Available online on www.ijtpr.com
International Journal of Toxicological and Pharmacological Research 2014; 6(4): 86-96

ISSN: 0975-5160

Review Article

A Review on Mechanism of Nitrosamine Formation, Metabolism and Toxicity in *In Vivo*

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ABSTRACT

Nitrosamines, an important class of carcinogens, are widely classified into volatile and tobacco specific compounds that contain various nicotine, nornicotine, anabasine, anatabine derived nitrosamines and also those that are specially found in smoke. Nitrosamines are formed via various nitrosating agents and also formed endogenously through pyrrolidine. During nitrosamine metabolism, several intermediates are formed that may be directly bind with DNA, alkylate them or react with another compound to form alkylating agents that may cause mutation and cancer. Literature reported that cytochrome (CYP) P450 subfamilies are involved in nitrosamines metabolism that catalyze specifically alpha but sometimes beta hydroxylation of nitrosamine to form hydroxylated product. Reports suggest the specific relationship between cytochrome and nitrosamine metabolism. Specific CYP 4502A members express in specific organs of body where nitrosamine metabolism takes place. To enlightened the complete background of nitrosamine formation, its metabolism and toxic mechanism to induce cancer or related obliterations in DNA to cause mutations in *In vivo* conditions. Data were collected from different books, internet and various reputed journals. Figures were drawn with the help of ChemDraw ultra 9.0 on the basis of previously published reports with some modifications. This review concluded that obliterations in cytochrome subfamilies esp. CYP P450 may lead to abnormal metabolic pathway of nitrosamines thus may responsible for severe health problems. This review provides a complete platform to understand the mechanism of nitrosamine toxicity in living beings thus may help in innovation of target specific drug against cancer or other cell obliterations.

Keywords: N-nitrosopyrrolidine, 4-(N-methyl-N-nitrosamine)-1-(3-pyridyl)-1-butanone, Cytochrome, Nitosating agents, alpha hydroxylation.

INTRODUCTION

N-Nitrosamines are a class of chemical compounds with the general structure $R^1N(-R^2)N=O$ (Figure 1). The essential feature of *N*-nitroso compounds is the N–N=O structure; the R1 and R2 groups attached to the amine nitrogen may range from a simple hydrogen (H) atom to more complex chemical substituents (including ring structures that incorporate the nitrogen atom) and may be alkyl or aryl groups[1]. Broadly, nitrosamines are classified into 2 categories. One category includes volatile nitrosamines that are found in large quantities in side stream smoke but not in tobacco. These are dimethylnitrosamine (DMN), nitrosopyrrolidine (NPYR), diethylnitrosamine (DEN) and methylethylnitrosamine (MEN)[2]. Another category is tobacco specific nitrosamines that found in various tobacco products and derived from tobacco alkaloids. Today, approximate 7 tobacco specific nitrosamines are determined and quantified. These are 4-(N-methyl-N-nitrosamine)-1-(3-pyridyl)-1-butanone (NNK) and its metabolic reduction product 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL), N-nitro-sonornicotine (NNN), iso-NNAL, iso-NNAC (4-(methylnitrosamino)-4-(3-pyridyl)butyric acid), nitrosoanabsine (NAB) and nitrosoanatabine (NAT)[3]. The detailed classification of nitrosamines, their structures and carcinogenicity are given in table 1.

Formation of Nitrosamines: Basically, N-nitrosamines are formed by an electrophilic substitution of secondary or tertiary amines, hydroxylamine and amine peroxides with a nitrosating agent (Figure 2) that can be derived from nitrates (NO_2-, NO_3-) and nitro compounds (-C-NO_2)[21,22]. It is well understood that nitrosonium cation that derived from above mentioned nitrosating agents attacks on a pair of free electrons on the amine nitrogen that results in the formation of n-nitrosoammonium cation plus nitrite cation. Basically, nitrosoammonium cation primarily derived from primary amines and further converted into another forms depends on environmental surroundings like pH, alkalinity of amines and temperature[23, 24].

Nitrosamines exposure in living beings: The nitrosating agent nitrous anhydride is usually used in food materials that are formed from nitrite in acidic or aqueous solution.

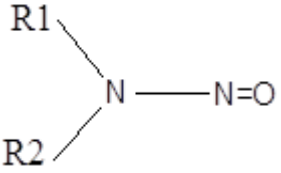

Fig. 1: General structure of nitrosamine

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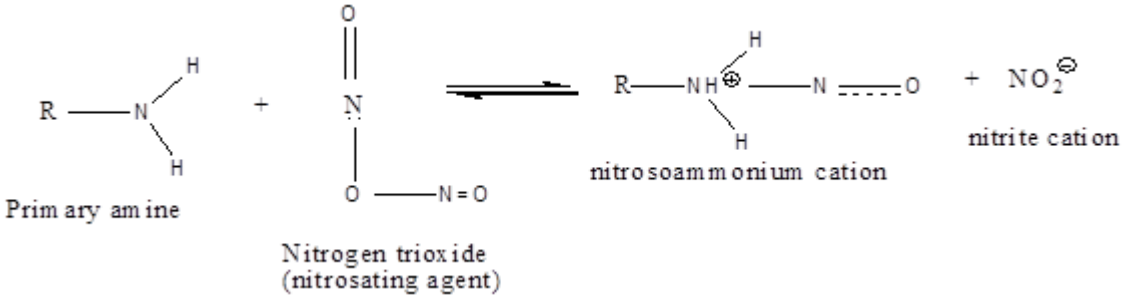


Fig. 2: Reaction involved in formation of nitrosamines from primary amine and nitrosating agent.

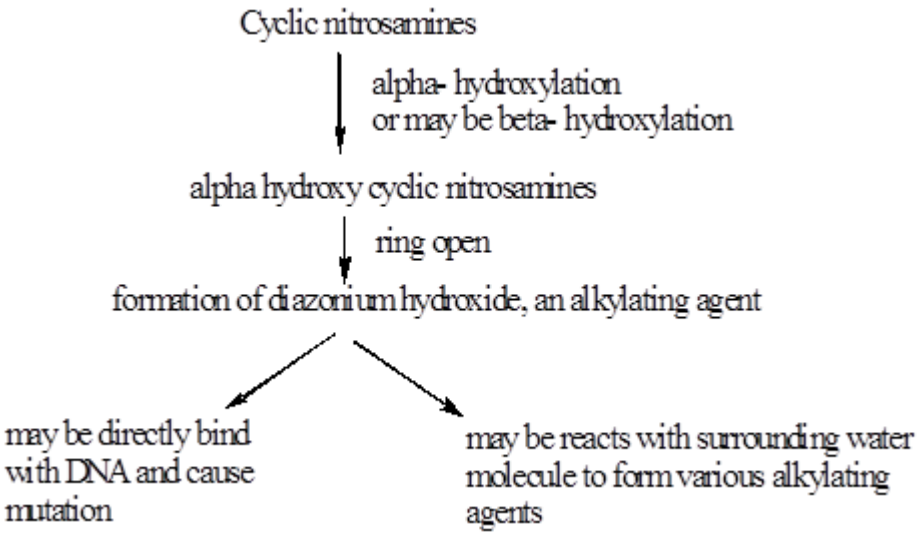


Fig. 3: General layout of adduct formation and metabolism of cyclic nitrosamines.

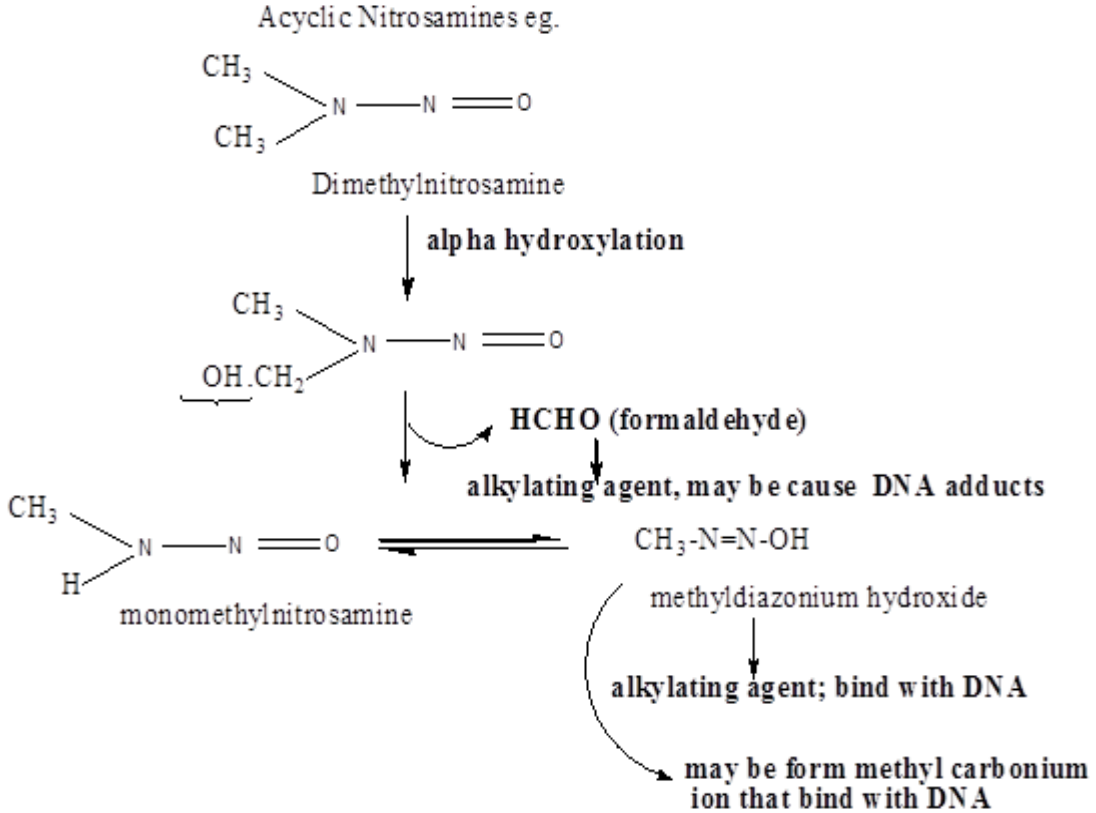


Fig. 4: General layout of adduct formation and metabolism of acyclic nitrosamine (e.g. Dimethylnitrosamine) (Lai and Arcos, 1980 with slight modification).

Foods that contain volatile nitrosamines include cured meats, primarily cooked bacon; beer; some cheeses; nonfat dry milk; and sometimes fishes. The volatile nitrosamine n-nitrosodimethylamine (NDEA) occurs to a major extent as nitrosopyrrolidine (Npyr) in food stuffs. It is estimated by several researchers that the average daily intake of volatile nitrosamines from foods is approximately 1 microgram/person[25]. High temperatures, as in frying, can also enhance the formation of nitrosamines. Nitrosamines can also be found in tobacco smoke, American dip snuff and to a much lesser degree, snus. (127.9 PPM for American dip snuff compared to 2.8 PPM in Swedish snuff or snus[26]. Sodium nitrite is used Table 1: A detailed classification of various nitrosamines, their molecular structure, formula, weight and carcinogenicity

basically in curing process of meat that is responsible for the formation of nitrosamines[27]**.** Ascorbic acid and sulphur dioxide are basically used to prevent the formation of nitrosamines[25]**.** Nitrosamines occurrence is a foremost concern in the emerging field of industries. Occupational safety and Health administration (OSHA) reported the occurrence of various amines in rubber, cork, linoleum and plastics products, act as major nitrosating agents thus helps in the formation of various types of toxic and carcinogenic nitrosamines. These amines are nitrosated with NO (nitric oxide) that are present in air via mixing, milling, extrusion, molding and curing processes. The target organs are liver, eyes, skin, kidney, lungs etc. One survey reported the level of NDMA, NDEA and other nitrosamines in various rubber and tire industries is approximate 50 µg nitrosamine/day. Due to the mixing of industrial waste products into rivers etc., presently ground water becomes also got crippled with nitrosamines contamination which is a somber problem that needs attention of people to reduce the risk of nitrosamine toxicity and cancer.

Metabolism Of Nitrosamines: Various reports suggested that nitrosamines like NPYR, NNN are carcinogenic to various laboratory animals thus possibly to humans because compounds are metabolized in a similar way in human as studied in animal tissues thus may be responsible to induce hepatocellular carcinoma[28,29,30,31,32,33]. So, it is important to understand the metabolism of nitrosamine compounds.

Generally, the metabolism of cyclic nitrosamines takes place via the α-hydroxylation of toxic substances. However, sometimes β-hydroxylation also has been seen in cyclic nitrosamines but the mechanism is unknown. α-hydroxylation of cyclic nitrosamines are catalyzed by cytochrome P450 or CYP-450 initiated monooxygenase reaction is as follows-

$$RH + O_2 + NADPH + H \longrightarrow ROH + H_2O + NADP^+$$

In this reaction, one O-atom is transferred into organic substrate. After the activation of nitrosamines, some alkylating agents like diazohydroxide and carbonium ions are formed that directly bind with the DNA or may generate a cascade of electrophiles that further reacts or bind with DNA and produce DNA adducts (Figure 3). For volatile or acyclic nitrosamines, HCHO (formaldehyde) may acts as an alkylating agent and produce DNA adducts[34] (Figure 4). It was previously reported that nitrosamines have good capability to cross the biological membranes thus easily enters and metabolized in tissues, thus damage the DNA. Various reports suggested that liver is very sensitive to metabolize the nitrosamines thus has more chance to cause damage and results in mutations[35].

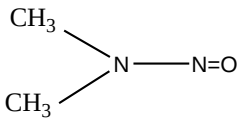
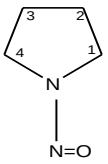
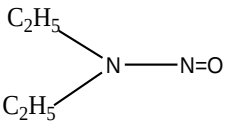
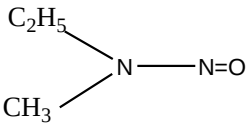
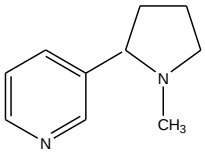
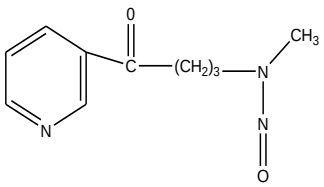
Metabolism of Tobacco specific nitrosamines: Some tobacco specific nitrosamines like NNN (N'-Nitrosonornicotine) is found most abundantly in tobacco and is very harmful for the human health. Previous reports suggest that NNN metabolic activation requires cytochrome P450 to catalyze α-hydroxylation at 2' and 5' carbon atoms. This hydroxylation gives rise to 2' and 5' hydroxy-NNN, very unstable intermediates. These intermediates cation results acyclic electrophilic derivatives (rings open) i.e. diazohydroxides. These derivatives may react with water molecule to form DNA alkylating agents i.e. 4-hydroxy-1-(3-pyridyl)-1-butanone and 5-(3-pyridyl)-2-hydroxytetrahydrofuran. These alkylating agents alkylate the DNA and generate adducts[32] (Figure 5).

Metabolism of Volatile nitrosamines (NPYR metabolism Pathway): This class of nitrosamines is also a very potent carcinogenic agent (eg. NPYR) which is responsible for tumor formation and several types of adducts formation in DNA. NPYR like NNN also require cytochrome P450 for its metabolic activation but the difference is that here the α-hydroxylation can only be done at 5'-carbon not on the 2' as a result of this unstable α-hydroxy NPYR is formed i.e. 4-oxobutanediazohydoxide which is a highly reactive intermediate compound formed in NPYR metabolism[36,37,38,39,40,41,42,43,44]. This electrophilic key intermediate may react to the surrounding water molecule or may directly generate a cyclic oxonium ion that further results in 2-hydroxytetrahydrofuran (2-OH-THF) and crotonaldehyde[40,42,45]. Both compounds have the capability to alkylate the DNA and also responsible for various adducts formation in DNA thus cause mutation or damage DNA. A report clearly indicates the formation of dThd, dAdo and dGuo in liver DNA of NPYR toxicated rats[45].

The possible mechanism of NPYR metabolism was also reported[39]. Author stated that the major product of NPYR metabolism, 2-OH-THF is found transitionally in tissue environment and may further convert into 3 different forms: butyrolactone, 4-hydroxybutanol and 1, 4-butanediol. All these forms are converted into a major and important compound knows as γ-hydroxybutyrate. This compound occurs in minute quantity in mammalian brain tissues and very rapidly metabolized via β-oxidation to glycolate and acetate and also via succinate[46,47]. Butyrolacone is converted into γ-hydroxybutyrate through an important liver enzyme lactonase. 1,4-butanediol is also a very important compound that preferably found in brain tissues (Figure 6).

Dna Adducts Formation Through Various Nitrosamines**:** The basic feature of nitrosamines to damage DNA is to cause various DNA adducts or defects in nucleotide base

Table 1: A detailed classification of various nitrosamines, their molecular structure, formula, weight and carcinogenicity

S. No.	Name and type of nitrosamine	Molecular structure of nitrosamines	Molecular formula and weight	Toxicity or Carcino-genicity/ experimental animal	References
1.	VOLATILE SPECIFIC NITROSAMINES				
	DMN		$C_2H_6N_2O$ 74.1	Liver is mainly affected (rats)	www.osha.gov
b.	NPYR		$C_4H_8N_2O$ 100.119	Liver (rats) and lungs (mice)	www.osha.gov
c.	DEN		$C_4H_{10}N_2O$ 102.14	Nasal cavity, trachea, lung, esophagus and liver in all animals	www.sigmaaldrich.com
d.	MEN		$C_3H_8N_2O$ 88.11	Interact with DNA and form adducts. Possible carcinogenic to humans. Tumorigenic property in liver, lungs, thorax and respiration (rats) Mutagenic property in Bacterial cells i.e. *S. typhimurium*; *E. coli.*	www.thsci.com, Lai et al., 1980; IARC CNREA8 Cancer Research, 1986 CRNGDP Carcinogenesis, 1984 MUREAV Mutation Research, 1981
2.	TOBACCO SPECIFIC NITROSAMINES				
a.	Nicotine derived nitrosamine				
I.	NNK		$C_{10}H_{13}N_3O2$ 207.23	Cause esp. mutations in gene that involved in cell growth, proliferation and differentiation; lung and transplacental carcinogen	www.wikipedia.com Hecht, 2002; Benowitz, 1998

Abbreviations: Dimethylnitrosamine (DMN), Nitrosopyrrolidine (NPYR), Diethylnitrosamine (DEN), Methylethylnitrosamine (MEN), 4-(N-methyl-N-nitrosamine)-1-(3-pyridyl)-1-butanone (NNK), 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL), N'-nitrosonornicotine (NNN), iso-NNAL, iso-NNAC (4-(methylnitrosamino)-4-(3-pyridyl)butyric acid), nitrosoanabsine (NAB) and nitrosoanatabine (NAT)

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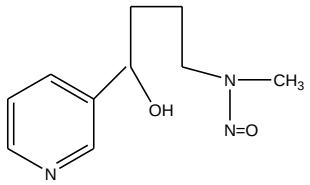
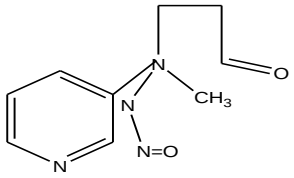
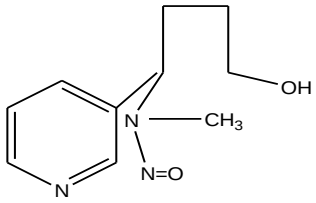
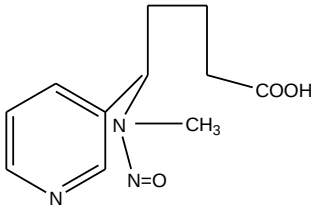
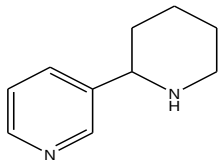
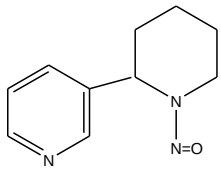
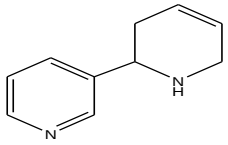
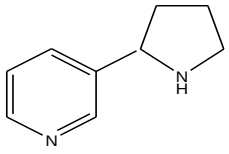
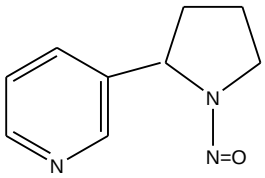
II.	NNAL (metabolic reaction product of NNK)			Potent pulmonary carcinogen (rats)	Hecht, 1998
III.	NNA			No mutagenic and carcinogenic activity shown in literature	IARC, 1985
IIIa.	Iso-NNAL			No strong evidence for its carcinogenic activity	Benowitz, 1998
IIIb.	Iso-NNAC			No carcinogenic activity	Benowitz, 1998
IV.	Anabasine derived nitrosamine				
	NAB		$C_{10}H_{13}N_3O$ 191.229	Cause tumors in esophagus	Boyland et al., 1964
V.	Anatabine derived nitrosamine		$C_{10}H_{11}N_3O$ 189.21	Little or no carcinogenic activity and mutation	Hoffman et al., 1984; Padma et al., 1989
	NAT				
VI.	Nornicotine derived nornicotine structure				

Abbreviations: Dimethylnitrosamine (DMN), Nitrosopyrrolidine (NPYR), Diethylnitrosamine (DEN), Methylethylnitrosamine (MEN), 4-(N-methyl-N-nitrosamine)-1-(3-pyridyl)-1-butanone (NNK), 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL), N'-nitrosonornicotine (NNN), iso-NNAL, iso-NNAC (4-(methylnitrosamino)-4-(3-pyridyl)butyric acid), nitrosoanabsine (NAB) and nitrosoanatabine (NAT)

Table 1: A detailed classification of various nitrosamines, their molecular structure, formula, weight and carcinogenicity

NNN		$C_9H_{11}N_3O$ 177.203	Cause cancer when exposure is long term	www.wikipedia.com

Abbreviations: Dimethylnitrosamine (DMN), Nitrosopyrrolidine (NPYR), Diethylnitrosamine (DEN), Methylethylnitrosamine (MEN), 4-(N-methyl-N-nitrosamine)-1-(3-pyridyl)-1-butanone (NNK), 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL), N'-nitrosonornicotine (NNN), iso-NNAL, iso-NNAC (4-(methylnitrosamino)-4-(3-pyridyl)butyric acid), nitrosoanabsine (NAB) and nitrosoanatabine (NAT)

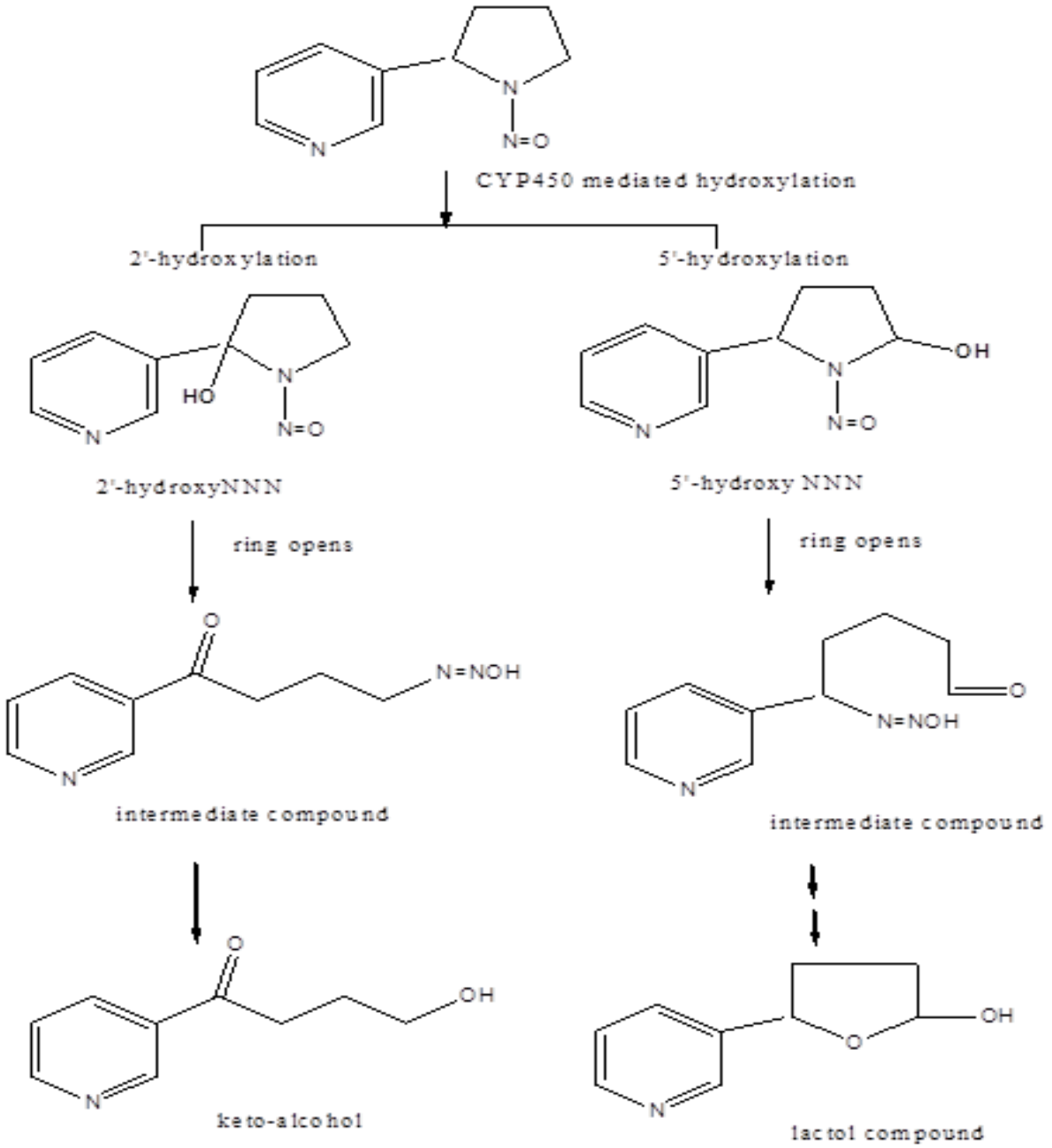


Fig. 5: Metabolism of Tobacco specific nitrosamine (NNN: N'-Nitrosonornicotine (Hansen et al., 2005 with minor modifications).

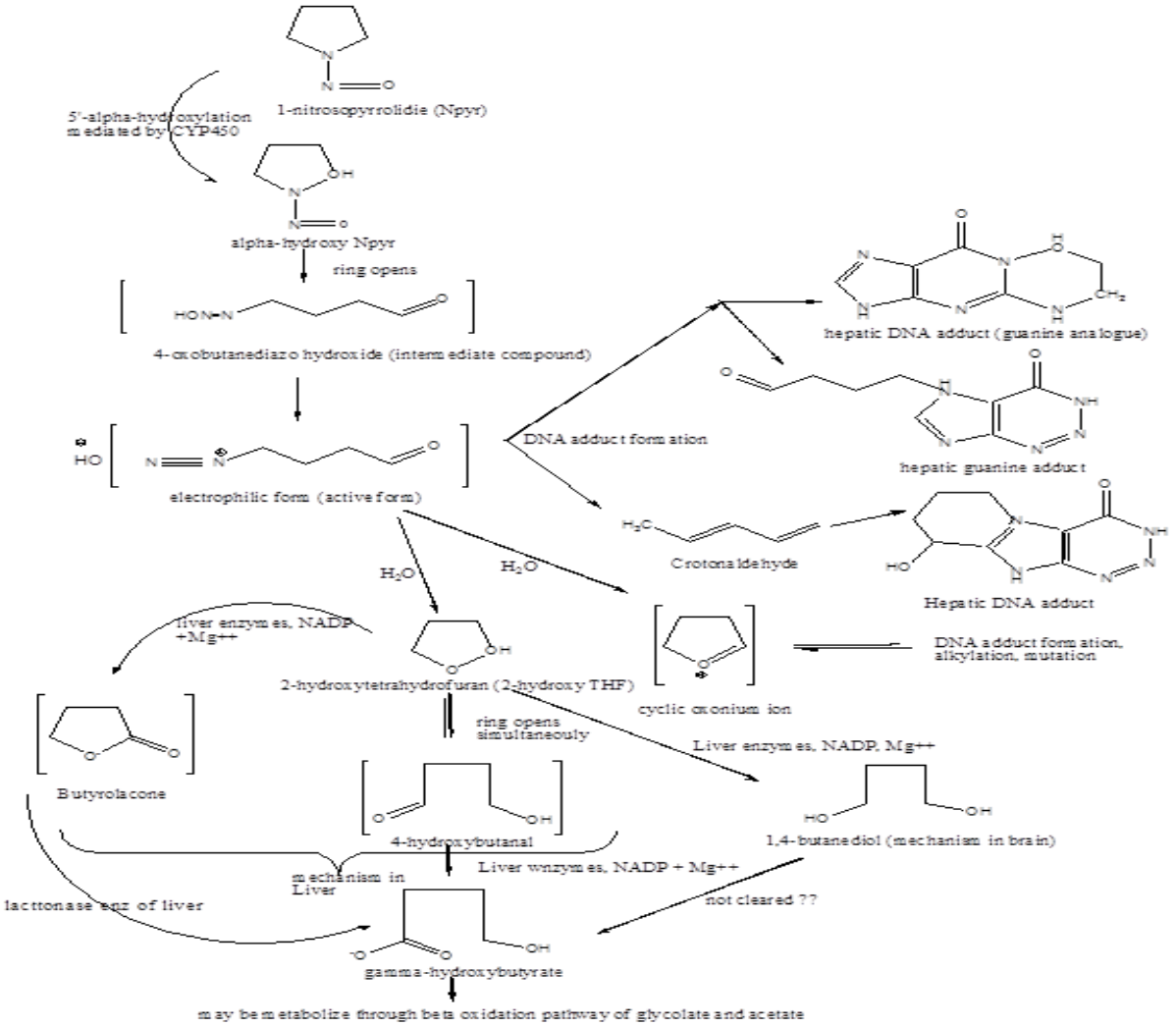


Fig. 6: A detailed mechanism of metabolism and adduct formation of Npyr (N-nitrosopyrrolidine), a volatile environmental carcinogenic nitrosamine (Hecker et al., 1979; Wang et al., 2007 with minor changes).

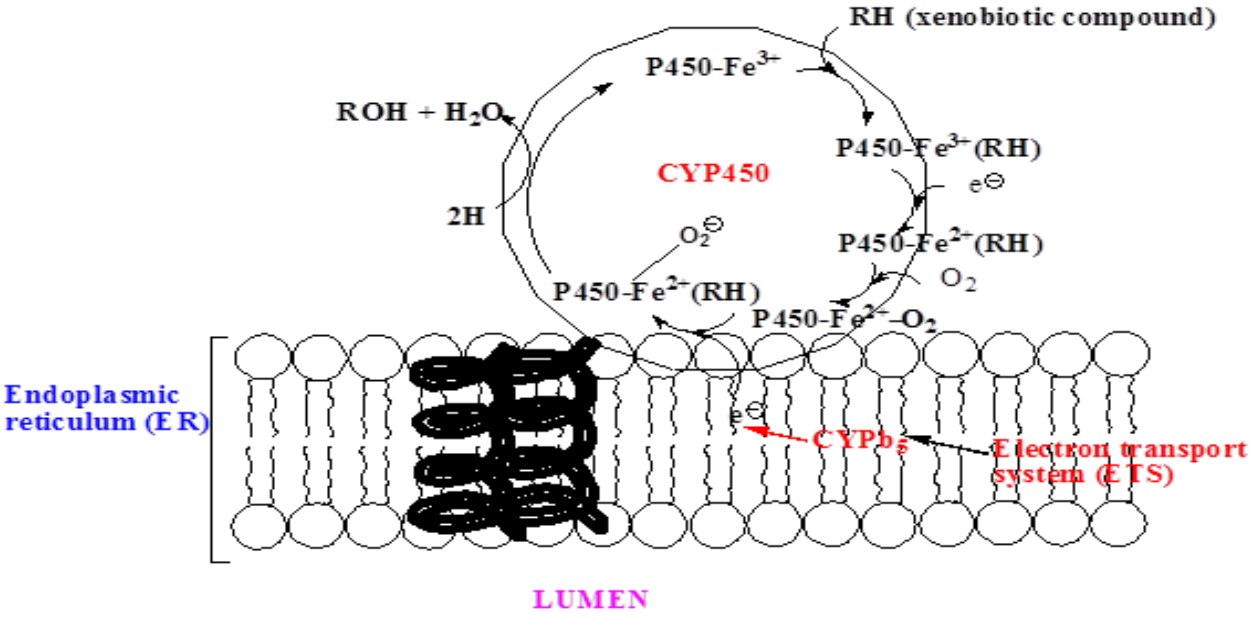


Fig. 7: Localization and Mechanism of hydroxylation of nitrosamine mediated by Cytochrome P450 (Zakrzewski, 1995with slight modifications).

Table 2: Expression of mRNA and Protein of Cytochrome P4502A subfamily in various organs of Rats, Mice and Human

S. No.	CYP2A family components	mRNA expression	Protein expression	Organs where CYPs expressed	References
1.	CYP2A3	yes	yes	Nasal mucosa and lungs of rats	Gopalkrishnan et al., 1999
2.	CYP2A4 and CYP2A5	yes	yes	Nasal mucosa, liver, lung and kidney of mice	Su et al., 1996
3.	CYP2A13		yes	Fetal nasal mucosa	Wong et al., 2004
4.	CYP2A13	yes		High level in nasal mucosa, trachea and lung but low level in liver	Su et al., 2000
5.	CYP2A6	yes	yes	Principally in human liver but also expressed in nasal mucosa, esophagus, trachea and lung in lesser amount	Shimada et al., 1994; Su et al., 2000; Godoy et al., 2002

pairing that further results in mutations thus disruption in cell functioning, metabolism and synthesis, apoptosis and finally cell damage. Available literature reported the complex pattern of adducts formation of cyclic nitrosamines in comparison to acyclic nitrosamines.

NPYR molecular structure states the two electrophilic centers in one molecule which are aldehyde and diazohydroxide whereas acyclic nitrosamines have these electrophilic centers on separate molecule which is responsible for complex adduct formation in DNA. Various literature showed the dAdo and dCyd adduct formation in DNA via NPYR toxicity. Some reports also showed the formation of dThd adducts in DNA but this compound is unstable.

Wang et al. 2007 reported the presence of dThd, dAdo and dGuo adduct in NPYR treated rats liver DNA that clearly indicate the carcinogenicity of NPYR, a cyclic nitrosamine. It was also reported that NPYR causes mutations and alterations in A: T base pairs in rat liver DNA which proved NPYR as a major hepatocarcinogenic compound[48]. A phenomenon of methylation has also been reported in various DNA bases via nitrosamine toxicity. The formation of O^6-methylguanine that cause the AT-GC transition in DNA, 3-methylguanine and 7-methylguanine adducts in esophageal cells of human tissue autopsy was also reported that showed the mutagenicity of nitrosamines [49].

Role of cytochrome p450 in nitrosamines activation or as a catalyst: Cytochrome P-450 (P stands for pigment) is a structurally similar hemo-protein natured having monooxygenase activity that plays a crucial role in various activation and detoxification process of various environmental carcinogens and xenobiotic compounds. The activity of carcinogens could be determined by determining the activities of CYP-P450 that have a fundamental role in chemoprevention and cancer prevention ways. Approximate 30 CYPs has been identified in human that are involved in various drug or compound metabolism. These belong to various subfamilies of CYPs are CYP1A, CYP2, CYP3A [50]. Various reports suggest that CYP2A subfamiliy is responsible to metabolize nitrosamines in humans[50,51,52,53]. CYP2A subfamily has mainly 3 gene family members that include nitrosamines metabolism: CYP2A6, CYP2A13 and CYP2A7. CYP2A6 is a hepatic coumarin hydroxylase that break down the substrate nicotine[54].

The active site of CYP450 have Fe^{++} center that tethered via a thiolate ligand derived from a cysteine residue that are highly conserved sequences in all known CYPs (PROSITE consensus pattern). The nitrosamine compounds when enters in body, they combine with oxidized CYP450 via heme prosthetic group and changes the spectral properties thus favors the transfer of an electron from ETC (Electron Transport Chain) via Cytochrome-p450 reductase (NADP-dependent). It allocate the oxygen molecule into the CYP450-Fe^{2+}-RH complex that further accept a second electron from Cytochrome b5 or flavoproteins via Cytochrome p450 reductase, NAD dependent enzyme that hydroxylated (R-OH) the compound and release a water molecule[24]. The Cytochrome turned into its original state and ready to take new one. This show the hydroxylation process in nitrosamines in which Cytochrome-P450 works as a catalyst (Figure 7). The expression of various CYP subfamilies is specific for different groups of animal tissues. Table 2 showed the expression level of mRNA and proteins of various CYP4502A subfamilies in organs of mice, rats and human. The changes in expression level of specific cytochromes are also dependent on nature, presence of functional groups and reactivity of environmental carcinogens (toxicants) or xenobiotic compounds.

CONCLUSION

Several evidences suggest that nitrosamines are potential environmental carcinogens that are suspected to cause cancer of various organs and mutation via various DNA adduct formation. Since mutations are inheritable character, so it must be possible that these health related problems may affect next progeny and might lead to severe problems. This review tends to elaborate the various metabolic pathways and DNA adducts formation of several major nitrosamines. Yet, no medications are available to cure nitrosamine causing cancers or health related problems. This review provides a basic line to

understand the mechanism of nitrosamine carcinogenicity and thus generate hope to have possible future aspects in medical field.

ACKNOWLEDGEMENTS

I am very thankful to the technical staff and Department of Bioscience and Biotechnology, Banasthali University for providing facilities to collect the literature and figure making.

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