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Cytidine does not affect acute toxicity of intravenously administered choline

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Abstract

Cytidine-5'-diphosphocholine (CDP-choline) is a key precursor for the intracellular synthesis of phosphatidylcholine and other phospholipids. Following either intravenous or oral application citicoline (CDP-choline of exogenous origin) undergoes quick decomposition to cytidine and choline, and for this reason it is frequently considered a prodrug. However, upon acute intravenous application in mice citicoline is, on a molar basis, 20 times less toxic than choline. To find out whether cytidine may attenuate toxicity of choline, in the present experiments we compared maximum tolerated doses of single intravenous injections of choline and equimolar mixture of choline and cytidine. We assumed that, if after oral intake a substantial part of citicoline is catabolised already in the intestine and its catabolites enter blood separately, intravenously applied equimolar mixture of cytidine and choline will be markedly less toxic than an equivalent molar dose of choline. However, the maximum tolerated single doses determined in our experiment for choline and equimolar mixture of choline and cytidine were similar. These data suggest that citicoline taken orally is not significantly decomposed in the intestinal lumen, but absorbed to blood as the intact molecule.

Key words: *citicoline, choline, cytidine, acute toxicity, rat.*

Introduction

Cytidine-5'-diphosphocholine (CDPCho) is an obligatory intermediate in the anabolism of phospholipids. Synthesized inside cells from choline and cytidine in the so-called Kennedy pathway, CDPCho plays a crucial role in the de-novo synthesis of phosphatidylcholine (PtdCho) [14]. Further, PtdCho is not an end-point but a central intermediate in the formation of other phospholipids, including phosphatidylserine, glycerophosphocholine, lysophosphatidylcholine, sphingomyelin, and ceramide [10].

Citicoline (CDP-choline supplied from an exogenous source) is being used in medicine since the 1970s. Its first episodic indication was acute pancreatitis [18], but soon after Lloyd A. Horrocks and his collaborators discovered that a mixture of citicoline and CDP-ethanolamine injected intracerebrally [8,19], or citicoline injected intracisternally [38] attenuate fatty acid release from brain phospholipids during ischemia. Further experiments indicated that intraperitoneal injections of citicoline improve recovery of spontaneous motor activity in a rat model of transient cerebral ischemia [21]. These observations paved the way for testing citicoline as a treatment of human acute brain ischemia, and the first placebo-controlled study performed with ischemic stroke patients in Japan [37] yielded seemingly positive results.

In the years that followed citicoline became widely used as a nootropic drug indicated for a variety of neurodegenerative diseases ranging from Parkinson's disease to senile cognitive impairment [33,35]. In particular, its positive effects in acute brain ischemia have

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been repeatedly confirmed in multiple preclinical studies in which citicoline was applied intraperitoneally (e.g. [4,17,34]). However, subsequent controlled clinical trials did reproduce neither the promising preclinical data, nor the initial success reported in the aforementioned Japanese study. The authors of a recent meta-analysis [24] juxtaposed results of 10 randomized controlled trials that included 4,281 ischemic stroke patients. In these trials citicoline was given orally, intravenously, or as a combination of an intravenous and oral application. The authors concluded that little to no difference was detected between citicoline-treated patients and controls in all-cause mortality, disability, and the assessment of the neurological function (although the evidence was rated as of low certainty).

When one looks at the history of citicoline as a drug for ischemic stroke, a question of equivalence between different routes of citicoline administration arises. In the aforementioned initial experiments performed by Horrocks's group, citicoline was given intrathecally. In the later animal experiments performed by several independent groups, citicoline was given almost always intraperitoneally. However, the largest clinical trials with citicoline for ischemic stroke involved oral or mixed (initially intravenous, followed by oral) drug application. In a pivotal article [7] the rationale for switching from parenteral to oral delivery route was presented as follows: citicoline will be considered a prodrug which, when given parenterally or orally, undergoes a quick hydrolysis in the intestine and in the circulation to cytidine monophosphate (CMP) and phosphocholine (PCho) followed by dephosphorylations to yield cytidine and choline. Therefore, the active principle of the citicoline prodrug is in fact cytidine and choline, compounds which separately enter the brain and are very efficiently used in the Kennedy pathway to generate phospholipids.

Indeed, relatively fast hydrolysis and dephosphorylation of citicoline upon its intravenous or oral application was described in experiments in which the fate of citicoline doubly labelled with radioactive isotopes 3H and 14C was followed in blood plasma [12,13]. On the other hand, it has never been solved whether following oral application citicoline is predominantly hydrolysed and dephosphorylated already in the intestinal lumen, or later in the circulation. Answering this question is important (Fig. 1). If ingested citicoline molecules are absorbed to blood prior to hydrolysis and dephosphorylation, intact citicoline may exert some specific pharmacodynamic activity, in particular it may enter the brain. Actually, upon oral application of citicoline to human volunteers, citicoline action as α7 nicotinic acetylcholine receptor agonist has been postulated [5]. However, there is no direct proof that intact citicoline interacts with these receptors, whereas choline is known as their selective agonist, although one order of magnitude less potent than acetylcholine (EC50 of choline and acetylcholine in the rat brain is 1.6 mM and 0.13 mM, respectively [3]). Conversely, if the catabolic decomposition of citicoline preferentially occurs already in the intestine, ingestion of citicoline would not be equivalent to citicoline given parenterally, but rather to ingestion of equimolar amounts of cytidine and choline. Not only such a mixture of simple ingredients would be less expensive than citicoline but, more importantly, oral intake of citicoline could be significantly less neuroprotective than citicoline given parenterally.

A straightforward answer to the aforementioned question would come from evaluating kinetics of intact citicoline in blood following oral intake of the compound. Unfortunately no such data have ever been published, and pharmacokinetics of citicoline was assessed only indirectly by assaying its catabolites [23,32,39].

When we tabulated published rodent acute toxicity data for choline and citicoline [36], it turned out that, for any route of administration (oral, intraperitoneal, intravenous), citicoline is, on a molar basis, less toxic than choline. When the compounds were applied intravenously, the difference was the largest, citicoline being more than 20-fold less toxic than choline. Our preliminary interpretation was that intact citicoline upon entering blood and tissues does not evoke acute cholinergic toxicity because it is not a substrate for acetylcholine synthesis. Later, however, it occurred to us that an alternative explanation is thinkable: low toxicity of oral citicoline compared to choline may occur when, following ingestion, citicoline is preferentially hydrolysed and dephosphorylated already in the intestine and cytidine entering blood along with choline somehow lowers choline toxicity. This could, for example, occur when cytidine stimulates choline uptake by the liver and flatten choline concentration-time profile in blood.

The aforementioned possibility prompted us to perform current experiments designed to verify whether cytidine may attenuate choline toxicity when both substances appear in blood simultaneously. Acute toxicity tests that have used mortality as the main observational endpoint to determine LD50 values following single dose are currently considered obsolete [26] and are also not allowed on ethical grounds. Instead, we chose to determine and compare maximum tolerated doses (MTD) of choline and choline plus cytidine over 2 weeks following intravenous application of single doses of the tested compounds. Various definitions of MTD can be found in the literature. For example Gad [11] defined it as estimated maximum dose that, when administered for the duration of a specific study, will not compromise the survival of the animals by causes other than car-

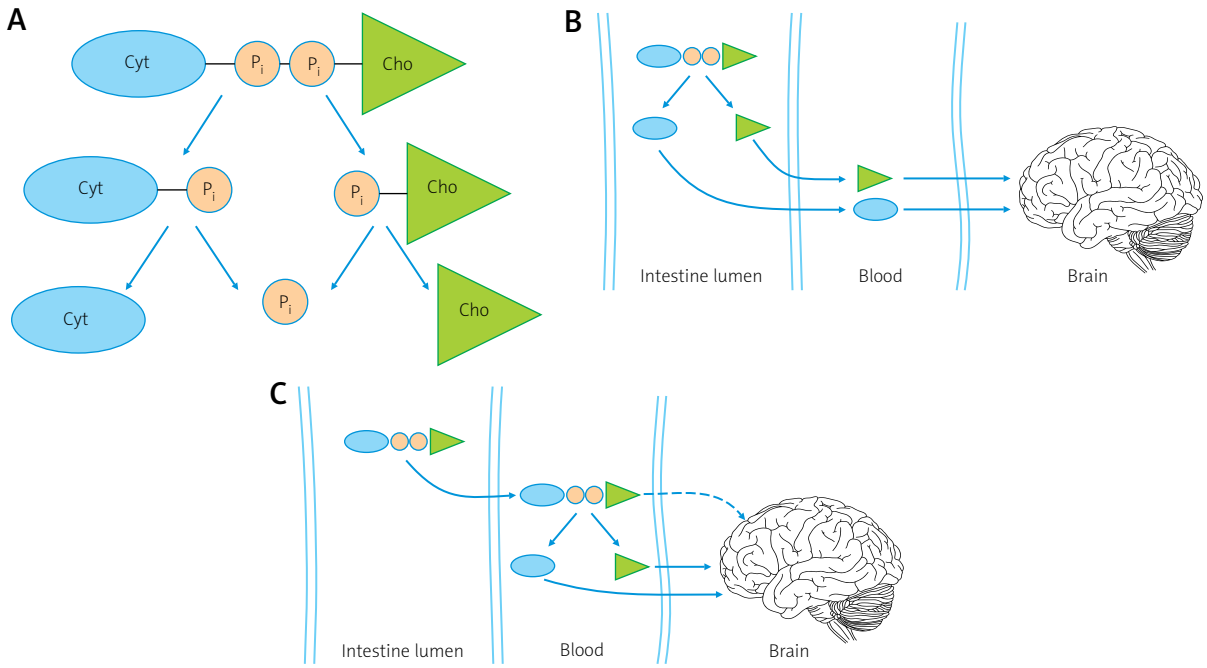

Fig. 1. Following oral delivery, citicoline is converted to choline and cytidine. It is not known whether this conversion takes place already in the intestine or only in blood. **A**) Two-step catabolism of citicoline consists of hydrolysis to CMP and PCho, and subsequent dephosphorylations. Blue ellipses – cytidine moiety (Cyt); pink circles – phosphate group (Pi); green triangles – choline moiety. **B**) If citicoline catabolism preferentially occurs in the intestine, cytidine and choline separately enter blood and are taken up by brain tissues. In such case, oral citicoline intake would be equivalent to concomitant oral intake of cytidine and choline. **C**) If intact citicoline crosses intestine walls and its catabolism preferentially occurs in blood, brain uptake of unhydrolysed citicoline may also occur.

cinogenicity, and in humans MTD was defined by the occurrence of severe toxic side effects during the first course of treatment [28]. For the sake of the present experiments, MTD was defined as any observable sign of toxicity, even if it was not severe and did not compromise survival of the animals.

Material and methods

Animal experiments were conducted in the animal facility at the Łukasiewicz Research Network – Institute of Industrial Organic Chemistry, Pszczyna Branch (Poland), following approvals of the Local Ethical Committee for Experiments on Animals in Katowice (approval number 11/2020 dated 9 March 2020, 33A/2020 dated 20 July 2020 and 33B/2020 dated 20 July 2020). As a biological test, system 8-week old male and female Wistar rats (Cmdb: WI; outbred) were used. The animals were obtained from the husbandry of laboratory animals of the Experimental Medicine Centre at the Medical University in Białystok, and kept behind the breeding barrier in standard conditions (air temperature 21-25°C, relative air humidity 41-60%, artificial fluorescent light with 12/12 lighting cycle).

The rats were acclimatized for 5 days under the experimental conditions. A detailed medical-veterinary examination was performed prior to the beginning of the experiment, and only healthy animals were used. Following injection of the tested compounds the animals were kept in cages with a plastic bottom (58 × 37 × 21 cm) covered with wire bar lids, with ad libitum access to the standard granulated fodder and tap water.

Choline chloride (purity > 99.9%) and cytidine (purity 99%) were purchased from Sigma-Aldrich. To estimate MTD of the tested compounds, test groups of 5 males and 5 females were planned for each dose of the tested compounds. Initial doses were chosen based on the literature data on LD50. We were unable to find scientific literature data on LD50 of cytidine, but according to industrial data (Sigma-Aldrich, 2021) LD50 of cytidine following intraperitoneal application in a mouse is 2,700 mg/kg b.w., therefore potential cyti-

dine toxicity is not an issue in our experiment. We were also unable to find scientific literature data on LD50 in a rat after single intravenous choline injection, but LD50 after single intraperitoneal choline injection in a rat was reported as 35-74 mg/100 g b.w., equivalent of 2.5 to 5.3 mmol/kg b.w., wherein all deaths occurred within 20 min after the injection [6]. We assumed that starting dose in a single dose MTD experiment should be considerably lower than the toxic dose, and chose 0.75 mmol/kg b.w., which is slightly lower than 1/10 of the LD50 of i.v. choline in mice (8.64 mmol/kg b.w.).

For intravenous injections, the rats were briefly anesthetized with isoflurane, and the substances tested, freshly dissolved in sterile apyrogenic water and heated to the body temperature, were injected into the tail vein. The injected volume was 0.1 ml/100 g b.w., far below the recommended limit of volume injected intravenously in rodent experiments, which is 5 ml/kg [27].

After injection, the rats were observed for 14 days for any detectable symptoms of toxicity. The observation involved evaluation of changes in the skin, fur, eyes, and mucosal membranes, assessment of the function of respiratory, circulatory, autonomic and central nervous systems, somatic activity, and behaviour. Particular attention was paid to the possible occurrence of tremors, convulsions, salivation, diarrhoea, lethargy, sleep, and coma. Food intake and body weight of the animals was also monitored daily. After 14 days, all surviving animals were euthanized and subjected to post-mortem examinations.

Results

Initially two males received doses of 3 mmol/kg b.w. choline chloride and one female received a dose of 3 mmol/kg b.w. choline chloride and cytidine. These animals died immediately after administration of the tested compounds, and the error in dosing was corrected.

The dose of 0.75 mmol/kg choline chloride evoked clinical signs in 3 of 4 males and 2 of 5 females. Salivation occurred in 3 males and 1 female, porphyrin depositions around the eyes ("red tears") were found in 2 males and 2 females, respiratory murmurs in 2 males, seizures in 1 male and 1 female, tremors in 1 male, and a decrease in locomotor activity in 2 males. The dose of 0.75 mmol/kg of choline chloride plus 0.75 3 mmol/kg of cytidine evoked clinical signs in 5 of 5 males and 2 of 5 females. Salivation was detected in 2 males and 2 females, respiratory murmurs in 2 males, porphyrin depositions around the eyes ("red tears") were found in 4 males, and a distinct decrease in locomotor activity in 1 male. All aforementioned clinical signs were classified as mild due to the fact that they were transient, lasting up to 10 minutes after administration of the test item.

In the next step, doses of the tested compounds were decreased to 0.6 mmol/kg. Choline chloride in this dose evoked salivation in 2 of 3 males and 4 of 5 females. The dose of 0.6 mmol/kg of choline chloride plus 0.6 mmol/kg of cytidine evoked clinical signs in 4 of 4 males and 5 of 5 females. In all cases the only detectable clinical sign of toxicity was salivation.

Finally, doses of 0.3 mmol/kg of choline and 0.3 mmol/kg of choline plus 0.3 mmol/kg of cytidine were injected, and they did not evoke any clinical signs in any animal.

Post-mortem examinations revealed that choline chloride as well as the mixture of choline chloride and cytidine in doses of 0.75 mmol/kg and 0.6 mmol/kg resulted in the presence of cecum flatulence in the majority of animals. No such lesions were found in animals that received 0.3 mmol/kg doses.

Collation of the prevalent signs noted following injection of choline chloride alone or together with cytidine in male and female rats is presented in Table I. The conclusion of the study is that in rats of the Wistar strain, MTD of choline chloride and MDT of equimolar mixture of choline chloride and cytidine are similar, estimated between 0.3 and 0.6 mmol/kg b.w.

Discussion

Overstimulation of acetylcholine receptors may produce muscarinic, nicotinic, and central nervous system effects. The prevalent toxicities observed in our experiment following single intravenous application of choline chloride or the mixture of choline chloride and cytidine appear to be muscarinic effects. Salivation is a typical effect of overactivation of muscarinic receptors [30]. The toxicity revealed in post mortem examinations, cecum flatulence, could also be related to overstimulation of the muscarinic receptors, which are present in intestines and elicit intestinal smooth muscle contraction [9,20]. Muscarine, a natural product found in certain mushrooms, is a non-selective acetylcholine receptor agonist which does not penetrate cellular membranes; oral or parenteral application of this compound in experimental animals has been shown to activate the peripheral parasympathetic nervous system, whereas effects on brain were negligible and no significant reduction in body weight of the animals in comparison to controls occurred [31].

As mentioned in the introduction, citicoline has been used in medicine for almost 5 decades. Over this time period its pharmaceutical formulations as well as clinical indications evolved. Initially the drug was applied parenterally, not only in the treatment of stroke, but also in other indications such as Parkinson's disease [25] and glaucoma [29]. When oral formulations of citicoline were developed, it was argued that

Table I. Prevalent toxicities observed following *i.v.* injection of the tested compounds

	Choline chloride			Choline chloride + cytidine		
Dose (mmol/kg)	0.75	0.6	0.3	0.75	0.6	0.3
Any clinical sign	**♂♂♂**♂♂ **♀♀**♀♀♀	**♂♂**♂ **♀♀♀♀♀**	♂♂♂♂♂ ♀♀♀♀♀	**♂♂♂♂♂** **♀♀**♀♀♀	**♂♂♂♂** **♀♀♀♀♀**	♂♂♂♂♂ ♀♀♀♀♀
Salivation	**♂♂♂**♂♂ **♀**♀♀♀♀	**♂♂♂** **♀♀♀♀**♀	♂♂♂♂♂ ♀♀♀♀♀	**♂♂**♂♂♂ **♀♀**♀♀♀	**♂♂♂♂** **♀♀♀♀♀**	♂♂♂♂♂ ♀♀♀♀♀
"Red tears"	**♂♂♂**♂♂ **♀♀**♀♀♀	**♂**♂♂ ♀♀♀♀♀	♂♂♂♂♂ ♀♀♀♀♀	**♂♂♂♂**♂ ♀♀♀♀♀	♂♂♂♂ ♀♀♀♀♀	♂♂♂♂♂ ♀♀♀♀♀
Cecum flatulence	**♂♂♂**♂♂ **♀♀**♀♀♀	**♂♂**♂ **♀♀♀♀♀**	♂♂♂♂♂ ♀♀♀♀♀	**♂♂♂♂♂** **♀♀♀**♀♀	**♂♂♂♂** **♀♀♀♀♀**	♂♂♂♂♂ ♀♀♀♀♀

citicoline is a prodrug which, following administration by either oral of parenteral route, releases its two main components, cytidine and choline [7,35].

Currently there is no reasonable doubt that parenteral and oral citicoline dosing exert similar effects in patients with open angle glaucoma; importantly no such effect has been reported following choline and other choline esters ([16] and the references cited). However, in ischemic stroke patients citicoline appeared beneficial when was given parenterally, but the benefits were not confirmed when the compound was given orally. A question whether citicoline, when used for the treatment of stroke, is acting as a prodrug or an active compound, has been discussed in the previous review [15], and the conclusion was that unhydrolysed (intact) citicoline may be pharmacologically active in this setting.

Relevant to this issue are studies of liposomal formulations of citicoline, which in preclinical experiments have been shown significantly more active in decreasing ischemic infarct volume than equivalent doses of free citicoline [1]. Recently it has been demonstrated that liposomes containing citicoline may be targeted specifically to the peri-infarct areas where this drug may be the most useful [2,22]. Such a method of intact citicoline delivery might be more similar to the intrathecal injections of this substance used by Horrocks and collaborators in the very first experiments performed 40 years ago. It remains to be confirmed that intact citicoline molecules targeted locally to the ischemic penumbra are able to cross the blood-brain barrier, reach cell membranes and efficiently suppress the decomposition of cellular phospholipids.

On the other hand, on the major world markets citicoline is currently accepted as a food ingredient, and recognized as a nontoxic source of bioavailable choline. As we have noticed recently [36], putative resistance of citicoline to intraintestinal hydrolysis is of importance also because the intestinal microbiome metabolizes a significant fraction of choline to trimethylamine (TMA), a gaseous metabolite further oxidised to its N-oxide (TMAO) which is suspected of being atherogenic. Tissue bioavailability of choline moiety from citicoline seems significantly higher than that of free choline, making this substance a safe and efficacious exogenic source of choline required for support of cellular phospholipid biosynthesis.

Conclusions

The result of the present study indicates that concomitant administration of cytidine does not dramatically increase the threshold of cholinergic toxicity evoked by intravenous choline chloride. Whereas such conclusion does not invalidate observations that in circulating blood citicoline undergoes quick hydrolysis and dephosphorylations to choline and cytidine, it supports the hypothesis that upon oral intake of citicoline a major part of the dose is absorbed to blood prior to hydrolysis. When a major part of the ingested citicoline dose enters blood as intact molecules, a fraction of these may escape hydrolysis and dephosphorylation, enter the brain, serve as an active compound and provide neuroprotection.

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Disclosures

The study was approved by the Local Ethical Committee for Experiments on Animals in Katowice (approval number 11/2020 dated 9 March 2020, 33A/2020 dated 20 July 2020 and 33B/2020 dated 20 July 2020).

The authors declare no conflict of interest.

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