INHIBITION OF *IN VITRO* NITROSATION OF NORNICOTINE

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Abstract. The inhibition of nornicotine nitrosation was studied. The concentration of nornicotine was 100 μM and nitrite 1000 μM. As inhibitors were used following compounds: ascorbic acid (as reference) (800, 1000, and 5000 μM), dihydroxyfumaric acid (1000 μM), (+)catechin (1000 μM), resveratrol (1000 μM), tartaric acid (1000 μM), quercetin (1000 μM), and grape seed extract (50, 100, 150 μg/ml). The best inhibitory effect was obtained for AAs at 5000 μM (90.7%), (+)Ct (95.5 %) and GSE at 150 μg/ml (96.1%). The small inhibitory effect was observed for TA – 22,5%.

Keywords: N-nitrosonornicotine, nitrosation, inhibition, grape seed extract, polyphenol.

1. INTRODUCTION

Smoking causes an estimated 430,000 deaths per year in the U.S., including 30% of all cancer deaths [1]. Lung cancer alone would kill over 150,000 people in the U.S. in 2000, and cigarette smoking is directly responsible for 87% of lung cancer mortality [1]. An understanding of nicotine metabolism provides a critical framework for deciphering the mechanisms by which tobacco products cause disease [2].

The reaction of nicotine with nitrous acid (derived from nitrite] results in the formation of three products, 4-(N-methylnitrosamino)-4-(3-pyridyl)-l-butanal(NNA), nitrosonornicotine (NNN), and 4-(N-methylnitrosamino)-1-(3-pyridyl)-l-butanone(NNK) [3].

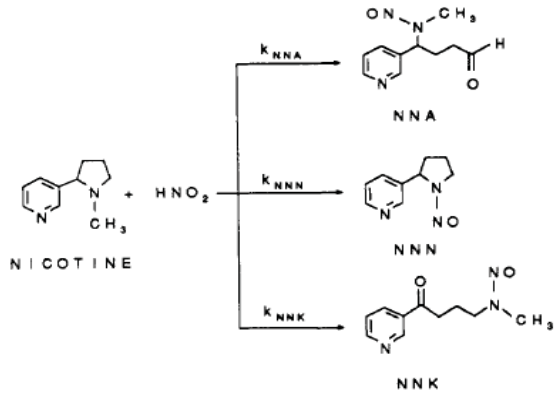

Fig.1. Reaction of nicotine and nitrous acid.

Humans, like rats, metabolize nicotine to nornicotine (0.4–2.7% of dose) and it is possible that nornicotine could also be concentrated in saliva [4].

Human exposure to nitrite occurs through diet, via reduction of dietary nitrate, and from endogenously produced nitric oxide [5,6].

N-Nitrosonornicotine (NNN) was the first tobacco specific nitrosamine (TSNA) for which tumorigenicity [7] and the occurrence in tobacco smoke [8] has been proven. NNK and NNN have been recently classified as "carcinogenic to humans" by the IARC [9].

Although enormous energies have been invested in treating existing cancer by chemotherapy, prevention of cancer is the preferred option. The reduction of human exposure to endogenously formed NOC, also as TSNA, as one of the ways of cancer chemoprevention, is possible through the use of inhibitors of the nitrosation process. Thus, ascorbic acid and ascorbate were shown to inhibit nitrosation by reacting with the nitrosating agents [10-13]. Polyphenolic compounds, which are present in high quantities in human foods and beverages derived from plant and fruits, are also potent blocking agents of NOC formation [14-17]. The polyphenols include a wide range of closely related compounds synthesized by plants; these include the flavonoids found in tea leaves (catechins), isoflavonoids in soybeans (genistein and daidzein), and stilbenes in red grapes (resveratrol). Each of these has been shown to have anticancer properties in cell culture models of cancer [18-20]. Because of the complexity of their action, the anticancer activity of polyphenols has yet to be clearly understood. Thus, complex polyphenols from grape seed extracts and red wine have been shown to possess in vitro antioxidant activity [21]; inhibit aromatase enzyme activity [22]; inhibit the

growth of cancer cells in cell culture [23,24]; and prevent or attenuate disease in various animal models of disease, including atherosclerosis [25], cataract formation [26], and skin cancer [27]. Some dietary polyphenols were shown to inhibit mutagenesis through inhibition of nitrosamine formation [28-32].

2. METHOD AND ANALYSIS

Chemicals. Nornicotine (NN), sodium nitrite, N-nitrosonornicotine (NNN), 5-methyl-*N*'-nitrosonornicotine (5-MeNNN), ethanol, ascorbic acid (AAs), dihydroxyfumaric acid (DFH_4), (+)catechin ((+)Ct), resveratrol (Resv), tartaric acid (TA), quercetin (Que), ammonium sulfamate were purchased from Sigma Chemical Co. (St. Louis, MO). Grape seed extract was ordered online www.wholehealtproducts.com

Equipment. The following main equipment was used for the urine analysis: SpeedVac centrifugal concentrator (Savant Instruments, Farmingdale, NY); HP 6890 gas chromatograph (Agilent Technologies, Wilmington, DE) interfaced to a model 543 Thermal Energy Analyzer (Orion Research, Beverly, MA); the separation was performed on a 30m x 0.25mm ID, 0.25µm film sickness, DB 1301 column (Agilent Technologies) and on a 15 m × 0.25 mm i.d. DB 5-MS column (0.25 µm film thickness) from J & W Scientific connected to a 2 m × 0.32 mm i.d. deactivated precolumn.

Method

For studying the inhibitory effect of NNN formation in watery system, the following compounds were used: ascorbic acid, dihydroxyfumaric acid, (+)catechin, resveratrol, tartaric acid, quercetin and GSE, vs control.

100 µM of NN to 1000 µM of $NaNO_2$ were used for control (without inhibitor). The same concentration of NN and $NaNO_2$ was used in the case of inhibitors, the ratio Inh: $NaNO_2$ = 1:1 for dihydroxyfumaric acid, (+)catechin, resveratrol, tartaric acid and quercetin, but for AAs the ratio was 1:0,5; 1:0,8; 1:1 and 1:5. For GSE we used the following concentration: 50, 100 and 150 µg/ml. Since inhibitors are not dissolvable in water, we considered to dissolve all reagents in ethanol (100%) The experiments have been done in the acid environment corresponding to that of gastric (pH 1-2), adding 100 µl HCl (1N) to each sample of 1 ml. The samples were kept for 1.5 hours in thermostat with shaker at temperature of 37°C.

After 1.5 hrs, the samples were kept in ice for stopping the reaction. To each samples 100 µl of ammonium sulfamate were added, for remaining nitrite destroying. After 15-20 min, 3 ml of KH_2PO_4 (pH 7) were added as buffer solution to each samples (1 ml).

Sample analysis includes several phases:

Internal standard: as internal standard used in a well-known concentration (20 ng), we used 5-MeNNN. Adding it to every sample excludes the error caused by NNN loses in the treatment process.

Sample work-up: in order to extract NNN from samples and its later purification, we used *ChemElut extraction cartridges* (packed with specially cleaned and sized diatomaceous earth), eluted 2 time with 8 ml methylene chloride ($MeCl_2$), collected in 15-ml centrifuge tubes and less in speed-vac to dryness (1.5 hrs).

Dry extract of NNN with internal standard were transferred with methanol to 200 µl plastic GC vials, and was less in speed-vac to dryness. The samples were re-dissolved adding 20 µl acetonitrile.

Analysis: after the samples have been purified, they have been analyzed. In this order, we used the GC-TEA (Gas Chromatography-ThermoEnergyAnalizer) method.

All samples have been analyzed twice, with duplicates.

3. RESULTS AND DISCUSSION

The standard curve for NNN and 5-MeNNN were analyzed. With these curves the concentration of NNN formed in system was calculate and re-calculate. (Fig.1.)

For all standard curves, high accuracy was obtained (R^2> 0,99).

Degree of nitrosation in all samples has an order of 10^{-4}- 10^{-5} % (Tab.1.).

In our study, AAs was used as reference, because ascorbic acid was shown to inhibit nitrosation over a pH range of 2–5 through a rapid reduction of nitrous acid to nitric oxide (NO) and formation of dehydroascorbic acid (Fig. 2) [23].

The experimental data showed that the degree of inhibition of NNN formation with Aas, as inhibitor, for the following concentration 800, 1000 and 5000 µM was 19.9, 31.6 and 90.7% vs control (Tab.1.). Medium recovery for these AAs concentration was 37%, for control – 94%. So, for rate Inh:Nitrite = 5:1, we have nearly complete inhibition of NNN formation.

It is possible that the same mechanism is involved in the inhibition of endogenous NNN formation by dihydroxyfumaric acid, observed in this study. DFH_4 showed a good inhibitory effect – 74.9% with 91%. Dihydroxyfumaric acid is formed by the oxidation of tartaric acid in the presence of iron (II) and hydrogen peroxide [33] and is found in grapes and secondary winery products. It was demonstrated that *N*-nitrosation of secondary amines in simulated gastric juice is effectively inhibited by dihydroxyfumaric acid [34,35], and the present study supports this finding. However, potential health effects of dihydroxyfumaric acid are unknown.

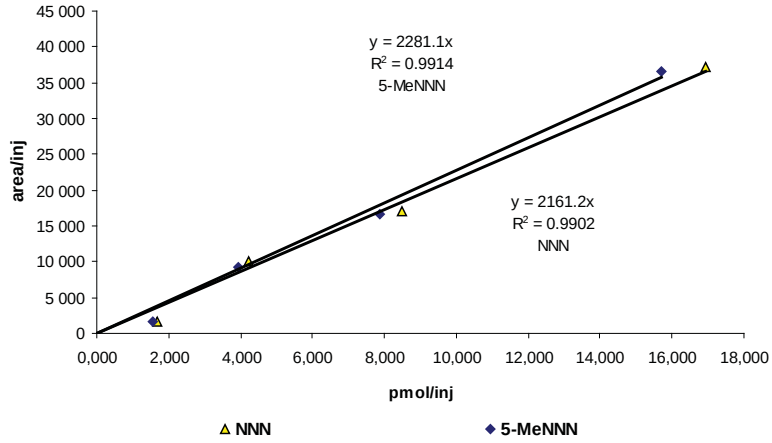

Fig.1. Standard Curve for NNN and 5-MeNNN.

TA practically showed small inhibiting properties, only 22.5% from control (Tab.1.). The absence of double bond in the chemical structure of acid may be the cause of small inhibiting properties.

Polyphenolic antioxidants were shown to inhibit or catalyze formation of NOC, depending on their structure and reaction conditions [reviewed in 36]. In our study, we observed effective inhibition of nornicotine nitrosation in the presence of catechin, resveratrol, and quercetin with 95.5, 46.5, and 78.7% respectively (Tab.1.). Is interesting that Que is known as very good antioxidant, much better than catechin are, and it must be better inhibitor. It was found that inhibition of N-nitrosation is directly correlated with the scavenging (electron-donating) property for nitrogen-centered free radical [37]. Quercetin has an identical number of hydroxyl groups in the same position as catechin, but contains the 2,3-double bond in the C ring and 4-oxo function. This structure advantage confers an enhancement of the TEAC (total antioxidant activity in trolox equivalent) value to 7.7 mM compared to the saturated heterocyclic ring of catechin with approximately half the antioxidant activity (2,4 mM) [38]. We suppose that incomplete dissolution may be the blocking factor for better inhibitory effect. The effect of polyphenolic compounds on *N*-nitroso compound formation depends on pH, the nature of nitrosated amine, and the relative concentrations of nitrite and phenolics [36].

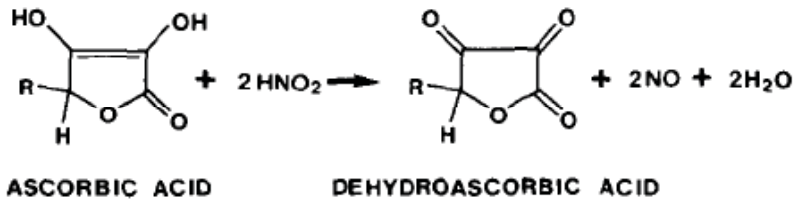

Fig.2. Reduction of nitrous acid by ascorbic acid to NO and production of dehydroascorbic acid (ref. 23).

GSE showed a very good inhibitory effect in the nitrosation of nornicotine. We obtained 66.0, 93.2, and 96.1% of inhibition of NNN vs. control for the concentration of GSE 50, 100, and 150 mg/ml (Tab.1.). GSE used is naturally extracted, so there are no chemical or solvent residues. GSE contain a full 90% Oligomeric Proanthocyanidins, additionally, there is 56% of Grape Seed extract, 27% of Grape Skin Extract and 17% of Red Wine Extract. Oligomeric Proanthocyanidins, in fact anti-oxidants in general, are structured in such a way that they are able to donate electrons freely without altering their valence (their electrons are not paired) - what this means is that anti-oxidants can stabilize free radicals without themselves becoming dangerous [39]. They are a class of phenolic compounds, which take the form of oligomers or polymers of polyhydroxy flavan-3-ol units, such as (+)-catechin and (-)-epicatechin [40]. Recently, epidemiological data have shown that red wine may reduce the mortality rate from coronary heart disease, the so-called ‘‘French paradox’’ [41.42]. Proanthocyanidins are the major polyphenols in red wine as well as in grape seeds, and they have potent antioxidant activity [43.44], inhibit low density lipoprotein oxidation [46], as well as a variety of biological activities [46-50].

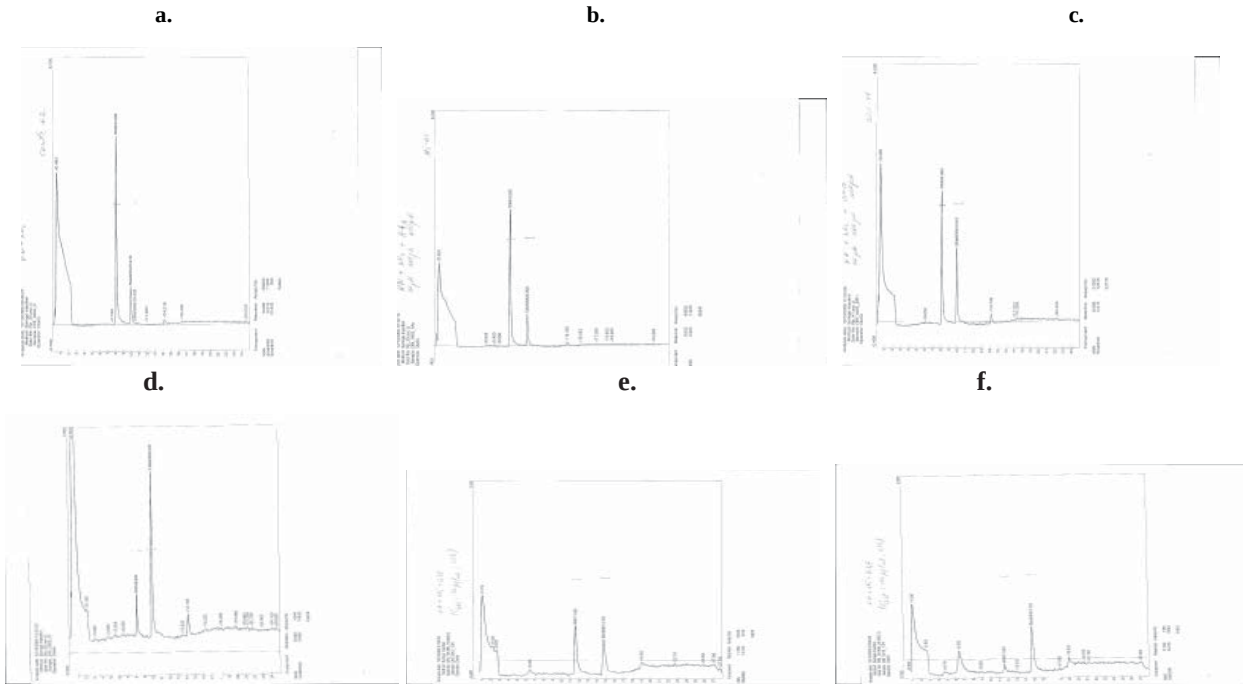

Fig. 3. GC-TEA traces of NNN and 5-MeNNN in the tested samples: (a) control $NN+NO_2^-$ (without inhibitor), (b-d) $NN+NO_2^-$ with 1000 μM AAs, DFH_4 and (+)Ct respectively; (e,f) $NN+NO_2^-$ with 50 and 150 GSE μg/ml respectively

In Fig.3. some GC-traces for sample are shown: control (without addition of inhibitor), with AAs, DFH_4, (+)Ct (each of 1000 μM) and GSE (50 and 150 μg/ml). Comparing the peaks area for NNN and 5-MeNNN in each trace with control, we can see an evident inhibitory effect of utilized compounds.

Table 1

Inhibitory effect of *in vitro* nitrosation of NN by different compounds

Sample	NNN of initial NN,%	NNN, pmol	Inhibition of NNN, %
Control	$3{,}12\cdot10^{-4}$	311,8	0,0
Aas, 800μM	$2{,}50\cdot10^{-4}$	249,7	19,9
Aas, 1000μM	$2{,}13\cdot10^{-4}$	213,3	31,6
Aas, 5000μM	$2{,}89\cdot10^{-5}$	28,9	90,7
TA, 1000μM	$2{,}42\cdot10^{-4}$	241,6	22,5
Rezv, 1000μM	$1{,}67\cdot10^{-4}$	166,8	46,5
DFH, 1000μM	$7{,}83\cdot10^{-5}$	78,3	74,9
Que, 1000μM	$6{,}64\cdot10^{-5}$	66,4	78,7
(+)Ct, 1000μM	$1{,}41\cdot10^{-5}$	14,1	95,5
GSE, 50μg/ml	$1{,}06\cdot10^{-4}$	106,1	66,0
GSE, 100μg/ml	$2{,}11\cdot10^{-5}$	21,1	93,2
GSE, 150μg/ml	$1{,}22\cdot10^{-5}$	12,2	96,1

4. CONCLUSION

The best inhibitory effect was obtained for AAs at the molar ratio AAs: NO_2^- = 5:1 (90.7%). From polyphenols (1:1 molar ration with nitrite), (+)Ct was the best result (95.5 %), DFH_4 and Que were the comparable inhibitory effect (74.9 and 78.7 % respectively). GSE at 100 and 150 μg/ml has shown a very good inhibition (93,2 and 96.1% respectively). The small inhibitory effect was observed for TA – 22,5%.

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