SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PhD)

EFFECTS OF ALIPHATIC ALCOHOLS OF ILLEGALLY PRODUCED SPIRITS ON GRANULOCYTE AND MONOCYTE FUNCTIONS

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Effects of aliphatic alcohols of illegally produced spirits on granulocyte and monocyte functions

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The Examination takes place at the Building “A”, Department of Internal Medicine, Faculty of Medicine, University of Debrecen, on 14th October 2014 at 11 am.

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The PhD defence takes place at the Lecture Hall of Building “A”, Department of Internal Medicine, Faculty of Medicine, University of Debrecen, on 14th October 2014 at 1 pm.
1. INTRODUCTION

Although ethanol containing beverages have been produced and consumed since the beginning of the human history, the level of alcohol drinking has increased remarkably from the 19th century, when spirits were started to be produced in large quantities. The volume of alcoholic beverages manufactured and sold was further enhanced during the 20th century and recently, acute and chronic diseases related to excessive alcohol-consumption have become a significant public health issue. The latest available report published by the World Health Organization (WHO) estimated that the worldwide adult per capita alcohol consumption was 6.13 litres in 2005. The amount of alcohol intake is the highest in Europe, where the average per capita pure alcohol consumption/year is almost 15 litres. Compared to the countries of the world and Europe, Hungary possess the third and second position with the value of 16.27 litres/capita per year, respectively.

Although most alcohol consumed worldwide is from commercial sources, with its manufacture and sale subject to regulation by government authorities, there is a certain amount (in some parts of the world it may be considerable) which is unregulated and thus unrecorded. Some are conventional beverages that have escaped the gaze of fiscal authorities as a result of smuggling or diversion of
legitimate production to the black market but others have avoided regulatory oversight from the beginning. They include home-made spirits, untaxed alcohols, counterfeit or relabelled alcohols, surrogate alcohols or non-beverage alcohols (perfumes, aftershaves, medicinal alcohols). Experts of the WHO estimated that the global adult per capita alcohol consumption from these unrecorded sources was 1.76 litres, equivalent to 28.6 % of alcohol consumed worldwide, although this varies considerably among countries, with the highest share (47.9 %) in low income countries but comprising a far from negligible share (11.2 %) even in the most developed countries. In 2005, the adult per capita alcohol intake from these unrecorded sources was 2.67 litres in Europe, equivalent to 22 % of alcohol consumed, although this varies considerably among countries, especially in Central and Eastern Europe, such as Hungary, Romania, Republic of Moldova, Ukraine, and Russia.

Unrecorded spirits have begun to attract attention from toxicological and public health researchers concerned about their potentially adverse health effects. Previous studies have demonstrated that illegally produced and home-made spirits are often contaminated with a variety of toxic heavy metals including lead, arsenic, antimony, cadmium, zinc, and copper which can release into these beverages from metal equipments used during distillation processes. The International Agency for Research on Cancer (IARC) classified
arsenic and cadmium as human carcinogens (IARC group 1.) and lead as possible human carcinogen (IARC group 2B). Besides heavy metals, acetaldehyde (IARC group 1.), the by-product of distillation, has been detected in illegal spirits. They have also been reported to be contaminated with ethyl-carbamate and water-derived nitrate and nitrite ions which belong to the group of probable human carcinogens (IARC group 2A). In addition, gas chromatographic studies have demonstrated that some illegally produced spirits and surrogate alcohols are often contain methanol and aliphatic alcohols comprising more than two carbon atoms, also called higher alcohols, such as 1- and 2-propanol, 1- and 2-butanol, isobutanol, and isoamyl alcohol. They are usually formed as by-products during alcoholic fermentation of maize, rice, wheat, millet, and, especially, many fruits.

Numerous epidemiological and clinical studies have demonstrated the etiologic role of excessive alcohol consumption in the development of several diseases affecting the gastrointestinal tract, pancreas, brain, heart and liver, among other organs. While ethanol in alcoholic beverages is primarily responsible for the liver damage, higher alcohols, have even more pronounced hepatotoxic effects. Therefore, compared to Western Europe, where drinking of illegal alcohol products is not significant, the markedly higher level of premature mortality from chronic liver diseases and cirrhosis in the
countries of Central and Eastern Europe may be associated with consumption of large volumes of home-made spirits containing aliphatic alcohols.

Alcohol does, however, have harmful consequences beyond hepatotoxicity and the effects of intoxication; clinical and laboratory studies have confirmed that both acute and chronic alcohol consumption can induce changes in immune cell functions including decreased antigen-specific T-lymphocyte activation and proliferation, modulation of monocytes, T-lymphocyte cytokine synthesis and B-lymphocyte immunoglobulin production, and reduced antigen presenting capacity of monocytes, macrophages and dendritic cells. These abnormalities can lead to increased susceptibility to bacterial and viral infections. As a consequence, alcohol abuse has been associated with higher incidence of a number of infectious diseases including tuberculosis, pneumonia, Human Immunodeficiency Virus-1 and Hepatitis C infections. Although functions of T- and B-lymphocytes, monocytes/macrophages have shown to be altered in chronic alcoholics, impaired granulocyte function has been proposed as the main factor in increased susceptibility to infections in heavy drinkers. Granulocytes, as the most abundant white blood cell population in humans, constitute the first line of host defence against bacteria, fungi, viruses and virally infected cells. They migrate rapidly to the site of infections, being
activated by a variety of chemotactic substances including N-formyl-methionyl-leucyl-phenylalanine (FMLP) and interleukin-8. Granulocytes, also known as polymorphonuclear leukocytes (PMNLs), play a pivotal role in phagocytosis and killing of invading pathogens via the generation of superoxide-anions ($O_2^-$) and related reactive oxygen species such as hydroxyl and perchlorate radicals and hydrogen peroxide. Beside granulocytes, monocytes play also a crucial role in engulfing and killing pathogenic microorganisms and defects in their functional state could contribute to the impaired antimicrobial defence observed in heavy drinkers.
2. AIMS OF THE STUDY

Although the exact mechanism is not known, alterations in functions of granulocytes and monocytes have been proposed as a mechanism in the impaired antimicrobial defence seen in alcohol abusers. It has been reported that ethanol can suppress the effector function of PMNLs and monocytes including $O_2^\cdot$ generation and phagocytosis. Less attention has been paid to the higher alcohols found in home-made spirits, among which only 1-butanol and isoamyl alcohol have so far been identified as reducing $O_2^\cdot$ production. However, it is possible that acute or chronic exposure to the other aliphatic alcohols in the home-produced spirits consumed in central and eastern Europe could alter $O_2^\cdot$ generation and phagocytosis, thereby contributing to alcohol-induced decreases in anti-microbial activity in affected individuals. To examine these possibilities, we investigated the effects of aliphatic alcohols of illegally produced spirits on these essential functions of human PMNLs and monocytes.
We intend to answer the following questions:

1. How do individual aliphatic alcohols alone and in combination with ethanol affect $\text{O}_2^{\cdot-}$ production by granulocytes as well as phagocytosis by PMNLs and monocytes?

2. Are aliphatic alcohols alone and in combination with ethanol able to influence $\text{O}_2^{\cdot-}$ production by granulocytes as well as phagocytosis by PMNLs and monocytes at biologically relevant concentrations?

3. Can aliphatic alcohols contribute to the ethanol-induced immunosuppression thereby increasing the susceptibility to infectious diseases in alcoholics and heavy drinkers?
3. MATERIALS AND METHODS

3.1. Separation of granulocytes for measurement of superoxide anion production

Granulocytes were isolated from human buffy coats produced by the Regional Blood Transfusion Centre of Debrecen. The buffy coat was mixed with dextran and the erythrocytes were allowed to sediment at room temperature for 60 min. PMNLs were separated from the leukocyte-rich supernatant with countercurrent centrifugal elutriation. The supernatant was loaded into the elutriation chamber and with stepwise increase of the flow rate, fractions of 100 ml were collected. Then the elutriation was stopped and granulocytes remaining in the elutriation chamber were washed out. The PMNL fraction was centrifuged and resuspended in Hanks’ solution. The viability of the cells was determined by trypan blue exclusion test and found to be 96-98%. The purity of granulocyte suspensions varied between 95-98%, as judged by morphology.

3.2. Treatment of granulocytes with aliphatic alcohols

Granulocytes were incubated in Hanks’ solution containing, separately, ethanol, 1-propanol, 2-propanol, 1-butanol, 2-butanol, isobutanol and isoamyl alcohol, at concentrations of 12.5 mM, 25.0 mM, 50.0 mM, 100.0 mM, 200.0 mM and 400.0 mM at 37°C for 60
min. PMNLs were also incubated in a mixture containing ethanol and each aliphatic alcohol at final concentration of 10 mM and 20 mM. Following incubation, the cells were centrifuged, the supernatant was removed and PMNLs were washed twice with Hanks’ solution. The viability of granulocytes was checked by trypan blue exclusion test after the treatments and was found to be 88-95%.

3.3. Stimulation and measurement of superoxide-anion production

Protein kinase C (PKC) mediated activation of reduced nicotinamide adenine dinucleotide phosphate oxidase, the $\mathrm{O}_2^\cdot$ generating enzyme in PMNLs, was investigated using phorbol 12,13-dibutyrate (PDBu) which is a widely used phorbol ester that stimulates $\mathrm{O}_2^\cdot$ production in a variety of cells. In addition FMLP, a chemotactic peptide, was applied to induce granulocyte $\mathrm{O}_2^\cdot$ release via the formyl peptide receptors. Superoxide-anion release was measured by superoxide dismutase (SOD) inhibitable reduction of ferricytochrome c. Granulocytes were incubated in Hanks’ solution, with PDBu and FMLP separately at 37°C for 15 min. The change in absorbance was measured spectrophotometrically at 550 nm with a double beam spectrophotometer at room temperature. The amount of $\mathrm{O}_2^\cdot$ secreted into the medium was determined on the basis of the molar extinction coefficient of reduced cytochrome c. The magnitude of effect of aliphatic alcohols on $\mathrm{O}_2^\cdot$ generation was calculated as follows: 100-
[(O$_2$\textsuperscript{$\cdot$} production by treated cells/ O$_2$\textsuperscript{$\cdot$} production by untreated cells) x 100].

3.4. Separation of granulocytes and mononuclear cells for investigation of phagocytosis by PMNLs and monocytes

After informed consent and the approval of the Institutional Ethical Committee at the University of Debrecen, peripheral blood was collected from healthy volunteers (n=11) in vacutainer test tubes containing EDTA. The age of the subjects varied between 23-54 years with a mean of 31.1 ± 4.3 years. All were non-smokers, non-alcohol abusers, had normal dietary habits, and were not taking any medications that could influence the results of the experiments. Blood sampling was performed between 8 and 9 AM and the samples were processed immediately. Blood samples were layered on the top of a discontinuous Ficoll gradient (1.077 and 1.119 g/ml) and centrifuged. Mononuclear and polymorphonuclear cells were collected from the top and interface of the Ficoll layers, respectively. Then the cells were washed twice with Hanks’ solution. Their viability was determined by trypan blue exclusion test and found to be 96-98%.
3.5. Preparation of FITC labeled and opsonized zymosan particles

Zymosan particles were labeled with fluorescein isothiocyanate (FITC) and opsonised with human AB serum. The particles were incubated in carbonate buffer containing FITC for 60 minutes at 37 °C. Then they were washed three times and opsonized in Hanks’ solution containing human AB serum at 37 °C for 30 minutes. The fluorescein isothiocyanate labeled and opsonized particles (FITC-OZ) were washed three times and stored at -20 °C in Hanks’ solution until the phagocytosis assay.

3.6. Phagocytosis assay

Granulocytes and mononuclear cells in Hanks’ solution containing heat inactivated human AB serum were placed into the wells of chamber slides to allow the cells to adhere for 30 minutes at room temperature. Subsequently, the non-adherent cells were removed by washing, the adherent cells and FITC-OZ were incubated in solution containing, separately, ethanol, methanol, 1-propanol, 2-propanol, 1-butanol, 2-butanol, isobutanol, and isoamyl alcohol at 37 °C for 60 min. Granulocytes and monocytes were also incubated in a mixture containing ethanol and each of the aliphatic alcohols. Untreated cells served as controls. The target/effector ratio was 10:1. Following incubation, the fluorescence of non ingested particles was quenched.
by trypan blue solution and the cells were fixed with paraformaldehyde. Monocytes were identified by indirect immunofluorescent method. The cells were labelled with anti-CD14 monoclonal antibody and then with immunoglobulin G-conjugated Dylight 594 fluorescent dye. The nuclei of the granulocytes and monocytes were stained with 4’,6-diamidino-2-phenylindol (DAPI) and the slide was then removed from the chamber for microscopic evaluation. The number of FITC-OZ/cell was determined with a fluorescent microscope by examining 1 x 100 cells in randomly selected microscopic fields. Then the phagocytosis index, the average number of ingested particles/cell, was calculated.

3.7. Statistical analysis

The results are presented as mean values obtained from six independent experiments. Differences in $O_2^{+}$ production by PMNLs as well as phagocytosis by untreated granulocytes and monocytes and cells treated with aliphatic alcohols were determined by repeated measures analysis of variance (ANOVA) using the Newman-Keuls post-hoc test. Values of $p < 0.05$ were considered to be statistically significant.
4. RESULTS

4.1. Effects of aliphatic alcohols on superoxide anion production by granulocytes

Granulocytes incubated in Hanks’ solutions containing 200 mM and 400 mM ethanol resulted a significantly decreased $O_2^-$ generation when the cells were stimulated with FMLP. PMNLs incubated with 200 mM 1-propanol, 400 mM 2-propanol, 1-butanol (50 mM, 100 mM) and 2-butanol (25 mM, 50 mM and 100 mM) released significantly lower amounts of $O_2^-$ in response to FMLP compared with control cells. Incubation of the cells with isobutanol (100 mM and 200 mM) and isoamyl alcohol (25 mM, 50 mM) resulted in a concentration dependent inhibition of the FMLP-induced $O_2^-$ generation. Our results indicates that the FMLP-induced $O_2^-$ production was also significantly decreased when PMNLs were exposed to a mixture containing ethanol and each aliphatic alcohol at final concentrations of 10 mM and 20 mM. There was no significant difference between the PDBu-induced $O_2^-$ production by untreated granulocytes and PMNLs treated with aliphatic alcohols at all concentrations tested.
4.2. Effects of aliphatic alcohols on phagocytosis by granulocytes

Granulocytes incubated with 0.5 mM, 1 mM, 10 mM ethanol, demonstrated a significantly reduced phagocytosis compared to control cells. PMNLs treated with 0.05 mM, 0.5 mM, 1 mM, 10 mM methanol, 2-propanol, 1- and 2-butanol showed significantly reduced phagocytic activity compared to control cells. Phagocytic function was significantly inhibited when granulocytes were incubated with 0.5 mM, 1 mM, 10 mM 1-propanol, isobutanol, and isoamyl alcohol. The phagocytosis index was also significantly decreased when the cells were exposed to a mixture containing each aliphatic alcohol at final concentrations of 0.05 mM, 0.5 mM, and 5 mM. Compared to the cells treated only with 10.0 mM ethanol, granulocytes incubated with a mixture containing 10.0 mM ethanol and each aliphatic alcohol at final concentration of 0.05 mM, 0.5 mM, and 5.0 mM showed significantly decreased phagocytosis.
4.3. Effects of aliphatic alcohols on phagocytosis by monocytes

Monocytes incubated with methanol, ethanol, 1- and 2-propanol, 1- and 2-butanol, isobutanol, and isoamyl alcohol at a concentration of 0.005 mM, 0.05 mM, 0.5 mM, and 5 mM demonstrated a significantly reduced phagocytosis compared to control cells. The phagocytosis index was also significantly decreased when the cells were exposed to a mixture containing each aliphatic alcohol at final concentration of 0.005 mM, 0.05 mM, 0.5 mM, and 5 mM. Compared to the cells treated only with 10 mM ethanol, monocytes incubated with a mixture containing 10 mM ethanol and each aliphatic alcohol at final concentration of 0.05 mM 0.5 mM, and 5 mM showed significantly decreased phagocytosis.
5. DISCUSSION

Consumption of unrecorded alcohols, particularly illegally produced spirits is one of the most common form of exposure to aliphatic alcohols affecting large populations worldwide. The effects of these alcohols on immune cell functions beyond that seen with ethanol have been less investigated. Therefore, it was reasonable to ask whether the ingestion of illicit spirits might pose an additional risk factor for development of ethanol-induced immunosuppression in heavy drinkers and alcoholics. To address this question, we investigated the effects of higher alcohols on those granulocyte and monocyte functions which play a pivotal role in the removal and killing of a variety of pathogenic microorganisms.

Our results have demonstrated that higher alcohols found in illegally distilled beverages could suppress the FMLP-induced \( \text{O}_2^\cdot \) generation by human PMNLs in a concentration dependent manner and that there were considerable differences in their inhibitory effects. Furthermore, they could act synergistically since combined exposure of the cells to a mixture of ethanol and higher alcohols resulted in a 2.5-40.0 fold decrease in the minimal inhibitory concentration and 1.9-12.5 fold increase in the magnitude of inhibition. In contrast, these alcohols did not reduce the \( \text{O}_2^\cdot \) release in response to PDBu in individual and combined exposures. In
addition, our results confirmed that aliphatic alcohols found in illegally distilled spirits could suppress the phagocytosis by human granulocytes and monocytes in a concentration dependent manner and that there were also considerable differences in their inhibitory effects. Furthermore, they could act synergistically with ethanol since combined exposure of the cells to ethanol and a mixture of higher alcohols resulted in a further decrease in the phagocytic activity of the cells.

Having shown that AAs can act in this way, the next step is to determine whether they might be expected to reach sufficient concentrations in the blood of those drinking illegal spirits? We have previously shown that the average concentrations of AAs in illegal spirits are as follows: methanol (68.2 mM), 1-propanol (10.5 mM), 1-butanol (2.0 mM), 2-butanol (5.0 mM), isobutanol (6.0 mM) and isoamyl alcohol (15.0 mM). Assuming these concentrations are present in a product also containing 40% ethanol, we can calculate how much would be needed to reach the minimal inhibitory concentrations of AAs in the blood using Widmark’s equation as follows: \( A = C \times p \times r \); where \( A \): the amount of alcohol consumed [grams], \( C \): blood alcohol concentration [g/liter], \( p \): body weight [kilograms], \( r \): Widmark’s factor [0.7 litre/kilogram for men; 0.6 litre/kilogram for women]. Applying Widmark’s factor for men to these levels of AAs and a product containing 40% ethanol, the
amount of spirits that would have to be consumed to reach the minimal inhibitory concentrations of AAs (0.05 mM and 0.005 mM in case of granulocytes and monocytes, respectively) in the blood varies from 35.0 to 1225.0 (granulocytes) and from 3.5 to 400.0 ml (monocytes) of spirits, according to the type of alcohol concerned.

The next question is whether anyone would actually drink these quantities, thereby ingesting sufficient quantity of AAs to inhibit phagocytosis by granulocytes and monocytes in vivo. These products produced illegally from fruits and grains are widely available in the CEE countries, going under different names include “pálinka” (Hungary), “sligovica” (Slovakia), and “samogon” (Russia, Ukraine, Belorussia). Someone meeting the WHO criteria for an episodic heavy drinker would consume at least 60 g or more of pure alcohol on at least one occasion in a seven day period. This would involve consumption of 190.0 ml of spirit containing 40% ethanol. This easily exceeds the quantities required to inhibit phagocytosis for all of the AAs studied except isobutanol. The other high risk group comprises teenagers and young adults who consume large volumes of alcoholic beverages on a single occasion resulting in severe drunkenness, commonly referred to as binge drinking and now reported from many parts of the world.

Illegally produced spirits are much cheaper than their commercially available counterparts. Therefore, it is reasonable to suppose that
alcohol abusers, heavy episodic and binge drinkers especially in the countries of Central and Eastern Europe can intake these spirits in such high quantity that the concentrations of ingested aliphatic alcohols in their blood can reach biologically relevant levels thereby decrease phagocytosis by granulocytes and monocytes. In this way, consumption of aliphatic alcohols of illegally produced spirits may contribute to increased susceptibility to infectious diseases in these subjects. Clearly, further studies are required to address this question.

On the other hand, it should be noted that some toxicokinetic studies have reported concentrations of higher alcohols and ethanol in the blood of alcoholics after ingestion of beverages containing aliphatic alcohols to be within the range of 0.01-0.10 mM and 10 mM, respectively. However, the peak concentrations of methanol and 1-propanol in the blood of alcoholics have been reported to be as high as 10.0 mM and 0.22 mM, respectively. Moreover, all of the eight alcohols tested inhibited the phagocytosis of monocytes at a concentration as low as 0.005 mM, which was below the range observed in alcohol abusers. In addition, they can increase the inhibitory effect of ethanol on phagocytic activity when combined with it.

Although the exact mechanism is not known, ethanol-induced inhibition of phagosome formation have been proposed as being implicated in suppression of phagocytosis. Granulocytes and
monocytes express several specialized receptors on their cell surface including Toll-like receptors (TLRs) and Fc gamma receptors (FcγRs). Engulfment of serum opsonised particles used in our experiments have been reported to be predominantly mediated by these receptors. After recognition and binding of these particles to TLRs and FcγRs, the receptors have been shown to recruit to lipid rafts, which are highly organised and tightly packed microdomains containing more sphingolipids and cholesterol than the surrounding membrane bilayer. Lipid rafts have been implicated as playing an important role in efficient receptor-ligand binding and signal transduction. Following binding of opsonin coated particles to FcγRs, the intracellular motifs of the receptors have been demonstrated to be phosphorylated by Src and Syk family of tyrosine kinases leading to actin polymerization, cytoskeleton rearrangement, and phagosome formation. Previous studies have indicated that ethanol can incorporate into the lipid bilayer and by increasing membrane fluidity can affect its integrity and disrupt the structure of lipid rafts. It has been suggested that this may reduce recruitment of FcγRs and TLRs to lipid rafts resulting in defects in receptor-mediated intracellular signal transduction and, in this way, reducing actin polymerization, cytoskeletal reorganization, and phagosome maturation. Given the structural similarity between ethanol and aliphatic alcohols, we hypothesize that the inhibition of phagocytosis could be mediated by similar mechanism.
6. SUMMARY OF NEW SCIENTIFIC RESULTS

1. Aliphatic alcohols of illegally produced spirits can inhibit the FMLP-induced superoxide anion production by granulocytes as well as phagocytosis by PMNLs and monocytes in a concentration dependent manner. They can act synergistically in combination with ethanol.

2. Aliphatic alcohols of illicitly produced spirits alone and in combination with ethanol can inhibit phagocytosis by granulocytes and monocytes at biologically relevant concentrations.

3. Aliphatic alcohols found in spirits from illegal sources can contribute to ethanol-induced immunosuppression thereby increasing the susceptibility to infectious diseases in alcohol abusers, episodic heavy drinkers and binge drinkers.
7. APPLICABILITY OF THE RESULTS

The consumption of aliphatic alcohols of illegally produced spirits is an important public health problem worldwide, especially in certain Central and Eastern European countries. Therefore, our investigation can provide evidence that consumption of aliphatic alcohols found in illegally produced spirits may contribute to the increased susceptibility to infectious diseases seen in alcohol abusers. The results of our study can be used for the characterisation of hazards associated with unrecorded alcohol consumption. Apart from chronic liver diseases and cirrhosis, alcohol-related injury, and immunosuppression, drinking of unrecorded spirits can be associated with increasing health expenditure. Thus, the social and economic consequences of hazardous alcohol consumption should also be considered by health professionals and policy makers. The results of this research can give information for decision makers to implement preventive measures for reduction of the harm caused by illicit alcohol consumption in the Central and Eastern European countries.
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List of publications related to the dissertation

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4. **Pál, L., Árnyas, E.M., Tóth, B., Ádám, B., Rácz, G., Ádány, R., McKee, M., Szűcs, S.:** Consumption of home-made spirits is one of the main source of exposure to higher alcohols and there may be a link to immunotoxicity.  
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9. PRESENTATIONS AND POSTERS

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