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STUDY ON HEPATOTOXICITY OF CYTISINE (TABEX®) COMPARED WITH NICOTINE IN FRESHLY ISOLATED RAT HEPATOCYTES

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Summary. The aim of the study was to investigate the effects of cytisine and nicotine in seven consequently increasing concentrations (5nM-250 μM) in isolated rat hepatocytes. The level of malondialdehyde (MDA) was assayed as index of lipid peroxidation (LPO). Lactate dehydrogenase (LDH) leakage, cell viability and reduced glutathione (GSH) depletion were used as signs of cytotoxicity. Our data indicate that cytisine has significantly less prominent toxicity on cell viability and GSH level than nicotine. At the same time, we found higher toxicity of cytisine on MDA level. The higher cytotoxicity exerted by nicotine on cell viability and GSH level might be due to its metabolites. The results from this study showed that nicotine did not affect MDA level in the same extent as cytisine. This might be explained with the ability of nicotine to inhibit superoxide anion and other reactive oxygen species generation, involved in the process of lipid peroxidation and oxidative stress.

Key words: isolated rat hepatocytes, cytisine, nicotine, hepatotoxicity

ПРОУЧВАНЕ НА ХЕПАТОТОКСИЧНОСТТА НА ЦИТИЗИН (TABEX®) В СРАВНЕНИЕ С НИКОТИН В ИЗОЛИРАНИ ХЕПАТОЦИТИ ОТ ПЛЪХ

М. Мичева, М. Кондева-Бурдина и В. Вичева

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Резюме. Целта на изследването е да се проследят ефектите на цитизин и никотин в изолирани хепатоцити на плъхове. Двете вещества са приложени самостоятелно в седем постепенно нарастващи концентрации (5 nM-250 μM). Като биомаркер на прекисното окисление на липидите е определяно нивото на малондиалдехид, а като маркери на цитотоксичността са определяни активността на ензима лактат дехидрогеназа (LDH), клетъчна жизненост и ниво на редуциран глутатион (GSH). Получените резултати показват, че цитизинът проявява статистически значим, по-слаб цитотоксичен ефект върху клетъчната жизненост и нивото на GSH в сравнение с никотина, докато върху количеството на MDA цитизинът проявява по-изявена токсичност. По-изявената цитотоксичност на никотина върху клетъчната жизненост и нивото на GSH най-вероятно се дължи на неговите метаболити, докато по-слабата му токсичност върху количеството на MDA в сравнение с цитизина най-вероятно е свързана със способността на никотина да инхибира продукцията на супероксиден анион и други реактивни форми на кислорода, участващи в процесите на прекисно окисление на липидите и оксидативния стрес.

Ключови думи: изолирани хепатоцити на плъхове, цитизин, никотин, хепатотоксичност

Introduction

The World Health Organization estimates that one-third of the global adult population smokes. Tobacco smoking is one of the main reasons for increased mortality by 20% [14]. Nicotine, the main addictive component of tobacco, initiates synaptic and cellular changes that underlie the motivational and behavioural alterations that culminate in addiction [6]. Nicotine addiction is mediated through nicotine binding to brain nicotinic acetylcholine receptor (nAChRs) [11]. It stimulates dopamine releasing in the limbic system with which its rewarding effects are associated. In nicotine dependence, the expression of N-cholinergic and μ-opioid receptors in the brain is increased. In addition to central effects, nicotine manifests also peripheral effects – tachycardia,

elevated blood pressure, reduced smooth-muscle tonus of the abdomen and intestines – that are due to activation of the vegetative ganglia.

Nicotine tolerance is formed under the influence of pharmacodynamic, metabolic and behavioural mechanisms [15]. Every attempt to reduction or cessation of smoking causes development of abstinent symptoms – general discomfort, irritability, anxiety, reduced concentration, strong desire for nicotine, manifested already during the first hours. Due to the increasing social importance of the "nicotine dependence", various approaches for coping with the problem are sought for and applied. Along with nicotine-maintenance therapy – nicotine chewing gums, patches or nasal spray, other medications are also used for treatment of abstinence in nicotine-dependent individuals, some of which are of plant origin, such as lobeline and cytisine.

Tabex® is an original Bulgarian preparation of plant origin, intended for treatment of nicotine dependence. The preparation was developed on the basis of the alkaloid cytisine contained in the plant *Cytisus laburnum* L. (Golden Rain acacia). The plant is common for the southern parts of Central Europe and Italy. The largest quantity of the alkaloid – up to 3%, was found in the seeds. Cytisine possesses similar mechanism of action to that of nicotine: it excites vegetative nervous ganglia; increases adrenaline release by the adrenal gland; stimulates the reflex locomotor centre; elevates arterial blood pressure.

The objective of this study was to investigate the possible hepatotoxic effects of cytisine on isolated rat hepatocytes, in accordance with "Guidance for Industry".

The hepatotoxicity study of cytisine, a medicinal product described in details by Sopharma Pharmaceuticals, was carried out in comparison with nicotine.

Materials and methods

Methods

Compounds used

Cytisine (Sopharma Pharmaceuticals, Sofia, Bulgaria)

Nicotine base (Sigma Aldrich, Germany)

Chemicals

In our experiments, pentobarbital sodium (Sanofi, France), HEPES (Sigma Aldrich, Germany), NaCl (Merck, Germany), KCl (Merck, Germany), D-Glucose (Merck, Germany), NaHCO3 (Merck, Germany), KH_2PO_4 (Scharlau Chemie SA, Spain), $CaCl_2x2H_2O$ (Merck, Germany), Mg SO_4x7H_2O (Fluka AG, Germany), Collagenase from *Clostridium histolyticum* type IV (Sigma Aldrich, Germany), Albumin Bovine Serum Fraction V, minimum 98% (Sigma Aldrich, Germany), EGTA (Sigma Aldrich, Germany), 2-Thiobarbituric acid (4,6-dihydroxypyrimidine-2-thiol; TBA) (Sigma Aldrich, Germany), Trichloroacetic acid (TCA) (Valerus, Bulgaria), 2,2'-dinitro-5,5'-dithiodibenzoic acid (DTNB) (Merck, Germany) and Lactate dehydrogenase (LDH) kit (Randox, UK) were used.

Animals

Male Wistar rats (body weight, 220-250 g) were used. Rats were housed in plexiglass cages (3 per cage) in a 12/12 light/dark cycle, temperature 20 ± 2°C. Food and water were provided ad libitum. Animals were purchased from the National Breeding Centre in Sofia, Bulgaria. All performed procedures were approved by the Institutional Animal Care Committee and were in accordance with the requirements of the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purpose (1991).

Isolation and incubation of hepatocytes

Rats were anesthetized with sodium pentobarbital (0,2 ml/100 g). In situ liver perfusion and cell isolation were performed as described by Fau [7], with modifications [13]. After portal catheterization, the liver was perfused with HEPES buffer (pH = 7,85) + 0,6 mM EDTA, followed by HEPES buffer (pH = 7,85), without any addition and finally HEPES buffer, containing collagenase type IV and 7 mM $CaCl_2$ (pH = 7,85). The liver was excised, minced into small pieces and hepatocytes were dispersed in Krebs-Ringer bicarbonate (KRB) buffer (pH = 7,35), containing 1% Bovine Serum Albumin. After filtration, the hepatocytes were centrifuged at 500 x g and washed 3 times with KRB buffer. Cells were counted under the microscope and the viability was assessed by Trypan blue exclusion (0,05%) [7]. Initial viability averaged 89%. Cells were diluted with KRB, to make a suspention of about 3x106 hepatocytes/ml. Incubations were carried out in 25 ml Erlenmeyer flask. Each flask contained 9x106 hepatocytes. Incubations were performed in a 5% CO_2 + 95%O2 atmosphere [7].

In order to evaluate the effects of the test substances, cytisine and nicotine in vitro, seven consequently increasing concentrations (5 nM-250 µM) were used [12, 17, 8, 16, 18].

Parameters studied

Cell viability

Cell viability was assessed by Trypan blue (TB) exclusion. Trypan blue is a dye that has the ability to penetrate via destroyed cellular membranes and strain dead cells in blue. The cell viability was expressed as the percentage of cells viable at the 1st hour after the incubation with the tested compounds [7].

Lactate dehydrogenase (LDH) leakage

LDH leakage was measured spectrophotometrically as described by Bergmeyer [2].

Determination of reduced glutathione (GSH) levels

At the end of the incubation, cells were recovered by centrifugation at 4°C, and used to measure intracellular reduced glutathione (GSH), which was assessed by measuring non-protein sulfhydryls after precipitation of proteins with TCA, followed by measurement of thiols in the supernatant with DTNB. The absorbance was measured at 412 nm [7].

Lipid peroxidation (LPO)

Hepatocytes suspension was taken and added to 20% (w/v) TCA. After centrifugation, 1ml of the supernatant was added to 0,67% (w/v) TBA and heated at 100°C. The absorbance was measured at 535 nm, and the amount of TBARS was calculated, using a molar extinction coefficient of 1,56 x $10^5 M^{-1} cm^{-1}$ [7].

Statisitical analysis

Statistical analysis was performed by applying the Student's t-test with $p < 0,05$ considered statistically significant. All results (n = 7) are expressed as mean ± SD.

Results

The results from the conducted experiments on the effects of cytosine on isolated rat hepatocytes are shown in Tables 1-4.

The effects of cytisine and nicotine in seven consequently increasing concentrations on the cell viability are given in Table 1.

The results demonstrate that after 1 hour of incubation with the tested compounds the cell viability was statistically significant reduced by both cytisine and nicotine in concentration dependant manner. Cytisine, in concentrations 5 nM-250 μM, decreased, statistically significant cell viability from 12% ($p < 0,001$) to 61% ($p < 0,001$) versus control, while nicotine, in the same concentrations, decreased viability from 35% ($p < 0,001$) to 67% ($p < 0,001$). It is visible that in the low concentrations, the effect of cytosine was less toxic, compared to that of nicotine.

The effects of cytisine and nicotine on LDH leakage into the medium are shown in Table 2.

LDH leakage into the medium was statistically significant increased in concentration-dependent manner as by cytosine, as well as by nicotine, compared to the control. The toxic effect of cytosine on this parameter was comparable to that of nicotine. Cytisine showed significantly lower cytotoxicity than nicotine in concentrations 30 μM and 250 μM.

The effect of cytisine and nicotine on the level of the cell protector GSH is presented in Table 3.

The data demonstrated that in concentrations from 5 nM to 250 μM GSH was depleted by cytisine from 17% ($p < 0,01$) to 71% ($p < 0,001$), while nicotine – from 41% ($p < 0,001$) to 93% ($p < 0,001$). It is visible that similarly to the effect, exerted on cell viability, cytisine was less toxic than nicotine.

The effect of cytisine and nicotine on the MDA quantity in isolated rat hepatocytes is presented in Table 4.

Table 1. *Effect of cytisine and nicotine in seven consequently increasing concentrations on the cell viability on isolated rat hepatocytes (values are presented as the mean ± SD, n = 7)*

Group	**Cell viability (%)**		
		Cytisine	**Nicotine**
Control	**83 ± 5,6**		
5 nM		$73 \pm 4,9^{***\,+++}$	$54 \pm 6,8^{***}$
500 nM		$57 \pm 7,8^{***\,+++}$	$49 \pm 3,6^{***}$
1 μM		$46 \pm 8,0^{***\,+++}$	$39 \pm 5,6^{***}$
10 μM		$43 \pm 6,9^{***\,+++}$	$37 \pm 4,2^{***}$
30 μM		$39 \pm 3,0^{***\,++}$	$36 \pm 4,8^{***}$
100 μM		$37 \pm 6,2^{***\,+}$	$35 \pm 4,0^{***}$
250 μM		$32 \pm 7,6^{***\,+++}$	$27 \pm 5,4^{***}$

***$p < 0,001$ vs control
$^{+}p < 0,05$; $^{++}p < 0,01$; $^{+++}p < 0,001$ vs nicotine

Table 2. *Effect of cytisine and nicotine in seven consequently increasing concentration on LDH activity on isolated rat hepatocytes (values are presented as the mean ± SD, n = 7)*

Group	**LDH activity (nmol/min/106 hepatocytes)**		
		Cytisine	**Nicotine**
Control	**0,152 ± 0,06**		
5 nM		0,192 ± 0,04 **	0,194 ± 0,05 ***
500 nM		0,225 ± 0,04 ***	0,231 ± 0,03 ***
1 μM		0,261 ± 0,04 ***	0,262 ± 0,03 ***
10 μM		0,269 ± 0,05 ***	0,277 ± 0,03 ***
30 μM		0,288 ± 0,06 $^{***++}$	0,315 ± 0,03 ***
100 μM		0,338 ± 0,07 ***	0,349 ± 0,03 ***
250 μM		0,364 ± 0,04 $^{***+++}$	0,404 ± 0,02 ***

$^{***}p < 0,01$; $^{***}p < 0,001$ vs control
$^{++}p < 0,01$; $^{+++}p < 0,001$ vs nicotine

Table 3. *Effect of cytisine and nicotine in seven consequently increasing concentrations on GSH level on isolated rat hepatocytes (values are presented as the mean ± SD, n = 7)*

Group	**Level of GSH (nmol/106 hepatocytes)**		
		Cytisine	**Nicotine**
Control	**41 ± 15,0**		
5 nM		34 ± 8,8 $^{**+++}$	24 ± 5,9 ***
500 nM		28 ± 6,4 $^{***+++}$	19 ± 4,9 ***
1 μM		26 ± 5,5 $^{***+++}$	16 ± 4,2 ***
10 μM		23 ± 5,6 $^{***+++}$	13 ± 3,2 ***
30 μM		20 ± 4,8 $^{***+++}$	9 ± 3,8 ***
100 μM		15 ± 3,8 $^{***+}$	5 ± 1,2 ***
250 μM		12 ± 2,6 $^{***+++}$	3 ± 1,1 ***

$^{**}p < 0,01$; $^{***}p < 0,001$ vs control
$^{+++}p < 0,001$ vs nicotine

Table 4. *Effect of cytisine and nicotine in seven consequently increasing concentration on MDA quantity on isolated rat hepatocytes (values are presented as the mean ± SD, n = 7)*

Group	**Level of MDA (nmol/106 hepatocytes)**		
		Cytisine	**Nicotine**
Control	**0,152 ± 0,1**		
5 nM		0,189 ± 0,07 *	0,172 ± 0,08
500 nM		0,221 ± 0,1 $^{**++}$	0,181 ± 0,08
1 μM		0,267 ± 0,1 $^{***+++}$	0,197 ± 0,09 *
10 μM		0,270 ± 0,1 $^{***++}$	0,217 ± 0,09 ***
30 μM		0,283 ± 0,1 $^{***+}$	0,240 ± 0,09 ***
100 μM		0,294 ± 0,1 ***	0,281 ± 0,1 ***
250 μM		0,373 ± 0,2 ***	0,333 ± 0,2 ***

$^{*}p < 0,05$; $^{**}p < 0,01$; $^{***}p < 0,001$ vs control
$^{+}p < 0,05$; $^{++}p < 0,01$; $^{+++}p < 0,001$ vs nicotine

The results showed that cytisine, in concentration 5nM, increased TBARS quantity by 24% ($p < 0,05$), and the effect of nicotine was comparable to that of the control group. By increasing the concentrations, the toxic effects of the tested compounds were increased as well. In the highest concentration 250 μM, cytisine exerted more pronounced effect.

Discussion

Tobacco smoking is regarded as a form of nicotine addiction and is one of the major reasons for increased disease incidence and untimely death. Though smokers are usually aware of the negative consequences of smoking and more than 80% of them state firmly that they would like to quit smoking, this seems to be a difficult process. Nicotine withdrawal is manifested about 24 hours after the last smoked cigarette, and is considered as one of the main reasons for the high percentage of relapse.

Due to the high addictive potential of nicotine and to its multiorgan toxicity, various approaches for coping with this problem are sought for and applied. One perspective approach for treatment of nicotine dependence is the use of alkaloids of plant origin, such as cytisine, the main substance of the medicinal product Tabex® that possesses nicotine-like effects without posing a risk of dependence development.

In relation to the toxicity, there are reports from the manufacturing company Sopharma Pharmaceuticals for the following effects of Tabex® after oral administration in experimental animals [1]:

- Prominent toxic effects of the substance
- Good resorption index

In the studies for chronic toxicity it has been established that:

- In oral administration to mice, rats and dogs, Tabex® does not cause toxic changes in hemopoiesis and internal organs.
- Statistically significant increase of transaminases, compared to control groups at dose of Cytisine 1,35 mg/kg after 90-day administration.
- No changes are observed in the behavior of experimental animals and the examined clinical and laboratory indices.
- Pathohistological examinations show various degree of liver dystrophy in mice at doses of 3,3 mg/kg and in dogs at doses of 0,45 mg/kg.

Since the cytisine toxicity has not been determined in vitro, the objective of this study was to study possible hepatotoxic effects of cytisine, in comparison with the alkaloid nicotine, on isolated hepatocytes.

The model of isolated hepatocytes provides a possibility to evaluate the effects of various xenobiotics both from chemical and plant origin by direct interactions with the endogenous factors in the cell. The principle reason for using liver cells is their capacity for xenobiotic biotransformation that preserves few hours after isolation. Indicators of cytotoxicity that have been utilized with hepatocytes generally fall into two categories: those that measure membrane integrity (LDH leakage and trypan blue exclusion), and those that monitor cell function (level of cell GSH, MDA quantity) [10]. The possible changes in membrane integrity could be assessed by morphological changes in the cells, which are usually measured by Trypan blue exclusion test. LDH is probably one of the most commonly used enzyme markers, as its increased release in the medium is an indicator of membrane damage [9]. Increased LDH activity was accompanied by a decrease of cell viability. The use of markers of membrane integrity was complemented by analysis of cellular function. Assessment of the quantity of cell GSH characterized the possible toxic hepatic metabolism of xenobiotics [3]. It is known that cell GSH is a tripeptide and the most important non protein thiol in mammalian cells and plays an important role in cell detoxification and protection. The quantitative assessment of MDA gives information of the possible cytotoxicity associated with the formation of free radicals and unlocking of lipid peroxidation processes.

Summarizing the results of our study, we found out that cytisine exerts lower cytotoxicity on cell viability and GSH level than nicotine in equivalent concentrations.

It is known that nicotine undergoes metabolic activation to cotinin and nornicotine, which are known to inhibit mitochondrial respiration [4]. The strongly manifested nicotine-induced GSH depletion suggests presence of metabolites leading to reduction of GSH level [19].

Considering the other two parameters – LDH activity and quantity of MDA, it is visible that cytisine shows measurable with nicotine cytotoxic effects. These data are supported by the in vitro studies of Cormier et al. (2003) [5], which established that nicotine inhibits the generation of free radicals in the brain mitochondria. Similar mechanism of action of nicotine on the liver mitochondria, presented in hepatocytes, might be possible and our results on the parameter lipid peroxidation might be explained.

There are literature data that cytisine is excreted approximately 90% unchanged in the urine. The more prominent cytotoxic effect of cytisine on LDH leakage and MDA quantity, observed in vitro, might be due to the presence of the whole molecule that does not undergo biotransformation in the hepatocytes.

Conclusions

Considering the experimental data of our study on cytisine and nicotine in equimolar concentrations on isolated rat hepatocytes, we conclude that cytisine has significantly less prominent toxicity on cell viability and GSH level than nicotine. At the same time, we found higher toxicity of cytisine on MDA level. In our experiments in vitro, this might be related with the presence of the cytisine molecule that does not undergo biotransformation but interacts with endogenous structures of the hepatocytes during the incubation that probably leads to the changes observed. The higher cytotoxicity exerted by nicotine on cell viability and GSH level might be due to its metabolism.

The results from our study showed that nicotine did not affect MDA level in the same extent as cytisine. This might be explained with the literature data about the ability of nicotine to inhibit reactive oxygen species, involved in the process of lipid peroxidation [5].

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