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Drinking "Vodka" or vodka – This is a question☆

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ABSTRACT

"Vodka" *i.e.* 2-methyl-2-butanol (2M2B) is growing in popularity as a substance of abuse, especially among East European youngsters. At present, there is not much data regarding its toxicity both in humans and animals.
The direct effect of 2M2B on human tissue was evaluated and compared to that of two other alcohols (ethanol, 3-methyl-1-butanol). The used concentrations corresponded to those obtained from consumers of 2M2B. The experiments were carried out on HEK293 cell line with the use of the following techniques: MTT test, phase contrast and fluorescent microscopy.
The MTT test indicated that the toxicity of 2M2B was comparable to that of ethanol, but it was much lower than that observed after 3-methyl-1-butanol (3M1B). The high toxicity of the latter alcohol was confirmed by the microscopy techniques. On the other hand, the toxicity of 2M2B – expressed by the reduction of the number of survived cells – was slightly higher compared to one induced by ethanol. Also, the values of pIC50 for each alcohol reflect its level of toxicity described above.
On the basis of the literature data it is possible to argue that the toxicity of the tested alcohols results primarily from membrane damage induced by their solvent properties.

1. Introduction

Ethanol is a powerful psychoactive substance and its recreational use has a long history and goes back to ancient times. Drinking alcoholic beverages is highly prevalent in many cultures and, for most adults, moderate usage is not harmful. However, because of ethanol's widespread availability, it is one of the most popular substances of abuse. At present, another alcohol such as 2-methyl-2-butanol ("Vodka", 2M2B, *tert*-amyl or "Bluelight") appeared on the "drug scene", and it is gaining great popularity, especially among young people in Eastern Europe. Both alcohols produce similar CNS effects by the stimulation of GABA-A receptor (Martin et al., 2002, 2004).

Among the main reasons for the replacement of ethanol with 2M2B is its high potency (20 times higher than that of ethanol). Thus, it is not surprising that a volume of 7.5 ml of this alcohol elicits a rapid state of inebriation (warm alcohol glow, a feeling of well being, increased talkativeness) equal to that obtained after 5–7 shots (about 150 ml) of normal vodka. Also, the resultant low cost per dose of 2M2B inspires the consumers to buy it. Another relevant advantage stressed by the public is that this alcohol neither produces a hangover (it is not metabolized to aldehydes) nor is detected by common screening devices (Anand et al., 2014).

Until now, the toxicity of 2M2B in human beings, which is based only on single case reports of acute intoxication (Snyder, 1992) or of occupational exposure, has not been completely recognized. Similarly, the data obtained in animals are also scarce and controversial (Strubelt et al., 1999; Lewis, 2004; Bingham et al., 2001; Sax, 1984).

At present, 2M2B, as a product of TAME (tertiary amyl methyl ether) metabolism, has gained attention with respect to a possible health risk because of its increasing usage as a gasoline oxygenate, which improves combustion and thereby reduces the level of CO and aromatic hydrocarbons in automobile exhaust (Le Gal et al., 2001). The utilization of oxygenates is expected to increase further over the next decade to reduce urban air pollution (Health Effects Institute, 1997).

Summing up all of the data presented above, it was reasonable to investigate the direct impact of this alcohol on human embryonic kidney cells (HEK293). The line was chosen because free 2M2B and its metabolites are excreted with urine. Moreover, its toxicity was compared to that of two other alcohols *i.e.* ethanol (low toxic) and 3M1B (moderate/very toxic). The latter one is a substrate for production of artificial flavours and an ingredient of fusel oil (Hori et al., 2003).

2. Methods

2.1. Cell line and culture conditions

HEK293 (human embryonic kidney – obtained from ATCC, http://www.lgcstandards-atcc.org/products/all/CRL-1573.aspx?geo_country=

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pl) cell line was cultured in DMEM medium with 4.5 g glucose/l (Sigma-Aldrich, Germany) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Sigma-Aldrich, Germany) and 1% PEN/STREP (Sigma-Aldrich, Germany). The cell line was grown in 5% CO_2 humidified atmosphere at 37 °C. The original line was checked for the presence of mycoplasma.

2.2. Concentrations of alcohols

Three different types of alcohols were used, *i.e.* 2M2B, ethanol and 3M1B. Their concentrations were as follows: 10 mM, 25 mM and 50 mM. The chosen concentrations were based on information obtained from patients who drank 2M2B as a substitute of ethanol. The control group was without any treatment.

2.3. Cell viability assay

The MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide] assay was based on the protocol described by Park et al. (1987). MTT (5 mg/ml) was dissolved in PBS, sterilized by filtration (0.22 μm Millipore® filter) and stored at 4 °C. HEK293 cells were seeded at a density of 10^4 cells/well in a 96-well plate in 100 μl of culture medium and allowed to grow for 24 h before adding 2M2B, ethanol or 3M1B. All agents were used at the final concentrations of 10 mM, 25 mM and 50 mM. The plates were sealed to avoid evaporation of the alcohol. After 24, 48 and 72 h of incubation, the cells were washed twice in PBS and next 100 μl of MTT (0.5 mg/ml) in serum free medium was added to each well. For MTT metabolism the incubation was continued at 37 °C for further 2 h. Formazan (a product of the MTT metabolism) was dissolved in 100 μl of acidified isopropanol and absorbance was measured at 570 nm using a DigiScan 340 microplate reader (AsysHitech GmbH, Austria). The results are presented as a percentage of the control value (100%) which was without any treatment.

2.4. Phase-contrast microscopy

Cells were seeded in a culture flask and cultured until gaining 70% of confluence. Next, the media were supplemented with 2M2B, ethanol or 3M1B in a concentration of 25 mM each. The incubation was carried out for 7 days. The cells were observed in inverted phase contrast microscope Delta Optical IB-100 equipped with an optical camera HDCE-50B at 40× objective (Delta Optical, Poland).

2.5. Fluorescent microscopy

The procedure of preparing fluorescent preparations was as follows. Coverslips (∅ 20 mm) after washing with concentrated HCl and rinsing with 70% ethanol were placed at the bottom of the 12-well plate. Cells were seeded at 10^5 per 22 mm well (12-well plate) and left for 24 h. Thereafter, they were washed three times in PBS and next, 2M2B, ethanol or 3M1B were added to the cells in the concentration of 25 mM. Samples were left for further 7 days.

To visualize the nuclei, the cells were treated with a blue dye Hoechst 3342 at the concentration of 1 μM (HO3342, Sigma-Aldrich, Germany). Then, the cells were fixed in a 4% formaldehyde solution for 15 min (diluted from 37% stock, Sigma-Aldrich, Germany), and placed upside down on a Superfrost glass plate (Roth, Germany). Vectashield mounting medium (Vector Laboratories, USA) was dropped between each mentioned glass plate and the coverslip. The edges of the coverslips were glued with a nail polish. The visualization was performed in a fluorescence microscope (Delta Optical IB-100, Delta Optical, Poland) and the fluorescence intensity emission for the dye was at 352 nm.

2.6. Statistics

The data of MTT test were expressed as a mean ± SEM of two independent experiments conducted in triplicates for each concentration. All the values obtained after exposition to each concentration of the alcohol in question were compared to the control group without any treatment. The data were analyzed by Friedman ANOVA & Kendall Concordance using STATISTICA version 9.1 data analysis software system and $p < 0.05$ was assumed to be statistically significant (StatSoft, Inc., 2010, www.statsoft.com). The pIC50 parameter was calculated by GraphPad Prism 7 (GraphPad Software Inc., 2016, www.graphpad.com). The data of fluorescent and contrast phase microscopy were obtained from experiments which were repeated 2-times.

3. Results

3.1. The impact of 2M2B on cell viability

Fig. 1 presents the cell viability of HEK293 cells after 24, 48 and 72 h incubation with three different concentrations of 2M2B, ethanol and 3M1B. Generally, the effect of 2M2B on cell viability was rather small. However, it was dependent on the concentration and time of exposition. Maximum drops (about 20%) appeared at the highest concentration (50 mM), both after 48 and 72 h of exposure. Moreover, the described impact of 2M2B on cell viability was comparable to that of ethanol, but entirely different from the one produced by 3M1B. The effect of the latter compound was much more pronounced and it was also concentration and time dependent. The maximum decrease in cell viability was 80% (50 mM, 72 h).

The impact of each alcohol on HEK293 cell survival was further confirmed by the calculation of pIC50 and the values are as follows: 2M2B – 1.699, ethanol – 2.025, 3M1B – 0.884.

3.2. The impact of 2M2B on cell morphology and mortality

The morphology of the cells, their number and survival rate were estimated by contrast phase or fluorescent microscopy (two different experiments) after 7 day incubation with the alcohols in question (Fig. 2). The effect of 2M2B became visible on cell mortality. In comparison to the control, the number of the cells was reduced (Fig. 2D), but the shape of those which survived and their connections were unchanged (Fig. 2C). On the other hand, neither the cell connections nor the number was affected by ethanol (Fig. 2E and F). More evident changes such as incorrect morphology, loss of connections (Fig. 2G) and a reduced number of cells (Fig. 2H) were caused by 3M1B.

7-day incubation with 2M2B at the concentration of 50 mM led to the death of all cells (not shown).

4. Discussion

The results of MTT assay presented here show that the toxicity of 2M2B was comparable to that of ethanol, but lower than that observed after incubation with 3M1B. The drops in cell viability caused by the usage of the former two alcohols did not exceed 20%, and those caused by the latter were within the range of 40–80%. In all cases, these drops were time and concentration dependent. However, the long-term toxicity (7 days) of 2M2B, visible in the images of the fluorescent and contrast phase microscopy as a decrease in the number of the survived HEK293 cells, is greater than that produced by ethanol. What is more, a further increase in the concentration of 2M2B up to 50 mM led to the death of all cells. The high toxicity of 3M1B found in MTT test has been confirmed by microscopic techniques.

The results presented above do not differ much from those obtained from animal experiments carried out *ex vivo* (isolated rat liver). Strubelt et al. (1999), investigating the effect of 23 alcohols (including those tested in this study) on versatile functional and biochemical hepatic