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COMPARATIVE EVALUATION OF STIMULATORY EFFECTS OF ORAL TOBACCO AND NICOTINE CONSUMPTION ON HEPATIC MICROSOMAL *N*-DEMETHYLATIONS

BASHEER ALI*, SWARAJ KAUR, A. KUMAR and K. P. BHARGAVA
Jawahar Lal Nehru Laboratory of Molecular Biology, Department of Pharmacology and Therapeutics, King George's Medical College, Lucknow 226003, India

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Abstract—The present study was designed to investigate and compare the effects of chronic oral tobacco and nicotine consumption on hepatic microsomal drug metabolizing enzymes (DME) responsible for *N*-demethylation of amidopyrine, morphine and pethidine in rat. Chronic administration of tobacco for 28 days resulted in a marked increase in the rate of *N*-demethylation of amidopyrine, morphine and pethidine. Such tobacco treatment stimulated amidopyrine and pethidine *N*-demethylations about 2.5-fold but that of morphine less than 2-fold. The *N*-demethylation of these drugs was not affected by tobacco treatment for 2 and 7 days. Attempts were made to evaluate the role of nicotine in stimulation of DME by oral tobacco intake. Tobacco was found to contain 4.3 ± 0.18% nicotine on a dry weight basis. Although the magnitude of elevation in microsomal *N*-demethylations of these drugs by chronic oral intake of nicotine for 28 days was comparable to that obtained by tobacco treatment, there was marked difference in the substrate specificity in stimulation of amidopyrine and morphine *N*-demethylations. Nicotine treatment for 2 and 7 days, like tobacco, was also devoid of any influence on the microsomal *N*-demethylations. Both tobacco and nicotine inhibited *in vitro* biotransformations of amidopyrine, morphine and pethidine to their demethylated metabolites and the degree of inhibition in the two cases did not differ much when compared for different substrates. Preincubation studies demonstrated that the inhibition of amidopyrine *N*-demethylation by tobacco increased with time but remained unaffected by nicotine. The nature of inhibition of amidopyrine *N*-demethylase by tobacco and nicotine was non-competitive and competitive respectively. The activities of hepatic microsomal *N*-demethylases were unaffected in rats killed after 1 hr of a single oral dose of tobacco or nicotine. Therefore, it may be interpreted that stimulation of DME is possibly due to *de novo* synthesis of DME.

A number of investigations have focused attention on the chemical environment of man and its deleterious effects. Despite the awareness of the serious health consequences, the abuse of tobacco for smoking, chewing or snuffing is indulged in by many millions of people throughout the world. In India and many East Asian countries, chewing of tobacco is quite popular. The air or sun-cured tobacco leaves, along with betel nut and slaked lime, are widely used for chewing [1]. In India, Pakistan, Bangla Desh and a couple of other countries, the percentage of tobacco chewing is greater than that of tobacco smokers since the former habit is popular in both sexes whereas the latter is mostly confined to males. It is well established that nicotine is the reinforcing constituent that gives tobacco its universal popularity [2].

Human beings are exposed to various xenobiotics which are mostly inactivated by hepatic microsomal mixed-function oxidase systems and are eliminated from the body [3, 4]. Hence, these enzymes are potential sites of drug interactions with other drugs and xenobiotics. Induction of these enzymes by cigarette smoke has been observed in animals [5–7] and man [8–10]. Polycyclic aromatic hydrocarbons (PAH) such as 3, 4-benzpyrene, 3, 4-benzofluorene, anthracene, fluoranthene, pyrene, chrysene, etc. have been implicated as the principal agents in tobacco smoke responsible for the induction of microsomal enzymes, because these agents when administered in pure forms induce microsomal drug metabolizing enzymes [5, 11, 12].

A number of studies dealing with the estimation of polycyclic aromatic hydrocarbons in tobacco leaves and their role in tumerogenic effects has revealed that the contents of these substances in tobacco ranged from 5–20 parts per billion (p.p.b.) and therefore their presence in traces does not contribute an appreciable amount to the aromatic hydrocarbons present in tobacco smoke [13]. Pyrolysis of paraffin hydrocarbons and other substances of tobacco leaves at high temperature leads to the formation of tumerogenic aromatic hydrocarbons [14]. This was further evident from the relatively weak carcinogenic activity of tobacco leaves extract [13].

The role of other tobacco smoke constituents in enzyme induction is much less well documented. Administration of nicotine to mice in drinking water increased metabolism of meprobamate [15]. Subcutaneous injections of nicotine for two weeks resulted in only slight stimulation of nicotine metabolism to cotinine [16]. A single intraperitoneal dose

* To whom correspondence should be addressed.

of nicotine (40 mg/kg) enhanced the metabolism of 2-acetylaminofluorene, 3,4-benzpyrene and 3-methylcholanthrene [12].

Studies dealing with the inhibition of the cigarette smoking habit by oral intake of nicotine tartrate (50 mg/24 hr divided into five doses) in man has provided substantial evidence that nicotine is responsible for the smoking habit [17]. However, the quantitative and qualitative contribution of nicotine in tobacco smoke-induced changes in drug metabolizing enzymes has not yet been critically evaluated. Moreover, there is also a gap in our knowledge about the role of other constituents of tobacco leaves [13] in the modification of pharmacokinetics of drugs and other xenobiotics. These observations prompted the present investigation, where the effect of chronic oral intake of sun-cured tobacco leaves on hepatic microsomal *N*-demethylation of amidopyrine, morphine and pethidine was evaluated in rats and compared with that obtained with an equivalent amount of pure nicotine base under identical experimental conditions. The effect of tobacco and nicotine intake on these enzymes was also studied after acute treatment and *in vitro* conditions.

MATERIALS AND METHODS

Glucose-6-phosphate and nicotinamide adenine dinucleotide phosphate (NADP) were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. Amidopyrine was supplied by Burgoyne Pharmaceutical, London, U.K. Pethidine hydrochloride and morphine sulphate were acquired from local manufacturers. All other chemicals used were of analytical grade and obtained from commercial sources.

Female albino rats (100–150 g) maintained on *ad lib.* diet were used throughout the study. Animals were treated with commercially available sun-cured tobacco leaves. This variety is grown in the South-West part of India and locally known as Gujrati Deshi Tobacco (Trade name—Ratna Chhap Zafrani Patti No. 64). A homogenate of tobacco leaves prepared in water was given orally with the help of a feeding cannula to rats twice a day (11 a.m. and 4 p.m.) at a dose of 250 mg/kg for 2, 7 and 28 days. The nicotine content of the tobacco leaves was estimated. A 10 mg/kg dose of pure nicotine base corresponding to a nicotine content of 250 mg tobacco leaves was also administered to rats orally twice daily for 2, 7 and 28 days. All the experimental and control rats were run under similar experimental conditions. The rats, unless otherwise mentioned, were killed after 16–20 hr from the last dose of tobacco or nicotine.

Determination of nicotine content in tobacco leaves. Nicotine was determined in the homogenate of sun-cured tobacco leaves by the spectrophotometric method reported earlier [18]. A suitable aliquot of homogenate was extracted with a heptane–isoamyl alcohol (98.5:1.5) mixture in alkaline medium. The drug extracted in heptane phase was returned to an aqueous phase of 0.1 N HCl and read at 259 nm in a Hitachi–Perkin–Elmer Spectrophotometer. Blanks and standards using nicotine base were also run under similar experimental conditions.

Assay of hepatic microsomal N-demethylases. Rats were decapitated and the livers were immediately removed, blotted dry, weighed and homogenized individually in ice cold 1.15% KCl solution in the ratio of 1:4 (w/v) with the help of a Potter–Elvehjem homogenizer. All subsequent tissue manipulations were conducted at 0–4°. The homogenates were centrifuged at 9000 *g* for 15 min in a Remi K-24 Centrifuge (India) and the resulting supernatant fractions were used as the source of drug metabolizing enzymes. The activities of drug metabolizing enzymes responsible for *N*-demethylation of amidopyrine, morphine and pethidine were assayed by the method described earlier [19]. An estimation of formaldehyde formed during *N*-demethylation of amidopyrine, morphine and pethidine was taken as the index of enzyme activities. An assay of DME was performed in individual 9000 *g* supernatant fractions from control and treated animals. The incubation mixture of 3 ml consisted of phosphate buffer (0.1 M, pH 7.4), 10 μmoles of glucose-6-phosphate, 0.72 μmoles of nicotinamide adenine dinucleotide phosphate (NADP), 15 μmoles of $MgCl_2$, 20 μmoles of nicotinamide, 10μmoles of different substrates and 9000 *g* supernatant equivalent to 44 mg protein. After a preincubation of 10 min without substrate at 37° the reaction was started by addition of substrate and run for 20 min in an atmosphere of oxygen. In contrast to the reaction conditions reported earlier semicarbazide hydrochloride was not added to the reaction mixture, since it was found to inhibit drug metabolizing enzymes. The reaction was stopped by addition of 2 ml of Nash reagent (30% ammonium acetate containing 0.4 ml of acetylacetone). The reaction mixture was heated in a boiling water bath for 10 min. The precipitated proteins were centrifuged out and the yellow colour was read at 415 nm in a Hitachi–Perkin–Elmer Spectrophotometer. Known amounts of formaldehyde carried through the incubation and assay procedures served as the standards. All reaction rates were determined when product formation was in the linear range with respect to protein concentration. Protein was determined by the method of Lowry *et al.* [20].

RESULTS

Determination of nicotine content in tobacco showed that it contained 4.3 ± 0.18% nicotine on a dry weight basis. The LD_{50} of nicotine has been reported to be 25.5 mg/kg and 40 mg/kg when administered intraperitoneally in saline and corn oil respectively [12, 21], but it was found to be 55 mg/kg when given orally to rats [22]. Hence, an oral dose of 250 mg/kg of tobacco equivalent to 10 mg/kg of nicotine base was selected to evaluate the effect of tobacco consumption on drug metabolizing enzymes.

Chronic oral administration of tobacco (250 mg/kg) twice daily for 28 days caused significant stimulation of hepatic drug metabolizing enzymes (DME) responsible for *N*-demethylation of amidopyrine, morphine and pethidine in rat (Table 1). The increase in amidopyrine and pethidine *N*-demethylations was 156.8 and 150.7 per cent respectively, whereas the rate of morphine *N*-demethylation was increased up to 68.6 per cent only.

An oral dose of nicotine base (10 mg/kg) was given

Table 1. Stimulation of hepatic microsomal *N*-demethylations by chronic oral tobacco treatment in rat.

Substrate	Formaldehyde formed* (ng/mg protein/20 min) Control	Experimental	Per cent increase
Amidopyrine	312.0 ± 17.2	801.4 ± 27.2	156.8†
Morphine	77.2 ± 5.0	130.2 ± 5.8	68.6†
Pethidine	107.5 ± 4.0	269.6 ± 12.0	150.7†

* Assay conditions were as indicated in the text. Each experiment was done in duplicate and values are mean ± S.E. of separate enzyme preparations obtained from 6–8 control or experimental rats.
† $P < 0.01$

to rats twice daily for 28 days and *N*-demethylations of amidopyrine, morphine and pethidine were followed under identical experimental conditions (Table 2). In contrast to tobacco, chronic nicotine-treated rats exhibited the least stimulation of amidopyrine *N*-demethylation. The degree of stimulation of pethidine *N*-demethylation was not significantly altered, whereas the increase in morphine *N*-demethylation was greater than that observed after tobacco intake.

The influence of acute tobacco and nicotine treatment for 2 and 7 days was investigated by determining the *N*-demethylation of amidopyrine, morphine and pethidine under the experimental conditions described for chronic studies. As is evident from Table 3 the activities of microsomal *N*-demethylases were not affected by acute treatment with either tobacco or nicotine.

Liver microsomal preparation from rats given an oral dose of tobacco (500 mg/kg) or nicotine (20 mg/kg) and killed after 1 hr exhibited no change in the activities of *N*-demethylases.

In vitro effects of tobacco and nicotine on DME are summarized in Table 4. Tobacco inhibited *N*-demethylations of all the three drugs when tested at a final concentration of 4.2 mg/ml of incubation mixture. The percentage inhibitions of biotransformation of amidopyrine, morphine and pethidine were 75.0 59.7 and 64.8 respectively. Nicotine also inhibited *in vitro* metabolism of drugs by hepatic microsomal *N*-demethylases. In these experiments nicotine was used at a final concentration of 1 mM, which on a weight basis was equivalent to 0.16 mg nicotine or 4.2 mg of tobacco per ml of incubation mixture. Inhibition of *N*-demethylases produced by nicotine was 66.5, 71.0 and 60.0 per cent with amidopyrine, morphine and pethidine respectively. However, like tobacco, nicotine also did not exhibit any remarkable specificity in inhibition of the metabolism of different substrates.

In order to determine the nature of inhibition of microsomal *N*-demethylases by tobacco and nicotine, preincubation and kinetic studies were performed. No change in the inhibition of amidopyrine *N*-demethylation was noticed when nicotine was preincubated with the enzyme preparation for varying periods before addition of the substrate to the reaction mixture, but the inhibition by tobacco increased with time (Table 5). Evaluation of the kinetics of molecular interaction of amidopyrine *N*-demethylase with tobacco and nicotine by the conventional reciprocal plot of Lineweaver–Burk [23] revealed the noncompetitive and competitive nature of enzyme inhibition respectively (Figs 1 and 2). The mean Michaelis constant (K_m) of the *N*-demethylase for amidopyrine, as calculated, from four different experiments, was 2.35. The inhibitor constant (K_i) of amidopyrine N-demethylase for tobacco and nicotine was 9.0 and 0.05 mg (or 1.0×10^{-4} M) respectively.

DISCUSSION

This study was designed to formulate the quantitative and qualitative relationship between oral intake of tobacco and one of its most important constituents, nicotine, with respect to their capacity to stimulate hepatic microsomal drug metabolizing enzymes catalysing *N*-demethylation of amidopyrine, a drug possessing anti-inflammatory, analgesic

Table 2. Stimulation of hepatic microsomal *N*-demethylations by chronic oral nicotine treatment in rat.

Substrate	Formaldehyde formed* (ng/mg protein/20 min) Control	Experimental	Per cent increase
Amidopyrine	312.0 ± 14.7	414.4 ± 30.2	32.8†
Morphine	83.3 ± 5.2	166.6 ± 10.4	100.0†
Pethidine	118.3 ± 6.0	312.5 ± 15.8	164.1†

* Mean values ± S.E. of duplicate experiments, $n = 6$–8.
† $P < 0.01$

Table 3. Effect of acute tobacco and nicotine treatment on microsomal *N*-demethylations

Substrate	Per cent change* Tobacco 2 days	Tobacco 7 days	Nicotine 2 days	Nicotine 7 days
Amidopyrine	Nil	Nil	Nil	Nil
Morphine	Nil	Nil	Nil	Nil
Pethidine	Nil	Nil	Nil	Nil

* N = 6 done in duplicate.

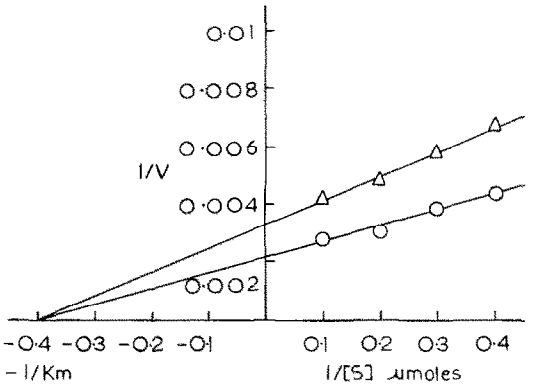

Fig. 1. Lineweaver–Burk plot showing non-competitive inhibition of hepatic amidopyrine *N*-demethylase by tobacco. In this study *I/S* represents reciprocal of the substrate concentration and *I/V* represents the reciprocal of the ng of formaldehyde formed/mg protein/20 min under the experimental conditions described in the text. Key: Control (○—○) and 4.0 mg tobacco (△—△).

and antipyretic properties and two narcotic–analgesics, morphine and pethidine, in rat. Chronic oral administration of tobacco resulted in significant stimulation of biotransformation of amidopyrine, morphine and pethidine to their demethylated products. Tobacco elicited an approximately two-and-a-half-fold increase in the metabolism of amidopyrine and pethidine, whereas *N*-demethylation of morphine was enhanced by less than two-fold.

For many years investigators have been looking for biochemical changes in the enzyme-catalysed molecular biotransformation of drugs, other xenobiotics and endogenous substrates that accompany exposure to tobacco smoke. It has been inferred from these studies that polycyclic aromatic hydrocarbons (PAH) such as 3,4-benzpyrene, pyrene, fluoranthene, anthracene etc. play the key role in tobacco smoke-induced enhancement of the metabolism of drugs, carcinogens and other xenobiotics [5, 24]. These substances are also present in polluted city air [25], certain smoked and cooked foods [26, 27], tars, pitches and soots [28] and a number of other environmental pollutants. Induction of drug metabolizing enzymes has been shown to reduce the carcinogenic effects of polycyclic aromatic hydrocarbons [29, 30].

Since tobacco leaves contain only traces of polycyclic aromatic hydrocarbons and nicotine has been shown to be an inducer of drug metabolizing enzymes, it was thought of interest to evaluate the role of nicotine in the stimulation of amidopyrine, morphine and pethidine *N*-demethylations by tobacco intake in rats. Chronic oral administration of nicotine equivalent to that present in tobacco increased *N*-demethylations of all three substrates. Elevation of *N*-dealkylation of ethylmorphine and norcodeine has been reported in post-mitochondrial fractions from rat liver after intraperitoneal administration of nicotine (4 mg/kg) 4 times a day for three days [31]. Nicotine oxidation to cotinine was increased to a lower extent. It is important to mention here that the magnitude of elevation in the metabolism of ethylmorphine, norcodeine and aniline produced by chronic oral administration of nicotine (5.9 mg/kg/day) in drinking water for 7, 14 and 21 days remained constant throughout the period and was comparable to that observed in rats after acute administration of nicotine in high doses (4 doses of 4 mg/kg) for 3 days. Recently, it was shown that only a 10–15 per cent increase in the metabolism of nicotine took place after continuous administration of nicotine for 10 days in rats [16]. However, the present study revealed that oral nicotine administration to rats (2 doses of 10 mg/kg/day) for 4 weeks enhanced *N*-demethylations of morphine and pethidine up to about 2- and 2.5-fold, respectively, whereas that of amidopyrine was less than one-and-a-half-fold. Studies dealing with the effect of semi-chronic administration of nicotine (2.28 mg/kg/day) in drinking water on the metabolism of meprobamate indicated an approximately 4-fold increase in its disposition [15]. The increase in disposition was completely blocked by ethionine showing the induction of microsomal drug metabolizing enzymes as the biochemical basis of enhanced meprobamate metabolism. Our results are contrary to those reported earlier [12] where a single high dose of nicotine (40 mg/kg) in corn oil given intraperitoneally caused 90 and 130 per cent induction of rat liver microsomal hydroxylases of carcinogens, 2-acetylaminofluorene and 3,4-benzpyrene, respectively, in 24 hr but depression if nicotine treatment was continued for another 2 or 3 days. Such increase in enzyme activities was inhibited by prior treatment of animals with ethionine, a protein synthesis inhibitor.

Induction kinetic studies showed that the animals treated with tobacco and nicotine for short periods, i.e. 2 and 7 days, did not cause any modification in

Table 4. *In vitro* effect of tobacco and nicotine on drug metabolizing enzymes

Substrate	Per cent inhibition* Tobacco	Nicotine
Amidopyrine	75.0 ± 2.5†	66.5 ± 0.8†
Morphine	59.7 ± 1.6†	71.0 ± 1.3†
Pethidine	64.8 ± 1.9†	60.0 ± 1.8†

* The activities of drug metabolizing enzymes were followed by determining the rate of *N*-demethylation of the drugs. Nicotine and tobacco were used at final concentrations of 0.16 mg (i.e. 1 mM) and 4.2 mg/ml, respectively, of incubation mixture. Values represent mean ± S.E.

† $P < 0.01$.

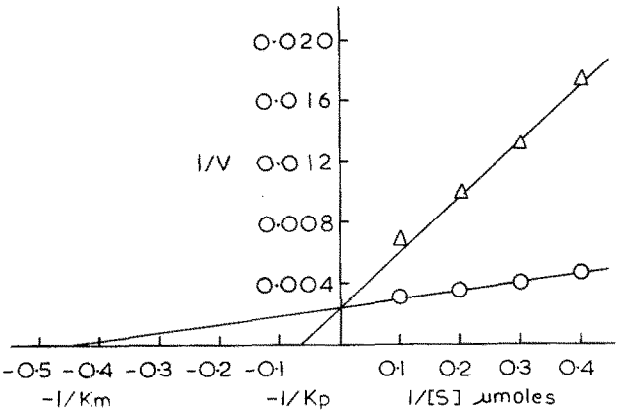

Fig. 2. Lineweaver–Burk plot of *I/S* vs *I/V* showing competitive inhibition of amidopyrine *N*-demethylase by nicotine. Key: Control (○—○) and 0.3 mg nicotine (△—△).

N-demethylase activities. This suggests that the stimulation of these enzymes by oral intake of tobacco and nicotine occurs after a prolonged period. Differences in the drug metabolism have also been reported between short-term and prolonged pretreatment of subjects with other xenobiotics [32, 33].

The degree of *N*-demethylation of the drugs by chronic oral intake of tobacco and nicotine was variable. The degree of stimulation of pethidine *N*-demethylation was of the same order in rats pretreated with nicotine and tobacco, whereas it was different for amidopyrine and morphine *N*-demethylations. It appears that in addition to nicotine, other constituents of tobacco also play an important role in the stimulation of amidopyrine *N*-demethylation. A number of investigations have indicated the existence of multiple *N*-demethylases in hepatic microsomes but their number and molecular differentiation is at present not fully worked out. Sex [34, 35] and species [36] differences have been shown to exist in rates of demethylation of several amines. The induction of microsomal *N*-demethylases is also markedly dependent upon the substrate investigated [3, 37]. Attempts have been made to elucidate the molecular basis of the different substrate specificities of microsomal mixed function oxidases. It has been suggested that, depending on the substrate used, the NADPH-cytochrome P-450 reductase component can play a marked role in determining the substrate specificity [38]. Recent studies demonstrated the presence of at least four to six forms of cytochrome P-450 which exhibit more or less distinct substrate specificities and immunological and spectral uniqueness [39, 40].

Table 5. Effect of preincubation of tobacco and nicotine on amidopyrine *N*-demethylase activity

Preincubation time in min	Per cent inhibition* Tobacco	Nicotine
0	40.4	52.6
5	46.2	47.6
20	65.1	54.9

* Values of per cent inhibition represent the mean values calculated from two separate experiments done in duplicate.

Animals killed after a single oral dose of tobacco or nicotine did not exhibit any modification in the activities of microsomal *N*-demethylases. *In vitro* addition of tobacco or nicotine in high concentrations inhibited *N*-demethylations of the drugs tested. It may be noted with interest that the magnitudes of inhibition of *N*-demethylases produced by tobacco and nicotine were comparable and there did not exist any significant substrate specificity in either case. These results are in accordance with those reported earlier where only high concentrations of nicotine were found to inhibit *in vitro* *N*-dealkylation of ethylmorphine and *O*-dealkylation of norcodeine [31]. Such *in vitro* inhibitory responses of various xenobiotics have been explained on the basis of an alternate substrate mechanism possibly due to the nonspecificity of the microsomal mixed function oxidase system [4].

It may be concluded from preincubation studies that the inactivation of *N*-demethylases by tobacco progresses slowly with time but remains unaffected by nicotine. Furthermore, kinetic studies demonstrated that tobacco and nicotine inhibited the *N*-demethylases by different biochemical mechanisms in that the nature of enzyme inhibition by the two xenobiotics was noncompetitive and competitive respectively. The mean Michaelis constant (K_m) of *N*-demethylase for amidopyrine was 2.35. The microsomal *N*-demethylases were found to possess significantly higher affinity for nicotine when compared to tobacco.

It may be concluded from the present study that oral administration of tobacco causes differential stimulation of hepatic microsomal *N*-demethylations of amidopyrine, morphine and pethidine. Since *in vitro* addition of either tobacco or nicotine inhibited the drug biotransformations and a single oral dose did not alter the DME activities, it may be interpreted that stimulation of DME activities is possibly due to *de novo* synthesis of one or more components of the microsomal oxidase system and not as a result

of direct activation of existing enzyme molecule. The magnitude of DME stimulatory effectiveness of tobacco, unlike tobacco smoke which induces the metabolism of xenobiotics several-fold due to the presence of PAH [8–11], is low and comparable to that observed after oral nicotine consumption. It appears from these observations that in addition to nicotine, which plays a major role, other constituents of tobacco may also be responsible for the enhanced metabolism of drugs. Further studies dealing with the comparative evaluation of the consequences of tobacco and nicotine intake on other DME might be helpful in the elucidation of the biochemical basis of possible tobacco interaction with drugs, carcinogens, pesticides, insecticides and other environmental pollutants.

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