Acetyl-L-carnitine diminishes the effects of chronic ethanol-induced endoplasmic reticulum stress in the cortex of rat brain

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Abstract:

Chronic alcohol consumption is one of the most important problems of our time. Many diseases, such as liver diseases and infectious diseases, are seen in humans due to chronic alcohol consumption. Acetyl-L-carnitine (ALCAR), an antioxidant molecule, has been found to be neuroprotective. Accordingly, this study investigated the effect of ALCAR on endoplasmic reticulum stress in the rat cerebral cortex in a chronic alcoholism model. Four groups consisting of 10 three-month-old albino Wistar rats were randomly created. The groups were designated as control (C), chronic ethanol (EtOH), acetyl-L-carnitine (ALCAR), and chronic ethanol+ALCAR (EtOH+ALCAR). During a 4-week period, the rats were given either distilled water, ethanol, ALCAR, or a combination of ethanol and ALCAR orally. After a duration of 4 weeks, the animals were euthanized, and the entire brain tissues were removed and preserved in formalin fixative for further embedding in paraffin. Endoplasmic reticulum stress-related proteins (GRP78, CHOP, and XBP1) were analyzed by the immunohistochemical method in rat brain cortex sections. Our results showed that GRP78, CHOP, and XBP1 expressions increased in the EtOH group compared to the control group, whereas they decreased in the EtOH+ALCAR group compared to the EtOH group. In conclusion, our study showed that ALCAR administration decreased the increase in ER stress caused by chronic ethanol exposure. Accordingly, ALCAR supplementation may be beneficial for ER stress-related diseases.

1. Introduction

One of the biggest health and social problems of our time is chronic alcohol consumption. Alcohol consumption is the seventh most significant risk factor in terms of its contribution to the overall burden of disease, and its negative impact on health is substantial [1]. In 2018, the World Health Organization released a research indicating that the excessive consumption of alcohol led to 3 million fatalities, recording for 5.3% of all deaths, and caused 132.6 million years of healthy life lost globally in 2016 [2]. According to the report, the death rate from alcohol consumption is excessive than the death rate from diseases such as HIV/AIDS, tuberculosis, and diabetes. According to the data, in 2016, injuries accounted for 28.7% of all alcohol-related deaths globally, come after by digestive system disorders (21.3%), infectious diseases (12.9%), cardiovascular diseases (19%),

and cancer (12.6%). There is a significant association between the quantity of alcohol consumed and the incidence of certain diseases, such as ischemia disorders and diabetes [3]. Also, long-term alcohol consumption triggers the TLR4/NF-κB pathway, leading to a series of pro-inflammatory reactions that contribute to tissue destruction in internal organs, including the liver [4]. Moreover, studies have demonstrated that alcohol use induces lipid peroxidation and the generation of free radicals in numerous essential organs [5]. Acetyl-L-carnitine (ALCAR) is a biological molecule that plays a crucial role in energy metabolism. It also possesses neuroprotective and neurotrophic properties [6,7]. Studies have demonstrated the preventive effects of Acetyl-L-carnitine on neurons in various conditions, including hypoxia-ischemia, Alzheimer's disease (AD), severe brain injury, and situations acting on the central nervous system (CNS) or the peripheral nervous system (PNS) [8]. ALCAR has antioxidant properties and defends mitochondria from oxidative damage by inhibiting the construction of scavenging free radicals and oxidizing agents [9]. ALCAR supplementation improved cognitive performance and facilitated neuronal signal transmission in aged rats [10]. Furthermore, ALCAR administration has been shown to have neuroprotective effects in a peripheral neuropathy model induced by chronic constriction injury in rat sciatic nerve [11]. One of the organelles found in cells, called the endoplasmic reticulum (ER), is involved in multiple physiological activities, including as protein synthesis and folding, intracellular calcium regulation, and lipid formation. ER stress occurs when the ER's normal functioning is disrupted by physiological and environmental stimuli, leading to the buildup of misfolded or unfolded proteins in the ER [12]. When cells experience ER stress, they initiate a complex signaling cascade known as the unfolded protein response (UPR). The UPR tries to restore ER homeostasis by decreasing the production of proteins, increasing the expression of ER chaperones, and making it easier for misfolded proteins to break down. Nevertheless, if ER stress continues or becomes excessive, the UPR can transition from a state that promotes cell survival to one that induces programmed cell death, known as apoptosis [13]. Long-term alcohol usage is commonly acknowledged as a causal factor for several disorders that impact vital organs, including the brain. This suggests that excessive alcohol use can have adverse effects on brain processes. Our literature review revealed no research on how ALCAR affects ER stress in the rat brain cortex in a long-term alcohol experimentation model. The aim of this study was to examine the impact of ALCAR administration on ER stress in the cortex of rats exposed to chronic alcohol.

2. Material and Methods

2.1 Subjects

In this study 40 three-month-old male Wistar rats were used. The experimental rats were housed in a controlled environment maintained at a temperature of 22°C±2°C. They were subjected to a 12-hour light/dark cycle and supplied with access without restriction to commercial rat food and water.

Rats were randomly separated into four groups (n = 10/group): Control (C), chronic ethanol (EtOH) group, acetyl-L-carnitine group (ALCAR), and chronic ethanol+acetyl-L-carnitine group (EtOH+ALCAR). The control group received distilled water for 4 weeks. The chronic EtOH group received 5 g/kg ethanol (25% w/v). The ALCAR group received 50 mg/kg of acetyl-L-carnitine, and the EtOH+ALCAR group received the same doses of ethanol and acetyl-L-carnitine. Rats received daily gavages of distilled water, ethanol, and ALCAR. After 4 weeks, the animals were sacrificed under ketamine (50 mg/kg) and xylazine (6 mg/kg) anesthesia. Total brain tissues were extracted and fixed in formalin fixative for paraffin embedding.

2.2. Collection and Processing of Tissue Samples

After being placed in 10% formaldehyde, the samples were left on a shaker for 12 hours. Afterwards, tissues were washed under running tap water for two hours. Afterwards, the samples underwent a sequence of alcohol solutions and were then embedded in paraffin.

2.3. Immunohistochemistry Assay

Paraffin-embedded 5-μm sections were obtained from total brain tissue samples. Paraffin sections attached on poly-L-lysine-coated slides were incubated overnight at 60 °C. For deparaffinization, the sections were treated twice for five minutes each in xylene and then dehydrated by passing through a series of alcohol solutions (100%, 90%, 80%, and 70%) for five minutes each, followed by two washes in distilled water for five minutes each. After that, the sections were washed three times in Tris-buffered saline for five minutes each (TBS, pH 7.2–7.3). To eliminate antigenic masking, sections were heated twice in a microwave oven (800 watts) for 5 minutes each in 200 ml citrate buffer (2.1 g citric acid in 900 ml distilled water; pH 6.0). After

this procedure, sections were allowed to cool in the citric acid solution at room temperature for 20 minutes. After being cooled, the sections underwent three rounds of washing, with each round lasting 5 minutes, using TBS solution. To eliminate endogenous peroxidase activity, sections were treated with hydrogen peroxide solution (peroxidase blocking reagent, S2001, DakoCytomation, Denmark) for 20 minutes. After washing three times for 5 minutes each in TBS, the tissue edges were circled with a hydrophobic pen (PAP Pen, H-4000, Vector Laboratories, Newark, CA, USA). The sections were incubated with blocking serum (Ultra UV Block, TA-060-UB, LabVision, Fremont, CA, USA) to prevent non-specific antibody binding at room temperature for 7 minutes. Sections were then incubated overnight at +4°C with antibodies (ER stress markers) against GRP78 (Bioss, bs-1219R; 1:250, Massachusetts, U.S.A), CHOP (Bioss, bs-1361R; 1:300, Massachusetts, U.S.A), and XBP1 (Abcam, ab37152; 1:250, Cambridge, UK). For the negative control, TBS was added instead of the primary antibody. Following incubation, sections were washed three times for 5 minutes each in TBS and incubated for 45 minutes with biotinylated secondary antibody (Vector Lab, BA-1000, Newark, CA, USA) and then for 20 minutes with streptavidin-peroxidase complex (TP-125-HL; Lab Vision, Fremont, California, USA), with three TBS washes for 5 minutes after each step. To develop the signal, tissues were treated with diaminobenzidine (DAB tablets, D-4168, Sigma-Aldrich, Missouri, USA) for approximately 3–5 minutes. The sections were then transferred to distilled water and counterstained with hematoxylin for 20 seconds. After washing in running tap water and placing in distilled water for 5 minutes, sections were dehydrated through a series of alcohol solutions (70%, 80%, 90%, and 100%) and cleared in xylene for 5 minutes each before being mounted with entellan. Sections taken from the control and experimental groups were then evaluated. The immunostaining was analyzed as the percentage of immuno-positive neurons for GRP78, CHOP, and XBP1 by counting 200 neurons from randomly selected cortex regions from each slide under an axioplan microscope (Zeiss, Germany). All slides were evaluated for immunostaining by two observers without any information about the experimental group.

2.4. Statistical Analysis

The statistical studies were carried out through the GraphPad Prism 10 software. The percentages of neurons that showed positive immunostaining were compared using a one-way analysis of variance (ANOVA), followed by Tukey's multiple comparisons post-hoc test. The results were reported as means ± SEM. A p-value below 0.05 was classified as statistically significant.

3. Results and Discussions

Our immunohistochemical analysis revealed that GRP78, CHOP, and XBP1 proteins were all expressed in neurons of the cortical region of the brain. It was found that the chronic EtOH group had a significantly higher percentage of GRP78-positive neurons in the cortical regions than the control group ($p<0.0001$). In addition, a significant reduction in the number of GRP78-positive neurons in the chronic EtOH + ALCAR group with respect to the chronic EtOH group was observed ($p<0.0001$) (Fig. 1). As observed for GRP78 protein, the number of CHOP-positive (Fig. 2) and XBP1-positive (Fig. 3) neurons in the cortical regions of the experimental group animals was significantly increased in the chronic EtOH group compared to the control group ($p<0.0001$), while there was a significant decrease in the number of CHOP-positive and XBP1-positive neurons in the chronic EtOH + ALCAR group with respect to chronic EtOH group ($p<0.0001$). It's known that chronic alcoholism has a negative impact on human health and social structure. Additionally, it leads to numerous diseases and creates interpersonal problems. Alcohol consumption can cause harm to multiple important organs, including the pancreas, intestines, liver, lungs, and brain. Tissue damage is induced by inflammation, oxidative stress, and compromised immunological responses owing to alcohol metabolites [14].
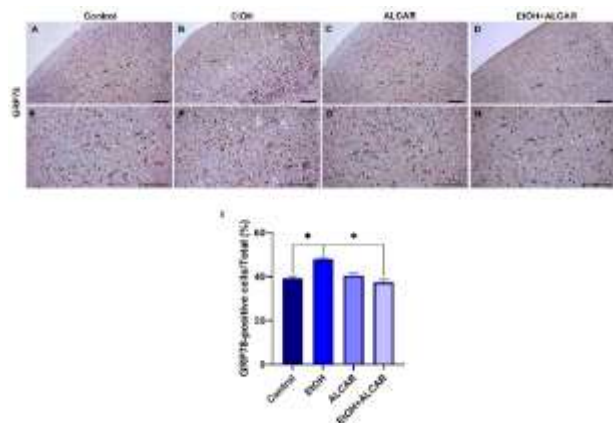

Figure 1. *GRP78 immunostaining in rat brain cortex tissue of control and experimental groups (A-H). Percentage of GRP78 positive cells of the groups (I). Arrows indicate GRP78 positive neurons. Scale bars indicate 50μm. EtOH: Chronic ethanol group, ALCAR: Acetyl-L-Carnitine group, EtOH+ALCAR: Chronic ethanol+Acetyl-L-Carnitine group. *: p<0.0001*
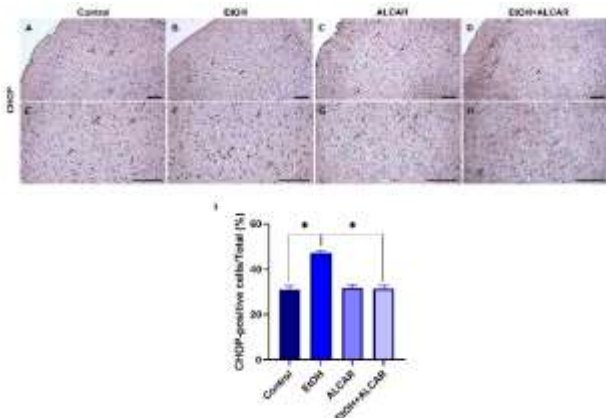
*Figure 2. CHOP immunostaining in rat brain cortex tissue of control and experimental groups (A-H). Percentage of CHOP positive cells of the groups (I). Arrows indicate CHOP positive neurons. Scale bars indicate 50µm. EtOH: Chronic ethanol group, ALCAR: Acetyl-L-Carnitine group, EtOH+ALCAR: Chronic ethanol+Acetyl-L-Carnitine group. *: p<0.0001*
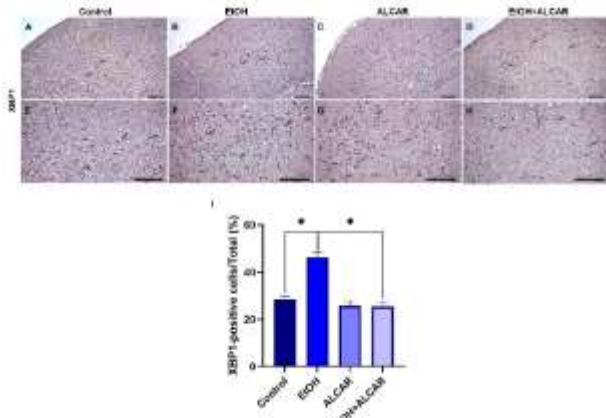

*Figure 3. XBP1 immunostaining in rat brain cortex tissue of control and experimental groups (A-H). Percentage of XBP1 positive cells of the groups (I). Arrows indicate XBP1 positive neurons. Scale bars indicate 50µm. EtOH: Chronic ethanol group, ALCAR: Acetyl-L-Carnitine group, EtOH+ALCAR: Chronic ethanol+Acetyl-L-Carnitine group. *: p<0.0001*

In addition, alcohol consumption has been directly linked to the induction of ER stress. Excessive alcohol use disrupts the normal operation of the ER, leading to hepatic symptoms such as liver fat accumulation (steatosis) and inflammation [15]. In this study, we investigated the effect of acetyl-L-carnitine on ER stress in the rat cerebral cortex in a chronic alcoholism model. GRP78 is upregulated in response to various types of cellular stress, including oxidative stress, hypoxia, and ischemia. By aiding in proper protein folding and reducing the accumulation of misfolded proteins, GRP78 helps protect neurons from damage [16]. Neurodegenerative illnesses such as amyotrophic lateral sclerosis (ALS), AD, and Parkinson's disease (PD), are linked to the rise in GRP78 [16,17]. The main site of protein folding, the endoplasmic reticulum, is also implicated in the creation of ROS. When proteins are misfolded, ER stress and excessive ROS production occur [18]. On the other hand, elevated CHOP levels have been observed in several neurodegenerative conditions following PD, AD, and traumatic brain injury (TBI). In these contexts, CHOP-mediated apoptosis can exacerbate neuronal loss and worsen disease progression [19]. When cells cannot cope with prolonged ER stress, they initiate cell death, a mechanism particularly evident in the brain cortex, where high metabolic demand and protein synthesis rates make neurons vulnerable to ER stress. In epilepsy and ischemic brain injury, CHOP expression significantly increases in the cortex and other parts of the brain that are affected. This shows that CHOP is a principal factor in cell death and connects ER stress to neuronal apoptosis [19]. Similarly, in our study, GRP78 and CHOP expressions increased in the EtOH group compared to the control group. However, the same protein expression decreased in the EtOH+ALCAR group compared to the EtOH group. After a TBI or stroke, XBP1 expression increases in affected brain regions, including the cortex. This upregulation is part of the cellular response to injury, aimed at promoting cell survival and recovery. XBP1's role in managing the cellular stress response helps reduce neuronal apoptosis and supports tissue repair processes following injury [20]. XBP1 is related to several neurodegenerative diseases, such as ALS and AD. In AD, XBP1 potentially slows the progression of neurodegeneration by helping manage the increased load of misfolded proteins and oxidative stress in neurons. In ALS, XBP1 contributes to cellular mechanisms that reduce the toxic consequences of protein aggregation and ER stress [21]. Similarly, in our study, it was observed that XBP1 expression increased in the EtOH group compared to the control group. Besides, XBP1 expression was found to decrease in the EtOH+ALCAR group compared to the EtOH group.

4. Conclusions

In conclusion, our study showed that acetyl-L-carnitine decreased ER stress-associated proteins in the rat cerebral cortex, which increased due to chronic alcohol consumption. This finding suggests that natural antioxidant acetyl-L-carnitine may help prevent disorders linked to ER stress.

Author Statements:

- **Ethical approval:** The rats were obtained from the Akdeniz University Experimental Animal

Care and Production Unit with the approval of the Akdeniz University Animal Experiments Local Ethics Committee (protocol #: 1679/2024.03.001).

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