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PI3K And mTOR Immunoreactivity In Testicular Tissue In Experimental Alcohol Addiction Model

Deneysel Alkol Bağımlılığı Modelinde Testis Dokusunda PI3K Ve mTOR İmmünoreaktivitesi

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ABSTRACT

Alcohol use disorder has negative effects on the reproductive system by increasing oxidative stress and causing damage to DNA integrity. Phosphatidylinositol-3-kinase (PI3K) and mammalian target of rapamycin (mTOR) levels, which are involved in oxidative stress, may play a key role in the reproductive system disorder caused by alcohol use. PI3K and mTOR immunoreactivities were evaluated in testicular tissue in acute and chronic alcohol intake model in male rats (n=21). Rats were divided into 3 groups control (n=7), acute model (n=7), and chronic model (n=7). Histopathological analysis of testicular tissues taken from the experimental groups was performed by hematoxylin-eosin (H&E) staining. Then, the experimental groups' testicular tissues were dissected and the immunohistochemistry method determined PI3K and mTOR expressions. According to the H&E staining results, when the experimental groups were compared with the control group, spermatozoa were less or absent in acute and chronic groups. mTOR and PI3K expressions were significantly increased in testicular tissues belonging to chronic and acute alcohol model groups. mTOR and PI3K expressions significantly increased in the chronic alcohol model compared to the other groups.This study reveals that PI3K and mTOR molecules, which participate in oxidative stress, increase short- and long-term alcohol consumption and that these molecules may be associated with damage to the reproductive system.

Keywords: Oxidative stress, Alcohol addiction, Reproductive system, Immunohistochemistry

ÖZ

Alkol kullanım bozukluğu, oksidatif stresi artırarak ve DNA bütünlüğünün bozulmasına neden olarak üreme sistemi üzerinde olumsuz etkilere sahiptir. Oksidatif streste rol oynayan fosfatidilinositol-3-kinaz (PI3K) ve rapamisin memeli hedefi (mTOR) düzeyleri, alkol kullanımının neden olduğu üreme sistemi bozukluğunda anahtar rol oynayabilir. Erkek sıçanlarda (n=21) akut ve kronik alkol alım modelinde testis dokusunda PI3K ve mTOR immünreaktiviteleri değerlendirildi. Sıçanlar kontrol (n=7), akut model (n=7) ve kronik model (n=7) olmak üzere 3 gruba ayrıldı. Deney gruplarından alınan testis dokularının histopatolojik analizi hematoksilen-eozin (H&E) boyama ile yapıldı. Daha sonra deney gruplarının testis dokuları disseke edildi ve immünohistokimya yöntemiyle PI3K ve mTOR ekspresyonları belirlendi. H&E boyama sonuçlarına göre deney grupları kontrol grubuyla karşılaştırıldığında akut ve kronik gruplarda spermatozoanın az olduğu veya hiç olmadığı görüldü. Kronik ve akut alkol model gruplarına ait testis dokularında mTOR ve PI3K ekspresyonları anlamlı derecede arttı. mTOR ve PI3K ekspresyonları kronik alkol modelinde diğer gruplarla karşılaştırıldığında anlamlı düzeyde arttı. Bu çalışma, oksidatif strese katılan PI3K ve mTOR moleküllerinin kısa ve uzun süreli alkol tüketimini artırdığını ve bu moleküllerin üreme sistemi hasarıyla ilişkili olabileceğini ortaya koymaktadır.

Anahtar Kelimeler: Oksidatif stres, Alkol bağımlılığı, Üreme sistemi, İmmünohistokimya

Ethics committee approval was obtained for this study (Protocol number: 23/112).

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INTRODUCTION

Excessive alcohol consumption is one of the causes of preventable deaths and kills approximately 3 million people worldwide every year. The high levels of alcohol intake in the population are essentially responsible for the elevated morbidity and mortality from alcohol-related diseases.[1-2]

The organ where ethanol is oxidized is the liver. Thus, ethanol causes the synthesis of toxic and carcinogenic metabolites acetaldehyde and acetate. Ethanol's ability to interact with lipids enables it to influence physiological cellular pathways.[3] There is a significant association that acute and chronic alcohol intake will lead to various types of cancer such as gastrointestinal cancer, kidney diseases such as chronic kidney disease, hypertensive heart diseases, and liver diseases such as hepatitis and cirrhosis.[4-5] In addition, alcohol use disorder has negative effects on the reproductive system by increasing oxidative stress and causing damage to DNA integrity.[3]

Acute and chronic alcohol use adversely affects the male reproductive system. Alcohol intake reduces semen parameters by lowering testosterone levels. The effect of alcohol on testosterone can be attributed to direct testicular toxicity and modifications of the hormone feedback mechanism in the pituitary gland and hypothalamus.[6] Also, alcohol use disorder is strongly associated with oxidative stress.

The situation that deteriorates in favor of oxidant products in our body is defined as oxidative stress. This stress is shown as the main cause of many diseases such as DNA damage, neurodegenerative disorders, and infertility.[7] Oxidative stress-related lipid peroxidation can cause cell injury, intracellular membrane, and cell destruction. Hypoxia causes loss of effectiveness of antioxidant mechanisms and increased free radical formation in the mitochondrial electron system.[8] Phosphatidylinositol-3-kinase (PI3K), a serine/threonine protein kinase (AKT), involved in oxidative stress in the cell, is a major target in the pathology of diseases.[9] Also, the PI3K protein mediates an essential role in cell growth, proliferation, metabolism, and tumor metastasis, it is regulated by various signaling proteins.[10] The PI3K-associated mammalian target of the rapamycin (mTOR) pathway is involved in various functions such as growth factor signaling and nutritional status to drive eukaryotic cell growth.[11] Alcohol use disorder has been associated with increased oxidative stress via catalase metabolic pathways, microsomal ethanol oxidation system, and alcohol dehydrogenase.[12]

With alcohol consumption, the production of free radicals increases or antioxidant levels decrease, which may cause oxidative damage.[13] Ethanol metabolism plays a direct role in the release of reactive nitrogen and oxygen species. Ethanol treatment reduces antioxidant activity by causing the depletion of induced glutathione (GSH) levels.[14] In addition, alcohol use disorder is also harmful to the germ cells in the testicle due to oxidative stress.[13]

In alcohol use disorder, pathologies in coexistent hormonal subsystems that interact with the hypothalamus-pituitary-gonadal axis also play a role in gonadal testosterone suppression.[15] In this context, PI3K and mTOR molecules, which are involved in oxidative stress related to alcohol use disorder, may accompany the cellular damage mechanism. In this study, histopathological evaluation as well as P13K and mTOR immunoreactivity were observed in testicular tissue in acute and chronic alcohol intake exposure.

MATERIAL AND METHODS

Experimental Groups

Control Group (n=7): No treatment was applied to the animals.

Acute Group (n=7): The protocol in the study of Nguyen et al. was used to build an acute alcohol model in this study.[16] Similarly, in this present study, McCormark et al. and Mugli et al. protocol was used.[17,18] To the animals in this group, a total volume (2ml) of 18% v/v ethanol prepared in distilled water was administered by oral gavage (o.g) at a total dose of 1 g/kg/bw.[16]

Chronic Group (n=7): To the animals in this group, a total volume (2ml) of 20% v/v ethanol prepared in distilled water was administered by o.g at a total dose of 4.5 g/kg/bw.[19] Afterward, the test animals were then sacrificed and the testicular tissues were removed.

Histopathological Study

Routine paraffin tissue follow-up was performed to examine the histological changes in testicular tissues of the experimental groups. Testicular tissues were fixed in 10% formaldehyde fixative for 12 hours. After washing in tap water (4 hours), the tissues were kept in 70%, 80%, and 90% alcohol series for 24 hours and in 100% alcohol for 3 hours to perform the water recovery process. The testicles were cleared in Xylol for 15 minutes. Then the tissues were paraffinized three times for 1 hour in an oven at 58^{o}C. At the end of the third hour, the tissues were embedded in clean paraffin in the appropriate orientation. Serial sections (5 μm) were taken from testicular paraffin blocks using a microtome, and were placed on normal slides with milling for H&E staining. 7 serial sections from the tissues taken from each animal in the experimental groups were evaluated under the Olympus BX51 light microscope and viewed with a DP71 model digital camera. Testicular tissues were evaluated using the Johnsen scoring method (Table 1).[20]

Immunohistochemistry Analysis

mTOR (sc-517464, Santa Cruz Biotechnology Inc), and PI3K (sc-1637, Santa Cruz Biotechnology Inc) immunoreactivities in the sections taken from testicular tissues of experimental groups were analyzed by the Avidin-Biotin peroxidase method.[21] Sections were examined with an Olympus BX53 light microscope. Assessment of immunoreactivity levels was performed with Image J Version 1.46.

Statistical Tests

The mean Johnsen score data and immunostaining intensities were compared with a one-way analysis of variance using the GraphPad Prism 8 Version 8.4.3 program. Differences between groups were determined by applying Tukey's multiple comparison test ($p<0.05$ significant).

RESULTS AND DISCUSSION

According to the H&E staining, the histopathological effects of acute and chronic alcohol intake on testicular tissues were compared with the control groups and evaluated under a light microscope. Haphazardly selected 15-20 seminiferous tubules in each section taken from the testicular tissues of each animal were examined histologically. When the experimental groups were compared with the control group; in acute and chronic groups, little or no spermatozoa, slightly impaired spermatogenesis, impaired integrity in the seminiferous tubules, and irregularity in the cells in the tubule were observed (Figure 1A). The damage observed in the chronic and acute groups was greater than in the control groups. The Johnsen score of the chronic group (6.84 ± 0.53) was significantly lower than the control group (8.38 ± 0.30) ($p<0.0001$), but similar to the acute group (6.99 ± 0.41) ($p=0.2276$).

mTOR and PI3K expressions increased significantly in testicular tissues belonging to

chronic and acute alcohol model groups. In the chronic alcohol model, mTOR expression increased 2.17-fold compared to the control group and 1.71-fold compared to the acute alcohol model. It was determined that mTOR expression increased 1.26-fold in the acute alcohol model compared to the control group. In the chronic alcohol model, PI3K expression increased 3.11-fold compared to the control group and 1.82-fold compared to the acute alcohol model. PI3K expression in the acute alcohol was 1.7-fold more increased than in the control group (Figs. 1C and 1D).

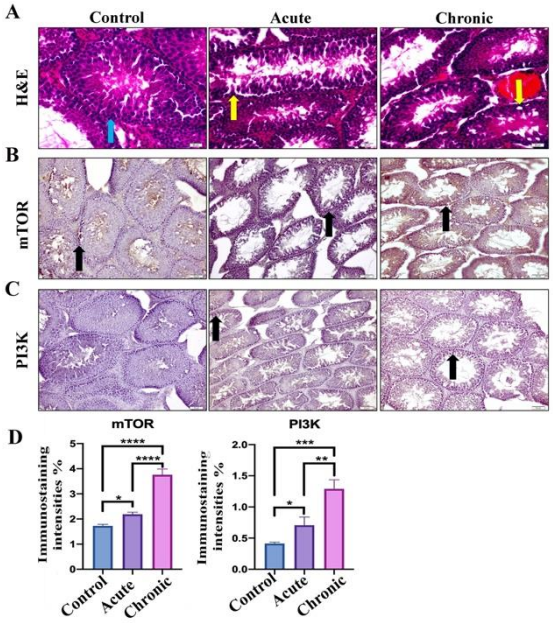

Figure 1. A) H&E staining images of testicular samples. The blue arrow shows complete spermatogenesis, and the yellow arrow shows disrupted seminiferous tubules and irregularity in the cells within the tubule. Magnification 40X and scale bar 20 µm. B) mTOR immunostaining images. C) PI3K immunostaining imaages. D) Bar graphs show the immunostaining intensity of mTOR and PI3K (%). Magnification is 20X and the scale bar is 50 µm.

Tablo 1. Johnsen Score In Testıs

Score	HISTOLOGICAL CRITERIA
1	There are no cells in the seminiferous tubules.
2	There are no germ cells, only Sertoli cells.
3	There are only spermatogonia as germ cells.
4	No spermatozoa and spermatids, few spermatocytes.
5	There are no spermatozoa and spermatids, there are a large number of spermatocytes.
6	No spermatozoa and few (<10) spermatids.
7	No spermatozoa, no late spermatids, but many early spermatids.
8	There are late spermatids that do not contain mature spermatozoa.
9	Slightly impaired spermatogenesis, many late spermatids, irregular epithelium.
10	Complete spermatogenesis.

This research revealed that PI3K and mTOR immunoreactivity in testicular tissue increased with both acute and chronic alcohol consumption. In the chronic alcohol model, mTOR expression was increased compared to the control and acute alcohol groups ($p<0.05$). PI3K expression was significantly increased in the chronic alcohol model compared to the control and acute alcohol groups. These data show that PI3K and mTOR in the PI3K/AKT/mTOR signaling pathway, which is one of the signaling pathways that regulate the oxidant balance in the body, can be histopathologically modulated in acute and chronic alcohol exposure. Hence, there may be an association between damage to the alcohol-induced reproductive system and PI3K and mTOR proteins, which are implicated in the oxidative stress reaction.

Alcohol undergoes dehydrogenation to acetaldehyde, producing acetyl and methyl radicals.[22,23] These metabolites are responsible for the of reactive oxygen species. Therefore, regular alcohol use triggers lipid peroxidation by overproduction of reactive oxygen species, lowers superoxide dismutase (SOD) antioxidant activity, and lowers GSH levels.[24] Alcohol use disorder also harms testicular germ cells related to oxidative stress.[13]

In a study in 2011, it was shown that alcohol causes degeneration in the

seminiferous tubules in the testis and morphological damage in the spermatogenic series cells starting from the early period and continuing in the adult period starting from the early development period.[25] It has also been shown by previous studies that ethanol reduces the reproductive activity of spermatogonia at all stages of the seminiferous tubule cycle, and that chronic ethanol use suppresses spermatogenic competence and leads to gonadal dysfunction, and inhibits male reproductive activity by preventing spermatogenesis.[26,27] The use of ethanol reduces the reproductive activity of spermatogonia and spermatogenic activity.[28] In this study, following the literature, when the experimental groups were compared with the control group; It was observed that spermatozoa were less or absent in acute and chronic groups, the integrity of the seminiferous tubules was impaired with impaired spermatogenesis, and there was irregularity in the cells in the tubule.

It has been reported that ethanol significantly reduces cell proliferation rates, culture growth, viability, and migration capacity, and these effects include PI3K and mTOR.[29] Among the factors that negatively affect the reproductive system in alcohol use disorder, PI3K and mTOR, which are involved in oxidative stress, can be included. The biological mechanisms of why alcohol will affect reproduction are still not fully elucidated. It has been suggested in the literature that alcohol may reduce reproduction by altering endogenous hormone concentrations. Compared to not drinking alcohol, 14 alcoholic drinks per week were found to be associated with suppressing folliculogenesis and ovulation by affecting FSH secretion and estrogen amount.[30]

Alcohol consumption may also be associated with the intake of other toxic substances found in alcoholic beverages, such as ethyl carbamates and food additives.[31] The evaluation of multi-factors in the mechanism of reproduction related to alcohol use disorder should be considered. For alcohol consumption and reproductive effects, different ethnicities, diagnostic methods and dietary habits can also be explained as part of the disparity in alcohol sensitivity.[32]

CONCLUSION AND RECOMMENDATIONS

This research revealed increased immunoreactivity of PI3K and mTOR in testis cellular damage in reproductive functions induced by acute and chronic alcohol consumption. In future studies, more detailed data can be obtained by showing protein and mRNA analysis of other components involved in oxidative stress in the same organs.

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