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TOXICOLOGICAL EVALUATION OF THE INTERACTION BETWEEN CIRCADIAN RHYTHM ACTIVATORS AND GENERAL ANESTHETICS

DOCTORAL THESIS

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This thesis has been deemed by the jury in accordance with the relevant articles of Yeditepe University Graduate Education and Examinations Regulation and has been approved by Administrative Board of Institute with decision dated ...16.03.2018... and numbered 2018/06-01

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DECLARATION

I hereby declare that this thesis is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person nor material which has been accepted for the award of any other degree except where due acknowledgment has been made in the text.

05.03.2018

Feyza Kelleci

Manevi dedem Tabip Kıdemli Albay Mehmet İhsan AKTAŞ' a,
Sevgileri kalbimde yaşayan büyükbabalarım ve büyükannelerime,
Desteklerini benden asla esirgemeyen,
Sahip olunabilecek en güzel aileye,
AİLEME ithaf ediyorum…

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LIST OF SYMBOLS AND ABBREVIATIONS

α-tocopherol-$O^{\cdot}$	Tocopheryl radical
8-OHdG	8-Hydroxy-2-guanosine
ALT	Alanine aminotransferase
ANOVA	Ordinary one-way analysis of variance
AST	Aspartate aminotransferase
ATP	Adenosine triphosphate
bHLH	Basic helix-loop-helix
Bmal1	Brain-Muscle-Arnt-Like protein-1
BSA	Bovine serum albumin
Ca	Calcium
CAPS	3-(Cyclohexylamino)-1-propanesulfonic acid
CAT	Catalase
CCGs	Clock-controlled genes
cGMP	Cyclic guanosine monophosphate
Ck	Casein kinase
Clock	Circadian Locomotor Output Cycle Clock
CNS	Central nervous system
$CO_3^{\cdot -}$	Carbonate radical
CO_3^{-2}	Carbonate ion
Cry	Cryptochrome
Cu	Copper
Cu^{+2}	Copper ions
$CuSO_4$	Copper sulfate
CuZnSOD	Copper-Zinc SOD
dCLK/CYC	dClock and Cycle
DNA	Deoxyribonucleic acid
Dpt	Doubletime
DSPS	Delayed sleep phase syndrome
E. coli	Escherichia coli
E-box	Enhancer box
ECG	Electrocardiogram

EDTA	Ethylenediaminetetra acetic acid disodium dehydrate
EEG	Electroencephalogram
ELISA	Enzyme-Linked Immunosorbent Assay
ER	Endoplasmic reticulum
ERS	Endoplasmic reticulum stress
FAD	Flavin adenine dinucleotide
FBXL	F-box protein
Fe	Iron
FeSOD	Iron SOD
FMN	Flavin mononükleotit
FRQ	Frequency
GABA	Gamma-aminobutyric acid
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase enzyme
GC-MS	Gas Chromatography-Mass Spectrometer
GR	Glutathione Reductase
GSH	Glutathione
GSH-Px	Glutathione peroxidase
Gsk3	Glycogen synthase kinases3
GSSG	Oxidized glutathione
GST	Glutathione S-Transferases
$H^{\cdot}$	Hydrogen radical
H_2O_2	Hydrogen peroxide
$HO^{\cdot}$	Hydroxyl radicals
HOCl	Hypochlorous acid
HPLC	High liquid pressure chromatography
HRP	Avidin-Horseradish Peroxidase
IARC	International Agency for Research on Cancer
INL	Inner nuclear layer cells
INT	Iodonitrotetrazolium chloride
inh	Inhalation
ipRGCs	Retinal ganglion cells
KCl	Potassium chloride
KH_2PO_4	Potassium phosphate monobasic

KO	Knockout
LH	Fatty acid side chain of membrane phospholipids
$LOO^{\cdot}$	Lipid peroxy radical
MDA	Malondialdehyde
MFO	Mixed-function oxidase
Mn	Manganese
MnSOD	Manganese SOD
Myc	Multifaceted oncogene
Na^{+} K^{+} ATPase	Sodium-potassium ATPase
Na_2CO_3	Sodium carbonate anhydrous
$Na_2HPO_4.2H_2O$	Sodium phosphate dibasic dihydrate
NAD	Nicotinamide adenine dinükleotid
NaKtartrate	Sodium potassium tartrate
NaOH	Sodium hydroxide
NiSOD	Nickel SODs
NO	Nitric oxide
NSSA	Non-enzymatic superoxide radical scavenger activity
$O_2^{-\cdot}$	Superoxide radical
OCl-	Hypochlorite ion
OD	Optical density
OR	Oxidation resistance
OSI	Oxidative stress index
P	Phosphor
PAS	PER-ARNT-SIM homology
Per	Period
RHT	Retinal-hypothalamic tract
RNA	Ribo Nucleic acid
RNS	Reactive nitrogen species
$ROO\cdot$ or $RO_2\cdot$	Peroxyl or peroxy radical
ROOH	Lipid peroxides
RORE	Retinoic acid-related orphan receptor response elements
ROS	Reactive oxygen species

SCN	Suprachiasmatic nuclei
SDS	Sodium dodecyl sulfate
Se	Selenium
Se-GSH-Px	Selenium-dependent GSH-Px
Singlet O_2	Singlet oxygen
SOD	Superoxide dismutase
TAC	Total antioxidant capacity
TBA	2-Thiobarbituric acid
TCA	Trichloroacetic acid
Tim	Timeless
TOS	Total oxidative status
TTFL	Transcription/translation feedback loops
UV	Ultraviolet
VIP	Vasoactive intestinal polypeptide
WC	White collar protein
YUDETAM	Research Center of Yeditepe University

ABSTRACT

Toxicological evaluation of the interaction between circadian rhythm activators and general anesthetics. Yeditepe University, Institute of Health Science, Department of Pharmaceutical Toxicology, Ph.D. Thesis, İstanbul.

The circadian rhythm lasts about 24 hours and is constituted by 4 main genes/proteins; Circadian Locomotor Output Cycle Clock (Clock), Brain-Muscle-Arnt-Like protein-1 (Bmal1), Cryptochrome (Cry) and Period (Per). Many pathologies, diseases, and medications including general anesthetic agents are thought to cause a change in these genes/proteins. On the other hand the activation or inhibition of the genes/proteins can contribute to the reduction of the oxidative stress potential of anesthetic agents. We investigated the effect of combination therapy of KL001 with isoflurane. Twenty-four mice were randomly divided into 4 groups of 6 animals each as a control, KL001, isoflurane and KL001 plus isoflurane group. Animals were exposed to isoflurane for 4 hours and KL001 was administered of 100 mg/kg at night hours. The Cry levels and oxidative stress parameters including malondialdehyde (MDA) level and superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px) activities were investigated in liver, kidney, brain, and erythrocyte. Our results showed decreased MDA level in erythrocyte and liver, increased GSH-Px activity in liver and brain, increased SOD activity in erythrocyte, kidney, and brain, decreased CAT activity in liver in isoflurane group. According to our findings, isoflurane changed the oxidative stress parameters remarkably in brain. We also found a decrease of Cry level in the plasma, liver, and brain in isoflurane group. KL001 increased Cry level inhibited by isoflurane and decreased the isoflurane-induced oxidative stress. In conclusion, our data indicated that we can reduce the potential oxidative stress of isoflurane or other anesthetic agents by triggering the Cry level with a synthetic activator or by arranging drug administration time at certain period of the day. Especially in early hours of the morning, KL001 may be protective against isoflurane-induced oxidative stress. In further research, the effects of Per, Clock, and Bmal1 on anesthetic agents toxicity can also be investigated. It can be determined at which time the operations are performed to create the least toxicity.

Keywords: Biological clock, isoflurane, cryptochrome, KL001

ABSTRACT (Turkish)
ÖZET

Sirkadiyen Ritim Aktivatörleri ile Genel Anestezik Maddeler Arasındaki İlişkinin Toksikolojik Açıdan Araştırılması. Yeditepe Üniversitesi Sağlık Bilimleri Enstitüsü, Farmasötik Toksikoloji ABD., Doktora Tezi. İstanbul.

Sirkadiyen ritim yaklaşık olarak 24 saat sürer ve ana olarak 4 gen/proteinden oluşmaktadır; Circadian Locomotor Output Cycle Clock (Clock), Brain-Muscle-Arnt-Like protein-1 (Bmal1), Cryptochrome (Cry) ve Period (Per). Bir çok patolojinin, hastalığın ve genel anestezikler de dahil olmak üzere kullanılan ilaçların sirkadiyen ritimin işleyişinden sorumlu olan gen/proteinlerin regülasyonunda değişime sebep olduğu düşünülmektedir. Ayrıca sirkadiyen gen/proteinlerinin aktivasyonu ya da inhibisyonunun anestezik ajanlardan kaynaklı oksidatif stresin azaltılmasına katkıda bulunabileceği bildirilmektedir. Bu tez çalışmasında Cry' un sentetik aktivatörü, KL001' in izofluran ile kombinasyon tedavisinin etkisi araştırıldı. 24 fare her grupta 6 hayvan olacak şekilde 4 gruba ayrıldı; kontrol, KL001, izofluran artı izofluran ve KL001 grubu. Hayvanlar izofluran' a 4 saat boyunca maruz bırakıldı ve KL001 gece saatlerinde 100 mg/kg olarak uygulandı. Karaciğer, böbrek, beyin ve eritrositte Cry düzeyleri ve oksidatif stres parametrelerinden malondialdehit (MDA) seviyesi, süperoksit dismutaz (SOD), katalaz (CAT), ve glutatyon peroksidaz (GSH-Px) aktiviteleri incelendi. İzofluran grubunda eritrosit ve karaciğerde MDA düzeyinde azalma, karaciğer ve beyinde GSH-Px aktivitesinde artış, eritrosit, böbrek ve beyinde SOD aktivitesinde artış, karaciğerde CAT aktivitesinde azalma saptandı. Bulgularımıza göre izofluran beyinde oksidatif stres parametrelerini önemli ölçüde değiştirdi. Ayrıca izofluran grubunda plazma, karaciğer ve beyinde azalmış Cry seviyesi bulundu. KL001 uygulaması ile izofluran ile indüklenen düşük Cry seviyesinin arttığı ve izofluran ile indüklenen oksidatif stresin de azaldığı saptandı. Sonuç olarak verilerimiz Cry düzeyini sentetik bir aktivatör ile tetikleyerek veya günün belli saatlerine göre ilaç uygulamasını düzenleyerek izofluran ve diğer anestetik ajanların meydana getirdiği oksidatif stresi azaltabileceğimizi gösterdi. Özellikle sabahın erken saatlerinde KL001 izofluran kaynaklı oksidatif strese karşı koruyucu olabilmektedir. Daha ileri araştırmalarda Clock, Bmal1 ve Per gen/proteinlerinin anestetik ajanların toksisitesi üzerine etkileri de incelenmelidir. Anestezik kaynaklı toksisiteyi azaltmak için günün hangi saatinde ameliyatların yapılacağı belirlenebilir.

Anahtar kelimeler: Biyolojik saat, izofluran, cryptochrome, KL001

1. INTRODUCTION and PURPOSE

There is a growing interest in chrono-modulated therapy, which modulates the treatment at specific hours of the day, especially in recent years. This therapy aims to increase the efficiency of the treatment and to tolerate the possible toxic effects. Because of these features, chrono-modulated therapy has begun to replace conventional drug treatment regimens (1). Primarily, the circadian rhythm and the relationship with the circadian rhythm and various disease states need to be deeply understood in order to practice chrono-modulation therapy (2).

All organisms have a mechanism called circadian rhythm that provides physiological and psychological adaptation to the environment (3). In mammalian cells, this rhythm is constituted and regulated by genes/proteins known as circadian genes. Circadian genes consist of 4 genes/proteins; Circadian Locomotor Output Cycle Clock (Clock), Brain-Muscle-Arnt-Like protein-1 (Bmal1), Cryptochrome (Cry) and Period (Per). These genes/proteins provide positive and negative feedback cycles of circadian rhythm (4, 5).

It is known that the rhythms in the body play a critical role in ensuring cellular activities in a healthy way. The synthesis, function, and destruction of every molecule in the cell take place a specific period. From this standpoint, the circadian genes/proteins which are responsible for controlling the timing of cellular events have a vital importance (1).

Unfortunately, many factors such as some medications, chemicals, pathologies, and diseases are known to disrupt circadian rhythm via breaking its genes/proteins release (6, 7). Clinical trials have shown that one of these factors can be anesthetic agents used in surgical operations. For example, the sevoflurane, as an inhalation (inh) anesthetic agent, has been found to suppress the Per2 expression in rats (8, 9). Moreover, disruption of circadian rhythm can be the source of oxidative stress in many tissues. In various studies, the operation itself or/ and anesthetic agents (10) including sevoflurane and isoflurane interfering with circadian rhythm are found to alter oxidative stress parameters (11). On the other hand, it is known that circadian rhythm parameters can prevent the development of oxidative stress. One of the main parameters is melatonin hormone (secreted according to dark light cycle) that exhibit antioxidant role in erythrocytes. As a result, deteriorated circadian rhythm needs to be corrected for the scavenging of the oxidative stress (12).

Circadian rhythm deterioration occurs when the genes/proteins responsible for the circadian rhythm are inhibited or activated. For this purpose, it is thought that two new types of therapies can be used to modify the expression of genes/proteins. As a first approach, light can be used for an inducing or inhibiting the expression of these genes/proteins. In another approach, newly synthesized gene activator and inhibitor agents can be used for the modification of circadian rhythm. These two new approaches together with adjustment of treatment regimens at specific hours of the day are crucial regarding the development of Chrono-modulated therapy. This could generate the significant changes in clinical practice and patient outcomes (2).

In the light of this information, in this thesis, we aimed to investigate the combination treatment strategy of a general anesthetic (inh isoflurane) with Cry activator (KL001) to reduce toxic side effects of anesthetic agents by determining the oxidative stress status in rats. Antioxidant effect and oxidative damage were evaluated with measuring the amount of malondialdehyde (MDA) as a marker of lipid peroxidation, the activity of superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) and catalase (CAT) as a marker of antioxidant status.

2. GENERAL INFORMATION

2.1. Overview of Biological Rhythms

Life involves a series of cyclic and rhythmic chemical processes. All living systems are under the control of these essential processes (13). Some of these cycles are called geophysical cycles. They consist of various periods such as a daily (24 hours ± 30 seconds), seasonal (365.24 days), lunar (29.53 days) and tidal (12.8 hour) cycles. The physiochemical events and behaviors such as feeding type, migration, growth, reproduction, hibernation and more are synchronized by them. For instance, the lunar cycle organizes the menstrual cycle in females. As the food availability varies according to seasonal changes, the dietary pattern of all species are affected, and some of the species hibernate. Besides, it is thought that the seasonal changes can modulate our biology, mental health and other behaviors (14).

As well as geophysical cycles, there are biological cycles in organisms and are regulated by endogenous rhythms; ultradian, infradian and circadian rhythms. Rhythms with more than one cycle per day are called ultradian rhythms that have a responsibility to regulate Electroencephalogram (EEG), Electrocardiogram (ECG), breath cycle (15), blood circulation, thermoregulation, blinking, pulse, hormonal release, heart rate, urination, bowel activity, nostril dilation and appetite (16). More than one day, for example, weekly, monthly (e.g., the menstrual cycle) or seasonally (e.g., hibernation) cycles are called infradian rhythms. Circadian rhythms last about a day (13) and are responsible for the organizing several biological processes. As being understood from functions of the rhythms, they supply perfect behavior and physiological adaptation of organisms to their environment (15).

2.1.1 Circadian Rhythms

All of the endogenous rhythms in all living systems are evolved by adapting to the light-dark cycle. It occurs as a result of daily rotation of the planet earth around its axis. This adaptation supports to develop an internal clock that controls circadian rhythms (from the Latin ‘circa diem’, meaning about a day) (17). The internal clock is synchronized to the daily light-dark cycle by light input through the eye and serves to create self-sustaining, cell-autonomous rhythms with a frequency of approximately 24 hours (18, 19).

2.1.1.1. History of Circadian Rhythms

Generation and control of circadian rhythms have attracted the attention of many researchers because of their vital importance. Circadian rhythms were initially explained by observing prokaryotes, eukaryotes and higher plants. The physiological and behavioral observation of the human has taken place immediately (20, 21). Discovery of the circadian rhythm in cyanobacteria has demonstrated that the circadian rhythm exists in prokaryotes as well (22). French scientist Jean-Jacques d'Ortous De Mairan first identified the relationship between geophysical cycles and the circadian clock in 1729. He demonstrated that the leaves of *Mimosa pudica* continued to open and close every 24 h even when the plant was in the enclosed box (23, 24). In 1832 Augustin de Candolle demonstrated a circadian rhythm lasted for 22 to 23 hours on the leaves of the same plant. The research resulted that endogenous clock oscillation has been independent of geophysical status. This idea is now generally accepted, but discussions about the topic have continued until the 1960s (24). Insights about eukaryotic clocks came up in the discovery of clock genes of *Neurospora* and *Drosophila* (20). In 1971, Konopka and Benzer (25) discovered one of the circadian clocks components, Per using *Drosophila melanogaster.* The study showed that mutation in the Per gene of *Drosophila* affected both circadian and ultradian rhythms (23, 26). Since the middle of the twentieth century, developing techniques in the field of molecular genetics have provided to increase the studies of circadian system (27). In 1994, Vitaterna et al. (28) identified and cloned the first mammalian gene related to clock component, Clock. By identification of the mice Clock gene, molecular analysis of mammalian clock components has begun investigated seriously (21). Another research demonstrated that Per and Timeless (Tim) genes of *Drosophila,* Frequency (FRQ) gene of *Neurospora* and Cry1-Cry2 in mammals compose negative parts, White collar protein (WC-1/WC-2) in *Neurospora,* dClock and Cycle (dCLK/CYC) heterodimer in *Drosophila* and Clock-Bmal1 in mammals compose positive parts of circadian clock (20). They reflected examples of the molecular basis of the transcription and translation feedback loop circadian oscillators (22).

Crosthwaite et al. (29) studied the effect of light on circadian clocks. For this purpose, the effects of light on the FRQ gene in *Neurospora* was investigated. They reported that short pulses of visible light induced FRQ rapidly. The FRQ induction takes a role in clock resetting. This induction can be inhibited by drugs that block the synthesis of protein or ribonucleic acid (RNA). On the other hand, rapid light-induced changes caused an oscillator state variable.

In addition to all this information, molecular studies also assert that mammals, *Drosophila, Neurospora* use similar components to generate circadian rhythms (20, 30).

After these investigations, researchers showed that circadian rhythms are produced endogenously through genetic control in organisms, ranging from bacteria to humans and the rhythms control vital aspects of the physiology (21, 31).

This vital issue has won two Nobel Prizes in recent years. The circadian rhythm is known that closely relates to deoxyribonucleic acid (DNA) repair. So that Prof. Dr. Aziz Sancar won a Nobel Prize in 2015 with his work on "Mechanistic studies of DNA repair". Furthermore, 2017 Nobel Prize was given to Jeffrey C. Hall, Michael Rosbash and Michael W. Young due to research on circadian rhythm genes/proteins; Per, Tim, and Doubletime (Dpt).

Timeline;

1729 - *Mimosa pudica*, Discovery of the circadian clock

1971 - *Drosophila melanogaster*, Discovery of the Per gene

1994 - Mice, Discovery of the Clock gene of the mammalian

1995 - *Neurospora*, Discovery of the FRQ gene

2001 - *Drosophila*, Discovery of the dCLK/CYC genes, positive parts of the cock

- *Drosophila*, Discovery of the Per and Tim genes, negative parts of clock
- *Neurospora*, Discovery of the WC-1/WC-2 genes, positive parts of clock
- *Neurospora*, Discovery of the FRQ gene, negative parts of clock
- Mammalian, Discovery of the Clock-Bmal1 genes, positive parts of clock
- Mammalian, Discovery of the Cry1 and Cry2 genes, negative parts of clock

2015 - Nobel Prize to Prof. Dr. Aziz Sancar, Mechanistic studies of DNA repair

2017 - Nobel Prize to Jeffrey C. Hall, Michael Rosbash, and Michael W. Young, Their research on circadian rhythm genes/proteins; Per, Tim, and Dpt.

2.1.1.2. Biological Importance

Oscillations of circadian rhythm in nearly every cell complete their circulation even in the absence of external factors (32-34). These daily oscillations such as mental alertness, physical strength (18), sleep-awake cycles, hormone secretion, body temperature, locomotor activity, clotting factors, immunological reactions, blood pressures, heart rhythm, glucose in the bloodstream, homeostasis, and so on are regulated by circadian rhythms (3, 13, 35). It is thought that the insufficient functions of circadian

oscillators are caused by various pathologies (6), some drugs, some diseases and desynchronization of timing such as chronic jet lag, shift work, or mental health disorders such as schizophrenia and depression which affect sleep timing and quality. Disruption of circadian rhythm results in genotoxic stress, cell cycle disruption, aging (7), obesity, neuropsychiatric disorders (19), insomnia, gastrointestinal diseases, metabolic syndrome, cardiovascular events and cancer (3, 17). Shift work with circadian rhythm disruption was designated as a Class 2A probable human carcinogen by the International Agency for Research on Cancer (IARC) in 2007 (36).

Since the circadian rhythm deterioration and the linkage of diseases have been understood, the treatments of these diseases have started to be rearranged accordingly. So understanding the molecular mechanism of the circadian rhythm is essential to enlighten the relationship between circadian rhythm disruption and diseases (19).

2.1.1.3. Organization

Circadian rhythm of multicellular developed organisms such as mammals' is established by a complex hierarchical organization of organs. This complex structure is responsible for the continuity of synchronization of the circadian system and it is mainly served by the brain and liver. Each cell of tissue is in harmony with each other to ensure the persistence of circadian system (19). As a theory, circadian rhythm has been generated by a pacemaker that receives various environmental signals via input pathways and organizes different periodic outputs.

Various factors affect the pacemaker as well as the oscillation of the circadian system. Some of them are light, nutrition, emotional stress, temperature cycles, and sleep privation but the light is considered the strongest of them (18). So it is regarded that the circadian rhythm is theoretically composed of three complex interconnecting pathways; an input component (afferent) from the light-dark cycle, a pacemaker that generates the oscillations, and an output component (efferent) that is measurable. The continuous synchronization of these three components or in other words synchronization of the circadian clock with environmental and internal body signals maintain the coordinated function of individual cellular clocks and the integration of network system (19, 24).

In summary, light is firstly perceived by eyes as an input signal from the environment, and then this signal is detected by the pacemaker, suprachiasmatic nucleus (SCN). Subsequently, outputs that are responsible for specific behaviors and

physiological activities are generated by the pacemaker. In this way, the body provides feedback information about the regulated internal environment to the clock (18).

2.1.1.4. Mechanisms of Circadian Rhythm

2.1.1.4.A. The Central Master Clock

In the brain of mammals, circadian rhythms are managed by ~15,000 neurons localized in the anterior hypothalamus, known as the SCN. SCN is composed of a pair of small nuclei located above the optic chiasm and laterally to the third ventricle (17, 34). This region is "central master clock" which is required to synchronize cellular clocks throughout the body (37). The SCN receives signals from the environment and controls peripheral clocks (33). Surgical removal of the SCN within the brain disrupts behavioral rhythmicity and rhythmic electrical activity in the central nervous system (CNS) (38).

A) Photoreception and Light

The circadian clock is mainly reset by the light. On the other hand, the light also changes the clock activity (29, 39). The light-activated photoreceptor molecules produce intracellular signals then they are converted into the body. Converted intracellular signals become elements of the circadian oscillator (29).

Mammalian circadian rhythms occur centrally via the eyes and optic nerves because of the light receptor function. Also, the pineal gland and deep brain have photoreceptor molecules. Blindness or optic nerve damage can lead the disruption of light cycle hence the circadian rhythm breakdown (18). The various type of cells and pigments located inside and outside of the eye play as photoreception. Both the inner and outer retina has two photoreceptor cells, rods and cones (34, 40). The rod and cone cells of the outer retina possess the photosensory pigments as rhodopsin and color opsins. Additionally, there are another intrinsic photoreception cells as retinal ganglion cells (ipRGCs) and inner nuclear layer cells (INL). The primary site of retinal projections is SCN. The rod and cone cells of outer and inner retina take the light input. After that, the input is transferred to the SCN through the retinal-hypothalamic tract (RHT). INL and ipRGCs express Cry which is one of photopigment containing blue light absorbing flavoprotein. Another remarkable circadian photopigment, melanopsin is located in the inner retina and expressed by ipRGCs. It is thought that melanopsin and Cry are the only known photopigments in the inner retina due to the only candidates for the circadian photoreceptors. Melanopsin primarily acts in the transduction of the light input to the

SCN. All of the light-sensitive cells and photosensory pigments found in the inner and outer part of retina play an essential role in photic entrainment (Figure 1) (18).

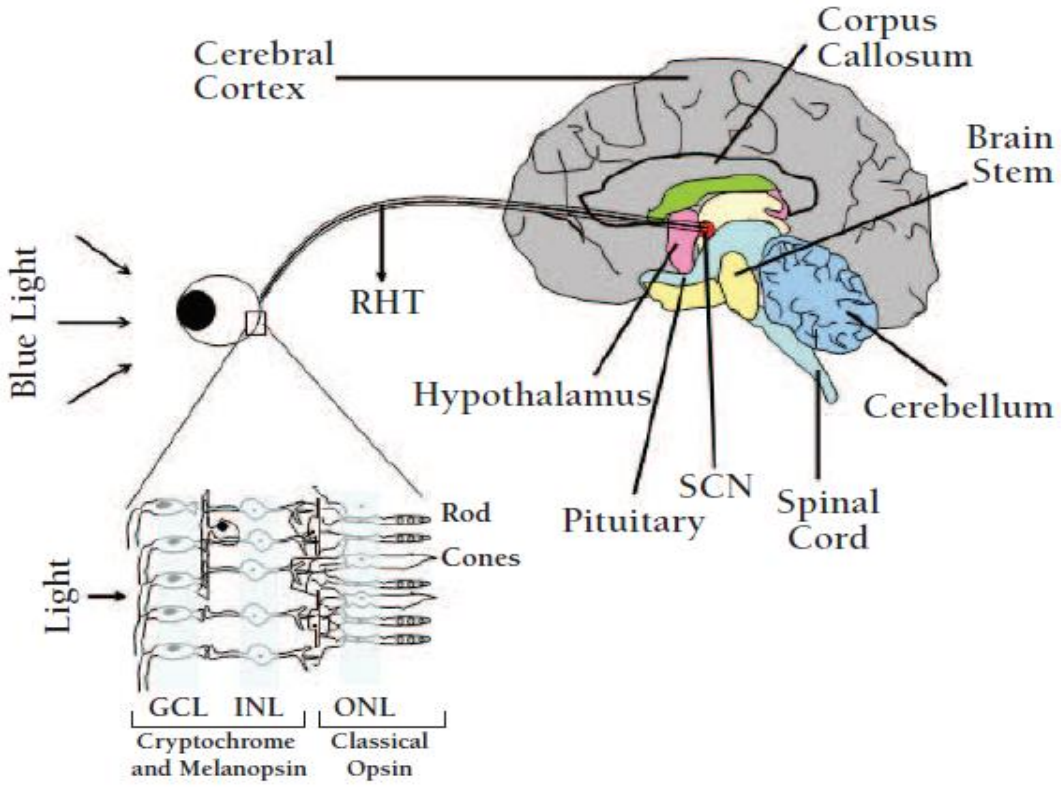

Figure 1: Light reception by eye (18).

B) Vasopressin

Vasoactive intestinal polypeptide (VIP) or vasopressin is the most common neuropeptide transmitter within the SCN. VIP closes potassium channels resulting to membrane depolarization of the SCN neurons. As a result, expression of Per1 and Per2 are elevated. The primary role of the VIP is to harmonize the oscillations in the circadian rhythm (16).

C) GABA

Inhibitory transmitter, gamma-aminobutyric acid (GABA) is another major neurotransmitter released by SCN neurons. GABA is essential synchronizer of SCN connecting individual clock cells to each other (41).

2.1.1.4.B. Peripheral Cellular Circadian Clock

Peripheral cellular circadian clocks are present in other parts of the brain and almost all other organs as the liver, heart, muscle (33), lung, kidney (42), white adipose tissue, pancreas, and ovary (33). This clock is responsible for different metabolic processes including in the metabolism of cholesterol and lipids, glucose, amino acid, and detoxification pathways (17). Besides they play a significant role in the regulation of cell cycle genes including multifaceted oncogene (Myc), Wee1, Cyclin D, and p21 (43).

The peripheral clock is self-sustaining. It does not respond to light-dark cycles (17) and needs the central pacemaker (SCN) for rhythm generation (42). There are other SCN-independent factors as food, temperature and hormonal changes that affect the peripheral clock (17).

2.1.1.5. Molecular Components of Circadian Rhythm

At the molecular level of the clock in mammalian cells, there are auto-regulatory interlocking transcription/translation feedback loops (TTFL), which work together. These loops are driven by four master integral clock genes/proteins: two activators (Clock and Bmal1) and two repressors (Per (1 and 2 isoforms) and Cry (1 and 2 isoforms)), also phosphatases and kinases that regulate the stability and localization of them. Circadian rhythms are generated and maintained through regulation of these clock genes/proteins (4, 44).

These genes/proteins constitute of positive and negative feedback loops, called clock-controlled genes (CCGs). Clock and Bmal1 form the positive feedback part, whereas Cry and Per constitute the negative feedback part of CCGs. It means the transcription factors Clock and Bmal1 form the heterodimer that treats as a transcriptional activator by recognizing Enhancer box (E-box) components and also interacting with each other. This heterodimer directly regulates Cry and Per translocation. They form a negative complex that completes the core feedback loop by repressing Clock: Bmal1-mediated transcription (45). E-boxes act as a protein-binding site and regulate specific gene expression in neurons, muscles, pancreas and other tissues. Its specific DNA

sequence, CACGTG, is recognized and bound by transcription factors to initiate gene transcription. E-boxes are promoter and enhancer elements in mammalian genomes. They are present in either one or multiple copies in the regulatory regions of CCGs. Namely, this regulatory system controls the expression of many CCGs via E-boxes (17, 46). Molecular transcriptional/translational feedback loop is shown in Figure 2.

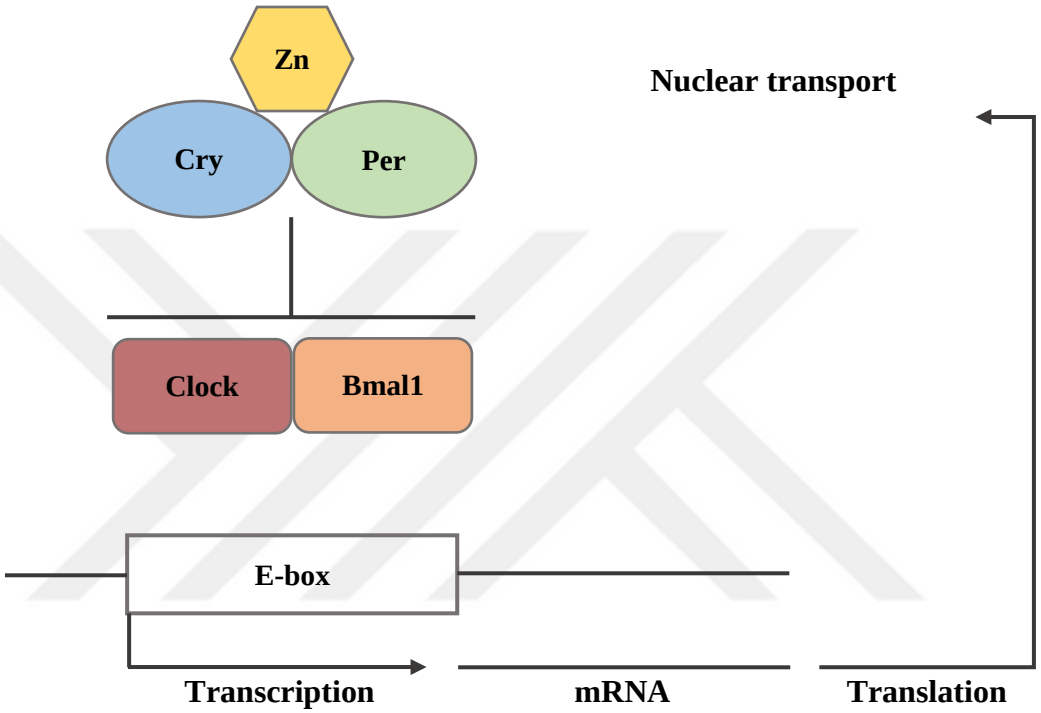

Figure 2: Molecular transcriptional/translational feedback loop
Zn: Zinc, Per: Period, Cry: Cryptochrome, E-box: Enhancer box, mRNA: Messenger RNA (47).

Clock: Bmal1 heterodimers activate transcription of retinoic acid-related orphan nuclear receptors, Rev-erbs and Rors that generate another regulatory loop. Rev-erbs suppress the transcription process of Bmal1 whereas Rors activate this process. Therefore Rors and Rev-erbs both positively and negatively regulate the circadian oscillation of Bmal1 (32).

In addition to these controls, some of the kinases are responsible for controlling of circadian rhythm via phosphorylation process of clock genes/proteins. One of them is

glycogen synthase kinases 3 (GSK3α and β) (48). Another control kinases, casein kinases1 (Ck1ξ and Ck1d) play a role of critical factors in the rhythm (32, 44). They positively regulate Bmal1 with phosphorylation. Also, Per proteins are phosphorylated and degraded by Ck1ε and Ck1d to reduce their stability (33, 49, 50). The molecular components of the circadian rhythm are shown in Figure 3.

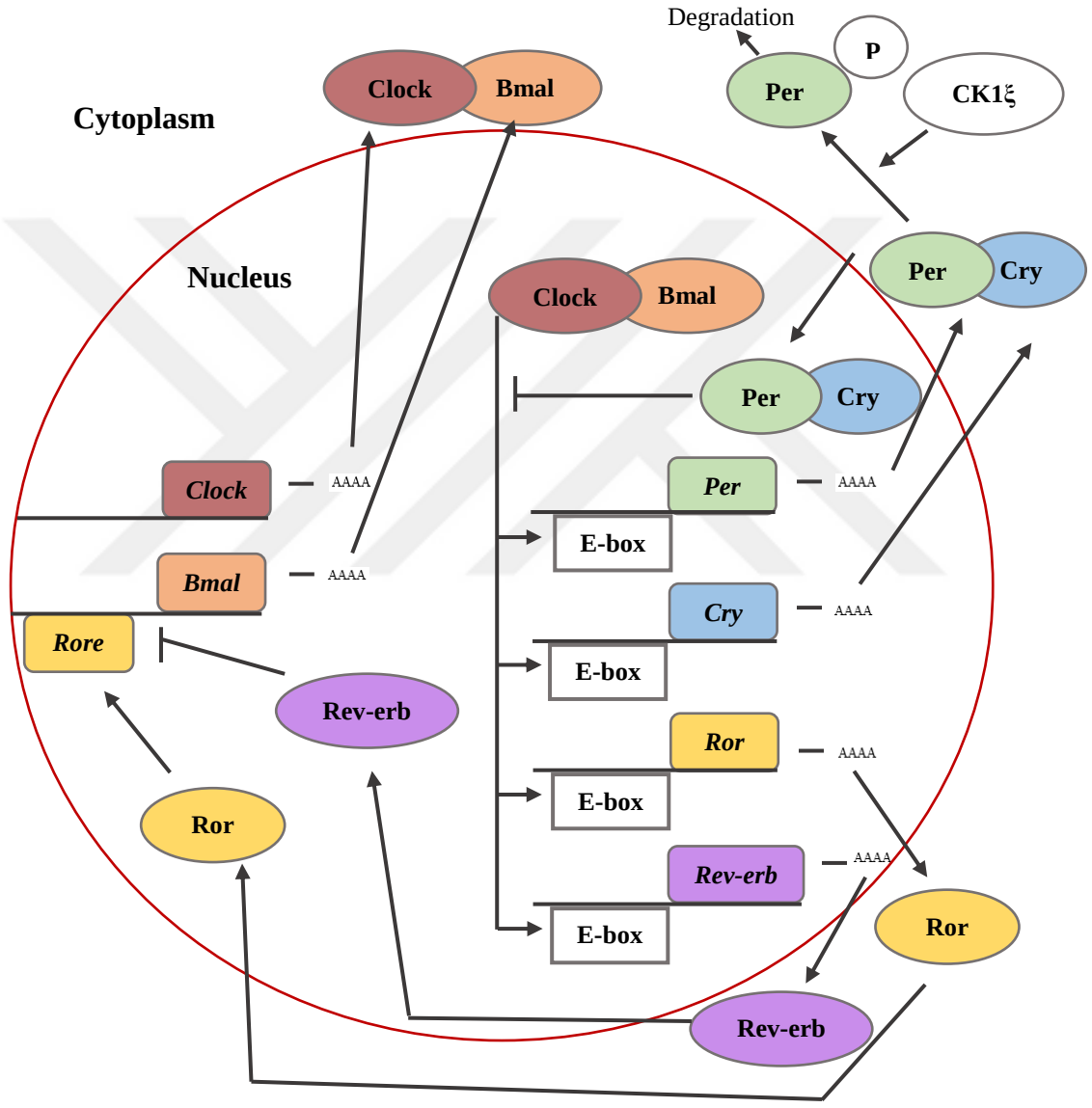

Figure 3: Schematic representation of the molecular components of circadian rhythm Per: Period, Cry: Cryptochrome, Ror, and Rev-erb: Retinoic acid-related orphan nuclear receptors, Rore: Retinoic acid-related orphan receptor response elements, Ck1ξ: casein kinases1 epsilon, P: Phosphor (33).

2.1.1.5.A. Clock and Bmal1

Clock and Bmal1 belong to the family of bHLH-PAS (basic helix loop helix- PER-ARNT-SIM homolog) containing transcription factors (42). Clock gene is not expressed rhythmically, whereas in rats Bmal1 is expressed rhythmically (13). Clock gene heterodimerizes with Bmal1, and they connect to the E-box. Their primary function is the induction of Per and Cry transcription. Bmal1 also takes part in RNA cycles (50). As mentioned above circadian elements are responsible for cell cycle control, and therefore they are associated with cancer. For example, the Clock: Bmal1 heterodimer stimulates the Wee1 gene, resulting in the arrest of the G2-M phase cell cycle and inhibition of mitosis. Cry also play a role in the induction of mitosis. The Myc oncogene, which is effective in G1 phase and tumor formation, is inhibited by Bmal1. Furthermore, Per blocks the transition from G1 to S in the cell cycle, so that the formation of mitosis have been prevented (43). The development of "late-shift diseases," including diabetes and cardiovascular disorders is seen by absent the Clock gene in mice. This gene is also directly a related endocrine function of the pancreas. If the Clock gene is obliterated, the pancreas loses the ability to secrete insulin and consequently develop type 1 diabetes (2). In addition, obesity, metabolic syndrome, hyperphagia, hyperlipidemia, and hepatic steatosis were seen in mice with the Clock gene/protein deficiency. Clock is responsible for the lipid and glucose metabolism and cell cycle regulation. Furthermore, hypoglycemia, impaired glucose clearance, reduced lifespan, premature aging, impaired steroidogenesis, reduced fertility, and impaired adipogenesis were seen in mice with the Bmal1 gene/protein deficiency. Bmal1 is responsible for glucose homeostasis, adipogenesis, and cell cycle regulation (33).

2.1.1.5.B. Cry

The Cry is a class of flavoprotein and belongs the family of blue-light photoreceptors found in plants and animals (40, 51). They participate in the RNA cycle. The stabilization of Per and inhibition of transcription of Per and Cry are among the other functions of Crys. Panda et al. (50) indicated that Cry is associated with Per in physically. Crys are expressed in plants and beetles by light and dark cycles. However, human Cry synthesis is thought to be regulated by light (13). Recent studies have demonstrated that Cry is a primary circadian photoreceptor in fruit flies and *Drosophila*. The inner retina of mice contains the Cry1 and Cry 2. They are also related to the light-activated repair enzyme DNA photolyase (40). Recently the "magnetoreception" function of Cry has

attracted the attention of many researchers. Cry, and magnetic field relationship was investigated in several of the organisms including fruit flies, *Drosophila*, plants and migratory birds (47, 52). Most importantly, the photochemical properties of Cry have been found to be favorable for detecting weak magnetic fields (53).

The ubiquitin system is used for degradation of Cry proteins. Cry binds to a ubiquitin ligase, FBXL3 (F-box and leucine-rich repeat protein) or FBXL21 and is stabilized (47). One of newly synthesized chemical is KL001 (54), which inhibits FBXL3-dependent degradation of Crys, causing lengthening of the period of the Crys (47).

A knockout (KO) studies in mice have played critical roles in determining the function of Crys in mammals. While Cry1 KO mice displayed a shorter period phenotype, Cry2 KO mice exhibited a more extended period phenotype than the wildtype control mice. The results suggest that these different types of Cry show different function in the regulation of circadian clock (47). Furthermore mutation of them alters the central circadian oscillator function in mice (50).

Crys play indispensable roles in the regulation of growth, development in plants, in the generation of the circadian clock, and in the sensing of magnetic fields among various species (51, 53). It has also been related to the appearance of the various disease. In the absence of Cry in mice display increased aldosterone production causing to arterial hypertension as well as impairment of hepatic gluconeogenesis and insulin resistance and diabetes was observed (2). In addition, low risk of tumor and cancer formation were seen in mice with the Cry gene/protein deficiency. Cry is responsible for xenobiotic detoxification and the cell proliferation. Furthermore, the cytochrome P450 2E1 (CYP 2E1) enzyme is the target genes of Cry (33).

2.1.1.5.C. Per

Per belongs to the family of PAS protein. They consist of 3 subgroups, Per1, Per2, and Per3. In addition to being involved in RNA and protein cycling, it is also the activator of Bmal1. There is a physical association with Cry and among Per proteins (50). It is temporarily induced by light in the retina and peripheral tissue (13).

Distortion of circadian rhythm genes is associated with the risk of acute myocardial infarction. Especially Per2 deficiency can cause myocardial ischemia. Per2 is also related to noncardiac peripheral ischemia and modulating the inflammatory response to tissue ischemia (2). In addition, the high risk of cancer formation, abnormal response

to gamma irradiation, increased cellular proliferation, and reduced muscular strength under stress were seen in mice with the Per gene/protein deficiency. Per is responsible for tumor suppression and cell cycle regulation (33). Circadian rhythm genes/proteins defect and biological consequences are demonstrated in Table 1.

Table 1: Circadian rhythm genes/proteins defect and biological consequences (2, 32)

Disrupted gene	Physiological effect (2, 32)	Disease (2)
Bmal1	Infertility, Progressive arthropathy, Abnormal gluconeogenesis, Abnormal lipogenesis, Altered sleep pattern (32) Decrease insulin secretion of pancreas (2)	Diabetes Obesity
Clock	Metabolic syndrome, Abnormal gluconeogenesis, Abnormal behavioral sensitization to psychostimulant, Altered sleep pattern (32) Decrease insulin (2)	Diabetes Obesity
Per1	Abnormal apoptosis/cancer development, Abnormal behavioral sensitization to psychostimulant (32)	
Per2	Improper cell division/cancer development, Abnormal behavioral sensitization to psychostimulant, Improper alcohol intake, Familial advanced sleep phase syndrome (32) Decrease oxygen efficiency on heart results in endothelial dysfunction (2) Decrease innate immunity (2)	Heart attack Edema Sepsis Inflammation
Per3	Associated with DSPS* (32)	
Cry1 and Cry2	Altered sleep pattern, Increase the secretion of aldosterone hormone in kidney (32) Increase the secretion of glucose in liver (2)	Hypertension Diabetes
Ror alfa	Cerebellar ataxia, Abnormal bone metabolism (32)	

Ror beta	Locomotor difficulties, Retinal degeneration/blind, Male reproductive abnormality during first six months of age (32)
Ror gamma	Lack of lymphoid organ development, Abnormal lymphocyte homeostasis (32)
Ck11/Ck1d	Familial advanced sleep phase syndrome (32)

* DSPS: Delayed sleep phase syndrome

2.1.1.6. Synthesized Circadian Rhythm Gene / Protein Activators and Inhibitors

Together with the discovery of these genes/proteins in recent years, a various synthetic chemical capable of inhibiting or activating them via different pathways has been synthesized (47). Some of the activators and inhibitors are demonstrated in Table 2.

Table 2: Synthesized circadian rhythm gene/protein activators and inhibitors

Gen/Protein	Activators	Inhibitors
Cry	KL001 (54)	KS15-KS25 (45)
Per	IC261 (5)	-
Bmal1	GSK126 (3), Selenyum (6)	DBC1 (7)
Clock / Bmal1	-	Ketamine (39)
Rev-erb		
Rev-erb-a	GSK4112 (55)	SR8278 (56), Mir-96 (57), polyethleneimine (58)
Rev-erb-b	SR9009 and SR9011 (59)	-
Ck1		
Ck1-e	-	PF-4800567 (60)
Ck1-a		Longdaysin (61)
Ck1-d		LH846 (49)
GSK3β	MicroRNA-3646 (62), Protocadhrin 9 (63) TSSC3 (64)	TDZD8 (65, 66), Tideglusib (66, 67), TWS119 (66, 68), peptide L803-mts (66), SB216763 (69, 70), Lithium (37)

2.2. General Anesthetic Agents

General anesthetic agents are used to inducing and to keep unconsciousness during surgery, to reduce psychological and physical distress in patients (9). During the 1960s, intravenous and inhaled anesthetic agents commonly called as general anesthetics are practiced to be achieved these clinical goals. However, now depending on the needs

of surgery, the anesthetic agents are used alone or in combination with adjuvant medications such as opioids and/or myorelaxants (71).

a. Inhaled Agents

Inhaled agents that produce an anesthetic effect are halothane (first clinically use in 1951), enflurane (1972), the isomer of enflurane, isoflurane (1980) and sevoflurane (1980). There is also desflurane, but it is used for only clinical trials. Nitrous oxide was used due to the analgesic effect in the 1800s. However, the effect of anesthesia was discovered in 1884 and started to use as an anesthetic agent (72). Nowadays isoflurane is highly preferred because of its rapid onset and rapid dispersal. Sevoflurane is also often preferred because it has less toxicity and is thought to have protective properties (73).

b. Intravenous Agents

The most common agents used for induction of anesthesia in clinically are barbiturates; thiopental (first clinically use in 1935) and methohexital (1957), the benzodiazepine; diazepam (1961) and midazolam (1979), etomidate (1975), propofol (1984) and ketamine (1966) (72).

2.2.1. Side Effect of Anesthetic Agents

Both inhaled and intravenous anesthetic agents are known to have various side effects. These side effects influence vital systems, especially the nervous system, cardiovascular system and respiratory system. Besides, almost all systems such as renal, muscular, reproductive, and liver can be affected by the anesthetic agents (72). Bradycardia, hypotension, ophthalmic and motor disorder (74), sleep and mood disorder (75), Alzheimer's disease, cognitive impairment (76), hepatitis (77), acute renal failure (78) and more are among the side effects. Main reasons underlying the side effect are the occurrence of DNA damage and alteration of oxidative status originated from anesthetic agents (79). Also, inhaled anesthetic agents have a risk of genotoxicity, cytotoxicity, and teratogenicity (80).

According to examinations in recent years, it is showed that the risk of mortality due to the anesthesia/surgery has been decreased (72). However, the decrease in the mortality rate should not be considered as a reduction in other side effects. For this reason, recently there has been rising attention in the development of risk assessment in anesthetic

agents to increase the patient's quality of life (81). Various studies are being carried out to reduce the possible side effects of anesthetic agents (72).

2.2.1.1. The Relationship of Anesthetic Agents with Oxidative Stress

Recent clinical reports support that the surgery under general anesthesia manipulates oxidative stress (10, 79). Oxidative stress is likely to be important regarding damages especially in DNA and chronic inflammation response by the accumulation of immune cells in the damaged areas. These factors generate the basis of the side effects (11). Kotzampassi et al. (10) investigated the relationship between anesthesia and oxidative stress markers in rat serum and liver tissue. The operation itself increased the level of nitric oxide (NO), MDA and decreased the level of SOD and GSH-Px (10). Many studies reported that oxidative stress, inflammation, and DNA damage were elevated by isoflurane and sevoflurane in patients under anesthesia/surgery (Table 3) (11). Likewise, the occupational exposure of anesthetic gases has been shown to lead to the depletion of glutathione (GSH) resulting in injury and cell death. The data indicated that isoflurane and sometimes sevoflurane increase the DNA damage and change the redox status (79).

Table 3: Effects of isoflurane and sevoflurane on oxidative stress in human

Subject of study	Anesthetics	Effects
Patients (elective lower abdominal surgery)	Isoflurane, sevoflurane	Increased DNA damage and oxidative stress (82)
Patients (orthopedic surgery)	Sevoflurane	Increased DNA damage and changes oxidative status (11)
Patients (elective hysterectomy)	Isoflurane	Increased inflammation response and oxidative stress (83)
Patients (elective cholecystectomy)	Isoflurane	Increased inflammation response and oxidative stress (11)
Patients (elective thoracotomy lobectomy)	Sevoflurane	Pulmonary dysfunction by increased inflammation response and formation of free radicals (84)
Operating room personnel	Isoflurane	Increased DNA damage (79, 85-87), changes in redox and oxidative status (79)

2.2.1.2. Impact of the Anesthetic Agents on Circadian Rhythm Synchronization

Since researchers found that the anesthetic effect and the duration of anesthesia of morphine were altered according to the specific time of day, systemic and local anesthetics begun to be utilized by anesthesiologists in consideration of daily cycling. There are many reports about strong relation between anesthetic agents and failure in circadian rhythms. This failure is most likely to cause postoperative sleep and cognitive disorders. Therefore, it is clinically significant to prevent symptoms of insomnia, confusion, and postoperative delirium resulting in morbidity and mortality, and other physiological disturbances by eliminating the damage in the circadian rhythm (2).

Several studies have shown that anesthesia/surgery disrupt the synthesis or function of circadian rhythm genes/proteins. For example, the inhaled anesthetic agent, sevoflurane repressed Per2 expression in rodents (8, 9). In another similar study in mice administration of sevoflurane-induced the increase of nicotinamide adenine dinükleotid (NAD(+)), and thereby caused to the suppression of Per2 expression (88). Kikuchi et al. (89) showed that propofol in both rodents and humans caused the modification of the circadian rhythms of physiological parameters as locomotor activity and body temperature. Moreover, isoflurane depressed hippocampal ACh release and corrupted the diurnal rhythm.

2.3. Oxidative Stress

2.3.1. Oxygen, Free radicals, and Oxidative Stress

The oxygen molecule is present in the earth atmosphere and constitutes 21% of the atmosphere. Furthermore, dissolved oxygen is found in seas, rivers, lakes and other water beds.

The oxygen molecule is vitally essential for all organisms except anaerobic species. While organisms need oxygen to survive, it is known that the excess concentration of oxygen in the environment shows toxic side effect. The toxic side effect of molecular oxygen is due to reactive products such as superoxide radical ($O_2^{-\cdot}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($HO^{\cdot}$), singlet oxygen (singlet O_2) and hypochlorous acid (HOCl). These products, which are the result of the metabolism of oxygen in biological systems, are called reactive oxygen species (ROS) (90, 91). The destructive effect of ROS causing potential biological damage is termed oxidative stress. Oxidative stress is also caused by nitrogen free radicals such as reactive nitrogen species (RNS), which is formed by cellular metabolism. When there is an overproduction of

ROS/RNS and deficiency of enzymatic and non-enzymatic defense systems, oxidative stress occurs in biological systems. Briefly, disturbance in the balance of oxidant/antioxidant reactions in favor of oxidants causes oxidative stress (92).

2.3.2. Free Radicals

A free radical is a molecule or molecular fragments containing one or more unpaired electrons, and this electron is present alone in the atomic or molecular orbital (90, 92). The presence of one or more unpaired electrons leads to the withdrawal of that material into a magnetic field, which sometimes makes it highly reactive (90, 93).

Free radicals are formed by the removal of an electron from a non-radical atom or molecule, or by the addition of an electron to a non-radical atom or molecule (90). The covalent bond of the molecule is broken, and a radical can easily be formed by leaving one of the electrons in one atom and the other in the other, which is known as a homolytic fission. The breaking of this covalent bond is an energy-requiring process and heat can be generated by forces such as electromagnetic radiation (93). The homolytic fission can be represented by the following equation;

(Presuming that A and B are two covalently bonded atoms)

$$\mathbf{A : B \rightarrow A^{\cdot} + B^{\cdot}}$$

The homolytic fission can also occur in the water molecule. As a result of reaction $H^{\cdot}$ and $OH^{\cdot}$ radicals occur.

In heterolytic fission, the opposite of homolytic fission, one of the molecular atoms takes two electrons during the opening of the covalent bond. It can be described as below (90);

$$\mathbf{A : B \rightarrow A^{:-} + B^{+}}$$

2.3.2.1. Reaction Mechanisms of Free Radicals (90)

A) In the case of reacting with two radicals, they can participate their unpaired electrons to constitute a covalent bond. Thus, they lose their radical properties.

$$\mathbf{H^{\cdot} + H^{\cdot} \rightarrow H_2}$$

B) However, biological molecules exhibit non-radical properties. If a free radical reacts with non-radical, a series of chain reactions may occur.

1) A radical may link another molecule. The resulting product still has an unpaired electron and acts as a radical.

$$\mathbf{A^{\cdot} + B \rightarrow (A\text{-}B)^{\cdot}}$$

2) A radical may become nonradical by donating a single electron. The radical behaves as a reducing agent, and the acceptor has an unpaired electron.

$$\mathbf{A^{\cdot} + B \rightarrow A^{+} + B^{\cdot -}}$$

3) A radical may become nonradical by taking a single electron. The radicals behave as an oxidizing agent, and the acceptor has an unpaired electron.

$$\mathbf{A^{\cdot} + B \rightarrow A^{-} + B^{\cdot +}}$$

4) A radical may separate a hydrogen atom from a C-H bond. As a result, carbon has an unpaired electron.

$$\mathbf{(C\text{-}H) + OH^{\cdot} \rightarrow C^{\cdot} + H_2O}$$

2.3.2.2. Sources of Free Radicals

The cell is exposed to ROS by external sources as well as endogenous sources. The primary source of ROS is an inevitable result of the human aerobic mechanism (94).

2.3.2.2.A. Endogenous sources of ROS

1. Mitochondrial electron transport chain

The mitochondria is known to serve as the significant organelle responsible for ROS production. The electron transport system in the mitochondria is responsible for this production (94).

The most important task of the animal mitochondrion is the oxidation or reduction of the NADH + H^+ and reduced Flavin adenine dinucleotide ($FADH_2$) produced during the Krebs cycle and β-oxidation of fatty acids and other metabolic events. The process of oxidation is localized in the inner membrane of the mitochondrial electron transport

chain. The energy is released by this oxidation and is used in adenosine triphosphate (ATP) synthesis. Every four electrons given on the cytochrome oxidase complex oxidize one molecule of oxygen to two molecules of water. In this electron transport chain mainly $O_2^{-\cdot}$ formation sources are NADH-coenzyme Q reductase complex and coenzyme Q themselves. The mitochondria isolated from some animal tissues has been shown to produce H_2O_2 in vitro. The vast majority of this is caused by mitochondrial SOD activity with O_2 dismutation. Some trypanosomes and plant tissues have been shown to produce H_2O_2 as animal mitochondria (91, 95).

2. Endoplasmic reticulum

The endoplasmic reticulum (ER) in many animals and some plant tissues contain mainly cytochromes known as cytochrome P-450. The name of the P-450 was given because the reduced cytochromes form a complex with carbon monoxide and this complex absorbs the light of 450 nm wavelength. Cytochrome P-450 oxidizes a multi-substrates using molecular oxygen. One atom of the oxygen molecule is connected to the substrate, and the other atom forms water, so it is also called the mono-oxygenase or mixed-function oxidase (MFO) reaction. Cytochrome P-450 requires an agent (RH_2) to be reduced during the reaction and utilizes reduced nicotinamide adenine dinucleotide phosphate (NADPH) for this purpose. The most important mechanism for the formation of free radicals from chemical agents is activation of xenobiotics by microsomal cytochrome P-450 system. This system creates free radicals by adding an electron (reduction) to the molecules (as in carbon tetrachloride and halothane) or by removing an electron (oxidation) from the target molecule (90, 91).

3. Redox cycle

Redox cycle is another mechanism for the formation of free radicals from chemicals. These reactions do not require P-450. Free radical formation from xenobiotics does not occur only with microsomal reactions. Menadione, paraquat, diquat, nitrofurantoin, adriamycin, bleomycin, and furosemide enter into an alternative redox cycle. These compounds tend to gain an additional unpaired electron. The radical of these agents is easily oxidized with oxygen to turn into the main compound and eventually $O_2^{-\cdot}$ is formed. Two situations arise as a result of the redox cycle; 1) Additional free radical production can lead to further cell damage, 2) GSH and NADPH release may occur. Oxidation of these two reducing agents causes oxidative stress in the cell (91, 95).

4. Arachidonic Acid Metabolism

Arachidonic acid metabolism is also an important source of ROS. Induction of phagocytic cells leads to activation of phospholipase and protein kinase. It also leads to release of arachidonic acid in the plasma membrane. Various free radical intermediates are formed by the enzymatic oxidation of arachidonic acid. Some xenobiotics also produce reactive intermediates at the same time. These intermediate products may also show toxicity by interacting with target structures (91).

2.3.2.2.B. Exogenous sources of ROS

1. Irradiation: ROS could also be generated by ionizing and nonionizing irradiation (96).
2. Drugs: Anesthetics and narcotic drugs are known principal participator of ROS formation. Many xenobiotics such as diquat, paraquat, dioxins, acetaminophen, iron (Fe), adriamycin, bleomycin, nitrofurantoin, furosemide, asbestos, ozone, menadione and dieldrin, mustard gas, alcohol, cigarette smoke, carbon tetrachloride also caused ROS production (91, 94).
3. Food: Food is one of the primary sources of oxidants and contains different kinds of oxidants such as peroxides, aldehydes, oxidized fatty acids, and transition metals (94).
4. Air pollution: Air pollutants including industrial pollutants, car exhaust, and cigarette smoke compose sources of ROS and NO derivatives. They attack and damage the organism by direct interaction with skin and inhalation (94).

2.3.2.3. Oxygen Derivatives Free Radicals

ROS occur during some physiological events in aerobic organisms. One of their sources is the mitochondrial electron transport chain. Often 95% of the oxygen used in cells is reduced to water by the gradual addition of four electrons in mitochondria. Under normal conditions, during this mitochondrial event, 1-2% of oxygen is converted to $O_2^{-\cdot}$. $O_2^{-\cdot}$, H_2O_2, $HO^{\cdot}$, singlet O_2 and hypochlorous acid (HOCl) are mainly ROS. These reactive species play a role in many pathological events by reacting with vital macromolecules such as enzymes and proteins, lipids and nucleic acids (DNA and RNA), disrupting cell structure and function (91, 92).

2.3.2.3.A. Superoxide radical ($O_2^{-\cdot}$)

$O_2^{-\cdot}$ is formed by attachment of one extra atom to molecular oxygen (90, 91). The main source of $O_2^{-\cdot}$ is mostly mitochondrial electron transport chain in a cell. During energy transmission, a few electrons may leak premature oxygen forming $O_2^{-\cdot}$. Another important source of $O_2^{-\cdot}$ is a respiratory burst of phagocytic cells such as neutrophils and macrophages (92). Peroxidases also found in plants and bacteria produce $O_2^{-\cdot}$ during oxidation. Thiol compounds such as glyceraldehyde reduced riboflavin, flavin mononucleotide (FMN), FAD, adrenaline, tetrahydropyridine, and cysteine are oxidized in the presence of oxygen to give $O_2^{-\cdot}$. $O_2^{-\cdot}$ may also be formed by the exposure of tryptophan amino acid and melanin to ultraviolet (UV) light. $O_2^{-\cdot}$ and H_2O_2 are also produced by photochemical separation of organic compounds in seawater. Tryptophan dioxygenase, galactose oxidase, aldehyde oxidase enzymes also produce $O_2^{-\cdot}$ during their catalytic functions (91).

This radical is a weak oxidant but rather a powerful reducer with a millisecond half-life. $O_2^{-\cdot}$ is an essential factor in oxygen toxicity, and the SOD enzyme protects the organism against it. It is unlikely that $O_2^{-\cdot}$ would lead to significant cell damage by itself due to its weak oxidant effect. It may react with thiol groups, thus causing depletion of GSH and inducing further oxidative stress on cells. When the $O_2^{-\cdot}$ reacts with thiol groups on the enzyme and other cellular proteins, this may induce its inactivation. However, $O_2^{-\cdot}$ can also initiate a chain of reactions that can consequently lead to remarkable oxidative stress. One of these critical reactions called as **Heber-Weiss** reaction. In this reaction, $O_2^{-\cdot}$ react with H_2O_2 in the presence of Fe metals. As a result, extremely reactive $OH^{\cdot}$ occurs.

$$\mathbf{O_2^{\cdot -} + H_2O_2 \rightarrow HO^{\cdot} + {}^{-}OH + O_2}$$

$O_2^{-\cdot}$ releases Fe from the intracellular Fe deposits. Ferritin contains +3 valent Fe. $O_2^{-\cdot}$ can convert it to +2 valent and leads to be free. The free Fe ion may be used in Fe-dependent and radical-producing reactions such as the Haber-Weiss reaction or may play a role in other significant free radical-mediated cell damages. In addition, $O_2^{-\cdot}$ reacts with Fe +3 to form the oxidizing perferril radical.

As mentioned before, $O_2^{-\cdot}$ has a very short half-life and it is readily turned into O_2 and H_2O_2 in a process, called dismutation reaction.

$$\mathbf{2O_2^{\cdot -} + 2H^{+} \rightarrow O_2 + H_2O_2}$$

Although this dismutation reaction may be spontaneous or accelerated by the SOD enzyme (90, 91).

2.3.2.3.B. Hydrogen peroxide (H_2O_2)

H_2O_2 does not contain unpaired electrons. Although not chemically radical, it is mentioned in the ROT and plays an essential role in free radical biochemistry. This radical can be formed by the result of the dismutation reaction from $O_2^{-\cdot}$ or directly by the reduction of O_2. In addition, some peroxisomal enzymes produce abundant amounts of H_2O_2 without the need for $O_2^{-\cdot}$. However, since the CAT activity is very high in peroxisomes, the amount of H_2O_2 in the cytosol is unknown. H_2O_2 is also produced during photosynthesis and phagocytosis. H_2O_2 is also detected in the human respiratory air (91, 94, 97, 98).

Although H_2O_2 is a powerful oxidizing agent, it reacts relatively slowly. After H_2O_2 has formed, it can induce either directly undergoes oxidative damage or cause more damage by creating more reactive free radicals such as $O_2^{-\cdot}$ (91). It shows solubility characteristics in aqueous media. Therefore, it can quickly pass through biological membranes. H_2O_2 can oxidize thiol groups of proteins. It has been shown that DNA chain breaks occur within seconds with exposure to H_2O_2 (94, 98). In this way, the glyceraldehyde-3-phosphate dehydrogenase enzyme (GAPDH), which is one of the glycolytic pathway enzymes, is inactivated. Exposure to H_2O_2 at high doses leads to ATP release due to inhibition of glycolysis (91).

Since H_2O_2 can pass through the cell membrane, it reacts with iron ion (Fe^{+2}) in the cell to form extremely strong oxidant radical, $HO^{\cdot}$ (90). This reaction is known as the Fenton reaction (reaction 10). Besides H_2O_2 can react with $O_2^{-\cdot}$ to form $HO^{\cdot}$. This reaction is known as the Haber-Weiss reaction. Thus, $HO^{\cdot}$ formation is considered to be the underlying cause of the toxic effect of H_2O_2. Co-exposure to H_2O_2 and UV radiation increase harmful effects, because UV radiation induces the homolytic fission of H_2O_2 to $HO^{\cdot}$. Damage of H_2O_2 is prevented by CAT and GSH-Px enzymes (90, 91, 99).

2.3.2.3.C. Hydroxyl radicals ($OH^{\cdot}$)

$OH^{\cdot}$ is the neutral form of the hydroxide ion. Although $OH^{\cdot}$ has a very short half-life (approximately 10^{-9} second), it is most reactive and most potent oxidant in biological systems. Because it has a short half-life, it reacts near to its site of formation area (92). There are several reactions which $OH^{\cdot}$ can be produced, such as:

1. $OH^{\cdot}$ is formed by the reaction of $O_2^{-\cdot}$ with H_2O_2 in the presence of Fe ions. This reaction is called the **Haber-Weiss** reaction (90, 92). The **Haber-Weiss** reaction is mentioned before.
2. Another mechanism for $OH^{\cdot}$ production is **Fenton** reaction. H_2O_2 generates $OH^{\cdot}$ by reacting with Fe^{+2} and possibly also with Cu^{+} (90, 99).

   $$\mathbf{Fe^{+2} + H_2O_2 \rightarrow Fe^{+3} + OH^{\cdot} + OH^{-}}$$
   $$\mathbf{Cu^{+} + H_2O_2 \rightarrow Cu^{+3} + OH^{\cdot} + OH^{-}}$$

3. Since one of the leading constituents of living cells is water, exposure of these cells to ionizing radiation, such as X-rays or γ rays induces homolytic fission of water and subsequent formation of the hydrogen radical ($H^{\cdot}$) and $HO^{\cdot}$. Ultra-sonication of aqueous solutions can also form $HO^{\cdot}$ and $H^{\cdot}$ (90, 91).

   $$\mathbf{H_2O \rightarrow H^{\cdot} + HO^{\cdot}}$$

4. The radical production can also be occurred by during freezing, drying and freeze-drying processes (91).
5. $HO^{\cdot}$ can also be produced by UV radiation or heat-induced homolytic fission of H_2O_2 (90, 91, 99).

   $$\mathbf{H_2O_2 \rightarrow 2HO^{\cdot}}$$

6. $HO^{\cdot}$ can also be formed by the biological system itself with the formation of $O_2^{-\cdot}$ and in the presence of H_2O_2 and redox-active Fe (91).

$HO^{\cdot}$ can react with almost all cellular macromolecules, but the basic and most important effects are on lipids, proteins, cytochromes and nucleic acids (DNA and RNA). $HO^{\cdot}$ can incorporate into the aromatic ring of purine and pyrimidine bases on the structure of DNA and RNA, and generate radicals (91, 99).

The reactivity of $HO^{\cdot}$ is so high that when it occurs in the living cell, it immediately reacts with every nearby biological molecule by forming different secondary radicals. For example, it reacts with carbonate ion (CO_3^{-2}) to give carbonate radical ($CO_3^{\cdot -}$), which is a potent oxidizing agent (90, 91, 99).

2.3.2.3.D. Hypochlorous acid (HOCl)

Even though HOCl is not radical, it is among the ROS, due to it is a robust two electron oxidizing species (90). HOCl plays an essential role in killing bacteria (via damaging DNA and ATP synthesis) in phagocytic cells because of its dominant antibacterial property. Activated monocytes, neutrophils, eosinophils and all macrophages produce $O_2^{-\cdot}$. Radical production is crucial for phagocytic cells to kill bacteria. HOCl is generated by combining of chloride ion with H_2O_2 which is formed by the dismutation of $O_2^{-\cdot}$ This reaction usually occurs in phagocytic cells and catalyzed by the myeloperoxidase enzyme (90, 100, 101).

$$\mathbf{H_2O_2 + Cl \rightarrow HOCl + OH^{\cdot}}$$

This can be illustrated by examining patients with chronic granulomatous disease as follows: the Chronic granulomatous disease is a congenital condition. In the presence of this disease NADPH-oxidase system which is an $O_2^{-\cdot}$ producing system in phagocytes does not work. Along with that phagocytes of these patients can take bacteria, but the $O_2^{-\cdot}$ cannot be produced and therefore H_2O_2 and HOCl cannot be generated. As a result, the bacteria does not die. When the phagocyte cell is destroyed, living bacteria are released again, and the patient is exposed to persistent infections (100).

Proteins are another target of HOCl. It can cause side-chain damage, fragmentation, and aggregation of proteins by multiple reactions. It also can able to cross membranes, causing damage to membrane proteins and lipids on its passage. HOCl can also participate in the formation of singlet O_2 (90).

HOCl readily oxidizes thiol groups, amines, thioethers, nucleotides, and hemoproteins (91). Hence GSH, N-acetylcysteine, and mercaptopropionyl glycine are very effective at protecting the human body from against oxidative damage by HOCl (100).

2.3.2.3.E. Singlet oxygen (singlet O_2)

Singlet O_2 is a more reactive species of the molecular oxygen. They have two singlet type, delta, and sigma (90). Sigma type is more reactive and converts to delta types rapidly. That is why there are more delta types in biological systems. Although delta-type is not radical, it may occur in some radical reactions.

Singlet O_2 is produced by the reaction of photosensitization. When a light of a particular wavelength is transmitted on several molecules as acridine orange, methylene blue, Bengal rose, and toluidine blue, flavin (FAD and FMN), chlorophyll a and b, bilirubin, retinal, porphyrin, singlet O_2 occurs. In this way, chlorophyll loses its green color and flavins loses their orange color. Singlet O_2 can lead damage to the mitochondria that has rich haem proteins and protein-containing flavins. Haem protein containing CAT enzyme can be inhibited in the same way as well.

If eye retina is exposed to light continuously and intensely, the retinal pigment present in the retina can produce singlet O_2, causing damage to the eye. Highly unsaturated lipids are present in the retinal pigment, and singlet O_2 can easily attack these lipids. Singlet O_2 causes significant damage by reacting biologically essential proteins and amino acids such as methionine, tryptophan, histidine or cysteine (90, 91).
Singlet O_2 can also be produced from the hypochlorite ion (OCl-) mixture formed by passing chlorine gas through cold alkali solution and H_2O_2 in the laboratory (90).

2.3.2.4. Non- Oxygen Derived Free Radicals

2.3.2.4.A. Nitric Oxide (NO)

NO is known as endothelium-derived relaxation factor (EDRF). NO is occurred by a combination of one atom of nitrogen and one atom of oxygen with a unpaired electron and thus conforms to the definition of radical. This lipophilic free radical gas is synthesized from 1-arginine via NO synthase enzyme in vascular endothelial cells. It passes quickly through the smooth muscle and binds hem Fe of guanylate cyclase enzyme. By this way, this process stimulates the synthesis of cyclic guanosine monophosphate (cGMP) and stimulates vascular relaxation (91). NO can also be generated non-enzymatically during UV exposure of the skin (90).

NO plays a vital role in the regulation of the cardiovascular system. It also plays significant roles in the skin, e.g., in regulating blood flow, hair growth, and wound healing (91).

The radical nature of NO is not enough to describe toxicity. In the case of NO reacts with $O_2^{\cdot-}$, a potent oxidant, peroxynitrite occurs. It is considered that decomposition of this radical can cause the formation of $HO^{\cdot}$ (91).

2.3.2.5. Target Structures for Free Radicals and Damages

2.3.2.5.A. Polyunsaturated Fatty Acids and Lipid peroxidation

Bio-membranes and intracellular organelles (mitochondria, ER) are susceptible to oxidative attacks of $HO^{\cdot}$ due to the presence of unsaturated fatty acids in membrane phospholipids. This situation is considered as the main factor for cellular oxidative damage and is called lipid peroxidation (90, 91).

Lipid peroxidation is composed of three stages: initiation, propagation, and termination. **Initiation stage** begins with the attack of $HO^{\cdot}$ on the fatty acid side chain of membrane phospholipids (LH). Lipid radical is generated at initiation stage (90, 91, 98). Other various Fe-oxygen complexes also can initiate lipid peroxidation such as reaction below (91).

$$\mathbf{LH + HO^{\cdot} \rightarrow L^{\cdot} + H_2O}$$

The resulting carbon-centered lipid radicals undergo molecular regulation to stabilize themselves, and they form more stable conjugated dienes configuration.

$$\mathbf{L^{\cdot} + L^{\cdot} \rightarrow L\text{-}L}$$

These conjugated dienes react with O_2 in aerobic medium to form a peroxyl, or so-called peroxy radical ($LOO^{\cdot}$ or $LO_2^{\cdot}$). $LOO^{\cdot}$ can break hydrogen atoms of neighboring lipid molecule and form a lipid peroxide. In this process, a new lipid radical is formed. This stage is called **propagation** stage. The newly formed lipid radical reacts with oxygen to form another $LOO^{\cdot}$. Hence, lipid peroxidation proceeds in the form of a chain reaction (91, 98).

1. The Role of transition metals and lipid peroxidation

As previously mentioned, Fe^{+2} takes part as a cofactor in the formation of $HO^{\cdot}$ in Haber-Weiss reaction. Besides, in the reaction known as the Fenton reaction, the Fe^{+2} ion forms $HO^{\cdot}$ from H_2O_2. This radical can also initiate the lipid peroxidation reaction. The lipid peroxidation can be prevented by using H_2O_2 removing enzymes CAT or selenium-dependent GSH-Px (Se-GSH-Px), $HO^{\cdot}$ scavengers or Fe chelating agents (90, 91, 98).

Fe plays a second important role in the lipid peroxidation reaction. Lipid peroxides (ROOH) are extremely unstable at physiological temperatures alone, but in the presence

of transition metal complexes such as Fe and copper (Cu^{+2}), the decomposition of these lipid peroxides are extended. Thus, Fe^{+2} or its chelates react with lipid peroxide, just like the reaction with H_2O_2, break O-O bond and generate the alkoxyl radical ($RO^{\cdot}$) (90, 91).

$$\mathbf{ROOH + Fe^{+2} \rightarrow Fe^{+3} + OH^{-} + RO^{\cdot}}$$

2. Enzymatic and non-enzymatic lipid peroxidation

Cyclooxygenase and lipoxygenase enzymes cause lipid peroxidation with their substrates such as in fatty acids. Xanthine-xanthine oxidase system also generates $O_2^{-\cdot}$. This radical can reduce Fe^{+3} to Fe^{+2} and initiate lipid peroxidation. Microsomal fractions in some animal tissues may also initiate lipid peroxidation in the presence of NADPH and Fe^{+3} salts. These reactions can be illustrated as examples of enzymatic lipid peroxidation. Fe^{+2} and Cu^{+2} ions, azo compounds, artificial lipid peroxides such as lipid hydroperoxides, tertiary butyl hydroperoxide, and cumulus hydroperoxide can initiate and stimulate peroxidation reactions without any enzymatic activity. It is called non-enzymatic lipid peroxidation (91).

3. The Consequences of Lipid Peroxidation

Radicals, aldehydes and other lipid peroxidation products cause severe damage to membrane proteins. In general, the occurrence of lipid peroxidation results in the impairment of normal membrane functioning, changes in fluidity, increasing the entry of substances that normally do not penetrate into the cell (such as the Ca^{+2} (Calcium ion)), inactivation of membrane-bound enzymes, inhibiting protein synthesis, inhibiting DNA replication and stopping mitochondrial respiration.

Cell membrane surface receptors can be inhibited during lipid peroxidation. Lipid peroxidation can damage enzymes such as glucose-6-phosphatase, Sodium-potassium ATPase (Na^{+} K^{+} ATPase), and ER Ca^{+} ATPase. Damage to the Ca^{+} ATPase enzyme causes deterioration of myocardium contractility and relaxation. In damage of voltage regulated K^{+} channels that responsible for generating electrical activity in nerve tissues and heart, can cause irregularities in heartbeat and death of neurons.

Continued oxidation of fatty acid side chains and producing aldehydes can cause loss of membrane integrity completely. Notably, the breakdown of lysosomal membranes causes the hydrolytic enzymes to drain into the rest of the cell and increase the damage of the cell.

It is known that peroxidation of liver or erythrocyte membranes leads to the formation of high molecular mass protein aggregates on the membrane. As a result, hepatocyte membrane permeability is increased, hepatocytes are dying, and erythrocytes are disintegrating. Furthermore, lipid peroxidation of the erythrocyte membrane results in the losing of their shape modification and compressing ability when erythrocyte passes through the small capillaries, resulting in hemolysis. It has been suggested that the loss of viability of mammal sperm by prolonged incubation at 37 °C is due to accumulation of peroxidation products.

Lipid peroxidation in foods can change their taste, the texture of food proteins and destroy their structure (90, 91, 98, 102).

4. By-products of Lipid Peroxidation

Isoprostanes: Group of prostaglandin-like compounds known as isoprostanes is produced in humans by a non-cyclooxygenase free-radical catalyzed mechanism involving peroxidation of arachidonic acid that is one of the fatty acids (102).

Decomposition products from lipid peroxides: Decomposition of lipid peroxides increased by Fe and Cu^{+2} ions or by heating generates a wide variety of toxic products including **epoxides**, **saturated aldehydes**, **unsaturated aldehydes**, **ketones**, and **hydrocarbons** such as **ethane** and **pentane. Es**pecially, toxic aldehydes as **MDA** (formed from peroxidation of linolenic, arachidonic, or docosahexaenoic acids), and **4-hydroxynonenal** (formed from linolenic and arachidonic acid peroxides) are produced. All of these products can bind membrane proteins, inactivating enzymes and receptors. They can also attack DNA, forming mutagenic lesions (103). Products of free radical damage are shown in Figure 4.

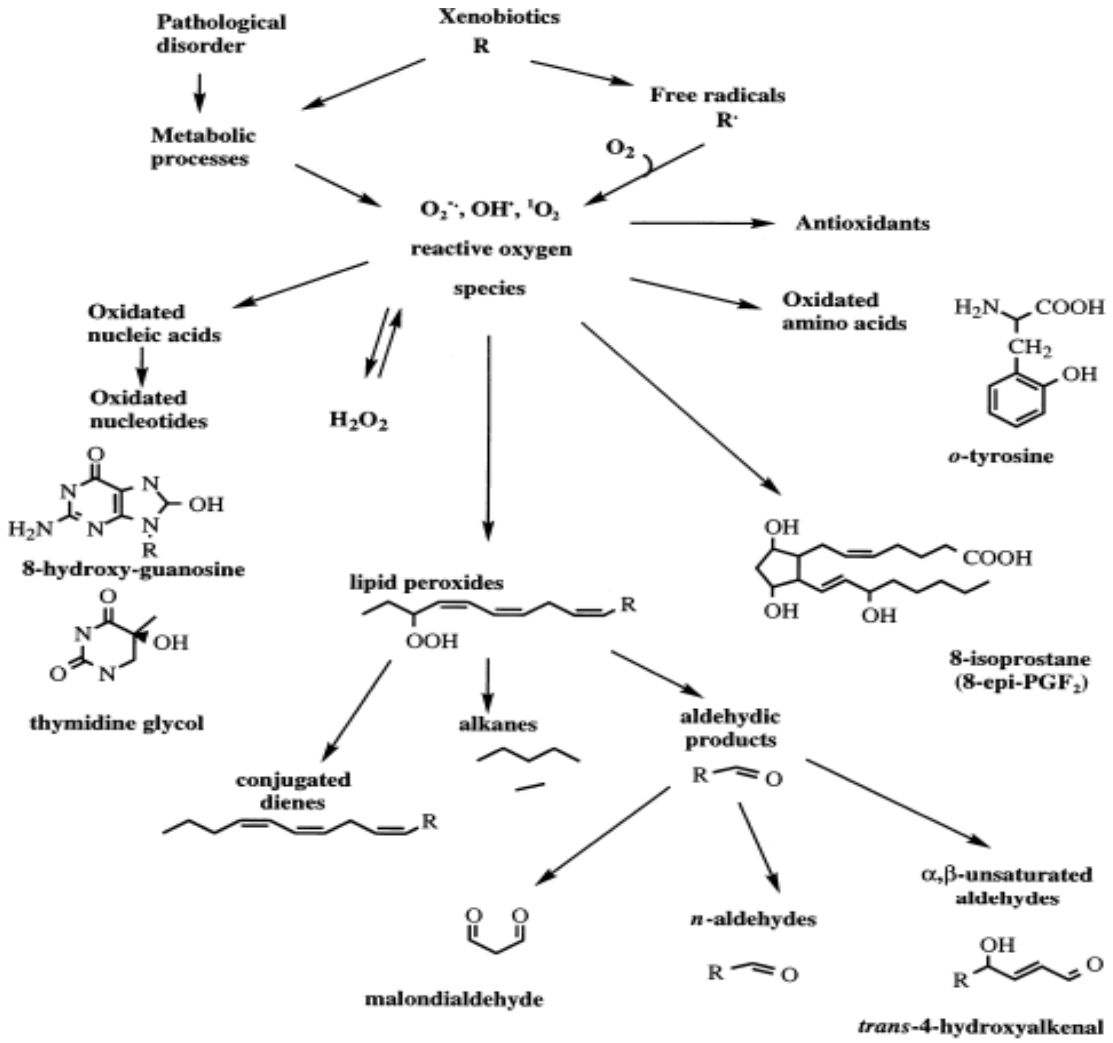

Figure 4: Products of free radical damage (102)

2.3.2.5.B. Protein and Protein Oxidation

Proteins are considered to be possible targets for free radicals ($HO^{\cdot}$, $RO^{\cdot}$ and nitrogen-reactive radicals) since they are the central member of the cell membrane (94). Secondary products derived from lipid oxidation can interact with the amino acid residues of proteins. This reaction can cause the formation of cross-linkage between proteins (104). Also, various damage such as aggregation fragmentation, fracture, modification of thiol groups can be encountered at the result of protein oxidation. Therefore lipid oxidation can be considered as a promoter of protein oxidation (91, 104). The lipid peroxidation products as alkoxy, peroxy, and other carbon-centered radicals can attack the tryptophan and cysteine part of proteins while aldehydes can react with -SH groups in proteins. Damaged protein structure causes changing ion transport, increasing Ca entry

into the cell, and changing enzyme activities (91). The primary marker for oxidative damage of protein is 3-nitrotyrosine (94).

2.3.2.5.C. DNA Damage

Oxidative damage caused by free radical in DNA leads to the development of cancer, aging, cardiovascular diseases, autoimmune disease and degenerative diseases, both by a direct effect on DNA (single and double chain fractures, damage of purine, pyrimidine bases or deoxysugar) or by modulating signal transduction, cell proliferation (105-107).

Among the free radicals, $HO^{\cdot}$ react with DNA by participation to double bonds of DNA bases and by separation of an H atom from the methyl group of thymine and each of C-H bonds of deoxyribose (106). It can also participate to guanine at the position of 4, 5 or 8 in purine ring. Participation to C-8 produces an OH-adduct radical that is oxidized to 8-hydroxy-2-guanosine (8-OHdG). This product is most commonly encountered biomarkers of oxidative stress-related DNA damage, and its mutagenesis is best known (105-107). Moreover, transition metals like Fe can show high-binding tendency to DNA thus ensuring repeated attack upon the DNA by mediated $HO^{\cdot}$ (94). Target structures for ROS are shown in Figure 5.

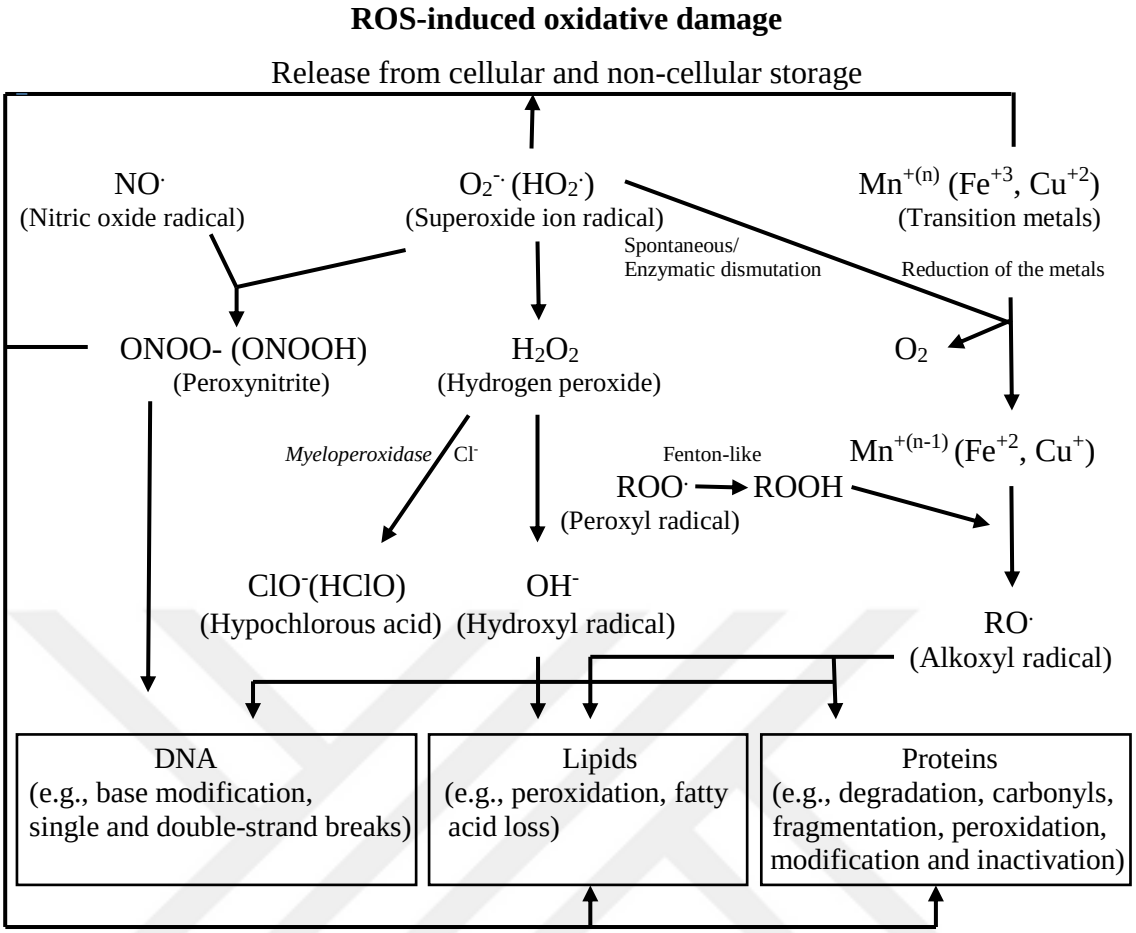

Figure 5: Target structures for ROS (94)

2.3.2.6. Free Radicals and Related Disease

Free radicals play a role in many pathologic events. Free radicals have been reported to be responsible for atherosclerosis, brain disorders as Alzheimer's disease, Parkinson's disease, Down's syndrome and multiple sclerosis, chronic granulomatous disease, diabetes mellitus, inflammatory diseases as asthma and rheumatoid arthritis, lung disorder as asbestosis and adult respiratory distress syndrome, radiation injury, post-ischemic reperfusion injury, hemolytic diseases as thalassaemia, skin disorders. Over and above, they are responsible for cancer and toxicity of various chemical substances such as cigarette smoke, alcohol paracetamol, paraquat, carbon tetrachloride, bleomycin, phenylhydrazine, adriamycin, Fe, and Cu (102). Oxidative stress biomarkers can be useful to estimate the effect of oxidative stress-related chronic diseases (11). Biological markers of oxidatively damaged macromolecules are shown in Figure 6.

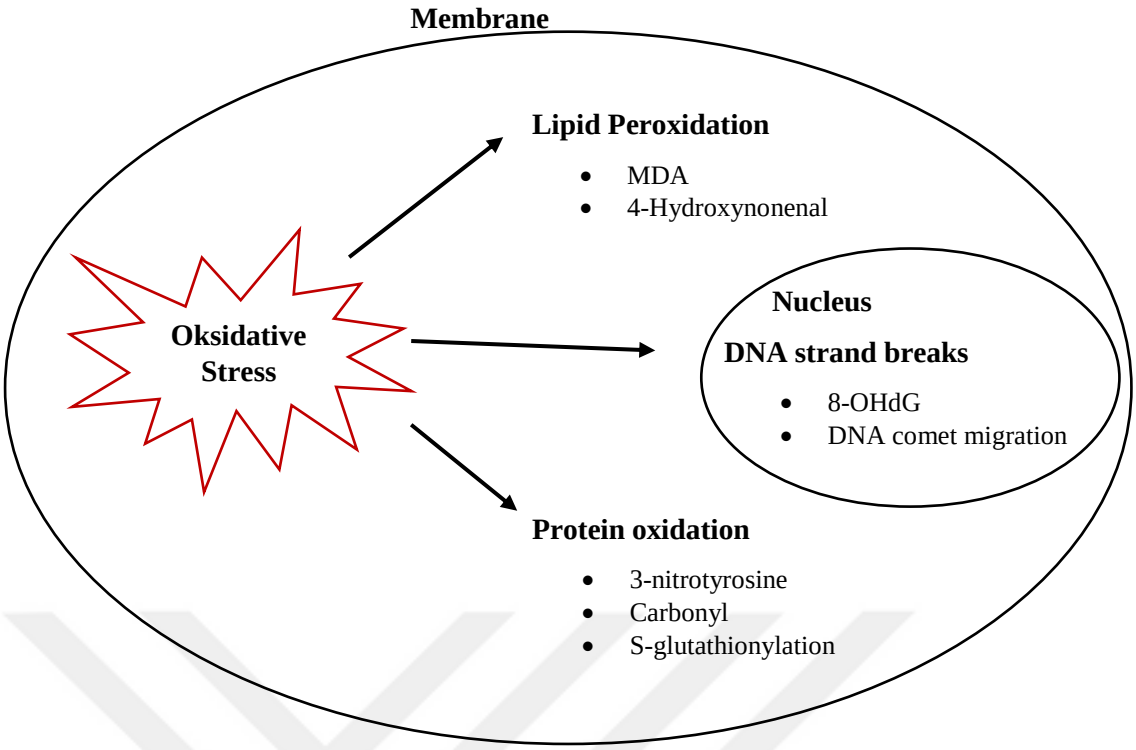

Figure 6: Biological markers of oxidatively damaged macromolecules (11)

2.3.2.7. Antioxidant Defense Systems Against Free Radicals

The antioxidant protection system components in biological systems are shown in Table 4 (91).

Table 4: Enzymatic and non-enzymatic antioxidant defense systems against free radicals (91)

Enzymatic Antioxidant Defense Systems	Non-Enzymatic Antioxidant Defense Systems
The Primary İmportant Ones	GSH
SOD	Vitamin C
CAT	Vitamin E
GSH-Px	Carotenoids
Glutathione S-Transferases (GST)	Uric acid
	Deferoxamine
Related Ones	Melatonin
Glutathione Reductase	Sistein
NADPH-Quinone oxidoreductase	Albumin
Epoxide hydrolase	Ceruloplasmin
UDP-glucuronyl transferase	Heptoglobulins
Sulfonyl transferase	Transferrin and Lactoferrin
Glucose-6-phosphate dehydrogenase	Ferritin
6-phosphogluconate dehydrogenase	Flavonoids
Isocitrate dehydrogenase	Butylated hydroxyanisole
GSSG and conjugate carriers	Butylated hydroxytoluene
	Ebselen
	Lycopene
	Metallothionein
	Bilirubin
	Ubiquinone
	Mannitol
	Oxypurinol
	Probucol

2.3.2.7.A. Enzymatic Antioxidant Defense Systems

1. Superoxide Dismutase (SOD)

a. Copper-Zinc SOD (CuZnSOD)

In 1938 T. Mann and D. Keilin, isolated a blue-green protein containing Cu from cattle blood and named it **hemocourethrin**. In 1953, a similar protein was isolated from the horse liver and was named **hepatocourethrin**. This type of protein was then isolated from the brain and was called **cerebocuprein**. In 1970 it was determined that this protein in erythrocyte contain Cu as well as zinc. Initially, no enzymatic activity of this protein has been detected, and it has been suggested that it serve as metal depots. Then Joe M. McCord and I. Fridovich have demonstrated that this protein catalytically degrades $O_2^{-\cdot}$ in erythrocyte and exhibits SOD activity.

CuZn SOD enzymes are very stable and can be easily isolated from erythrocytes. This enzyme is lysed by erythrocytes. The hemoglobin is removed by the addition of chloroform and ethanol. The enzyme passes through the organic phase. Cold acetone application precipitates this enzyme. It is further purified by ion exchange chromatography. Many enzymes cannot withstand these procedures. These enzymes are also highly resistant to heat.

The CuZn SOD is a key enzyme in the detoxification of $O_2^{-\cdot}$ and converts $O_2^{-\cdot}$ to H_2O_2 (dismutation reaction of $O_2^{-\cdot}$)(108).

$$\mathbf{2O_2^{-\cdot} + 2H^+ \rightarrow H_2O_2 + O_2}$$

This reaction causes a generation of H_2O_2. Generated H_2O_2 is scavenged by the enzyme, which will be explained later (91).

CuZn SOD enzyme prevent the generation of $HO^{\cdot}$ by scavenging $O_2^{-\cdot}$ and inhibiting it to react with H_2O_2 in the presence of metal ions (109).

Cyanide is potently CuZn SOD enzyme inhibitor. These enzymes are also inactivated by long-term incubation by a compound called diethyldithiocarbamate. The compound diethyldithiocarbamate combines with Cu in the active area of this enzyme, removing it from its place (90).

The majority of CuZn SOD enzymes are found in the cell cytosol, as well as in the lysosomes, the inner and outer membranes of the mitochondrion, and the nucleus (110).

b. Manganese SOD (MnSOD)

MnSOD isolated from Escherichia coli (E. coli) is completely different from CuZn SOD. It has been demonstrated that this enzyme is pink in place of blue-green, is not inhibited by cyanide or diethyldithiocarbamate. The molecular mass of MnSOD is not same CuZn SOD and is not resistant to chloroform-ethanol and contains Mn^{+3} in the active site. Although it appears different in many respects to CuZn SOD, MnSOD like CuZn SOD also catalyzes the dismutation reaction of $O_2^{-\cdot}$ (90, 91, 110).

c. Iron SOD (FeSOD)

Another enzyme purified from E. coli is FeSOD. Fe SOD usually contains two protein subunits. There are one or two Fe ions in each protein molecule (90, 111). During the enzyme is in a normal state, the Fe is found as Fe^{+3}. Fe SOD also carries out the same 37 dismutation reaction but is slightly slower than other types of SODs. This enzyme cannot be inhibited by cyanide and display decreased catalytic activity at raised pH, like MnSODs (91, 110). Although Fe SOD has not been found in any animal tissue yet, it has been detected in some bacteria and some high plants such as tomato (91), mustard leaf and Ginkgo biloba (112).

d. Nickel SODs (NiSOD)

It has been reported that several aerobic Streptomyces species and Actinomycetes and Micromonospora species contain a new type of SODs with nickel-containing at the active site. They have a different structure and amino acid sequence from the other SOD types (113).

2. Catalase

The pure and crystalline CAT from beef liver has been isolated by J.B. Sumner and A.L. Dounce in 1937 (114). This enzyme takes shape with four protein subunits. There are a Fe^{+3}-protoporphyrin at its active portion and NADPH of each of these subunits. NADPH stabilizes CAT for the protection it from its substrate, H_2O_2 (115). Storage, freeze drying or acid and alkaline exposure lead to separation of subunits and loss of enzyme activity. CAT enzyme is present in almost all aerobic organisms except some bacteria and blue-green algae. It is found in all body organs in animals but mainly concentrated in the liver and red blood cells. It is found in small quantities in the brain, heart and skeleton muscles (90). It is used to prevent the cells from the harmful effects of

H_2O_2 (115) and $HO^{\cdot}$ production related from H_2O_2 (91). The reaction mechanism of catalysis occurs in the case of the below reaction (91).

CAT-Fe(III) + $H_2O_2^-$ → compound I + H2O

compound I + $H_2O_2^-$ → CAT - Fe(III) + H_2O +O_2

Net $2H_2O_2^-$ → $2H_2O + O_2$ (90)

The exact structure of "compound I" has not yet been known the CAT also carries out some peroxidase type reactions in the presence of H_2O_2. Thus alcohols such as methanol and ethanol are oxidized to formaldehyde and acetaldehyde by compound I (90). It has been suggested that CAT oxidize NO^{-2} to NO^{-3} in vitro as well as to oxidize elemental mercury to $mercury^{+2}$ (91).

CAT activity is non-specifically inhibited by azide, cyanide, peroxynitrite, and HOCl that also inhibits many other enzymes. The most commonly used inhibitor is aminotriazole (90). The CAT is not a circulating enzyme, and its plasma half-life is expressed in minutes only (116).

3. Selenium-dependent Glutathione Peroxidase (SeGSH-Px)

In 1957 Gordon C. Mills was first explored in animal tissues (117). It is present in some algae and fungi (91), rarely found in high plants and bacteria (90). The substrate of this enzyme is GSH, a small molecular weight thiol compound (91). Se-GSH-Px catalyzes the oxidation of GSH to GSSG in the presence of excess H_2O_2, while H_2O_2 is also detoxified by conversion to water (118), as shown below:

H_2O_2 + 2GSH $\xrightarrow{GPx}$ GSSG + $2H_2O$

This enzyme is found in large quantity in liver (119) although it shows moderate activity in heart and lung, low activity in the muscle (91). It is a tetrameric enzyme composed of four protein subunits, and each unit contains an element Se, at its active site. Molecular weight is about 85 000. It is mainly present in cytosol and mitochondria. GSH reduces Se part in the enzyme, and the reduced enzyme reacts with H_2O_2. While the selenolate form ($E\text{-}Se^-$) of Se-GSH-Px reduces the peroxide substrate (ROOH) to alcohol, it converts to oxyacid (E-Se-OH). GSH participates in the reaction in this cycle to form selenosulfide (E-Se-S-G). By binding the second GSH to the selenosulfide, the GSH is

oxidized to GSSG while the enzyme converts in the form of the active form, selenolate (90, 91).

Inhibitory agents of Se-GSH-Px are mercaptosuccinate and other thiols, such as mercaptopropionylglycine (90).

There are also other peroxidases as Cytochrome c peroxidase (120), NADH peroxidase (121), myeloperoxidase (122), that reduce H_2O_2.

4. Glutathione Reductase

GR enzyme is FAD-containing flavor protein that obtained from E. coli (123, 124). This enzyme often contains two protein subunits. The FAD is found in its active site of each subunit (90). As explained before, during the reduction of H_2O_2 or other lipid peroxides by GSH-Px, GSH was transformed into GSSG (oxidized GSH). However, GSH amount of the organism is limited, and it must be used again in reactions. For this reason, GSSG is reconverted to GSH by GR enzyme that carries out this reduction in the presence of NADPH (125). The reaction mechanism operates in the following way. During NADPH reduces FAD, $NADP^+$ occurs, and the electrons are transferred to the disulfide (-S-S-) bridge in GSSG. Disulfide bond break and convert GSSG to GSH (90). The rate changes of NADPH/ NADP+ and GSH/ GSSG reflect the balance between oxidative stress and antioxidant defense systems (124).

GR can be inhibited by some chemicals as arsenic (126), antimalarial drugs (127), N,N-bis(2-chloroethyl)-N-nitrosourea,2-Acetylamino-3-[4-(2-acetylamino-2-carboxyethylsulphanylthiocarbonylamino) phenylthiocarbamoylsulphanyl] propionic acid) (2-AAPA) (90).

5. Glutathione S-Transferases (GST)

GSTs with many new isozymes are a family of enzymes composed of two protein subunits and were first discovered in 1961. GSTs, display anti-oxidant activity against lipid peroxides by showing Se-independent GSH-Px activity. It links covalently to some strong alkylating agents such as azo-type carcinogens to protect cellular proteins and macromolecules from the toxic effects of these agents. GSTs act in the intracellular transport system as well as the antioxidant effect (91).

2.3.2.7.B. Non-enzymatic Antioxidant Defense Systems

1. Glutathione (GSH)

GSH, γ -glutamylcysteinyl glycine, is an atypical tripeptide. The reason for the atypical presentation is that glutamyl binds to the cysteine at the gamma position (91). It presents in most plants, microorganisms, and all mammalian tissues. GSH is found in two forms, the thiol-reduced (GSH) and disulfideoxidized (GSSG) forms. The ratio of these can vary depending on their location in the cell. Eukaryotic cells have three major reservoir areas of GSH. Approximately 90% of cellular GSH is in the cytosol, 10% in the mitochondria and a small quantity of it in the ER. It has been mentioned that GSH is a substrate of GSH-Px, which is H_2O_2-cleaving enzymes, and dehydroascorbate reductase enzymes. For this reason, it does not react directly with H_2O_2.

GSH serves many vital functions such as being the cofactor of the enzymes in the metabolic pathways, hormone synthesis, detoxifying xenobiotics, preventing the oxidation of –SH groups, scavenging free radicals including H_2O_2, $HO^{\cdot}$, HOCl and singlet O_2.

Detoxification of xenobiotics is one of the leading function of GSH. Some xenobiotics such as chloroform, bromobenzene, naphthalene and paracetamol form conjugates with GSH. Afterwards, these conjugates are metabolized to mercapturic acid and excreted in urine.

As stated above in the case of excess H_2O_2 in the environment, it is reduced by GSH in the presence of GSH-PX. As a result, GSH is oxidized to GSSG. Accumulating GSSG inhibits some enzymes activity as well as protein synthesis. Glutathione reductase reconverts GSSG to GSH, consuming NADPH during this process (91, 128, 129). GSH-GSSH conversion mechanism is shown in Figure 7.

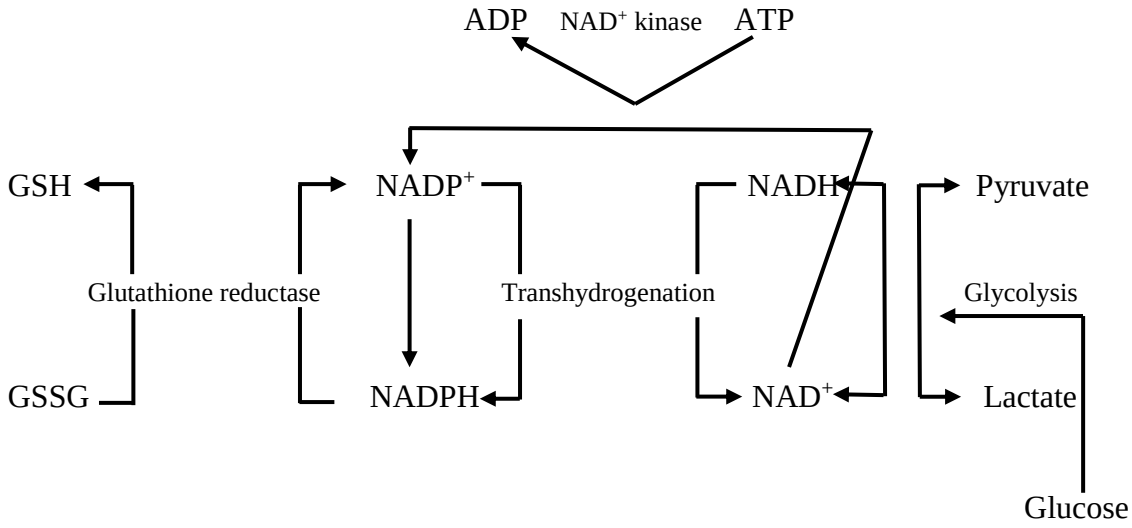

Figure 7: GSH-GSSH conversion mechanism (8)

GSH is also capable of chelating and detoxifying metals. The increase in cytoplasmic Cu can stimulate H_2O_2 production which could lead to forming $HO^{\cdot}$. GSH prevents this formation because of its chelation property (130).

2. Vitamin C (Ascorbic acid)

Ascorbic acid is water-soluble vitamins and mainly found in fruits and vegetables. Humans do not have the gulonolactone enzymes necessary for the synthesis of ascorbic acid so that they can obtain it only exogenously. It is present in the form of ascorbate (known as the salt of ascorbic acid) in most tissues and plasma. Ascorbic acid deficiency may lead to scurvy disease which can have fatal consequences.

Ascorbic acid acts as a cofactor for eight different enzymes in human. Three of them participate in collagen hydroxylation reactions. Other two ascorbic acid-dependent enzymes are required for the synthesis of carnitine that is necessary for the transport of fatty acid into mitochondria for ATP generation. The remaining three ascorbic acid-dependent enzymes have the following functions: one participates in the norepinephrine biosynthesis from dopamine, one adds amide groups to peptide hormones, and one takes part in tyrosine metabolism.

Ascorbic acid is a potent reducing agent because of its electron donor property. The species formed after the loss of one electron are semidehydroascorbic acid or ascorbyl radical. This radical is relatively stable with a half-life of 10^{-5} seconds and is

also unreactive. In the case of the reaction of a possibly harmful free radical with ascorbic acid, the reactive free radical is reduced, and the ascorbyl radical formed in its place is less reactive (131).

Ascorbic acid reacts easily with $O_2^{-\cdot}$ and $HO^{\cdot}$ and scavenges them due to its intense antioxidant activity (91). Furthermore, the tocopheroxyl radical can be reduced to alpha-tocopherol by ascorbate. By inhibiting radical formation, ascorbate can prevent protein or amino acid oxidation (131).

Another feature of ascorbic acid is that it has an oxidative effect besides antioxidant effect. Because it is the only cellular agent (except $O_2^{-\cdot}$) that reduces Fe^{+3} to Fe^{+2}. In this way, the ascorbate prepares a suitable environment for the Fenton reaction. That is, it contributes to $O_2^{-\cdot}$ production. Because of this property, vitamin C is considered to be a strong catalyst or a pro-oxidant of free radical reactions. In addition, the oxidation of ascorbic acid can also directly lead to H_2O_2. However, it has been noted that this type of effect is only seen at low concentrations (less than 0.2 mM) and it shows a strong antioxidant effect at higher concentrations (91, 131).

3. Vitamin E

Vitamin E, a necessary dietary factor, is composed a mixture of four tocopherols called α, β, γ and δ. The natural distribution of α-tocopherol is the largest, and its biological activity is the greatest. It is also a very potent antioxidant. It protects polyunsaturated fatty acids found in cell membrane phospholipids from free radical effects and lipid peroxidation. Vitamin E scavenges $O_2^{-\cdot}$, $HO^{\cdot}$, singlet O_2, lipid peroxy radicals ($LOO^{\cdot}$) and other radicals. GSH-Px and vitamin E can show complementary to each other's antioxidant effect. GSH-Px destroys peroxides that are already formed while vitamin E inhibits peroxides synthesis.

Vitamin E breaks lipid peroxidation chain reactions by breaking down lipid $LOO^{\cdot}$.

$LOO^{\cdot}$ + α-Tocopherol → LOOH + α-Tocopherol-$O^{\cdot}$

The resulting tocopheryl radical (α-tocopherol-$O^{\cdot}$) is stable and does not have sufficiently reactive to initiate lipid peroxidation on its own. In this way, the chain reaction of lipid peroxidation is terminated. For this reason, vitamin E is known as a

chain-breaking antioxidant. This property explains why vitamin E may be a preferred antioxidant.

Vitamin E can be re-reduced by ascorbic acid and GSH after it has been oxidized.

VitE-O + Ascorbic acid → VitE-OH + Dehydroascorbic acid

$2VitE\text{-}O^{\cdot} + 2GSH \rightarrow 2VitE\text{-}OH + GSSG$

Vitamin E plays an essential role in Se^{+2} metabolism. Se^{+2} is necessary for the absorption of vitamin E and lipids. It also helps keep vitamin E in lipoproteins. Vitamin E also reduces the need for Se^{+2} by preventing the loss of Se^{+2} from the organism and keeping it active (90, 91).

4. Carotenoids

β-carotene, the metabolic precursor of vitamin A, is a component of the chloroplast membrane in plants. It is an extremely powerful singlet O_2 cleaner and can also prevent the lipid peroxidation chain reaction by directly reacting with $HO^{\cdot}$, peroxy, and alkoxy radicals. Therefore carotenoids act as antioxidants in the body (91).

5. Uric acid

Uric acid is the final product of the purine catabolism catalyzed by xanthine oxidase or xanthine dehydrogenase. Uric acid is converted to its more polar metabolites by urate oxidase but humans' tissue does not contain this enzyme. Thus uric acid accumulates in plasma (90).

Uric acid is a potent scavenger of singlet O_2, $LOO^{\cdot}$, $HO^{\cdot}$, ozone and HOCl, and is regarded as an in vivo antioxidant. It is reported that lipid peroxidation is inhibited by uric acid (132).

Uric acid is also capable of chelating metal ions. It can bind Fe and Cu^{+2} ions and stimulate low-density lipoprotein oxidation (90).

6. Deferoxamine

This chelator agent is mainly specific for Fe, but it can also bind other metals in minimal quantities. It inhibits $HO^{\cdot}$ formation from Fe-dependent H_2O_2. Besides, it increases the oxidation of Fe^{+2} solutions and combines with the resulting Fe^{+3}.

It is also a good cleanser for $LOO^{\cdot}$. In many systems, deferoxamine prevents lipid peroxidation (91).

7. Melatonin

Melatonin is generated by the pineal gland at the base of the brain. It has crucial roles in regulating the circadian rhythm (90).

Melatonin is a powerful antioxidant that cleans $HO^{\cdot}$. It is considered the strongest of antioxidants. After melatonin reacts with $HO^{\cdot}$, it transforms into an indolylcation radical which shows antioxidant activity by keeping the $O_2^{-\cdot}$ in the environment (91). Due to its lipophilic nature, it enters a wide range of compartments including the blood-brain barrier and protects the brain against oxidative damage (133). As melatonin declines with aging, it has been reported that it may play a prime role in the pathogenesis of some diseases related to aging and aging (91).

Melatonin is also useful as scavengers of other radicals (133, 134). Furthermore, it has been demonstrated that melatonin increases activities of GSH-Px and SOD in the brain (134).

8. Sistein

Cysteine is known to be $O_2^{-\cdot}$ and $HO^{\cdot}$ cleanser (91).

9. Albumin

Albumin is multifunctional plasma protein that participates in many significant physiological and pharmacological reactions. Its main functions are to maintain of osmotic pressure and transport bile pigments, cholesterol, fatty acids, and metals. Albumin binds Cu^{+2} ions tightly and inhibits Cu-dependent lipid peroxidation and $HO^{\cdot}$ formation. It is also an active HOCl cleanser (135, 136).

10. Ceruloplasmin

The Cu-containing protein, ceruloplasmin, oxidizes Fe^{+2} to Fe^{+}. This reaction results in the inhibition of Fenton reaction and further formation of free radicals. It also inhibits Fe and Cu-dependent lipid peroxidation. An essential part of the antioxidant activity of human plasma is due to ceruloplasmin (91, 137).

11. Heptoglobulins

Hemoglobin can stimulate lipid peroxidation either by providing Fe in the decomposition or by directly interacting with peroxides. The isolated hem protein is a potent peroxidation stimulator. Plasma includes hemoglobin-binding protein, haptoglobin, and heme-binding protein, hemopexin. These binding reactions prevent Fe-induced lipid peroxidation (91).

12. Transferrin and Lactoferrin

Transferrin is present notably in serum but is also found at lower concentrations in other body fluids. Its fundamental function is the transport of Fe to proliferating cells, and it is also an important growth factor.

Transferrin acts as an antioxidant by reducing the concentration of free ferrous ion that takes part in Fenton reaction (137). The protein lactoferrin released from phagocytic cells also has antioxidant effect by the same mechanism (91).

13. Ferritin

Ferritin binds to Fe and avoids it from participating in free radical reactions (91).

2.3.2.8. Assays Used to Measure Oxidative Damage

There are various analyses related to the evaluation of oxidative damage. Briefly shown in Table 5 (94, 138).

Table 5: Assays used to measure oxidative damage (94, 138)

	Types of Analysis
1. Measurement of radicals (direct approach)	Electron Paramagnetıc Resonance Spectroscopy
2. Measurement of oxidative damage biomarkers	a. Determination of lipid peroxidation products MDA Aldehydes b. Determination of protein damage (e.g., carbonyls, peroxidation) c. Determination of DNA damage 8-OHdG d. Immunohistochemically markers
3. Antioxidant defense system measurement	a. Evaluation of antioxidant enzymes SOD GSH-Px CAT GST GR b. Determination of total antioxidant activity c. Measurement of low molecular weight antioxidants (e.g., HPLC (High liquid pressure chromatography), GC-MS (Gas Chromatography-Mass Spectrometer)) Alpha-tocopherol Ascorbic acid GSH Melatonin
4. Measurement of enzyme cofactors	Cu, Zn, Mn, Se, and Fe elements

3. MATERIALS and METHODS

3.1. Materials

3.1.1. Chemicals

1,1,3,3-Tetramethoxypropane	Sigma Aldrich; USA
2-Thiobarbituric acid (TBA)	Sigma Aldrich; USA
3-(Cyclohexylamino)-1-propanesulfonic acid (CAPS)	Sigma Aldrich; USA
Acetic acid	Riedel de Haen; Germany
Bovine serum albumin (BSA)	Sigma Aldrich; USA
CAT from bovine liver	Sigma Aldrich; USA
Copper sulfate ($CuSO_4$)	Riedel-de Haen; Germany
Ethylenediaminetetra acetic acid disodium dehydrate (EDTA)	Sigma Aldrich; USA
Folin-Ciocalteu's phenol Reagent	Sigma Aldrich; USA
Glutathione reductase from baker's yeast	Sigma Aldrich; USA
H_2O_2 (30% solution)	Riedel de Haen; Germany
Iodonitrotetrazolium chloride (INT)	Sigma Aldrich; USA
Isoflurane	Piramal; ABD
Isotonic saline	
Cry activator KL001	Sigma Aldrich; USA
L-Glutathione (reduced)	Sigma Aldrich; USA
NADPH	Sigma Aldrich; USA
Phosphoric acid	Merck; Darmstadt, Germany
Potassium chloride (KCl)	Riedel- de Haen; Germany
Potassium phosphate monobasic (KH_2PO_4)	Sigma Aldrich; Germany
Sodium azide	Sigma Aldrich; USA
Sodium carbonate anhydrous (Na_2CO_3)	Riedel-de Haen; Germany
Sodium dodecyl sulfate (SDS)	Merck; Germany
Sodium hydroxide (NaOH)	Riedel-de Haen; Germany
Sodium phosphate dibasic dihydrate ($Na_2HPO_4.2H_2O$)	Merck; Germany
Sodium potassium tartrate	Sigma Aldrich; USA

Tert-Butyl hydroperoxide	Sigma Aldrich; USA
Trichloroacetic acid (TCA)	Riedel-de Haen; Germany
Trizma base	Sigma Aldrich; USA
Xanthine oxidase from bovine milk	Sigma Aldrich; USA
Xanthine sodium	Sigma Aldrich; USA

3.1.2. Equipment

Centrifuge	Sigma 3-16 PK, UK & Ireland
Hairdryer	Arçelik, Turkey
Heparin tube	
Incubator	Heidolph Unimax 1010, Germany
Micropipette (100-1000 mL)	Isolab
Micropipette (500-5000 mL)	Rainin
Micropipette (20-200 mL)	Transferpipette
Microplate Reader	Thermo Labsystems Multiskan Ascent, Finland
Microplate Washer	Bio-Tek ELx50 ,USA
pH meter	Mettler-Toledo MP220, US
Polypropylene tubes	
Refrigerator	Arçelik, Turkey
Spectrophotometer	Thermo Scientific, Evolution 300, USA
Ultrasonic bath	Sonorex RK156BH, Germany
Volumetric flasks (10, 25, 50, 100, 200, 500, 1000 mL)	
Vortex	Heidolph Reax, Germany
Water bath	GFL, Germany

3.2. Methods

3.2.1. Experimental Protocol on Animals

The experimental protocol has been approved by the Animal Ethics Committee of Yeditepe University. A total of 24 male albino mice were obtained from Experimental Research Center of Yeditepe University (YUDETAM). The animals were eight weeks

old and weighted approximately 25-30 gram. All mice received food and water ad libitum during the entire experiment. The animals were housed at 21±1ºC with a 12:12 h light-dark cycle in Plexiglas cages. They were allowed to acclimate to the facilities for one week.

3.2.2. Experimental Design

Twenty-four animals were randomly divided into 4 groups of 6 animals each as a control, KL001, isoflurane and in combination KL001 and isoflurane group. The dosage of KL001 was determined as 100 mg/kg. The exposure time of isoflurane was determined as 4h. The animals were administered as single dose intra-peritoneal 0,4 mL olive oil (control group), intra-peritoneal 100 mg/kg KL001 (group 2), inh isoflurane for 4 h. (group 3), in combination intra-peritoneal 100 mg/kg KL001 and inh isoflurane for 4 h (group 4). The day and night cycle of rats is exactly the opposite of human. For this reason, KL001 was applied at night. During the experimental period, the animals were observed for clinical signs and symptoms of toxicity and mortality.

3.2.3. Preparation of Blood Samples and Tissue Collection

At the end of the experimental period, animals were euthanized by decapitation and blood samples were collected immediately into tubes containing heparin/lithium and centrifuged for 10 min at 4500 rpm at 4°C. After separation of plasma, the packed cells were washed three times with two volumes of isotonic saline. Then, erythrocyte samples were lysed with cold distilled water (1:4), stored at 4°C for 15 min and the cell debris was removed by centrifugation (3200 rpm at 4 °C for 10 min). Plasma and erythrocyte samples were stored at -80°C (139).

The brain, liver, and kidney organs were dissected, weighed and frozen on dry ice. All samples were stored at -80 °C until analysis.

3.2.4. Preparation of Tissue Homogenates

Tissue samples (1g) were homogenized in 9 mL (4 mL for brain tissue) of cold 1.15% KCl solution. During homogenization, the tissue glass Dounce homogenizer was held in a bucket with ice. After homogenization, the tubes with solutions were centrifuged for 10 minutes at 4000 rpm at 4°C. Supernatants were divided into Eppendorf tubes and stored at -80°C until analysis (139). The supernatants were then used to analyze oxidative stress biomarkers (protein content, MDA, SOD, GSH-Px, and CAT).

3.2.5. Determination of Protein Content in Erythrocyte Samples and Tissue Homogenates

Samples protein concentration was measured according to the method described by Lowry et al. (140). This method is a Cu-based assay depending on biuret reaction. In an alkaline environment, the Cu^{+2} ion forms a complex with the peptide bonds in the proteins and is reduced to Cu^{+1}. This complex cause color formation by reducing the Folin reagent. The intensity of the resulting color is directly proportional to the protein concentration and is measured spectrophotometrically at 640 nm. Protein levels are necessary for correction of enzyme levels in tissue.

The Chemicals and Reagents

- Alkaline Cu reagent
    A. Na_2CO_3 (in 0.1 N NaOH) 2%
    B. $CuSO_4$. $5H_2O$ 0.5%
    C. Sodium potassium tartrate 1%

To obtain alkaline Cu reagent, 50 mL A, 1 mL B, and 1 mL C are mixed immediately before use. The mixture is freshly prepared for use within 1 hour.

- Folin-Ciocalteu Reagent 1:2 v/v diluted
- BSA

The Procedure

Standards were prepared with BSA in concentrations of 0.625, 1.25, 2.5, 5, 10 and 20 mg/mL. For measurement, 10 µl of each standard/erythrocyte samples/tissue homogenates, 1000 µL ultrapure water and 4500 µl alkaline Cu reagent were mixed and kept at room temperature for 10 minutes. At the end of this process, 500 µl of diluted Folin's Reagent was added to the mixture and incubated at room temperature for 20 minutes. The absorbance of standards and samples were measured at 640 nm by using UV/VIS spectrophotometer. Results were expressed as mg/mL.

3.2.6. Antioxidant Enzyme Activities in Erythrocyte Samples and Tissue Homogenates

3.2.6.1. Determination of CAT activity

This assay was performed according to the method of Aebi (141) which relies on the conversion of H_2O_2 to H_2O by CAT in a time-dependent manner. The decrease in the absorbance of H_2O_2 is monitored at 240nm.

The Chemicals and Reagents

- Phosphate buffer 50 mM, pH 7.0
- H_2O_2 solution 30 Mm

The Procedure

3 mL phosphate buffer was used as a blank. Before the measurement, 1 mL H_2O_2 solution and 2 mL phosphate buffer in quartz cuvette were measured by using UV/VIS spectrophotometer at 240 nm. The absorbance was arranged 0.500 (± 0.05). Stock solution of CAT was used to prepare different concentration of standard solutions (0.00125, 0.0025, 0.005, 0.01, 0.015, 0.02, 0.025, 0.03, 0.035 kU/mL) with phosphate buffer.

Take 100 µL of each erythrocyte samples/tissue homogenate in an Eppendorf were added 900 µl phosphate buffer. After dilution, 500µL diluted brain homogenates, 200 µl of diluted liver homogenates, 50 µL of diluted kidney homogenates and 50 µL of diluted erythrocyte samples were diluted again to 4950 µL with phosphate buffer. 2 mL of each standard/sample and 1 mL of H_2O_2 solution were added to the quartz cuvette. The absorbance of each standard/sample was measured at 240 nm. The reduction rate of H_2O_2 was monitored at 240 nm for 4 times at every 15 s. CAT activity was expressed in kU/g protein.

3.2.6.2. Determination of SOD activity

SOD activities of erythrocyte samples and tissue homogenates were measured using the method described by Aydın et al. (142). In this method, $O_2^{-\cdot}$ was generated by xanthine/xanthine oxidase. The generated radical reacts with iodonitrotetrazolium (INT), and a violet-colored formazan dye is formed. In the presence of SOD, the colored complex formation is decreased. The absorbance of this color was measured by UV/VIS

spectrophotometer at 505nm. Depending on the CuZn-SOD activity in the medium, this reaction is prevented and the % inhibition is calculated.

The Chemicals and Reagents

- Xanthine sodium 0.05 mM
- INT 0.025 mM
- CAPS 50 mM
- EDTA 0.94 mM
- Xanthine oxidase 80 U/L

The Procedure

The mixture substrate was prepared in a volume of 100 mL containing 0.05 mmol/L xanthine sodium and 0.025 mmol/L INT in a buffer solution containing 50 mmol/L CAPS and 0.094 mmol/L EDTA. The mixture pH was adjusted to 10.2 by adding NaOH pellets.

A stock solution of SOD was diluted to the concentration 1 µg/mL. Diluted SOD was used to prepare different concentration of standard solutions (30, 90, 150, 210, 300, 420 ng/mL). 25 µl deionized water as blank was measured before the start and after every ±10 measurements. The erythrocyte samples were diluted 80 times, and the tissue homogenate was diluted 40 times before the assay. 25 µL diluted erythrocyte samples and tissue homogenates were mixed with 850 µL of substrate mixture. Then 125 µL of xanthine oxidase (80 U/L) was added to the mixture. The absorbance at 0, 0.5, 1, 1.5, 2, 2.5 and 3 minutes was noted at 505 nm using UV/VIS spectrophotometer. SOD activity was expressed in ng/mg protein.

The absorbance change of standards/samples per minute was calculated as:

$$\Delta A/min = \frac{A2-A1}{3}$$

A2-A1 = The difference between 7th and 1st absorbance or 6th and 0th absorbance

The inhibition percentage was calculated as shown below;

$$\text{Inhibition (\%)} = 100 - \frac{\Delta A\text{samp or std/min} \times 100}{\Delta A\text{blank/min}}$$

Log concentration of standard and sample were calculated. The calibration curve was plotted with the percent inhibition of standard solutions against the logarithm of their concentration.

3.2.6.3. Determination of GSH-Px activity

The measurement of GSH-Px activity was performed as described by Pleban et al. (143). Basically, GSH-Px is catalyzed to convert reduced GSH to oxidized glutathione (GSSG) in the presence of *tert-butyl* hydroperoxide. The generated GSSG is re-converted to GSH by glutathione reductase. During this process NADPH is consumed; therefore decrease in the NADPH concentration is expected. This amount of NADH used is monitored at a wavelength of 340 nm with decreasing absorbance.

The Chemicals and Reagents

- The reaction mixture

Trizma base	50 mM, pH 7.6
EDTA	1 mM
GSH	2 mM
NADPH	0.2 mM
Sodium azide	4 mM
Glutathione reductase	1000 U/L

- *tert*-Butyl hydroperoxide 1:1000 diluted

The Procedure

Erythrocyte samples were diluted 7 times, and tissue homogenates were diluted 17 times before the assay. 10 µL of the samples and 990 µL of the reaction mixture were mixed into a 1 mL cuvette and incubated at room temperature for 5 min. The reaction was initiated with the addition of 10 µL of *tert-butyl* hydroperoxide and the decrease in NADPH absorbance was measured at 340 nm as 7 readings for 3 min. The difference in absorbance per minute is calculated. GSH-Px activity was expressed in U/g protein.

3.2.7. Determination of MDA level

This method relies on the reaction of MDA with thiobarbituric acid (TBA). The pink-colored complex formation was measured at 532 nm. Detail of the method for erythrocyte samples is described by Aydın et al. (142) and for tissue homogenates by Jamall and Smit (144).

A) Determination of MDA in erythrocyte samples

The Chemicals and Reagents

- TCA 15%
- Phosphate buffer 330 mM, pH 7.5
- TBA (in 0.05 N NaOH) 1%
- EDTA 0.1 M
- 1,1,3,3-tetramethoxypropane 1:2000 diluted

The Procedure

10µl 1, 1, 3, 3-tetramethoxypropane was completed by 20 mL ultra-pure water. This solution was used as a standard for plotting the calibration curve. The standard solution was prepared in a concentration range of 0.0038-0.076 nmol/mL.

0.5 mL of each of erythrocyte samples, 0.5 mL phosphate buffer and 0.5 mL TCA were mixed and vortexed. The mixtures were kept on ice bags in the refrigerator (4 °C) for 2 h. At the end of the period, the tubes were centrifuged at 4400 rpm and 4 °C for 10 min. 1mL of the supernatant was transferred to another propylene tube and mixed with 75 µL of 0.1 M EDTA and 250 µL of 1% TBA solution. The mixtures were vortexed and kept on boiling water bath for 15 min. 1mL of phosphate buffer was used as a blank instead of the supernatant under the same conditions. The absorbance was recorded at 532 nm. The results were expressed as nmol/g protein.

B) Determination of MDA in tissue homogenates

The Chemicals and Reagents

- SDS 8.1 %
- Acetic acid sol. (pH 3.5) 20 %
- TBA (in 0.05 N NaOH) 0,8%
- 1,1,3,3-tetramethoxypropane 1:2000 diluted

The Procedure

Different concentrations of standard solutions were prepared as mentioned for MDA measurement in erythrocytes.

The reaction mixture (0.2 mL each standard/sample, 0.2 mL dodecyl sulfate sodium salt (8.1%), 1.5 mL of acetic acid (20%), 1.5 mL of TBA (0.8 %) and 600 µL ultra-pure water) were prepared in a closed-cap polypropylene tube and incubated in a water bath (95°C) for 1 hour. During this hour the tubes were mixed carefully every 15 minutes. After 1 hour they were allowed to cool. 2 mL of these solutions were transferred to a new tube and 2 mL of TCA (10%) was added. They were mixed by vortex. The tubes were centrifuged for 10 minutes at 1000g. Lipid peroxidation product, MDA was measured at 532 nm. The results were expressed as nmol/g protein.

3.2.8. Determination of Cry level

Enzyme-Linked Immunosorbent Assay Kit (ELISA kit) was applied for the quantitative determination of Cry concentration in plasma and tissue homogenates. This Elisa kit uses Sandwich-ELISA as the method. The micro ELISA plate provided in this kit has been pre-coated with an antibody specific to Human Cry1. Standards or samples are added to the micro ELISA plate wells and combined with the specific antibody. Then a biotinylated detection antibody specific for Human Cry1 and Avidin-Horseradish Peroxidase (HRP) conjugate are added to each microplate well successively and incubated. Free components are washed away. The substrate solution is added to each well. Only those wells that contain Human Cry1, biotinylated detection antibody and Avidin-HRP conjugate will appear blue in color. The enzyme-substrate reaction is terminated by the addition of stop solution and the color turns yellow. The optical density (OD) is measured spectrophotometrically at a wavelength of 450 nm ± 2nm. The OD value is proportional to the concentration of Human Cry1.

The Kit Components

- Micro ELISA Plate
- Reference Standard
- Concentrated Biotinylated Detection Ab
- Concentrated HRP Conjugate
- Reference Standard and Sample Diluent

- HRP Conjugate Diluent
- Concentrated Wash Buffer
- Substrate Reagent
- Stop Solution

Specification

Sensitivity: 0.19 ng/mL

Detection Range: 0. 31-20 ng/mL

Reagent Preparation

All reagent were brought to room temperature before use. The microplate reader was preheated 15 minutes before OD determination.

Wash buffer: 30 mL concentrated wash buffer was diluted in 750 mL of wash buffer with deionized or distilled water.

Standard working solution: The standard was centrifuged at 10.000 X g for 1 minute. 1 mL of Reference Standard & Sample Diluent was added, waited for 10minutes and inverted several times. After it dissolved fully, it was mixed thoroughly with a pipette. This reconstitution produced a working solution of 20 ng/mL. Then serial dilutions were prepared as follows: 20, 10, 5, 2.5, 1.25, 1.63, 0.31, and 0 ng\mL as blank solution.

Biotinylated Detection Ab working solution: Before the experiment, the required amount was calculated (100 µL/well). The stock tube was centrifuged before use. 100 X Concentrated Biotinylated Detection Ab was diluted to 1 X working solution by Biotinylated Detection Ab Diluent.

Concentrated HRP Conjugate working solution: Before the experiment, the required amount was calculated (100 µL/well). 100 X Concentrated HRP Conjugate was diluted to 1 X working solution by HRP Conjugate Diluent.

The Procedure

Different concentrations of Standard working solution were added to the first two columns: Each concentration of the solution was added to wells side by side (100 µL for each well). The samples were added to other wells (100 µL for each well). The plate was covered with a sealer. It was incubated for 90 minutes at 37 °C. The liquid of each well was removed. Immediately 100 µL Biotinylated Detection Ab working solution was

added to each well. The plate was covered with a sealer. It was mixed and incubated for 1 hour at 37 °C. The solution was aspirated from each well. 350 μL wash buffer was added each well. It was soaked for 1-2 minutes. The solution was aspirated from each well. It was dried against the clean absorbent paper. This wash step was repeated three times. 100 μL HRP Conjugate working solution was added each well. The plate was covered with a sealer. It was incubated for 30 minutes at 37 °C. The solution was aspirated from each well. The wash process was repeated for five times as conducted in the previous step. 90 μL Substrate Reagent was added to each well. The plate was covered with a sealer. It was incubated for 15 minutes at 37 °C. The plate was protected from light. 50 μL Stop Solution was added to each well. The OD of each well was determined at once, using a microplate reader set to 450 nm.

3.2.9. Statistics

The results were expressed as a mean ± standard deviation. Statistical comparisons were made using ordinary one-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons test. Statistically, a significant difference was defined as $p < 0.05$.

4. RESULTS

4.1. Results of Antioxidant Activity Studies

4.1.1. Results on Erythrocyte Samples

4.1.1.1. MDA Levels in Erythrocyte Samples

MDA levels in erythrocyte samples in study groups are given in Figure 8.1 and Figure 8.2. The first graph contained the results obtained by adding all the values we found (n=6). On the other hand, we did not have the opportunity to repeat the experiments, so we excluded the values, which are different from the total group in the second graph (n=5). In the first graph, the MDA level of the KL001 and isoflurane group significantly decreased compared to both control and the KL001 group ($p < 0.05$). Besides, the isoflurane group also showed a significant decrease compared to the KL001 group ($p < 0.05$). In the second graph, there was a significant decrease in the KL001 and isoflurane group comparing with all other groups ($p < 0.05$). MDA level of the isoflurane group was significantly lower than the control group and the KL001 group ($p < 0.05$).

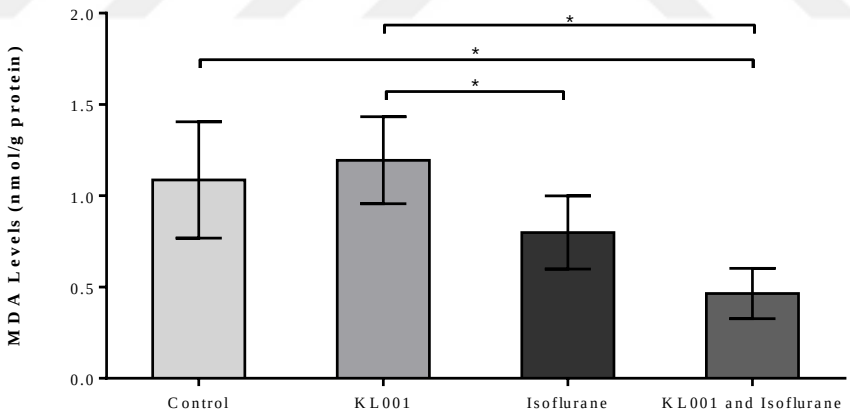

Figure 8.1. MDA levels in erythrocyte samples in study groups.

Results were presented as mean ± standard deviation

MDA levels were expressed as nmol/g protein.

n= 6

* $p < 0.05$

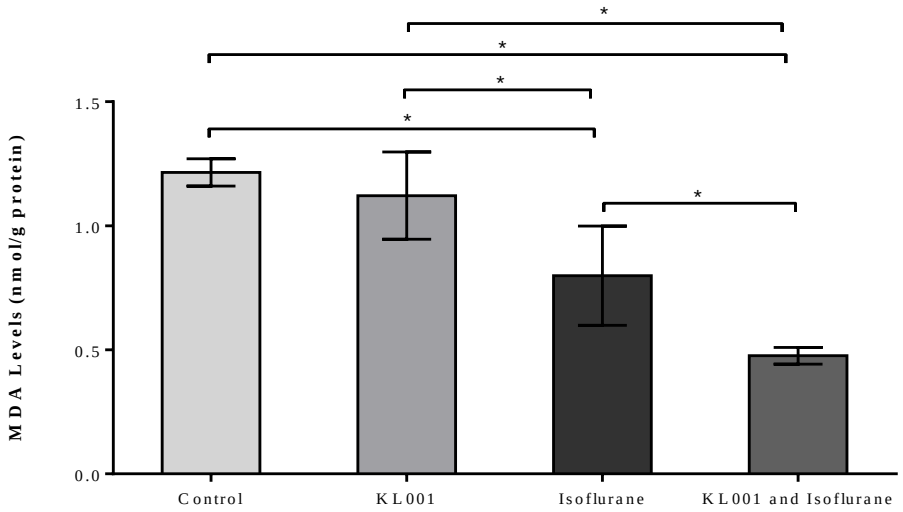

Figure 8.2. MDA levels in erythrocyte samples in study groups.

Results were presented as mean ± standard deviation ($p < 0.05$)

MDA levels were expressed as nmol/g protein.

n= 5

* $p < 0.05$

4.1.1.2. CAT Activities in Erythrocyte Samples

CAT activities in erythrocyte samples in study groups are given in Figure 9.1 and Figure 9.2. The first graph contained the results obtained by adding all the values we found (n=6). On the other hand, we did not have the opportunity to repeat the experiments, so we excluded the values, which are different from the total group in the second graph (n=4). According to the first graph, no statistically significant change was observed in any groups. In the second graph, the KL001 and isoflurane group exhibited a significant decrease compared with the KL001 group and isoflurane group ($p < 0.05$).

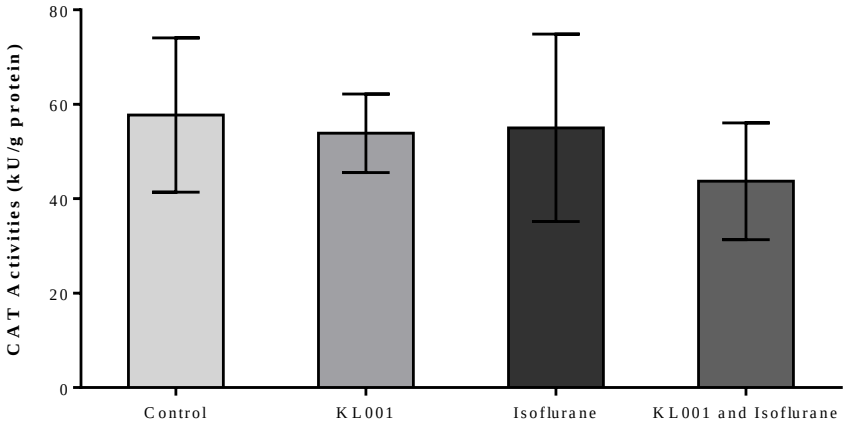

Figure 9.1. CAT activities in erythrocyte samples in study groups

Results were presented as mean ± standard deviation

CAT activities were expressed as kU/g protein.

n=6

* $p < 0.05$

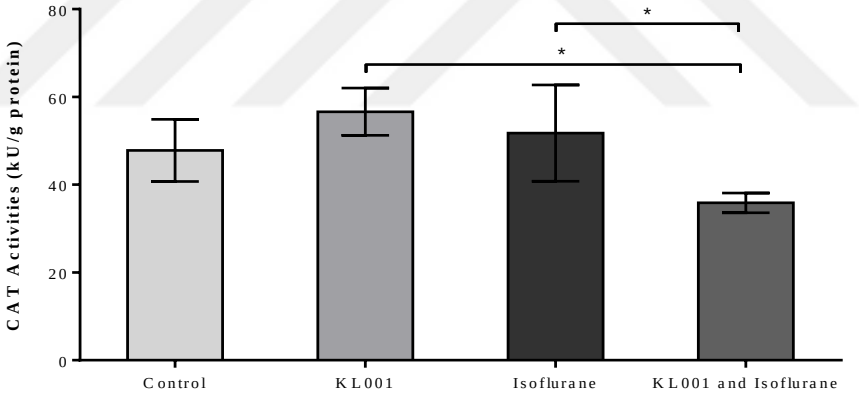

Figure 9.2. CAT activities in erythrocyte samples in study groups

Results were presented as mean ± standard deviation

CAT activities were expressed as kU/g protein.

n=4

* $p < 0.05$

4.1.1.3. CuZnSOD Activities in Erythrocyte Samples

CuZnSOD activities in erythrocyte samples in study groups are given in Figure 10.1 and Figure 10.2. The first graph contained the results obtained by adding all the values we found (n=6). On the other hand, we did not have the opportunity to repeat the experiments, so we excluded the values, which are different from the total group in the second graph (n=4). When all the values were taken into account, the KL001 group and the isoflurane group showed a significant rise compared to the control group ($p < 0.05$). In the second graph, there was a significant increase in the KL001 group and the isoflurane group with comparing the control group and there was a significant increase in the isoflurane group with comparing the KL001 group ($p < 0.05$). The KL001 and isoflurane group showed a significant decrease compared to isoflurane group ($p < 0.05$).

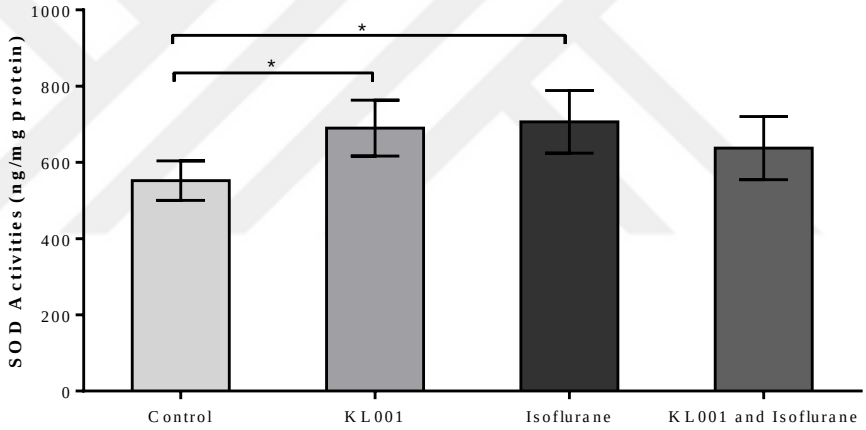

Figure 10.1. CuZnSOD activities in erythrocyte samples in study groups

Results were presented as mean ± standard deviation

CuZnSOD activities were expressed as ng/mg protein.

n=6

* $p < 0.05$

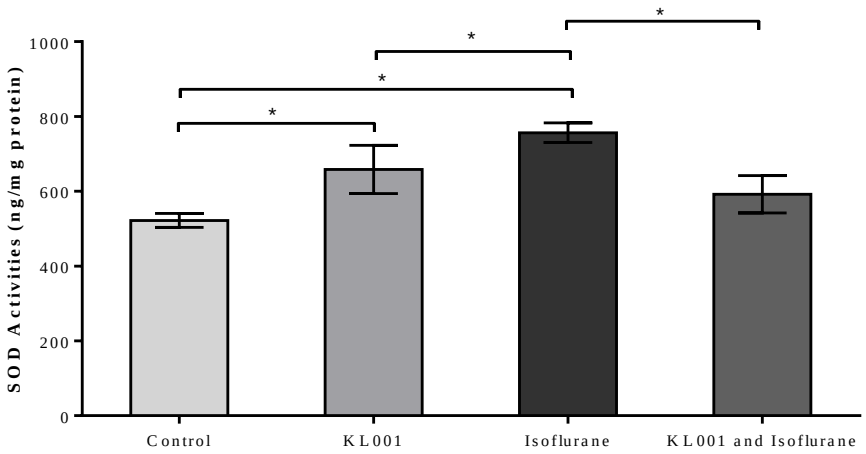

Figure 10.2. CuZnSOD activities in erythrocyte samples in study groups

Results were presented as mean ± standard deviation

CuZnSOD activities were expressed as ng/mg protein.

n=4

* $p < 0.05$

4.1.1.4. GSH-Px Activities in Erythrocyte Samples

GSH-Px activities of erythrocyte samples in study groups are given in Figure 11.1 and Figure 11.2. The first graph contained the results obtained by adding all the values we found (n=6). On the other hand, we did not have the opportunity to repeat the experiments, so we excluded the values, which are different from the total group in the second graph (n=4). According to the first graph, no statistically significant change was observed in any groups. In the second graph, the GSH-Px activity significantly decreased at only the KL001 and isoflurane group compared to the isoflurane group ($p < 0.05$).

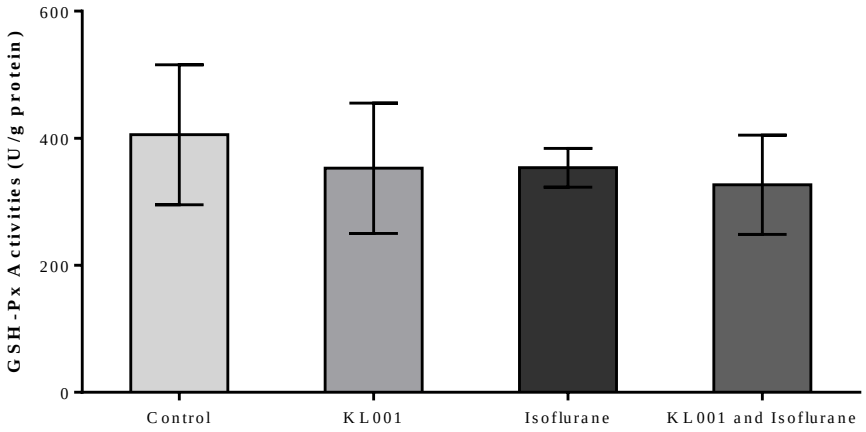

Figure 11.1. GSH-Px activities in erythrocyte samples in study groups

Results were presented as mean ± standard deviation

GSH-Px activities were expressed as U/g protein.

n=6

* $p < 0.05$

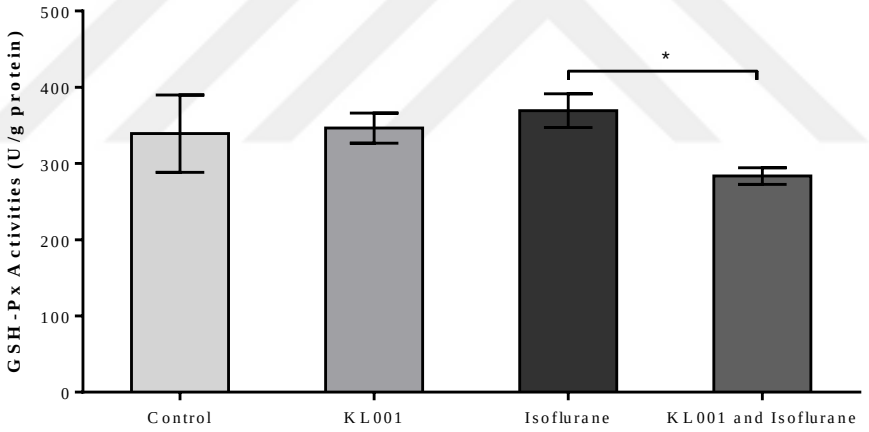

Figure 11.2. GSH-Px activities in erythrocyte samples in study groups

Results were presented as mean ± standard deviation

GSH-Px activities were expressed as U/g protein.

n=4

* $p < 0.05$

MDA levels, CAT, SOD and GSH-Px activities in erythrocyte samples in study groups were summarized in Table 6.1.

Table 6.1. MDA levels, CAT, SOD, and GSH-Px activities in erythrocyte samples in study groups

	Control	**KL001**	**Isoflurane**	**KL001 and Isoflurane**
Erythrocyte MDA	1,09 ± 0,32	1,19 ± 0,24	0,80 ± 0,20[b]	0,46 ± 0,14[ab]
Erythrocyte CAT	57,72 ± 16,34	53,86 ± 8,33	55,02 ± 19,86	43,69 ± 12,35
Erythrocyte SOD	552,17 ± 51,79	690,06 ± 73,40[a]	706,60 ± 82,10[a]	637,40 ± 82,95
Erythrocyte GSH-Px	405,47 ± 110,18	352,84 ± 102,64	353,49 ± 30,39	326,78 ± 78,25

Results were presented as mean ± standard deviation

[a-d] Values with different letters within a row were significantly different ($p < 0.05$)

[a] $p<0.05$ compared to the control group

[b] $p<0.05$ compared to the KL001 group

[c] $p<0.05$ compared to the Isoflurane group

MDA levels were expressed as nmol/g protein.

CAT activities were expressed as kU/g protein.

CuZnSOD activities were expressed as ng/mg protein.

GSH-Px activities were expressed as U/g protein.

MDA levels, CAT, SOD and GSH-Px activities in erythrocyte samples in study groups were summarized in Table 6.2. We excluded the values, which are different from the total group.

Table 6.2. MDA levels, CAT, SOD, and GSH-Px activities in erythrocyte samples in study groups

	Control	KL001	Isoflurane	KL001 and Isoflurane
Erythrocyte MDA	1,22 ± 0,05	1,12 ± 0,18	0,80 ± 0,20[ab]	0,48 ± 0,03[abc]
Erythrocyte CAT	47,82 ± 7,09	56,63 ± 5,38	51,76 ± 10,99	35,86 ± 2,27[bc]
Erythrocyte SOD	522,11 ± 18,89	658,49 ± 64,41[a]	756,51 ± 26,34[ab]	591,92 ± 49,75[c]
Erythrocyte GSH-Px	339,16 ± 50,83	346,42 ± 19,88	369,44 ± 22,16	283,56 ± 10,83[c]

Results were presented as mean ± standard deviation

[a-d] Values with different letters within a row were significantly different ($p < 0.05$)

[a] $p<0.05$ compared to the control group

[b] $p<0.05$ compared to the KL001 group

[c] $p<0.05$ compared to the Isoflurane group

MDA levels were expressed as nmol/g protein.

CAT activities were expressed as kU/g protein.

CuZnSOD activities were expressed as ng/mg protein.

GSH-Px activities were expressed as U/g protein.

4.1.2. Results on Liver Homogenates

4.1.2.1. MDA Levels in Liver Homogenates

MDA levels in liver homogenates in study groups are given in Figure 12.1 and Figure 12.2. The first graph contained the results obtained by adding all the values we found (n=6). On the other hand, we did not have the opportunity to repeat the experiments, so we excluded the values, which are different from the total group in the second graph (n=4). According to the first graph, no statistically significant change was observed in any groups. In the second graph, the level of MDA in the isoflurane group and in the KL001 and isoflurane groups significantly reduced when compared with the control group ($p < 0.05$).

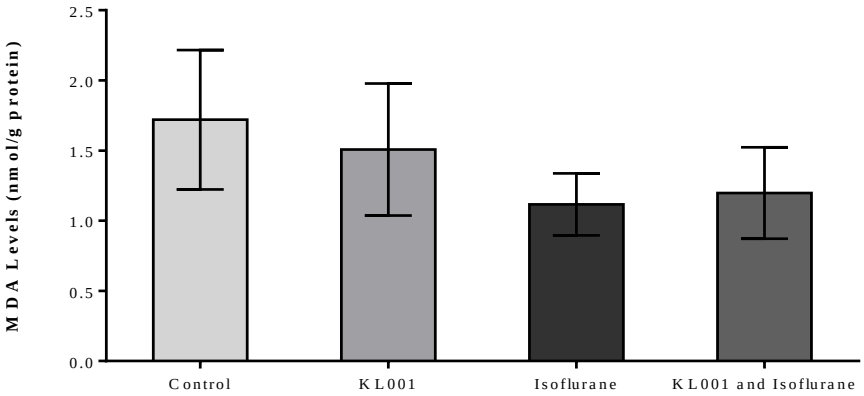

Figure 12.1. MDA levels in liver homogenates in study groups

Results were presented as mean ± standard deviation

MDA levels were expressed as nmol/g protein.

n=6

* $p < 0.05$

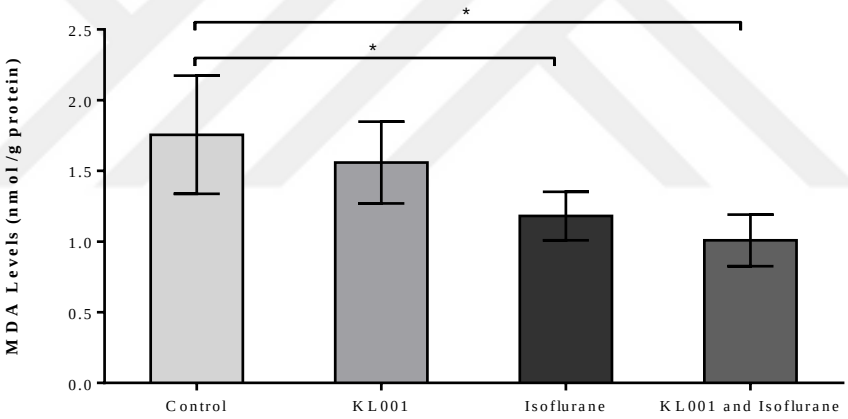

Figure 12.2. MDA levels in liver homogenates in study groups

Results were presented as mean ± standard deviation

MDA levels were expressed as nmol/g protein.

n=4

* $p < 0.05$

4.1.2.2. CAT Activities in Liver Homogenates

CAT activities of liver homogenates in study groups are given in Figure 13.1 and Figure 13.2. The first graph contained the results obtained by adding all the values we found (n=6). On the other hand, we did not have the opportunity to repeat the experiments, so we excluded the values, which are different from the total group in the second graph (n=4). In the first graph, all groups showed a significant decrease compared to the control group ($p < 0.05$). In the second graph, all groups also showed a significant decrease compared to the control group. In addition, there was a significant decrease in the KL001 and isoflurane group with comparing with both the KL001 group and the isoflurane group ($p < 0.05$).

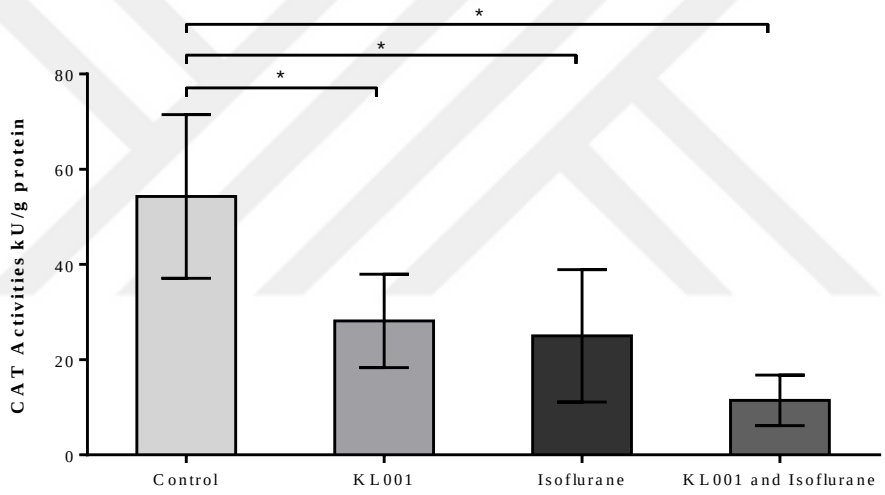

Figure 13.1. CAT activities in liver homogenates in study groups

Results were presented as mean ± standard deviation

CAT activities were expressed as kU/g protein.

n=6

* $p < 0.05$

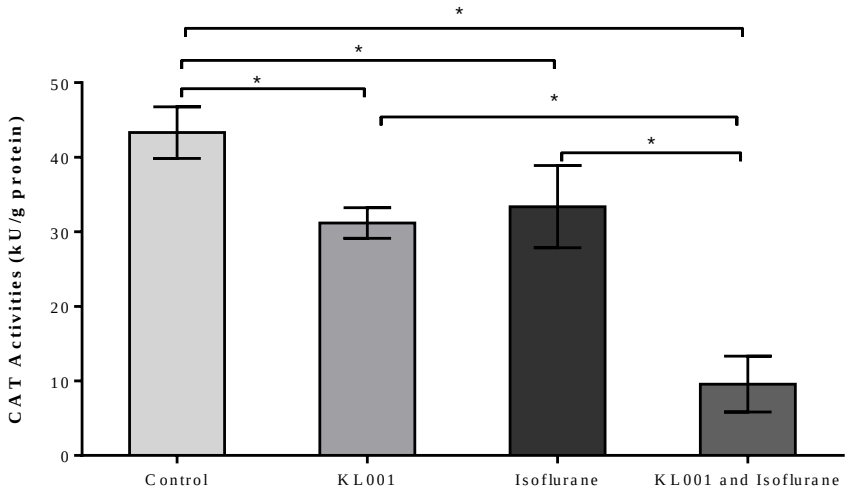

Figure 13.2. CAT activities in liver homogenates in study groups

Results were presented as mean ± standard deviation

CAT activities were expressed as kU/g protein.

n=4

* $p < 0.05$

4.1.2.3. CuZnSOD Activities in Liver Homogenates

CuZnSOD activities in liver homogenates in study groups are given in Figure 14.1 and Figure 14.2. The first graph contained the results obtained by adding all the values we found (n=6). On the other hand, we did not have the opportunity to repeat the experiments, so we excluded the values, which are different from the total group in the second graph (n=4). According to both graphics, no statistically significant change was observed in any groups.

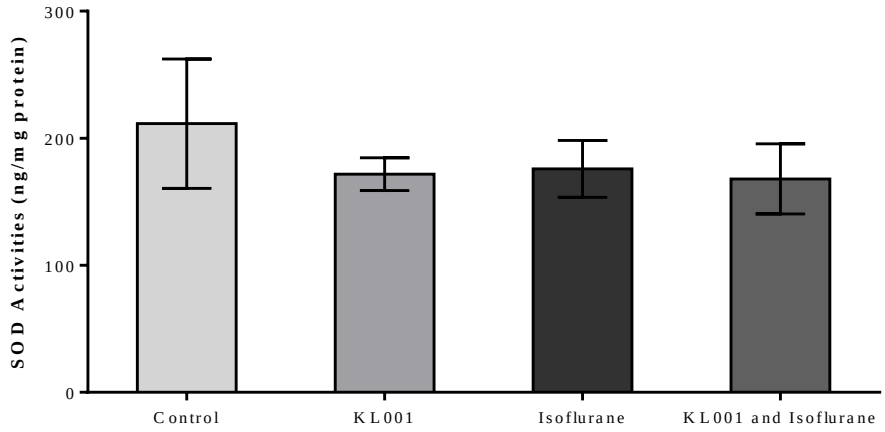

Figure 14.1. CuZnSOD activities in liver homogenates in study groups

Results were presented as mean ± standard deviation

CuZnSOD activities were expressed as ng/mg protein.

n=6

* $p < 0.05$

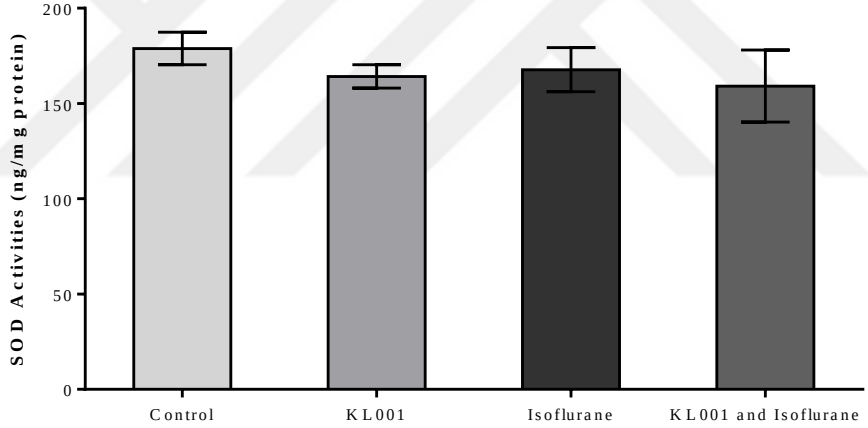

Figure 14.2. CuZnSOD activities in liver homogenates in study groups

Results were presented as mean ± standard deviation

CuZnSOD activities were expressed as ng/mg protein.

n=4

* $p < 0.05$

4.1.2.4. GSH-Px Activities in Liver Homogenates

GSH-Px activities in liver homogenates in study groups are given in Figure 15.1 and Figure 15.2. The first graph contained the results obtained by adding all the values we found (n=6). On the other hand, we did not have the opportunity to repeat the experiments, so we excluded the values, which are different from the total group in the

second graph (n=4). When all values are considered, the activity of GSH-Px in the KL001 and isoflurane group increased comparing with the control group and the isoflurane group ($p < 0.05$). In the second graph, both the isoflurane group and the KL001 and isoflurane group exhibited a significant increase compared to the control group and the KL001 group ($p < 0.05$).

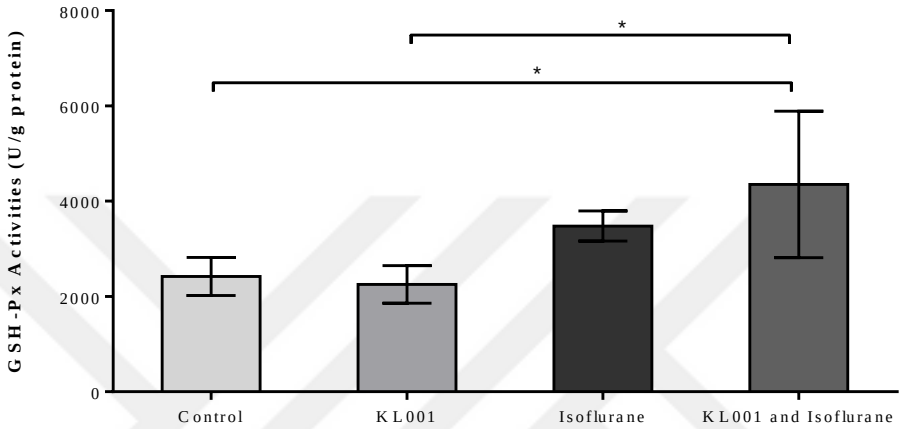

Figure 15.1. GSH-Px activities in liver homogenates in study groups

Results were presented as mean ± standard deviation

GSH-Px activities were expressed as U/g protein.

n=6

* $p < 0.05$

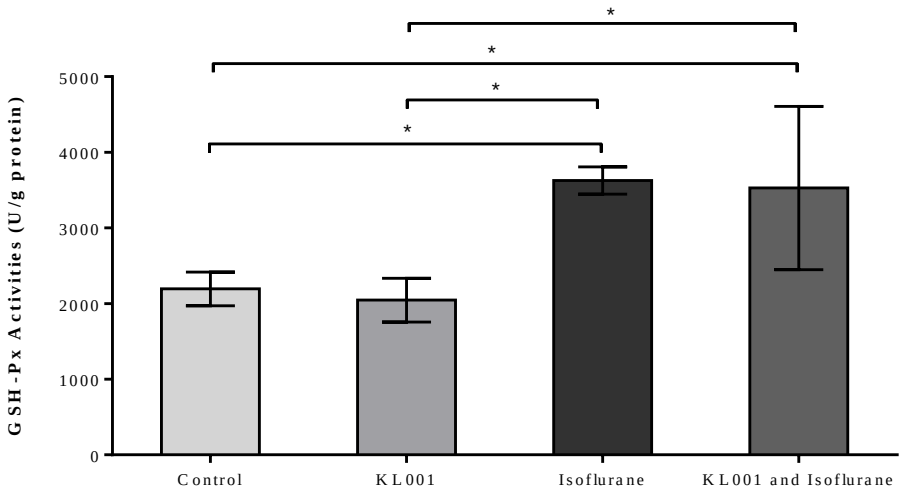

Figure 15.2. GSH-Px activities in liver homogenates in study groups

Results were presented as mean ± standard deviation

GSH-Px activities were expressed as U/g protein.

n=4

* $p < 0.05$

MDA levels, CAT, SOD and GSH-Px activities in liver homogenates in study groups were summarized in Table 7.1.

Table 7.1. MDA levels, CAT, SOD, and GSH-Px activities in liver homogenates in study groups

	Control	KL001	Isoflurane	KL001 and Isoflurane
Liver MDA	1,72 ± 0,50	1,51 ± 0,47	1,12 ± 0,22	1,20 ± 0,33
Liver CAT	54,27 ± 17,20	28,15 ± 9,82 [a]	25,01 ± 13,92[a]	11,46 ± 5,32[a]
Liver SOD	211,46 ± 50,99	171,74 ± 12,87	175,85 ± 22,42	168,01 ± 27,50
Liver GSH-Px	2419,58 ± 399,77	2253,93 ± 394,78	3477,36 ± 315,99	4352,69 ± 1536,78[ab]

Results were presented as mean ± standard deviation

[a-d] Values with different letters within a row were significantly different ($p < 0.05$)

[a] $p<0.05$ compared to the control group

[b] $p<0.05$ compared to the KL001 group

[c] $p<0.05$ compared to the Isoflurane group

MDA levels were expressed as nmol/g protein.

CAT activities were expressed as kU/g protein.

CuZnSOD activities were expressed as ng/mg protein.

GSH-Px activities were expressed as U/g protein.

MDA levels, CAT, SOD and GSH-Px activities in liver homogenates in study groups were summarized in Table 7.2. We excluded the values, which are different from the total group.

Table 7.2. MDA levels, CAT, SOD, and GSH-Px activities in liver homogenates in study groups

	Control	KL001	Isoflurane	KL001 and Isoflurane
Liver MDA	1,76 ± 0,42	1,56 ± 0,29	1,18 ± 0,17[a]	1,01 ± 0,18[a]
Liver CAT	43,31 ± 3,45	31,17 ± 2,05[a]	33,37 ± 5,51[a]	9,57 ± 3,74[abc]
Liver SOD	178,86 ± 8,55	164,17 ± 6,14	167,74 ± 11,60	159,16 ± 18,90
Liver GSH-Px	2194,21 ± 223,24	2045,35 ± 289,83	3626,72 ± 179,34[ab]	3527,61 ± 1079,63[ab]

Results were presented as mean ± standard deviation

[a-d] Values with different letters within a row were significantly different ($p < 0.05$)

[a] $p<0.05$ compared to the control group

[b] $p<0.05$ compared to the KL001 group

[c] $p<0.05$ compared to the Isoflurane group

MDA levels were expressed as nmol/g protein.

CAT activities were expressed as kU/g protein.

CuZnSOD activities were expressed as ng/mg protein.

GSH-Px activities were expressed as U/g protein.

4.1.3. Results on Kidney Homogenates

4.1.3.1. MDA Levels in Kidney Homogenates

MDA levels in kidney homogenates in study groups are given in Figure 16.1 and Figure 16.2. The first graph contained the results obtained by adding all the values we found (n=6). On the other hand, we did not have the opportunity to repeat the experiments, so we excluded the values, which are different from the total group in the second graph (n=4). According to both graphics, no statistically significant change was observed in any groups.

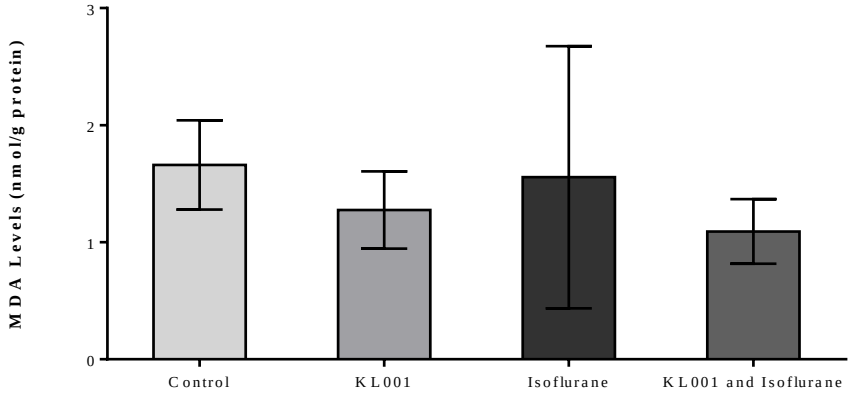

Figure 16.1. MDA levels in kidney homogenates in study groups

Results were presented as mean ± standard deviation

MDA levels were expressed as nmol/g protein.

n=6

* $p < 0.05$

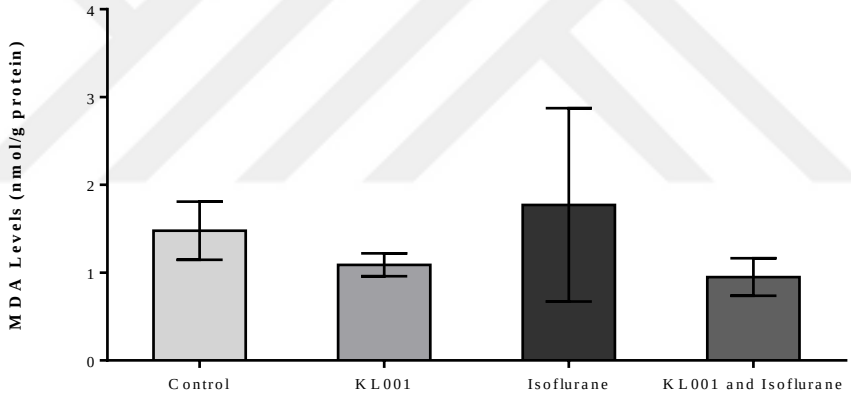

Figure 16.2. MDA levels in kidney homogenates in study groups

Results were presented as mean ± standard deviation

MDA levels were expressed as nmol/g protein.

n=4

* $p < 0.05$

4.1.3.2. CAT Activities in Kidney Homogenates

CAT activities in kidney homogenates in study groups are given in Figure 17.1 and Figure 17.2. The first graph contained the results obtained by adding all the values we found (n=6). On the other hand, we did not have the opportunity to repeat the experiments, so we excluded the values, which are different from the total group in the second graph (n=4). According to the first graph, no statistically significant change was

observed in any animal groups. In the second graph, there was a significant decrease in only the KL001 and isoflurane group compared to the isoflurane group ($p < 0.05$).

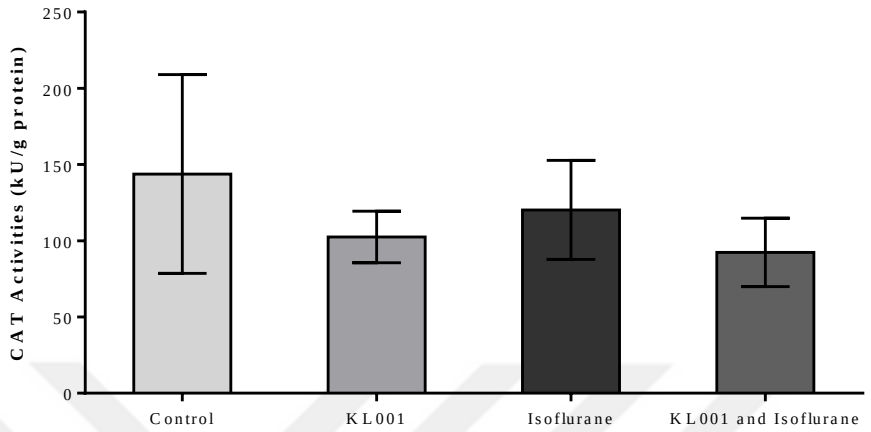

Figure 17.1. CAT activities in kidney homogenates in study groups

Results were presented as mean ± standard deviation

CAT activities were expressed as kU/g protein.

n=6

* $p < 0.05$

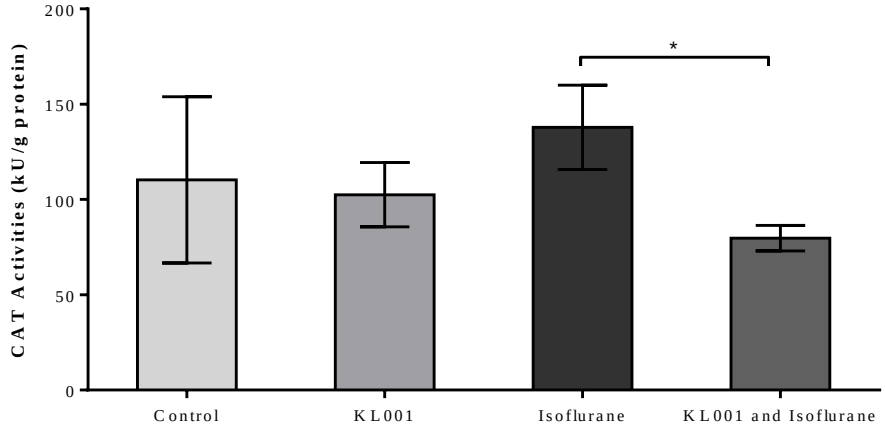

Figure 17.2. CAT activities in kidney homogenates in study groups

Results were presented as mean ± standard deviation

CAT activities were expressed as kU/g protein.

n=4

* $p < 0.05$

4.1.3.3. CuZnSOD Activities in Kidney Homogenates

CuZnSOD activities in kidney homogenates in study groups are given in Figure 18.1 and Figure 18.2. The first graph contained the results obtained by adding all the values we found (n=6). On the other hand, we did not have the opportunity to repeat the experiments, so we excluded the values, which are different from the total group in the second graph (n=4). According to the first graph, no statistically significant change was observed in groups. In the second graph, the activity of CuZnSOD significantly decreased in the KL001 and isoflurane group compared to the isoflurane group ($p < 0.05$). Besides CuZnSOD activity of the isoflurane group significantly increased compared to the control group ($p < 0.05$).

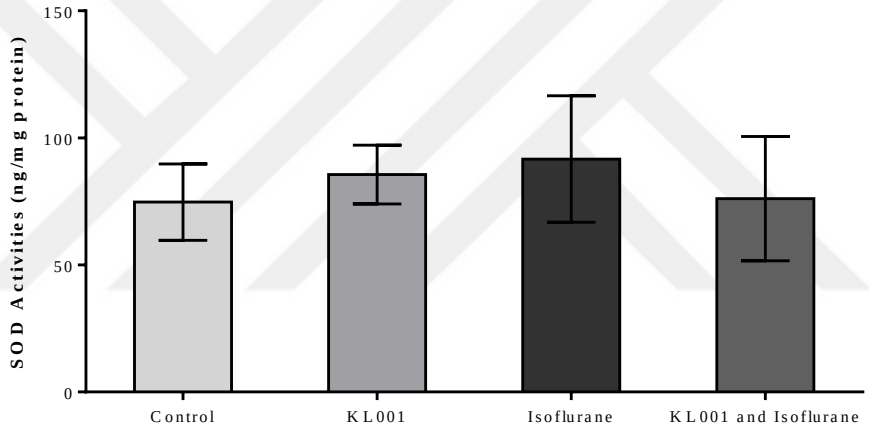

Figure 18.1. CuZnSOD activities in kidney homogenates in study groups

Results were presented as mean ± standard deviation

CuZnSOD activities were expressed as ng/mg protein.

n=6

* $p < 0.05$

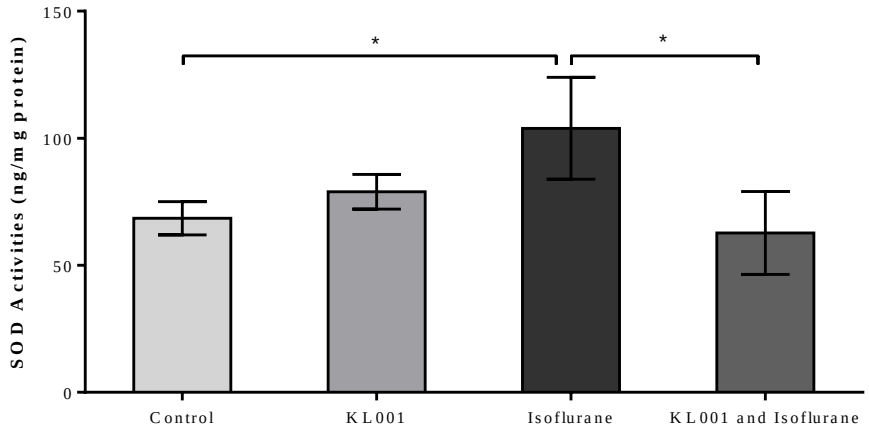

Figure 18.2. CuZnSOD activities in kidney homogenates in study groups

Results were presented as mean ± standard deviation

CuZnSOD activities were expressed as ng/mg protein.

n=4

* $p < 0.05$

4.1.3.4. GSH-Px Activities in Kidney Homogenates

GSH-Px activities in kidney homogenates in study groups are given in Figure 19.1 and Figure 19.2. The first graph contained the results obtained by adding all the values we found (n=6). On the other hand, we did not have the opportunity to repeat the experiments, so we excluded the values, which are different from the total group in the second graph (n=4). According to both graphics, no statistically significant change was observed in any groups.

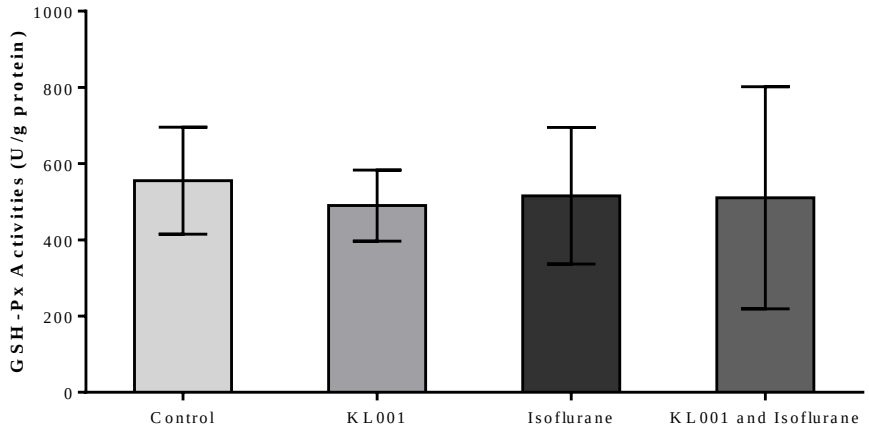

Figure 19.1. GSH-Px activities in kidney homogenates in study groups

Results were presented as mean ± standard deviation

GSH-Px activities were expressed as U/g protein.

n=6

* $p < 0.05$

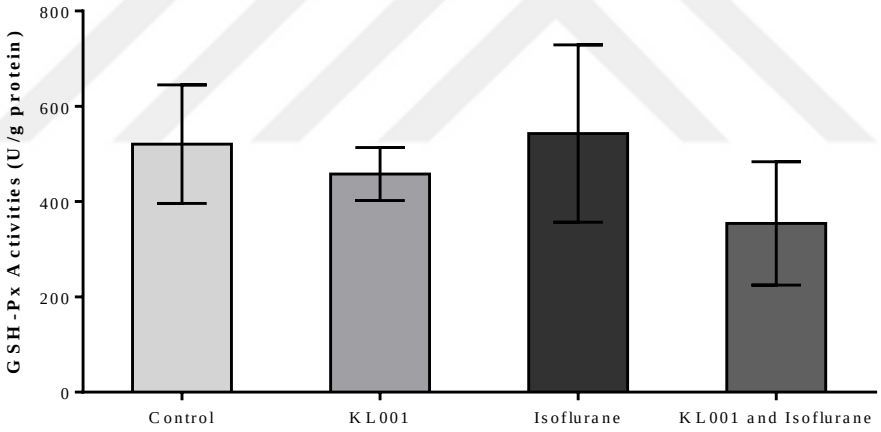

Figure 19.2. GSH-Px activities in kidney homogenates in study groups

Results were presented as mean ± standard deviation

GSH-Px activities were expressed as U/g protein.

n=5

* $p < 0.05$

MDA levels, CAT, SOD and GSH-Px activities in kidney homogenates in study groups were summarized in Table 8.1.

Table 8.1. MDA levels, CAT, SOD, and GSH-Px activities in kidney homogenates in study groups

	Control	KL001	Isoflurane	KL001 and Isoflurane
Kidney MDA	1,66 ± 0,38	1,28 ± 0,33	1,56 ± 1,12	1,09 ± 0,28
Kidney CAT	143,79 ± 65,23	102,50 ± 16,90	120,24 ± 32,53	92,36 ± 22,38
Kidney SOD	74,73 ± 15,05	85,57 ± 11,56	91,68 ± 24,87	76,09 ± 24,46
Kidney GSH-Px	555,42 ± 140,14	490,05 ± 93,14	515,60 ± 179,26	510,38 ± 291,15

Results were presented as mean ± standard deviation

[a-d] Values with different letters within a row were significantly different ($p < 0.05$)

[a] $p<0.05$ compared to the control group

[b] $p<0.05$ compared to the KL001 group

[c] $p<0.05$ compared to the Isoflurane group

MDA levels were expressed as nmol/g protein.

CAT activities were expressed as kU/g protein.

CuZnSOD activities were expressed as ng/mg protein.

GSH-Px activities were expressed as U/g protein.

MDA levels, CAT, SOD and GSH-Px activities in kidney homogenates in study groups were summarized in Table 8.2. We excluded the values, which are different from the total group.

Table 8.2. MDA levels, CAT, SOD, and GSH-Px activities in kidney homogenates in study groups

	Control	KL001	Isoflurane	KL001 and Isoflurane
Kidney MDA	1,48 ± 0,33	1,09 ± 0,13	1,77 ± 1,10	0,95 ± 0,21
Kidney CAT	110,31 ± 43,60	102,50 ± 16,90	137,86 ± 22,12	79,70 ± 6,69[c]
Kidney SOD	68,50 ± 6,57	78,93 ± 6,81	103,90 ± 20,03[a]	62,72 ± 16,33[c]
Kidney GSH-Px	520,65 ± 124,43	457,90 ± 55,62	542,78 ± 186,08	354,21 ± 129,27

Results were presented as mean ± standard deviation

[a-d] Values with different letters within a row were significantly different ($p < 0.05$)

[a] $p<0.05$ compared to the control group

[b] $p<0.05$ compared to the KL001 group

[c] $p<0.05$ compared to the Isoflurane group

MDA levels were expressed as nmol/g protein.

CAT activities were expressed as kU/g protein.

CuZnSOD activities were expressed as ng/mg protein.

GSH-Px activities were expressed as U/g protein.

4.1.4. Results on Brain Homogenates

4.1.4.1. MDA Levels in Brain Homogenates

MDA levels in brain homogenates in study groups are given in Figure 20.1 and Figure 20.2. The first graph contained the results obtained by adding all the values we found (n=6). On the other hand, we did not have the opportunity to repeat the experiments, so we excluded the values, which are different from the total group in the second graph (n=4). According to both graphics, no statistically significant change was observed in any groups.

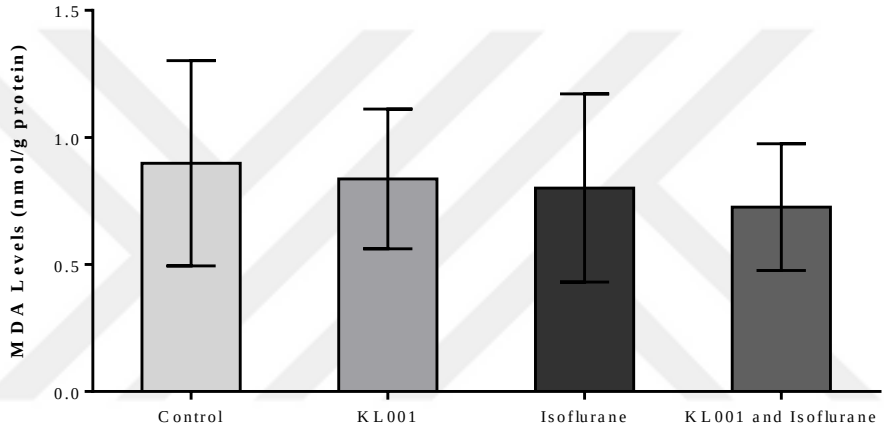

Figure 20.1. MDA levels in brain homogenates in study groups

Results were presented as mean ± standard deviation

MDA levels were expressed as nmol/g protein.

n=6

* $p < 0.05$

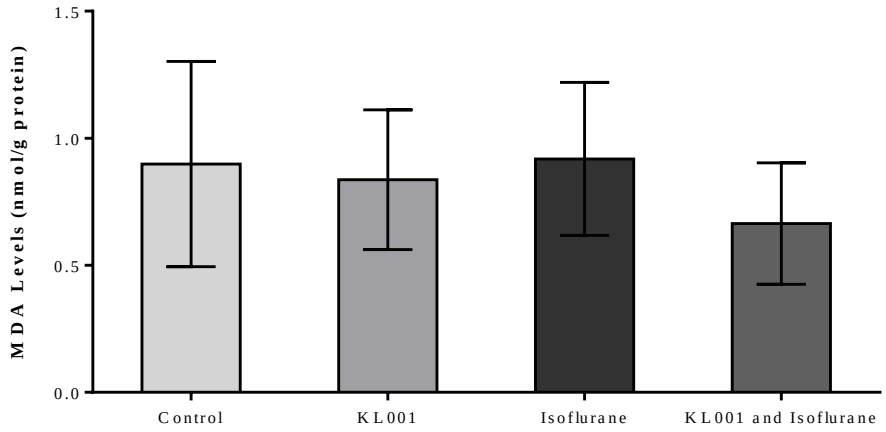

Figure 20.2. MDA levels in brain homogenates in study groups

Results were presented as mean ± standard deviation

MDA levels were expressed as nmol/g protein.

n=4

* $p < 0.05$

4.1.4.2. CAT Activities in Brain Homogenates

CAT activities in brain homogenates in study groups are given in Figure 21.1 and Figure 21.2. The first graph contained the results obtained by adding all the values we found (n=6). On the other hand, we did not have the opportunity to repeat the experiments, so we excluded the values, which are different from the total group in the second graph (n=4). According to the first graph, no statistically significant change was observed in any groups. According to the second graph, the activity of CAT significantly decreased in the KL001 and isoflurane group compared to the isoflurane group ($p < 0.05$).

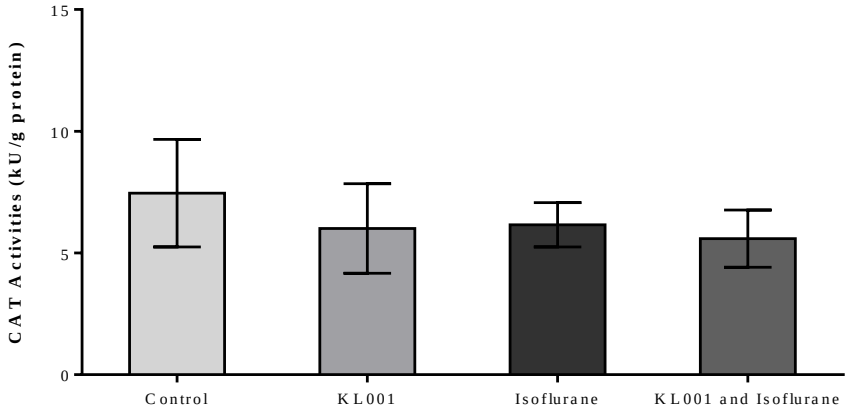

Figure 21.1. CAT activities in brain homogenates in study groups

Results were presented as mean ± standard deviation

CAT activities were expressed as kU/g protein.

n=6

* $p < 0.05$

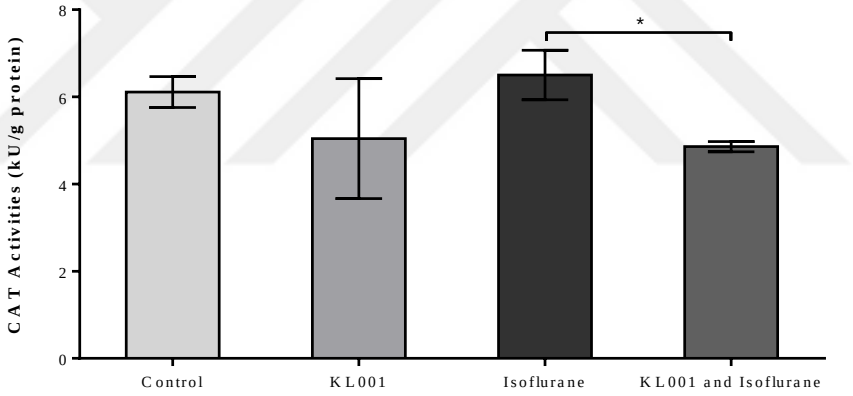

Figure 21.2. CAT activities in brain homogenates in study groups

Results were presented as mean ± standard deviation

CAT activities were expressed as kU/g protein.

n=4

* $p < 0.05$

4.1.4.3. CuZnSOD Activities in Brain Homogenates

CuZnSOD activities in brain homogenates in study groups are given in Figure 22.1 and Figure 22.2. The first graph contained the results obtained by adding all the values we found (n=6). On the other hand, we did not have the opportunity to repeat the experiments, so we excluded the values, which are different from the total group in the second graph (n=4). According to the first graph, no statistically significant change was observed in any groups. According to the second graph, the CuZnSOD activity of the isoflurane group significantly increased compared to the KL001 group. On the other hand, the CuZnSOD activity of the KL001 and isoflurane group significantly decreased compared to the isoflurane group ($p < 0.05$).

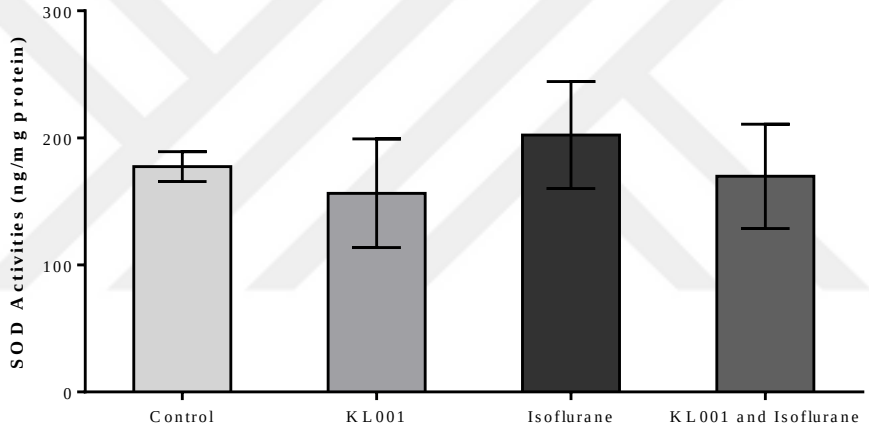

Figure 22.1. CuZnSOD activities in brain homogenates in study groups

Results were presented as mean ± standard deviation

CuZnSOD activities were expressed as ng/mg protein.

n=6

* $p < 0.05$

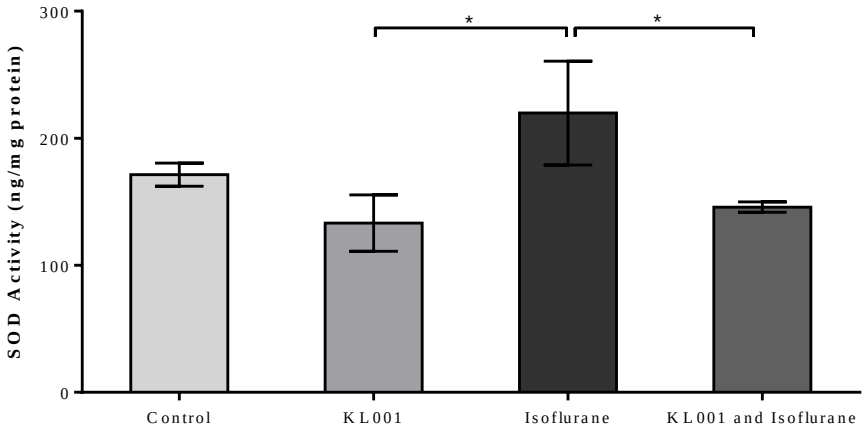

Figure 22.2. CuZnSOD activities in brain homogenates in study groups

Results were presented as mean ± standard deviation

CuZnSOD activities were expressed as ng/mg protein.

n=4

* $p < 0.05$

4.1.4.4. GSH-Px Activities in Brain Homogenates

GSH-Px activities in brain homogenates in study groups are given in Figure 23.1 and Figure 23.2. The first graph contained the results obtained by adding all the values we found (n=6). On the other hand, we did not have the opportunity to repeat the experiments, so we excluded the values, which are different from the total group in the second graph (n=4). In the first graph, there was a significant increase in the isoflurane group with comparing the control group ($p < 0.05$). According to the second graph, the GSH-Px activities of the isoflurane group and the KL001 and isoflurane group significantly increased compared to both the control group and the KL001 group ($p < 0.05$).

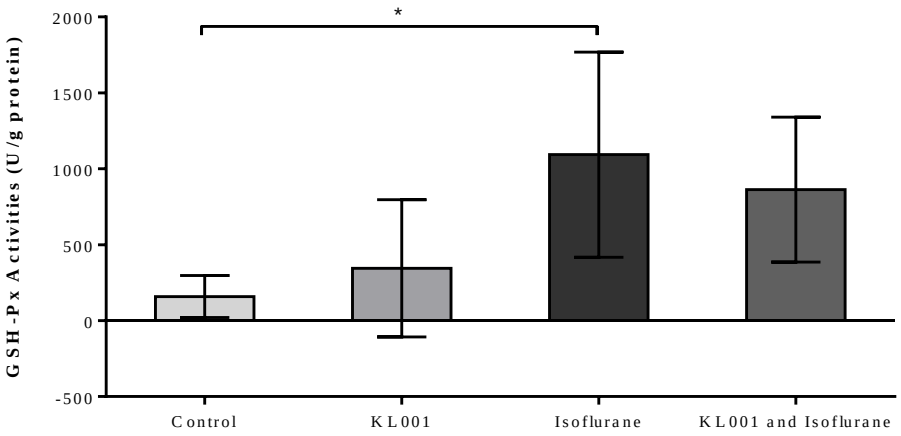

Figure 23.1. GSH-Px activities in brain homogenates in study groups

Results were presented as mean ± standard deviation

GSH-Px activities were expressed as U/g protein.

n=6

* $p < 0.05$

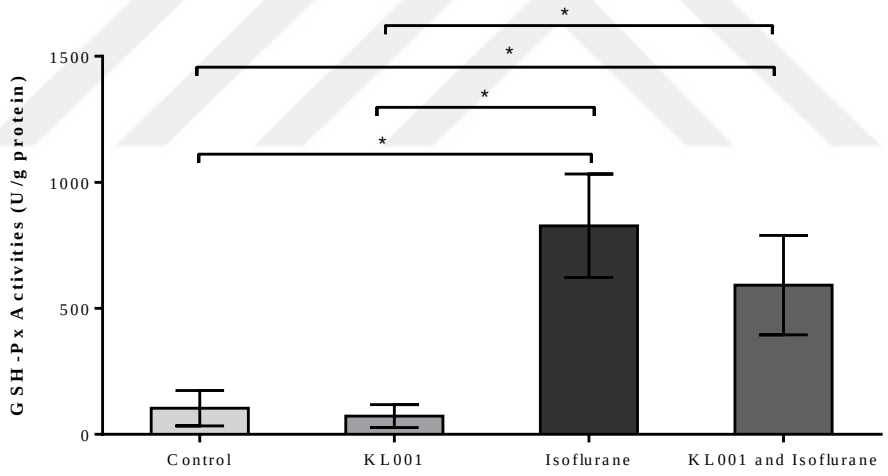

Figure 23.2. GSH-Px activities in brain homogenates in study groups.

Results were presented as mean ± standard deviation

GSH-Px activities were expressed as U/g protein.

n=4

* $p < 0.05$

MDA levels, CAT, SOD and GSH-Px activities in brain homogenates in study groups were summarized in Table 9.1.

Table 9.1. MDA levels, CAT, SOD, and GSH-Px activities in brain homogenates in study groups

	Control	KL001	Isoflurane	KL001 and Isoflurane
Brain MDA	0,90 ± 0,40	0,84 ± 0,27	0,80 ± 0,37	0,73 ± 0,25
Brain CAT	7,46 ± 2,21	6,01 ± 1,84	6,16 ± 0,91	5,59 ± 1,17
Brain SOD	177,37 ± 11,78	156,41 ± 42,74	202,16 ± 42,10	169,78 ± 41,07
Brain GSH-Px	158,92 ± 137,64	344,39 ± 452,05	1093,13 ± 675,99[a]	863,15 ± 477,40

Results were presented as mean ± standard deviation

[a-d] Values with different letters within a row were significantly different ($p < 0.05$)

[a] $p<0.05$ compared to the control group

[b] $p<0.05$ compared to the KL001 group

[c] $p<0.05$ compared to the Isoflurane group

MDA levels were expressed as nmol/g protein.

CAT activities were expressed as kU/g protein.

CuZnSOD activities were expressed as ng/mg protein.

GSH-Px activities were expressed as U/g protein.

MDA levels, CAT, SOD and GSH-Px activities in brain homogenates in study groups were summarized in Table 9.2. We excluded the values, which are different from the total group.

Table 9.2. MDA levels, CAT, SOD, and GSH-Px activities in brain homogenates in study groups

	Control	KL001	Isoflurane	KL001 and Isoflurane
Brain MDA	0,90 ± 0,40	0,84 ± 0,27	0,92 ± 0,30	0,66 ± 0,24
Brain CAT	6,11 ± 0,35	5,04 ± 1,37	6,50 ± 0,57	4,86 ± 0,12[c]
Brain SOD	171,29 ± 9,07	133,20 ± 22,13	219,80 ± 40,88[b]	145,80 ± 4,05[c]
Brain GSH-Px	103,76 ± 70,52	72,80 ± 45,67	827,52 ± 205,08[a b]	592,24 ± 197,39[ab]

Results were presented as mean ± standard deviation

[a-d] Values with different letters within a row were significantly different ($p < 0.05$)

[a] $p<0.05$ compared to the control group

[b] $p<0.05$ compared to the KL001 group

[c] $p<0.05$ compared to the Isoflurane group

MDA levels were expressed as nmol/g protein.

CAT activities were expressed as kU/g protein.

CuZnSOD activities were expressed as ng/mg protein.

GSH-Px activities were expressed as U/g protein

MDA levels, CAT, SOD and GSH-Px activities in erythrocyte, liver, kidney, and brain in study groups were summarized in Table 10.1.

Table 10.1. MDA levels, CAT, SOD, and GSH-Px activities in erythrocyte samples, liver, kidney, and brain homogenates in study groups

	Control	KL001	Isoflurane	KL001 and Isoflurane
Erythrocyte MDA	1,09 ± 0,32	1,19 ± 0,24	0,80 ± 0,20[b]	0,46 ± 0,14[ab]
Erythrocyte CAT	57,72 ± 16,34	53,86 ± 8,33	55,02 ± 19,86	43,69 ± 12,35
Erythrocyte SOD	552,17 ± 51,79	690,06 ± 73,40[a]	706,60 ± 82,10[a]	637,40 ± 82,95
Erythrocyte GSH-Px	405,47 ± 110,18	352,84 ± 102,64	353,49 ± 30,39	326,78 ± 78,25
Liver MDA	1,72 ± 0,50	1,51 ± 0,47	1,12 ± 0,22	1,20 ± 0,33
Liver CAT	54,27 ± 17,20	28,15 ± 9,82 [a]	25,01 ± 13,92[a]	11,46 ± 5,32 [a]
Liver SOD	211,46 ± 50,99	171,74 ± 12,87	175,85 ± 22,42	168,01 ± 27,50
Liver GSH-Px	2419,58 ± 399,77	2253,93 ± 394,78	3477,36 ± 315,99	4352,69 ± 1536,78[ab]
Kidney MDA	1,66 ± 0,38	1,28 ± 0,33	1,56 ± 1,12	1,09 ± 0,28
Kidney CAT	143,79 ± 65,23	102,50 ± 16,90	120,24 ± 32,53	92,36 ± 22,38
Kidney SOD	74,73 ± 15,05	85,57 ± 11,56	91,68 ± 24,87	76,09 ± 24,46
Kidney GSH-Px	555,42 ± 140,14	490,05 ± 93,14	515,60 ± 179,26	510,38 ± 291,15
Brain MDA	0,90 ± 0,40	0,84 ± 0,27	0,80 ± 0,37	0,73 ± 0,25
Brain CAT	7,46 ± 2,21	6,01 ± 1,84	6,16 ± 0,91	5,59 ± 1,17
Brain SOD	177,37 ± 11,78	156,41 ± 42,74	202,16 ± 42,10	169,78 ± 41,07
Brain GSH-Px	158,92 ± 137,64	344,39 ± 452,05	1093,13 ± 675,99[a]	863,15 ± 477,40

Results were presented as mean ± standard deviation

[a-d] Values with different letters within a row were significantly different ($p < 0.05$)

[a] $p<0.05$ compared to the control group

[b] $p<0.05$ compared to the KL001 group

[c] $p<0.05$ compared to the Isoflurane group

MDA levels were expressed as nmol/g protein.

CAT activities were expressed as kU/g protein.

CuZnSOD activities were expressed as ng/mg protein.

GSH-Px activities were expressed as U/g protein.

MDA levels, CAT, SOD and GSH-Px activities in erythrocyte, liver, kidney, and brain in study groups were summarized in Table 10.2. We excluded the values, which are different from the total group.

Table 10.2. MDA levels, CAT, SOD, and GSH-Px activities in erythrocyte samples, liver, kidney, and brain homogenates in study groups

	Control	KL001	Isoflurane	KL001 and Isoflurane
Erythrocyte MDA	1,22 ± 0,05	1,12 ± 0,18	0,80 ± 0,20[a b]	0,48 ± 0,03[abc]
Erythrocyte CAT	47,82 ± 7,09	56,63 ± 5,38	51,76 ± 10,99	35,86 ± 2,27[b c]
Erythrocyte SOD	522,11 ± 18,89	658,49 ± 64,41[a]	756,51 ± 26,34[ab]	591,92 ± 49,75[c]
Erythrocyte GSH-Px	339,16 ± 50,83	346,42 ± 19,88	369,44 ± 22,16	283,56 ± 10,83[c]
Liver MDA	1,76 ± 0,42	1,56 ± 0,29	1,18 ± 0,17[a]	1,01 ± 0,18[a]
Liver CAT	43,31 ± 3,45	31,17 ± 2,05[a]	33,37 ± 5,51[a]	9,57 ± 3,74[abc]
Liver SOD	178,86 ± 8,55	164,17 ± 6,14	167,74 ± 11,60	159,16 ± 18,90
Liver GSH-Px	2194,21 ± 223,24	2045,35 ± 289,83	3626,72 ± 179,34[ab]	3527,61 ± 1079,63[ab]
Kidney MDA	1,48 ± 0,33	1,09 ± 0,13	1,77 ± 1,10	0,95 ± 0,21
Kidney CAT	110,31 ± 43,60	102,50 ± 16,90	137,86 ± 22,12	79,70 ± 6,69[c]
Kidney SOD	68,50 ± 6,57	78,93 ± 6,81	103,90 ± 20,03[a]	62,72 ± 16,33[c]
Kidney GSH-Px	520,65 ± 124,43	457,90 ± 55,62	542,78 ± 186,08	354,21 ± 129,27
Brain MDA	0,90 ± 0,40	0,84 ± 0,27	0,92 ± 0,30	0,66 ± 0,24
Brain CAT	6,11 ± 0,35	5,04 ± 1,37	6,50 ± 0,57	4,86 ± 0,12[c]
Brain SOD	171,29 ± 9,07	133,20 ± 22,13	219,80 ± 40,88[b]	145,80 ± 4,05[c]
Brain GSH-Px	103,76 ± 70,52	72,80 ± 45,67	827,52 ± 205,08[a b]	592,24 ± 197,39[ab]

Results were presented as mean ± standard deviation

[a-d] Values with different letters within a row were significantly different ($p < 0.05$)

[a] $p<0.05$ compared to the control group

[b] $p<0.05$ compared to the KL001 group

[c] $p<0.05$ compared to the Isoflurane group

MDA levels were expressed as nmol/g protein.

CAT activities were expressed as kU/g protein.

CuZnSOD activities were expressed as ng/mg protein.

GSH-Px activities were expressed as U/g protein.

4.2. Results in Cry Levels

4.2.1. Cry Levels in Plasma Samples

Cry levels in plasma samples in study groups are given in Figure 24. The significant changes were found in Cry level of the isoflurane group when it was compared both control and KL001 groups ($p < 0.05$). Besides, the KL001 and isoflurane groups showed a significant decrease in Cry level compared to the KL001 group ($p < 0.05$).

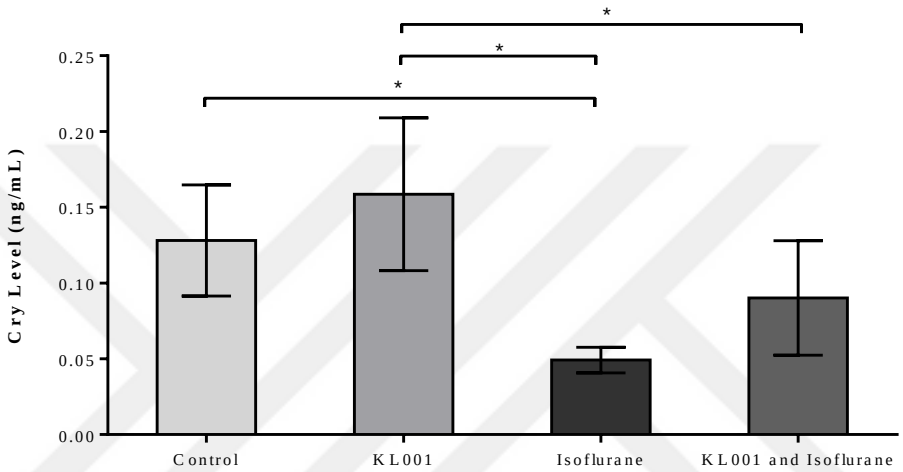

Figure 24. Cry levels in plasma samples in study groups

Results were presented as mean ± standard deviation

Cry levels were expressed as ng/mL

* $p < 0.05$

4.2.2. Cry Levels in Liver Homogenates

Cry levels in liver homogenates in study groups are given in Figure 25. Cry level of the isoflurane group significantly decreased compared to both the control group and the KL001 group ($p < 0.05$). Moreover, Cry level of the KL001 and isoflurane group significantly increased compared to isoflurane group ($p < 0.05$).

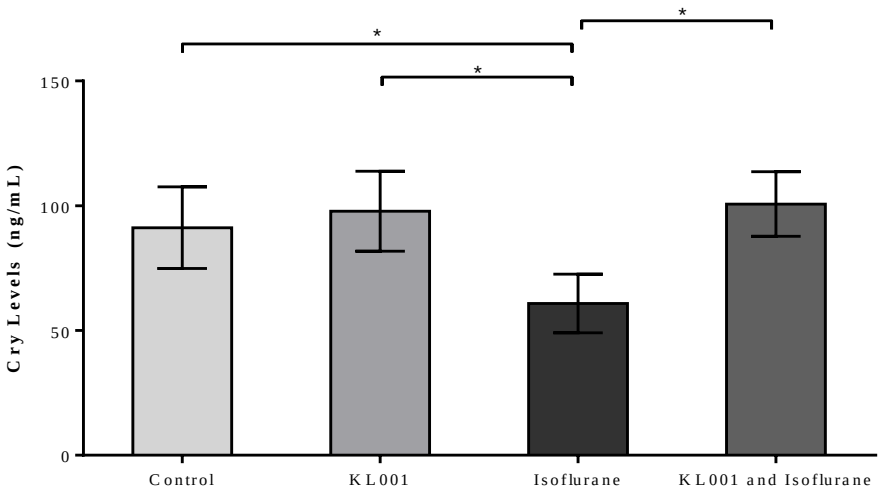

Figure 25. Cry levels in liver homogenates in study groups.

Results were presented as mean ± standard deviation

Cry levels were expressed as ng/mL

* $p < 0.05$

4.2.3. Cry Levels in Kidney Homogenates

Cry levels in kidney homogenates in study groups are given in Figure 26. There was no significant change in the kidney Cry level between the groups.

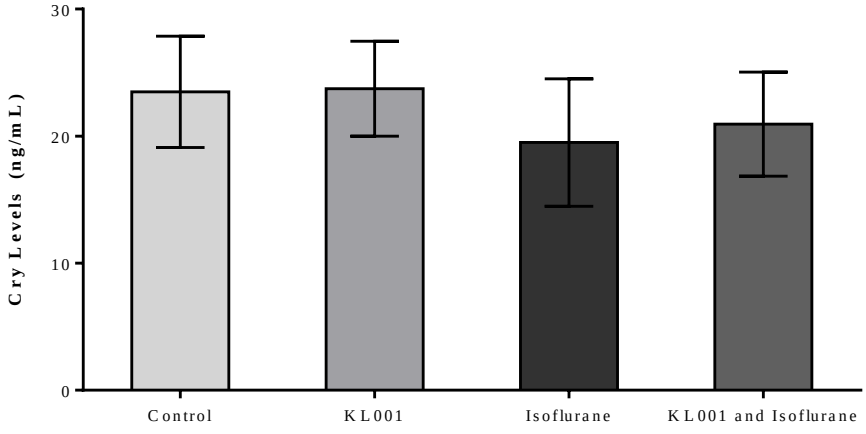

Figure 26. Cry levels in kidney homogenates in study groups.

Results were presented as mean ± standard deviation

Cry levels were expressed as ng/mL

* $p < 0.05$

4.2.4. Cry Levels in Brain Homogenates

Cry levels in brain homogenates in study groups are given in Figure 27. When compared with the control group, a significant increase in Cry level was detected in the KL001 group ($p < 0.05$). It was observed that both isoflurane and KL001 and isoflurane groups were significantly decreased in Cry level compared to KL001 group ($p < 0.05$).

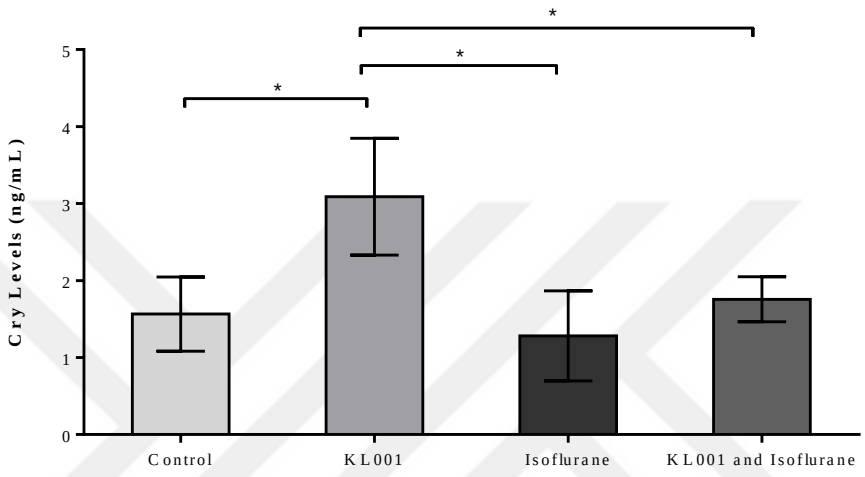

Figure 27. Cry levels in brain homogenates in study groups.

Results were presented as mean ± standard deviation

Cry levels were expressed as ng/mL

* $p < 0.05$

Cry levels in plasma, liver, kidney, and brain in study groups were summarized in Table 11.

Table 11. Cry levels in plasma samples, liver, kidney, and brain homogenates in study groups

	Control	**KL001**	**Isoflurane**	**KL001 and Isoflurane**
Cry levels in plasma	0,13 ± 0,04	0,16 ± 0,05	0,05 ± 0,008[ab]	0,09 ± 0,05[b]
Cry levels in liver	91,19 ± 16,37	97,84 ± 16,02	60,82 ± 11,73[ab]	100,68 ± 12,91[c]
Cry levels in kidney	23,49 ± 4,39	23,73 ± 3,74	19,5 ± 0,52	20,94 ± 4,10
Cry levels in brain	1,56 ± 0,48	3,09 ± 0,76[a]	1,28 ± 0,58[b]	1,76 ± 0,29[b]

Cry levels were expressed as ng/mL.

Results were presented as mean ± standard deviation

[a-d] Values with different letters within a row were significantly different ($p < 0.05$)

[a] $p<0.05$ compared to the control group

[b] $p<0.05$ compared to the KL001 group

[c] $p<0.05$ compared to the Isoflurane group

5. DISCUSSION and CONCLUSION

The circadian rhythm is a vital system that protects both our homeostasis and adaptation to the outside environment (145). There are 4 genes/proteins mainly responsible for the rhythm synchronization, and there are also various control systems (19). When any part of this system is broken down by endogenous or exogenous factors, many unwanted situations appear. The most important of them is oxidative stress, which is indicated as the underlying cause of many pathological conditions. The stress emerges in imbalances when the production of oxidants is higher than their degradation (145). As mentioned earlier, oxidative stress is both outcome and reason for the failure of the circadian rhythm. These two conditions trigger the formation of each other. Besides the circadian rhythm failure causes the endogenous defense systems to become inactive.

It is considered that it is possible to reduce the risk of developing oxidative stress by any antioxidant substances or preventive treatment by exogenously. To this end, several new approaches have been tested on new pathways. One of the new approaches is the intervention to circadian rhythm genes/proteins. The new substances have been synthesized for this purpose. They inhibit or activate the circadian rhythm by mimicking circadian rhythm genes/proteins (54). We thought that oxidative stress can be prevented by correcting circadian rhythm which is deteriorated. Based on this approach, we used the synthetic activator of Cry gene of the circadian rhythm genes to inhibit isoflurane-induced oxidative stress. For this purpose, we evaluated the studies about anesthesia toxicity and circadian rhythm conducted by examining the organs separately and discussed our results.

In the biological process, oxidants are formed in the body regularly. The energy required for the organism is produced by oxidation of organic substance using molecular oxygen. Normally the molecular oxygen is reduced entirely during this process. However, in some cases, the molecular oxygen is partially reduced, superoxide and various reactive oxidant intermediates as MDA are generated, causing to secondary reactions. Endogen sources that produce the radicals, as well as a variety of external sources such as food, drugs, tobacco, air pollutants, UV and more contribute to the formation of radical. In normal condition, the free radicals are destroyed by the endogenous antioxidant defense system in a healthy organism (146). If this defense system fails to function, many pathological conditions arise. In some cases, the endogenous antioxidant defense systems may not be sufficient to combat oxidative stress. In the body, the antioxidant enzyme (as SOD, CAT, GSH-Px, and more) activities change in response to the disrupted balance.

As we have dealt with in our thesis, the anesthesia used in surgery was shown as one of the main reasons for this imbalance. For instance, it is demonstrated that isoflurane induced MDA production and suppressed of GSH-Px and SOD enzyme activity in the brain (147). In addition, both ischemia-reperfusion injury and inflammation response are caused by ROS and are the leading causes of toxicity of anesthesia (148).

Anesthetic agents create anesthesia by affecting the CNS, especially the brain. For this reason, the brain is the main target of anesthetic agents, among the body parts (149). Postoperative cognitive dysfunction including delirium, declining in cognitive functioning, chronic impairment in memory, attention, and speed of information is one of the most common postoperative complications associated with brain damage. There is no effective treatment for that yet. However, the cause of the dysfunction has begun to be elucidated in order to develop new treatment methods. The new studies are aimed to discover new chemicals to treat and/or prevent dysfunction (150).

Recent animal studies advocate that the main reason for the development of cognitive dysfunction is the neuroinflammation resulting from volatile anesthetics under surgery (151, 152). Besides, volatile anesthetics especially isoflurane is reported to cause the neurotoxicity associated with Alzheimer's disease (153). The underlying mechanisms of isoflurane toxicity include *in vivo* and *in vitro* caspase-3 pathways activation (as a consequence of the accumulation of ROS, mitochondrial dysfunction, and reduction in ATP levels (154)), endoplasmic reticulum stress (ERS) (76) and overexpression of GSK3β (155). Furthermore many studies have shown that volatile anesthetic agents can raise the secretion of inflammatory cytokines in the brain (151, 156). The underlying reason of apoptosis (157) and neuroinflammation from overexpression of inflammatory cytokines is the formation of reactive oxygen radicals (150). The neuroinflammation may cause the synapse dysfunction resulting in cognitive dysfunction (150, 158). In the light of this information, Miao et al. (150) exhibited that ginsenoside Rb1, known as a neuroprotective agent inhibited neuroinflammation and ROS formation in H4 human neuroglia cells and also regulated isoflurane/surgery induced caspase-3 activation by ameliorating mitochondrial dysfunction. Thus, they demonstrate that cognitive dysfunction can be alleviated. The combination of Mg^{2+} and propofol (159), vitamin C (160) and glucose (154) also inhibited isoflurane / surgery-induced toxicity by the same pathway as in the previous study and the same *in vitro* cell culture model was used (159). These chemicals inhibited the toxicity by reducing the ROS production (154, 159, 160).

In addition to these, 2-Deoxy-D-Glucose was shown to diminish isoflurane / surgery-induced cytotoxicity and ROS level (161).

In another study of neurotoxicity caused by general anesthetics, rat pups at postnatal day 7 were applied to 1.5% isoflurane for 6 h along with elamipretide known to be antioxidant. In the brain of isoflurane administered groups ROS and level of an oxidant parameter namely, MDA levels were increased, while a decrease in SOD activity was detected. Treatment with elamipretide showed a protective effect against oxidative stress and mitochondrial damages, and also reduced isoflurane-induced cognitive deficits. According to research, the pediatric group receiving general anesthesia was thought to benefit from elampiride in patients (162). Another study on the use of anesthesia in the pediatric group compared desflurane and isoflurane. It was found that isoflurane was more likely to generate developmental neurotoxicity from desflurane. The toxicity occurred via GSK3β over-activation that is known to be inhibited by lithium. For this reason, these study suggested that lithium can reduce isoflurane-originated damage (155). Furthermore, since glycyrrhizin has an anti-inflammatory effect, has shown favorable results at inflammation induced by isoflurane in neonatal rodents (163).

Combination therapy of dexmedetomidine and isoflurane decreased the isoflurane/surgery-induced the alteration of TNF-α, IL-1β, MDA level, SOD activity and caspase-3 pathway thereby improve cognitive impairment (164). In mice a new antioxidant, hydrogen was investigated against isoflurane-originated ROS increase and cognitive impairment in terms of preventive effect. As a result, hydrogen showed preventive feature against neurotoxicity (165). As in the above examples, various antioxidant substances as salidroside (147), plumbagin (166), astragaloside IV (167) have exhibited a protective effect against isoflurane / surgery-induced neurotoxicity in many studies. Salidroside inhibited MDA production and ameliorated the suppression of GSH-Px and SOD enzyme activity by isoflurane (147). Astragaloside IV treatment like salidroside lowered the level of isoflurane‑induced MDA formation and regulated the depression of iNOS and SOD enzyme activity and NO production by isoflurane (167). For a similar purpose, in our thesis, we used the synthetic activator of the Cry gene, KL001 to reduce isoflurane-induced toxicity. According to our results, after administration the combination of KL001 and isoflurane, there was a decrease in the brain MDA level but the changes were not significant. When we added all the results to the account, we did not obtain significant changes between the groups for CAT and SOD activities. On the contrary, GSH-Px activity showed a significant increase in isoflurane

group compared with control ($p < 0.05$). But when we excluded the very different results, we found that KL001 depressed isoflurane-induced CAT and SOD enzyme activity in the group of KL001 and isoflurane ($p < 0.05$). Additionally, a significant increase of SOD enzyme activity was observed in the isoflurane administered group compared to the group KL001 ($p < 0.05$). In isoflurane group, the activity of GSH-Px significantly increased compared both the control and KL001 groups ($p < 0.05$). In summary, after excluding very different results, we found that KL001 significantly reduced the increased antioxidant enzyme activity caused by isoflurane in the brain ($p < 0.05$).

Besides, salubrinal was used as another substance for preventing anesthetic toxicity but showed a different pathway. Another toxicological pathway of anesthetics was considered ERS. ERS was thought as first response of cells under stress and related to neuronal death in neurodegenerative diseases. It has been shown that ERS was triggered by 1.3% 4h isoflurane-administered rats. Neuronal apoptosis was also induced. Pretreatment of salubrinal prevented overexpression of ERS-mediated apoptotic pathway (168).

In addition, mild hypothermia may help to cure isoflurane-induced DNA damage and cytotoxicity by reducing the isoflurane-derived depletion of ATP levels (153). In a study with a murine brain, low dose carbon monoxide was shown to prevent isoflurane-induced neurotoxicity that occurs via the oxidative stress associated mitochondrial apoptosis pathway (169). Similarly, in another study investigating the effect of carbon monoxide on isoflurane toxicity, it was demonstrated that isoflurane disrupted the function of cytochrome oxidase that plays a role in the mitochondrial electron transport chain. Low dose carbon monoxide restored this cytochrome and inhibited the formation of MDA (170). Isoflurane-induced cytosolic Ca increase is another toxicity pathway in the nervous system. Taking substances like high glucose increasing Ca flux with isoflurane increased the toxicity caused by anesthesia (171). Interestingly unlike these studies, some studies supported that inhaled anesthetic gases including isoflurane (172), and sevoflurane (173) attenuated oxidative stress and brain damage which was induced by ischemic and hemorrhagic stroke (157). For example, Li et al. (173) found that sevoflurane decreased the concentration of MDA level and SOD enzyme activity.

When we examine the issue from a circadian rhythm perspective, it seems that the strong association between anesthesia/surgery and disturbances in circadian rhythm is already known (9). Many studies with mice, rats and cell culture support that anesthesia impeded Per2 expression in the SCN reversibly. So Per2 could be considered as an

anesthetic effect biomarker. This effect was observed only in SCN, but not in the other organs (9, 174, 175). Kobayashi et al. (149) found that sevoflurane, applied for 2-6 hours, deteriorated the circadian rhythms and altered expression of circadian genes including Per2 in whole rat brain. Similarly, iv propofol caused depression in Per2 genes in the rat brain (176). Sevoflurane also inhibited light-induced expression of Per2 in mice SCN (81). In contrast to these studies, isoflurane was used instead of sevoflurane as an anesthetic agent. Inh isoflurane was applied during 4 hours. We preferred the Cry gene instead of Per gene and we aimed to trigger Cry gene/protein with synthetic Cry activator KL001. As a result, the Cry protein level in the rat brain was significantly decreased only in the isoflurane group, whereas The Cry protein level significantly increased in the combined administration of KL001 and the isoflurane ($p < 0.05$). So we found that isoflurane suppressed Cry protein in the rat brain. As a data of our study, we can also refer to the Cry gene/protein in addition to the Per gene/protein, which was thought to be the biomarker of brain anesthetics, as mentioned above.

One of the other receptors in the signal pathways of the circadian system is GABA receptor in the brain. Anesthesia increases $GABA_A$ receptor activity (9). It has been shown that sevoflurane could stimulate depolarization by $GABA_A$ receptor-associated intracellular signal transduction and raise Ca accumulation in the mice cell (9, 174). This situation led to a reduction of histone acetylation related to the E-box in the Per2 promoter region. Simultaneously a prevention of Clock-E-box interaction in the PER2 promoter region was observed. As a result, the expression of Per2 was inhibited via disruption of Clock status (174). Matsuo et al. (8) found that administration of both $GABA_A$ and $GABA_B$ receptor antagonists entirely stopped the inhibitory effect of sevoflurane on Per2 expression (8). Propofol known as GABA agonist suppressed Per gene expression in SCN (75).

It was suggested that melatonin was also used in the treatment of cognitive impairment due to isoflurane/surgery (177). This hormone belongs to the neurohormones synthesized by the pineal gland in the brain. While the light inhibits melatonin synthesis, the darkness stimulates the secretion (71). It is one of the primary hormones of circadian rhythm and can be synthesized endogenously and also taken from the outside. Melatonin can prevent the occurrence of major diseases such as cancer, Alzheimer's disease, a cardiovascular disease which are caused by dysfunction of circadian rhythm. Its receptors allow routine physiological functioning of cardiovascular systems, immune systems, and the brain. It is thought that this hormone can be used to cure in cognitive dysfunctions

because it improves learning, memory, and nerve root cell proliferation. Furthermore, it has been reported that anesthesia/surgery-induced circadian rhythm dysfunction may be more frequent in individuals with melatonin deficiency (177).

While this is the case in the brain, the liver as a major drug metabolism organ is another target of anesthetic agents. The relationship between halogenated inhaled anesthetic agents and hepatic damage has known about more than a half century. Chloroform is one of the first detected anesthetic agents to responsible for postoperative hepatotoxicity. Chloroform and ether left the place in halothane due to their high toxicity risk. Halothane had begun to be used in the 1950s. Although halothane was initially thought to be less toxic, it has been shown to cause fatal hepatotoxicity. Later discovered new generation halogenated anesthetics including enflurane, isoflurane and desflurane are also shown to lead similar damage, but less than halothane (73).

Hepatocytes are among the primary targets of anesthetics (78). Although case severity and frequency are variable, their toxicity (except sevoflurane) is characterized by nausea, lethargy, local hypoxia (73), fever, jaundice, rash, arthralgia, activation of the immune system via elevation of peripheral eosinophilia (73, 178). Rarely severe acute hepatitis (77), increased liver enzymes including alanine aminotransferase (ALT) and aspartate aminotransferase (AST) can be detected. These enzymes have been reported to increase after during 1 or 2 weeks following halothane exposure (179). Immunoallergic reaction (180) and apoptosis (80) are the underlying mechanisms of damage. These undesired effects generally occur with repeated anesthetic exposure (73).

On the other hand, toxicity in the liver depends on the biotransformation of the anesthetics by the enzymes in the liver. They undergo both oxidation (by CYP2E1 and CYP2A6) and reduction (by CYP2A6 and CYP3A4) reactions. The oxidation reaction many anesthetics results in the production of the trifluoroacetic acid metabolite, which causes immuno-toxicity in the liver, while the reduction reaction produces free radical. Specifically, CYP2E1 is responsible for the biotransformation of each halogenated anesthetic agents, and hepatic damage relates about how much an anesthetic agent is metabolized by this enzyme (20-30% for halothane, 2% for enflurane, 1% for sevoflurane, and 0.2% or less for isoflurane and desflurane) and the type and amount of their metabolites formed. Moreover being over forty years of age, obesity (because of accumulation of halothane in adipose tissue) and the use of drugs (phenobarbital, alcohol, isoniazid etc.) can induce CYP2E1 and increase the risk of hepatitis due to anesthesia

(73). In addition to these enzymes, GST is another enzyme responsible for the detoxification of anesthetic agents (74).

Although hepatotoxicity due to anesthesia is not yet prevented by current drug technology, efforts are being made to reduce it. For this purpose one of the new anesthetic agents, sevoflurane was produced. Sevoflurane does not induce an elevation in ALT and AST activity. The metabolism of sevoflurane does not result in the formation of trifluoroacetic acid. Instead of the toxic metabolite, less protein binding capability metabolite, hexafluoroisopropanol is formed. It does not accumulate and quickly passes phase II biotransformation, especially glucuronidation (73). Sevoflurane was shown to be more appropriate than isoflurane in inducing apoptosis in hepatocytes by Ruxanda et al. (181).

Inhaled anesthetic agents also stimulate severe hepatic insulin resistance which is another postoperative adverse outcome. The study conducted with isoflurane and sevoflurane showed that glucose metabolism and insulin resistance may alter by anesthesia (182).

In an experimental study with guinea pigs, halothane-induced hepatotoxicity, ROS defense system in hepatic tissue and probable protective role of vitamin E against the toxicity were investigated. In the halothane administered group, low activity of SOD and GSH-Px were found in hepatic tissue while the high activity of CAT and high concentration of MDA were encountered. Also in the same group, a high level of ALT and AST enzyme was obtained in serum. Vitamin E did not show any effect for present damage. However, it was argued that preoperative vitamin E could be used to prevent possible oxidative damage (183). When compared to isoflurane and sevoflurane-induced hepatotoxicity in patients, the increase in AST and ALT levels in the isoflurane-treated group was found even on the 7th postoperative day. The rise total bilirubin peaked 1st postoperative day in both isoflurane and sevoflurane groups. Additionally, sevoflurane was found to enhance hepatic arterial blood flow (184). In a study conducted on dogs, it was determined that sevoflurane reduced portal blood flow relative to isoflurane (185).

Serum and urine GST have been used in many studies due to the biochemical marker of anesthesia toxicity both in the liver and kidney. High serum level of GST indicated hepatic damage while high urine level of GST indicated renal damage and ROS formation in the kidney. Six different anesthetic agents as halothane, enflurane, sevoflurane, desflurane, propofol, and isoflurane were selected for a detailed investigation of the hepatocyte integrity deterioration resulting from anesthesia. As a

result of the study, it was stated that all the inhaled anesthetics agents increased the level of GST in the liver (74).

As already mentioned, acute hepatitis is a rare complication of anesthetics induced toxicity. However, according to the case report, acute hepatic failure could be created by repeated isoflurane administration (77). Kusuma et al. (186) supported severe acute hepatic failure could develop with repeated exposure of isoflurane. Another case report also notified that repeated administration of sevoflurane caused hepatic failure, with massive necrosis of hepatocytes (187). Exposure of isoflurane possibly induced liver necrosis and apoptosis. It was thought that the insulin‑like growth factor 1 as a new treatment approach could prevent isoflurane/surgery induced perioperative liver damage (188).

As shown in the examples in various animal species studies and case reports prove a very good relationship between hepatotoxicity and anesthesia. On the contrary, in some studies with sevoflurane, the opposite data is found. Sevoflurane has been shown not to cause hepatic impairment (189). Amazingly, some new researchers indicated that sevoflurane might have protective property against hepatic ischemia/reperfusion damage in liver surgery or liver transplantation (73). An exciting study of liver donors with undergoing hepatectomy was performed in terms of the antioxidant effect of two different anesthetic agents, isoflurane and propofol. It is known that surgery-induced ischemia-reperfusion injury and surgical stress generate ROS. In the direction of this information, they evaluated SOD, MDA, total oxidative status (TOS), total antioxidant capacity (TAC), and oxidative stress index (OSI) in donors which were exposed to isoflurane or propofol. Decreased levels of MDA and TAC in propofol applied groups in patients undergoing hepatectomy suggested that propofol has antioxidant effects. Isoflurane increased in OSI value, TAC, and MDA level but did not affect SOD in plasma. Nevertheless, the decreased SOD activity was found in erythrocytes (190). In another similar study, instead of isoflurane, desflurane and propofol were compared. Unlike the previous study dropped the level of MDA was not found in propofol exposure group. Nevertheless, propofol was reported to be a protective effect of hepatic injury and hepatic function when compared to desflurane (191). It was demonstrated that inhaled isoflurane had hepatotoxic effect while iv form of isoflurane showed a protective effect against hepatic damage via decreasing level of MDA and activity of SOD (80). The iv form of isoflurane also exhibited the same protective effect on the kidney. This issue will be mentioned later. On the other hand, we obtained interesting results in the liver. The

interesting part was that while we were waiting for the level of MDA to increase in the group of isoflurane, we determined a significant decrease when we excluded the very different results ($p < 0.05$). Furthermore, we found that CAT enzyme activity in the isoflurane group also significantly decreased with respect to the control group ($p < 0.05$). This made us thought that isoflurane could suppress CAT enzyme activity. Again, the SOD activity of isoflurane group reduced but it was not significant. When we excluded the very different results, only a significant increase in GSH-Px activity was observed in the isoflurane treated group compared to the control and KL001 groups ($p < 0.05$). These unexpected results suggest that isoflurane may be due to a low percentage of liver metabolism compared to other anesthetic agents. From a different perspective, these results suggest that isoflurane may exhibit hepatoprotective properties, as in some other studies. However, another confusing point is that isoflurane reduced Cry protein of liver in our study ($p < 0.05$). That is, we expect that the isoflurane, which affects the circadian rhythm, will also destroy the oxidative stress parameters.

When we look at circadian gene-based effects of anesthetics, we are faced with surprising results. Sakamoto et al. (192) studied not only the brain but also the clock genes in peripheral organs. They demonstrated that sevoflurane influenced the expression of 1.5% of 10,000 genes including circadian genes, Per2, Cry2, Per3, Bmal1, Clock, Rev-erb a, by using the microarray technology in brain, lung, spleen, blood, heart, liver, and kidney. Exposure to sevoflurane did not affect Per2 expression in the liver. Kubo et al. (75) studied the effect a veterinary anesthetic agent, 2,2,2-tribromoethanol on the liver circadian clock. Exposure of this anesthetic agent decreased the expression of Rev-erb-a, Bmal1, and Clock, but not Per2 in the liver.

In another study investigating the circadian rhythm effect of isoflurane, isoflurane was administered to the rats divided into two groups at night and daytime. The expression of Bmal1, Clock, Per2 and Cry2 genes were found to be repressed obviously in daytime administration group both brain and liver. These circadian genes were slightly influenced by isoflurane at night time administration group. The toxicity of isoflurane was demonstrated to be light dependent. (193). For this reason, isoflurane, which shows light-dependent toxicity, was applied daytime in our study. Nevertheless, we implemented the Cry activator substance, KL001 at night, considering that the mice day and night cycle is different from humans. As a result of the liver, we found that the amount of Cry protein significantly decreased in the isoflurane given group when compared to other groups ($p < 0.05$). According to this result, we could interpret that isoflurane depresses the

expression of Cry protein in the liver. We also found that when we added KL001 in addition to isoflurane treated group, Cry protein significantly increased ($p < 0.05$). For this reason, we could say that KL001 elevated the level of low Cry protein from isoflurane-induced in the liver. As a further study, we think that this may be clarified by repeated dose application of isoflurane.

The renal system encounters chemicals due to its excretion function. Proximal tubular cells of the renal system are one of the other targets of anesthetics. The fluorinated anesthetics such as isoflurane, sevoflurane and desflurane could play a role in the development of acute renal failure due to a decrease in arterial blood pressure. This type of nephrotoxicity was thought to be caused by anesthetics induced oxidative stress (78, 89). For this purpose, Durak et al. (78) evaluated oxidative stress parameters in guinea pigs' renal tissue treated with isoflurane. SOD, CAT, and GSH-Px enzymes, non-enzymatic $O_2^{-\cdot}$ scavenger activity (NSSA) and oxidation resistance (OR) and, MDA level were measured. NSSA, SOD and CAT activities were declined whereas MDA level, OR quantity and GSH-Px activity raised in isoflurane treated group. Hereby, their outcomes demonstrated that isoflurane caused damage of not only enzymatic antioxidant defense systems but also nonenzymatic antioxidant defense systems in renal tissue. They suggested that antioxidant therapy may be beneficial. Unlike this examples, Elena et al. (189) obtained that sevoflurane did not alter renal function. In addition to the administration of isoflurane to the inhalation, there are also studies on different routes of administration such as iv. For instance, Qin et al. (194) examined the antioxidant effects of the new iv isoflurane form on the renal tissue in rats. Levels of interleukin-6 and MDA and SOD activity was decreased by iv form of isoflurane. According to the result of that study, iv form of isoflurane might be useful against ischemia/reperfusion damage of renal tissue and may also reduce inflammation and show antioxidant properties (194). On the other hand, in this thesis, we investigated that isoflurane could cause oxidative stress in the kidney. We did not get any significant data when we evaluated all the results. When we excluded the very different results, we could not obtain a significant change in MDA level and GSH-Px activity in the kidney but we found a significant decrease in the activity of both CAT and SOD enzymes in the isoflurane group where KL001 participated in compared only isoflurane treated group ($p < 0.05$). And also high SOD activity was significantly found in isoflurane group comparing with the control group ($p < 0.05$). Our results suggest that isoflurane could not cause a serious oxidative stress on the kidney.

One of the severe complications of anesthesia/surgery is hypotension which may occur during operation. This situation is associated with damage to heart, kidney, and brain and increases postoperative morbidity and mortality rates, especially in risky patient groups. In the study on this subject, norepinephrine was administered with general anesthesia to the patient for arterial pressure control during the operation. Controlled arterial blood pressure was found to reduce the renal damage that can be caused by anesthesia/surgery (195).

As shown in many studies, glycosuria and proteinuria are related to exposure volatile anesthesia including sevoflurane, isoflurane, and desflurane. They could be thought as a renal biomarker of proximal tubule impairment induced by volatile anesthetics (196).

There are a few studies on the protective effect of volatile anesthetics on the kidney, which is still one of the controversial topics (196). The effect of xenon from volatile anesthetics on renal function in partial nephrectomy in patients with renal cancer was investigated. Xenon showed protective property on kidney (197). Similarly, isoflurane also was exhibited protection property against renal ischemia/perfusion by Menting et al. (198).

In addition, the kidney is an organ that has important functions for the circadian rhythm at the same time. It is responsible for the secretion of many steroid hormones in the event of daily rhythm or stress. Some of this hormone is cortisol and corticosterone which belong glucocorticoid hormones. They are controlled by a pituitary adrenocorticotropic hormone, and these hormones play a role in circadian rhythm (71). The human studies exhibited that 30-45 min exposure of propofol was related to a decline in plasma cortisol secretion in patients entering various operations (199, 200). The serum corticosterone secretion was increased by propofol in mice (201). In another study, the exposure to sevoflurane and isoflurane raised cortisol secretion for up to 12 hours postoperatively in-patient, but adrenocorticotropic hormone secretion was raised the only interoperation. Another secreted hormones from the adrenal gland, epinephrine and norepinephrine were increased by isoflurane exposure both inter and post operation. These same hormones increased postoperatively in sevoflurane exposure (202). Therefore it was considered that disturbance of hormone secretion by administration of anesthetic agent could cause deterioration of the circadian rhythm. On the other hand, it may be thought that circadian rhythm may also be disturbed by application of anesthetic agents, result in alteration hormone synthesis. But in contrast to other studies, we found that

isoflurane had no significant effects on the oxidative stress parameters and did not alter the Cry level.

Another target structure in the body in terms of anesthetic agents is blood. Oxidative damage of halothane, enflurane, and isoflurane were assessed in erythrocyte and plasma. While halothane and enflurane induced to increase GSH-Px activities in plasma, on the other hand, they decreased SOD and GSH-Px activities in erythrocytes. Isoflurane did not effect on plasma SOD and GSH-Px activities, but reduced SOD activity in erythrocyte (203). Contrary to these findings, in our present study, the isoflurane significantly increased SOD activity in erythrocyte samples compared to the control group ($p < 0.05$). Decreased MDA level was obtained in isoflurane administered group ($p < 0.05$). When KL001 was applied to the isoflurane treated group, it was found that the SOD activity significantly decreased in our results when the different values were excluded ($p < 0.05$). As a result, SOD enzyme activity was increased by isoflurane in erythrocyte samples and we concluded that KL001 suppressed this increased activity. When we look at the effect of isoflurane on the circadian rhythm in the plasma, it causes a significant decrease of the Cry protein to decrease as in other examined organs ($p < 0.05$), resulting in deterioration of the circadian component.

In another study with blood samples, exposure to isoflurane in rats resulted in hyperglycemia, high blood pressure, deterioration of circadian rhythms, and impaired blood concentration of melatonin, potassium, cortisone, lactate, and leptin (204).

Meantime interestingly it was demonstrated that some volatile anesthetics could generate oxidative stress in some organs, whereas in others they could reduce oxidative stress. For example, when the effect of isoflurane in rats with acute respiratory distress syndrome is compared with sevoflurane, sevoflurane had no effect on lung inflammation in intraperitoneally administration group, but it did reduce lung inflammation in an intratracheal group. Sevoflurane reduced inflammation by decreasing IL-6 expression. Over and above, sevoflurane diminished static lung elastance, alveolar collapse, inflammation, oxidative stress, and epithelial cell damage. Contrary to sevoflurane, isoflurane shown an enhancing effect on inflammation (205). Inflammation of airway is a severe situation. It can lead to further lung problems such as asthma and chronic obstructive pulmonary disease (148). Sevoflurane and isoflurane were found to inhibit hypoxic pulmonary vasoconstriction and hypoxemia, although the mechanism was not fully elucidated. However, some studies have concluded that lung hypoxemia inhibition

does not interfere with MDA production. In that situation, decreased SOD activity and NO concentration were revealed (206).

In a study comparing xenon and isoflurane for cardiovascular changes, xenon did not affect contractility while isoflurane declined the contractile index. Thus, xenon was suggested to be more suitable for use for cardiac stability (207).

The oxidative/antioxidative effects of desflurane and sevoflurane were compared with various rat tissue in vivo. When looking at the MDA levels in different tissues, reduced MDA level was found in the liver, kidney, brain in the group of sevoflurane and the only liver of the desflurane group. But increased MDA level was found in the lung of both anesthetic exposed groups. As for SOD, in the desflurane group, there was an increase in the brain and a decrease in the liver. In the sevoflurane group, there was no change in SOD activity. In respect of GSH-Px, it was determined reduced activity in kidney only the group of sevoflurane (148). For reasons like these mentioned, in clinical practice anesthetic choices are addressed by considering possible anesthetic toxicities.

In conclusion, the toxicity of isoflurane in the viewpoint of oxidative stress and the effect of co-administration of KL001 were researched in this thesis. Moreover, the possible relationship between isoflurane and the change in Cry gene/protein expression were investigated. Our aim was to reduce oxidative stress induced by isoflurane using Cry activator, KL001. Our results showed decreased MDA level in erythrocyte cells and liver tissue, increased GSH-Px activity in liver and brain tissues, increased SOD activity in erythrocyte cells, kidney, and brain tissues, decreased CAT activity in liver tissue in isoflurane group. According to our findings, isoflurane changed the oxidative stress parameters remarkably in brain tissue. The underlying mechanism of toxicity of isoflurane is a metabolism of volatilized anesthetics, that is, it forms a toxic metabolite. This suggests the isoflurane was subjected to the extrahepatic metabolism, especially in the brain tissue. In addition, a decreased level of Cry in the plasma, liver, and brain tissues were observed in isoflurane group indicating rhythm deterioration by acting on the Cry gene/protein. Cry gene/protein could be considered as one of the biomarkers of isoflurane toxicity. It was determined that the KL001 increased Cry level inhibited by isoflurane. Besides, KL001 also decreased the isoflurane-induced oxidative stress. As a possible pathway, the CYP2E1 enzyme responsible for the isoflurane metabolism and the toxic metabolite formation is inhibited by the Cry gene/protein. In addition, Cry also induces GST enzyme which is responsible for the detoxification of volatile anesthetic agents. We thought that oxidative stress could also be prevented by this pathway. For this purpose, it

would be helpful to investigate CYP2E1 and GST enzyme levels in further studies to elucidate the toxicity mechanism of the volatile anesthetic agents. The investigation of the Cry effect on toxicity pathways of isoflurane, including apoptosis pathways involving caspase-3 and GSK3β, as well as an expression of inflammatory factors including cytokines, TNF-a and IL-6 will provide important data to reduce the potential side effects of the isoflurane. Also, the effect of Cry on the toxicity of other anesthetics such as sevoflurane can also be included in the further study. Our data indicated that isoflurane-induced oxidative stress could be reversed with the circadian rhythm modification. This can be achieved to reduce the potential oxidative stress of isoflurane or other the anesthetic agents and medications by triggering the Cry level with a synthetic activator or by arranging drug administration time according to over-expressing of Cry gene at certain period of the day. Especially in early hours of the morning, KL001 may be protective against isoflurane-induced oxidative stress. Besides, in further research, the effects of other circadian genes/proteins including Per, Clock, and Bmal1 on anesthetic agents toxicity should also be investigated. It is necessary to determine at which time of day the other circadian gene/proteins reach the peak level. In this way, it can be determined at which time the operations are performed to create the least toxicity.

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7. APPENDICES

7.1. Ethical Approval

YEDİTEPE ÜNİVERSİTESİ

T.C. YEDİTEPE ÜNİVERSİTESİ, DENEY HAYVANLARI ETİK KURULU
(YÜDHEK)

ETİK KURUL KARARI

Toplantı Tarihi	Karar No	İlgi	Proje Yürütücüsü
25.07.2016	548	22.07.2016	Uzm.Ecz.Feyza KELLECİ

'Sirkadiyen Ritim Aktivatörleri ile Genel Anestezik Maddeler Arasındaki İlişkinin Toksikolojik Açıdan Araştırılması' adlı bilimsel çalışma etik kurulumuzda görüşülmüş olup, çalışmanın etik kurallara uygun olduğuna oy birliğiyle karar verilmiştir.

Etik Onay Geçerlilik Süresi: 2 Yıl	**Hayvan Türü ve cinsiyeti:** Fare ♂♀	**Hayvan Sayısı:** 72

GÖREVİ	ADI SOYADI	İMZA
Başkan	Prof. Dr. M. Ece GENÇ	
Başkan Yardımcısı	Prof. Dr. Erdem YEŞİLADA	
Raportör	Prof. Dr. Işıl AKSAN KURNAZ	
Üye	Prof. Dr. Bayram YILMAZ	
Üye	Prof. Dr. Başar ATALAY	
Üye	Doç. Dr. Soner DOĞAN	
Üye	Doç. Dr. Ediz DENİZ	
Üye	Doç. Dr. C. Narter YEŞİLDAĞLAR	KATILMADI
Üye	Sumru KİRAZCI	

8. CURRICULUM VITAE

Adı	Feyza	**Soyadı**	Kelleci
Doğum Yeri	Karaman	**Doğum Tarihi**	04.05.1987
Uyruğu	T.C.	**TC Kimlik No**	69769156830
E-mail	feyza-kelleci@hotmail.com	**Tel**	05059147210

Öğrenim Durumu

Derece	Alan	Mezun Olduğu Kurumun Adı	Mezuniyet Yılı
Doktora	Farmasötik Toksikoloji	Yeditepe Üniversitesi	2018
Yüksek Lisans	-	-	-
Lisans	Eczacılık Fakültesi	Yeditepe Üniversitesi	2012
Lise	-	Karaman Anadolu Lisesi	2005

Başarılmış birden fazla sınav varsa(KPDS, ÜDS, TOEFL; EELTS vs), tüm sonuçlar yazılmalıdır

Bildiği Yabancı Dilleri	Yabancı Dil Sınav Notu (#)
İngilizce	60 (YDS)

İş Deneyimi (Sondan geçmişe doğru sıralayın)

Görevi	Kurum	Süre (Yıl - Yıl)
Eczacılık Müzesi Sorumlusu	Yeditepe Üniversitesi	2015-2018
İlaç ve Zehir Danışma Koordinatörlüğü	Yeditepe Üniversitesi	2012-2018
Araştırma görevlisi	Yeditepe Üniversitesi	2012-2018

Bilgisayar Bilgisi

Program	Kullanma becerisi
Window Ofis Programları	Çok iyi

*****Çok iyi, iyi, orta, zayıf olarak değerlendirin

Bilimsel Çalışmaları

SCI, SSCI, AHCI indekslerine giren dergilerde yayınlanan makaleler

- Algul D, Sipahi H, Aydin A, Kelleci F, Ozdatli S, Yener FG. Biocompatibility of biomimetic multilayered alginate-chitosan/beta-TCP scaffold for osteochondral tissue. Int J Biol Macromol. 2015 Aug;79:363-9.

Diğer dergilerde yayınlanan makaleler

• Dursun D, Kelleci F, Aydın A. Biyolojik ve Biyobenzer İlaçlarda Güvenlilik Değerlendirmesi. Turk Farmakope Dergisi. 2(2). (2017)
• Sarı TE, Kelleci F, Charehsaz M, Aydın A. Possible Health Risk of Electromagnetic Fields. Acta Pharm. Sci. 55(3):81-92 (2017).
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Uluslararası bilimsel toplantılarda sunulan ve bildiri kitabında (*Proceedings*) basılan bildiriler

• Sarı B, Kelleci F, Efeoglu E, Charehsaz M, Sipahi H, Culha M, Aydin A. Oxidative stress after exposure to gold nanoparticles in rat. Does oligonucleotide coating change its effects? EUROTOX 2017 53rd Congress of the European Societies of Toxicology, 10-13 September 2017, Bratislava.
• Charehsaz M, Hougaard KS, Sipahi H, Kelleci F, Ekici AID, Kaspar Ç, Çulha M, Bucurgat ÜU, Aydın A. Reproductive and Developmental Toxicity of Silver Nanoparticles. International Nanotoxicology Congress, 23-26th April 2014, Antalya, Turkey.
• Charehsaz, M, Hougaard KS, Sipahi H, Kelleci F, Ekici AID, Kaspar Ç, Çulha M, Bucurgat ÜU, Aydın A. Developmental Toxicity of Silver Nanoparticles. 10th Nanoscience and Nanotechnology Conference, 17-21 June 2014, İstanbul. (Sözlü sunum).
• Algul D, Kelleci F, Ozdatli S, Sipahi H, Yener FG. Biocompatibility of biomimetic multilayered alginate-chitosan/beta-TCP scaffold for osteochondral tissue. 17th International Pharmaceutical Technology Symposium, 8-10 September 2014, Antalya, Turkey (Abstract Book p 52). (Kontrol No: 1171997
• Ozdatlı S, Kelleci F, Chareshaz M, Sipahi H, Aydın A. Gold Nanoparticles Induce Cytotoxicity: A Review of In Vitro and In Vivo Studiy, 1st International Congress and Workshop of Forensic Toxicology. 29-30 November 2014, Ankara.

Hakemli konferans/sempozyumların bildiri kitaplarında yer alan yayınlar

• Kelleci F. Yeditepe Üniversitesi, Eczacılık Fakültesi Müzesi. III. Uluslararası Üniversite Müzeleri Birliği Platformu. Trakya Üniversitesi, Edirne, 2016.

Diğer (Görev Aldığı Projeler/Sertifikaları/Ödülleri)

Projeler
• Kelleci F, Güldağ G, Aslan I, Duman G. Characterization Studies of Hydrogels. Haziran 2012.
• Sosyal sorumluluk projesi kapsamında Yeditepe Üniversitesinde ‘’Kullanılmayan atık ilaçların toplatılması’’ projesinde proje sorumlusu olarak görev alınmıştır. Ocak-Ağustos 2017.
Kurslar
• Yeditepe Üniversitesi Deney Hayvanları Kursu
• Tubitak, Workshop on 3Rs, 2D, 3D Liver, Eye, and Gastrointestinal Regulatory Models