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Effect of Copper Overload Together with Ethanol Uptake on Hippocampal Neurons

GÜNFER TURGUT, ILGAZ AKDOĞAN,[1] ESAT ADIGUZEL[1] and OSMAN GENÇ

Physiology and [1]Anatomy Departments of Medical Faculty, Pamukkale University, Denizli, Turkey

TURGUT, G., AKDOĞAN, I., ADIGUZEL, E. and GENÇ, O. *Effect of Copper Overload Together with Ethanol Uptake on Hippocampal Neurons.* Tohoku J. Exp. Med., 2003, **199** (4), 239-245 —— Copper is an essential trace element which forms an integral component of many enzymes. While trace amounts of copper are needed to sustain life, excess copper is extremely toxic in the brain. Also, ethanol intake causes morphological changes in the brain. The present study aims to investigate effects of copper overload with ethanol intake in hippocampal neuron numbers of rat brain. Control and experimental group of rats (n=6 for each group) were fed ad libitum. Experimental group were given ethanol with copper in drinking water each day for ten days. Control group animals were given only drinking water during this period. Afterwards, animals were decapitated and their brains were removed by craniotomy. Frozen brains were cut by a cryostat. Sections collected via systematic random sampling were stained with hematoxylin and eosin. On microscopic images obtained from pyramidal cell layers in hippocampus, total neuron numbers were estimated using the optical fractionator method. We observed that pyramidal neuron numbers in the subdivisions of hippocampus were significantly lower in the experimental group than in the control group. These results suggest that copper overdose with ethanol intake can cause neuronal loss in hippocampus of rat brain. ———— copper; ethanol; hippocampus; rat; stereology
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Copper is an essential element for the activity of a number of physiologically important enzymes. Enzyme-related malfunctions may contribute to severe neurological symptoms and neurological diseases: copper is a component of cytochrome c oxidase, which catalyzes the reduction of oxygen to water, the essential step in cellular respiration. Copper is a cofactor of Cu/Zn-superoxide-dismutase which plays a key role in the cellular response to oxidative stress by scavenging reactive oxygen species. Furthermore, copper is a constituent of dopamine-beta-hydroxylase, a critical enzyme in the catecholamine biosynthetic pathway (Strausak et al. 2001).

While trace amounts of copper are needed

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Address for reprints: Dr. Günfer Turgut, Pamukkale Üniversitesi Tip Fakültesi, P.K. 33, 20020 Denizli, Turkey.

e-mail: gturgut@pamukkale.edu.tr

to sustain life, excess copper is extremely toxic (Sarkar 2000). Copper plays a fundamental role in the biochemistry of the nervous system (Waggoner et al. 1999). Recent studies have implicated copper in the pathogenesis of neuronal injury in Alzheimer's disease and the prion-mediated encephalopathies (Waggoner et al. 1999). Moreover, short-term (1 hour) exposure to copper also is neurotoxic and copper can be neurotoxic at physiologically relevant concentrations (Horning et al. 2000).

Copper can exist as Cu^{+} or Cu^{2+} in physiologic conditions and is highly redox reactive, and therefore very toxic to the cell as a free ion (Tiffany-Castiglioni and Qian 2001). Copper homeostasis in the central nervous system can be disrupted by environmental stresses and in neurodegenerative diseases (Tiffany-Castiglioni and Qian 2001). Cell-specific compartmentation of metals in the brain is an important consideration for developmental neurotoxicity induced by metals (Bush 2000).

Using a microscopic approach in animal model systems, alcohol-induced morphological changes in the brain have demonstrated significant cell loss in various neuronal populations including purkinje and granule cells in the cerebellum, mitral cells in the olfactory bulb, pyramidal cells in the hippocampus, neurons in the inferior olive, and cerebral cortical neurons (Wei-Jung et al. 2001). A reduction in brain size, and in particular the white matter, has been previously demonstrated in chronic alcoholics during life, as measured by magnetic resonance imaging and CT scans (Mann et al. 1995).

In recent years industrial, agricultural and zootechnic development has been responsible for the diffusion of copper in the environment, causing pollution of water, soil and atmosphere (Shallari et al. 1998; Barranguet et al. 2002; Gong et al. 2002). This study was designed to investigate the effects of ethanol and copper ingestion in the pyramidal neuron numbers of hippocampus of rat brain using the optical fractionator method.

Materials and Methods

Animals and experimental design

Twelve 15 weeks Wistar rats were randomly allocated to two groups of 12 rats each: an experimental group ($n=6$) and a control group ($n=6$). All rats were fed ad libitum. Experimental group were given ethanol 20% (0.5 ml) (Somani and Husain 1997) with copper sulphate (100 mg) (Zhang et al. 2000) in 25 ml drinking water (Fields and Lewis 1995; Prohaska 1997; Rouach et al. 1997) each day for ten days. Control group animals were given only drinking water during this period. At the end of experimental period all the animals were anaesthetized with light ether and then decapitated. The brain of rats were removed and frozen in cryostat (Leica CM3050, Bensheim, Germany) at $-50°$ C. Frozen brains sections were cut in the horizontal plane at a thickness of 150 μm by a cryostat chamber at -15°C. Sections collected via systematic random sampling were stained with hematoxylin and eosin.

Microscopic images obtained from pyramidal cell layers in CA1, CA2 and CA3 subdivisions of the hippocampus using $\times 100$ oil objective (N.A.$=$1.25) with a microscope (Nicon Eclipe E 600, Tokyo) were transferred to a monitor (Sony Trinitron Color Video Monitor PVM-14N1MDE, Weybridge UK) using a video camera (Hitachi OSP Color Video Camera VK-C22OE, Tokyo).

Animal care and all experimental procedures used were in accordance with those detailed in the Guide for Care and Use of Laboratory Animals published by the U.S. Department of Health and Human Services.

Neuronal counts and optical fractionator

Sections. Sections were chosen according to systematic random sampling scheme. The first section in the series to be analyzed was chosen randomly from the first 2–4 sections. This section and every 2th-4th section thereafter

was stained for use in the analysis. The section sampling fraction, ssf, is thus 1/2–1/4.

Sectional area. In each of the sections to be sampled, neurons were counted with optical disectors at regular predetermined x, y axis within the CA1, CA2, CA3 subdivisions. "x" step was predetermined 300 μm and "y" step was predetermined 300 μm. Microscopic images obtained from pyramidal cell layers in CA1, CA2, CA3 subdivisions of the hippocampus using $\times 100$ oil objective were transferred to a monitor. An unbiased counting frame (Gundersen's unbiased counting frame) (West et al. 1991) was then superimposed on the monitor image of the section. The area of the counting frame of the dissector, a (frame), was known (25 μm$\times$25 μm$=$625 μm^2) relative to the area associated with each x, y movement, a (x, y step). Thus, the areal sampling fraction (asf)$=a$ (frame)$/a$ (x, y step).

Section thickness. At each step in the pyramidal cell layers to be sampled the neuronal nuclei were first observed under the frame, then the plane of focus was moved 10 μm from into the section. The counting frame was then focused through 30 μm of the thickness of the section and the number of neuronal nuclei was counted with unbiased counting rules (Q^-). With the optical disector it is only necessary to determine the first recognizable profile of the nucleus to come into focus within the counting frame. The height of the disector was 30 μm for this study ($h=30$ μm).

At each step in the pyramidal cell layers to be sampled the distance between the positions of the stage where the neuronal nuclei of first layer came into focus from above and below the section (i.e., the top and bottom surfaces) was determined. The distance in between the top and the bottom surfaces was measured. The mean thickness of the section was calculated for each of the section used in the analysis. Thus, the fraction of the section thickness sampled, here referred to as the thickness sampling fraction (tsf)$=h$ (the height of the disector)$/t$ (the mean of the section thickness).

Neurons were directly counted in a known fraction of CA1, CA2, CA3 subdivisions of the hippocampus. The number of neurons in the CA1, CA2, CA3 subdivisions of the hippocampus (N) was estimated as;

$N=\Sigma Q^- \times (1/ssf) \times (1/asf) \times (1/tsf)$ (25)

ΣQ^-: The total number of neurons counted in the disectors on the sampled sections.

ssf: The section sampling fraction or the fraction of the sections sampled.

asf: The areal sampling fraction is then a (frame)$/a$ (x, y step).

tsf: The section thickness sampling fraction is then h/t.

h: The height of the dissector (30 μm)

t: The mean thickness of the sections).

RESULTS

Neurons were counted by using the optical dissector method in the frames determined with fractionator sampling scheme. We observed that pyramidal neuron numbers in hippocampus CA1, CA2 and CA3 subdivisions were signifi-

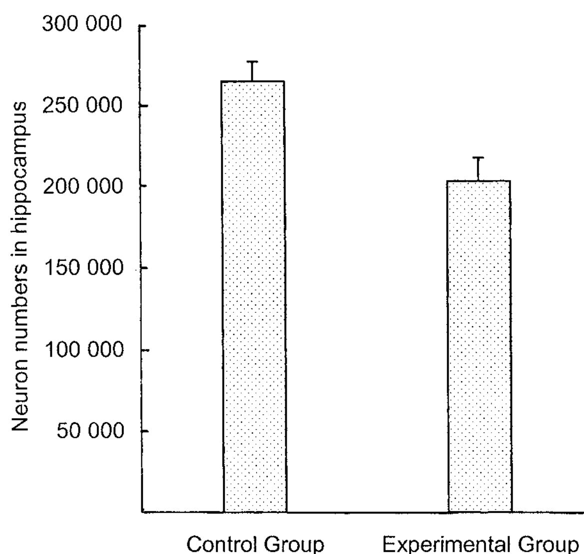

Fig. 1. Total pyramidal neuron numbers in hippocampus CA1, CA2 and CA3 subdivisions in the experimental and control group (Mann Whitney's U-test, difference $=23.27\%$, $p<0.01$).

TABLE 1. *Total number of neurons of CA1, CA2, CA3 subdivisions in the left hippocampus in the experimental group (Exp.) and parameters estimating of total number of neurons*

	Exp.					
	1	2	3	4	5	6
Q^- (The number of disectors)	148	151	155	174	176	164
$1/ssf$ (The section sampling fraction)	4	4	4	4	2	4
$1/asf$ (The areal sampling fraction)	144	144	144	144	144	144
$1/tsf$ (The section thickness sampling fraction)	2.08	2.25	2.34	2.12	2.11	2.23
t (The mean thickness of the section) (μm)	62.40	67.40	70.10	63.76	63.32	67.00
h (The height of the disector) (μm)	30	30	30	30	30	30
a (The unbiased counting frame) (μm^2)	625	625	625	625	625	625
N (The total number of neurons)	177 315	195 696	208 915	212 474	213 903	210 654

TABLE 2. *Total number of neurons of CA1, CA2, CA3 subdivisions in the left hippocampus in the control group rats and parameters estimating of total number of neurons*

	Control					
	1	2	3	4	5	6
Q^- (The number of disectors)	189	193	210	197	203	217
$1/ssf$ (The section sampling fraction)	4	4	4	4	4	4
$1/asf$ (The areal sampling fraction)	144	144	144	144	144	144
$1/tsf$ (The section thickness sampling fraction)	2.35	2.38	2.23	2.31	2.15	2.28
t (The mean thickness of the section) (μm)	70.60	71.40	67.00	69.50	64.50	68.60
h (The height of the disector) (μm)		30	30	30	30	3030
a (The unbiased counting frame) (μm^2)	625	625	625	625	625	625
N (The total number of neurons)	255 830	264 579	269 740	262 120	251 395	284 981

cantly lower in the experimental group (Mean$\pm$S.E.$=$203 159$\pm$14 249, Table 1) than in the control group (Mean$\pm$S.E.$=$264 774$\pm$11 824, Table 2), (Mann Whitney's U-test, difference$=$23.27%, $p<0.01$) (Fig. 1).

DISCUSSION

The individual effects of either copper (Armstrong et al. 2001) or ethanol (Bengoechea and Gonzalo 1991) on brain has been extensively studied previously. The goal of this study was to establish the effects of copper and ethanol together on neuronal cell number in rat hippocampus.

It is now generally accepted that ethanol can induce in vivo changes in membrane lipid

composition and fluidity which may effect cellular functions (Chin and Goldstein 1977). As ethanol increases the membrane fluidity of nerve cells (Edelfors and Jonsen 1990) and has been shown to alter the permeability of the blood brain barrier (Gulati et al. 1981, 1985). Thus these changes may play an essential role in copper uptake of the adult rat brain through blood brain barrier. In addition, it has also been reported that a copper transporting ATPase (Menkes protein) has been reported to be involved in the transport of copper across the blood brain barrier (Tiffany-Castiglioni and Qian 2001). However, this effect of ethanol on copper transporting ATPase has not been studied extensively yet. The above mentioned alterations, i.e., the increased copper uptake through blood brain barrier due to ethanol exposure or changes in the activity of copper transporting ATPase in blood brain barrier, may either contribute individually or together in this mechanism. On the other hand, the cell bodies of astroglia are juxtaposed between neuronal cell bodies and the capillary endothelium that forms the blood-brain barrier. Thus, astroglia are cytoarchitecturally positioned to be the first cells of the brain parenchyma to encounter metals crossing the blood-brain barrier (Tiffany-Castiglioni et al. 1996).

The mechanisms underlying the cellular toxicity of ethanol have been widely sought, but remain poorly understood. One mechanism that appears to contribute to this pathology is ethanol-related induction of free radicals/oxidative stress processes, and/or the down-regulation of protective antioxidants (Heaton et al. 2000). Ethanol-induced cell death and dysmorphogenesis in whole mouse embryos grown in culture have been found to be diminished by inclusion of free-radical scavengers in the culture media (Kotch et al. 1995).

Heaton et al. (1994) showed that relatively high alcohol concentrations reduced neurite extension and viability in primary cultured hippocampal neurons. Daily exposure to alcohol resulted in a dose-dependent decrease in the seizure threshold and in the selective loss of CA1 pyramidal cells. Reduction in the seizure threshold was significantly correlated with loss of CA1 pyramidal cells (Bonthius et al. 2001).

Acute or chronic ethanol administration can cause generation of oxygen free radical and oxidative stress to the brain. The generation of free radicals is thought to be one of the causes of cell injury after ethanol administration (Suzuki and Cherian 2000).

Alcohol exposure during the brain growth spurt in a rat model system produces severe damage to the developing central nervous system ranging from microencephaly (small brain for body size) (Goodlett et al. 1990) to significant loss of cerebellar Purkinje cells Hamre and West 1993; Marcussen et al. 1994), pyramidal cells in hippocampal CA1 field (Bonthius and West 1990) and glial cells in the cortex (Moony et al. 1996).

Astroglia also have emerging roles as major sites for the localization of brain copper (Tiffany-Castiglioni and Qian 2001). The brain concentrates metals for metabolic use, and therefore, brain cells possess physiologic properties for the protection of their cytoplasm from metal toxicity (Tiffany-Castiglioni et al. 1996). Normal brain contains potentially toxic levels of copper, iron, zinc and manganese (Bush 2000). Because these metals have high redox activity in their unbound ionic state, brain cells have protective mechanisms that allow them to exist in the cytoplasm without causing oxidative damage (Tiffany-Castiglioni et al. 1996). Furthermore, metals may share some mechanisms for entry, distribution, and storage in brain cells, which is an important consideration for neurotoxic xenobiotic metals (Tiffany-Castiglioni and Qian 2001). Also, some metals accumulate in astroglia, a circumstance that gives these cells potential roles as either metal depots or sites for toxic damage (Tiffany-Castiglioni and Qian 2001).

The results of the present study demonstrate that the effects of ethanol with copper ingestion in hippocampus of rat brain showing significant neuron loss. The neuronal loss may be resulting from neurotoxicity induced by copper and generation of oxygen free radical and oxidative stress caused by ethanol administration in hippocampus of rat.

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