepatic CYP1A2 protein content of these animals, as assessed on immunoblots cross-reacted with CYP1A2. Hepatic mRNA level of diabetic rabbits for CYP1A2 was determined by Real-time PCR analysis in this study for the first time. Hepatic CYP1A2 mRNA level was increased significantly 1.81-fold in the diabetic rabbits with respect to control animals. The present study demonstrated, for the first time, that diabetes induced CYP1A in rabbit liver The effect of benzene treatment on CYP1A protein level and its associated enzyme activities were also determined in present study. Benzene caused statistically insignificant decreases in ethoxyresorufin–O-deethylase (EROD), caffeine N-demethylation and CYP1A2 protein level in liver microsomes. Unlike the diabetes and benzene treatment, there was no significant change in the CYP1A dependent enzyme activities, CYP1A2 protein level and mRNA level of liver microsomes as a result of benzene treatment of diabetic rabbits.

In addititon to CYP2E1, CYP2B4 and CYP1A isozymes, the effects of diabetes, benzene treatment and combined treatment (diabetes and benzene) on CYP3A6 associated enzyme activity, erythromycin N-demethylase and protein level were determined in rabbit liver and lung. Induction of diabetes by single dose of alloxan caused increase in CYP3A6 associated enzyme activity (1.85-fold) and induction CYP3A6 protein level (1.50-fold) in rabbit liver, significantly ($p<0.05$). Similarly, erythromycin N-demethylase activity and CYP3A6 protein level were increased 1.64- and 1.51-fold in rabbit lung, respectively. To best of our knowledge, these results demonstrated, for the first time, that diabetes induced CYP3A6 in rabbit liver and lung. Relative liver CYP3A6 mRNA level was increased 13.55-fold

in diabetic with respect to control animals, respectively ($p<0.05$). Unlike diabetes, benzene treatment caused suppression of CYP3A6 protein (1.50-fold) and inhibition of its associated erythromycin N-demethylase activity (1.83-fold) in rabbit liver. Besides these, benzene treatment was caused 1.51-fold reduction of CYP3A6 mRNA level but this decrease was not found statistically significant. On the other hand, upon benzene treatment of rabbits, erythromycin N-demethylase activity and CYP3A6 protein level of pulmonary microsomes did not change significantly.

The effects of diabetes, benzene and benzene treatment of diabetic rabbits on Glutathione S-Transferase isozymes and catalase were determined throughout this study, also. There were no significant changes in activities both total GST and GST isozymes (Mu, Pi and Theta) in liver cytosols. On the other hand, catalase activity was increased 1.46-fold with respect to control animals activity ($p<0.05$). Upon benzene treatment of rabbits, catalase enzyme activity of liver cytosols was increased 1.40-fold; this increase was not found to be statistically significant.

The results of the present work indicate that both diabetus mellitus and benzene exposure stimulate metabolic activation toxic chemicals metabolized by CYP2E1 such as *N*-nitrosodimethylamine, *p*-nitrophenol, benzene by inducing CYP2E1 which results in increased amounts of reactive metabolites formation. Application of benzene to the diabetic rabbits further elevates expression and activities of the CYP2E1. As a result of additive induction of the CYP2E1 by two situations (diabetes and benzene treatment), further increase the risk of hepatotocixity produced by toxins may be observed when compared to the separate treatments. This may in turn further potentiate the risk of organ toxicity, mutagenesis and malignant transformation in liver and kidney of these subjects. As in the case of CYP2E1, the risk of carcinogenesis due to benzo(a)pyrene, aromatic and heterocyclic amines may be increased due to induction of CYP1A in diabetic subjects. Moreover in diabetic and benzene exposed subjects, alteration of drug clearance and clinical drug toxicity may be observed due to induction or suppression of CYP3A.

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PUBLICATIONS

1. MSc Thesis

Arslan S. (2003) Cytochrome P450 dependent drug metabolism in diabetic rabbits. *MS. Thesis.* Middle East Technical University, Ankara.

2. Articles in Science Citation Index

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