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TURKISH REPUBLIC
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GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCE
DEPARTMENT OF BIOLOGY

IMPACT OF ETHANOL INDUCED OXIDATIVE STRESS ON FOLLICULAR AND UTIRINE CELLS IN FEMALE REPRODUCTIVE SYSTEM

M. Sc. Thesis

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July 2017
KAYSERI

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KAYSERI

COMPLIANCE WITH SCIENTIFIC ETHICS

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"**Impact of Ethanol Induced Oxidative Stress on Follicular and Uterine Cells in Female Reproductive System**" named Erciyes University Graduate Thesis has been prepared accordance with Erciyes University Graduate Thesis Proposal and Thesis Writing Guidelines.

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IMPACT OF ETHANOL INDUCED OXIDATIVE STRESS ON FOLLICULAR AND UTERINE CELLS IN FEMALE REPRODUCTIVE SYSTEM

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ÖZET

Bu çalışmada etanolün foliküler ve rahim hücrelerine etkisi sitolojik ve moleküler yöntemlerle araştırılmıştır. Sığırlardan elde edilen foliküler (FC) ve rahim hücreleri (UC), *in vitro* bir model olarak kullanılmıştır. Artan etanol konsantrasyonları 6 ve 24 saatlik uygulamalardan sonra FC ve UC sayısında azalma ile hücre şekli, boyutu ve polaritesi üzerinde değişimlere neden olmuştur. Yine artan konsantrasyona ve uygulama süresine bağlı olarak hem foliküler hem de rahim hücrelerinde mitokondriyal aktivite artmıştır. Fakat etanolün 1000 mM'lık konsantrasyonu hem foliküler ve hem de rahim hücrelerinde mitokondriyal aktiviteyi azaltmıştır. Gerçek zamanlı kantitatif polimeraz zincir reaksiyonu (qRT-PCR) yöntemi kullanılarak oksidatif stresle ilgili *NRF2, KEAP1, CAT, HMOX1, PRDX1* ve *SOD1* genlerinin ifadesinde doz ve maruziyet süresine bağlı belirgin değişimler gözlenmiştir. Çalışma sonucunda elde edilen bulgular, alkol tüketiminin üreme organlarında oluşturduğu hasarları moleküler düzeyde ilk kez ortaya koymuştur. Ayrıca bu çalışmadan elde edilen bulgular, alkol tüketiminin oksidatif stresin artmasında kritik bir rol oynadığı ve üreme sisteminde ve dolayısıyla üreme potansiyelinde olumsuz etkilere yol açacağı anlaşılmıştır.

Anahtar Kelimeler: Etanol, oksidatif stres, foliküler hücreler, rahim hücreleri, gen ifadesi, dişi üreme sistemi

IMPACT OF ETHANOL INDUCED OXIDATIVE STRESS ON FOLLICULAR AND UTERINE CELLS IN FEMALE REPRODUCTIVE SYSTEM

Mustafa Abdulkareem SALMAN

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MSc Thesis, July 2017

Supervisior: Prof. Dr. Abdurrahman AYVAZ

ABSTRACT

In this study, the impact of ethanol on the follicular (FC) and uterine cells (UC) was investigated by using cytological and molecular methods. Follicular and uterine cells obtained from cattle and have been used as *in vitro* model. Increasing ethanol concentrations caused a reduction in the number of FCs and UCs after 6 and 24 h exposure, it also caused a change in the cell proliferation, shape, size and polarity of the cells. Dose dependent mitochondrial activity was determined for both FCs and UCs cells, and it was found that increasing concentrations and exposure time caused an increase in the mitochondrial activity. However, 1000 mM concentration caused a reduction in the mitochondrial activity both for FC and UCs. Expression of different genes related to oxidative stress has been investigated using real time quantitative polymerase chain reaction (qRT-PCR) method. Dose and exposure period dependent change in the expression levels of *NRF2, KEAP1, CAT, HMOX1, PRDX1* and *SOD1* genes was observed for both FCs and UCs. The result of the proposed project provides for the first time after hazardous effects of alcohol consumption at the molecular level of female reproductive system which may be correlated with infertility and several other reproductive diseases. Findings from this study provide important information how alcohol consumption increase the oxidative stress which would lead to adverse effects on reproductive system and therefore on the reproductive potential of the human being.

Keywords: Ethanol, oxidative stress, follicular cells, endometrium stromal cells, gene expression, female reproduction.

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LIST OF ABBREVIATIONS

FCs	Follicular cells
UCs	Uterine cells
WHO	World health organization
OS	Oxidative stress
ROS	Reactive oxygen species
MAPK	Mitogen Activated Protein Kinase
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
NRF2	Nuclear factor (erythroid-derived 2)-like 2
KEAP1	Kelch-like ECH-associated protein 1
HMOX1	(heme oxygenase (decycling) 1
CAT	Catalase
PRDX1	Peroxiredoxin-1.
SOD1	Superoxide dismutase
p38	mitogen-activated protein kinases
JNKs	*c-Jun N-terminal* kinases
ADH	Antidiuretic hormone
$NADH^+$	Nicotinamide adenine dinucleotide
ADH1B genes	Alcohol dehydrogenase 1B
CYP2E1	Cytochrome P450 2E1
NAD^+	fatty acid ethyl esters
FAEEs	phosphatic acid
PA	phospholipase D enzymes
GI	Gastrointestinal
ZO-1	granulocyte/macrophage colony–stimulating factor
GM–CSF	Polymorphonuclear leukocyte
PMNL	polymorphonuclear leucocyte
WBCs	White blood cells
HIV	human immunodeficiency virus
CCR5	natural killing cells
NK	Natural killing cells

CD4	cluster of differentiation 4
CD8	cluster of differentiation 8
TNFα	Tumor necrosis factor alpha
IL1β	Interleukin- 1 beta
INF	interferon
IgA	Immunoglobulin A
BAC	blood alcohol concentration
CA1	long-term potentiation
LTP	Long-term potentiation
NMDA receptor	N-methyl-D-aspartate receptor
MRI	magnetic resonance imaging
CT	computed tomography
WKS	Wernicke–Korsakoff syndrome
GH	growth hormone
PTH	Parathyroid hormone
EDTA	Ethylenediaminetetraacetic acid
HRT	hormonal replacement therapy
MDA	malondialdehyde

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INTRODUCTION

Alcohol consumption became a trait in the most social events in numerous regions of the world because it gives some relaxation and fake happiness. On the other hand, alcohol consumption causes health risks and social effects, poisoning and addiction. Chemically, alcohol is an organic compound containing a hydroxyl group (-OH) presents in alcoholic beverages like beer, wine and distilled liquor [1].

Alcohol was discovered thousands of years ago in Mesopotamia (Iraq now) especially in the Sumerians era (3000-2000 BC). Beer production began in northern Syria and the Aegean region between (1800-1500 BC). In (800-900 BC) Assyrian was the first who discovered the wine specifically in Ashurbanipal era at the same time India manufactured the beer by distilling rice and barley.

The discoveries rolled in wide regions of the world; such as China, Italy, Germany and England [2]. Between rejection and acceptance, religions have various opinions regarding alcohol. Some religions indicated that alcohol helps to strengthen the concupiscence and fertility; on the other hand, other religions put restrictions on alcohol usage [3]. It is worthy to mention that alcohol usage has advantages and disadvantages, but its harms are more than its benefits, the problem concerns addiction [4]. However, addiction is just the tip of the iceberg concerning the health issues and diseases associated with alcohol consumption. [5]. Where, it is noted that the volume refers to the overall pure alcohol taken within a specified duration [6]. The codes provided by the International Classification of Diseases are conventionally associated with the total alcohol volume [6]. Most notably, alcohol identified as a causative constituent of 200 International Classification of Disease-10 illnesses with a three-digit code [6]. Furthermore, it is asserted that the drinking pattern is related to groupings of the disease

such cardiovascular risk and injuries [6], because of that, the International classification of Disease associates a lcohol with adverse influence on over fifty key injury and ailment groups [7]. Besides, by 2004, alcohol intake was linked to around 2 million demises and ranked number eight in the mortality risk factors categorization [8].

Gender is vital in our present study because of its focus on FCs and UCs found in female reproductive organs. It is noted that in comparison with males, females are more susceptible to alcohol-related health problems [5]. The suggestion for this aspect include a bigger percentage of body fat leading higher concentration of alcohol in the blood, lesser liver volume for alcohol metabolism causes a reduction in average body weight [5].

Finally, it is not secret that much of the women suffer from many physiological problems in their reproductive system like delay or pregnancy weakness, menstrual disorder and abortion which have psychological effect on their family life. In our current study, we investigated the effect of ethanol that induce oxidative stress increasing inside the female reproductive system to prove its negative phenotypic and Genotypic effect. A limited number of studies are available in the literature on the effects of alcohol on FCs and UCs, and this study will provide an important contribution to this issue at the molecular level.

CHAPTER 1

LITERATURE REVIEW

1.1.Cultural history of alcohol

Numerous scientific and historical articles discussed the history of alcohol manufacturing. Where the word of alcohol related with vine tree, as stated in the Ancient manuscripts, that ADAM was the first human that implanted the vine tree. Furthermore, it was stated in Genesis of the Old Testament that Noah after the flood, worked as a farmer and planted the first tree, the vine, in order to obtain the wine [9].

There is a lot of myths in the literature about wine and gods [10], especially in the ancient Greeks culture, Africa, India, and Eastern Asia cultures, also mentioned the Vogue and wine drinking rituals.

From all the above, we can see that the wine has a great Vogue in the ancient cultures where the vine tree was a symbol of immortality, and wine effect positively on youth, immortal life, and wisdom.

1.2.Types of Alcohol and Alcoholic Beverages

There are many types of alcohol which are used in the laboratories and industrial fields like isopropyl and methyl. where isopropyl alcohol is utilized in industrial processes as well as in cleaning products and skin lotions. Methanol has been used as manufacturing solvent. It is found in cleaning solvents, paint removers, anti-freeze solutions.

The other type of alcohol is used in human consumption such as Ethyl which also known as ethanol. Human beings have been consuming Ethanol for its intoxicating and mind-altering effects. The specifications of ethyl alcohol are thin, clear liquid with harsh burning taste and high volatility, the concentration of absolute ethyl alcohol must be

diluted for human consumption. Ethyl alcohol is utilized as a reagent in some manufacturing applications [11]. As examples for alcohol consumed by humans are:

-Wines which synthesized from different fruits, such as grapes, peaches, plums or apricots, but the main type of fruits to produce wines are grapes. Wine contain 10-22% Alcohol [12].

-Beer Is liquid mix also manufacturing by the process of fermentation. It known as wort, is prepared by merge yeast and malted cereal, such as wheat, rye, corn. Beer concentration from 4 - 8 % alcohol [12].

-Whisky is synthesized from corn, rye or barley by distilling the fermented juice of these grains. Whisky contain 40-55 % Alcohol [12].

-Rum is a harsh taste distilled beverage synthesized from fermented molasses or sugarcane juice most Rum contain 40-55 %Alcohol [12].

-Brandy is distilled from fermented fruit juices most Brandy contain 40-50 % Alcohol [12].

-Gin is a distilled beverage. It is a mixture of alcohol, various flavors and water. Gin contain 40-50 % Alcohol [12].

-Liqueurs Synthesized by addition of sugar and flavoring such as fruits, flowers to brandy or to a mixture of alcohol and water. Most liqueurs contain 20-65 % Alcohol. [12].

-Tequila is a beverage made from the blue agave plant. Tequila contain 38 %Alcohol [12].

1.3.Alcohol and human body metabolism

Numerous studies found that 2-8% of alcohol consumption goes out of human body via urine, sweat and breath. 92% will remain in the body and metabolized by special enzymes [13]. Furthermore, the remaining alcohol will convert to acetaldehyde then convert to acetic acid where is called Acetyl radicals. This Acetyl radical merges with

Coenzyme A to be acetyl-CoA. Acetyl-CoA is important to start the Krebs Cycle, which is the main energy source of the human body. When the Krebs Cycle begin, the acetyl radical will finally be divided into carbon dioxide and water [14].

1.3.1.Oxidative Pathways

Figure 1.1. shows the main three enzymes which are in various types of body cells. These enzymes able to be grabbing tow hydrogen atoms of alcohol molecules.

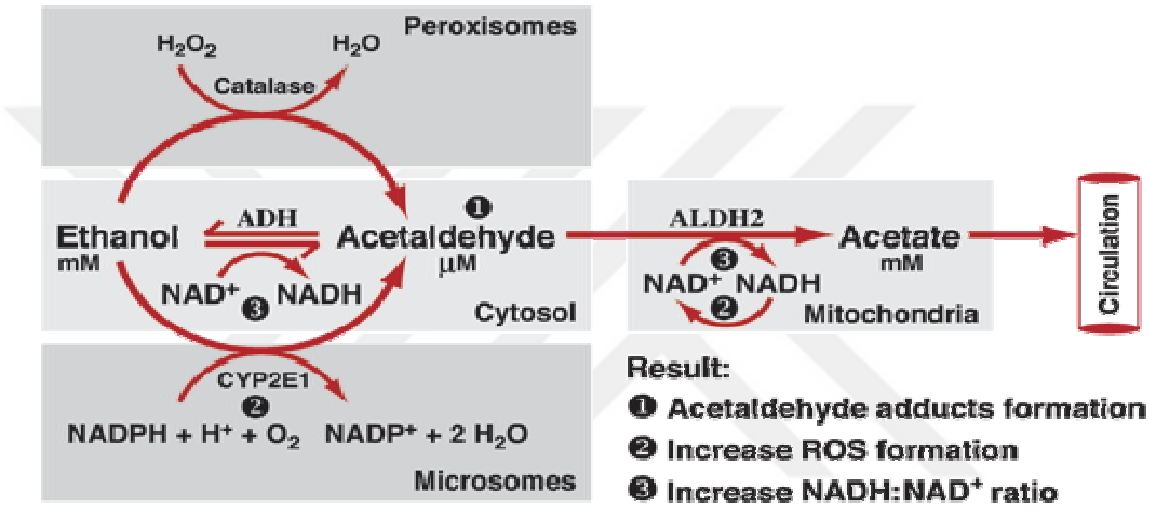


Figure 1.1. Oxidative pathways of alcohol metabolism [14]

1.3.1.1.ADH (Alcohol dehydrogenase)

They are found in various parts of body organs especially in stomach and liver (present in cell fluids i.e. cytosol). ADH play significant role in metabolism alcohol for producing acetaldehyde [15]. There are five types of ADH genes responsible in these metabolizing of alcohol and they are categorized according to their structural and kinetic properties.

Class II ADH, β-ADH (encoded by ADH4 and ADH1B genes) and CYP2E1. ADH can eliminate high concentrations of alcohol. The oxidation processes include an intermediate carrier of electrons (NAD^+) Nicotinamide Adenine Dinucleotide, which is converted to ($NADH^+$) by losing 2 electrons. Because of this, alcohol oxidation

produces a highly reduced cytosol in the liver cells. These reactions cause liver damage via some of ethanol metabolism products such as ROS [16].

1.3.1.2.Cytochrome P450

Which consist of CYP2E1,1A2 and 3A4 where are mainly found in microsomes and vesicles, it is more active in chronic alcohol cases, also CYP2E1 is important in converting alcohol to acetaldehyde primarily in liver. In addition, CYP2E1 can be present in other tissues that oxidize the ethanol (i.e. Brain). When the activity of ADH is low then lead to producing of ROS which cause a highly tissue damages [17].

1.3.1.3.Catalase

Catalase is another enzyme present in tiny organelles cells known as peroxisomes. It has the ability to oxidize the alcohol especially in the brain by converting alcohol to acetaldehyde which is in turn combined with neurotransmitter to synthesize tetrahydroisoquinolines THIQs [18].

1.3.2. Non-Oxidative pathways

Despite of the oxidative stress low levels, still its pathological products is more effective. Non-oxidative pathways consist of two pathways for metabolizing alcohol [18].

First, they convert alcohol to fatty acid ethyl esters (FAEEs) molecules which occurred by reaction of alcohol with weak fatty acids which have an important function in human cells. FAEEs is present in serum and other tissues after alcohol consumed. It remains for long time after alcohol elimination and causes damages.

The second pathway lead to the production of fat molecules (i.e. lipids) which are composed of phosphorus (i.e. phospholipid). These phospholipids primarily phosphatic acid (PA), will degraded by phospholipase D enzymes (PLD) lead to produce poorly metabolized of phosphatidyl ethanol. Although, their effects will be remaining on the cells for long time after chronic alcohol consumption period [19].

Finally, there is a relationship between oxidative and non-oxidative pathways, the inhibition of ADH, CYP2E1 and Catalase lead to increase the activity of Non-oxidative metabolism of alcohol. Furthermore, the liver and pancreas will increase the FAEEs production [20].

1.4.Impact of alcohol on organs of the body

Numerous studies proved that alcohol could influence of the body organs. These influences include functional damages, physiological damages and structural damage. We will review these impacts according to body organs.

1.4.1.Alcohol and Immune System

Alcohol abuse represses various arms of the immune response, causing an increased risk of infections. It is known that the immune system contains from many types of barriers, such as, physical barriers, mechanical barriers and chemical barriers. Consequently, all of them complete each other. Alcohol abuse has an impact on adaptive immune system and innate immune system directly [21].

The first line of host defense is the physical barriers which include structure such as epithelial cells and immune cells (i.e., macrophages and dendritic cells). Epithelial cells have two main function: a physical barrier as well as regulators of the innate and adaptive immunity. Particularly the epithelial cells play a role as immune barriers of the gastrointestinal tract. Researchers suggest that chronic alcohol abuse causes excessive intestinal permeability.

There is a Structures called tight junctions in the epithelial cells these junctions allow the epithelial cells together preventing passage water and other molecules from intestine into the blood directly. Therefore, activity of alcohol impacts these tight junctions and inhibits their function by damages tight junction proteins such as (ZO-1 & Occludine) thus allowing water and substances to passage into blood stream freely. Due to the level of bacterial infection will increase inside blood stream [22]. Furthermore, some researchers found that alcohol might have an influence on epigenetics of (ZO-1 & Occludine) proteins [23].

In respiratory system, chronic/acute alcohol consumption can decrease the functional activity of granulocyte/macrophage colony–stimulating factor (GM–CSF) and reduced their expression [24]. Moreover, a chronic/acute alcohol consumption able to inhibits cell division and recognition [25]. Other studies found that alcohol consumption may suppresses Mononuclear phagocytes cells that are present in the blood stream and macrophages in the tissues, especially in the case of tuberculosis pathogens (i.e., mycobacteria). In healthy persons when the tuberculosis pathogens attacked their bodies the macrophages will directly destroy it. Conversely, for the persons who is consuming alcohol, the activity of the macrophage will be suppressed, in spite of the high levels of macrophages [26].

Previous studies have been conducted on HIV patients, who are at the same time drinking acute alcohol, they found that alcohol has an influence on specific chemokine receptor present on the surface of macrophage known as CCR5, which is plays an important role in patients with concurrent HIV infection. Subsequently, alcohol abuse influences the expression of CCR5 leading to enhanced infectivity of these HIV strains in the macrophages [27]. Alcohol has an impact on NK quantitatively and qualitatively. Studies proved that alcohol interferes in expression of several types of NK proteins such as (e.g., proteins called perforin and granzymes A and B). Alcohol decrease the ability of NK to destroy target cells. Also, this failure in activity of NK cells plays an essential role in alcohol-associated tumor development, viral infection [28] and decrease the ability of NK to suppress liver fibrosis [29].

Several lines evidence shown that chronic alcohol can decrease the ability of B-cells to produce antibodies in blood stream [30]. Moreover, alcohol reduce numbers of B-cells that produce IgA antibodies particularly in mucous membrane (i.e., the lamina propria) [31]. Finally, alcohol prevents the response B- cells at developmental stages to different cytokines, especially to IL-2 and IL-4 [32].

1.4.2.Alcohol and Nervous System

Commonly, the impact of alcohol abuse starts as the primary feelings of relaxation and exuberance giving way to hangover, exhaustion, depression, vomiting and loss of

consciousness in elevated doses level [33]. Alcoholism has destructive consequences such as risk for brain and neuro- behavioral [34]. Furthermore, alcohol abusers have a difficulty mild neuropsychological [35].

1.4.2.1.Memory

The rapid rise of blood alcohol concentration (BAC) May be causing amnesia and blackouts thought that BAC influenced on the mechanism of transfer information from short-term memory to long-term store.

Alcohol may overlap with the activation of the NMDA receptor, a key prerequisite for establishing LTP. Other possible mechanism through were alcohol can stimulate its impact it by damaging the messages from structures that influence hippocampal function, like the medial septum and the prefrontal cortex [36].

1.4.2.2.Trauma

Alcohol abuse lead to confusion, staggering and loss of social relationships and occasionally making the drinkers aggressive thus increasing risk of traumatic injury. This could be due to falls, fights or road traffic accidents [37]. The unexpected cessation of chronic alcohol abuse releases the irritability, anxiety, tremors, agitation and seizures [38]. If these unexpected cessations are repeated, the severity of symptoms will increase. This phenomenon is called kindling. It is believed that kindling participates to a patient's relapse risk and to alcohol-related brain injury and cognitive weakness [39].

Several pathological and radiological (i.e. MRI and CT) investigative studies on cerebral atrophy, especially including the frontal cortex, has been documented in patients suffering from chronic alcohol abuse. These studies found that atrophy may occur in the hippocampus, corpus callosum, mammillary bodies, thalamus and cerebellar cortex [40, 41].

1.4.2.3.Dementia

Few studies have sparked controversy about the correlation of alcohol abuse with dementia, where, researchers proved that acute alcohol abuse has the ability impaired

the memory and cause syncope. Furthermore, chronic alcohol consumption concurrent with thiamine deficiency lead to (WKS). In addition, alcohol drinking may cause distortion of brain matter that is related to the decrease in perceptual and cognitive functions [40]. Studies found that alcohol abuse causing factor to cerebellar degeneration. In addition, chronic alcohol consumption has an impact on the central pontine and extrapontine myelinolysis, by influencing the osmotic demyelination and the changes in plasma sodium concentrations from hypernatremia. This an acute condition that is frequently fatal: mild forms can be asymptomatic [42]. The excess alcohol abuse may lead to Michiana–Bignami disease, cause seizures, ataxia, confusion, dysarthria or coma which can be mortal [42].

1.4.3.Alcohol and Bone

Numerous studies utilizing animal's laboratory propose that it is possible that the results of alcohol consumption will mostly affect bone development by reducing the osteoblast, bone strength, density, and volume. during long-term alcohol consumption. The rate of proliferation of cells in the growing region near the ends of long bones (i.e., growth plates) will stop due to alcohol consumption. If those impacts occur in humans they will significantly decrease bone mass thus leading to an increase in fracture and early onset of osteoporosis [43, 44]**.**

The bone supplies a structural support to the body, it is also the main storage depot of calcium. Alcohol may disrupt the balance of calcium in bone and blood by impacting the hormones that regulate calcium metabolism [43].

Study was noted that mild drinkers of men and women for 3-hour of periods, each person was receiving approximately 5 to 11 typical drinks [45], the PTH levels for each person were aggressively reduced until the end of the drinking time and rose over the next 9 h. Eventually the overriding levels were measured before alcohol consumption, the excretion of calcium in urine increased during the first 3 hours and subsequently decreased [45]. Furthermore, long-term of alcohol consumption was related with low blood calcium level (i.e., hypocalcemia) but normal PTH levels [45]. The researchers noticed that the hypocalcemia does not occur by reducing PTH secretion and they

propose that alcohol consumption suppressed the ability of the parathyroid glands to increase PTH production in response to the presence of hypocalcemia [45].

Few studies suggested that acute and short-term alcohol consumption may increase the levels of Calcitonin for short period [46]. Furthermore, alcohol may influence remodeling of the bone that occurs in small areas that were dispersed on the surface of the bone. Osteoclasts corrode the cavity on the bone surface in a process called resorption. When the resorption cavity is complete, the osteoclasts hid, the layer of the cavity is smoothed off, and a thin layer of matrix is deposit. Bone-forming cells (i.e., osteoblasts) fill in the newly formative cavity by new bone [46].

1.4.4.Cardiovascular system

In the cardiovascular diseases, aldehydes are released in huge amounts due to the presence of alcohol in the blood leading to inhibiting their reabsorption. An alcohol-based interference with spleens functionality causes a decrement in immunity level [47].

Both chronic and occasionally heavy drinking increases the risk of coronary artery disease. It also associated with sudden death caused by heart failure, with irregular heartbeats and chronic disease of the heart muscle (dilated cardiomyopathy) which leads to heart failure, where the heart can no longer pump blood around the body effectively. Heavy chronic alcohol use is also linked with high blood pressure, particularly in men. Blood pressure increases with drinking more than two or three doses (13g per dose) a day on average, on the other hand restricting alcohol would lower blood pressure [48].

1.4.5.Digestive system

Wang, Zankhan et al. indulged in extensive studies and published an article in in relation to the effects of excessive consumption of alcohol on the consumers gut [49]. Based on their studies, it is clear that the gut is infested with microflora that sustain a high level [49]. The presence of high levels of the alcohol can be dangerous to microflora due to excessive alcohol levels in the gut because it leads to the increased translocation of lipopolysaccharide of microflora. In turn, the gut is infected with inflammation because of the reduced levels of the microflora [49]. Consuming large

amount of alcohol causes the gut to harbor an increased amount of acetaldehyde leading to permeable environment to the endotoxins [50].

Szabo and Mandrekar stress that the presence of high levels of ethanol where is leading cause of liver cirrhosis [51]. Various traditional studies had disputed the fact that alcohol is not to blame for various cases of liver failure. Most of them suggested that the malnutrition and dietary deficiencies were the primary factors to blame. However, Szabo and Mandrekar disputed such beliefs by proving that the presence of ethanol cause a major disruption in the liver functionality [51]. Sequentially, the organ develops complications such as liver cirrhosis.

The pancreatic diseases have for long time, been associated with excessive consumption of alcohol. However, the direct link between the two aspects, alcohol consumption and pancreatic illnesses, lacks a definitive relationship. Gathering information from herreros et.al, [52]. In their article, the authors explored by observing the alcoholic beverages, duration and dose consumed. Using the mentioned factors, they observed that an excessive amount of alcohol in the body has 2.5% to 3% chances of causing pancreatic diseases among the heavy drinkers [53]. However, it was also established that about 70% of the pancreatic diseases are caused by the heavy presence of alcohol in the body.

1.4.6.Reproductive system

Studies show that the reproductive system can be affected by elevated amounts of ethanol [54]. The alcohol constrains the proper function of the reproductive organ, which can easily lead to impotence and barrenness [54]. Tests conducted to investigate the effect of alcohol in reproductive system show that the ethanol has inhibitory effects [55]. As such, heavy doses of the substance in male life causes inhibition in the reproductive tract, also some are reversible upon a reduction in the consumed amount [55]. Further research reveals that the exposure to huge amount of alcohol over person's life time profound effects on the sperm parameters.

Hypotestosteronemia is among the common infections that occur among the heavy alcohol drinkers [55]. This defect has direct effects on the sperms structures, which leads to a reduction in fertility levels. Gonadotropin levels are another area of interest that the heavy presence of alcohol in the body affects. In some instances, the alcohol causes low or elevated gonadotropin levels, which affects the testes. In turn, the tested are constrained in sperm production, and in some cases, the victim become infertile [55].

Alcohol abuse can also cause adverse effect on female reproductive system in various ways. It is notable that the alcohol can interfere with the female puberty, causing its delay. Heavy female drinkers also face the risks of having abnormal menstrual cycles as the substance affects the hormonal levels in their bodies [56]. Ovarian failure is yet another effect that alcohol can induce in females. In such cases, the females suffer from infertility because they do not undergo normal menstrual cycle or produce ovaries for fertilization [56]. Indeed, the effects are extensive and damaging, but with proper treatment and reduction in alcohol consumption, one can recovery [56].

1.4.7.Oxidative stress

Apparently, oxidative stress (OS) is a phenomenon which is triggered by a complete imbalance of pro-oxidant, the inclusion of oxidant defenses, and the responsive nitrogen and oxygen class [57]. A 2012 study by Agarwal et al. provides a sound basis for the description of oxidative stress emanating from alcohol consumption. Oxidative stress described as the result of a disproportion between antioxidants and pro-oxidants [58]. Much of it has to do with the augmented synthesis of metabolites, which are toxic and lead to the severe damage of the integral fundamental control mechanisms, as well as the destruction of biomolecules [59]. The most notable metabolites, which are known to influence the balance between the mentioned oxidants is the reactive oxygen species (ROS) [58]. During female reproduction. Meiosis I hindered by antioxidants and affected by a sugar in ROS, while meiosis II is enhanced by antioxidants [58]. Aerobic metabolism is credited with producing ROS [59]. In addition, a rise in P450 emanating from increased production of steroids within the developing follicle causes the formation of ROS [58]. They are known to comprise hydrogen peroxide, hydroxyl. As

well as, additional forms of reactive radials [59]. Primarily, in the course of synthesizing adenosine triphosphate and oxidative phosphorylation, the inner membrane of the mitochondrion discharges electrons [59]. The discharge described as the primary origin of ROS, ln addition; ROS are producing by extra processes.

The use of alcohol is linking to ROS generation. It noted that an oxidative means is involve in the removal of ethanol through hepatic metabolism [58]. Dehydrogenation of consumed alcohol yields acetaldehyde [58]. Additional acetaldehyde dehydrogenation leads to the production of ethanoic acid, as well as methyl and acetyl radicals [58]. Subsequently. The metabolites cause the production of ROS [58]. It implies that intake of alcohol increases the ROS release [58]. Moreover, it noted that alcohol use decreases the levels of glutathione. Cuts down the antioxidant role of superoxide dismutase, and initiates peroxidation of lipids [58]. Because of the chemical reaction of ROS with major macromolecules in the cells, the cells react to the production of ROS through an activation of gene expression. Which encodes for specific enzymes [58]. These enzymes are involved in oxygen radical's neutralization and processes, which involve direct ROS neutralization [59]. It asserted that the right equilibrium regarding the antioxidants and oxygen radical production is integral in sustaining a balanced level of homeostasis within the cells [59]. Consequently, having the right balance permits sufficient function and development of cells. [59] Nonetheless, disproportionate equilibrium. Which inclines towards ROS. Linked to augmented cellular damage [59]. Subsequently, biochemical pathways, a secondary reaction, activated to renew and repair the damaged cellular molecules [59].

The oxidative stress is responsible for the broad cellular damage: hence, resulting in a rise in gene expression [59]. Because of oxidative stress, there are several pathways involved in signaling that triggered. One of them is the Mitogen Activated Protein Kinase (MAPK) that majorly controls the transcription of genes [59].

Residues of serine, as well as threonine, undergo dephosphorylation and phosphorylation, which utilized in regulating the signaling cascade of this pathway [59]. Moreover, there is the NRF2¬ARE pathway, which is robust defense co-ordination

mechanism [60]. It involved in the control of gene expression, which takes part in anti-inflammatory and cellular antioxidant defense [60].

Oxidative stress is one of the major processes, which known to trigger this pathway as it adversely changes the signaling approaches used by cells [61]. Besides, ROS also activates the p38 and c-Jun N-terminal kinase pathways [61]. Oxidative stress can occur in different ways as follows,

1.4.7.1.Oxidation of lipids

Poly unsaturated fatty acids are capable of formation of free radicals and the lipid peroxyl free radicals which are produced react with other lipids resulting in the oxidation of substrates. consequences of this process include the alteration of cell permeability, membrane fluidity and other cellular functions of the body [62].

1.4.7.2.Oxidative damage to proteins

Oxidation of proteins consequently produces altered amino acids, disruption of polypeptide chain, increased proteolysis etc. whole metabolism of proteins and enzymes gets distracted due to the oxidation of proteins [62].

1.4.7.3.Oxidative damage to DNA

Some ionizing radiations produce oxygen free radicals as a result numerous mutations occur in the DNA altering the base pairs, degradation of base pairs and single strand breakage. Aging or senescence: the production of free radicals causes disruption of the proteins, damage the lipid, protein and DNA thus resulting in aging [62].

1.4.8.Reactive Oxygen Species (ROS)

ROS are chemically reactive molecules derived from molecular oxygen such as free radicals containing oxygen atoms, singlet oxygen, hydrogen peroxide and superoxide radicals. These are produced during metabolic reactions [63]. Sources and formation, occurrence: ROS are formed as by products in metabolic reactions and formed during releasing energy, oxidative stress etc. First step of formation includes reduction of

oxygen, second step involves formation of hydrogen peroxide in presence of enzyme superoxide dismutase, which is further reduced to hydroxide radical or fully reduced to water, by the action of another enzyme catalase [64]. The formed radicals cause damage to the cells and cellular metabolism. Thus, it is important to clear such reactive species from system which is done by these enzymes which prevent aerobic organisms from oxidative damage of the cell. ROS occur as exogenous (outside the cells) and endogenous; inside the cells as seen in immune response [64]. ROS reacts with electron chains and disturb them and cause metabolic disintegration in cells. Anaerobic organisms lack enzymes such as catalase, superoxide dismutase and hence cannot grow in presences of oxygen due to oxidative damage caused to cell by ROS [64].

1.4.9.Antioxidant

Antioxidant is a chemical molecule which produces free radicals, leading to chain reactions that damages cells. An antioxidant is a molecule that inhibits the oxidation of other molecules. Antioxidants are useful for preventing damage produced by reactive oxygen species (ROS). Few examples of antioxidants include fruits and vegetables, beta-carotene, lutein, lycopene, selenium, zinc and Vitamins A, C and E. There are two types of antioxidants [65]. Preventive antioxidants; inhibit the initial production of free radicals. They include catalase, glutathione peroxidase, ethylene diamine tetra acetate (EDTA) [65]. Chain breaking oxidants; inhibit the propagation phase of the peroxyl radicals [65].

1.4.10.Oxidative stress and female reproductive system

Subsequently, ethanol-induced oxidative stress is caused by dehydrogenase, which is a catalyst present in alcohol. Ideally, this condition occurs after consuming excess alcohol [66]. However, different studies illustrate that ethanol is present in human body at some stable proportions. Research indicate that this situation has a negative impact on the pathogenesis of sub-fertility and the oocytes process in female [67]. However, it should be pointed out the oxidative stress also affects male reproductive system to a smaller magnitude compared to female [67].

Undoubtedly, oxidative stress has been pointed out as the leading cause of fertility related complications such as frequent pregnancy loss, preeclampsia, and premature abortion cases. Specifically, alcohol and nicotine abuse have been singled out by modern researchers as the causative agents of oxidative stress [68, 69]. Additionally, it has been noted that acute exposure to alcohol in women who are past their menopause leads to increased estradiol levels and especially in menopausal women undergoing HRT. This is due to the impairment of estradiol metabolism as a result of reduced conversion of estradiol to estrone [70].

Subsequently, recent studies show that oxidative stress also affects follicle development and conversely the development of ovary. However, high concentrations of melamine in the DNA of animals may also lead to increased level of reactive oxygen (ROS) that may lead to induced granulose in cells and may lead to follicle atresia condition [71, 72]. According to study melamine influenced oxidative stress through deliberation of antioxidant enzymes such as glutathi-peroxide and superoxide dismutase. researchers analyzed malondialdehyde between an ovary treated with melamine and a control specimen and realized that prenatal and perinatal acrylamide led to the disruption of the cerebellum in rats [73].

Furthermore, absorption and metabolizing of ethanol in the liver leads to the formation acetaldehyde due to degradation of threonine pathways, betalamine pathways, and deoxyribose phosphate pathways [74, 75].

Ethanol can be easily converted in the human body into acetate, which affects the human body biologically and physiologically because of decreasing antioxidants level resulting from oxidative stress [76]. Also, consumption of alcohol at the onset of puberty life may lead to reproductive hormone production suppression [77]. Undeniably, this may cause delayed maturity of vital hormone such as estradiol, which helps in timing and regulation of female ovulation cycle [78]. Other key hormones that foster female development and maturity may also be impaired by consumption of alcohol.

1.4.11.Female reproductive system

The female reproductive system is made up of the internal and external organs. Internal organs include the ovaries, fallopian tubes, cervix, uterus, and the vagina. While externally there is the periurethral region, vestibular glands, labia majora and minora. Urinary meatus, clitoris, mons pubis. and the Perineum [79]. The ovaries are located on both ends of the uterus the hold the ova and release them in response to hormonal changes. The fallopian tubes are vital in the transportation of the ova und sperm [79]. The inferior part which makes up the uterus is called the cervix [79]. ln response to hormone changes the walls of the uterus which arc muscular and thick undergo variation [79]. However, in general it can be divided into the cervix as earlier noted also the corpus uteri [79]. As for the external organs naming them is enough because of the focus of this research.

Microscopically. Numerous types of cells constitute both the external and internal organs, of interest is the microscopic structure of the uterus, three layers form the uterine corpus include the serosa, myometrium and endometrium. The cells in the endometrium are characterizing by large nuclei and small amounts of cytoplasm. It is composed of the outer stratum functional and inner stratum basal [79]. The stratum functional is responsible for reacting to hormone variations. Moreover, the main kinds of cells making up the endometrium include the stromal and epithelial cells. The endometrial stroma has cells, the cells are known to go through a distinctive cellular change termed as decidualization [80]. In the female reproductive system, this occurs during pregnancy or the menstrual cycle [80]. Consequently, they transform into decidual stromal cells [80]. The follicular cells surround the oocyte. When follicle-genesis is taking place, it is stipulating that the cumulus and mural cells constitute the follicular cells [81]. Additionally, during the ovarian phase the follicular cells induced to secrete estrogen when the follicular phase is occurring [81]. Hence, it is evident that the endometrial fibroblast and follicular cells play critical role in female reproduction [81].

CHAPTER 2

MATERIALS AND METHODS

2.1. Materials

The materials that have been used in our experiments according to the stages required contain a various chemicals, kits and tools that were purchased from different manufacturers. Furthermore, before starting our experiment, we prepared all solutions with deionized and demineralized Millipore water (ddH2O) as required. In addition, the pH was adjusted with hydrochloric acid (HCl) or sodium hydroxide (NaOH). As well as, the buffers were filtered through 0.2 μ filters and autoclaved at 120°C for 20 minutes.

2.1.1. Chemicals and biological reagents

Chemicals and Biological reagents	Manufacturer	Aim
Biological Dissection tools	Nicholson Road, Ambala, Haryana (indiamart)	were utilized for collecting uterus from slaughtered animals.
Collagenase type 2	sigma/ USA	Digest and separate the tissue into single cells.
Ethanol 70%	**emsure / germany**	has been prepared from 700ml of absolute ethanol mixing with 300ml of distillated water. Using for sterilizing and preventing contamination.
Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12	sigma-aldrich /USA	using for seeding the follicular and endometrium fibroblast cells.
Fetal bovine serum	Pan-Biotech GmbH, P30-8500, Aidenbach, Germany	10% of total media contain from fetal bovine serum Food supplement.
Antibiotic, anti-mycotic	Sigma-Aldrich, P4333, Steinheim, Germany	1% of total media contain from (AA). To avoid bacterial and fungal infection during cell culture
Dimethyl sulfoxide	sigma-aldrich /USA	Regular laboratory use, cell culture and dissolving special powder.
Trypsin EDTA (0.25%), phenol red	Gibco® / USA	Detach the cells from culture plate or flask.
Trypan blue	sigma-aldrich /USA	Cell counting for cell culture
Mitotrecker	Thermofisher / USA	for mitochondrial staining
Triton-X, X100-100ML	Thermofisher / USA	Increase the permeability of the cells

		during different examination
Cell proliferation reagent WST-1	sigma-aldrich /USA	Quantification detection purpose.
Total RNA purification RNA purification kit	EURx®/ Poland	universal RNA/miRNA Isolation of total RNA from cultured cells
Maxima H Minus First Strand	thermo / EU Lithuania	Convert the total RNA in to cDNA for real time quantitative PCR
QuantiFast SYBR Green PCR Kit	(Bio-Rad Laboratories, 10032041, California, USA)	Real time quantitative PCR for target genes
Quantitative Real Time PCR primers (21+21 BP)	(Bio-Rad Laboratories, 10032041, California, USA)	Quantify the target genes in response to different treatment
2-Mercaptoethanol	Sigma /Aldrich Chemie GmbH, Munich, Germany	Total RNA isolation
Buffers	**Preperation**	
Sodium chloride	physiological saline solution has been prepared from 9g of NaCl in 1000ml of distillated water.	
RBCs lysis buffer	has been prepared by adding 8.26g of NH_4Cl, 1.19g of $NaHCO_3$, 200ml of EDTA (0.5M, pH8), distillated water was added up to 100ml and p H has been adjusted in 7.3, buffer was sterilized by autoclave to store up to 6 months.	
Phosphate buffer saline 1X	was prepared by adding 8.00g of NaCl, 0.2g of KCl, Na_2HPO_4 was 1.44g, KH_2PO_4 was 0.24g and distillated water has been added up to 1000ml.	
PFA	was prepared by adding 80ml of PBS, 4g of paraformaldehyde powder, 1N of NaOH and HCl for adjusting p H.	

2.1.2.Equipment

Following equipment were used in different phase of the experiment.

Name of Equipment	Manufacturer
CO2-incubator (MCO-17AI)	Sanyo, Japan
Biological safety cabinet	Telstar
Centrifugation	hettich centrifuge, USA
Microcentrifugation	Beckman Coulter/ germany
Refrigerator centrifugation	Sigma 1-15pk
Thermocycler	Senso quest labcycler /Germany
microplate (ELISA)	Glomax Multi Detection System, Promega Bio Systems Sunnyvale, CA, USA
Quantitative real time PCR	Roche / Switzerland
Fluorescent microscopy	Nikon Eclipse T*i*-S microscope (Nikon Instruments Inc., Tokyo, Japan)
Nanodrop 8000 spectrophotometer	Thermo Fisher Scientific, DE, USA
Ultra-low freezer (-80oC)	Labotect GmbH, Göttingen, Germany
Water bath	Memmert GmbH / Germany
Semi-micro Balance	Sanyo / Japan
Laboratory oven	Panasonic biomedical / Japan
Hemacytometer	Fisher scientific / USA
lab shaker (vortex)	Thermofisher / USA
Inverted microscopy	Nikon Eclipse TS100 microscope (Nikon, Tokyo, Japan).

2.2.Methods

2.2.1. Collection and uterine cells

The collection of UCs has been conducted by Kirk, D. 1978. In this experiment, we did not use live animals for experimental samples and samples will be collected from slaughtered animals. Therefore, ethical permission is not required to conduct this experiment. The first step of the experiment was to collect and culture endometrial fibroblast cells. For this, uterus collected form slaughtered animals by using sterilized Biological Dissection tools and kept in the ice during transportation until processing. In the laboratory, uterine body horn cut by surgical scissors into very small pieces putted in glass beaker 1000 ml and washed three times with 0.9% NaCl to remove bloods and other debris. After that, the samples rinsed via 70% ethanol for 30 seconds to removing bacteria then washed again three times with 0.9% NaCl and samples have been kept in DMEM-F12 media containing 10% FBS and 1% AA (Antibiotic, Anti-mycotic) during further processing. Endometrium has been opened by surgical scissors, after that by anatomical method, the inner endometrium tissue was isolated and transferred to a petri dish containing 3-4 ml 1X digestion media (collagenase type 2), Samples have been chopped by surgical scalpel blade into 1mm3 size. Then, the minced tissue samples were collected in a 15 ml sterile falcon tube and added another 6 ml digestion media and incubated for 1 - 1.5 h in an incubator at 37˚C with gentle rotating. The sample was shaken manually every 15 min for proper digestion. At the end of digestion period, samples were transferred to 50 ml sterile falcon tube and 20 ml of DMEM-F12 media containing 10% FBS were added to stop the activity of digestion media. After that, samples were passed through 40 µm pore sized filter to separate single cells from the cell clump. The flow has been collected by centrifuged at 400 × g for 5 min, the supernatant discarded and the cell pellet were subjected to RBC lysis buffer to remove the red blood cells also isotonocity were restored by adding DMEM-F12 media containing 10% FBS. Finally, samples were a gained centrifuged at 400 × g for 5 min and the supernatant were discarded. Uterine cells were seeded on the cell culture plate and cultured for 24 h in DMEM/F-12 media supplemented with 10% FBS, anti-biotic 100 U/ml and anti-mycotic 100 µg/ml at 37°C in a humidified atmosphere of 5% CO_2.

2.2.2.Collection and Culture of follicular cells

According to sohel, et. al. 2013 [82]. Ovaries n= 120 were obtained from local abattoir and transported to the lab in 0.9% saline solution NaCl at a temperature of 37°C in thermos-flask within 2 h after collection. Follicular cells were collected from follicles 5-8 mm washed once in calcium-magnesium free PBS (CMF). These cells were collected by aspiration method using syringe and needle in 50 ml falcon tube, followed by centrifugation at 500 x g for 5 min and resuspended in 2 ml of RBC lysis buffer for 1 min to remove erythrocytes.

Isotonicity was restored by adding 8 ml of DMEM F-12 media containing 10% FBS. After two additional washes with media. Follicular cells were seeded on the cell culture plate and cultured for 24 h in DMEM/F-12 media supplemented with 10% FBS, anti-biotic 100 U/ml and anti-mycotic 100 µg/ml at 37°C in a humidified atmosphere of 5% CO_2.

2.2.3.Treatment with ethanol

In order to assess the effect of alcohol consumption on cells from female reproductive organs, Follicular and Uterine cells were cultured up to 60 – 70 % confluence and treated with ethanol. Before that, experiment was made to select the optimal concentration, in addition, the time period for exposure was checked. five different concentrations of ethanol were selected are (50, 100, 200,500 and 1000 mM), as well as, the period for cell exposing was 6 and 24 h. After that, media was removed and cells were either collected using trypsin and investigated for different phenotypic and genotypic parameter. Figure 2.1. and 2.2. shows the experimental layout to achieve mentioned goals of the whole experiment for follicular and uterine cells separately.

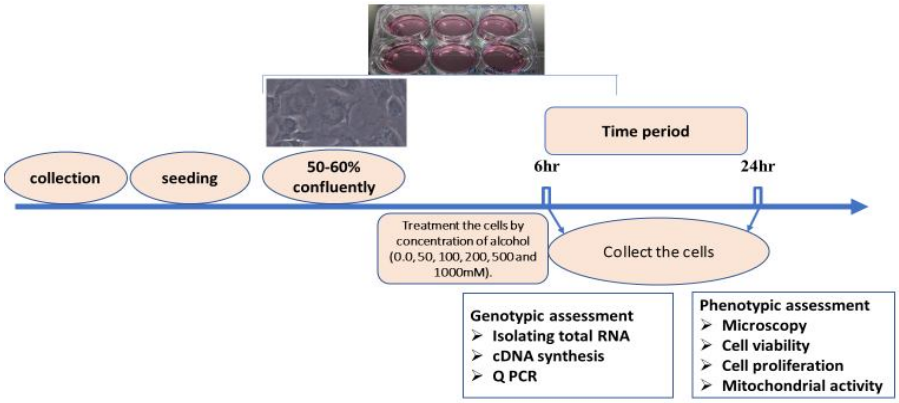


Figure 2.1. Experimental layout of follicular cells

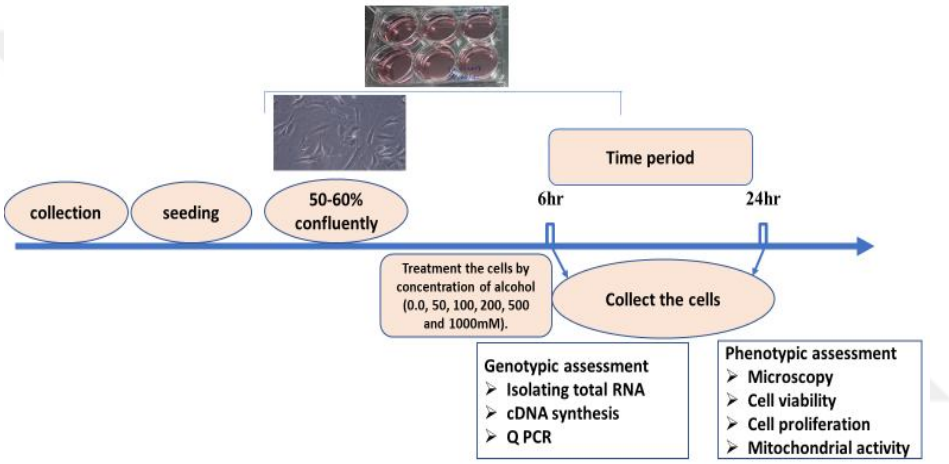


Figure 2.2. Experimental layout of uterinecells

2.2.4.Cell Morphology and Viability

Size, shape and polarity of follicular and uterine cells were checked after treated by different concentrations of alcohol under inverted microscopy manufactured by Nikon Eclipse TS100 microscope Nikon, Tokyo, Japan.

For calculating both dead and alive cells which are treated and non-treated by alcohol. trypan blue staining was used to check live and dead cells according to Strober, 2001, 24 well plates form was used to this experiment, old culture media that consist of dead cells were kept in 15 ml falcon tube, live cells were trypsinized by adding 200 µl of trypsin enzyme to detach the live cells by incubating for 5 min in 37˚C, 5%CO2. Subsequently, 800 µl of fresh warm media was added to stop trypsin reaction. Alive and dead cell were collected in 15 ml of falcon tube separately for each concentration, cells were centrifuged

in 500 xg for 5 min. Then, the supernatant was removed. 250 µl of fresh media, 150 µl of PBS and 100 µl of trypan blue were added and we waited 5 min, Zahlkammer counting chamber (hemocytometer) with cover slip were used to counting, 10 µl of Cell Suspension were transferred to hemocytometer by using micro pipette to both of chamber.

2.2.5.Mitochondrial Activity Detection

In order to detect Mitochondrial activity in FCs and UCs MitoTracker® Red CMXRos (M7512; Invitrogen) has been used for mitochondrial activity detection. The media contain alcohol was removed, 500 µl of fresh warm media without FBS containing 400 nM of mitotricker were added to each well, after that cells have been incubated in 37˚C, 5%CO2 and dark place for 30 min for mitochondrial staining purpose. After that, cells were washed by 500 µl of PBS twice for 5 min each. 300 µl of 4% PFA have been added to each well for fixing the cells and incubated for 25 min. Cells were washed again three times 5 min each by 500 µl of PBS. Thereafter, old PBS was removed and 200 µl of fresh PBS was added to keep the cells moist. Images have been acquired with the suitable wavelength for the dyes at 40x magnification under a fluorescence microscope.

2.2.6.Cell proliferation assay

For investigating the extent of cell proliferation which FCs and UCs were exposed. Cell proliferation reagent WST-1 was utilized to detect the quantification of cell proliferation. 96 well plates form was used. Old media were removed, 100 µl of fresh warm media contained from (10% of FBS, 1% P/S & 89%of DMEM) and 10 µl of cell proliferation reagent WST-1 was added to each well. Subsequently, cells were incubated for 4 h at 37˚C, 5%CO2 and dark place. Microplate (ELISA) reader with a filter for wavelength 420-480 nm was used to check the proliferation of FCs and UCs.

2.2.7.Total RNA purification

Total RNA has been extracted from cells (n = 3) by EURx®/ Poland universal RNA/miRNA according to the manufacturer's protocol. Firstly, 2 ml Eppendorf tube of the cells culture have been centrifuged in 1000 xg for 5 mins, supernatant was removed.

After that, 400 µl of RL buffer containing 40 µl of B-ME to the cell pellet and has been mixed thoroughly by vigorous vertexing and pipetting for homogenization. Also, 100 µl of lysis buffer consisting of 1 µl of B-ME was added gto cell pellet to homogenized the sample. The sample were centrifuged at maximum speed for 2 min. Subsequently, the supernatant has been transferred carefully to the homogenization spin-column placed in the 2 ml receiver tube and centrifuged at maximum speed for 2 min. 600 µl of Absolut ethanol was added to the sample and mixed thoroughly without centrifuging. After that, up to 700 µl of sample mixture have been applied to the RNA binding spin-column and centrifuged for 1 min in 11000 xg. After that, the spin-column were removed, the supernatant has been poured off and placed back in to receiver tube. the mixture which is remained has been transferred to the same RNA binding spin-column and we did the step again, then 600 µl of wash miRNA buffer was added to mixture and centrifuged at 11000 xg for 1 min, the spin-column was removed and supernatant poured off with placed back into the receiver tube to centrifuged again at 11000 xg for 1 min to removing residual wash buffer. Spin-column has been placed into new receiver tube and 40-80 µl of RNase-free water was directly added onto the membrane. Finally, the mixture was centrifuged at 11000 xg for 2 min, spin-column was removed also the receiver tube was capped. The concentration and purity of total RNA have been determined using a Nano Drop ND-1000 spectrophotometer.

2.2.8.cDNA synthesis and analysis

For mRNA analysis 320 ng total RNA have been reversed transcribed to cDNA using cDNA synthesis with Maxima H minus first strand kit (K1681, Thermo scientific EU Lithuania) in accordance with the manufacturer's protocol. After total RNA concentration were checked by spectrophotometer and found a high concentration and quality of total RNA. All reagents have been kept on ice, 1µl of 10x dsSNase buffer, 1µl of DsDNase, 320 ng of our total RNA samples have been mixed gently and completed the volume up to 10 µl by adding water nuclease free. Subsequently, the contents were incubated for 2 min at 37˚C in water bath then we added to each sample a composition of 0.25 µl of oligo dT primer, 0.25 µl of Random hexamer primer, 1 µl of 10 mM dNTP mix and 3.5 µl of water nuclease free with mixing gently. Thereafter, samples have been centrifuged and

incubated in 65˚C for 5 min by using labcycler. After that, 4 µl of 5x RT buffer and 1 µl of maxima H Minus enzyme ix added to each sample, the samples have been incubated for 10 min at 25˚C followed 15 min at 50˚C. Finally, we terminated the reaction by heating for 5 min in 85˚C. The concentration and purity of the complementary DNA have been determined using a Nano Drop ND-1000 spectrophotometer.

2.2.9.Real-Time Quantitative PCR

An ABI PRISM® 7000 sequence detection system (Applied Biosystems, Foster City, CA) instrument has been utilized, According to (Bio-Rad Laboratories, 10032041, California, USA) protocol, the primer sequences of the genes used in this experiment were designed via Primer3 online Software v.0.4.0 (http://frodo.wi.mit.edu/primer3/) and listed in supplementary for (GAPDH, NRF2, KEAP1, HMOX1, PRDX1 and SOD1). Primers have been delivered and prepared for our target genes Table 2.1. Before running quantitative RT-PCR reaction, the suitability of endogenous control was confirmed by investigating the consistency of GAPDH expression among the experimental samples (data not shown). Firstly, the content for each sample has been prepared separately, which consist of 10 µl of SYBR green, 0.3 µl of forward primer also 0.3 µl of reverse primer and 7.4 µl of RNase free water to be the volume 18 µl. The reaction content has been distributed to each well, then 2 µl of our cDNA samples were added to be in total volume 20 µl. The plate has been covered by special nylon over and closed carefully, as well as, we vortexed the plate thoroughly. Subsequently, the real time quantitative PCR was programmed according to protocol steps; Preincubation, qRT-PCR has been programmed for 2 min at 50˚C and for 10 min at 95˚C per 1 cycle. Amplification; was seated for 45 cycles at 95˚C and 60˚C with holding 15 and 30 seconds Respectively, also 72˚C for 30 second. Melt curve; temperature was programmed 60˚C with holding 15 seconds followed 95˚C in continuous mode and 20 acquisitions (per ˚C). Relative expressions of each mRNA were analyzed using a comparative CT ($2^{\Delta\Delta CT}$) method.

Table 2.1. List of primers that used for qRT- PCR experiment

Gene	Accession number	Primer sequences	Annealing temp.(°C)	Product size
GAPDH	NM_001034034.2	F5'-GAAGCTCGTCATCAATGGAAA R5'-CCACTTGATGTTGGCAGGAT	**58**	**67**
NRF2	NM-205117.1	F5'-ATCACCTCTTCTGCACCGAA R5'-GCTTTCTCCCGCTCTTTCTG	**58**	**258**
KEAP1	NM_001101142.1	F5'-TCACCAGGGAAGGATCTACG R5'-AGCGGCTCAACAGGTACAGT	**55**	**199**
CAT	NM_001035386.1	F5'-TGGGACCCAACTATCTCCAG R5'-AAGTGGGTCCTGTGTTCCAG	**51**	**178**
HMOX1	NM_001014912	F5'-CAAGGAGAACCCCGCTACA R5'-CCAGACAGGTCTCCCAGGTA	**55**	**225**
PRDX1	NM_174431.1	F5'-TGGATCAACACACCCAAGAA R5'-GTCTCAGCGTCTCATCCACA	**55**	**217**
SOD1	NM_174615	F5'-AGAGGCATGTTGGAGACCTG R5'-CAGCGTTGCCAGTCTTTGTA	**53**	**189**

2.3.Statistical analysis

All experiments were performed with a minimum of three biological replicates. Statistical differences between treatment group means (quantitative variables) were analyzed by one-way ANOVA followed by a multiple pairwise comparison (Tukey test, $\alpha=0.05$). For statistical analyses and graphs SPSS version 20.0 and GraphPad Prism were used. Data is expressed as mean ± SD of three biological replicates. Differences were considered significant at $P<0.05$.

CHAPTER 3

RESULTS

In this study, the follicular (FC) and uterine cells (UC) obtained from ovarian and uterus of cattle, respectively. The cells seeded in the culture medium were exposed to different ethanol concentration ranging between 0-1000 mM and the results obtained were given in this section. And the data obtained from all these in vitro experiments are given below for each cell culture.

3.1.Follicular Cells (FCs)

3.1.1.Cell collection and seeding

Ovaries have been collected from slaughtered cattle, subsequently, GCs were isolated and seeded in six wells plate consisting of 10% of FBS., 1% of AA and DMEM-F12 media as in the Figure 3.1.

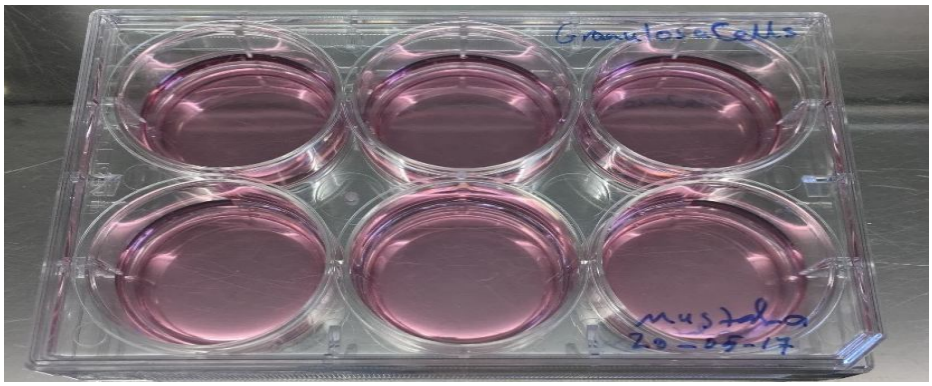


Figure 3.1. Follicular cells seeded in six wells culture plate consist of 10% of FBS. 1% of AA and DMEM-F12 media

We checked the cells morphology daily under inverted microscopy (health, shape and size). We found FCs are healthy in the culture suspension and attached to plate surface. These FCs mostly round or fusiform in shape, italic to Steller with unequal normal size. After two days of cultivation, it was found that the number of viable cells per mL was 1.5×10^5. The image of the FCs on the second day of the seeding is given in Figure 3.2.

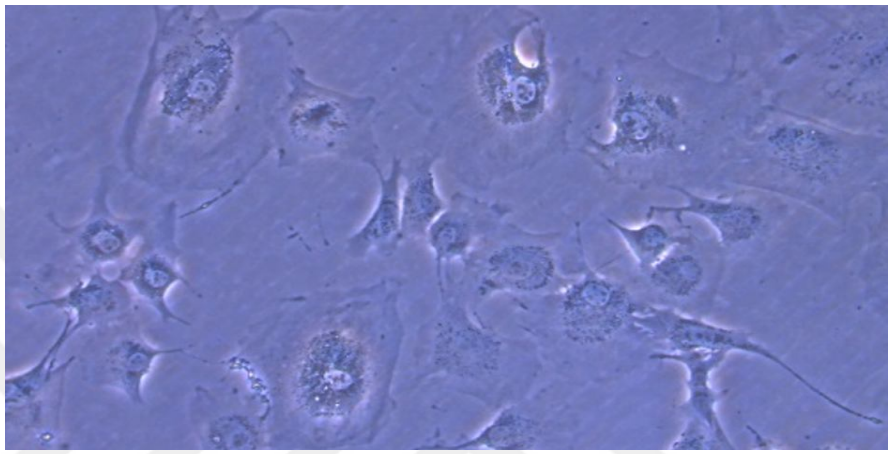


Figure 3.2. Follicular cells after two days of seeding, (10x)

At the third days of the examination, it was found that the cells are in good health and normal shape with normal unequal size. Cells proliferation increased with increasing period and the number of viable cells found as 2.8×10^5 per ml. The image of the FCs on the third day of the seeding is given in Figure 3.3.

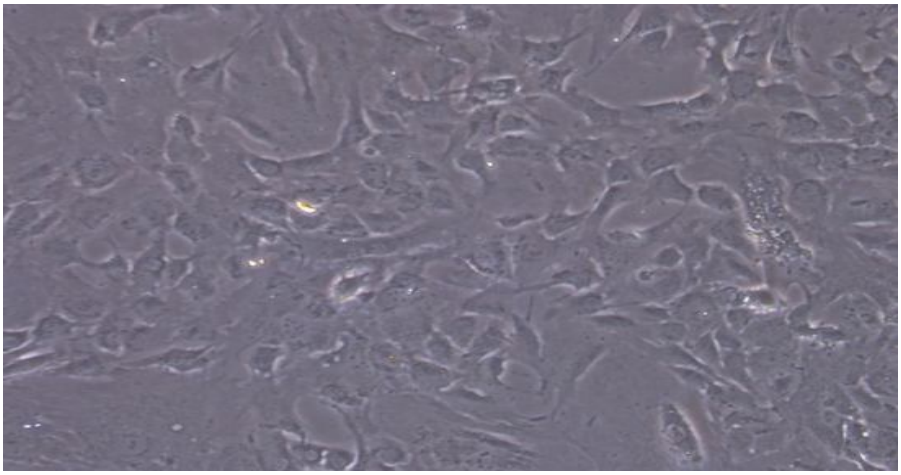


Figure 3.3. Follicular cells after three days of seeding, (10x)

At the end of the fifth day, when the cells were examined, no cell degradation was observed, and the cells maintained their healthy state. The number viable cell was 5.1×10^5 viable cells per ml. The image of the FCs on the fifth day of the seeding is given in Figure 3.4. The growing rate of FCs in the culture media lasted longer than fibroblast cells.

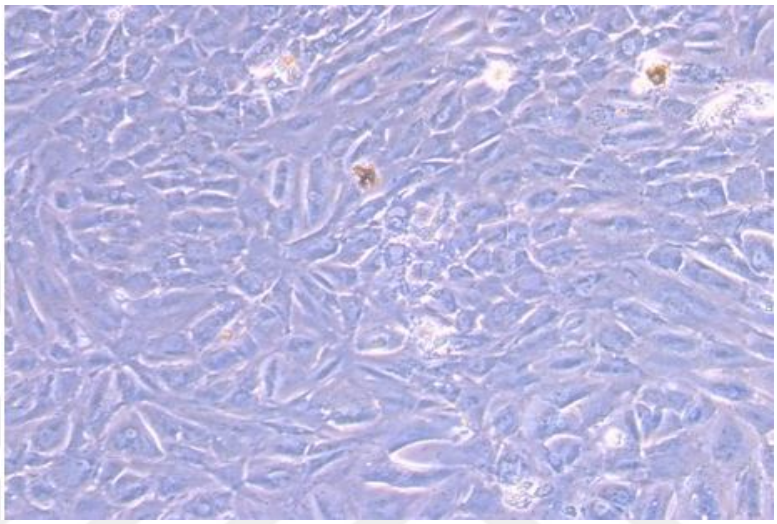


Figure 3.4**.** Follicular cells in fifth day of the seeding (10x)

3.1.2.Effects of ethanol on follicular cell morphology and cell viability

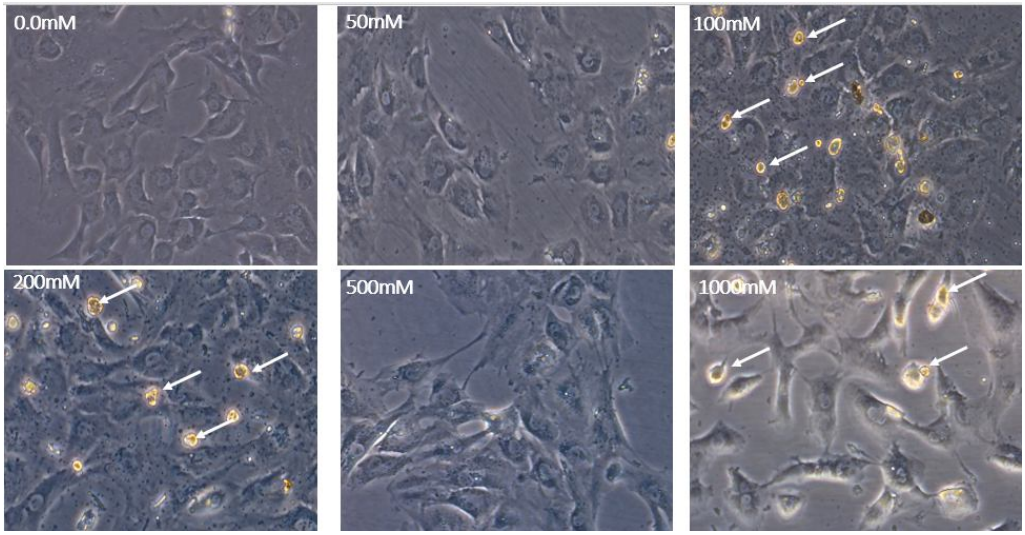


Figure 3.5. Polarity, size, shape and viability of the ethanol treated follicular cells after 6 h at different concentration

FCs exposed to different ethanol concentration and the results obtained in this experiment were recorded after 6 and 24 h exposure. As seen in the Figure 3.5, 50 mM of ethanol caused very little cell damage, but the resulting defects were at minimal level after 6 h when the FCs exposed to different ethanol concentration. However, the size of the cells increased after exposure and significant cell degradation was observed at 100 mM and above. The elongation and fibrillation of the cells were observed depending on the increasing concentration of ethanol. As can be seen in the figure, agregation was observed after the cells treated with 500 mM concentration. Besides, ethanol caused an increase in the polarity of the cells and multipolar cells were observed at the higher concentrations. Morever, the number of dead cells began to increase after the cells exposed to 100 mM and above concentrations. A large number of black spots were observed scattered around in the cell cultures exposed to 100 and 200 mM ethanol suggesting that cell fragments of the dead cells may be degraded and be scattered around. Cells shrunken and dead by the action of ethanol are shown with white arrows in the Figure 3.5.

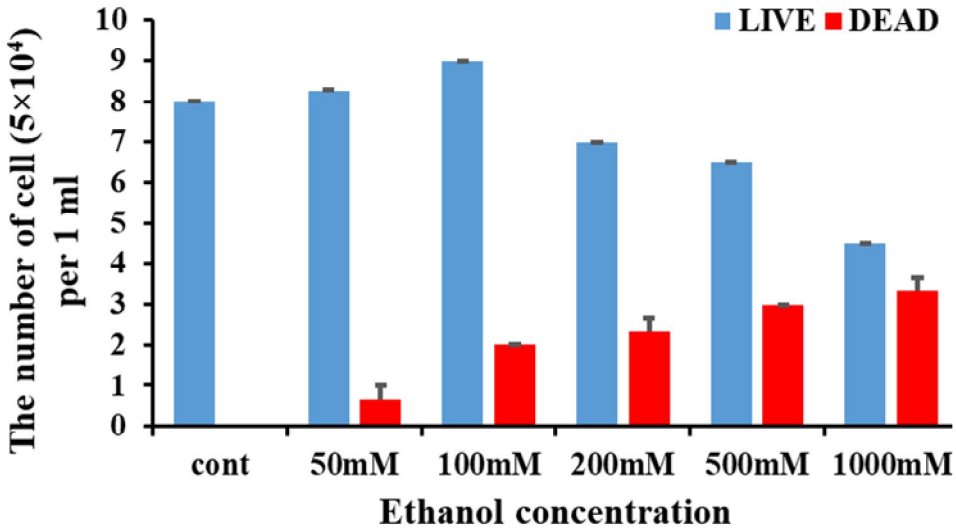


Figure 3.6. Cell viability of follicular cells after 6 h

After 6 h, viability of the cells were also investigated by using trypan blue and the number of dead and living cells were counted and given in Figure 3.6. When the cells exposed to lower concentration of ethanol (50 and 100 Mm) cell viability increased when compared the control, however at the higher concentrations (200, 500 and 100 Mm) cell viability significantly decreased with increasing ethanol concentration (F=

13864, P<0.05). The number of dead cells significantly increased when exposed to different ethanol concentarions (F= 30.933, P<0.05).

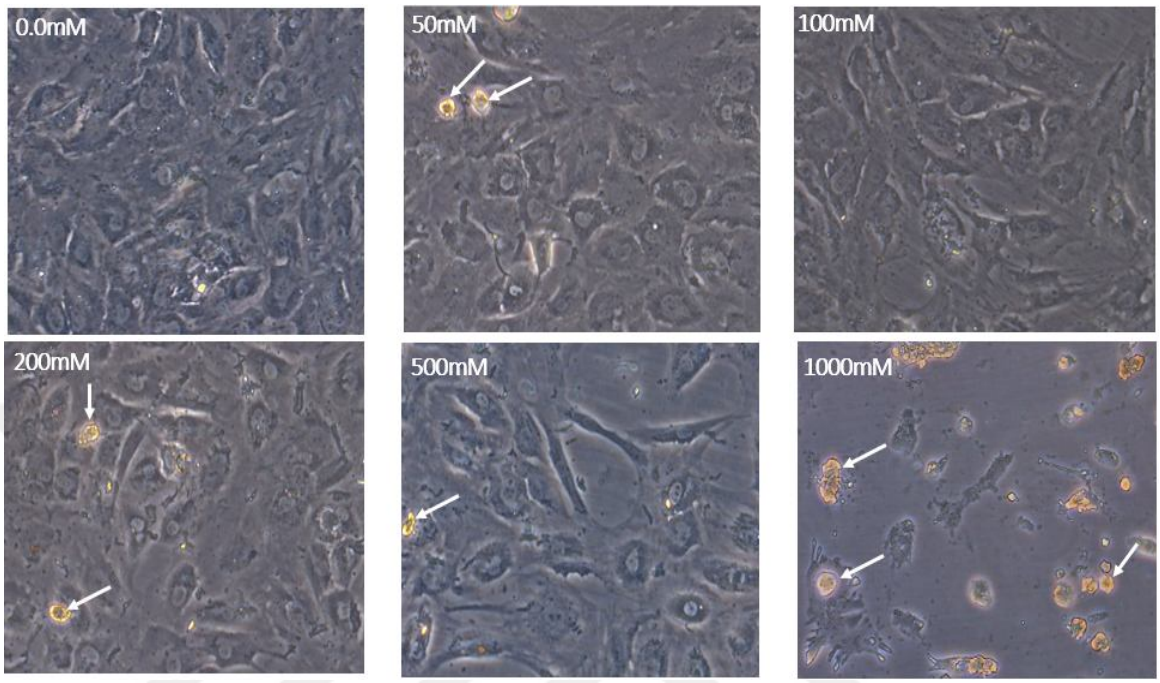


Figure 3.7. Polarity, size, shape and viability of the ethanol treated follicular cells after 24 h at different concentration

When the FCs are exposed to ethanol for 24 hours, cell death has begun to be noticeable in the smallest concentration applied. Interestingly, there was a significant increase in viable cell count when compared to other exposures at 200 mM exposure. Elongation, size and polarity of the cells increased with increasing ethanol exposure. Aggregation of the cells observed with 500 mM exposure.

It has been observed that cell degeneration levels at 6 and 24-hour ethanol exposure are quite different for 100mM ethanol exposures. After 24 h ethanol exposure Figure 3.7.

When the effects of 1000mM ethanol were examined in 6 and 24-hour ethanol exposures, a significant difference was observed in factors such as cell shape, polarity, cell size and cell death. When 1000mM ethanol was applied for 6 hours, live cells were still evident despite cell death. However, when applied at the same concentration for 24 hours, it was observed that the cell shapes changed and almost no living cells remain Figure 3.8.

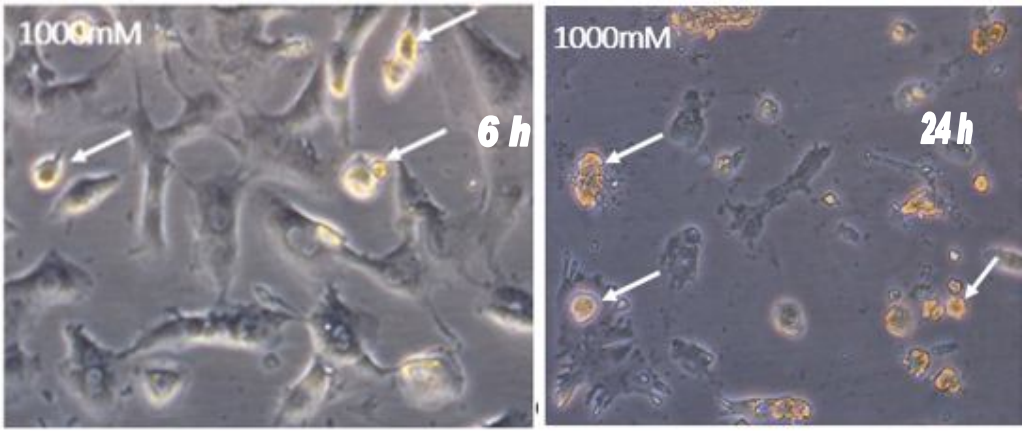


Figure 3.8. The effects of the same concentration of ethanol on the follicular cells, depending on exposure time

The number of dead and living cells were counted and given in figure 3.9. When the cells exposed to different concentration of ethanol cell viability increased untill 200mM when compared control but at the higher concentrations (500 and 1000 mM) cell viability significantly decreased with increasing ethanol concentration ($F=1300.8$, $P<0.05$). The number of dead cells significantly increased when exposed to different ethanol concentarions after 24 h exposure ($F=79.61$, $P<0.05$).

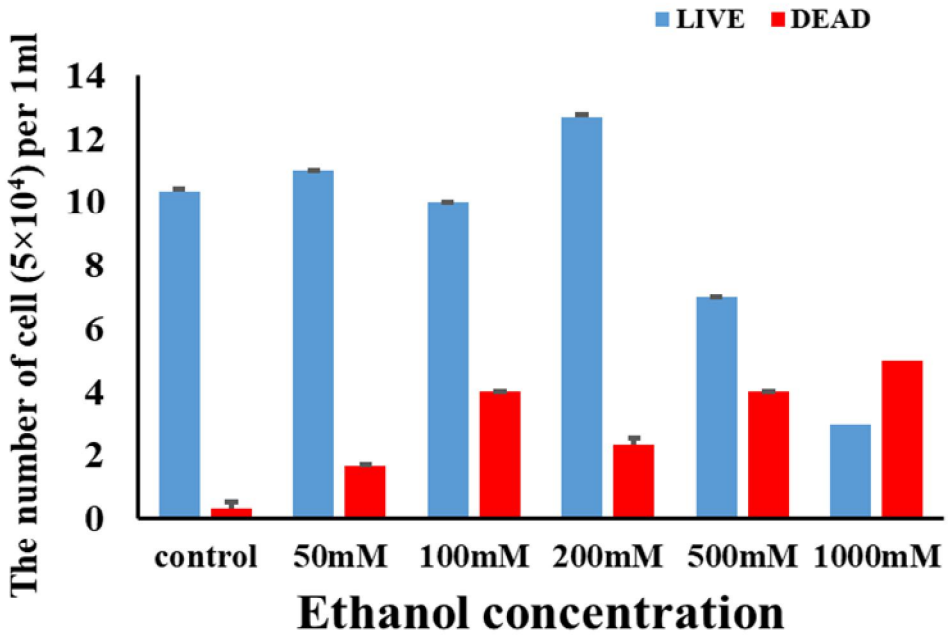


Figure 3.9. The cell viability of follicular cells after 24 h

3.1.3.Effects of ethanol on proliferation of follicular cells

After the FCs were exposed to ethanol in varying concentrations, cell proliferation was observed for each administration period and the data were recorded. Cell proliferation significantly increased with increasing concentration of ethanol up to 100 mM ($F=560.923$, $P<0.05$), in 6 h exposure. But the increasing alcohol concentration showed a significant inhibitor on FCs proliferation in 200, 500 and 1000 mM. After 24-h 50, 200 mM of ethanol exposure for FCs showed a significant increasing in cell generation. the cells treated by 100, 500 and 1000 mM of ethanol exposure was observed a significantly reduced in generation ($F=337.09$, $P<0.05$) as seen in the figure 3.10.

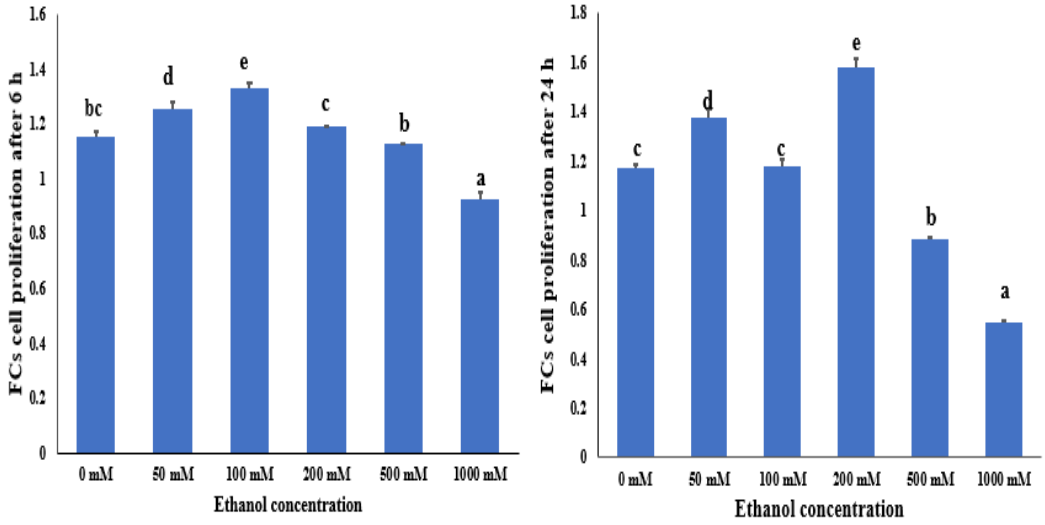


Figure 3.10. The cell proliferation of follicular cells after 6 and 24 h (Means with different letters on the bars were significantly different, ANOVA, $P<0.05$)

3.1.4.Effects of ethanol on mitochondrial activity in follicular cell

Mitochondria participate in defense against releasing ROS, it also targets for oxidative stress. Mitochondria also contribute in the controlling mechanism of oxidative stress inhibits in the cell. Therefore, we next investigated for mitochondria in FCs that are treated of different concentrations of ethanol for various period of time. When the FCs were exposed to different ethanol concentrations the mitochondrial activities were

observed under confocal microscope and the images of the cells are given in Figure 3.11. As seen from the images, normal signals were obtained indicating normal mitochondrial activity. At 50, 100 and 200 mM concentrations of ethanol treatment exhibited higher signal of mitochondrial activity than that of untreated control. On the other hand, at 500 and 1000 mM of ethanol exposure the signal of mitochondrial activity was lower than that of untreated control.

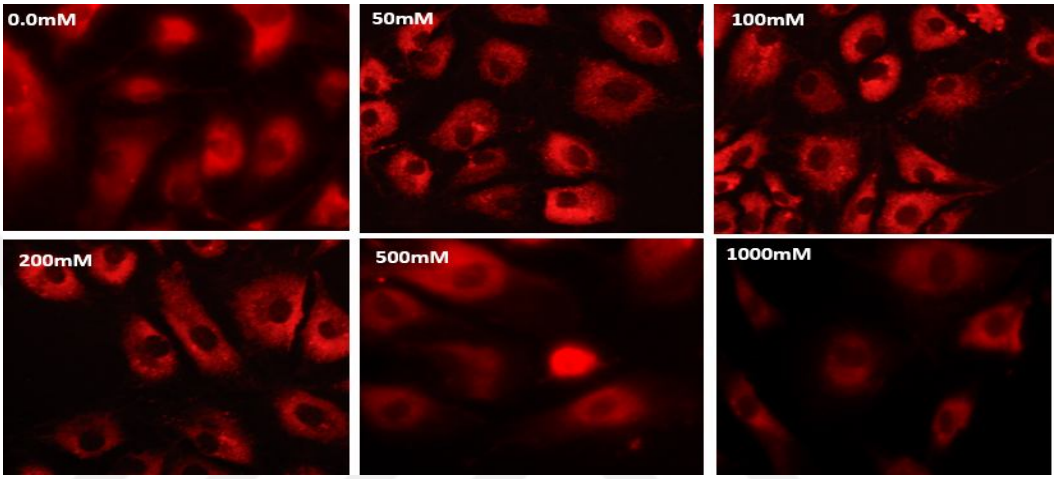


Figure 3.11. Mitochondrial activity after 6h ethanol exposure for follicular cells

When the FCs were exposed to ethanol for 24 hours, the mitochondrial activities of the cells were shown in the Figure 3.12. The signals of mitochondrial activity for the cells exposed to different concentration of ethanol increased, except in 1000 mM. In this concentration, mitochondrial activity was reduced.

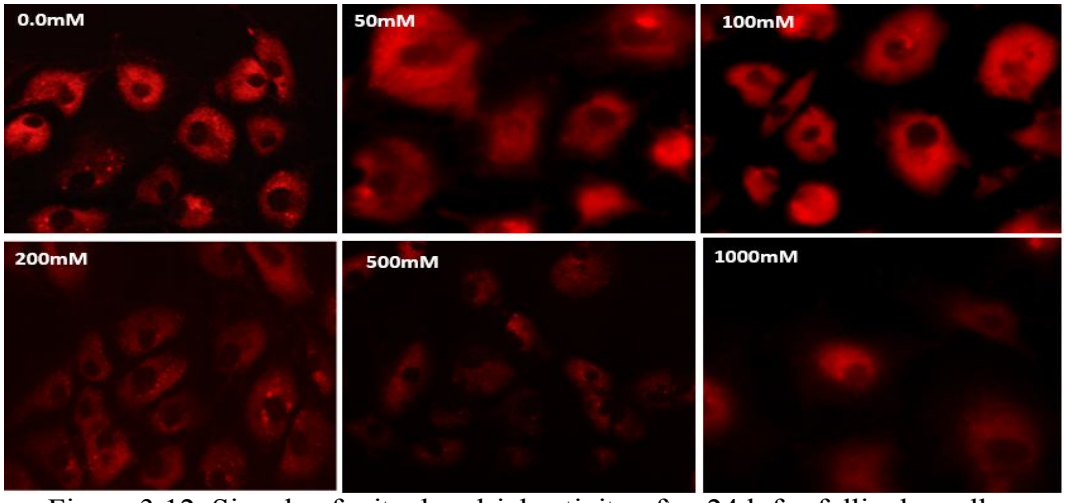


Figure 3.12. Signals of mitochondrial activity after 24 h for follicular cells

3.1.5.Effects of ethanol on antioxidant genes in follicular cells

In order to assess ethanol induced oxidative stress and determine expression levels of antioxidant genes in FCs, quantitative real-time PCR was used. Expression levels of *NRF2, KEAP1, CAT, HMOX1, PRDX1* and *SOD1* genes were determined after the cells were exposed to different concentrations of ethanol for 6 and 24 h. In order to determine the expression level of this gene, fold change values were estimated and interpreted. The findings from this study are described below for each gene and exposure time.

3.1.5.1.*NRF2* expression

The expression of *NRF2* gene was decreased in the FCs treated with 50, 200 and 500 mM of ethanol when compared to untreated control after 6 h exposure (Figure 3.13). Fold change values indicate that when the cells are exposed to ethanol the expression level of the gene decreased with the increasing concentrations. This trend was also the case for the cells up to 200 mM concentration, however, a sudden increase was observed with 500 mM of ethanol after 24 h exposure. It is noted that when the cells are exposed to high concentration of ethanol for a longer period, a remarkable increment is observed in the expression of the gene.

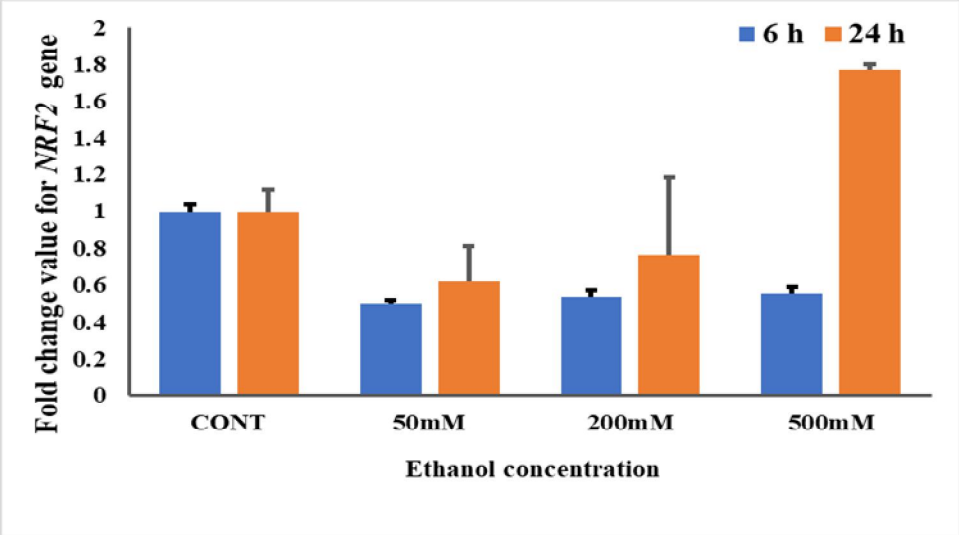


Figure 3.13. Estimated fold change values for *NRF2* gene in follicular cells after being exposed to ethanol for 6 and 24 h.

DCT values of the *NRF2* gene were estimated after exposing the follicular cells to ethanol and the results were given in Figure 3.14. DCT values were significantly higher ($F=180.094$, $P<0.05$) than control after 6h exposure indicating a decrease in gene expression. Although this increasing trend in DCT values was also the case for 24 h exposure with 50 and 200 mM concentrations, statistically significant decrease was observed with 500 mM when compared to untreated control ($F=8.007$, $P<0.05$).

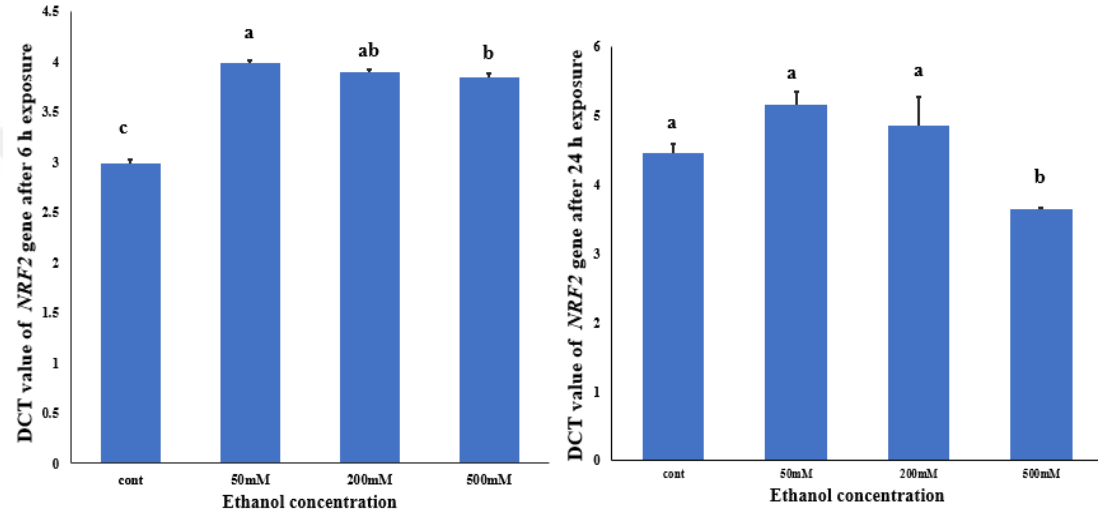


Figure 3.14. DCT values of *NRF2* gene in follicular cells after ethanol exposure (Means with different letters on the bars were significantly different ANOVA, $P<0.05$)

3.1.5.2.*KEAP1* expression

When evaluated the fold change values of the *KEAP1* gene expression after short-term exposure 6 h of ethanol showed a reduction when compared to control. On the other hand, a high level of gene expression was observed for long term exposure 24 h with 500 mM of ethanol. It is noted that both high concentration and longer exposure cause striking increase in the expression of *KEAP 1* gene. Long-term high-concentration ethanol increased the level of *KEAP 1* gene expression to very high levels (Figure3.15.).

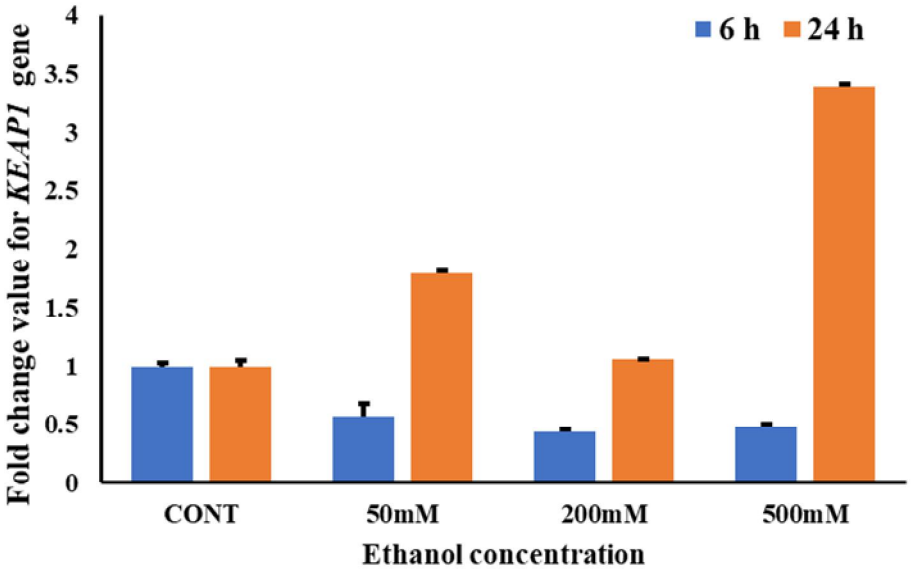


Figure 3.15. Estimated fold change values for *KEAP1* gene in follicular cells after being exposed to ethanol for 6 and 24 h.

When the DCT values were estimated for both 6 and 24 h of exposure statistically significant results were obtained. While this value incraesed with increasing ethanol concentration after 6 h, significant reduction was observed for 24 h of exposure (For 6h: $F=84.625$, $P<0.05$ and for 24h: $F=774.141$, $P<0.05$)(Figure 3.16.).

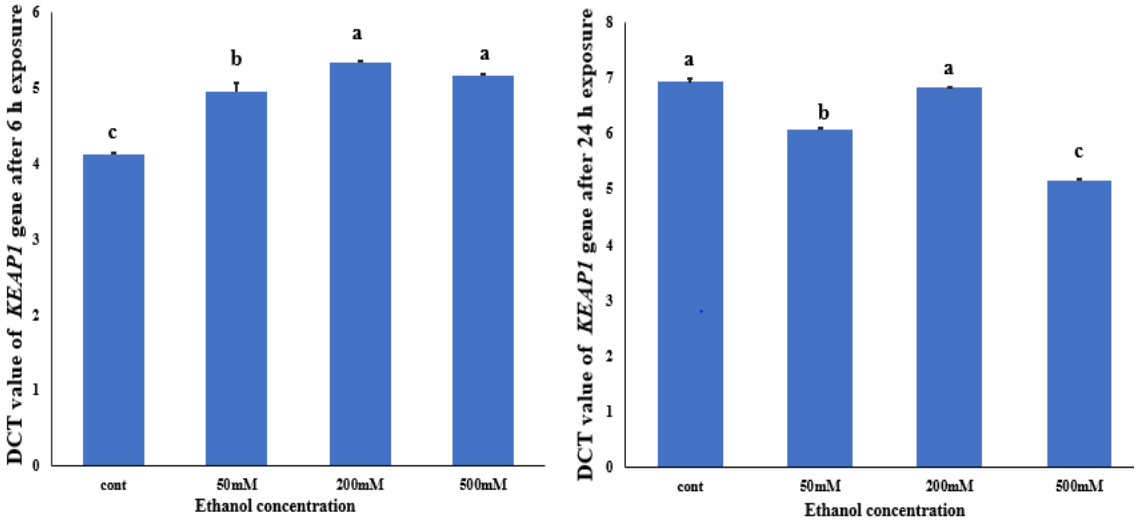


Figure 3.16**.** DCT values of *KEAP1* gene in follicular cells after ethanol exposure (Means with different letters on the bars were significantly different, ANOVA, $P<0.05$).

3.1.5.3.*CAT* expression

For short term exposure 6 h, all treatment concentrations for *CAT* gene expression shows a decreasing compared with untreated control. While for long term exposure 24 h, 50 mM concentration shows a slight increase, however, in gene expression the other concentration showed a high increase in *CAT* gene expression compared to the untreated control Figure 3.17.

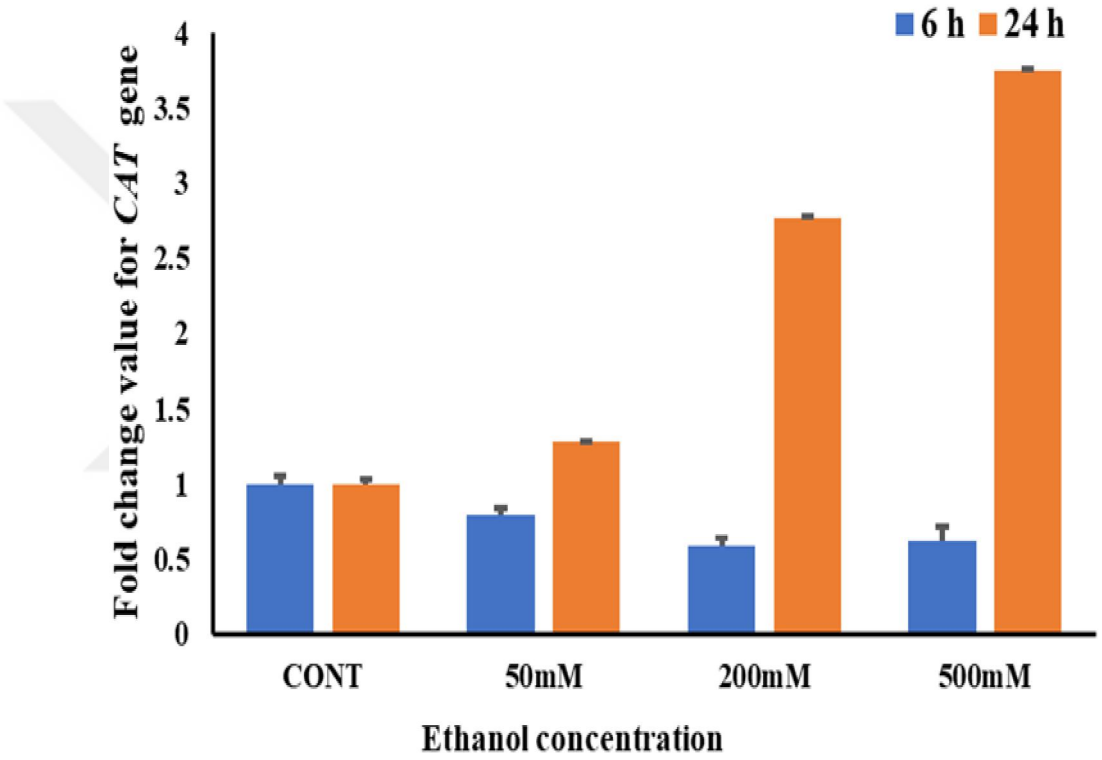


Figure 3.17. Estimated fold change values for CAT gene in follicularcells after being exposed to ethanol for 6 and 24 h.

DCT values for *CAT* gene were obtained after exposing GCs to ethanol as shown in the Figure 3.18. It appeared that DCT values for 6 h exposure were significantly higher ($F=28.403$, $P<0.05$) than control thus announcing a decrease in *CAT* expression.

For 24 h of ethanol exposure the DCT values showes a decrease for all ethanol concentrations, indication increasing in *CAT* gene expression level ($F=2292.149$ $P<0.05$; Figure 3.18.).

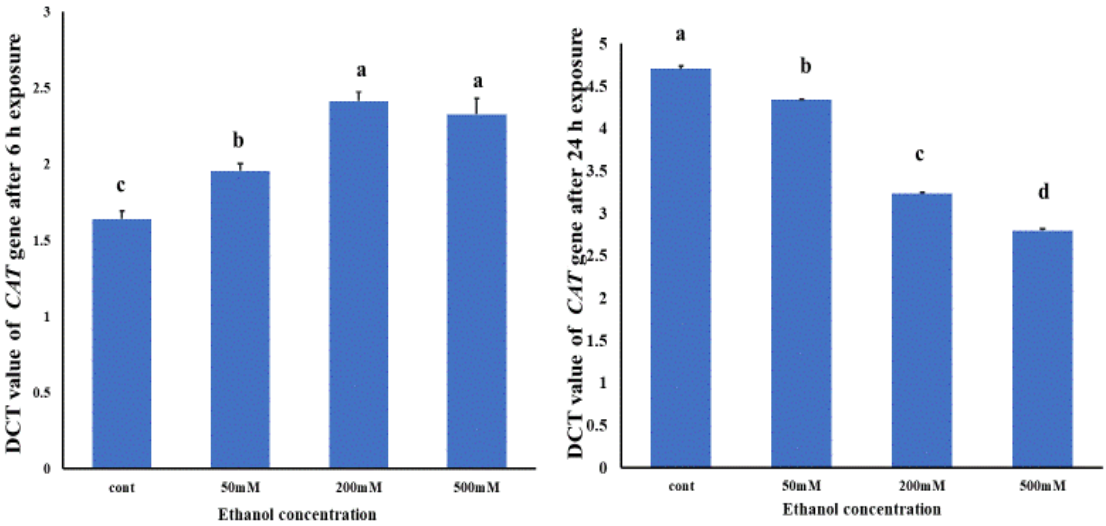


Figure 3.18. DCT values of *CAT* gene in follicular cells after ethanol exposure (Means with different letters on the bars were significantly, ANOVA, P<0.05)

3.1.5.4.*HMOX1* expression

After 6 h of ethanol exposure the *HMOX1* gene expression shows a decrease for all ethanol concentrations (Figure3.19.). On the other hand, after 24 h of ethanol exposure there was a slight increase when the cells were treated with 50 mM. Whereas at 200 mM of ethanol exposure, we found a decrease in *HMOX1* gene expression. Furthermore, in 500 mM a huge increase was obtained in expression level of *HMOX1* gene (Figure 3.19.).

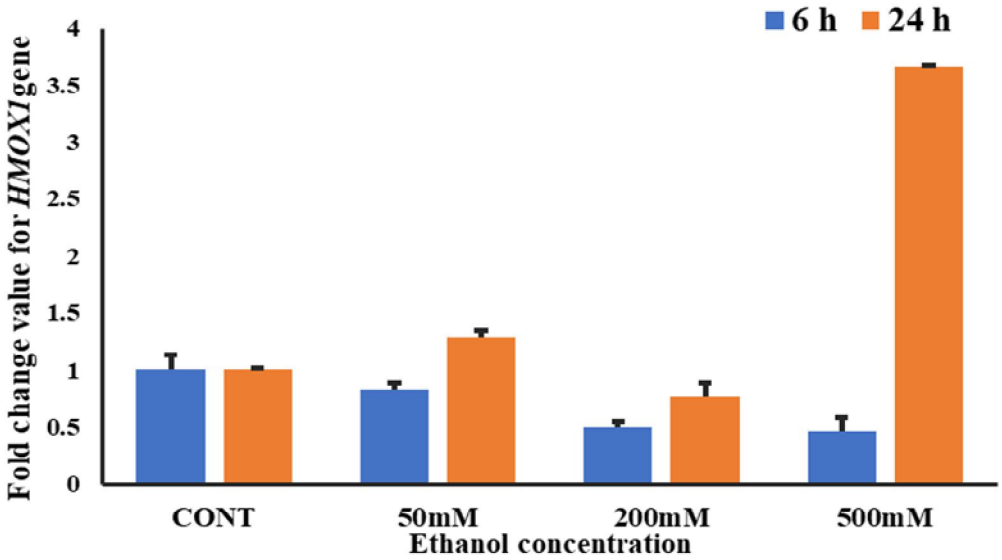


Figure 3.19. Estimated fold change values for HMOX1 gene in follicular cells after being exposed to ethanol for 6 and 24 h.

For DCT values in case of 6 h of ethanol exposure, all *HMOX1* DCT values appeared to be higher than the control value (F=30.759, P<0.05, Figure 3.20.) which express the reverse relationship with fold change. For 24 h of ethanol exposure, a small decrease appeared in 50 mM and significant decrease in 500 mM with slight increase when treated with 200 mM (F=248.771, P<0.05, Figure 3.20.).

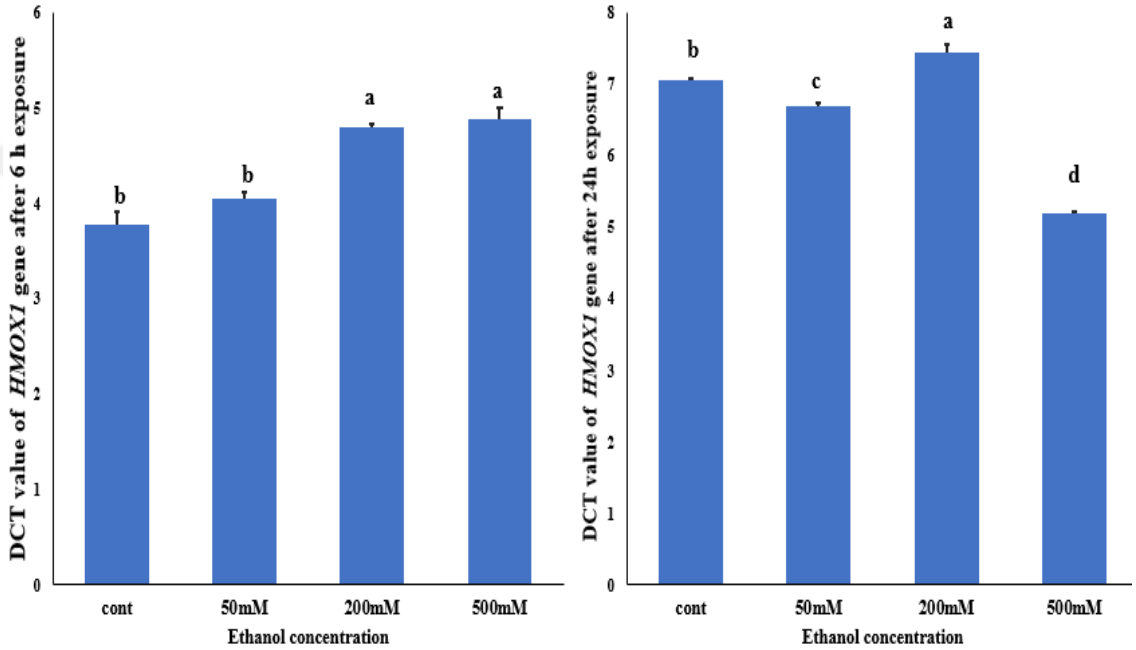


Figure 3.20. DCT values of *HMOX1* gene in follicular cells after ethanol exposure (Means with different letters on the bars were significantly different ANOVA, P<0.05)

3.1.5.5.*PRDX1* expression

Shown in Figure 3.21. after 6 h the gene expression of *PRDX1* for GCs exposed to various ethanol concentration was observed to be lower than untreated control. The lowest rate was found at 50,200 and 500 mM of ethanol exposure.

On the other hand, after 24 h the level of *PRDX1* expression at FCs have been increased at 50 and 200 mM of ethanol exposure and it showed a huge increase in the cells exposed to 500 mM concentration (Figure 3.21.).

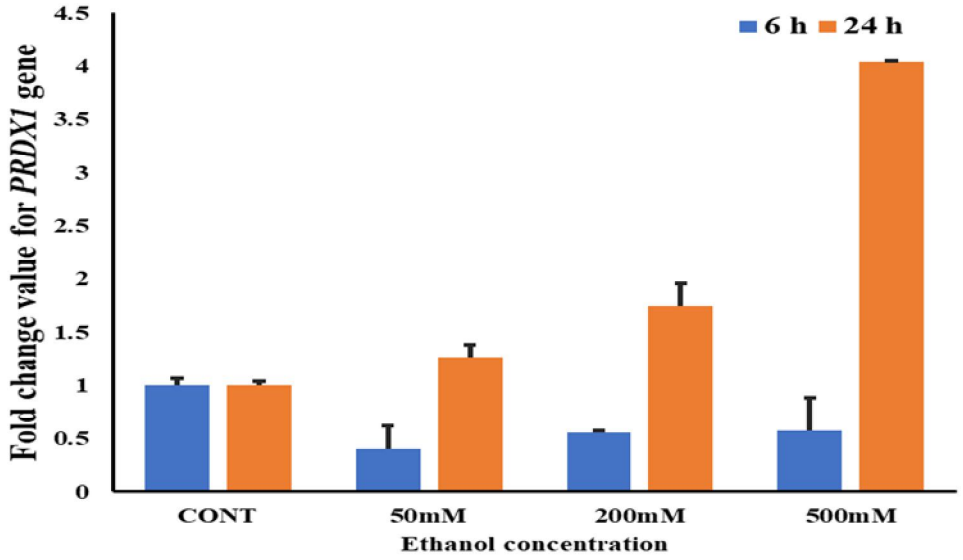


Figure 3.21. Estimated fold change values for *PRDX1* gene in follicular cells after being exposed to ethanol for 6 and 24 h.

All DCT values of *PRDX1* gene at 6 h exposure was highly increase for all of ethanol concentration ($F=8.389$, $P<0.05$; Figure 3.22.). On the other hand, after 24 h of ethanol exposure DCT values were calculated and showed a decrease ($F=53.450$, $P<0.05$, Figure 3.22.) compared to the control value.

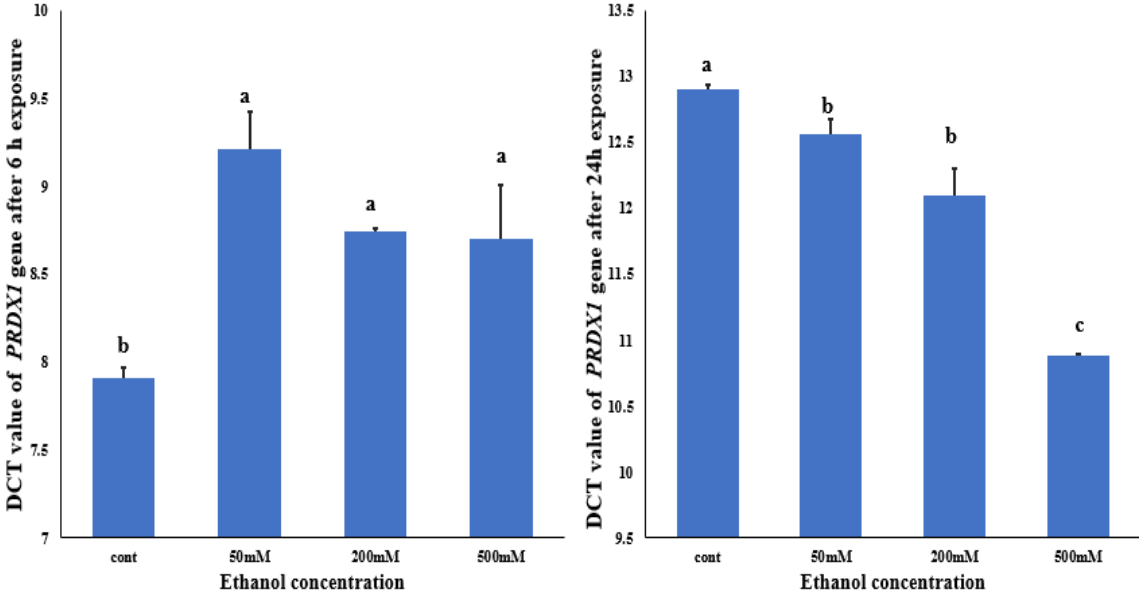


Figure 3.22. DCT values of *PRDX1* gene in follicular cells after ethanol exposure (Means with different letters on the bars were significantly different, ANOVA, $P<0.05$)

3.1.5.6.*SOD1* expression

After 6 h of exposing FCs to various ethanol concentrations. It was observed that the expression level of *SOD1* gene appeared to be low, there was a decrease in its expression levels FCs treated by 50, 200 and 500 mM of ethanol compared to the untreated control Figure 3.23.

However, the expression of *SOD1 gene* after 24 h has been increased when treated by 50 and 200 mM with ethanol. Moreover, the cells treated by 500 mM of ethanol showed a huge increase in the expression level of *SOD1* (Figure 3.23.).

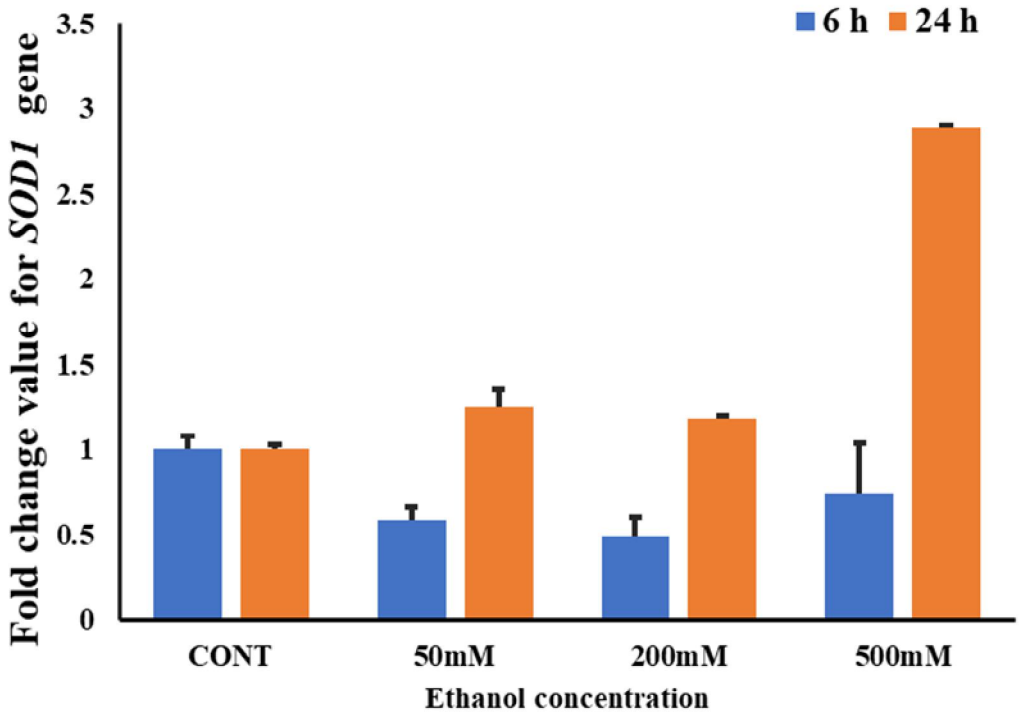


Figure 3.23. Estimated fold change values for *SOD1* gene in follicular cells after being exposed to ethanol for 6 and 24 h.

The DCT values for short term exposure 6 h showed a decrease in gene expression for all concentrations ($F=6.683$, $P<0.05$, Figure 3.24). Also after 24 h there was a decrease in all values for all concentrations ($F=170.675$, $P<0.05$, Figure 3.24.).

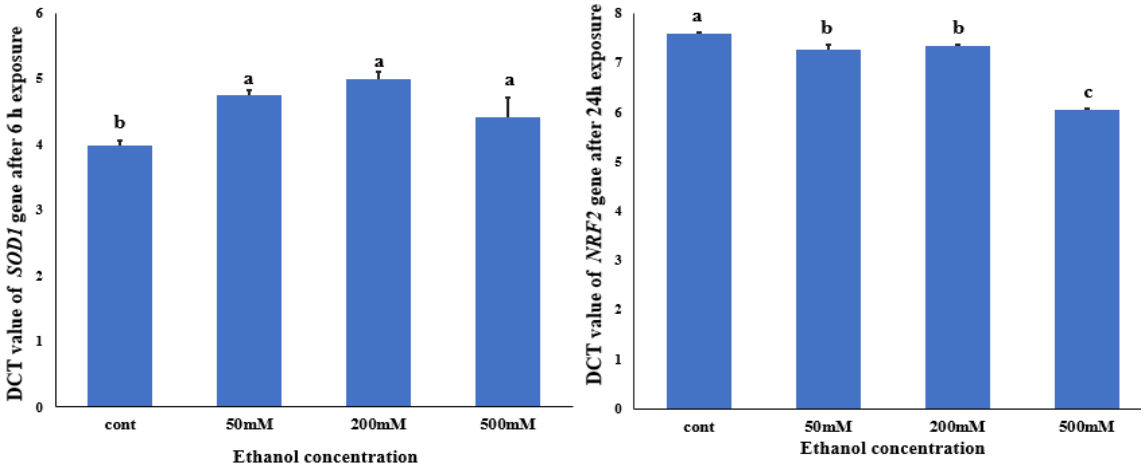


Figure 3.24. DCT values of *SOD1* gene in follicular cells after ethanol exposure (Means with different letters on the bars were significantly different, ANOVA, P<0.05)

3.2.Uterine cells

3.2.1.Cell collection and seeding

Uteruses have been collected from slaughtered cattles, later, UCs have been isolated and seeded in six wells plates contain from 10%FBS, 1% of AA and DMEM F-12 as shown in the figure 3.25.

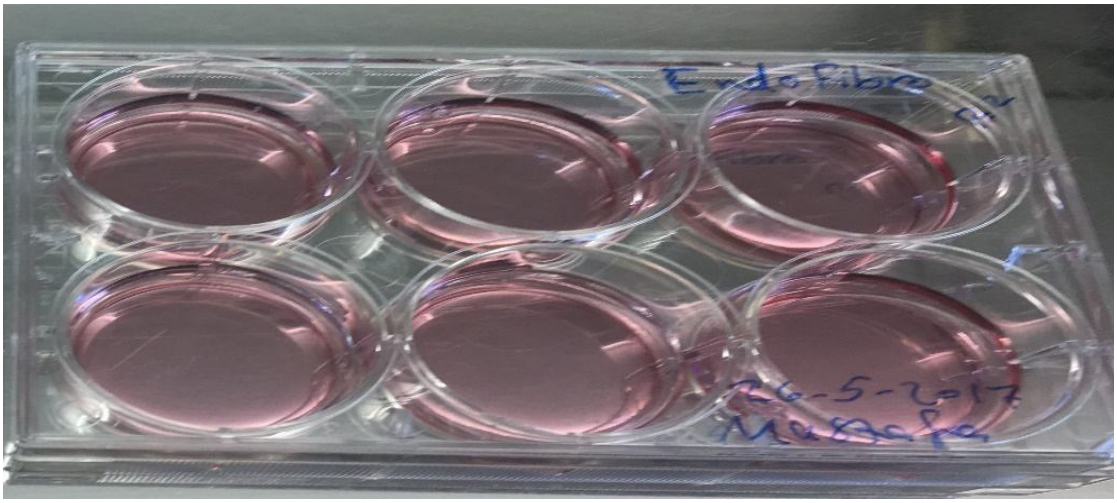


Figure 3.25. Uterine cells seeded in six wells culture plate consist of 10% of FBS. 1% of AA and DMEM-F12 media

After one day, cells have been checked by using inverted microscopy for morphological investigation. The UCs was found to be in good health, cells were flat spindle-shaped, they have normal size, also attaching to plate surface. We checked fibroblast cells viability by using trypan blue, it was 2.9×10^5 cell viable per ml, as in the figure 3.26.

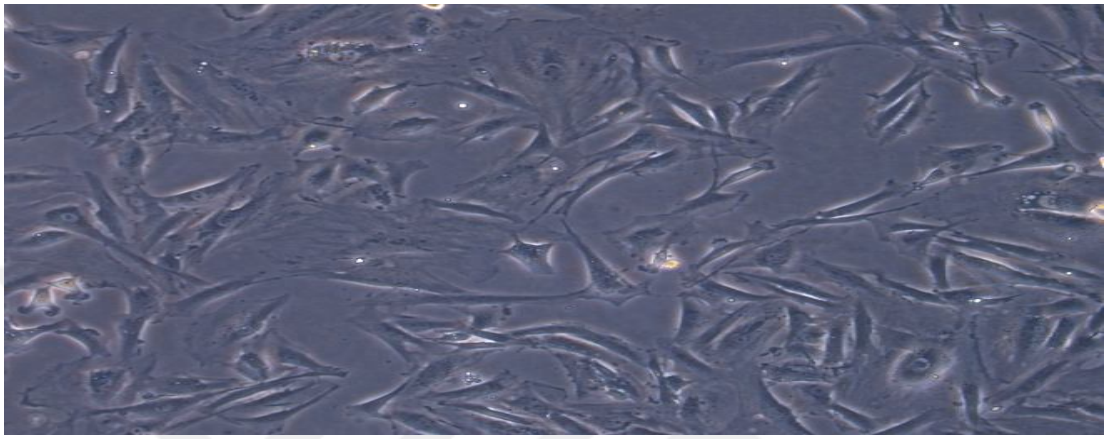


Figure 3.26. Uerine cells after one days of seeding, 10x objectives

In third day, cells were found to be 100% confluent without any morphological changes, furthermore, cell viabilty has been calculated and cell count was found to be 5.3×10^5per ml as shown in figure 3.27.

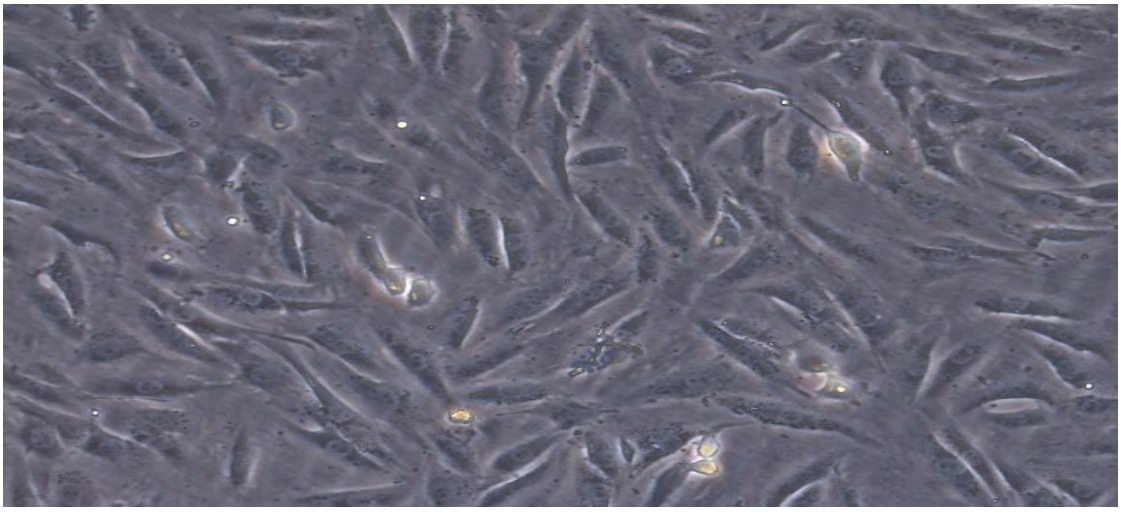


Figure 3.27. Uterine cells after third day of seeding, 10x objectives

Finally, cells have been harvested and passaged to other plate to start the cells treatment.

3.2.2.Effects of ethanol on morphology and viability of uterine cells

The treated and control groups of UCs were examined using inverted microscope for their morphological changes after each exposure. As seen in Figure 3.28., after 6 h of 50 mM of ethanol exposure caused very little morphological changes in UCs, some of cells have been elongated, furthermore, few dead cells have appeared. At 100 mM and above, the cell size was completely altered, it became more elongated and cell was degraded and the dead cells appeared to be more than the dead cells at 50 mM of ethanol concentration. However, at 200 mM of ethanol exposure, the cells increased in their polarity and cells became more elongated. As can be seen in the Figure3.28., aggregation was observed after the cells were treated with 500 mM concentration. Thus, causing an increase in the polarity of the cells. Moreover, the cells shrunken and died by the action of ethanol are shown with white arrows in the Figure 3.28.

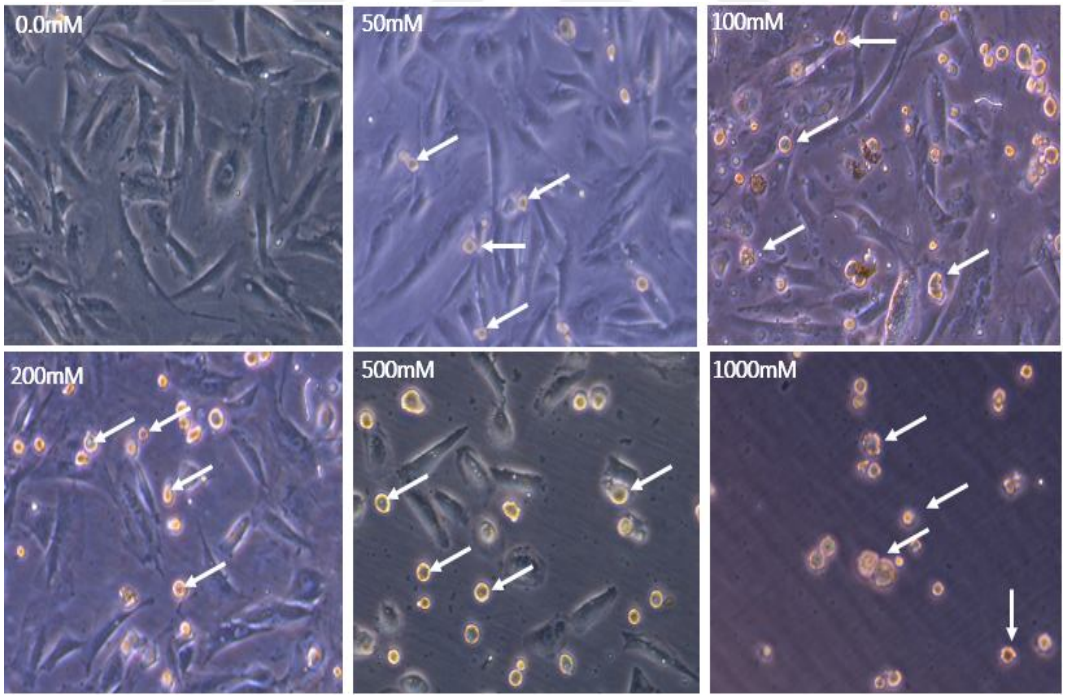


Figure 3.28. Polarity, size, shape and viability of the ethanol exposed UCs after 6 h

UCs viability were also investigated and observed. The number of dead and living cells were counted and given in Figure 3.29. When the cells were exposed to lower concentrations of ethanol (50, 100 and 200 Mm), cell viability increased compared to

untreated control (F= 13864, P<0.05). On the otherhand, at higher concentrations (500 and 1000 Mm) cell viability significantly decreased with the increase of ethanol concentration (F= 30.933, P<0.05).

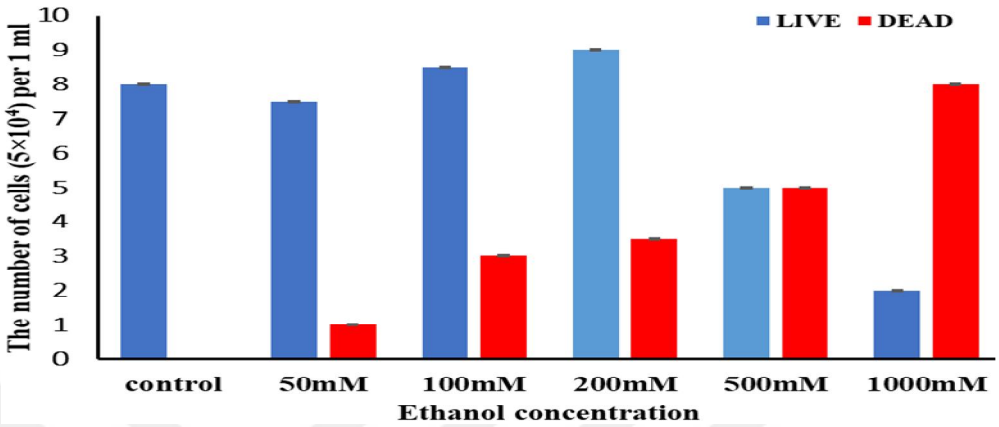


Figure 3.29. Cell viability of uterine cells after 6 h of ethanol treatment

After 24 h of exposing UCs to the ethanol, the cells which were treated by 50 mM nearly began to recover its shape and a few dead cells were notable while compared to the cells treated with the same concentration after 6 h. The cells treated by 100, 200 and 500 mM of ethanol retain these changes in their elongation, aggregation, polarity and size. Moreover, the dead cells also existed. As for the cells treated by 1000 mM of ethanol, we found that most of the cells were dead. The decayed and dead cells are shown with white arrows in the Figure 3.30.

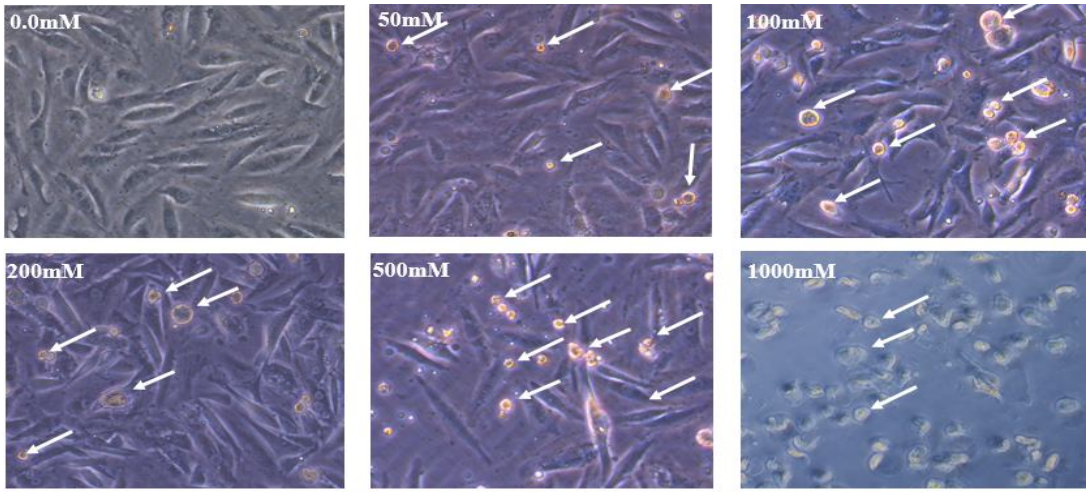


Figure 3.30. Polarity, size, shape and viability of the ethanol exposed uterine cells after 24 h

The dead and living cells were counted after 24 h and given in Figure 3.31. 50 and 100 mM of ethanol exposure the cell viability significantly decreased. On the other hand, at 200 mM of ethanol exposure showed significant increase in cell viability. 500 and 1000 mM cell viability significantly decreased with increase in ethanol concentration ($F=1300$, $P<0.05$). The number of dead cells significantly increased when exposed to different ethanol concentrations after 24 h exposure ($F=79.61$, $P<0.05$).

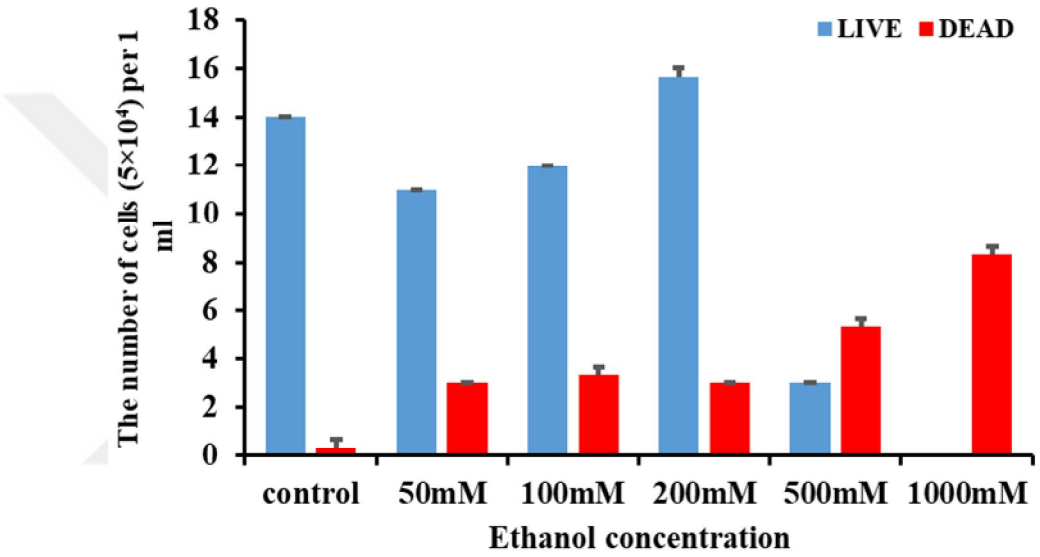


Figure 3.30. cell viability of uterine cells after 24 h of ethanol treatment

3.2.3.Effects of ethanol on proliferation of uterine cells

FCs were exposed to various ethanol concentrations. The significant acute effect on cell proliferation is shown in Figure 3.32. After 6-h period, at 50 mM of ethanol exposure the cells proliferation was slightly increased, while at 100 and 200 Mm, cell proliferation showed a significant increase, in the cells that were treated by 500 and 1000 mM of ethanol there was a significant decreasing ($F=823$, $p<0.05$) compared with the untreated control. After 24 hours, it was surprisingly found that the UCs treated by 100 and 200 mM of ethanol had a huge significant increase in its proliferation, while there was a significant decreasing at 500 and 1000 mM of ethanol exposure ($F=829.281$, $P<0.05$) compared to untreated control.

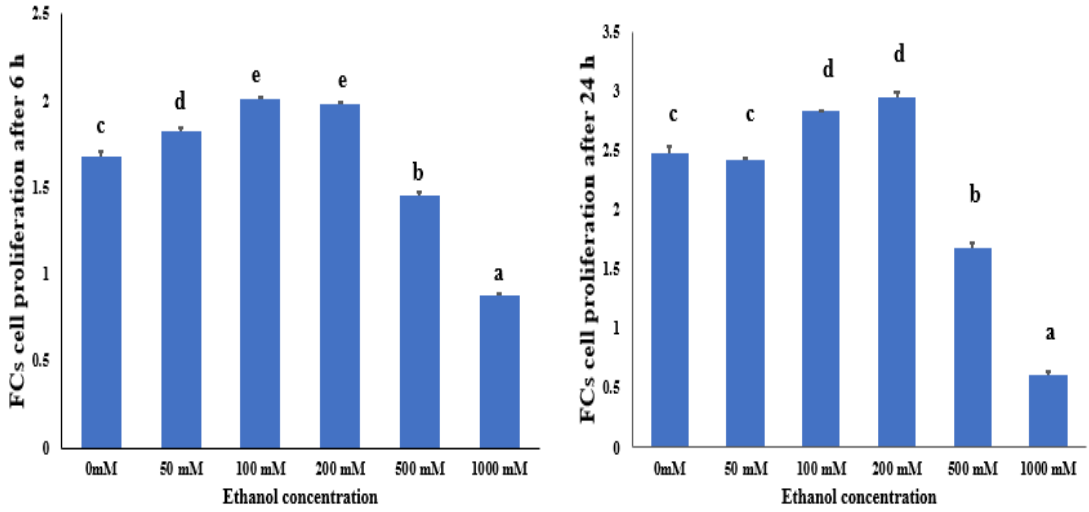


Figure 3.31. The cell proliferation of uterine cells after 6 and 24 h (Means with different letters on the bars were significantly different, ANOVA, $P<0.05$)

3.2.4.Effects of ethanol on mitochondrial activity in uterine cells

Mitochondria itself is a target of oxidative stress, also it is contributing in energy releasing mechanisms, which also executes an oxidative stress inside the cell by releasing ROS. Therefore, the activity of mitochondria in UCs treated by various ethanol concentrations have been investigated and shown in Figure 3.33. After 6-h, high mitochondrial activity was observed in 50,100,200 and 500 mM of ethanol exposure detected by higher fluorescence signals intensity detected by confocal microscopy (Figure 3.33). While there was no signal of mitochondrial activity in cells treated by 1000 mM of ethanol compared to untreated control

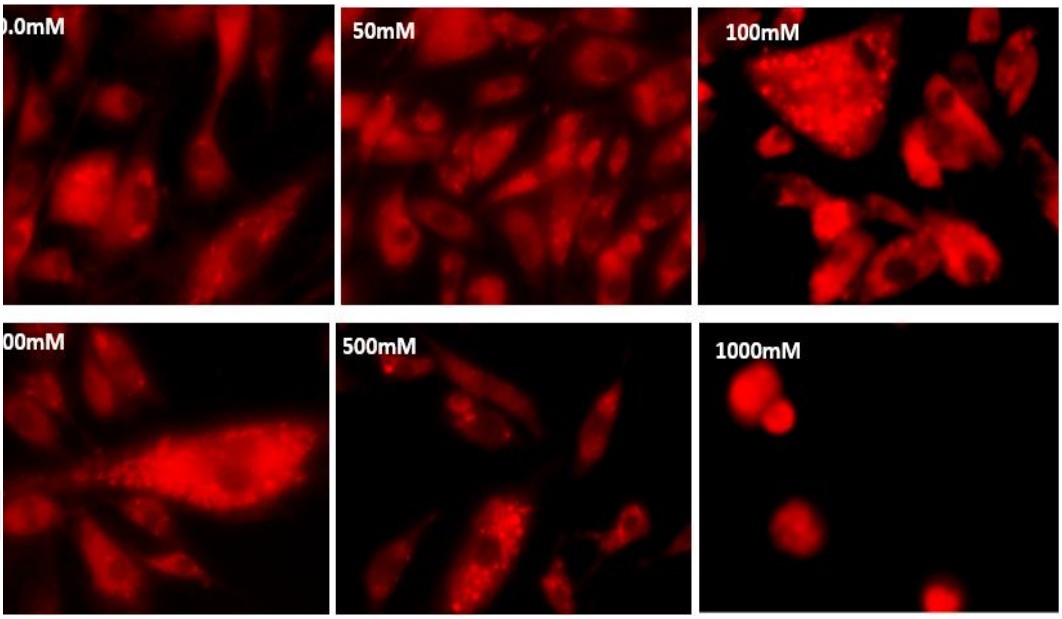


Figure 3.32 Signals of mitochondrial activity after 6-h for uterine cells

After 24-h, in all of the treated cells groups we found an increase in their mitochondrial activity even at 1000 mM of ethanol exposure compared to untreated control as seen in the figure 3.34.

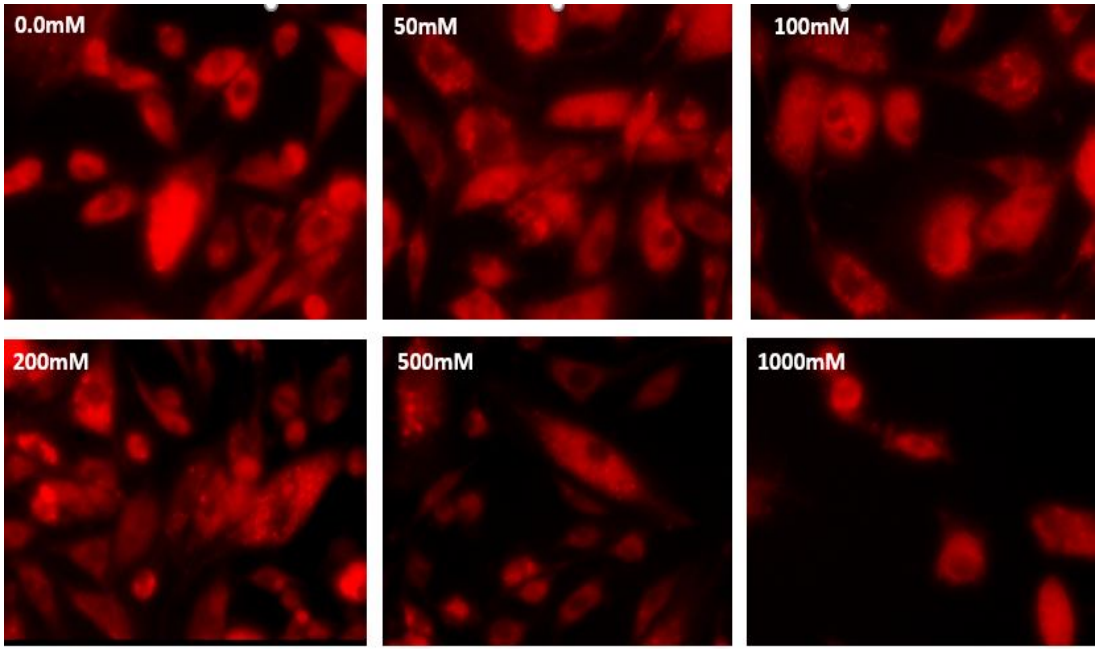


Figure 3.33. Signals of mitochondrial activity after 24-h for uterine cells

3.2.5.Effects of ethanol antioxidant genes in uterine cells (UCs)

NRF2, KEAP1, CAT, HMOX1, PRDX1 and *SOD1* genes was investigated by qRT-PCR, hence to understand the changes in expression levels of these antioxidant genes caused by ethanol. Different concentrations of ethanol were used on UCs for 6 and 24 h and the results obtained were recorded and interpreted in this section.

3.2.5.1.*NRF2* expression

When the UCs are exposed to different concentrations of ethanol ranging between 50 to 500 mM, the expression level of *NRF2* gene was estimated as fold change values and the results obtained are given in Figure 3.35. As can be seen from the figure, gene expression decreased with increasing concentration after 6h exposure. However, when these cells are exposed to the same ethanol concentrations for 24h, the expression level increased with increasing concentrations.

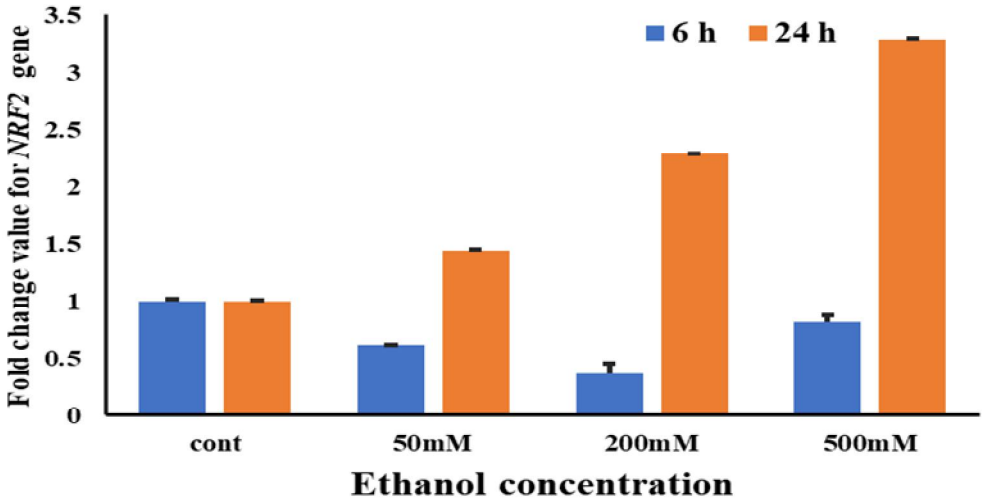


Figure 3.34. Estimated fold change values for *NRF2* gene in uterine cells after being exposed to ethanol for 6 and 24 h

We have also estimated DCT values of the *NRF2* gene by comparing the reference gene after the UCs exposed to different ethanol concentrations and the results were given in Figure 3.36. As can be seen from the figure, DCT value was found at 500 mM higher than 50 and 200 mM ethanol treatment after 6h, but these values decreased with increasing ethanol concentrations after 24 h exposure (For 6h: $F=151.035$, $P<0.05$ and for 24h: $F=5418.662$, $P<0.05$)

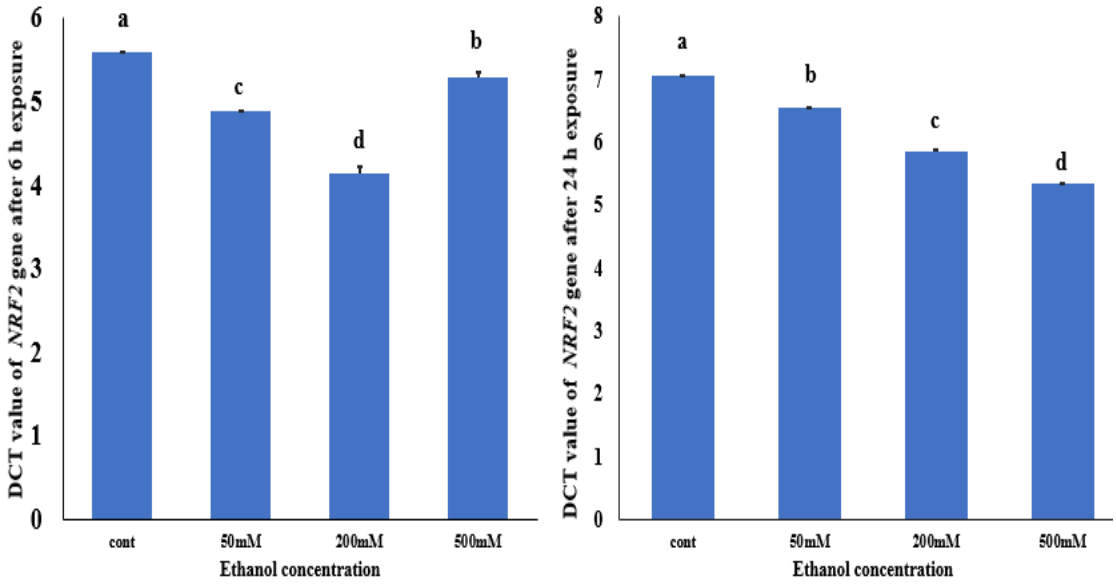


Figure 3.35. DCT values of *NRF2* gene in UCs cells after ethanol exposure (Means with different letters on the bars were significantly different, ANOVA, $P<0.05$).

3.2.5.2.*KEAP1* expression

When the UCs are exposed to ethanol, the expression level of *KEAP1* gene was estimated as fold change values and the results obtained are given in Figure 3.35. As can be seen from the figure, after 6 h exposure, gene expression slightly decreased at 50 mM of ethanol, remarkably increased at 200 mM and decreased again at 500 mM doses. However, when these cells are exposed to the same ethanol concentrations for 24h, the expression level increased with concentrations higher than 50 mM.

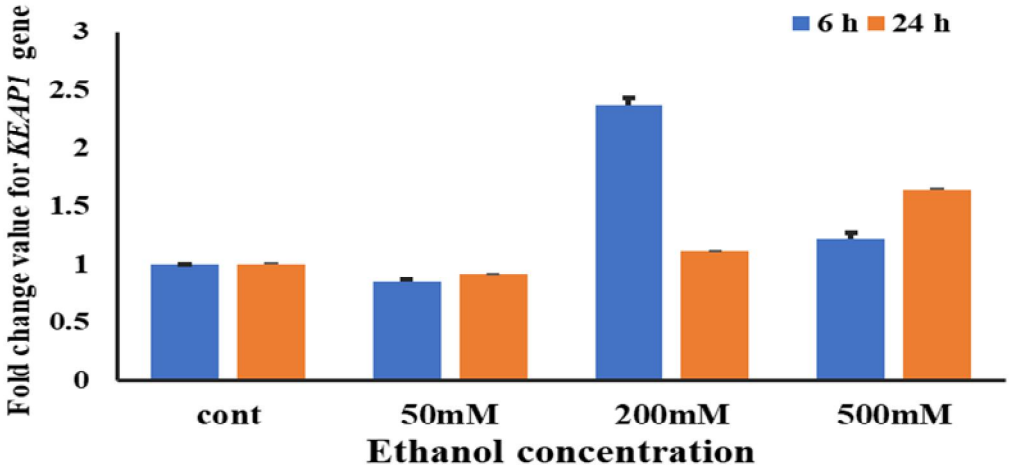


Figure 3.36. Estimated fold change values for *KEAP1* gene in uterine cells after being exposed to ethanol for 6 and 24 h

DCT values have also been estimated for the *KEAP1* gene after the UCs were exposed to ethanol and the results were given in Figure 3.38. As can be seen from the figure, DCT value was the lowest at the 200 mM ethanol exposure after 6h. However, after 24h exposure DCT value increased in a way that indicates a diminish in gene expression, but this value decreased with 200 mM and above after 24h (For 6h: F=238.899, P<0.05 and for 24h: F=1982.459, P<0.05)

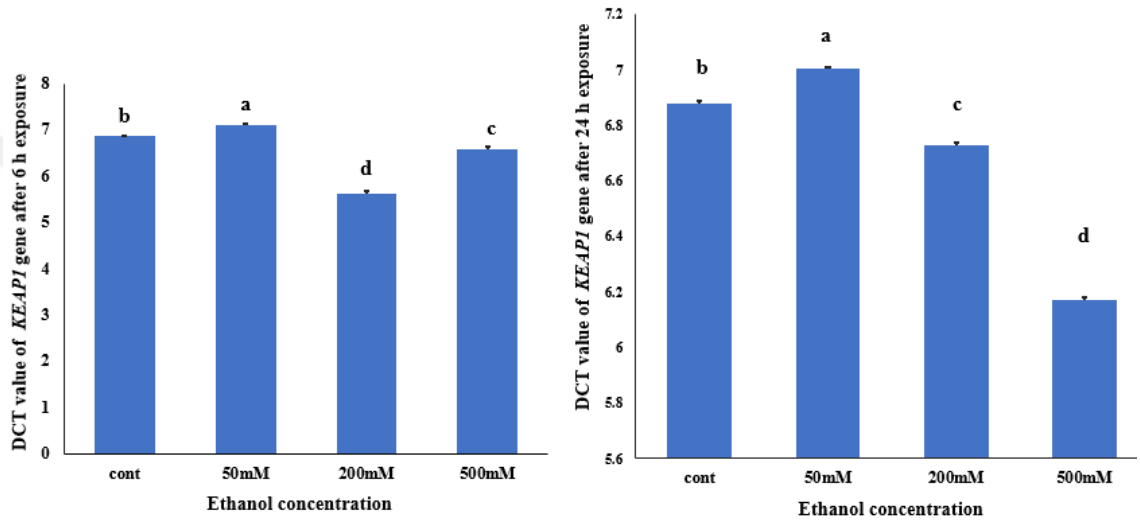


Figure 3.37. DCT values of *KEAP1* gene in uterine cells after ethanol exposure (Means with different letters on the bars were significantly different, ANOVA, P<0.05)

3.2.5.3.*CAT* expression

The expression level of *CAT* gene was estimated and fold change values are indicated in Figure 3.35. There was no significant change in gene expression up to a concentration of 200 mM in both 6 and 24 h of exposure, whereas at 500 mM, expression level was increased. The DCT values for the *CAT* gene are also calculated and shown in the Figure 3.40. As can be appreciated, a remarkable decrease was observed for both 6 and 24 hour exposure at the 500 mM concentration, which was consistent with the increase in gene expression, while the DCT values showed no significant change up to the 200 mM application (6h: F=23.755, P<0.05 and 24h: F=1346.147, P <0.05).

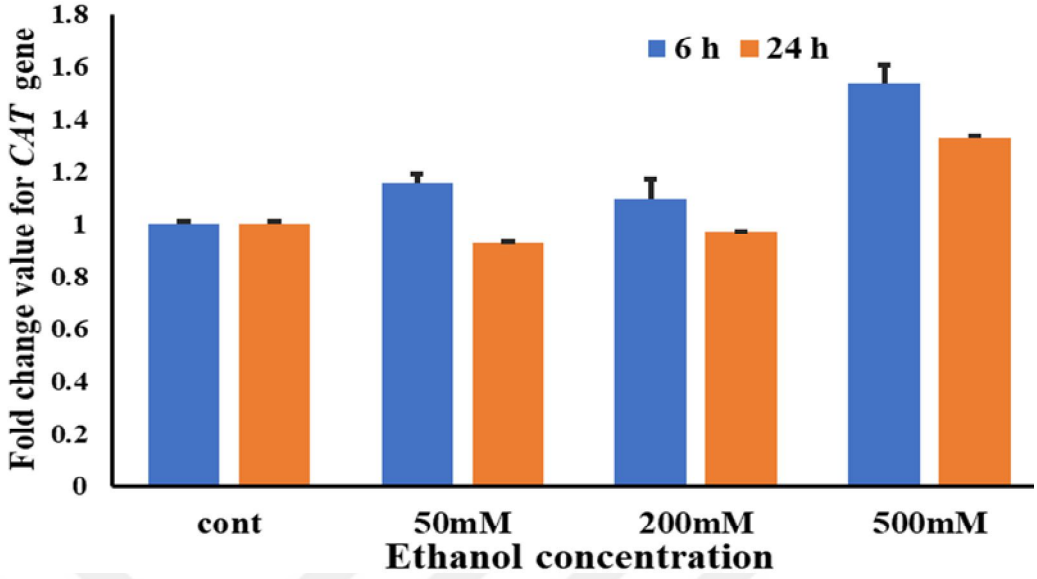


Figure 3.38. Estimated fold change values for *CAT* gene in uterine cells after being exposed to ethanol for 6 and 24 h

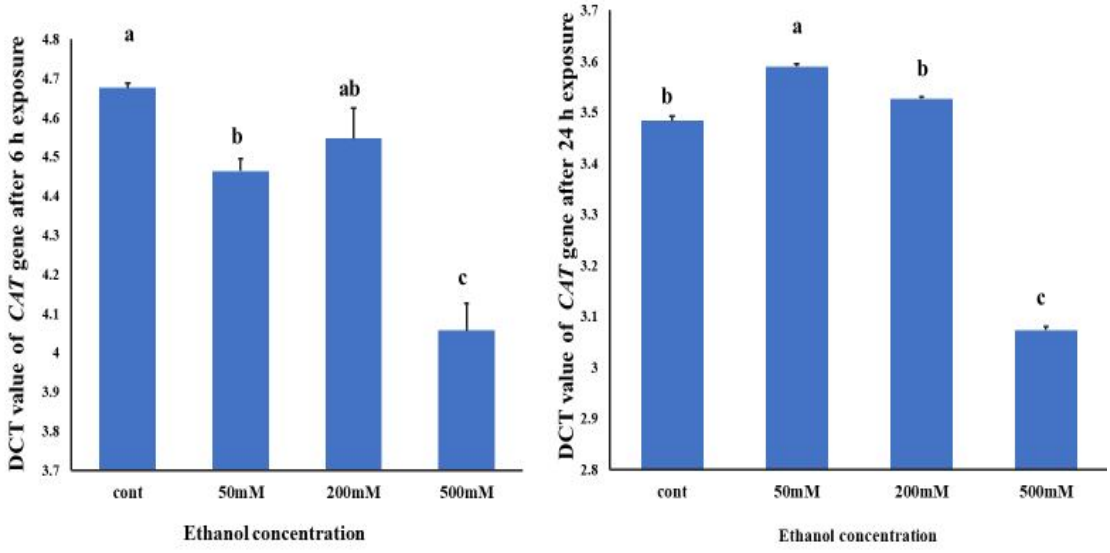


Figure 3.39. DCT values of *CAT* gene in uterine cells after ethanol exposure (Means with different letters on the bars were significantly different, ANOVA, $P<0.05$)

3.2.5.4.*HMOX1* expression

When the expression level of the *HMOX1* gene was investigated using real time q PCR, gene expression was observed to increase markedly at the 500 mM treatment while the 50 and 200 mM ethanol treatment caused decrease in the gene expression when

compared to control (Figure 3.41). A similar trend was observed for both 6 and 24-hour applications.

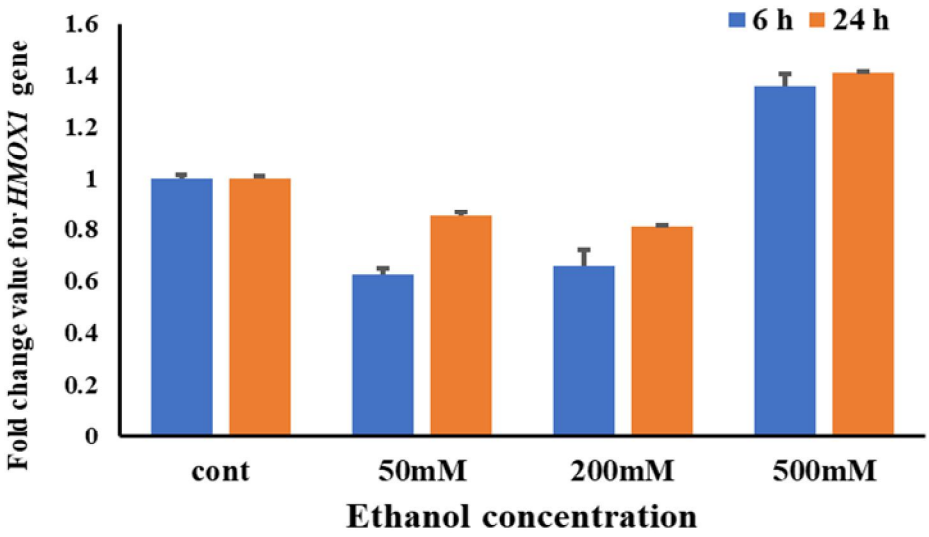


Figure 3.40. Estimated fold change values for *HMOX1* gene in uterine cells after exposed to ethanol for 6 and 24 h

Consistent with the fold change values, DCT values increased up to 200 Mm ethanol but this increase was not significant between 50 and 200 mM concenrations for 6h exposre. The DCT values suddenly dropped below the control value for both 6 and 24 h exposures (Figure 3.42). This decrease in DCT values was significant for 6 and 24h exposure (For 6h: $F=161.551$, $P<0.05$ and for 24h: $F=1727.037$, $P<0.05$).

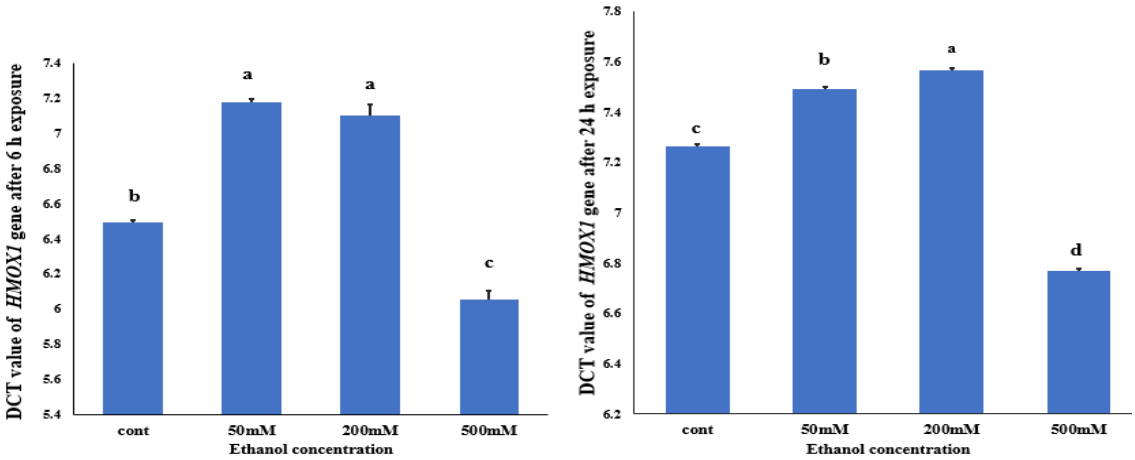


Figure 3.41. DCT values of *HMOX1* gene in uterine cells after ethanol exposure (Means with different letters on the bars were significantly different, ANOVA, $P<0.05$)

3.2.5.5. *PRDX1* expression

When we observed the results related with *PRDX1* gene after real time q PCR the most striking situation was observed in the 200 mM application after 24 h exposure. The estimated fold change value was the highest in this treatment showing the highest gene expression level (Figure 3.43). For this experiment fold change value after 500 mM ethanol exposure was lower than that of control.

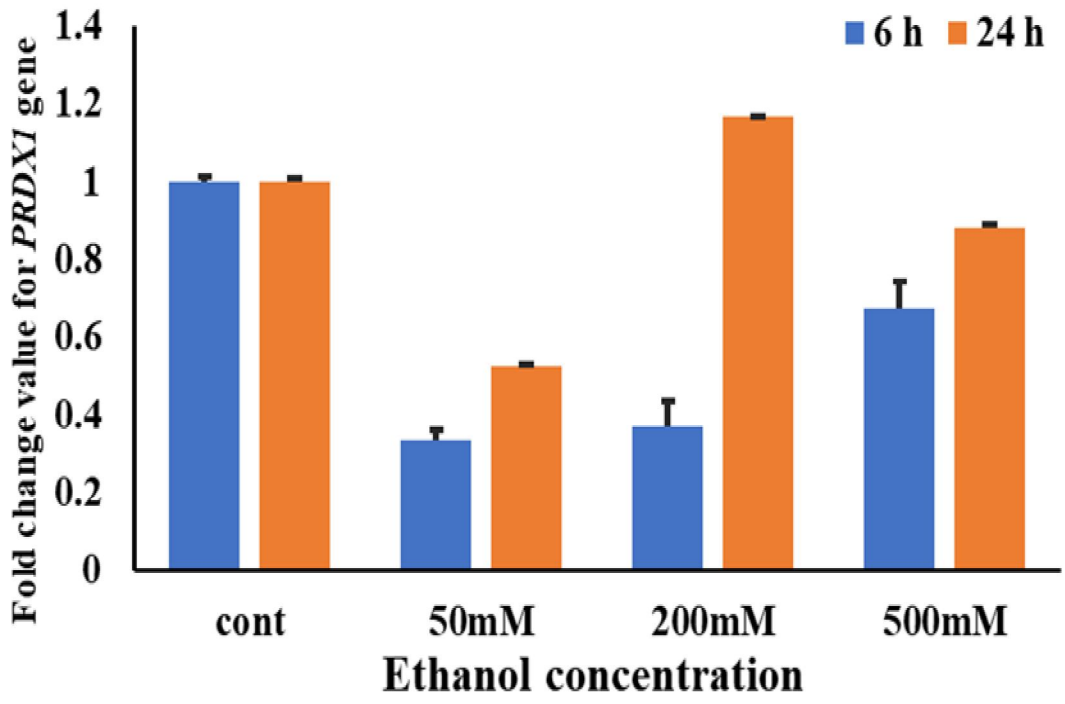


Figure 3.42. Estimated fold change values for *PRDX1* gene in uterine cells after exposed to ethanol for 6 and 24 h

After the observations of the expression of PRDX1 gene, the DCT values estimated and shown in Figure 3.44. Consistent with the fold change results the lowest DCT values was observed with 200 mM ethanol after 24 h exposure. However, none of this value was higher than that of control after 6h exposure. Statistically significant differences were observed among different ethanol concentrations (For 6h: $F=217.500$, $P<0.05$ and for 24h: $F=4365.534$, $P<0.05$).

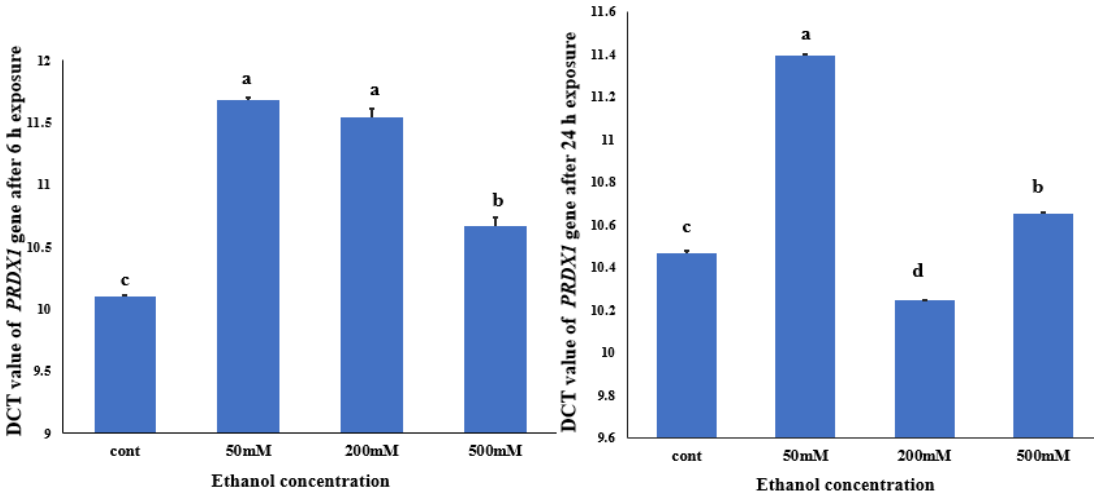


Figure 3.43. DCT values of *PRDX1* gene in uterine cells after ethanol exposure (Means with different letters on the bars were significantly different, ANOVA, $P<0.05$)

3.2.5.6.*SOD1* expression

Expression levels of the SOD1 gene were analyzed and the results were recorded and showed in Figure 3.45. With respect to the experiments made and the results obtained with this gene, the gene expression increased when compared to control at a concentration of 200 and 500 mM. The highest fold change value was obtained for the highest applied concentration after 6 h exposure.

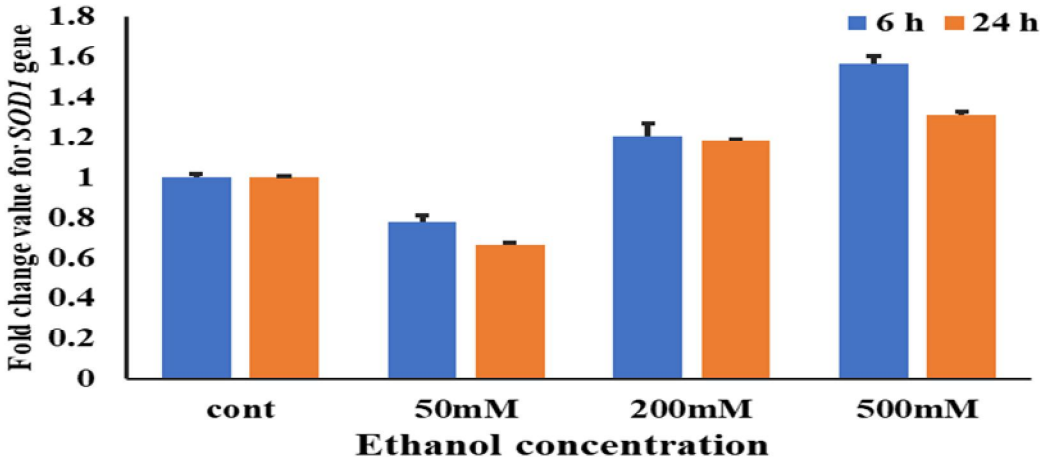


Figure 3.44. Estimated fold change values for *SOD1* gene in uterine cells after being exposed to ethanol for 6 and 24 h.

When we observed DCT values after q PCR experiments similar trends were observed for both 6 and 24 h exposures. When we compare the results of these applications, it can be said that a slight change was observed when comparing 6 and 24 h exposures. There were statistically significant changes among the ethanol concentrations for both 6 and 24 h of exposure (For 6h: F=108.74, P<0.05 and for 24h: F=1724.182, P<0.05).

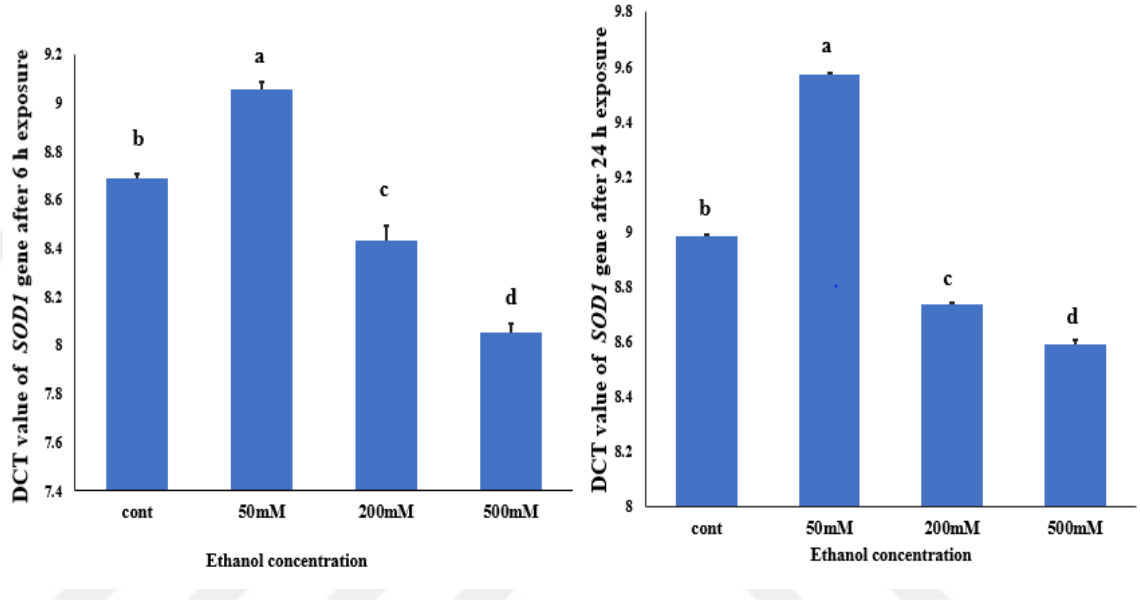


Figure 3.45. DCT values of *SOD1* gene in uterine cells after ethanol exposure (Means with different letters on the bars were significantly different, ANOVA, P<0.05)

CHAPTER 4

DISCUSSION

In this study the impact of ethanol on the follicular and uterine cells was investigated by using cytological and molecular methods.

The follicular cells are the primary cellular part which execute many serious actions throughout follicular growth and ovulation. During these actions, these cells encounter many types of stresses (exogenous and endogenous) which leads to increase the ROS and lipid accumulation, furthermore, it causes cell damage [83].

Uterine cells are the main supporting part of uterus which play an essential role through implantation and pregnancy by giving a balance between ROS and antioxidants. When the pregnancy occurs, these cells are able to decrease oxidative stress by inhibiting ROS by the SOD1 enzyme. If there is no pregnancy, the progesterone will inhibit SOD1 activity leading to increase oxidative stress resulting in menstruation [84].

Ethanol exposure caused a huge morphological alteration to the follicular and uterine cells. Ethanol caused changes in cell size for FCs and UCs, these alterations could be normal physiological response occurs due to increased workload, functional demand, stimulation by hormones and growth factors. According to studies, the hormone-induced hypertrophy occurs within the endometrium and myometrium of the uterus. Because during the follicular stage of the menstrual cycle, estrogen upregulation stimulates an increase in muscle proteins of the stroma of the endometrium and the large smooth muscle layer of the myometrium [85]. On the other hand, pathological hypertrophy occurs due to an abnormal stressor in defecting cellular regulation, hormone stimulation and growth factor. Up to now, a number of studies have been conducted on adverse effects of ethanol on the female reproductive system such as fertility problems, delayed/absent menstrual cycle, reduced ovarian weight, lowered

hormone concentrations and inhibited ovulation [85]. It can be said that ethanol directly interfered with the physiology and functions of the cell leading to convert the hypertrophy from normal physiologic to a pathologic.

In our study, we found that FCs in the ovaria and UCs of endometrial tissue have changed dramatically in their shapes because of ethanol exposure and it may cause cell dysplasia. This result could be normal physiologic case or pathologic case [86]. It is stated that these defects in FCs and UCs will lead to abnormal cell divisions over time and thus to cancer because there is a link between the morphological abnormalities of cells and tumour formation [86].

The present study investigated cell viability and cell proliferation for follicular and uterine cells which were treated by different concentrations of ethanol for different periods of time. Results in this study have shown that ethanol increases cell proliferation at some concentrations and decreases it at some other concentrations. This increasing could be normal physiological response and it is common because of hormones or growth factors actions when there is a need to increase functional capacity of hormone sensitive organs, or when there is a need for functional compensatory increase after damage or resection of an organ [87]. This ethanol induced oxidative stress possibly led the cells to quickly respond by increasing cell number due to reduce oxidative stress.

On the other hand, the increasing of proliferation may be pathologic, because of unorganized hormones activity and growth factors acting on target cells. Ethanol exposure to FCs and UCs could cause stress which led to excessive action of hormones or for growth factors that led to activate hyperplasia for these cells [87].

In our results, a significant increase in mitochondrial activity was observed for both types of cells treated with different concentrations of ethanol for different exposure periods. It is not secret that the mitochondria is the main target for the oxidative stress in addition to the ROS accumulation. Also, ethanol enhances the oxidative stress and simultaneously induce an increase in ROS, so does the mitochondria, which starts to release ROS due to energy production [88]. The reason for this increase could be due to two possible reasons, first is the high activity of mitochondria to reduce the level of

intracellular oxidative stress and resistance. Second is that ethanol causes a damage to the mitochondrial DNA which leads to a significant reduction in defences against oxidative stress. Therefore, a negative effect on mitochondria leads to excessive production of mitochondrial activity [88].

In this study, different concentrations of ethanol are different exposure periods caused significant changes in the activity of antioxidant genes such as *NRF2, KEAP1, CAT, HMOX1, PRDX1* and *SOD1*. However, these gene expressions showed an increase or decrease depending on cell line, duration of exposure and concentrations. For example, in follicular cells a very pronounced increase was observed in the expression of all genes at the highest dose and highest exposure times. However, at short exposure period 6 h, gene expression was observed to be reduced in comparison with the control despite an increase in concentration. We can say when the cells are exposed to ethanol for prolonged periods of time, the oxidative stress increases with increasing duration, thus leading to an increase in the expression of oxidative stress genes in follicular cells. The same trend was also the case for UCs, however the increase in the expressions of the genes were not so high when compared with follicular cells. Previous studies related with ethanol exposure found that there was an overlap in the action of the genes for example, *KEAP* and *NRF2* overlapping. The ethanol could cause *NRF2* translocation to the nuclear compartment. Another reason is that *KEAP1* could represses *NRF2* activation, this repression will increase by ethanol [89]. In general, after 24 hours of exposure, we observed that there is an increasing in gene expression for detected genes of FCs and UCs compared to the untreated control cells, but specifically the increase in the 200 and 500 mM concentrations were pronounced.

In conclusion, our study has achieved the results for evaluating the ethanol induced oxidative stress in FCs and UCs. Ethanol had a high toxic effect even in low concentrations. These results show that increasing ethanol concentration and prolonged exposure to ethanol increase the oxidative stress in the cells leading to the formation of free radicals. In addition, it can be said that it causes dramatical changes in parameters such as shape, size, mitochondrial activity and gene expression of the cells.

Initially, the cells were not prepared to resist this large amount of oxidative stress caused by ethanol. Due to the normal physiological state of the cell as well as the lack of excess organelle production responsible to the reduction of the oxidative stress such as mitochondria, the cells are sensitive against ethanol exposure. Therefore, the apparent toxic effect on cells after 6 hours caused a significant alteration in the shape, size, proliferation, physiological and molecular activity of the cell where led to damage the cells. However, after 24 hours of exposure, the cells began to reorganize its physiological and molecular activity by increasing the expression of the genes responsible for antioxidant enzymes, mitochondrial activity and increasing physiological adaptations. These changes in the activity of the cells were different depending on the concentrations and exposure periods of ethanol. There is a need for deep study on these cells at these concentrations. In order to better understand the level of oxidative stress caused by ethanol, the effects of different concentrations and exposure times need to be investigated in detail.

According to WHO statistics, there is anxiety about the increase of adolescents who are consuming ethanol, especially females [90], where the report classified alcohol consumers as adults and pregnant females. In our current study, we found a strong relationship between ethanol toxicity and the oxidative stress.

Our suggestions are:

The community should take important steps including the prevention or establishment of special rules for alcohol consumption.

Add an educational curriculum or workshops to educate the community, especially females, about the risks of alcohol consumption. Also concern to issuing booklets and distributing it in hospitals and universities to raising the awareness of the community about the risks of alcohol consumption.

Most of studies focused on the investigation of the effects ethanol on organs physiology, few of them handled the molecular aspect, they were focused on specific organs such as the liver. After our results, we recommend expanding the investigations especially on the female reproductive system because of its special importance.

Interestingly, we have found a significant alteration in follicular and uterine cells when exposed to 200 and 500 mM ethanol concentrations, we also found the survival and even the increase of the cell proliferation. In the future, the causes of increased cellular proliferation and gene expression will be investigated in terms of apoptosis and growth factor genes, also the genes responsible for cell adaptation. In addition, genetic mutations that may be caused by ethanol on these genes should be investigated.

Given the adverse effects of alcohol and other factors which increase the oxidative stress, it is important to conducting studies to clarify the relationship of oxidative stress and its effect on the immune activity of the cell in the resistance of pathological infection especially in female reproductive system.

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