The effects of ethanol on insulin-like growth factor-I immunoreactive neurons in the central nervous system

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ABSTRACT

الأهداف: تقييم أثر عقار الايثانول على تركيب خلايا (IGF–I) ومقارنة ذلك بين الأدمغة الطبيعية والأخرى التي تلقت المعالجة المزمنة بعقار إيثانول، باستعمال طريقة الكيمياء النسيجية المناعية.

الطريقة: أجريت هذه الدراسة الحالية بجامعة كوسيلي الطبية خلال الفترة ما بين مارس2006م إلى أكتوبر 2007م. تمت الاستفادة من التسريب الوعائي لإصلاح أدمغة الجرذان البالغة 10 لكل مجموعة. بعد تطبيق التقنيات النسيجية الروتينية، تم تثبيت العينات النسيجية بواسطة عقار بارافين. تم تطبيق الإجراء الكيميائي النسيجي المناعي لمقدار10ميكروجرام من السماكة وتمت ملاحظة ظهور الخلايا الموجبة (IGF–I) في المناطق العصبية المشرحة.

النتائج: اختلف توزيع التفاعل المناعي لخلايا التركيب (IGF–I) بين الطبقات للقشرة الدماغية الطبيعية والمناطق المهادية في الدماغ الكحولي. انخفض مقدار التفاعل المناعي (IGF–I) مقارنة مع المناطق العصبية المشرحة المفحوصة في الأدمغة الطبيعية.

خاتمة: تسيطر حضور التفاعل المناعي (IGF–I) في العصبونات للمناطق المشرحة المختلفة بوضوح أن هذه العصبونات الخاصة فعالة في تركيب (IGF–I). يظهر الانخفاض في التفاعل المناعي لتركيب (IGF–I) في المناطق الدماغية لدى الجرذان البالغة التي تمت معالجتها لفترة مزمنة بعقار إيثانول بوضوح أن عقار الإيثانول يؤثر بشكل سلبي على تركيب (IGF–I).

Objectives: To evaluate the effect of chronically ethanol treatment on insulin-like growth factor-I (IGF-I) synthesis in various adult brain regions using immunocytochemistry.

Methods: We performed this study at the Faculty of Medicine, Kocaeli University, Kocaeli, Turkey from March 2006 to October 2007. The vascular perfusion was utilized to fix the adult rat brains (10 for each group). After applying the routine histological techniques, the tissues were embedded in the paraffin. The immunohistochemical protocol was applied to the 10 μm thick sections and the expression of IGF-I positive cells were observed in the neuro-anatomic areas.

Results: The distribution of IGF-I immunoreactive cells differed between the layers of the normal cerebral cortex and in the thalamic areas. In the alcoholic brain, the amount of IGF-I immunoreactive cells were decreased compared to the similar neuro-anatomical areas examined in the normal brains.

Conclusion: The presence of IGF-I immunoreactivity in the neurons of the various neuro-anatomic areas demonstrates clearly that, these particular neurons are active in IGF-I synthesis. The decrease in the immunoreactivity of IGF-I in the chronically ethanol treated adult rat brain areas, show clearly that, ethanol effects negatively on the IGF-I synthesis.

Cell proliferation and cell death are independently regulated by both intracellular programs and by extracellular signal molecules. Proliferation, differentiation, maturation, migration, and viability of various cell groups are regulated by specific protein so-called 'growth factors'.[1,2] These factors that stimulate cell growth bind to receptors on the cell surface and activate intracellular signaling pathways.[1,3] Insulin-like

growth factor-I (IGF-I) is one of the growth factors.[1,3] Insulin-like growth factor-I is a mitogenic polypeptide which, along with ß-nerve growth factor and relaxin, is a member of a family of bio-active protein structurally homologous to insulin.[4] Although the main source of IGF-I (somatomedin-C) is the liver, other organs of the body including the brain are known to synthesize this peptide.[5] Evidence is accumulating which indicates that IGF-I can act by endocrine, paracrine and autocrine mechanisms.[6] Insulin-like growth factor-I influence growth as well as differentiation in ovary, lens, muscle, erythroid, thyroid, and central nervous system (CNS).[7] Previous studies, employing competitive displacement and affinity cross-linking techniques, demonstrated insulin and IGF-I receptors in the CNS.[8-10] However, information on the precise cellular distribution of IGF-I in the brain is still lacking. It presents in the brain throughout life. While their role as modulators of brain growth; differentiation during development is well known. Their possible involvement in adult brain function is less known. In addition, accumulating evidence indicate important roles for IGF-I in brain plasticity processes. Specifically, IGF-I modulates synaptic efficiency by regulating synapse formation neurotransmitter release and neuronal excitability in an adult brain. It also provides contact trophic support to target cells in the brain, and in this way the appropriate neuronal function was maintained.[10-13] Pathological dearrangement of this trophic input may lead to brain disease.[13,14] It is well known that chronic ethanol influences[15,16] a variety of metabolic processes and induces a generalized catabolic state, for example body weight and lean body mass of chronically ethanol (ETOH)-treated rats are diminished compared to pair-fed controls. Similarly, a decline in muscle strength, diminished muscle metabolism, and rearrangement of muscle fibers has been reported in alcoholic men. A decrease in bone density has also been observed after chronic ETOH intake. It is also known that the CNS is particularly susceptible to ethanol toxicity.[16,17] It was hypothesized that chronic ETOH abuse causes the degeneration of myelin and axons.[18] Metabolism of ETOH results in the production of the toxin acetaldehyde in the liver[19] and brain,[20] the accumulation of hydrogen peroxide, and the propagation of free-radicals. The neurodegenerative effects of ETOH are mediated by glutamate excitotoxity and changes in the calcium levels.[21] Increasing evidence shows that ethanol interferes with the action of growth factors.[22] The results of a study suggest that ethanol neurodegeneration in humans is associated with insulin and IGF resistance with attendant impairment of neuronal survival mechanisms and acetylcholine homeostasis.[23] It has been demonstrated that there is a decrease in plasma IGF-I levels in chronically ETOH administrated rats. This decrease in IGF-I may be due to the direct effect of ETOH on IGF-I producing cells or on growth hormone releasing hormone producing cells.[24,25] The aim of the present study was to evaluate the effect of chronically ethanol treatment on IGF-I synthesis in various adult brain regions using immunocytochemistry.

Methods. The experiments reported in this study were carried out in accordance with the Declaration of Helsinki. The ethical approval was granted by the Kocaeli University Ethics Committee, Kocaeli, Turkey. The present study was performed at the Medical School, Kocaeli University, Kocaeli, Turkey, from March 2006 to October 2007. We used adult male Wistar rats (220-260 g at the beginning of experiments). We placed them in a quiet, temperature and humidity-controlled room (23 ± 1°C and 60 ± 5%) in which 12-12 hours light-dark cycle was maintained. Rats were individually housed in plexiglass cages.

Administration of ethanol. As previously described,[26] we presented the modified liquid diet (MLD) to the rats (n=10) without ETOH for 5 days. We added 2.4% ETOH for 3 days. Then, we increased the ETOH concentration to 4.8% for 3 days. Finally, ETOH (7.2% v/v) was given to rats FOR 16 days. The MLD was prepared daily and they were fed at the same everyday (10:00 am). The final composition of the MLD with ETOH was: low fat cows milk (925 ml), ETOH (75 ml) (95.6% purity ethyl alcohol, Tekel, Turkey), low fat cows milk (925 ml, MİS Süt, Turkey), vitamin A (5000 IU), and sucrose (17 g) (tea sugar). This mixture supplies was 1000.7 kcal/l. While control rats (n=10) were pair-fed by an isocaloric MLD containing sucrose as a caloric substitute for ETOH. The weight of the rats was recorded everyday, and the daily ETOH intake was measured and expressed as grams per kilogram per day. Blood alcohol concentrations were determined by headspace gas chromatography method. On the 21st day, before removing ETOH from diet, we took blood samples from 3 rats using intracardiac puncture, under a very light ether anesthesia.

Daily ETOH consumption of the rats was in a range of 12.3-18.2 g/kg. Blood alcohol concentration (BACs) were 293.6 ± 5.2 mg/dl (mean ± SEM, n=10). After 21 days, there was a 3.8% decrease in weight of rats that received modified liquid diet. The technique of the administration of ETOH as part of a totally liquid diet is obviously preferable in the animal models for the development of ETOH tolerance and dependence.

Immunohistochemistry. Each rat were deeply anesthetized with ether, and transcardial perfused with saline, and then 4% paraformaldehyde in 0.1 M phosphate buffer. Fixed brains were removed

and post-fixed overnight in the same fixative. The brains were embedded in paraffin. Serial brain sections (10 μm) were cut, deparaffinized, and rehydrated. Immunohistochemistry was performed using the avidin-biotin-peroxidase method. The rehydrated sections were pretreated with 3% hydrogen peroxide for 10 minutes to eliminate endogenous peroxidase activity. Sections were then washed in PBS-Triton X (Tx) 100 (Sigma, Germany). To eliminate the nonspecific binding, sections were pretreated with normal rabbit serum. Sections were incubated in anti-IGF-I polyclonal antibody (UB2-495) and antihuman IGF-I antibody (dilution; 1:8000, obtained through the National Hormone and Pituitary Program) for 24 hours at 4°C in a humidified chamber. After PBS-Tx washing, biotinylated anti IgG secondary antibodies (100 μl, Histostain Plus Kits, Zymed, San Francisco, CA, USA) were applied for 15 minutes at room temperature. After PBS-Tx washing, streptavidin-peroxidase conjugate (Histostain Plus Kits, Zymed, San Francisco, CA, USA) was applied to the sections for 15 minutes at room temperature. Excess conjugate was removed with Tris buffer, and the tissue was immuno-treated with a solution of 0.6% hydrogen peroxide and 0.02% of diaminobenzidine (DAB) for 5 minutes at room temperature. The control staining for immunocytochemistry was carried out by omitting the primary antibody and replacing it with non-immune serum. The immunopositivity was examined by light microscopy (BX50F-3; Olympus, Tokyo, Japan).

Quantitative and statistical analysis. The number of IGF-I immunoreactive cells were quantified in 2.5 mm^2 fields of coronal brain sections in the immunoreactive areas of 10 rats of both groups with a x40 objective using an ocular micrometer system (Olympus, Tokyo, Japan). Quantification and measurements of the diameter of immunostained IGF-I cells in the distinct brain areas were performed by 3 investigators using the microscopic observation in a blinded approach. Similar levels of brain sections were maintained between the ETOH exposed and the control brains according to the atlas of Paxinos and Watson (1994).[27] Quantification and measurements of IGF-I immunoreactive cells were presented as mean ± SD. As for statistical analysis; Mann-Whitney U test was used. A *p*-value of less than 0.001 was interpreted as a statistically significant.

Results. Ethanol consumption and blood alcohol concentration were measured. The daily ETOH consumption of the rats ranged from 12.3-18.2 g/kg. Blood ethanol concentrations were 293.6 ± 5.2 (mean ±SEM). Insulin-like growth factor-I immunoreactivity was observed in the adult rat brains of the control (**Figures 1a & 2a**), and ETOH treated rats (**Figures 1b & 2b**). The neuronal cell bodies showed an intense immunoreactivity whereas the dendrites and the proximal part of the axons showed low staining (**Figures 1a & 2a**). In the brain of the control group (**Figures 1a & 2a**), IGF-I immunoreactive cells were present in the cerebral cortex (frontal, parietal and temporal cortex) (**Figures 1a & 1b**) and in the area surrounding the third ventricle corresponding to the thalamic area (**Figures 2a & 2b**). Among these neuroanatomic areas, IGF-I immunoreactive cells were more than half in the thalamus (**Figure 2**). The distribution of IGF-I immunoreactive cells differed between layers of the cortex of the control (**Figure 1a**) and ETOH treated (**Figure 1b**) rat brains. In the ETOH treated brains, IGF-I immunoreactive cells were decreased in number compared to the similar neuro-anatomical areas examined in the brains of the control group (**Table 1**).

In the control group of the cortical layers (frontal parietal and temporal), the localization of the IGF-I positive cells were observed in all layers except in the lamina I (lamina molecularis), but the number
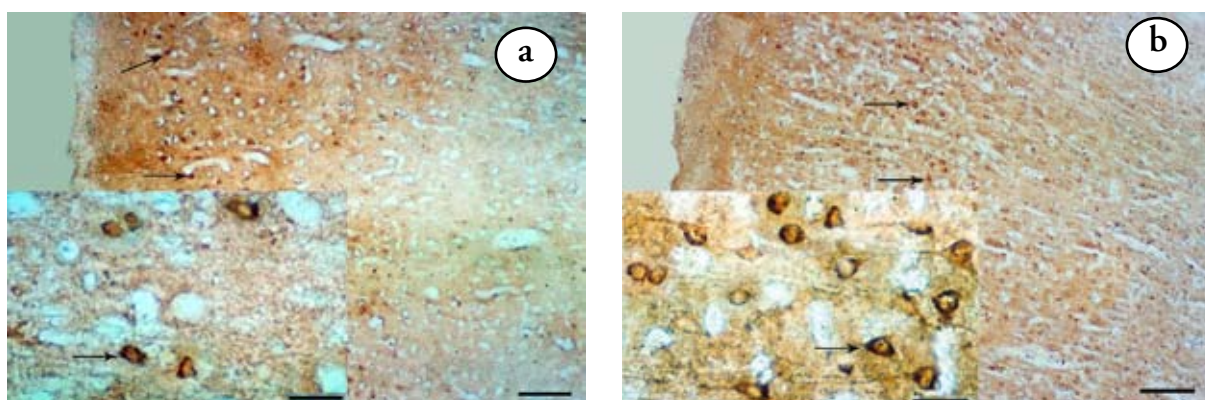

Figure 1 - Photomicrographs of insulin-like growth factor-I (IGF-I) immunoreactivity in the cortex. Note the significant decrease in density of IGF-I immunoreactive neurons in **a**) ethanol treated compared to the **b**) control rats. At a higher magnification, neuronal processes were evident (inset). Scale bar: 100 μm.
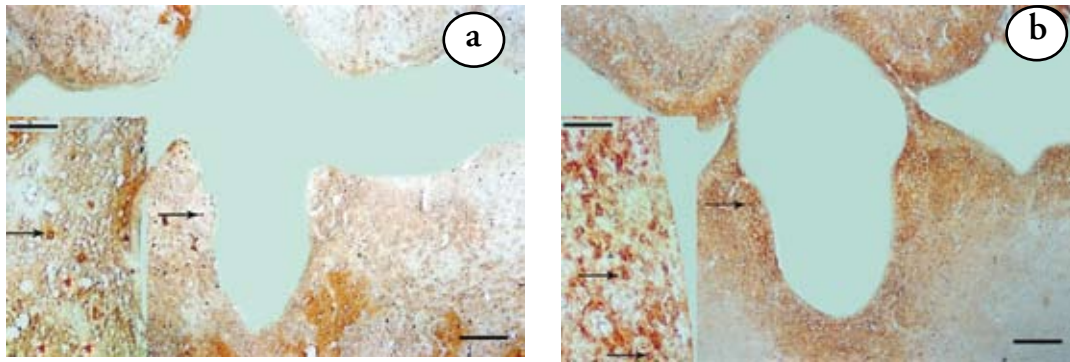
Figure 2 - Photomicrographs of insulin-like growth factor-I (IGF-I) immunoreactivity in the thalamic area. Note the significant decrease in density of IGF-I immunoreactive neurons in **a)** ethanol treated compared to the **b)** control rats. At a higher magnification neuronal processes were evident (inset). Scale bar: 250 µm.

Table 1 - An intensity of insulin-like growth factor-I (IGF-I) immunoreactive cells in the different neuroanatomical region of the brain. The data are expressed as mean ± SD.

Brain area	Control group (n=10)	Chronically ethanol group (n=10)
Frontal cortex	877.7 ± 30.7	103.2 ± 9.6*
Parietal cortex	768.8 ± 34.6	310.2 ± 64.5*
Temporal core	623.4 ± 19.1	405.3 ± 14.9*
Thalamus	1187.5 ± 40.13	7.6 ± 4.6*

*Significantly different from control value, $p < 0.001$

of stained cells were decreased throughout the deep layers (**Figure 1a**). However, in the ETOH treated rat brains, the number of IGF-I immunoreactive cells were distinctly decreased from the superficial layers to the deep layers (**Figure 1b**) compared to the control brains. In addition, there was also a distinct decrease in the IGF-I immunoreactive cells in the thalamus of the ETOH treated (**Figure 2b**) compared to the control rat brains (**Figure 2a**).

Discussion. Previous studies have determined the presence of IGF-I in the rat brain.[8,28,29] Noguchi at al,[34] demonstrated somatomedin C (IGF-I) in many regions including the thalamus, but they did not show any immunoreactivity in the cortex. Garcia-Segura et al,[35] observed IGF-I immunoreactivity in the olfactory bulb, hippocampus, striatum, septal nucleus, amygdala including cerebral cortex. On the other hand, Zhou at al,[36] detected IGF-I mRNA in basal hypothalamus including paraventricular nucleus and supraoptic nucleus using in situ hybridization. Our results show that IGF-I immunoreactivity is widely distributed throughout the cortical and thalamic brain regions of the adult rat brain. There are consistencies and discordant across studies in regards to the localization.[28,35] This may be due to the specificity of the primary antibody. It is considered that IGF-I produced in the brain is not growth hormone-dependent, and that it is produced via local stimulatory mechanisms. Localization of binding sites for IGF-I in the rat brain by quantitative autoradiography was demonstrated.[37] There are wide interest and growing amount of evidence on IGF-I as a neurotrophic factor.[30] Insulin-like growth factor-I is known to influence prenatal and postnatal growth.[31-33] In the present study, IGF-I immunoreactivity was observed in both the controls and the ETOH treated adult rat brains. Insulin-like growth factor-I immunoreactive cells were more distinct in all cortical areas and the thalamus. In the cerebral cortex, IGF-I immunoreactive cells were higher in layers I, II, and VI. Their role as modulators of brain growth and differentiation during development is apparent while their possible involvement in adult brain function is less known. The presence of IGF-I positive cells in these areas of the cerebral cortex further supports to the hypothesis that the IGF-I may have specific biological functions in the CNS, specifically it is thought that it may play a role as a neuromodulator in various cellular columns and nuclei of the brain.[30] These results suggest that the integration of somatomotor pathways, synaptic plasticity, and the remodeling of synaptic connectivity in these areas of the cerebral cortex may be influenced by IGF-I. In addition, we know that thalamus receives the termination of the somotosensory pathways from the head and neck, the body and from cerebellum and the basal ganglia. These are reciprocally connected in topographically organized with the somotosensory and somotomotor cortex.[34] These results suggest that the integration of all these pathways in the thalamus and in the cortex may be influenced by IGF-I. It was observed that, ETOH exerts multiple toxic effects on body growth and metabolism in humans.[31] Chronic ETOH abuse is frequently associated with marked

weight loss, which results primarily from malnutrition and liver disfunction, although other adverse metabolic effects of ETOH are also important.[19,21] Animals fed ETOH-containing diets exhibit decreased growth even in sufficient food intake. In the present study, we showed that IGF-I immunoreactive cells in the ETOH treated brains were present in all cortical and thalamic areas; however, the amount of IGF-I immunoreactive cells were decreased compared to the similar neuro-anatomical areas examined in the brains of the control group. Ethol exerts toxic effects in almost all organs, particularly in the liver and brain. It is known that ETOH interferes with growth factor action at 3 different sites, ligand production, receptor expression or signal transduction.[2,3] Growth-promoting effects of the IGF-I on all types of brain cells in culture are well documented.[16] It has been demonstrated that, neuronal survival is stimulated by insulin and IGF, whose signaling pathways are major targets of ETOH neurotoxicity.[38] Interestingly, chronic ethanol treatment caused dose-dependent decreases in the secretion and synthesis of IGF-I in liver and blood in vivo.[39] In addition, ETOH may affect receptor activation, signal transduction or intracellular signaling components. For example, ETOH may inhibit thyrosine autophosphorylation of IGF-I receptor. As a result, it is thought that the integration and the regulation of all the neuronal pathways including the cortex and the thalamus may be affected by the decreased IGF-I peptide. Recently, it was noted that the inhibitory effects of the ETOH-induced IGF-I system are related to increased JNK1/2 activity, JNK1/2 activity has been linked to proliferation and apoptosis of various cells.[40] Therefore, our results are similar with those of previous studies mentioned above, and that ETOH may exert a negative effect on IGF-I synthesis.

In conclusion, this study suggests that ETOH-induces a decrease in the immunoreactivity of IGF-I in the rat brain areas, this show clearly that, ETOH affects negatively on the IGF-I synthesis. These findings might be helpful to understand the pathogenesis of brain damage induced by ETOH, and may lead to a therapeutic approach against ETOH toxicity.

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