REPUBLIC OF TURKEY
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DEPARTMENT OF PHARMACEUTICAL TOXICOLOGY

TOXICOLOGICAL EVALUATION OF COMBINED EXPOSURE TO BISPHENOL A AND DIETHYL HEXYL PHTHALATE AND INVESTIGATION OF POSSIBLE PROTECTIVE EFFECT of CARNOSIC ACID

DOCTOR OF PHILOSOPHY THESIS

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APPROVAL

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 ..... and numbered ..2018/16-03

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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.

Date

Signature

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ABSTRACT

Özdatlı K. Ş. (2018) Toxicological Evaluation of Combined Exposure to Bisphenol A and Diethyl Hexyl Phthalate and Investigation of Possible Protective Effect of Carnosic Acid. Yeditepe University, Institute of Health Science, Department of Pharmaceutical Toxicology, Ph.D. thesis, İstanbul.

DEHP and BPA, the main components of plastic products, are the important endocrine-disrupting chemicals which can mimic or disrupt the actions of estrogen by inhibiting normal functions of the body. Because of this widespread usage of DEHP and BPA, not only alone exposure but also combined exposure is considered as an important issue. Nowadays, researches are increased related to the effects on neurological functions instead of endocrine disrupting effects. This study aims to investigate combined exposure of DEHP and BPA and the possible protective effect of carnosic acid as an antioxidant. In this study, C-57BL/6 mice aged 16 weeks (25 g to 35 g of body weight) were randomly divided into 15 groups (6 mice/group) as BPA (16, 5, 1.6 mg/kg), DEHP (16, 5, 1.6 mg/kg), 20 mg/kg CA, BPA+DEHP (16, 5, 1.6 mg/kg) and BPA+DEHP+20 mg/kg CA (16, 5, 1.6 mg/kg). Chemical exposure was assessed for subacute toxicity and application was made for 28 days by oral gavage. The animals were sacrificed 24 h after the last administration. Before and after drug administration passive avoidance and accelerated rotarod tests were conducted to examine the effects on behavior. And also, level of MDA, GSH-Px, CAT and SOD activities in the cortex were analyzed. Then Bcl-2, Bax, BACE and p-tau levels in the cortex were detected by western blot. There was not observed any change in passive avoidance and rotarod tests. The SOD activity of all dosage of BPA+DEHP and BPA+DEHP+20CA significantly decreased according to control. In CAT activity, while combined administration of 5 and 16 BPA+DEHP significantly increased as compared with control, combined administration of 1.6 and 16 BPA+DEHP+20CA significantly decreased as compared with 16 BPA and 1.6 DEHP. The same changes were observed in GSH-Px activity levels of BPA+DEHP+20CA and BPA+DEHP groups like CAT activity. Although there were no significant changes observed in BPA and DEHP groups, combined administration of BPA+DEHP caused a significant decrease in MDA levels according to control. While Bcl-2 and Bax levels were significantly increased at combined administration of BPA+DEHP, addition of CA to BPA+DEHP combination caused significantly decrease as compared to BPA+DEHP administration. Although the level of BACE enzyme expression significantly increased

in combined administration of BPA+DEHP, BACE levels were significantly decreased in the combined administration of BPA+DEHP+20CA. However, there was an opposite effect between dose and response. And also, in our studies, no relevance was observed in the caspase-3, APP and amyloid beta tests. While p-tau levels of BPA+DEHP groups were significantly lower than other groups, p-tau levels of BPA+DEHP+20CA groups significantly increased compared to combined administration of BPA+DEHP groups. In conclusion, when BPA and DEHP used together, they damaged the cortex in terms of neurodegeneration by oxidative stress and also carnosic acid has been shown protection by radical scavenging properties.

Key words: **BPA, DEHP, Combined, Neurotoxicity, Carnosic acid**

This study was supported by Medipol University BAP (Project no: 201704).

ÖZET

Özdatlı K.Ş. (2018) Bisfenol A ve Dietil Hegzil Ftalata Kombine Maruz Kalmanın Toksikolojik Açıdan Değerlendirilmesi ve Karnozik Asidin Muhtemel Koruyucu Etkilerinin Araştırılması. Yeditepe Üniversitesi Sağlık Bilimleri Enstitüsü, Farmasötik Toksikoloji ABD., Doktora Tezi. İstanbul.

Plastik ürünlerin ana bileşenlerinden olan DEHP ve BPA östrojenin etkilerini taklit ederek veya bozarak vücut üzerindeki etkilerini inhibe eden önemli endokrin bozucudardır. BPA ve DEHP'nin bu kadar yaygın kullanılmasından dolayı tek başlarına maruz kalma değil aynı zamanda kombine maruz kalma da önemli bir konu olarak kabul edilmektedir. Günümüzde endokrin bozucu etkilerinin yanı sıra nörolojik fonksiyonlar üzerindeki etkileri ile iligili araştırmalar da artmaktadır. Bu çalışmada BPA ve DEHP'ye kombine maruz kalmanın toksikolojik açıdan değerlendirilmesi ve karnozik asidin muhtemel koruyucu etkilerinin araştırılması amaçlanmıştır. Bu çalışmada, 16 haftalık (25 ila 35 g vücut ağırlığı) C-57BL / 6 fareleri BPA (16, 5, 1.6 mg/kg), DEHP (16, 5, 1.6 mg/kg), 20 mg/kg CA, BPA+DEHP (16, 5, 1.6 mg/kg) ve BPA+DEHP+20 mg/kg CA (16, 5, 1.6 mg/kg). olacak şekilde rasgetle 14 gruba (6 fare/grup) ayrılmıştır. Kimyasal maruziyet subakut toksisite açısından değerlendirilmiş ve 28 gün boyunca oral gavaj yoluyla ilaç uygulaması yapılmıştır. Son uygulamadan 24 saat sonra fareler dekapite edilmiştir. İlaç uygulamasına başlanmadan önce ve sonrasında davranış üzerindeki etkileri incelemek için farelere hızlandırılmış rotarod ve pasif sakınma testleri yapılmıştır. Ayrıca kortekste MDA seviyesi ile GSH-Px, CAT ve SOD aktiviteleri incelenmiştir. Daha sonra korteksteki Bcl-2, Bax, BACE ve p-tau seviyeleri western blot yöntemi ile tespit edilmiştir. Pasif sakınma ve rotarod testlerinde anlamlı bir sonuç elde edilememiştir. BPA+DEHP ve BPA+DEHP+20CA'nin bütün gruplarında SOD aktivitesi kontrol grubuna göre anlamlı olarak azalmıştır 5 ve 16 BPA+DEHP gruplarının CAT aktivitesi kontrole kıyasla anlamlı şekilde artarken, 1.6 ve 16 BPA+DEHP+20CA gruplarının CAT aktivitesi 16 BPA ve 1.6 DEHP gruplarına kıyasla anlamlı şekilde azalmıştır. BPA+DEHP ve BPA+DEHP+20CA'nin bütün gruplarının GSH-Px aktivitelerinde CAT aktivitesine benzer değişimler gözlemlenmiştir. Her ne kadar BPA ve DEHP gruplarında anlamlı bir değişim olmamasına rağmen, BPA+DEHP beraber verildiği gruplarda MDA seviyeleri kontrole karşı anlamlı bir şekilde azalmıştır. BPA+DEHP kombine verildiği grupların Bcl-2 ve Bax seviyeleri anlamlı şekilde artarken; karnozik asidin BPA+DEHP kombinasyon gruplarına eklenmesi BPA+DEHP

gruplarına kıyasla anlamlı bir azalmaya sebep olmuştur. Her ne kadar BPA+DEHP kombine veridiği grupların BACE enzim ekspresyon seviyesi anlamlı ölçüde artarken BPA+DEHP+20CA kombine veridiği grupların BACE enzim ekspresyon seviyesi anlamlı şekilde azalmıştır. Fakat doz-cevap arasında ters etki vardır. Ayrıcaçalışamalarımızda kaspaz-3, APP ve amiloid beta (1-42) testlerinde herhangi bir ilişki görülmemiştir. BPA + DEHP gruplarının p-tau düzeyleri diğer gruplardan anlamlı derecede düşük olmakla birlikte, BPA + DEHP + 20CA gruplarının p-tau seviyeleri, BPA + DEHP gruplarına kyasla anlamlı olarak artmıştır. Sonuç olarak, BPA ve DEHP beraber verildiği zaman, kortekste oluşan nörodeneretif hasarın oksidatif stress kaynaklı olduğu ve karnozik asidin radikal süpürücü özelliği ile koruma sağladığı görülmüştür.

Anahtar Kelimeler: **BPA, DEHP, Kombine, Nörotoksisite, Karnozik asit**

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LIST OF SYMBOLS AND ABBREVIATIONS

AD	Alzheimer
ALS	Amyotrophic Lateral Sclerosis
BACE	B-Secretase
BPA	Bisphenol A
CA	Carnosic Acid
CAPS	3-(Cyclohexylamino)-1-Propane Sulfonic Acid
CAT	Catalase
DEHP	Diethyl Hexyl Phthalate
DTNB	5,5'-Dithiobis-2-Nitrobenzoic Acid
EDC	Endocrine-Disrupting Chemicals
EDTA	Ethylenediaminetetraacetic Acid Disodium Salt Dihydrate
GSH-Rd	Glutathione Reductase
GSHG	Oxidized Glutathione
GSH-Px	Glutathione Peroxidase
GSSG	Oxidized Glutathione
HD	Huntington's Disease
HMW	High Molecular Weight Compounds
IR	Immunoreactive
LC_{50}	Lethal Concentration 50
LD_{50}	Lethal Dose 50
LDL	Lipoproteins
LDS	Lithium Dodecylsufate
LMW	Low Molecular Weight Compounds
MBP	Monobutyl Phthalate
MBzP	Monobenzyl Phthalate
MEHHP	Mono-2-Ethyl-5-Hydroxyhexyl
MEHP	Monoethyl Hexyl Phthalate
MEHP	Mono-2-Ethylhexyl
MEOHP	Mono-2-Ethyl-5-Oxohexyl Phthalate
MEP	Mono-Ethyl Phthalate

MMP	Mono-Methyl Phthalate
MS	Multiple Sclerosis
NAD^+	Nicotinamide Adenine Dinucleotide
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NOAEL	No Observed Adverse Effect Level
PD	Parkinson's Diseases
PP	Postpartum
PVC	Polyvinyl Chloride
$R^{\cdot}$	Lipid Radicals
$RO^{\cdot}$	Alloxyl
$RO_2^{\cdot}$	Peroxyl
$ROO^{\cdot}$	Lipid Peroxyl Radical
ROONO	Alkyl Peroxynitrites
ROS	Reactive Oxygen Species
SDS	Dodecyl Sulfate Sodium Salt
Se-GSH-Px	Selenium-Dependent GSH-Px
SOD	Superoxide Dismutase
TBA	Tribromo Acetic Acid
TCA	Trichloro Acetic Acid
Vitamin C	Ascorbic Acid
XOD	Xhantin Oxidase

1. INTRODUCTION AND AIM

Endocrine-disrupting chemicals (EDC) are man-made or natural chemicals which can mimic actions of estrogen or disrupt by inhibiting the normal function of the body. There are many materials used in everyday life particularly medical products like plastic materials, foods, cosmetic products, therefore lifelong exposure is concerned. Recently, exposure to these products is dramatically increasing. Diethyl hexyl phthalate (DEHP) and bisphenol A (BPA) have an important place among exposed chemical substances. Nowadays, effects of DEHP and BPA on the health have become vitally important. Because of this widespread usage of DEHP and BPA, not only alone exposure but also combined exposure is considered as an important issue. Although the studies are mostly about endocrine disrupting effects on hormonal levels, researches related to their effects on neurological functions are increased. Most of the reported studies showed that both BPA and DEHP exposure have toxic effects on neurological development during pregnancy, prenatal and postnatal stages and also same studies on adult mice and rats showed that cerebral cortex and hippocampus are adversely affected by insulin homeostasis, neurogenesis, learning and memory functions that cause neurodevelopmental toxicity which has been reported to be a risk factor for neurodegenerative disorders (Alzheimer's, Parkinson's, etc.)[1-12].

In the literature, there are lots of studies about health protective effects of food components which are important for human health. In this regard, studies on the protective effect of rosemary against neurotoxicity have remarkably increased. These studies are also included carnosic acid (CA) which is obtained from rosemary[13–15]. CA prevents neurotoxicity such as amyloid- β induced Alzheimer's disease and 6-hydroxydopamine induced Parkinson's disease by preventing the formation of oxidative stress[16–20]. However, the content of these studies is not sufficient to clarify the mechanism showing the protective effect of rosemary on neurotoxicity. This study aims to investigate toxicity which occurs as a result of the combined exposure of DEHP and BPA and also aims to investigate the possible effect of CA in preventing this toxicity.

2. GENERAL INFORMATION

While the use of plastic is increasing every day, the use of polyvinyl chloride (PVC) in plastics is also increasing due to its low cost and durability. Endocrine disruptor chemicals (EDCs) are natural and synthetic compounds, exogenously taken, disrupting endocrine functions and possibly cause adverse effects in the organism. A definition for a potential endocrine disruptor was given alongside the OECD Endocrine Disrupters Testing and Assessment (EDTA) meeting in April 2011. "A possible endocrine disruptor is a chemical that is able to alter the functioning of the endocrine system but for which information about possible adverse consequences of that alteration in an intact organism is uncertain" [21]. EDCs have agonist and/or antagonist effects on hormones by mimic or block them. This effect may occur by affecting the production or transporting of hormones or by binding hormone receptors by altering its metabolism and elimination. The EU report on EDCs published in 2002 contains 60 endocrine disrupting substances that are clearly shown to be harmful to the environment and human health in hundreds of chemical substances. Some of the important EDC may be listed as phthalates (diethyl phthalates, dihexyl phthalates, di-propyl phthalates), industrial products (bisphenol A, polybrominated biphenyls), pesticides (carbaryl), heavy metals (arsenic, cadmium, mercury, uranium and lead), phytoestrogens (daidzein, genistein, formononetin, equol), organohalogens (dioxins, furans, pentachlorophenol) and some drugs (contraceptives, cimetidine, diethylstilbesterol). Here, bisphenol A and diethyl hexyl phthalate will be discussed in detail[22].

2.1. Bisphenol A

Although Bisphenol A (BPA) which is named as chemically 2,2-bis(4-hydroxyphenyl)propane was firstly synthesized in 1891 and announced as synthetic estrogenic agent in the 1930s[23], it remained at backplane because of stronger estrogenic activity of diethylstilbesterol up to the 1950s. BPA is an organic synthetic compound consisting of two hydroxyphenyl groups in its structure. It belongs to the family of diphenylmethane derivatives and bisphenols. BPA is colorless solid soluble in organic solvents and it has been commercially available in the market since 1957[24]. BPA is the most widely used industrial chemical to produce plastic containing polycarbonate, polymers and epoxy resins. Generally, it is used in as a protective coating in food and beverages containers, in adhesives, toothpaste, flame retardants, digital and electronic

materials[25, 26]. While polycarbonate plastics are widely used in the production of baby bottles, sports equipment, and medical products, epoxy resins are mostly used as preservatives in food and beverages. Both BPA in epoxy resin and polycarbonate is able to pass through food and beverages. As an estimated 6.6 μg/day is consumed from containers containing epoxy resin. Heating of plastics increases passage of BPA[24]. Exposure to BPA can rapidly distribute body fluids and tissues and cause accumulation. For example, the placenta, amniotic fluid, blood, serum, saliva, mother milk, reproductive system tissues are mainly BPA distributed tissues. According to some reported studies, BPA can pass from mother to the fetus[25].

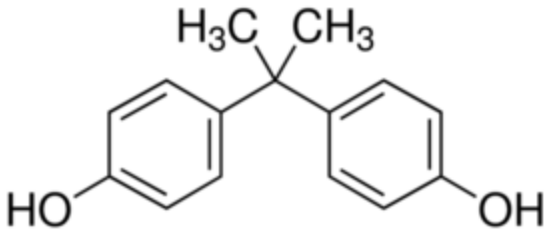

Figure 2. 1. The molecular structure of BPA

2.1.1. Exposure Levels of BPA

Although the amount of BPA taken with daily foods is very different, only the passages from the foods can be measured. BPA level is measured in infants fed breast milk as 0.2 μg / kg, in infants fed formula without baby's bottle as 2.3 μg/kg, in infants fed formula with baby's bottle as 11 μg/kg and in adults as 1.5 μg/kg in Europe. Changes in the bio-monitorization of BPA metabolites in urine are observed when evaluated in the aspect of daily human exposure. The estimated exposure level in the USA is 0.16 μg/kg, and in Japan is between 0,04-0.08 μg/kg. Because of BPA is completely absorbed, it is thought that the data obtained from the urine reflect exactly the truth[27]. In 2014, according to the risk assessment report prepared by Joint Emerging Science Working Group of FDA, the optimal NOAEL (no observed adverse effect level) of BPA is determined as 5 mg/kg in multigeneration studies using sub-chronic oral exposure in rats[28].

2.1.2. Toxicokinetic of BPA

In human and reptiles, orally taken BPA is quickly absorbed and rapidly metabolized by the highest rate in CYP2C18 and slightly in CYP2C19 and CYP2C9 enzymes following oral absorption. It is converted to major metabolite as BPA-

glucuronide (69.5%) by first pass effect in hepatic and intestine. At the same time, a little amount is converted to sulfate conjugate[29]. 80 percent of orally taken BPA is eliminated in 6 hours and it has been determined that it have passed to the urine nearly at 42 hours. Conjugates of BPA don't have endocrine activities[30,31]. In rats, orally taken BPA is quickly converted to the glucuronide conjugate however high incidence of BPA binds to plasma proteins, is eliminated via bile and then reabsorbed from the intestine by returning to free BPA. This enterohepatic cycle is repeated a few times till BPA eliminated completely. Elimination is slower and also exposure to free BPA is much more in rats. In the body, BPA can be found in urine, blood, adipose tissue, semen, breast milk, amniotic fluid, placenta, colostrum, umbilical cord blood and saliva[32]. In mice, after low dose exposure, oxidation metabolites of BPA are identified, and it is thought that these metabolites have much higher estrogenic activity. Because of the first past effect is an important pathway, the risk assessment studies are made by different routes. The concentration of BPA in fetal tissues is the same amount as in the mother's blood, this indicate that BPA is able to pass into the placenta. Moreover, BPA is able to pass maternal milk approximately at 1-3 μg/L concentration. This amount is higher when compared to the amount in the mother's blood[33,34]. Oral bioavailability in both of mother and fetus is very low. At the same time, infants are exposed by feeding with formula or baby bottles[28,35].

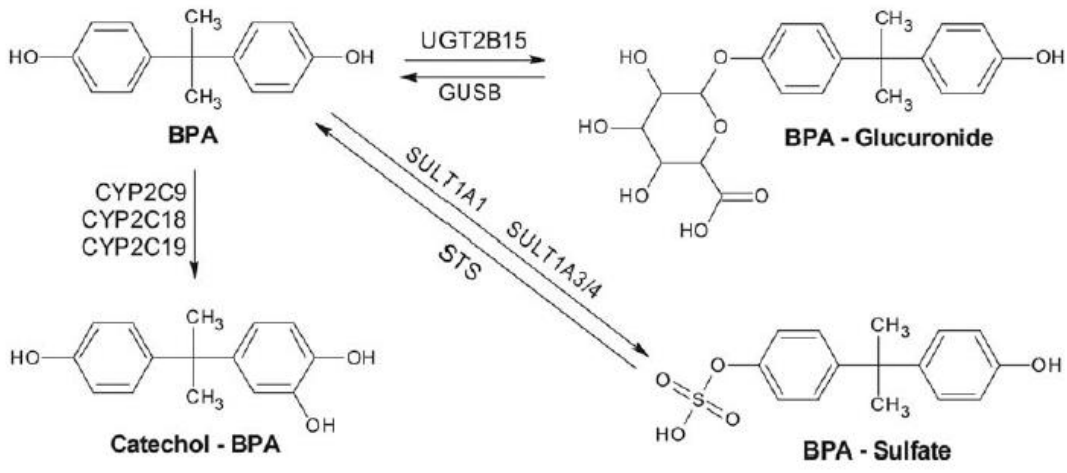

Figure 2.2. BPA metabolism in human[36]

2.1.3. Toxicity of BPA

2.1.3.1. Acute Toxicity

According to toxicity tests, BPA shows low acute inhalation toxicity. In a study conducted with 10 female and male, 170 mg/m³ BPA was administered via inhalation and LC_{50} (Lethal Concentration 50) level was determined as >170 mg/m³. In another study which made to determine acute toxicity, 5 female and male rats were administrated orally 2000–5000 mg/kg BPA. Consequently, reported that BPA given at 2000 mg/kg concentration did not cause death however one male and all female rats were died at a concentration of 5000 mg/kg. It has been reported that female mice were more sensitive than male, and when all the males and females were evaluated, the Lethal Dose 50 (LD_{50}) value was 5000 mg/kg[37].

2.1.3.2. Neurotoxicity

There is a continuous exposure to BPA due to releasing from containers of food and beverages and can be stored in adipose tissue. Some toxicological studies reported that BPA exposure may cause memory problems by affecting brain development in elderliness but the outcome of adult exposure is not fully determined. In recent studies on the neurotoxic effect of BPA in rats have reported that BPA has dose-dependent effect on behavior, memory and learning problems[3]. Furthermore, it has been reported that BPA can cross placenta and blood-brain barrier and cause permanent damage by affecting neurogenesis, synaptogenesis, myelination in the brain development of the fetus[12]. Although the mechanisms involved in the molecular studies on how these effects of BPA effects are not fully understood, several pathways have been reported including oxidative stress, neuroinflammation, activation of the MAPK pathway[11,12].

Human Studies

Neurological studies on BPA have mainly explored the effect on children. Harley et al. conducted a study in which were investigated neurologic development at 5,7, and 9 years children during pregnancy and after borne and they were compared urinary metabolites with information taken from their teachers[38]. While there was no significant relationship between exposure and neurodevelopmental effects on girls during pregnancy, there was a relationship between urinary concentration in the prenatal period and anxiety and depression in males at 7 years. Moreover, they found that there was a significant

correlation between childhood urinary concentration in 7 years girls and extrovert behaviors in 7 years girls. Similarly, hyperactivity was reported to increase in both boys and girls at 7 years of age[38]. In a similar study conducted by Perera et al, while there was a relationship between emotional reactions and aggressive behavior syndrome in boys exposed to BPA in the prenatal period, there was associated with low scores in all syndromes, anxiety and aggressive behaviors in girls[39]. In another study, the scores of extrovert behaviors were found higher for all children, especially girls in prenatal BPA exposure[40]. In another study conducted by Braun et al, more anxiety and depression while less emotional control reported in girls exposed to BPA during pregnancy[41]. Yolton et al. reported that when BPA and DEHP were investigated in the aspect of the neurobehavioral effect in infants, BPA is not effective, but DEHP is associated with nonoptimal reflexes in male infants[42].

Animal Studies

It has been reported in several studies that BPA is effective on energy metabolism and insulin, so this effect causes neurodegenerative disorders. In a study conducted by Li et al. investigated the effect of low dose BPA in glucose transport and insulin. 100 μg/kg BPA was administrated subcutaneously for 30 days and examined the effect on glucose pathway in the hippocampus. As a consequence, they found that insulin receptor sensitivity and glucose transport level were decreased. At the same time, they observed that phosphorylated tau and β-APP were increased[8]. In a study, BPA was administrated 50 mg/kg BPA and 10 mg/kg melatonin was administrated as a protector to adult mice. There was reported that BPA caused oxidative stress and induced apoptotic genes in the brain. Also, it was reported that given melatonin had a protective effect against the effects of BPA[26].

It has been reported that exposure to environmental pollutants affect behavior, memory, and learning. Moreover, pre- and perinatal exposure to BPA has also been reported caused neurotoxic effects. In a study, the mice in the tenth day after birth were administrated a single dose of BPA and changes in abrupt and conscious behavior have been observed in adult mice. It has been reported that the effects were depending on dose and lasted for a long time[25]. In a study conducted by Castro et al, animals were administrated 50 μg/kg BPA subcutaneously for 4 days to investigate BPA induced plasticity and memory on the prefrontal cortex of the brain. And then, they performed in

the molecular experiments that change in genes affecting plasticity and memory-related functions on the prefrontal cortex[43]. In another study reported by Castro et al., investigated the effects of dopamine and serotonin in low-dose BPA-exposed male in pregnancy and after pregnancy and they found that BPA is effective on both serotonin and dopamine-releasing genes in both childhood and adulthood. Therefore, it has been reported that it can cause psychopathological impacts[44].

Some studies, indicated that exposure to BPA in newborns caused neurotoxic effects. In a study, alternation in both hippocampal and cortex protein levels which are important for normal brain development was reported in both male and female rats exposed to BPA on a postnatal day 10[3]. Another study investigated the effect of BPA exposure in neonatal and found that BPA exposure in pregnancy caused abnormal distributions and stratification in nerve and abnormal dopaminergic projections in the neonatal neocortex[4].

Some studies have also reported that BPA develops toxicity on rats by affecting neurogenesis in the hippocampus. In this context, Mathisen et al. investigated the effect of prenatal exposure to BPA on the development of the cerebellum. They reported that female mice were exposed to BPA in drinking water to investigate the effect of BPA in the development of the granule cerebellum in the period of preconception, pregnancy, and lactation[7]. Tiwari et al researched the effect of BPA on neurogenesis and they determined decrease in cell proliferation, in the amount and size of oligodendrocytes, the expression of genes responsible for myelinization, and protein levels[45].

Stump et al observed neurodevelopmental effects during pregnancy and lactation, rats were exposed to BPA at various doses and examined their effects on offspring. Although they have not found any evidence about BPA being a neurodevelopmental toxicant, weight loss has been observed in both the mother and the offspring. At the same time, in the study, NOAEL level was identified as 5.85 mg/kg in pregnancy and 13 mg/kg in lactation[46]. In a study conducted by Kimura et al., they examined the effect of neural morphology in the hippocampal CA1 region at 3 weeks and 14 months of age. The pregnant mice were exposed to 0, 40, 400 μg / kg BPA and the hippocampal CA1 region was examined by brains of 3-week and 14-month-old pups after birth. It has been determined that spinal density in the hippocampal CA1 region of 14 months old mice decreases. The length and branching of basal dendrites were decreased in the 3-week-old mice were exposed to the dose of 400 μg / kg. The exposure to low-dose BPA during

pregnancy indicated that the developmental CA1 region of the hippocampus was morphologically affected and persisted[47]. In another study reported that exposure to BPA in the early postpartum period increased apoptosis and oxidative stress and the level and genes of autophagic proteins[11].

2.2. Phthalates

The report on endocrine disruptors published by the European Union in 2002 included phthalates among hundreds of chemical substances which are clearly shown harm to the environment and human health[48]. Plastic additives are added to hard plastics like PVC to soften them, to be processable and moldable named as plasticizers. Phthalate esters are plasticizers which have a wide usage area and generally used to increase their softness and elasticity of polymers such as PVC, polyvinyl acetate, celluloses, and polyurethanes[49].

Phthalates are industrial synthetic compounds which have been used in wide range area since the 1920's. Phthalates or phthalate esters are consists of dialkyl or alkyl aryl esters of phthalic acids (1,2-benzedicarboxylic acid) and they are named depending on the length of alkyl chains (Figure 2.3)[50].

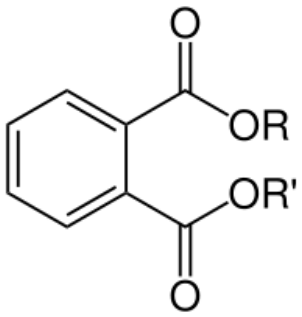

Figure 2.3. General Molecular Structure of Phthalates

Phthalates are composed of an aromatic chain and usually two aliphatic side chains. They are colorless, odorless (or low odor) and oily liquids. While they are low soluble in water, well soluble in oil. When they are added to plastics, they allow long polyvinyl molecules to slide over each other. Because phthalate esters don't bond to polymers with covalent bonds, they are not stable and can easily be separated from their polymers by some physical processes. Especially they can easily be separated at high temperatures and can be evaporated[51].

Long of side chain determines the properties of phthalate such as solubility in different polymers, volatility, behaviors at different temperatures. These chains may be straight or branched aliphatic as well as cycloaliphatic and aromatic side chains. Biodegradation is the main destruction process of phthalate esters in the environment except for the atmosphere. It is thought that they are quickly undergoing photooxidation with hydroxyl radicals in the atmosphere (R40) [52] Aerobic and anaerobic microbial degradation of phthalate esters begins with hydrolysis to form monoester and alcohol. Monoester forms phthalic acid by a series of enzymatic degradation. Different phthalates are produced by branching with a different number of alcohol esters for different purposes. Among these, mostly used are diethyl hexyl phthalate (DEHP), diisodesyl phthalate and diisononyl phthalate, lower molecular weight diethyl phthalate and dibutyl phthalate. High efficiency and low cost of DEHP is the main reason why its usage is higher than the others[53]. This situation led to further investigations to observe the effects of this phthalate compared to other phthalate derivatives[48].

Phthalates are in a wide chemical class which contains both Low Molecular Weight (LMW) and High Molecular Weight (HMW) compounds. LMW phthalates are mainly used as raw materials or auxiliary chemicals in the production of cosmetic and personal care products, paper, cardboard, automotive, construction and building materials, medical bags, tubes, and paints and also used in non-vinyl resins such as cellulosic, acrylics and urethanes as plasticizers. HMW phthalates are used in 80% of the industries and they are mainly found in plastic materials especially PVCs [54–56]. Phthalates and their field of use are listed in Table 2.1.

In briefly, phthalates are chemicals that are widely used as plasticizers to provide flexibility and durability of PVC type plastic products including in construction and building materials, food materials, textile products, toys, baby care products (including baby's bottle and teat), blood bags, iv fluid bags, infusion sets and medical equipment. They are widely used in personal care products including cosmetics, perfumes, and soap and produced and consumed in high volumes. Community phthalates exposure usually occurs through diet and cosmetic care products or contact with medicinal applications due to leaks from the plastic matrix. DEHP is the most commonly used derivative and for example, 20-40 percentage of PVC-medical equipment is consisting DEHP [57,58]. Moreover, most of these phthalates do not need to be informed on the label [59,60].

Table 2.1. Phthalates and their field of use

Phthalates	Usage
Dimethyl Phthalate	Repellent, plasticizer
Diethyl Phthalate	In cosmetics (Shampoo, perfume, soap lotion), as an industrial solvent, in medicines (tablet coating, capsule production).
Dibutyl Phthalate	As industrial solvents in adhesives and cosmetics.
Butyl benzyl Phthalate	As industrial solvents in adhesives and vinyl floor coverings, the seal production
Dicyclohexyl Phthalate	As a stabilizer in the production of rubber and polymers.
Di(2-Ethylhexyl) Phthalate	As a plasticizer in soft plastics (iv bags, toys, home products, food industry, food covering boxes), paper industry, electrical capacitors, paints/pigments, resins, rubber industry, textile products
Dioctyl Phthalate	As a plasticizer in soft plastics
Diisononyl Phthalate	As a plasticizer in soft plastics, the usage area of DEHP

2.2.1. Di-(2-Ethylhexyl) Phthalate

DEHP is a benzendicarbocylic acid ester. DEHP is the most commonly used phthalate. The formula is $C_{24}H_{38}0_4$. It is a colorless-yellowish, oily liquid at room temperature and normal atmospheric pressure[61]. While it is low soluble in water and lipophilic. The relative solubility in blood is more than water. Soluble in many organic solvents[62].

It was firstly produced in 1939 in the United States. Main use (%95) is the production of PVC. It is found the percentage of 1-40 in PVC. In the first years, it was used more than 50% of PVC products, this amount has decreased in recent years. The use of DEHP in the early 2000s is prohibited in Europe and America by determining the adverse effects on human health, especially in food containers, baby rattle and toothpicks and baby toys[48].

Because of DEHP meddles to the environment in the process of production, usage, and disposal, an essential part of it is found in foods, inner room air and soil[63]. The situation with people is most frequently affected with DEHP by way of food. Foods are

contaminated many times with plastic materials, and therefore DEHP during production, packaging, and storage. Although DEHP is determined in many nutrients, due to lipophilic property, the transition to oily nutrients like dairy products, fish, meat, olive is more common. By showing that found in the mother's milk and in the foods, it has been determined that influencing this substance starts in the first years of life[48].

The other way of entering the body is directly transfused to blood during the medical treatment. DEHP is the most commonly used phthalates in the medical equipment and plastic parts of technical tools. Especially, it has been shown that DEHP levels are increased at infants at intensive care units (taking blood, thrombocyte, and total parenteral feeding by intravenously), patients who are mechanically ventilated and dialyzed[63]. Studies have indicated that each blood transfusion may affect 3300 μg/ kg (3.3 mg/ kg) DEHP[64]. In a study conducted that influence is approximately 47 μg/kg in the dialysis patients[65]. American Environmental Protection Agency and European Union are reported that the average daily tolerable intake of DEHP is orderly 20 μg/kg and 37 μg/ kg[66,67]. Opinion on phthalate migration from soft PVC toys and child-care articles expressed at the 6th CSTEE plenary meeting, Brussels, 26/27 November 1998). Daily intake of DEHP per kilogram is higher for infants and little children (0.5 to 4 years of age). This group is expected to consume more calories per kilo, to have more dairy products and fatty foods in its diet, to have more respiration per minute than adults, and to be more influenced by contact with plastic toys[67].

2.2.1.1. Toxicokinetic of DEHP

Most of the biotransformation studies of DEHP have been performed with the animal experiments. More frequently, oral route (90%) is conducted due to the main route of exposure[68]. It is thought that 20-25 % of DEHP is absorbed from the gastrointestinal system. Animal studies have been showed that it can be absorbed by oral, dermal and inhalation routes[69]. Orally taken DEHP is absorbed by converting the same amount of monoethyl hexyl phthalate (MEHP) or 2-etylhexanole by hydrolysis or esterification with especially pancreatic lipases in the intestine. It is thought that in people with less lipase enzyme, conversion to MEHP and 2-etylhexanole is very low so less toxic substances may be absorbed. There are no human studies about inhalation but the detection of DEHP in the urine and lung tissue of infants who are dependent on the respiratory device, suggesting that it might also be absorbed in this way [69]. Some studies showed that blood

elimination occurs in a very short time. After thrombosis transfusion, the half-life of taken DEHP is 28 minutes. The half-life of DEHP in the hemodialysis patient is 5-7 hours[69]. It has also been shown that the bioavailability of orally taken DEHP is higher in young rats than in older rats[61].

80 percentage of phthalates are bounded to lipoproteins in the bloodstream. There is a weak bond between albumin. The main metabolite is MEHP, don't bond to lipoproteins and found as free or albumin bonded. In mouse experiment, 24 hours after the single dose and tagged iv DEHP administration, it was shown that metabolites are found in urine and feces[69].

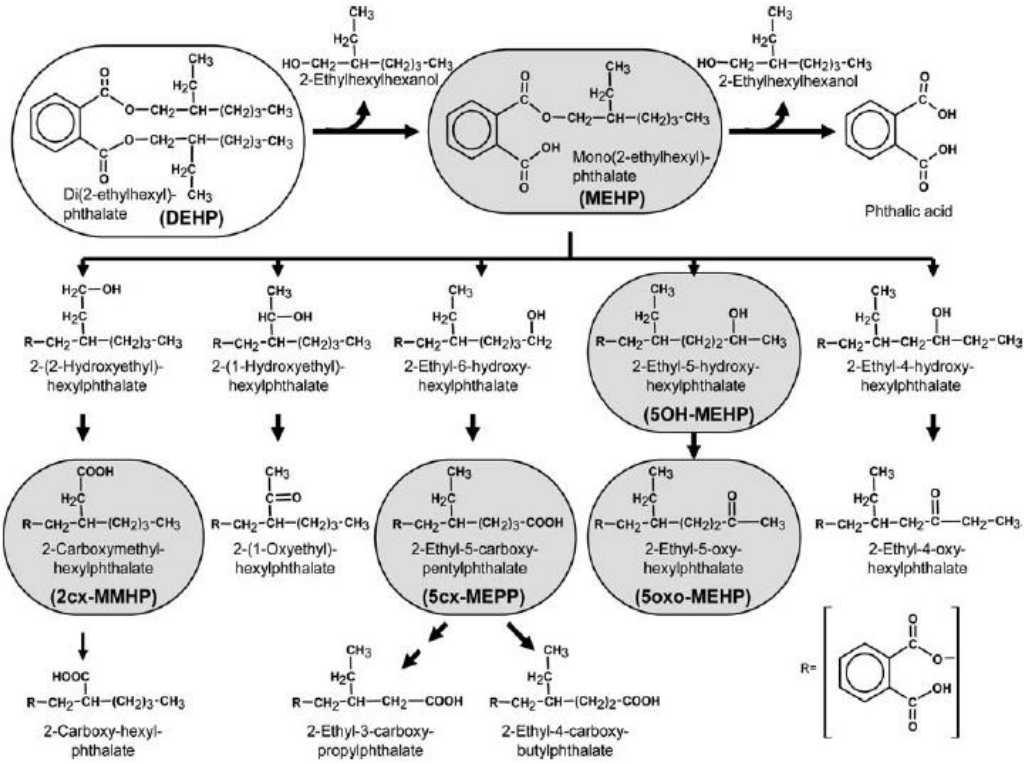

Figure 2.4. DEHP metabolism and metabolites[71]

DEHP transforms into main metabolite MEHP by lipase enzyme found in the liver, kidney, skin, lung and plasma and especially in the intestine and pancreas (Figure 2.4). An important part of MEHP is divided into more than 30 metabolites with the advance metabolism by the several oxidative reactions[71].

Furthermore, the presence of DEHP in the adipose and renal tissues of post-mortems was demonstrated. It is thought that DEHP may be stored in adipose tissue due to lipophilic properties[70]. The presence of congenital malformations in the offspring with

DEHP administration to pregnant animals suggests that DEHP may be transported through the placenta[64].

Approximately %65 of these metabolites are excreted in urine by conjugation with glucuronic acid. Other metabolites and non-conjugated DEHP are excreted by feces. Another metabolite of DEHP, 2-ethyl hexanol, is excreted as 2-ethyl hexanoic acid and some ketoacid derivatives by oxidation in the urine[71]. The half-life of DEHP is 24 hours and %80 of DEHP is eliminated in 5-7 days from the body[64,72].

At the same time, DEHP is metabolized by CYP4A, alcohol, aldehyde dehydrogenases and forms aliphatic side chains of 2-ethyl hexanol and MEHP[73].

2.2.1.2. Toxicity of DEHP

Because of LD_{50} levels of phthalates are between 1-30 g/kg, they show low acute toxicity. Short and long-term dose studies in rats have shown dose-dependent adverse effect of phthalates on liver, kidney, thyroid, and testis. Genotoxicity and mutagenicity studies on all phthalates are reported that they did not show such an effect. Different results were obtained in carcinogenicity studies. For example, while carcinogenic effect of diethyl phthalate is suspicious, carcinogenic effect of DiNP was not observed. In the carcinogenetic studies of DEHP conducted in rats, proliferation in mitochondria and peroxisome, increased CYP4A1 and PcoA activity, proliferation in liver tissues, suppression of apoptosis was observed in hepatocellular carcinoma and other pathways related to cancer. Underlining mechanisms almost all of these effects have been mediated through the induction of PPAR-alfa receptors.

New studies have shown that phthalates showed their carcinogenic effect by activating PPAR-alfa in rats but it was invalid in human[74] According to evaluation of International Agency For Research On Cancer (IARC), DEHP is class III (non-classified as human carcinogen) carcinogen[75].

2.2.1.3. Neurotoxicity

Human Studies

Although phthalates have been extensively studied for reproductive and developmental toxicity, information on neurodevelopmental toxicity is very limited. Oxidative stress is an important factor in the formation and progression of

neurodevelopmental disorders. In a study on neurotoxic effect of DEHP and BPA was evaluated the formation of free radicals in the plasma of in autistic children and it has been found that oxidant parameters were higher in their plasma[76]. Likewise, Testa et al. investigated metabolite levels in the urine to compare phthalate levels between autism spectrum disorder and healthy children and they reported that urinary levels of 5-OH-MEHP and 5-oxo-MEHP were higher in autism spectrum disorder children[77].

Many human studies on phthalate are mostly made with pregnant mothers. The reason of this, there is continuous exposure and the effect on future generations is not known clearly. In recent years, the increase in neurological disorders seen in children makes it difficult to link this effect to a single factor. In this aspect, Kim et. al. and Engel et. al. investigated relationship between DEHP exposure and attention-deficit/hyperactivity disorder. Kim et.al. reported that a significant relationship was found between mono-2-ethyl phthalate and 5-OH-MEHP which are urinary metabolites of DEHP and hyperactive children identified by the teacher[78]. Engel et. al. investigated the effect of DEHP on cognitive and behavioral effects development by taking urinary samples from pregnant mothers in the third trimester pregnant and their 4-5 aged children as a result of this pregnancy. As a result, they reported that behavioral effects were due to prenatal LMW phthalate exposure before birth and these effects were mostly found in clinically diagnosed with attention deficiency[79].

In a similar study, Lien et. al. investigated several phthalates [mono-methyl phthalate (MMP), mono-ethyl phthalate (MEP), monobutyl phthalate (MBP), monobenzyl phthalate (MBzP) and three di-(2-ethylhexyl) phthalate (DEHP) metabolite: mono-2-Ethylhexyl, mono-2-ethyl-5-hydroxyhexyl and mono-2-ethyl-5-oxohexyl phthalate (MEHP, MEHHP and MEOHP)] in urinary samples taken from pregnant mothers in the third trimester and evaluated behavioral syndromes in children born from these mothers and who are 8 years old as regards to List of Child Behavior. When the gender, intelligence, and family income were optimized and the relationship between the concentration of MBP, MEOHP and MEHP in mother’s urine and the scores of outward orientation problems were evaluated, were found to be significantly higher a relationship. When the gender, intelligence and family income of the children were optimized, the scores of the outward orientation problems when the relationship between MBP, MEOHP and MEHP concentrations in the mother's urine was evaluated. Moreover, MBP and

MEOHP were significantly associated with Delinquent Behavior and Aggressive Behavior scores[80].

In a study conducted by Tellez-rojo et. al., urinary samples were taken from pregnant mothers in the third trimester and their children who are 3 years old. Also, neurodevelopmental effect of DEHP in children were evaluated according to mental and psychomotor development index at specific time intervals for 3 years. At the end of the experiment, while there was not found a significant relationship in all children, they investigated different effects depend on gender. While negative relationship was investigated between metabolites of DEHP in girls, there was no a specific relationship in boys[81]. Similar results were obtained by Polanska et al.[82].

Kim et al investigated neurodevelopmental effects of prenatal DEHP exposure. Like other studies, the neurodevelopmental effects of cognitive and behavioral development of DEHP and DBP were evaluated according to mental and psychomotor development index (MDI and PDI) by taken urinary samples from pregnant mothers in the third trimester and children born from these mothers and who are 6 months. The metabolites of DEHP and DBP (MEHHP, MEOHP, MBP) were investigated in urinary samples taken from pregnant mothers in the third trimester. As a result of the analyzes, there was an inverse relationship between MDI and PDI scores of 6-month-old infants and pre-natal phthalate exposure. Also, they found an inverse relationship between MDI and PDI scores and all metabolites (MEHHP, MEOHP and MBP) analyzed in urinary samples of male infants while no significant relationship was found in female infants[83].

Animal Studies

In some studies, postpartum DEHP exposure has been evaluated to investigate axonal and neuronal development in the hippocampus. A study was conducted by Smith et al, it was investigated the effect of post-natal DEHP exposure on lipid structure of the hippocampus, postpartum (pp) pups were given DEHP as acute 10 mg / kg, it (from day 16 to day 22). As a result, they found that the levels of systemic phosphatylcholine and sphingomyelin were increased in hippocampus of female rats but it has been shown that lysophosphatidylcholine were not change in both females and males. According to this result, DEHP exposure damaged hippocampal development in males but not in females (R71)[84].

Xiujuan et al., investigated effect of DBP exposure on brains of early and normal born rats. Pregnant rats were given 500 mg/kg DBP, by orally, from gestational day 6 to postnatal day 21. Levels of estradiol increased in both early and normal birth infants, level of testosterone decreased in both early and normal birth male infants. At the same time, when investigated the effect on hippocampus was reported to induce neurotoxicity in early neonates and not to affect normal neonates[84].

Hoshi et al. investigated the effect of low level of DBP on developmental neurotoxicity. Pregnant rats were given less than 1.0 mg/kg dose of DBP during pregnancy. Although there was not observed adverse effects on motor functions and weight of offspring and their mothers, offspring whose mothers exposed to 10 μg/kg DBP not to be able to clean their fur and take care themselves. That is, it has been reported that low dose of DBP exposure adversely affected emotional stability[85].

In a study was investigated the effect of BPA, DEHP and TCDD on the midbrain dopaminergic system functions which are motor activity, emotion and cognitive behaviors on the neuropsychological disorder such as attention deficiency and hyperactivity. Mouse dams and their pups were orally administrated BPA (5 mg/kg), DEHP (1 mg/kg) and TCDD (8 ng/kg) alone or combination to investigate their effects. Tyrosine hydroxylase and Fos-immunoreactive (IR) were used as biomarker for dopamine and neuronal activity. As a result, cell number of Tyrosine hydroxylase and Fos-IR and their intensity in dopaminergic core in the midbrain were different from control groups. There was not found any differences in combinations groups and they considered it as the interaction between the chemicals[85].

Min et al. investigated the effect of BBP exposure on brain and underlying mechanism of these effects. Kunming mice were orally administrated BBP at dosage of 0, 50, 250 and 1250 mg/kg during 14 days. According to Morris maze test results, mice received 250 and 1250 mg/kg BBP were observed poorer learning and memory performance. At the same time, they observed that reduction in locomotor activity and depression in forced swimming test. Changes in the hippocampus and oxidative damage have also been reported in pathological examinations. BBP were tested to understand toxicity on serotonin and pCREB and it has been found alterations in levels of serotonin in hippocampus and reduction in level of CREB phosphorylation. Consequently, BBP exposure affected in aspect of CREB, oxidative stress and impaired behavioral performance[1].

In a study conducted by Tang et. al., Kunming mice were administrated DEHP (0, 5, 50, 500 mg/kg) and Vit E as a protective to investigate the effect of DEHP exposure on brain and their cognitive effects were observed by Morris maze test, levels of Malondialdehyde (MDA) and ROS. Moreover, 500 mg/kg DEHP were given a depression group and applied open field test, tail suspension test and forced swimming test. They observed that dysfunction in spatial learning, memory and Morris maze test, oxidative damage in groups exposed to 50 ve 500 mg/kg DEHP and increased in levels of Reactive Oxygen Species (ROS) and MDA in groups 50 mg/kg DEHP and vit A received groups. When evaluating behavioral results, it was reported that while Vit E has a protective effect against cognitive impairment in combination with 5 mg/kg DEHP, Vit E, combined with 5 mg / kg DEHP, caused behavioral disturbances in mice. They concluded that exposed to DEHP has neurotoxic effects on cognitive and locomotor activities[86].

DEHP exposure, especially in critical periods, can cause insulin resistance by affecting glucose and insulin homeostasis. Some studies reported that there is an association between insulin resistance and Alzheimer (AD) pathogenesis. Sun et. al. showed in a such study that prenatal DEHP exposure affected hippocampal insulin expression and insulin pathway. Moreover, they reported that impairment in cognitive abilities and increase in level of phosphor-tau[5].

2.3. Carnosic Acid

CA found in rosemary *(Rosmarinus officinalis)* and sage *(Salvia officinalis)* in the Lamiaceae familia is a phenolic (Figure 2.5) diterpene. Some studies revealed that CA has several biological properties such as antioxidant, antiandrogenic, anticarcinogen and neuroprotective[13–15].

Rosmarinus officinalis is traditionally known as rosemary and mostly range in Mediterranean climate. Because of essential oil of rosemary is used in perfume and used as specie, often is cultivated. Furthermore, the usage of *Rosmarinus officinalis* has recently increased in neurodegenerative disorders due to antioxidant properties[87]. CA is a main substance found in the leaves of *Rosmarinus officinalis* responsible from antioxidant activity[16].

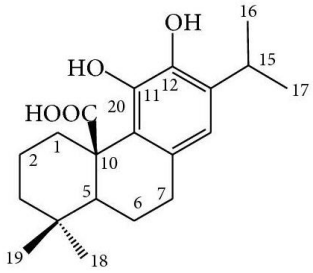

Figure 2.5. Molecular structure of Carnosic acid [88]

2.3.1. Mechanism

Radical scavenging activity of CA occurs because of the presence of hydroxyl groups found at atoms C11 and C12 (Figure 2.6)[16]. With this property prevents lipid peroxidation and rupture of biological membranes[17]. CA is able to penetrate through blood brain barrier and shows protective effect after conversion from its catechol from to quinone form by stress induced oxidation. This conversion makes CA an electrophilic that it binds to keep 1 protein found in the cytoplasm of neuronal cell which releases Nrf2 transcription factor. Nrf2 activates and enters nucleus to stimulates endogenous antioxidant enzymes known as phase 2 enzymes[89].

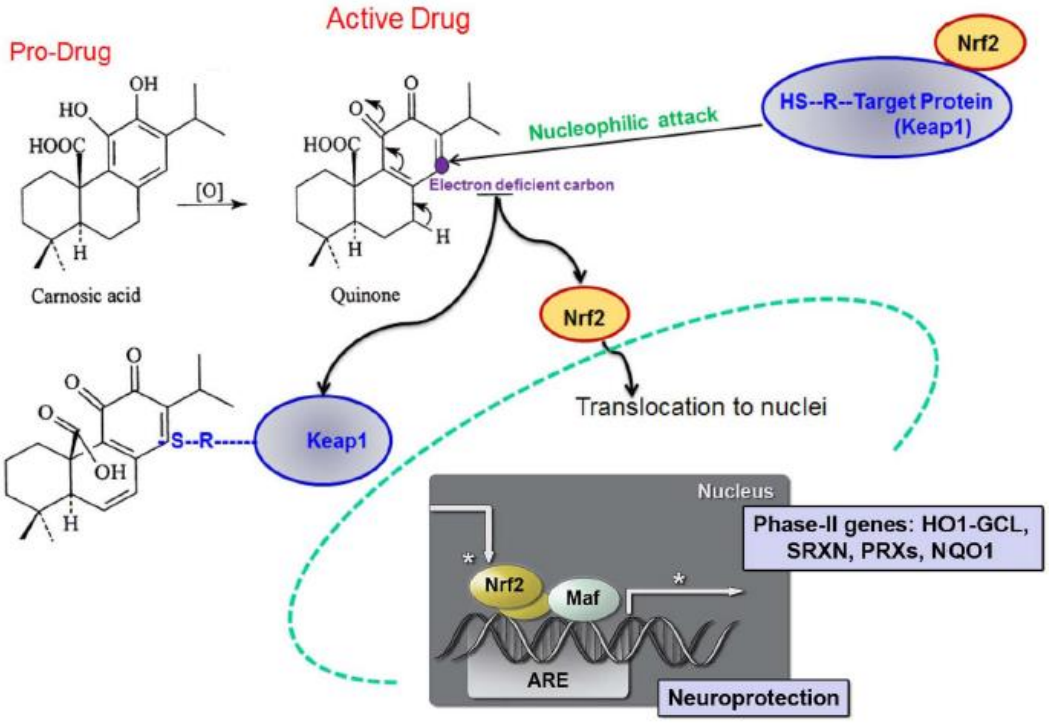

Figure 2.6. Neuroprotection of carnosic acid[89]

A study showed that CA protects neuronal cells against ischemic damage[90]. In another study reveal that CA dieldrin induced apoptotic molecules decreased after the use of CA[20]. Also, It has been shown that CA prevents neurotoxicity by preventing oxidative stress in Alzheimer's caused by amyloid-beta and 6-hydroxydopamine-induced Parkinson's disease in animal studies[14,16–18].

2.3.2. Bioavailability

A pharmacokinetic study made in rats, CA is administrated oral and iv routes. The result of the study showed that iv bioavailability was found %40 after 6 hours. At the same time, free CA was determined in several organs after 24 hours. The analysis is performed after 24 hours was identified that CA elimination was 15% in feces taken by oral route. And also, the main elimination route of CA is found as feces[91]. In another study conducted by Vaquero et al, the main metabolite of orally taken CA was identified as glucuronide conjugate in liver, gut, and plasma. Other metabolites were 12-methyl ether and 5,6,7,10-tetrahydro-7-hydroxy rosmariquione of CA. These metabolites were detected in 25 minutes after taken orally. Interestingly, free CA and its metabolites were also found in brain[92].

2.4. Free Radicals and Oxidative Stress

Free radicals can be defined as molecules that carrying unpaired electrons (e-) on atomic orbital[93–95]. This definition includes hydrogen atom (unpaired e-), transition metals and oxygen molecule[95]. Uncoupled e- is the electron that occupies an atomic orbital alone[96]. Most of free radicals, due to these unpaired electrons has high reactivity, as can give electrons to other molecules, can get electrons from them. Therefore, they can act as a reductant or oxidant[93,95]. It was first discovered by Gomberg in 1900. These molecules are continuously formed in the body and are annihilated by antioxidant defense system. Impairment of this balance leads to increased free radicals and cell damage (Table 2.2 and 2.3). This situation defined as oxidative stress. Oxidative stress is caused by an inadequate antioxidant defense mechanism and increased free radicals in various states. As a result of this, DNA, proteins, carbohydrates, lipids, and enzymes can be damaged [97–99]. The production of free radical is a part of pathophysiology and toxicity of many xenobiotics are related to the production of excess amounts of free radicals[100]. Oxidative stress, have gained importance in recent years as an important mechanism playing a role in the pathophysiological events such as atherosclerosis, diabetes, cancer, chronic

inflammatory diseases, central nervous system disorders, in cell senescence and thus cellular destruction, cell damage and cell death[101–104].

Table 2.2. Reactive oxygen species and reactive nitrogen spices[105]

Radicals	Non-Radicals
Reactive oxygen species (ROS)	
Superoxide, $O_2^{\cdot -}$	Hydrogen peroxide, H_2O_2
Hydroxyl, $HO^{\cdot}$	Hypochlorous acid, HOCl
Peroxyl, $RO_2^{\cdot}$	Ozone, O_3
Alloxyl, $RO^{\cdot}$	Singlet oxygen, $^1O_2$
Reactive nitrogen species (RNS)	
Nitric oxide, $NO^{\cdot}$	Nitrous acid, HNO_2
Nitrogen dioxide, $NO_2^{\cdot}$	Nitrosyl cation, NO^+
	Nitrosyl anion, NO^-
	Peroxynitrite, $ONOO^-$
	Alkyl peroxynitrites, ROONO

2.4.1. Types of Free Radicals

2.4.1.1. Superoxide Radical

Superoxide radical ***($O_2^{\cdot -}$)*** occurs when the cell undergoes normal metabolic functions, or in a pathological state, by reducing oxygen by taking an electron ($O_2 + e^- \rightarrow O_2^{\cdot -}$). Molecular oxygen is the first intermediate product. Two percentage of taken oxygen is converted to $O_2^{\cdot -}$ in the body. It is formed during all oxidation reactions and phagocytic events but mainly it is formed due to the presence of oxygen electron leaks from electron carriers such as NADH dehydrogenase and coenzyme Q during energy metabolism in the mitochondria.

Although its reactivity is less then hydroxyl radical, it can damage DNA. Superoxide leads to the formation of $OH^{\cdot}$ by Haber-Weiss reaction;

$$O_2^{\cdot -} + H_2O_2 \longrightarrow O_2 + OH^{\cdot} + OH^- \text{ (Haber-Weiss reaction).}$$

$O_2^{\cdot -}$, is detoxified by converting to H_2O_2 by superoxide dismutase enzyme or by self-dismutation in mild acidic medium[106–108].

Table 2.3. Molecules mediating oxidative stress[99]

Name	Structure	Main reactions
Superoxide	•O-O$^-$	Catalysis of Haber-Weis reaction by recycling Fe^{2+} and Cu^+ ions; formation of hydrogen peroxide or peroxynitrite
Hydrogen peroxide	HO-OH	Formation of hydroxyl radical; enzyme inactivation; oxidation of biomolecules
Hydroxyl radical	•OH	Hydrogen abstraction; production of free radicals and lipid peroxides; oxidation of thiols
Ozone	$^-$O-O$^+$=O	Oxidation of all kinds of biomolecules, especially those containing double bonds; formation of ozonides and cytotoxic aldehydes
Singlet oxygen	O=O	Reaction with double bonds, formation of peroxides; decomposition of amino acids and nucleotides
Nitric oxide	•N=O	Formation of peroxynitrite; reaction with other radicals
Peroxynitrite	O=N-O-O$^-$	Formation of hydroxyl radical; oxidation of thiols and aromatic groups; conversion of xanthine dehydrogenase to xanthine oxidase; oxidation of biomolecules
Hypochlorite	ClO$^-$	Oxidation of amino and sulfur-containing groups; formation of chlorine
Radical	R•	Hydrogen abstraction; formation of peroxyl radicals and other radicals; decomposition of lipids and other biomolecules
Peroxyl radical	R-O-O•	Hydrogen abstraction; formation of radicals; decomposition of lipids and other biomolecules
Hydroperoxide	R-O-OH	Oxidation of biomolecules; disruption of biological membranes
Copper and iron ions	Cu^{2+}, Fe^{3+}	Formation of hydroxyl radicals by Fenton and Haber-Weis reactions

2.4.1.2. Hydrogen Peroxide

Hydrogen peroxide (H_2O_2) is produced by many reactions in the cell by reducing the oxygen molecule by taking two electrons or during dismutation of $O_2^{\cdot -}$. H_2O_2 which plays a role in the many cellular metabolic functions such as macrophage activities and thyroid hormone biosynthesis is produced especially during oxidative electron transport in the mitochondria and is also present in very small amounts in cells. H_2O_2 is an oxidizing

agent, although not radical. It reacts with NO to form $OH^{\cdot}$ and it allows the formation of peroxynitrites. It also allows the formation of $OH^{\cdot}$ in the presence of transition metals such as iron and copper by Fenton reaction:

$$H_2O_2 + Fe^{+2} \longrightarrow Fe^{+3} + OH^{\cdot} + OH^{-}$$

It enables the formation of radicals such as hypochlorous acid by enzymatic (myeloperoxidase) oxidation with chlorite ions:

$$H_2O_2 + CI^{-} \longrightarrow HOCI + OH^{-}$$

Hypochlorous acid can cause the formation of singlet oxygen ($^1O_2$):

$$H_2O_2 + HOCI^{-} \longrightarrow {}^1O_2 + H_2O + CI^{-}$$

Also, hydrogen peroxide can convert hydrogen carbonate ion to hydrogen peroxycarbonate:

$$H_2O_2 + HCO_3^{-} \longrightarrow H_2O + HCO_4^{-}$$

Hydrogen peroxide is detoxified by conversion to water by catalase and peroxidase[107–109].

2.4.1.3. Hydroxyl Radical

Hydroxyl radical ($OH^{\cdot}$) is the most reactive radical between oxygen radicals. It has been thought to cause DNA modification in more than a hundred ways. $OH^{\cdot}$ is basically produced by three different mechanisms:

1) Ionizing radiation causing water decomposition

   $2H_2O \longrightarrow H_2O^{+} + e^{-} + H_2O^{\cdot}$ Reaction results with the formation of $OH^{\cdot}$
2) Photolytic decomposition of alkyl hydroperoxides
3) According to Fenton reaction, it is formed by the decomposition of H_2O_2 in the catalysis of the metals[106,107].

2.4.1.4. Peroxyl Radical

Briefly, the peroxyl radical ($ROO^{\cdot}$) is the conjugate acid of the hydroperoxyl radical, so it is the dioxyl radical. It initiates lipid peroxidation, causes modification of proteins. It causes damage to DNA by causing a synergistic effect with $O_2^{\cdot-}$[100,106,110].

2.4.1.5. Nitric Oxide

Nitric oxide (NO) is a free radical gas contains an unpaired electron that is commonly found in the organism, naturally synthesized in many cells and tissues, lifespan expressed in seconds.

It is produced by nitric oxide synthase enzyme, especially in vascular endothelial cells. Nitric oxide synthase enzyme has three known isoforms, neuronal, endothelial and inducible. NO increases apoptosis, cytotoxicity, mutagenesis and DNA damage under oxidative stress and also causes lipid oxidation. NO reacts with $O_2^{\cdot -}$ to form $OH^{\cdot}$ and nitrogen dioxide radicals through peroxynitrite ($NO + O_2^{\cdot -} \longrightarrow ONOO^-$).

$$ONOO^- + H^+ \longrightarrow ONOOH \text{ and } ONOOH \longrightarrow NO_2 + OH^{\cdot}$$

The formed peroxynitrite activates the nuclear enzyme, poly (ADP-ribose) synthase (PARS) in the cell, leading to ATP consumption and cell death. Most of the physiological effects of NO are due to its easy binding to some transition metal ions. If response of the cells to nitrosative stress are similar to those of oxidative stress, NO reacts with proteins to form protein-S-nitrosothiols[109,111].

2.4.2. Source of Free Radicals

Free radicals can occur during the oxidation and reduction reactions that occur as a consequence of metabolic activity in the organism or by various external factors[112,113].

2.4.2.1. Endogenous Free Radical Sources

Plasma Membranes

Plasma membranes have great prominence because of both free radical formation and the chain reactions targeted to free radicals and also the place where lipid peroxidation takes place. In prostaglandin synthesis, OH radicals are formed during the oxidation of arachidonic acid, catalyzed by cyclooxygenase enzymes(Figure 2.7)[114].

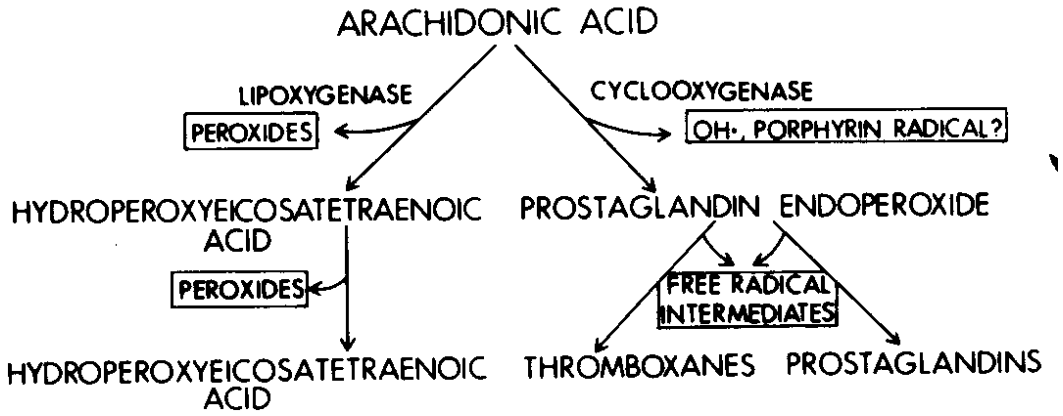

Figure 2.7. The relationship between prostaglandin synthesis and free radicals[112]

Enzymes

Inactivated phagocytic cells, reduced nicotinamide adenine dinucleotide phosphate oxidase (NADPH oxidase) in the plasma membrane mediates the production of $O_2^{\cdot -}$ radical[115]. One of the most studied enzymes is xanthine oxidase. *In vivo,* the xanthine/hypoxanthine oxidation is catalyzed by the enzyme xanthine dehydrogenase. This enzyme; the electrons are taken from the substrates are carried to the nicotinamide adenine dinucleotide (NAD^+) much more than oxygen.

XD is converted to xanthine oxidase by proteolytic enzymes during its purification or by oxidation of thiol (-SH) groups. Under normal in vivo conditions, the $O_2^{\cdot -}$ radical is not formed the result of the xanthine/hypoxanthine metabolism catalyzed by this enzyme, which is located in the cytosol of animal and plant tissues. Nevertheless, dehydrogenase-oxidase transformation occurs when tissue damage occurs. Xanthine oxidase catalyzes the oxidation of uric acid from hypoxanthine and xanthine and meanwhile reduces O_2 to $O_2^{\cdot -}$ and H_2O_2 [96]. Furthermore, cytochrome P450 causes detoxication reactions during the formation of $O_2^{\cdot -}$ radicals[116].

Autoxidation Reactions

Some biological molecules can be oxidized in the presence of O_2, in this situation O_2 is converted to $O_2^{\cdot -}$. These molecules are glyceraldehyde, reduced flavin mononucleotide, reduced flavin adenine dinucleotide, adrenaline, noradrenaline, L-dopa, dopamine, tetrahydro pteridines and thiol compounds such as cysteine. Tetrahydro Pteridines is the cofactor of oxygenase enzymes such as nitric oxide synthase, phenylalanine hydroxylase, and tyrosine hydroxylase. Since O_2 is not very reactive these autoxidations are accelerated by the addition of transition metal ions such as iron and copper, that is catalyzed by metal ions[96,112].

Hem Proteins

Iron is found as Fe^{2+} in the rings of hemoglobin and myoglobin and remains in this state until the oxygen is bound.

$$\mathbf{hem - Fe^{2+} - O_2 \leftrightarrow hem - Fe^{3+} - O_2^{\cdot -}}$$

while Fe^{2+} is bound to O_2, Fe^{3+} is bound to $O_2^{\cdot -}$. $O_2^{\cdot -}$ is released with the decomposition of the oxyhemoglobin molecule.

$$\mathbf{hem - Fe^{2+} - O_2 \rightarrow hem - Fe^{3+} + O2^{\cdot -}}$$

Fe^{3+} in the ring of hem cannot bind to O_2 so it is inactive and known as methemoglobin (the situation is the same for myoglobin and methoglobin). 3% of hemoglobin in human erythrocytes was detected to undergo this oxidation every day. The oxidation of hemoglobin and myoglobin is accelerated in the presence of nitrite ions or transition metal ions (especially copper)[117,118].

Mitochondrial Electron Transport

The most important source of superoxide radicals in most aerobic cells is electron transport chains. These are found in bacterial membranes and in mitochondria and endoplasmic reticulum of eukaryotic cells. In mitochondria, electrons escaping from the electron carriers in the respiratory chain can combine with molecular oxygen to form $O_2^{\cdot -}$.

In cases where mammalian cytochrome oxidase activity is saturated with oxygen, the rate of electron transport and hence the formation of superoxide radicals is increased by mitochondria. At physiological oxygen levels, about 1-3% of the oxygen is converted to superoxide in the mitochondria. Mitochondrial damage increases electron transport and superoxide generation[119].

Mitochondrial DNA

The formation of oxygen radicals by the mitochondria in the respiratory chain damages mitochondrial proteins, lipids, and DNA. Mutations in mitochondrial DNA accumulate in aged tissues[120,121].

Bacterial Superoxide Formation

Studies on *E. coli* have shown that some cytosolic enzymes form $O_2^{\cdot -}$, but the main $O_2^{\cdot -}$ source is the electron transport chain[122].

Peroxisomes

Peroxisomes are important sources of cellular hydrogen peroxide because they contain oxidases at high concentrations and have been shown to form superoxide as a precursor to hydrogen peroxide. Proximal hydrogen peroxide forming enzymes include D-amino acid oxidase, urate oxidase, L-α-hydroxy acid oxidase and fatty acid-acyl-CoA oxidase[123].

Endoplasmic Reticulum

Microsomal fractions isolated from different tissues and containing the endoplasmic reticulum have been shown to form superoxide and hydrogen peroxide when incubated with NADPH. This formation rate increases at high oxygen concentrations. These reactive oxygen species are largely due to the cytochrome P-450 system[116,124].

Nucleus

The membrane surrounding the cell nucleus contains the electron transport chain, which forms superoxide with an increase in oxygen concentrations in the presence of NADH or NADPH. This pathway is crucial for living things because the formed oxygen radicals are close to the nuclear DNA[96].

Disintegration of Metal Ions

Transition metals, especially iron and copper, are found in various oxidation steps in physiological conditions. The electron exchange between the oxidation steps is occurred by oxidation and reduction reactions.

$$Fe^{3+} + e^{-} \leftrightarrow Fe^{2+}$$

$$Cu^{2+} + e^{-} \leftrightarrow Cu^{+}$$

Transition metals accelerate free radical reactions due to these properties and catalyze the reaction. They catalyze synthesis of hydrogen peroxide and superoxide hydroxyl radical by Haber-Weiss and Fenton reactions. The main effects of metal ions on free radical reactions are those on lipid peroxidation[95,96,113].

Exogenous Free Radical Sources

Exogenous free radical sources include radiation, natural gas, environmental pollutants such as chlorine and ozone in the water, nitric oxide, and aldehydes in cigarette smoke, as well as drugs, pesticides and other xenobiotics (Figure 2.8)[110,125].

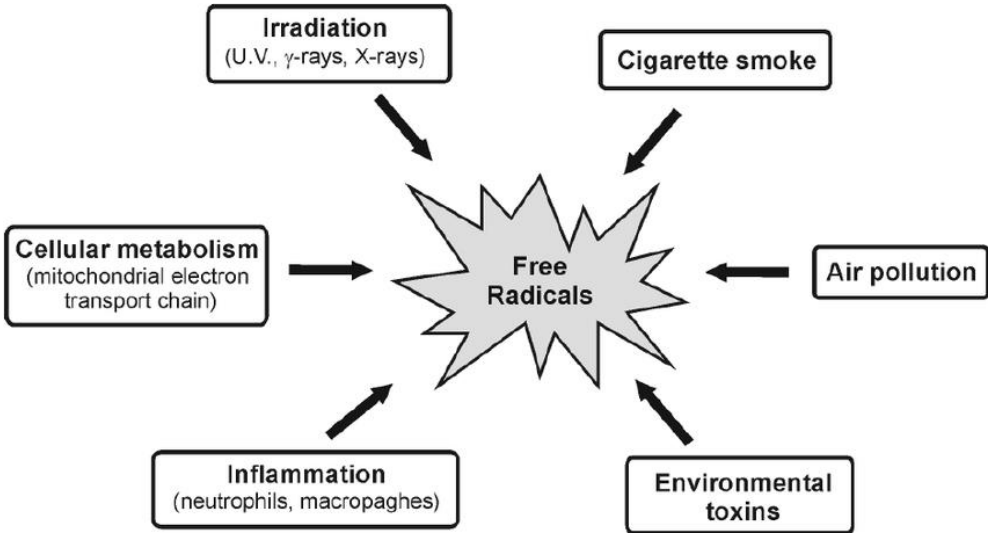

Figure 2.8. Illustration of exogenous free radical sources [126]

2.4.3. Damaging Effects of Free Radicals

Free radicals have a capacity of making damage to proteins, lipids, carbohydrates, nucleic acids, and DNA (Figure 2.9). Therefore, when they reach a sufficient concentration to inactivate the intracellular defense mechanisms, they may react with intracellular components and cause metabolic and cellular disorders. The goal of oxidative stress depends on the type of cell, the stress, and severity of the exposure. For example; carbon tetrachloride causes damage by lipid peroxidation in cells whereas cellular damage of H_2O_2 is on DNA[112,113,127].

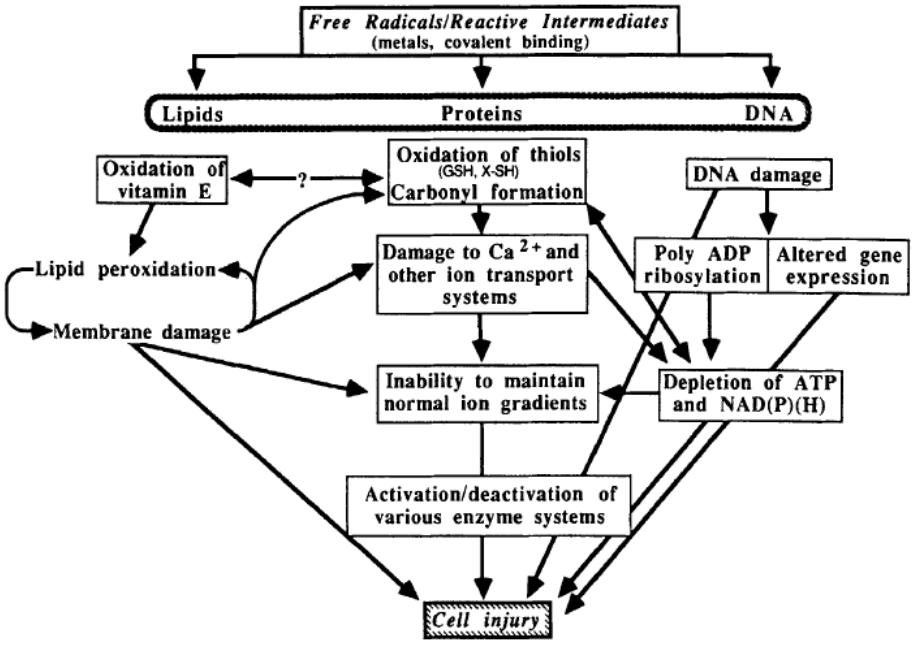

Figure 2.9. Effects of free radicals on cellular components[113]

2.4.3.1. Effects on Nucleic Acids and DNA

Free radicals have also damaging effects on DNA[128]. Particularly free radicals formed by exposure to ionizing radiation affect DNA, causing mutations and deaths in cells. Radicals facilitate dipyramids, single chain breaks and the formation of DNA-protein cross-links. Hydrogen peroxide released from active neutrophils easily diffuses from the membranes into the cell nucleus, causing DNA damage, cell dysfunction and eventually cell death. Again, the hydroxyl radical readily enters the reaction with deoxyribose and bases, causing damage. Thus, DNA is an important target that can easily be damaged by free radicals[129-130].

Over-production of reactive oxygen radicals leads to modifications on all components of the DNA molecule. $OH^{\cdot}$, damages both purine and pyrimidine base and causes cleavage and chain breakage in the deoxyribose core. Continuous modification of genetic material is the first step of carcinogenesis, aging, and mutagenesis [131,132]. DNA repair enzymes and DNA polymerases are also affected by free oxygen species[130]. Damage to the DNA molecule leads to chronic inflammation, infection, aging, carcinogenesis, neurodegenerative and cardiovascular diseases[133].

2.4.3.2. Effects on Proteins

The susceptibility of proteins to free radical damage depends on the amino acid composition, the location of amino acids responsible for protein activation or structural regulation, and the repairability of the damaged protein. Covalent modification of proteins with reactive oxygen species or oxidative stress products results in post-protein oxidation[134]. Reactive oxygen species react either with peptide bonds or with side chains of amino acid. These reactions are also affected by cations such as iron and copper. Oxidatively modified proteins are either separated by low molecular weight products or form crosslinked high molecular weight products[135]. Since unsaturated and sulfur-containing molecules have a high reactivity to free radicals, proteins containing amino acids such as tryptophan, tyrosine, phenylalanine, and cysteine are easily affected by free radicals. In particular, sulfur and carbon-centered radicals form. As a result of these reactions, the three-dimensional structures of the proteins that contain a large number of disulfide bonds are distorted. Therefore, they cannot perform their normal functions[136,137]. As a result of the reduction of the thiol groups of the proteins, loss of enzymatic functions, membrane, ion and metabolite transport, and contractile functions[135,138]. Pyrroline and

lysine can undergo nonenzymatic hydroxylation when exposed to H_2O_2, $O_2^{-}\cdot$, OH radical-generating reactions. Oxidative molecules, such as hypochlorous acid, catalyze the oxidation of tyrosine, 3-chlorotyrosine and histidine to 2-oxo histidine in metal catalysis (binding metal to protein structure). Hem proteins are also molecules that are affected by free radicals. Oxyhemoglobin is converted to methemoglobin by the effect of H_2O_2 and O_2^{-} radical[118]. Protein oxidation has been suggested to be associated with the development of a number of pathologic diseases such as atherosclerosis, diabetes, ischemia-reperfusion injury, rheumatoid arthritis, Alzheimer, Parkinson and aging[135,139].

2.4.3.3. Effects on Carbohydrates

Free radicals also have important effects on carbohydrates. The result of auto-oxidation of monosaccharides; H_2O_2, peroxides and oxoaldehyde products in the structure occur. They play an important role in pathological processes such as diabetes, cancer, and chronic diseases associated with smoking. Oxoaldehydes have antimitotic effects due to their ability to bind to DNA, RNA and proteins and form cross-links between them. This is thought to play a role in cancer and aging[140].

2.4.3.4. Effects on Membrane Lipids

Biological membranes and intracellular organelles (mitochondria, endoplasmic reticulum) are susceptible to oxidative attacks due to the presence of polyunsaturated fatty acids in membrane phospholipids. Therefore, a series of reactions called lipid peroxidation are started as a result of the attacks of free radicals. Lipid peroxidation is the disruption of cell membrane structure as a result of oxidative damage of membrane lipids. Integrity and fluidity of cell membranes are important for the continuity of the cell[96].

2.4.3.5. Non-enzymatic Lipid Peroxidation

Lipid peroxidation occurs in three steps (Figure 2.10) [125,141–144]

1. Initiation Phase: A free radical formed in the organism starts with the breakdown of the H^+ from a methylene group in the polyunsaturated fatty acid (RH) chain in the membrane structure. The double bonds in the fatty acids weaken the C-H bond and facilitate the breakdown of the H + atom. Radicals that are active in the first H + atom; OH, alkoxyl, peroxyl, and hydroperoxyl radicals. As a result of the hydrogen breakage, the fatty acid chain acquires a carbon-centered lipid radical ($R^{\cdot}$). Fatty acids with one or two double bonds are more resistant than such attack polyunsaturated fatty acids.

2. Propagation Stage: The lipid radical is stabilized by converting it into a conjugated diene form with a molecular arrangement. This is followed by the reaction of the molecular oxygen with the resulting lipid peroxyl radical ($ROO^{\cdot}$).

$$\mathbf{R^{\cdot} + O_2 \rightarrow ROO^{\cdot}}$$

The lipid peroxyl radical also affects neighboring fatty acids, leading to the formation of new lipid radicals ($R^{\cdot}$), while it is transformed into a lipid hydroperoxide (ROOH) by taking up released $H^{\cdot}$. Thus, chain reactions continue by self-catalyzing.

$$\mathbf{ROO^{\cdot} + RH \rightarrow ROOH + R}$$

As a result, many fatty acid chain hydrocarbons are converted into lipid hydroperoxides. Hydrophilic lipid hydroperoxides exert considerable harmful effects on integrity, fluidity, and permeability in membranes.

Lipid hydroperoxides are highly resistant molecules. However, degradation increases at high temperatures and in the presence of metal ions, thus accelerating lipid peroxidation.

$$\mathbf{2\ ROOH \xrightarrow{Cu+2,\ Fe+2} ROO^{\cdot} + RO^{\cdot} + H^{+} + OH^{-}}$$

The termination of the reaction chain depends on factors such as the concentration of oxygen and the amount of chain-breaking antioxidants.

3. Termination stage: Lipid peroxidation chain reactions continue until the formation of a non-radical product with the interaction of two lipid peroxide radicals.

$$\mathbf{ROO^{\cdot} + R^{\cdot} \rightarrow ROOR}$$

$$\mathbf{R^{\cdot} + R^{\cdot} \rightarrow RR}$$

The degradation of lipid hydroperoxides results in the formation of hydrocarbon gases and aldehydes, which are secondary lipid peroxidation products. These products damage the cell components by diffusing from the area of peroxidation to other parts of the cell.

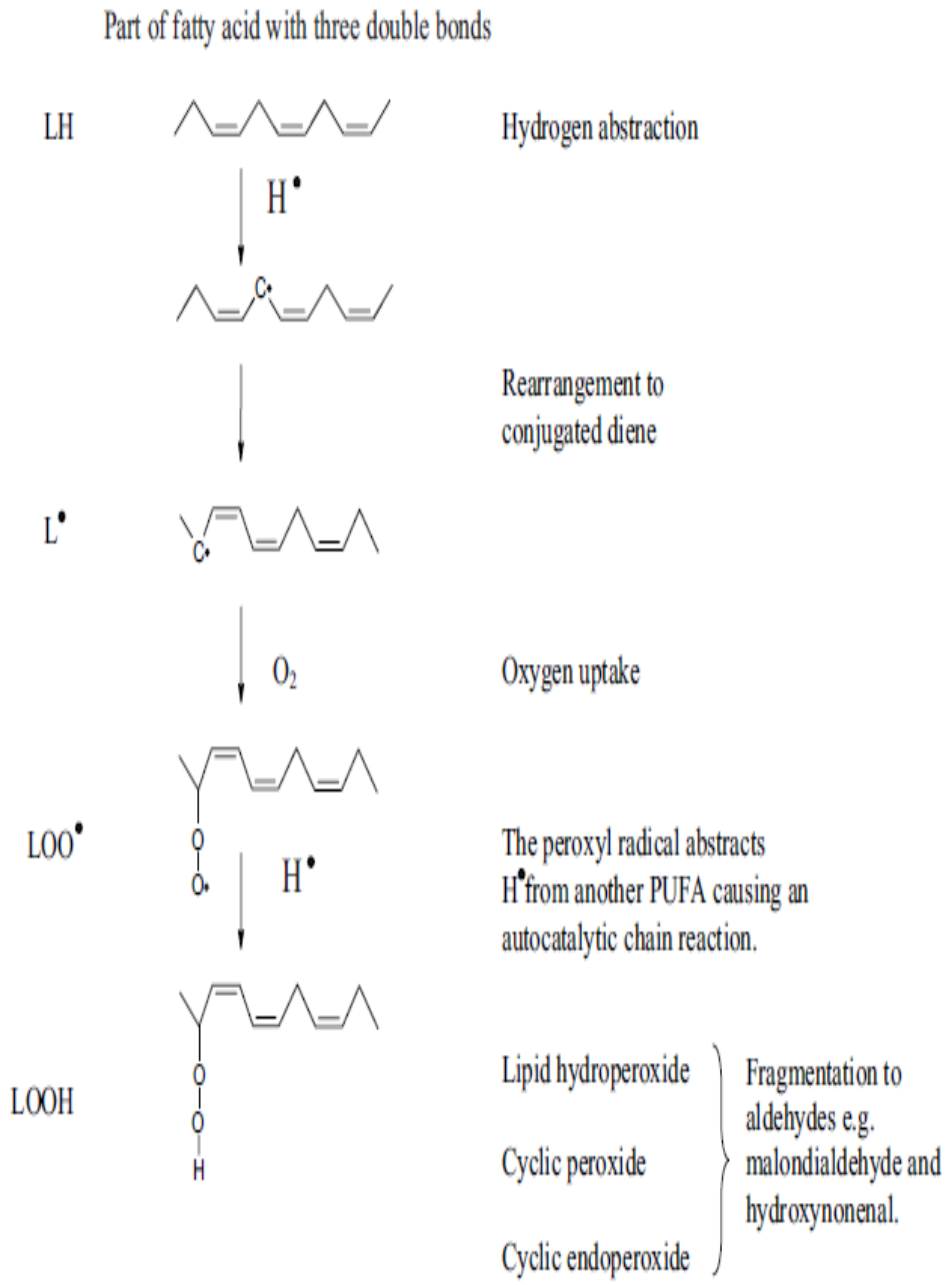

Figure 2.10. Mechanism of lipid peroxidation[141]

2.4.3.6. Enzymatic Lipid Peroxidation

Cyclooxygenase and lipoxygenase enzymes cause a certain percentage of lipid peroxidation in fatty acids which are their substrates, resulting in hydroperoxides and endoperoxides with important biological functions. Prostaglandins are formed as a result of arachidonic acid peroxidation catalyzed by cyclooxygenases[145,146]. During lipid

peroxidation, MDA, which is a determinant of peroxidative damage, can be easily detected from biological structures[115].

MDA results in the oxidative peroxidation of polyunsaturated fatty acids (linolenic acid, arachidonic acid, docosahexaenoic acid) containing more than one double bond. It is a moderate reactant under physiological conditions and may act as both electrophilic and nucleophilic. It reacts with protein containing the primary amine group, physiological molecules such as nucleic acid, amino phospholipids. MDA rapidly metabolizes in mammalian tissues. It is oxidized to malonic acid by aldehyde dehydrogenases. Malonic acid is a competitive inhibitor of mitochondrial succinate dehydrogenase enzyme[96,147].

MDA assay is widely used as an indicator of lipid peroxidation in biological samples. MDA is found in free and bound forms in biological materials. The amount of free MDA is normally very low. The bound MDA can be liberated with hot acid or alkali[147–149].

2.5. Antioxidant Defense Systems

The rate of formation of free radicals and their rate of removal from the environment is in balance in metabolism, which is called oxidative balance. As long as this oxidative balance is established, the organism is not affected by free radicals. Increase in the rate of formation of free radicals or disruptions that may occur at the rate of removal will cause to deteriorate at this balance. This is called as 'oxidative stress'. Oxidative stress results in an imbalance between the formation of free radicals and antioxidant defense system and leading to tissue damage[150]. Reactions of free radicals and tissue damages in metabolism that occur as a result of cause many diseases such as chronic heart diseases, arteriosclerosis, diabetes, stroke, neurodegenerative disorders, Alzheimer, cataract, cancer[151,152]. Defense mechanisms have been developed in metabolism to prevent these damages caused by free radical species. These mechanisms are called antioxidant defense systems or briefly antioxidants[153,154]. Antioxidant defense systems try to annihilate the oxidative stress by reducing the production of reactive oxygen species or by eliminating the damages caused by reactive oxygen species[155]. Antioxidants are molecules that stabilize free radical species without damaging the cells in metabolism and minimize the damage they can give[153]. A good antioxidant;

- can specifically remove the efficacy of free radicals,
- chelate with redox metals,

- must be available in tissue and body fluids at suitable levels,
- should be easy to absorb,
- should be able to function easily in both liquid media and membranes[155].

Hydrophilic and lipophilic antioxidants are needed for free radicals in various properties. Hydrophilic antioxidants are found in the cytosol and extracellular fluids, and lipophilic properties are found in membranes and lipoproteins[156]. Classification of antioxidants can be done in different forms. According to where they are in metabolism and functioning, plasma antioxidants (beta-carotene, ascorbic acid, uric acid, ceruloplasmin, etc.), antioxidants found in the cell membrane (alpha-tocopherol,) and intracellular antioxidants (superoxide dismutase, catalase, glutathione peroxidase, etc.) or as enzymatic antioxidants and non-enzymatic antioxidants according to their structure and activities. While enzymatic antioxidants include enzymes such as glutathione peroxidase, catalase, superoxide dismutase, non-enzymatic antioxidants can be included general nutritional origin such as carotenoids, tocopherols, ascorbic acid, phenolic compounds or may be classified as metabolic origins, such as glutathione, ceruloplasmin, albumin, and bilirubin[154,157] .

2.5.1. Enzymatic Antioxidants

2.5.1.1. Superoxide Dismutase

Superoxide dismutase (SOD) is one of the most important antioxidant enzymes found in the cells where oxygen is metabolized[158]. In 1939, Mann and Keilin isolated a blue protein containing copper from bovine erythrocytes, called hemocuprein, but the enzymatic activity of this crystallized protein has not been determined. Subsequently, another copper-containing protein similar to hemocuprein was catalyzed from human erythrocytes and named as erythrocuprein, but no activity was observed[159]. The superoxide dismutase enzyme (SOD) catalyzes the dismutation of superoxide, hydrogen peroxide, the first step in the defense of antioxidants[160,161].

$$2O_2^{\cdot} + 2H^+ \xrightarrow{SOD} O_2 + H_2O_2$$

In the enzymatic dismutation reaction, the superoxide anion first binds to the guanido group of arginine in the enzyme. During this bonding, it converts Cu^{+2} to Cu^{+1} by giving one electron. It turns into molecular oxygen. The other superoxide anion picks up the electrons and the protons in the medium and provides hydrogen peroxide

formation. Thus, the dismutation reaction is completed[162]. Superoxide dismutase can be studied in four different classes according to the metal ion contained as the enzyme cofactor. These are superoxide dismutase enzymes containing dinuclear Cu / Zn or mononuclear Fe, Mn and Ni[163]. Superoxide dismutase containing copper and zinc are generally found in the cytosol of eukaryotic cells, in the intermembrane region of nuclear membranes and mitochondria and in some prokaryotes[162]. The Cu / Zn SOD enzyme located in the cytosol of animal cells consists of two similar subunits. The molecular weight of each subunit is approximately 16 kDa. In prokaryotic cells, Mn-SOD consists of two identical subunits and each subunit contains a manganese atom. The mitochondrial dismutase in eukaryotes is a tetramer, the dismutase in prokaryotes is similar but the molecular weight is twice that of prokaryotes[163]. Fe-SOD is found in some aerobic and anaerobic bacteria and plants, whereas Ni-containing SOD is generally detected in bacterial strains such as Streptomyces[158,164].

2.5.1.2. Glutathione Peroxidase

Glutathione Peroxidase (GSH-Px) enzyme was first discovered in 1957 by Mills in animal tissue. It has been reported to be present in some algae and fungi, although it is usually not found in higher plants and bacteria[96,165].

Two main types of this enzyme have been identified. One of these types is "selenium-dependent GSH-Px (Se-GSH-Px)" which contains covalently bound selenium in the active site in the form of selenocysteine. Se-GSH-Px is active against organic hydroperoxide and H_2O_2. Catalyzes the degradation of various hydroperoxides, including H_2O_2, by oxidation of GSH. Oxidized glutathione (GSSG) is again reduced to GSH by the enzyme GSH-Rd [96,166,167].

$$\mathbf{H_2O_2\ (ROOH) + 2GSH \rightarrow GSSG + 2H_2O\ (ROH)}$$

$$\mathbf{GSSG + NADPH + H^+ \xrightarrow{GSH\text{-}Rd} 2GSH + NADP^+}$$

The other type of enzyme is composed of non-selenium-dependent proteins for catalysis and shows negligible activity against H_2O_2. This group of enzymes is called GST and is defined as proteins that catalyze the conjugation of GSH with electrophilic compounds[96].

Se-GSH-Px consists of four protein subunits containing an atomic Se element in the active site of each unit. The molecular weight is about 85,000. It is found in cytosol

and mitochondria. The Se element is in the form of selenocysteine, and Se is present instead of the sulfur in the normal cysteine (R-SeH instead of R-SH).

GSH reduces Se in the enzyme and reduced enzyme reacts with H_2O_2. The catalytic cycle of Se-GSH-Px enzyme is shown in Figure 2.11. While the selenolate form of Se-GSH-Px reduces the peroxide substrate (ROOH) to alcohol, it transforms itself into oxyacid (E-Se-OH). GSH is added to the reaction in this cycle to form selenosulfide (E-Se-S-G). while the enzyme is converted the active form selenolate with the binding of a second GSH to selenosulfide, the GSH is also oxidized to GSSG[96].

Se-GSH-Px enzyme has high in liver, moderately in heart, lung, and brain and low activity in muscle. In the liver, it is located in the cytosol and mitochondria[169].

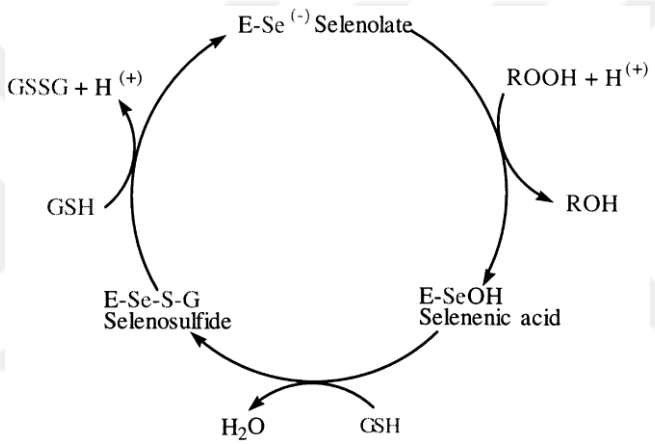

Figure 2.11. The catalytic cycle of Se-GSH-Px enzyme[168]

Other than Se-GSH-Px, there is also phospholipid hydroperoxide GSH-Px enzyme (PLGSH-Px) and the molecular weight is 20,000. It is a cytosolic enzyme containing a selenium atom that reduces the membrane phospholipid hydroperoxides to alcohols. When vitamin E deficiency which is the most important membranes antioxidant, provides protection against peroxidation of the membrane[96].

2.5.1.3. Glutathione Reductase (GSH-Rd)

It has been mentioned in 2.5.1.2 that GSH is converted to GSSG during the reduction of H_2O_2 or other lipid peroxides by Se-GSH-Px. It is necessary to use this oxidized form again in the reaction by converting it into a reduced form. Because the GSH deposit of the organism is limited. The GSH-Rd enzyme is responsible for this reduction in the presence of NADPH[96,169,170–172].

$$\mathbf{GSSG + NADPH + H^+ \xrightarrow{GSH\text{-}Rd} 2GSH + NADP^+}$$

Nicotinamide adenine dinucleotide phosphate (NADPH) required herein can be provided by various enzyme systems in animal or plant tissues, but the most well-known system is the oxidative pentose phosphate pathway. The first enzyme in this pathway is glucose-6-phosphate dehydrogenase (G6PD) enzyme.

$$\textbf{G6PD + NADP}^{+} \longrightarrow \textbf{6-Phosphogluconate + NADPH + H}^{+}$$

The resulting 6-phosphogluconate is also converted to NADPH via the 6-phosphogluconate dehydrogenase enzyme.

$$\textbf{6-Phosphogluconate + NADP}^{+} \rightarrow \textbf{CO}_2 \textbf{ + NADPH + H}^{+} \textbf{ + Ribulose-5-phosphate}$$

The GSH-Rd enzyme with a molecular weight of 120,000 contains two protein subunits and each subunit contains a FAD in its active site. NADPH probably reduces FAD and the electrons in this FAD are also transferred to the disulfide (-S-S-) bridge in GSSG, breaking the disulfide bond and converting the molecule to GSH[169].

2.5.1.4. Non-Enzymatic Antioxidants

Glutathione

GSH, is a low molecular weight tripeptide consisting of L-glutamic acid, L-cysteine and glycine formed by a two-step reaction. these reactions are mediated by δ-glutamyl cysteine synthetase and glutathione synthetase. In each step, one mole ATP is involved. It is found mostly in the liver. In the cell is mostly found in cytosol, mitochondrion and nucleus.

The majority of intracellular glutathione is present in the form of thiol (reduced glutathione, GSH), and less frequently in the form of disulfide (oxidized glutathione, GSSG) [96,173–177]. Figure 2.12 shows the formula of GSH.

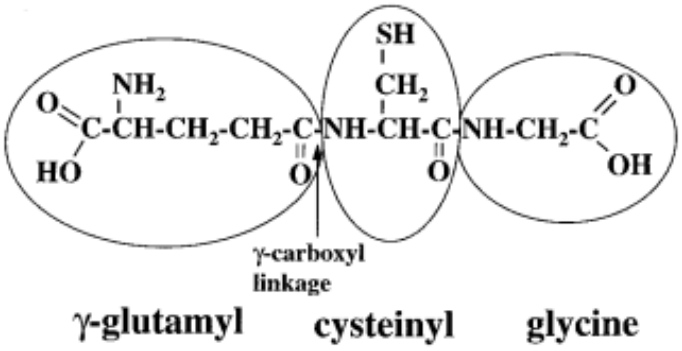

Figure 2.12. The structure of GSH[178]

GSH, which is a strong nucleophilic property, enters into conjugation with electrophilic compounds to provide detoxification thereof. This reaction is terminated by the formation of thioether (mercapturic acid and N-acetyl-cysteine derivatives) and the resulting products are excreted in the urine. Conjugation of GSH with electrophilic material begins with the catalysis of GST enzyme (Figure 2.13)[96,177].

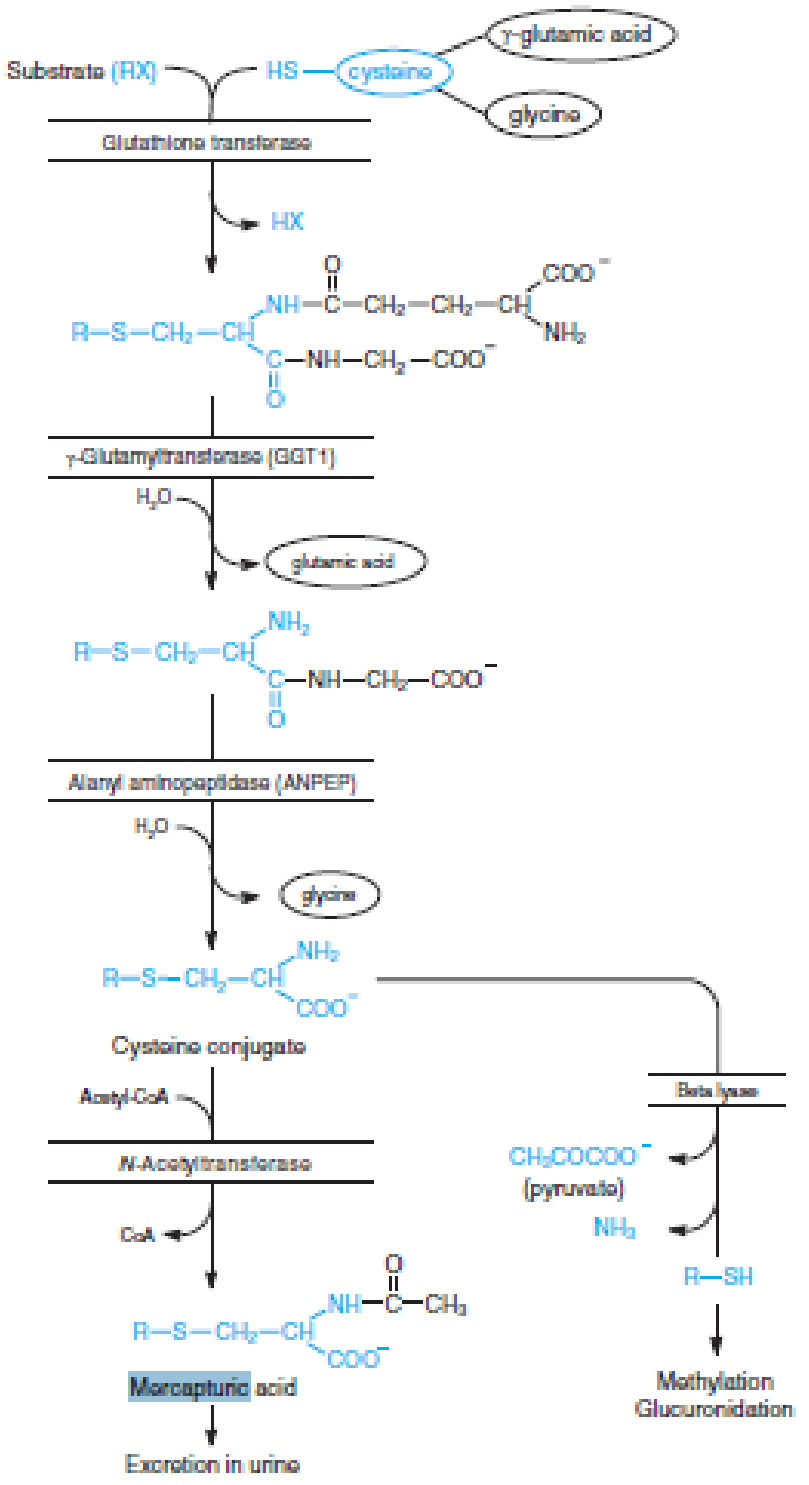

Figure 2.13. GSH conjugation and mercapturic acid biosynthesis[179]

GSH is a substrate of Se-GSH-Px and dehydroascorbate reductase enzymes and is also scavenger of $OH^{\cdot}$ and singlet O_2. GSH is also a reducing agent for various peroxides and free radicals. While the reduction of free radicals is non-enzymatically marching, peroxides are reduced by GSH-Px. As already mentioned, metabolism of

hydroperoxides and H_2O_2 with GSH-Px is related to the oxidation of GSH. GSSG is reduced by GSH-Rd and GSH recreates in the presence of NADPH[96,169].

$$\mathbf{H_2O_2\ (ROOH) + 2\ GSH \rightarrow GSSG + 2H_2O\ (ROH)}$$

The balance between GSH and GSSG in the liver and other organs is regulated by GSH-Rd and GSH-Px enzymes (Figure 2.14).

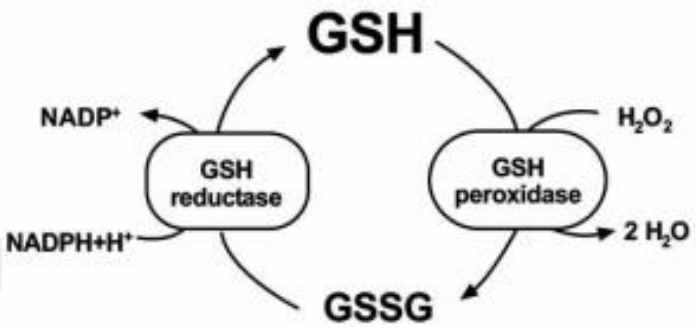

Figure 2.14. The GSH and GSSG cycle[168]

GSH is also involved in tasks such as providing membrane permeability, DNA synthesis, protein conformation, and regulation of enzyme activity[179]. The method which is developed by Ellman is widely used to determine glutathione in tissues and blood. This method, developed with various modifications, is based on the measurement of the absorbance of the color product of the resulting colorant at 412 nm by reaction of GSH with 5,5'-dithiobis-2-nitrobenzoic acid (DTNB)[180–182].

2.5.1.5. Catalase

Catalase (CAT) was obtained from the bovine liver by Sumner and Dounce in 1937. The molecular weight is around 240,000. CATs consist of four protein subunits that are attached to the active moiety by the iron group (Fe^{3+} - protoporphyrin).

Each subunit also contains a molecule-reduced NADPH that protects the enzyme against its substrate H_2O_2[96,169,183]. Storage, freeze drying, exposure of acid and alkaline lead to separation of subunits and loss of enzyme activity. CAT is found in all organs in animals, but mainly in the liver and red blood cells. They are mainly located in peroxisomes within the cell and are found in mitochondria, chloroplasts and in the endoplasmic reticulum [184].

$$\mathbf{2H_2O_2} \xrightarrow{\mathbf{CAT}} \mathbf{O_2 + 2H_2O}$$

CAT provides protection against H_2O_2, which is harmful to biological systems by breaking down H_2O_2 into O_2 and H_2O by a dismutation reaction mechanism similar to SOD [96,185,186].

$$\mathbf{CAT\text{-}\ Fe^{+3} + H_2O_2 \rightarrow Compound\ I}$$

$$\mathbf{Compound\ I + H_2O_2 \rightarrow CAT\text{-}\ Fe^{+3} + 2\ H_2O + O_2}$$

The CAT enzyme also carries out some peroxidase type reactions in the presence of H_2O_2. Thus, compounds I, oxides alcohols such as methanol and ethanol and aldehydes to formaldehyde and acetaldehyde[169].

$$\mathbf{AH2 + H_2O_2 \rightarrow A + 2H_2O}$$

CAT can also convert nitrite (NO_2^-) to nitrate (NO_3^-) under in vitro conditions, it has been suggested that absorbed elemental mercury in the body (Hg) can also be oxidized to mercury II (Hg^{2+})[186].

Ascorbic Acid (Vitamin C)

Vitamin C acts as a reducing agent in many hydroxylation reactions in the organism. Vitamin C, a very potent reducing agent, is readily dehydroascorbic acid (DHA) oxidized via the semihydrocascorbate radical intermediate product[187] (Bates CJ, Counsell JN, Hornig DH, editor. The function and metabolism of vitamin C in man. It is also a powerful antioxidant due to its strong reducing properties. It cleans them by easily reacting with $O2^-$ and $OH^\cdot$ It is known that vitamin C protects food containing oils such as vegetable and animal fats, fish, margarine and milk from oxidative degradation.

Vitamin C causes radical production both for the formation of H_2O_2 and for the formation of Fe^{2+} for the Fenton reaction. However, an optimum Fe^{3+} / Fe^{2+} ratio is required for this type of effect. For this reason, the factor that determines whether vitamin C acts like prooxidant or antioxidant is an iron reduction[96,188].

α-Tocopherol

A-tocopherol, the only fat-soluble antioxidant, is found in the phospholipid layer of cell membranes and in plasma lipoproteins (LDL)[189]. The H atom in the OH group of α-tocopherol can be removed very easily. For this reason, the peroxyl and alkoxyl radicals formed during lipid peroxidation are preferably combined with α-tocopherol instead of joining with a neighboring fatty acid.

Carotenoids

B-carotene, the metabolic precursor of vitamin A, is a component of the chloroplast membrane in plants. It is a highly potent singlet O_2 cleaner and can also prevent lipid peroxidation chain reaction by directly reacting with $OH^{\cdot}$, peroxy and alkoxy radicals. α-Tocopherol exists at a lower concentration, providing protection against the hydrophobic moieties that the tocopherols cannot affect due to their solubility. The most effective carotenoid lycopene known as singlet O_2 repellent[96,190].

Melatonin

It is a very powerful antioxidant with a lipophilic property that lifts the radical of $OH^{\cdot}$ It has antioxidant activity in a wide distribution reaching all organelles and cell nucleus of the cell and crossing the blood-brain barrier. Like other antioxidants, melatonin has no prooxidant effect[136,191].

Uric Acid

Human tissues do not contain urate oxidase, so uric acid, which is the final product in purine metabolism, accumulates. Uric acid is a powerful cleanser for singlet O_2, peroxyl radicals, $OH^{\cdot}$, ozone and HOCl and is considered an in vivo antioxidant[192].

Uric acid reacts with $OH^{\cdot}$ and forms carbon-centered radicals and they react with oxygen to form urate peroxyl radicals. These radicals, derived from uric acid, cannot be considered completely harmless even if they are less reactive than $OH^{\cdot}$[96].

Deferoxamine

Deferoxamine is a specific chelating agent for iron. Deferoxamine binds to Fe^{3+} and reducing the iron in these complexes is extremely difficult. It reacts slowly with $O_2^{\cdot -}$ or peroxides. In many systems inhibits lipid peroxidation. The iron-dependent H_2O_2 inhibits the formation of $OH^{\cdot}$ Increases the oxidation of Fe^{2+} solutions and combines with the formed Fe^{3+}. It is also a good cleanser for peroxyl radicals [169].

Ubiquinol

Ubiquinol (Coenzyme Q) plays a role in establishing proton exchange between the two sides of the inner mitochondrial membrane in the mitochondrial electron transport

chain. In the two-step oxidation, ubiquinol forms both $O_{2^{\cdot -}}$ and destroys free radicals, especially in the lipid phase[193].

Lipoic Acid

Lipoic acid is formed by dihydrolipoic acid reduction. Both destroy metal chelate builders and reactive oxygen species. Dihydrolipoic acid also plays a role in the regeneration of vitamins C and E[194].

Metallothionein

Metallothioneins are small proteins bound to cysteine which binds to transition metal ions. While they metabolize metals, they also prevent metals from catalyzing the reactions of News-Weiss and Fenton, which lead to OH radical formation [195]. In addition to these, melanin, albumin, cysteine and bilirubin have antioxidant activity[96].

2.6. Neurodegeneration and Neurodegenerative Mechanisms

Neurodegenerative diseases include a group of pathologies characterized by progressive and irreversible loss of neurons in specific regions of the brain. Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), multiple sclerosis (MS) and amyotrophic lateral sclerosis (ALS) are some of the neurodegenerative diseases. In Parkinson and Huntington's disease, neuronal loss in the basal ganglia is accompanied by abnormalities in the control of movements, whereas in Alzheimer's, cognitive functions and memory impairments are observed due to the loss of hippocampal and cortical neurons. Multiple sclerosis is a demyelinating disease of the central nervous system. In amyotrophic lateral sclerosis, muscle weakness is observed due to degeneration of spinal, bulbar and cortical motor neurons[196,197].

Mechanisms underlying the pathology of neurodegenerative diseases; oxidative stress, mitochondria, apoptosis, excitotoxicity, neurotrophic factors, defects in protein modification, dysfunction of the ubiquitin-proteosome system, oxidative DNA damage, poly (ADP-ribose) polymerase-1 activation and[198,199].

2.6.1. Mitochondria and Effect of Related Cell Death Pathways

Mitochondria, responsible for energy production and the provision of intracellular ion balance, also play a role in the regulation of free radical production and cell death

pathways. Regulation of intracellular calcium levels is carried out by mitochondria. Changes in Ca^{+2} levels lead to increased mitochondrial respiration or free radical production through various signaling pathways[200,201].

Mitochondria play important roles in necrosis and apoptosis. Intracellular ATP levels determine which path of death the cell will enter. Unlike necrosis, Apoptosis is an active process that is dependent on ATP. For a neuronal apoptosis to occur, it is necessary that mitochondrion partially functions. Severe mitochondrial damage results in necrosis, while less severe damage leads to the development of apoptosis[202].

In classical apoptotic cells, nuclear chromatin condensation and fragmentation, contraction of cytoplasmic organelles, decrease in cell volume and changes in plasma membrane are observed. Apoptosis induction in the cell occurs either outside or inside the cell. Death receptors, mitochondria and caspases play a central role in the development of apoptosis[203].

Mitochondria trigger apoptotic cascades with proteins released from the intermembrane gap. Cytochrome c, apoptosis inducer factor (AIF), endonuclease G, Smac / DIABLO, HtrA2 / Omi and procaspase 2, 3, 9 are released from mitochondrial as apoptosis regulatory proteins[204].

Bcl-2 protein family members regulate the release of regulatory proteins from intermembrane spacing. It consisting of the proapoptotic members such as Bax, Bak, Bok, Bik, Bnip3, Bmf, Bcl-xS, Bad, Bim, Bid, Noxa and antiapoptotic members such as Puma and Bcl-2 Bcl-xL, Bcl-w, Bcl-B, Boo[205]. Pore formation at the membrane surface is required to release regulatory proteins from the intermembrane gap. One of the suggested mechanisms for the pore formation is the formation of the Bax-VDAC (voltage dependent anion channel) complex with Bax or Bak oligomerization. Antiapoptotic Bcl-2; It is heterodimerized with Bax or Bak and prevents pore formation[206]. Another mechanism is the increase in Ca^{+2} and in the levels of free radical or mitochondrial permeability transition which occurs in the outer membrane of mitochondria depending on the ratio of ADP / ATP[207].

The cytochrome c released from the intermembrane gap and procaspase 9 combine with APAF-1 (apoptosis protease activating factor) in the cytosol to form the apoptosis complex. Activated caspase 9; affects procaspase 3[204]. Active caspase 3, one of

the major apoptosis enhancers; initiates chromatin condensation and DNA fragmentation via the nuclear "apoptotic chromatin condensation inducer (ACINUS)" and "caspase-active DNase (CA DNase)"[208]. Smac / DIABLO and HtrA2 / Omi inhibit apoptosis inhibitor proteins (IAP) in the cytosol. There is also serine protease activity of HtrA2 / Omi[209]. AIF and endonuclease G catalyze the separation of DNA into high molecular weight fragments by a mechanism independent of caspases[210].

Factors such as decreased growth factors, UV damage, free radical production and cytokine signaling lead to the induction of p53, the key regulator of cell cycle. p53 activates Bax and caspase 8. And also, activation of PIG3 (p53-induced protein) stimulates the production of mitochondrial free radicals[211].

Caspases from cysteinyl aspartate proteases play a role in the cell death and inflammation. They are synthesized and stored as an inactive proenzyme in the cell. They are divided into two main groups as initiator and effectors. Initiator isoforms (Caspases 1, 4, 5, 8, 9, 10, 11, 12) act by binding to adapter proteins. More than 400 substrates were identified in which the effector caspases (Caspases 3, 6, 7) interacted[212].

Fas / CD95 and TNFR1 (tumor necrosis factor receptor-1), the death receptors on the cell membrane, are responsible for maintaining the apoptotic exogenous pathway. Activation evolves by binding specific ligands such as Fas / CD95 and TNF-a to the receptors. The intracellular death sites of the receptors (DD) are combined with adapter proteins containing the death effector regions (DED). The result of this coupling is that the adapter proteins activate procaspase 8. The active caspase-8 provides activation of other caspases directly or indirectly. While the apoptotic exogenous pathway plays an important role in the immune and cancer cells, the activation of caspase 8 has been shown in paralysis, Huntington and Parkinson[213-214].

In Parkinson, Alzheimer, Huntington and ALS; release of cytochrome c from mitochondrial to cytosol, activation of caspases and display of truncated products of some substrates by active caspases suggest that the cell death form developed in neurodegenerative diseases involves apoptosis-related processes[213,215–217].

2.6.2. Exotoxic Amino Acids and The Role of Exotoxicity

Glutamate and aspartate from the excipient amino acids; are the main neurotransmitters of the mammalian central nervous system. They are found in very high

concentrations in the brain. They direct the synaptic transition at the nerve terminals and control ion entry into the neuron. Glutamate is a neurotransmitter necessary for learning, memory, synaptic plasticity, proliferation and differentiation of immature neurons. Glutamate and aspartate show effects by interacting with ionotropic and metabotropic receptors on the postsynaptic cell surface. onotropic receptors (NMDA, KA, AMPA) bound to ion channels trigger ion entry into the cell when stimulated. Metabotropic receptors are related to G protein and show their effect via secondary reporters (IP3, intracellular Ca^{+2} release)[218,219].

High affinity glutamate transporters (EAAT1-5) play a role in terminating the excitatory neurotransmission. These carriers, which are found in neurons and astrocytes, are influenced by ion gradient. Providing glutamate homeostasis depends on the ability of the glutamate transporters to maintain their quantity and function properly[220].

Excitatory amino acids normally responsible for neurotransmission are also potential sources of neurotoxicity. Excitotoxicity; is a process that results in over-stimulation of glutamate receptors and causes neurodegeneration[221]. Disruption of intracellular ion balances, formation of NO and peroxynitrite are among the mechanisms underlying glutamate toxicity. Increased the input of Na^{+}, Cl^{-} and Ca^{+2} into the cell.

Increase in intracellular Ca^{+2} levels; It activates Ca^{+2}-dependent lipolytic (lipase, phospholipases) and proteolytic enzymes (proteases), leading to cell necrotic or apoptotic cell death[222]. The NMDA receptor co-agonist D-serine has also been reported to affect neurodegenerative processes[223]. Neurodegeneration triggered by exotoxic damage plays a role in the pathogenesis of chronic neurodegenerative diseases such as Parkinson, Huntington, Alzheimer and ALS[224].

2.6.3. Oxidative Damage of DNA

The oxidative damage of DNA is one of the basic mechanisms in the pathogenesis of chronic neurodegenerative diseases such as Alzheimer's, Parkinson's, Huntington's[225,226,227]. Because most of the free radical production is mitochondria, more oxidative stresses are exposed than mitochondrial nuclei. For this reason, mitochondrial DNA has more damage than nuclear DNA[228].

Oxidative DNA damage is not only caused by oxidative stress. The inadequacy of DNA repair mechanisms also contributes to the development of damage. DNA repair mechanisms follows two paths while maintaining genome integrity. One of these Prevention of the participation of oxidatively modified deoxynucleotide triphosphates to the genome and the other is the removal of lesioned regions in nuclear and mitochondrial DNA. The most frequently investigated oxidative DNA lesion is 8-hydroxy-deoxyguanosine (8-OHdG)[229].

Removal of oxidative lesions from DNA is responsible for base excision repair (BER), nucleotide excision repair (NER), and transcription-repaired repair mechanism (TCR). BER and NER, transcriptionally independent, are global genomic DNA repair [225].

The enzyme involved in BER is DNA glycosylase. DNA glycosylase recognizes inappropriate base. Deoxyribose is removed by hydrolysis of the N-glycosidic bond between sugar and base. The lesion-specific glycosylase enzyme form is used. Apyrimidinic or apurinic region (AP region) is formed. Specific AP endonuclease enzymes recognize the AP region and open a notch in this chain. The exonuclease enzyme separates phosphate and sugar. The resulting gap is filled with DNA polymerase. Phosphodiester junction is provided by DNA ligase[230]. NER is an important defensive mechanism against damages that create large attachments on DNA bases. TCR is a transcription-dependent repair that RNA polymerase is activated when compared to a DNA lesion[225].

2.6.4. Neuroinflammation

Neuroinflammation is a process involving the responses of brain tissue to various damages, infections and disease agents. Cells involved in the process of neuroinflammation are the hematopoietic system cells such as lymphocytes, monocytes and macrophages, and the microglial cells of the central nervous system[231]. As a result of deterioration of the blood brain barrier, circulating hematopoietic system cells come to the damaged area. Immune system cells provide immunoresponse by release or by synthesis phagocytosis and regulatory elements. Complex system components, cytokines, chemokines, interleukins, transforming growth factors, glutamate, nitric oxide and free radicals are some of the substances released from immune cells. Microglial cells are mononuclear phagocytic cells of the central nervous system. When they are activated

similar to macrophages in other tissues, they release phagocytosis, antigen presentation, various inflammatory cytokines and neurotoxic factors. Activation of astrocytes in the central nervous system is necessary for the continuation of the immunological response. Astrocytes are also responsible for ensuring the integrity of the blood-brain barrier[232,233].

Recent studies have shown that microglial cells play a role in the pathogenesis of chronic neurodegenerative diseases such as Alzheimer's, Parkinson's, MS and ALS[234]. Therapeutic approaches to suppress the microglial activation process have been shown to have a slowing effect on neurodegeneration, especially in Alzheimer's disease[235].

3. MATERIAL AND METHODS

3.1. MATERIALS

3.1.1. Chemicals

Name	Brand
Bisphenol A	Sigma
Diethyl hexyl phthalate	Sigma
Carnosic acid	Sunfull
Corn oil	Cigdem
Lysis buffer: sodium hydroxide	Merck
Triton-X	Santa Cruz
Trizma base	Sigma
Protease/phosphatase inhibitor cocktail	Cell Signaling Technology
Dodecyl sulfate sodium salt (SDS)	Sigma
Tribromo acetic acid (TBA)	Sigma
Trichloro acetic acid (TCA)	Riedel-de Haen
Acetic acid	Sigma
1,1,3,3-Tetramethoxypropane	Sigma
Potassium dihydrogen phosphate	Sigma
Disodium hydrogen phosphate	Sigma
Hydrogen peroxide	Sigma
Catalase from bovine	Sigma
Ethylenediaminetetraaceticacid disodium salt dihydrate (EDTA)	Sigma
Sodium azide	Sigma
NADPH	Sigma
L-Glutathione reduced	Sigma
Glutathione Reductase	Sigma
Tert-butyl hydroperoxide	Sigma
Xhantin sodium	Sigma
3-(Cyclohexylamino)-1-propane sulfonic acid (CAPS)	Sigma

2-(4—iodophenyl)-3-(4-nitrophenol)-5 phenyltetrazolium chloride (INT)	Sigma
Xhantin Oxidase (XOD)	Sigma
SOD from bovine erythrocytes	Sigma
4-12% Bis-Tris Gel	Invitrogen
PVDF Membrane	Invitrogen
Running Buffer	İnvitrogen
LDS Sample Buffer	Invitrogen
Reduce Agent	Invitrogen
Antioxidant	Invitrogen
Sodium chloride	Merck
Tween-20	Sigma
TritonX-100	Sigma
Beta mercaptoethanol	Sigma
non-fat dry milk	Chem Cruz
β-actin antibody	Cell signaling
Anti-mouse antibody	Cell signaling
Anti-Rabbit antibody	Cell signaling
p-tau antibody	Cell signaling
t-tau antibody	Cell signaling
Bcl-2 antibody	Cell signaling
Bax antibody	Cell signaling
B-secretase (BACE) antibody	Cell signaling
ECL solution	Bio-Rad

3.1.2. Equipment

Name	Brand
Passive Avoidance	Ugo Basile, 40550
Rota-rod	Ugo Basile, 47600
Ultrasonic Bath	Isolab,
Balance	Ohaus
Micropipette	Gilson
Vortex	Heidolph,
Eppendorf tubes (2 ml)	Isolab
Falcons (15 ml)	Isolab
Blood collecting tubes (including EDTA)	B&D
Nano-spectrophotometer Protein concentration	IMPLEN P330
Calculator	
Deionized water	ELGA
Microcentrifuge	
96-well plate	Corning costar
Biorad heating and cooling block	Biorad
Plate reader	SpectrMax İ3
quartz cuvette	Agilent
Deep freezer (-20 °C)	Siemens
Deep freezer (-80 °C)	Aucma
Spectrophotometer	Thermo Fisher

3.2. METHODS

3.2.1. Experimental Design

All procedures involving the use of laboratory animals were reviewed and approved by the Experimental Animal Ethics Committee of Medipol University (Decision number: 22/06/2016- 60). Eighty-four male C-57BL/6 mice aged 16 weeks (25 g to 35 g body weight) obtained from Medipol University Medical Research Center (MEDITAM). The mice were housed in cages under standard hygienic conditions with 12 h light and dark cycles. They fed with standard diet and had free access to water.

Mice were randomly divided into 14 groups (6 mice/group) as vehicle (corn oil), BPA (16, 5, 1.6 mg/kg), DEHP (16, 5, 1.6 mg/kg), 20 mg/kg CA, BPA+DEHP (16, 5, 1.6 mg/kg) and BPA+DEHP+20 mg/kg CA (16, 5, 1.6 mg/kg). Doses of BPA, DEHP were determined according to the NOAEL values reported in the European Union risk assessment reports and selected as 3.2 times lower and 3.2 times higher than the NOAEL values and also administered by oral gavage. The mice were observed daily for changing in the oars and nails and gross clinical signs and symptoms of toxicity and mortality. Body weights of mice were recorded weekly. Chemical exposure was assessed for subacute toxicity and application was made for 28 days. The animals were decapitated 24 h after the last application. Brain was dissected and frozen on dry ice block immediately. All samples were kept at -80 °C for further testing.

3.2.2. Passive Avoidance Test

Light-dark passive avoidance device (Ugo Basile model 40550, Italy) was used to evaluate conditioned emotional memory with fear. The device consists of two different compartments at 22x21x22 dimension (Figure 3.1). And also, it has a structure divided into two chambers. The illuminated white compartment is connected to the dark compartment which has a grill floor with an electric current (0.5mA). Unavoidable electrical current is applied to feet of experimental animal via a shock generator.

Figure 3.1. Illustration of passive avoidance test apparatus

At the beginning of the experiment applied trail training to mice. The first day of this training, mice were first placed in the bright compartment. After 30 seconds, the door between compartments was opened allowing them to move freely into the dark area. Acquisition training was applied after 15 mins. At this state, mice were again placed in the bright compartment and after 30 seconds, the door between compartments was opened to move into the dark area. This time was recorded as training latency. If the passage of the experimental animal from the light partition to the dark chamber does not occur within 300 seconds, the experimental animal was eliminated from the experiment. When experimental animal completely entered the dark area, the door between compartments was automatically closed and 0.5 mA electrical current through grill floor was applied to feet of experimental animal for 3 seconds. After that experimental animal was placed in its cage. Among the trails, each compartment was cleaned to remove possible fear tips. 24 hours after acquisition training, memory trails were applied. The memory obtained with painful stimulations was be assessed by recording the delay in the entrance to the dark chamber of the experimental animals. During this test was not applied electrical current. Experimental animal was waited maximum of 300 seconds to enter dark compartment.

3.2.3. Rotarod Test

Rotarod test used to evaluate motor coordination and balance of mice. In the rotarod test, each mouse was expected to perform two of three tests on the rotating cylinder (Figure 3.2.). The speed of the rotating cylinder was continuously increased from 4 rpm to 40 rpm for a period of 5 minutes. Delaying time from rotarod was measured and the time between tests was 10-15 minutes for each mouse.

Figure 3.2. Illustration of rotarod test apparatus

3.2.4. Tissue Preparation

Cortex samples were minced and homogenized in cold lysis buffer (1:3) which contains 150 mM NaCl, 1% Triton-X 100, 50 mM Tris-HCl (pH=8.0) and 1% protease/phosphatase inhibitor cocktail solution and centrifuged at 14,000 rpm for 15 minutes at +4 °C. The supernatant was aliquoted and stored at -80°C until assay. At the same time, protein concentration measured with help of nanophotometer spectrophotometric protein concentration calculator (IMPLEN P330). That is, supernatants were diluted with water in 1:50 ratio. After that 1 ul of each sample was taken on the nanophotometer to measure protein concentration (ug/ml). Then all samples were stored at -80°C. The supernatants were then used to analyze oxidative stress and neurodegeneration parameters as described below.

3.2.5. Measurement of MDA Levels

The MDA levels in the cortex homogenates were determined according to the method developed by Jamall and Smith and adapted to 96-well plate[236]. Briefly, the reaction mixture was prepared as 15 µL of each sample, 15 µL SDS (8.1%), 112,5 µL acetic acid (20%), 112,5 µL TBA (0.8%) and deionized water and incubated at 95 °C on plate heating and cooling block for 1 hour. Then, the mixture and TCA were added equal rates as 150 µL. The plate was centrifuged at 1000x g for 10 minutes. In this assay it was observed the formation of red adduct in acidic medium between TCA and MDA and measured at 532 nm. MDA level was expressed in nmol/g protein. Tetra methoxy propane solution was used as standard.

3.2.6. Determination of SOD Activity

Superoxide $O_2^{\bullet-}$ radicals are produced during oxidative energy processes and SOD enzyme is responsible from dismutation of this toxic radical to hydrogen peroxide and molecular oxygen. Experimental design is based on using I.N.T to form a red formazan dye when react with xanthine and XOD to generate superoxide radicals. Inhibition rate of this reaction determinates the SOD activity.

SOD activities were measured using the method described by Aydin et al[237]. Briefly, each cortex homogenate was diluted 1:17 with deionized water respectively. 25 µL of diluted sample was mixed with 850 µL of substrate solution 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 (pH 10.2). Then,125 µL of xanthine oxidase (80 U/L) was added to the mixture and absorbance increase was followed at 505 nm for 3 minutes against air. SOD activity was expressed in U/mg protein. The calibration curve was prepared from the absorption readings of the standards. Standard solutions were prepared from 100mg/L stock solution in the concentration range between 30 ng/mL to 420 ng/mL and deionized water was used as a blank solution.

The absorbance change of the standards and samples per minute was calculated as described below:

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

The inhibition percentage of both samples and the reference was calculated against the control samples as follows:

$$\text{Inhibition } (\%) = \frac{\Delta Asample/\min X\ 100}{\Delta Acontrol/\min}$$

3.2.7. Determination of GSH-Px Activity

GSH-Px enzyme is responsible for the removal of hydrogen peroxides which occur in the cell. GSH-Px catalyzes the partial conversion of reduced glutathione (GSH) to oxidized glutathione GSSG) in the presence of peroxide. GSSG is reduced to GSH by the help of GSH-Rd and NADPH, which is formed in the presence of partially xanthine oxidase. GSH-Px activity is measured by reading the absorbance difference at 340 nm while the NADPH oxidation to NADP.

GSH-Px activity was measured by using the mixture of 1 mmol/L sodium EDTA, 2 mmol/L reduced GSH, 0.2 mmol/L NADPH, 4 mmol/L sodium azide and 1000 U GSH-Rd in 50 mmol/L Tris buffer (pH 7.6). Cortex homogenates were diluted 3 times before the assay. Ten µL of sample and 990 µL of mixture were mixed in a quartz cuvette "and incubated at 37 °C for 5 min. The reaction was initiated by adding 10 µL tert-butyl hydroperoxide solution (1:1000). And the decrease in NADPH absorbance was recorded at 340 nm for 3 min. The absorbance of each sample was measured at every 30 seconds and the difference in absorbance per minute was calculated. GSH-Px activity was expressed in U/g protein[237].

3.2.8. Determination of CAT Activity

Catalase activity was determined according to the method developed by Aebi[185]. The principal of this method relies on the conversion of H_2O_2 to H_2O and molecular oxygen by CAT in a time-dependent manner. The decrease in the absorbance of H_2O_2 can be monitored at 240 nm.

Cortex homogenate was diluted 250 times in the 50 mM phosphate buffer pH 7.0 before the assay. 1000 µL of diluted sample and 500 µl of hydrogen peroxide solution were mixed in a glass cuvette. The reduction rate of H_2O_2 was measured at 240 nm for 4 times at every 15 s at room temperature. Phosphate buffer was used as a blank solution and the same procedure was followed for the calibration curve. Catalase activity was expressed as kU/g protein. Standard CAT solution was prepared from CAT stock lyophilized powder in a concentration range of 0.01 – 0.035 kU/mL. The inhibition percentage was calculated as follows:

$$\text{Inhibition } (\%) = 100 - \frac{\Delta Asample/std/\min X\ 100}{\Delta Ablank/\min}$$

3.2.9. Western Blot Analysis

For western blot analysis, cortex tissue samples belonging to the same group were pooled, homogenized, sonicated, and treated with protease /phosphatase inhibitor cocktail. Total protein content was evaluated nanophotometer spectrophotometric protein calculator (IMPLEN P330). Samples were mixed with NuPAGE lithium dodecyl sulfate (LDS) sample buffer (Invitrogen) and NuPAGE sample reducing agent (Invitrogen) and then incubated at 70°C for 10 min. The proteins were electrophoresed by loading into 4-12% Bis-Tris gel and the proteins were separated by their weight. And then proteins were transferred to

polyvinylidene difluoride (PVDF) membranes by using the iBlot Dry transferring system (Invitrogen). After that, blocking of membranes was performed by using 5% non-fat milk dissolved in TBST (137 mM NaCl, 20 mM Tris, 0.1% Tween-20) for 1 hour at room temperature. Membranes were incubated with primary monoclonal antibodies for 16-18 hours at 4 °C at on a shaker. Antibodies are monoclonal rabbit anti-phospho tau (Thr181) (12885S, cell signaling); monoclonal anti-total tau (Tau46) (4019S; cell signaling), monoclonal rabbit anti-Bcl-2 (2870; Cell Signaling), rabbit polyclonal anti-Bax (2772; Cell Signaling), monoclonal rabbit anti-BACE (5606; Cell Signaling), rabbit anti-APP (2452; Cell Signaling), rabbit polyclonal anti-cleaved caspase-3 (active) (AB3623; Merck Millipore), mouse monoclonal anti-amyloid beta (sc-28365; Santa Cruz), mouse monoclonal anti-B-actin (cst-3700s; Cell Signaling) (all diluted 1:000). The following day, the membranes were washed 3 times for 5 minutes with TBST and then incubated with HRP-conjugated secondary antibody (anti-rabbit 1: 2500; anti-mouse 1: 2500) which dissolved in TBST containing 5% non-fat milk powder for 1 hour at room temperature. Membranes were washed 3 times with TBST for 5 minutes. Membrane images were obtained using ECL HRP substrate (Biorad Clarity) with the help of ChemiDoc MP System (Bio-Rad). Protein levels were analyzed using the ImageJ program and values normalized by using β-actin blots[238].

3.2.10. Statistical Analysis

The data was analyzed by using Graphpad Prism 7. The Kolmogorov–Smirnov test was used to test normal distribution assumption for continuous variables. Descriptive statistics for continuous variables were shown as mean ± standard deviation. For comparing more than two groups of variables, data with normal distribution and homogeneity of variance were analyzed using ANOVA. For pair wise comparisons, Tukey test was used for data with normal distribution and homogeneity of variance. The dependent sample t test was used to compare of the differences between levels of variables before and after. Statistically significant difference was defined as $p < 0.05$.

4. RESULTS

4.1. Results of Behavioral Tests

4.1.1. Results of Passive Avoidance Test

Mice were given medication by oral gavage for 28 days that had been trained with passive avoidance test before administration. According to this, mice that entered the dark chamber were provided learning-memory by applying feed-shock at 0.5 mA. On the 28th day after the drug administration, passive avoidance test for learning-memory was performed again to determine if they remembered or not. Results were shown in Figure 4.1.

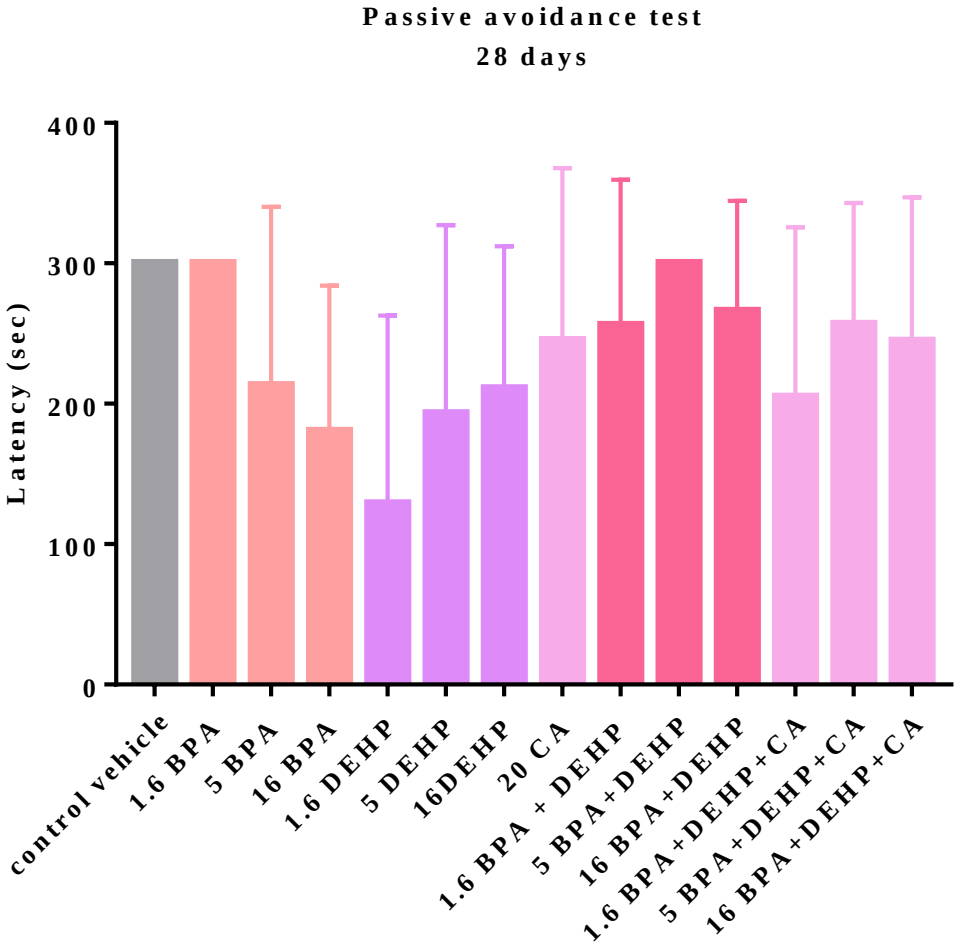

Figure 4.1. Performance in the passive avoidance test.
Mice treated with control (corn oil), BPA (16, 5, 1.6 mg/kg), DEHP (16, 5, 1.6 mg/kg), 20 mg/kg CA, BPA+DEHP (16, 5, 1.6 mg/kg) and BPA+DEHP+20 mg/kg CA. All administrations were given by per oral after punishment. The results were expressed as the mean ± SD (n=6).

Insignificantly, we observed mice that received high dose of BPA, they forgot feed-shock and passed dark chamber in a shorter time. Therefore, while low dose of BPA has no effect on memory, and high dose of BPA has an adverse effect on learning-memory.

Insignificantly, in mice that received low dose of DEHP, forgot feed-shock and passed dark chamber in shorter time. A proportional effect was observed that as time passed, the dark chamber increased in high dose received mice.

In combination of BPA and DEHP, mice that received low dose, they forgot feed-shock and entered the dark chamber in a shorter time. That is, adverse effect was observed on learning-memory. However, this effect was less than alone DEHP usage. Inversely proportional to dose, an increase in entering times to the dark chamber was observed in high doses of BPA+DEHP, like DEHP. The same effect was seen in CA which was given to decrease adverse effect of BPA and DEHP on learning-memory.

Furthermore, at low dose of BPA+DEHP+20CA was observed a further reduction in entering the dark chamber compared to mice receiving BPA and DEHP. When given low dose of DEHP adverse effect was seen, whereas when BPA+DEHP were given combination this effect significantly reduced.

4.1.2. Results of Rotarod Test

The motor coordination of the mice was assessed by the rotarod test. In the accelerated rotarod, each mouse was placed on a rotating cylinder and the speed of the rotating cylinder was continuously increased from 4 rpm to 40 rpm over a period of 5 minutes. The delay time of the mice over the rotarod was calculated. The results were as follows and shown at Figure 4.2 and Figure 4.3 respectively.

Impairment in motor coordination was observed non-significantly in high doses of BPA and DEHP administration. It had been observed that in the case of high doses of BPA+DEHP combination, this effect was improved but the effect decreased with the addition of CA. In the case of 5BPA + DEHP administration, it was observed that there was insignificantly a coordination disturbance compared to BPA and DEHP administration and CA addition to BPA+DEHP was provided a protective effect. Also, it has been observed that low dose BPA + DEHP and BPA+DEHP+20CA administration did not cause any change in motor coordination.

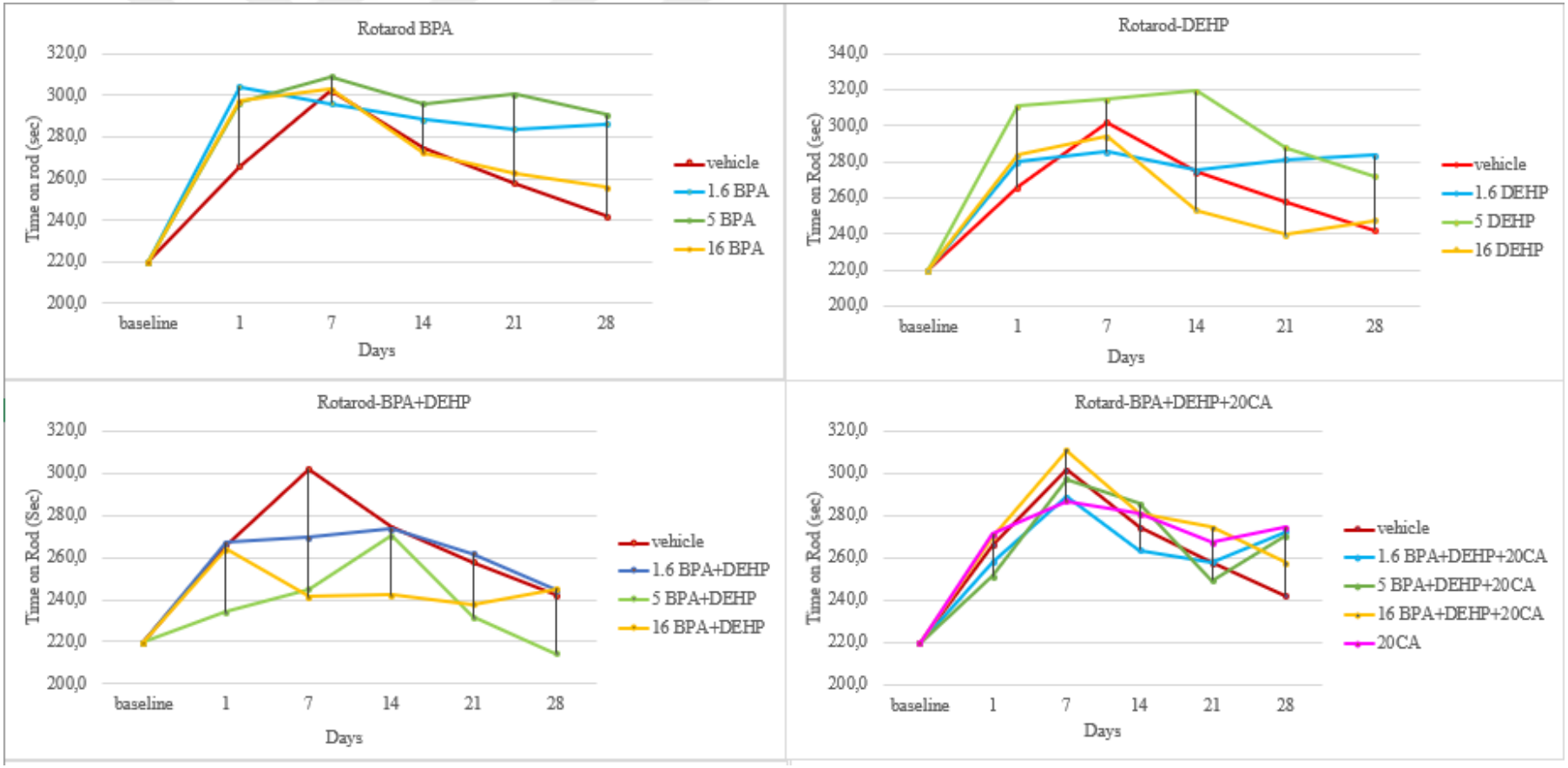

Figure 4.2. The Results of Rotarod.

Mice treated with control (corn oil), BPA (16, 5, 1.6 mg/kg), DEHP (16, 5, 1.6 mg/kg), 20 mg/kg CA, BPA+DEHP (16, 5, 1.6 mg/kg) and BPA+DEHP+20 mg/kg CA. All administrations were given by per oral after punishment. The results were expressed as the mean ± SD (n=6). Compared to control, *p < 0.05

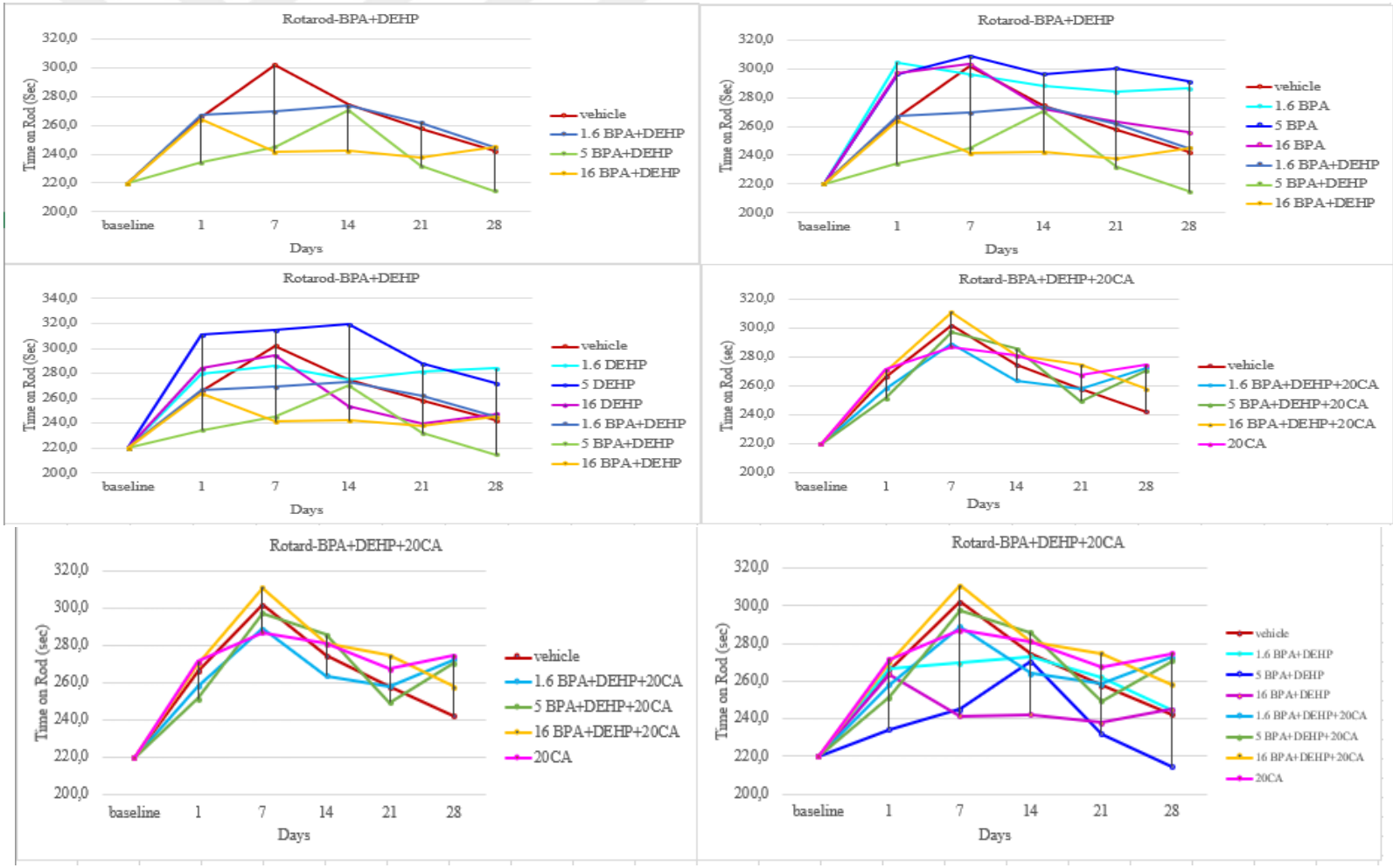

Figure 4.3. The Results of Rotarod for BPA+DEHP treatment.

Mice treated with control (corn oil), BPA (16, 5, 1.6 mg/kg), DEHP (16, 5, 1.6 mg/kg), 20 mg/kg CA, BPA+DEHP (16, 5, 1.6 mg/kg) and BPA+DEHP+20 mg/kg CA. All administrations were given by per oral after punishment. The results were expressed as the mean ± SD (n=6). Compared to control, *p < 0.05

4.2. Results of Oxidative Stress Parameters

4.2.1. Results of MDA Levels

MDA levels in cortex homogenates in mice were represented in Figure 4.4. Mice treated with BPA, DEHP and CA were given in different doses and different combinations during 28 days.

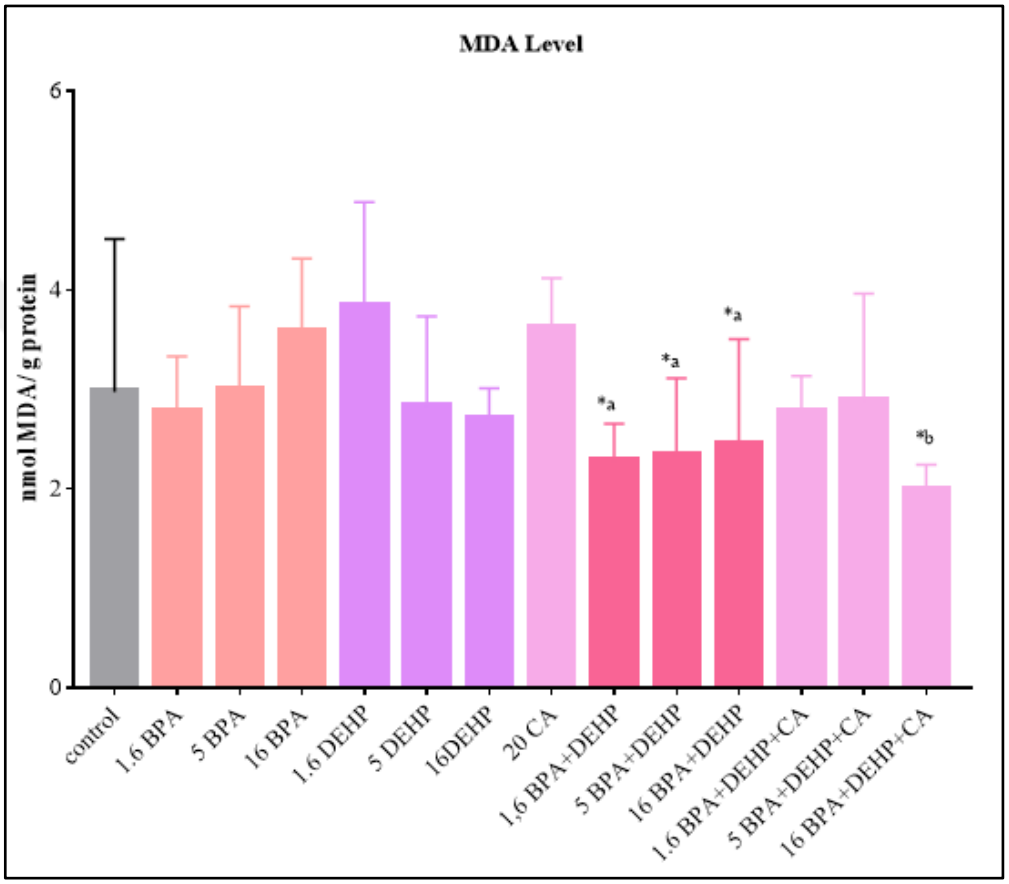

Figure 4.4. Results of the MDA levels in the cortex of mice (nmol MDA/ g protein).

The results were expressed as the mean of duplicates ± SD. Mice treated with control (corn oil), BPA (16, 5, 1.6 mg/kg), DEHP (16, 5, 1.6 mg/kg), 20 mg/kg CA, BPA+DEHP (16, 5, 1.6 mg/kg) and BPA+DEHP+20 mg/kg CA. *a, significantly different from control; *b significantly different from 1,6 DEHP *$p \leq 0.05$,)

Although there were no significant changes observed in BPA and DEHP groups, combination of BPA+DEHP caused a significant decrease in MDA levels according to control. MDA levels of all dosages of BPA+DEHP significantly decreased compared to control. And according to 1.6 DEHP, MDA levels of 16 BPA+DEHP+20CA significantly decreased.

4.2.2. Results of SOD Activity

SOD activities of cortex homogenates in mice were represented in Figure 4.5. Mice treated with BPA, DEHP and CA were given in different doses and different combinations during 28 days.

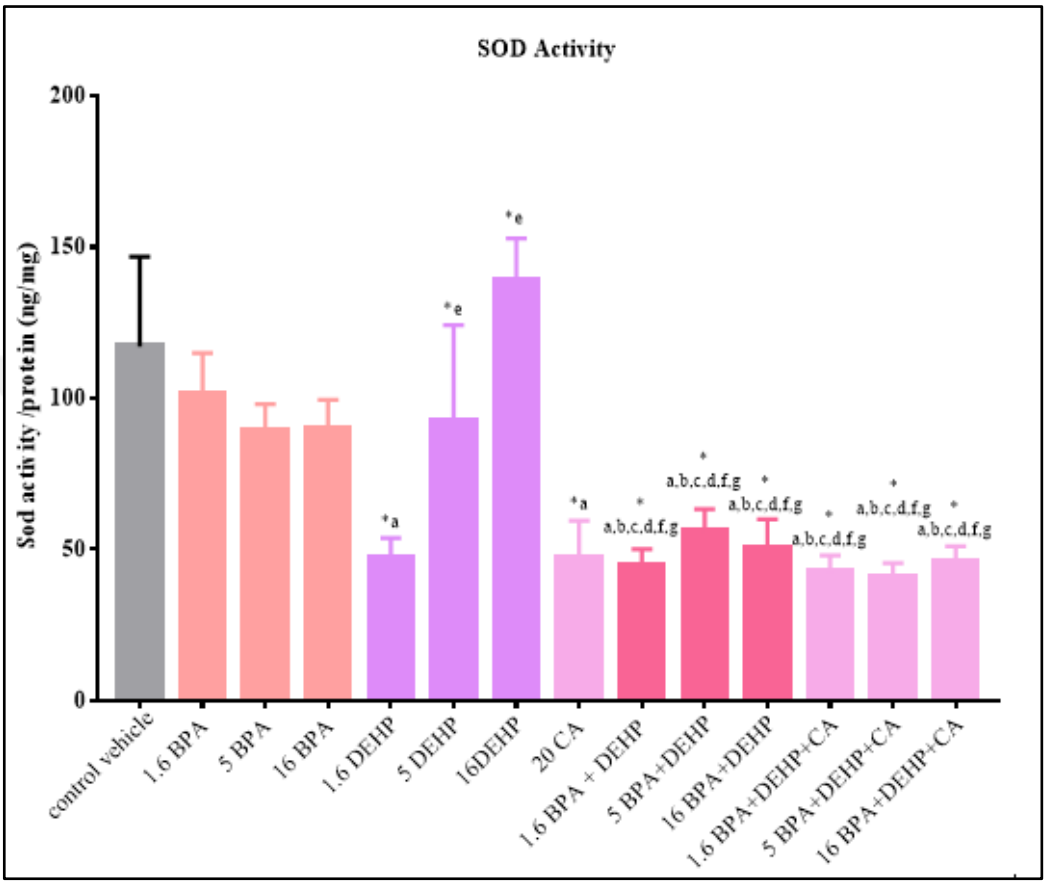

Figure 4.5. Results of the SOD activities (kU/g protein) in the cortex

The results were expressed as the mean of duplicates ± SD. Mice treated with control (corn oil), BPA (16, 5, 1.6 mg/kg), DEHP (16, 5, 1.6 mg/kg), 20 mg/kg CA, BPA+DEHP (16, 5, 1.6 mg/kg) and BPA+DEHP+20 mg/kg CA. (*$p \leq 0.05$). *a significantly different from control, *b significantly different from 1.6 BPA, *c significantly different from 5 BPA, *d significantly different from 16 BPA, *e significantly different from 1.6 DEHP, *f significantly different from 5 DEHP, *g significantly different from 16 DEHP

The SOD activity of all dosage of BPA+ DEHP and BPA+DEHP +20 CA, 1.6 DEHP and 20 CA significantly decreased compare to control. According to all dosages of BPA, 5 and 16 DEHP SOD activity of all dosage of BPA+ DEHP and BPA+DEHP +20 CA significantly decreased. Interestingly, SOD activity of 5 and 16 DEHP significantly increased according to 1.6 DEHP.

Despite the fact that no significant change was observed between BPA groups, there was a decrease in the dose-dependent effect. While BPA and DEHP did not have an adverse effect on SOD activity when used alone; their combined use led to a significant

decrease in SOD activity. This decrease in SOD activity may be due to increased oxidative stress or radical scavenging activity of CA.

4.2.3. Results of CAT Activity

CAT activities of cortex homogenates in mice were represented in Figure 4.6. Mice treated with BPA, DEHP and CA were given in different doses and different combinations during 28 days.

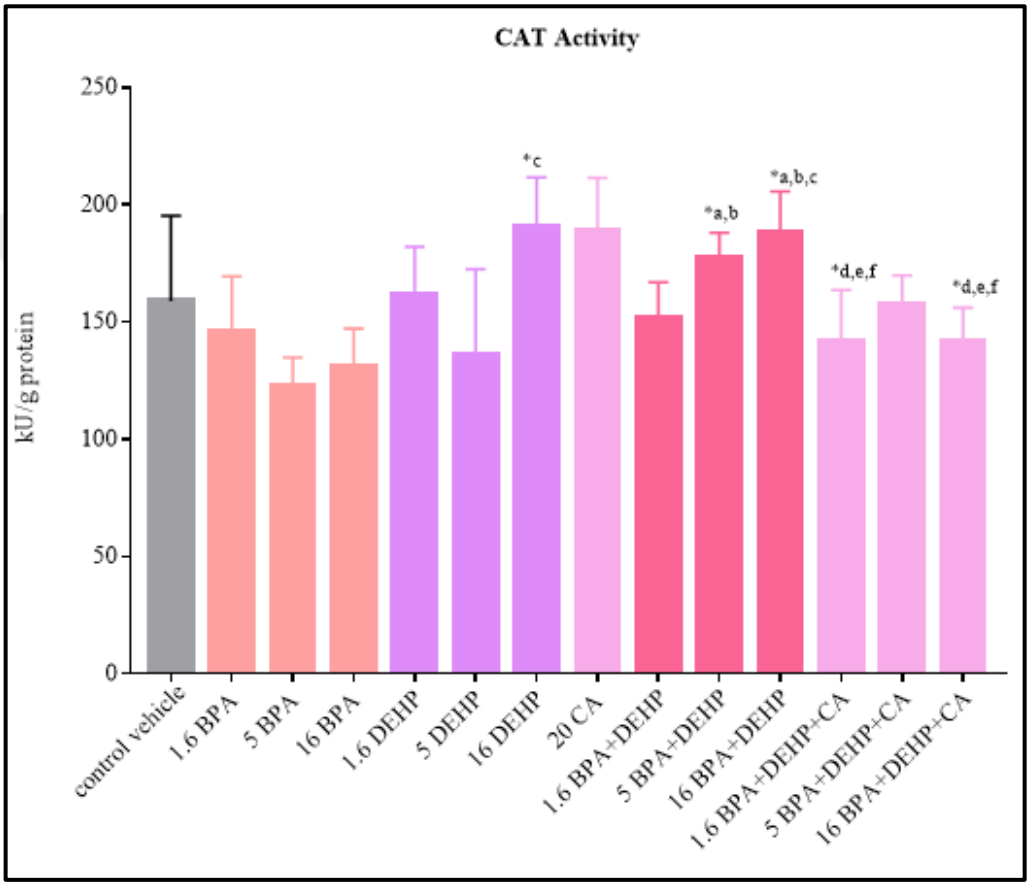

Figure 4.6. Results of the CAT activities (kU/g protein) in the cortex of mice.
The results were expressed as the mean of duplicates ± SD. Mice treated with control (corn oil), BPA (16, 5, 1.6 mg/kg), DEHP (16, 5, 1.6 mg/kg), 20 mg/kg CA, BPA+DEHP (16, 5, 1.6 mg/kg) and BPA+DEHP+20 mg/kg CA. (*$p \leq 0.05$). *a significantly different from 5 BPA, * b significantly different from 16 BPA, *c significantly different from 5 DEHP, *d significantly different from 16 DEHP, *e significantly different from 20 CA, *f significantly different from 16 BPA + DEHP

In CAT activity, according to 5 and 16 BPA, significantly increased in the activity of 5 and 16 BPA+DEHP. And also, significantly decreased activity was seen in the combined administration of 1.6 and 16 BPA+DEHP+20 CA versus 16 DEHP, 20 CA and 16 BPA+DEHP in order. A significantly increased activity was observed in 16 DEHP and 16 BPA+DEHP in terms of 5 DEHP.

Although an insignificant decrease in CAT activity was observed when BPA and DEHP were administrated alone, their combined administration led to a significant increase in CAT activity. In this case, formed hydrogen peroxide was removed from the medium by the CAT instead of the GSH-Px and thus increased the CAT activity. Also, we found that insignificantly decreased CAT activity with combined administration of BPA+DEHP+20 CA may be occurred because of enhanced the radical formation due to the inability of the body to compensate the free radical formed during use. Therefore, increased oxidative stress will disrupt the oxidant / antioxidant balance. In our study, we found that BPA+DEHP or BPA+DEHP+20 CA may show its toxicity by increasing hydrogen peroxide.

4.2.4. Results of GSH-Px Activity

GSH-Px activities of cortex homogenates in mice were represented in Figure 4.7. Mice treated with BPA, DEHP and CA were given in different doses and different combinations during 28 days.

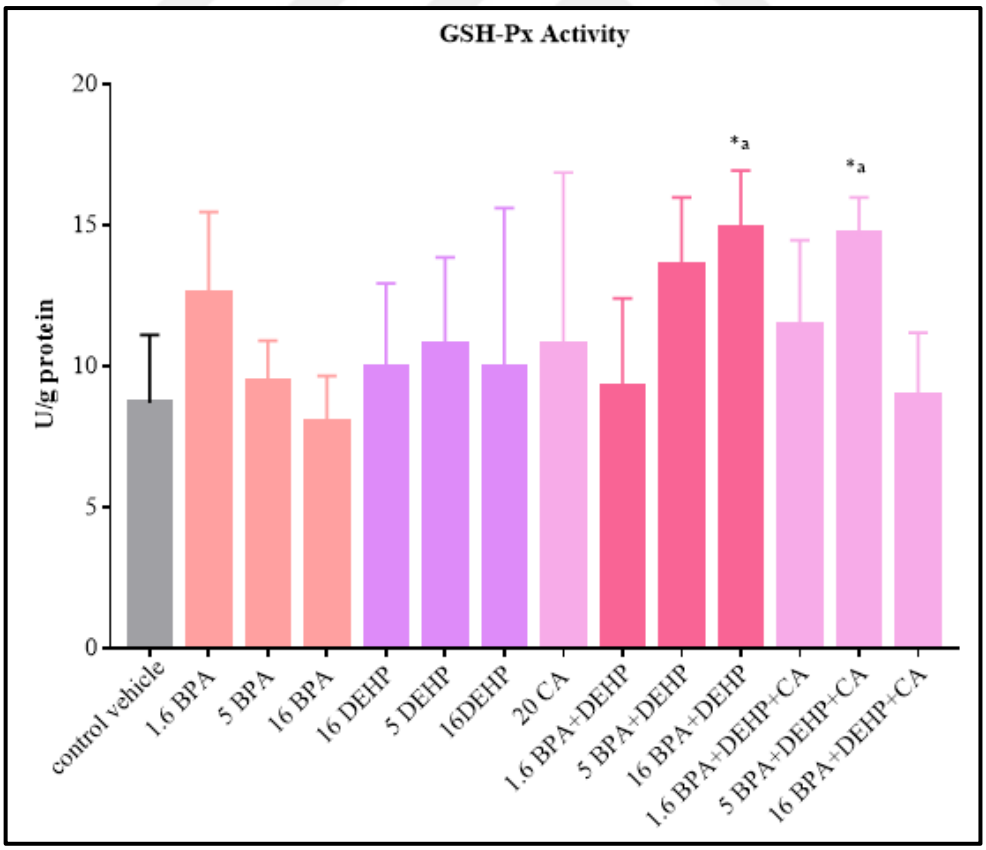

Figure 4.7. Results of the GSH-Px activities (U/g protein) in the cortex of mice.
The results were expressed as the mean of duplicates ± SD. Mice treated with control (corn oil), BPA (16, 5, 1.6 mg/kg), DEHP (16, 5, 1.6 mg/kg), 20 mg/kg CA, BPA+DEHP (16, 5, 1.6 mg/kg) and BPA+DEHP+20 mg/kg CA. (*$p \leq 0.05$) *a significantly different from 16 BPA

A significant decrease in GSH-Px activity was observed in 16 BPA versus 16 BPA+DEHP. Also, the GSH-Px activity of 16 BPA+DEHP was increased according to 16 BPA. While BPA and DEHP did not have an adverse effect on GSH-Px activity when they were administrated alone; their combined administration led to an increase in GSH-Px activity. Furthermore, although no significant change was observed between BPA groups, there was a decrease in a dose-dependent manner.

4.3. Results of Western Blot Analysis

Mice treated with BPA, DEHP and CA were given in different doses and different combinations during 28 days. Later on, Bcl-2, Bax, p-tau, t-tau and BACE protein levels were calculated by Western Blot technique with tissues taken from the cortex of mice. The image of membrane were given in Figure 4.8.

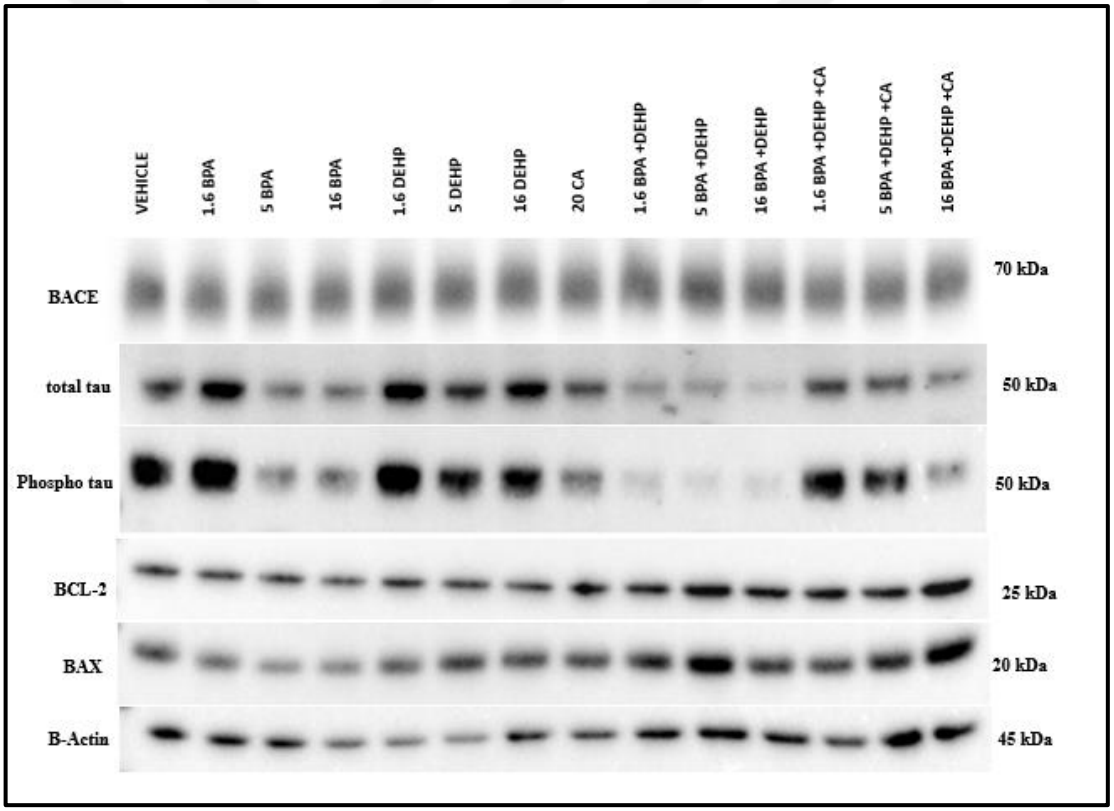

Figure 4.8. Bcl-2, Bax, phospho tau, total tau, BACE and β-actin protein expressions in the cortex

Mice treated to BPA, DEHP, and CA at different dosage and combination regime for 28 days. The results were expressed as the mean of duplicates ± SD from three independent experiments with proteins of six mice. Protein expression levels were normalized relative to β-actin control and phosphorylated protein expression levels were normalized relative to their total protein. Control (corn oil), BPA (16, 5, 1.6 mg/kg), DEHP (16, 5, 1.6 mg/kg), 20 mg/kg CA, BPA+DEHP (16, 5, 1.6 mg/kg) and BPA+DEHP+20 mg/kg CA

4.3.1. Results of Anti-Apoptotic and Pro-Apoptotic Proteins

4.3.1.1. Analysis of Bcl-2 Protein

Mice treated with BPA, DEHP and CA were given in different doses and different combinations during 28 days. Later on, Bcl-2 protein level was calculated by Western Blot technique with tissues taken from the cortex of mice. The results were represented in Figure 4.9.

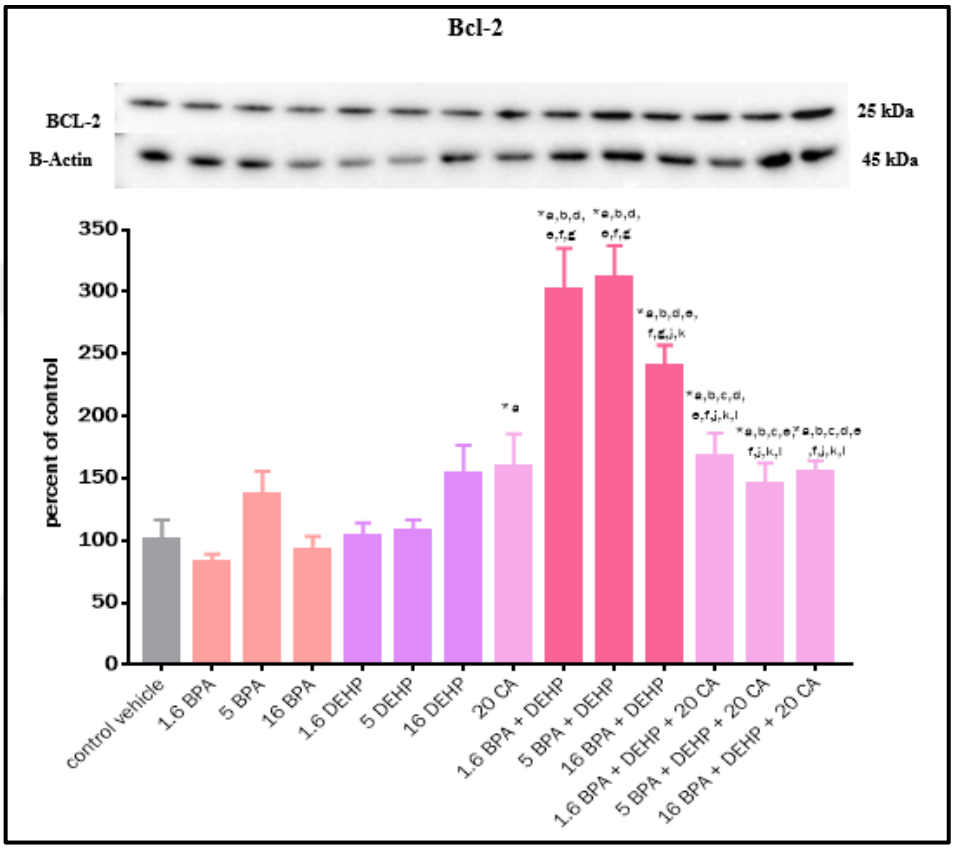

Figure 4.9: The results of Bcl-2 Protein expression level

The results were expressed as the mean ± SD of three independent experiments. Protein expression levels were normalized relative to β-actin control. ($p \leq 0.05$) *a significantly different from control, *b significantly different from 1.6 BPA, *c significantly different from 5 BPA, *d significantly different from 16 BPA, *e significantly different from 1.6 DEHP, *f significantly different from 5 DEHP, *g significantly different from 16 DEHP, *h significantly different from 20 CA, *j significantly different from 1.6 BPA+DEHP, *k significantly different from 5 BPA+DEHP, *l significantly different from 16 BPA+DEHP. Protein measurements were made in three replicates.

Measurements of Bcl-2 protein were calculated by proportioning them with the control group. According to this, control group 100 ± 23.30, 1.6 BPA group 82 ± 14.12, 5 BPA group 136±28.18, 16 BPA group 94±17.26, 1.6 DEHP group 107 ± 14.56, 5 DEHP 112 ±14.91, 16 DEHP group 162±30.1, 20 CA group 170±31.90, 1.6 BPA+DEHP group 313 ± 32.11, 5 BPA+DEHP group 326±41.16, 16 BPA+DEHP group 249±26.49, 1.6

BPA+DEHP+20CA group 173±28.33, 5 BPA+DEHP+20CA group 148±15.98 and finally 16 BPA+DEHP+20CA group 160±22.82 were calculated.

Bcl-2 level significantly increased in 20 CA and all combined groups of BPA + DEHP and BPA + DEHP + 20CA as compared to control ($p < 0.05$). Bcl-2 levels of 1.6 BPA + DEHP and 1.6 BPA + DEHP + 20CA increased significantly as compared to 1.6 BPA and 1.6 DEHP groups ($p < 0.05$). Additionally, Bcl-2 levels of 16 BPA + DEHP and 16 BPA + DEHP + 20CA groups significantly increased as compared to 16 BPA and 16 DEHP groups ($p < 0.05$). Bcl-2 levels of 1.6 BPA+DEHP+20CA groups significantly decreased as compared to 1.6 BPA+DEHP groups ($p < 0.05$). Bcl-2 levels of 5 BPA+DEHP+20CA groups significantly decreased as compared to 5 BPA+DEHP groups ($p < 0.05$). Bcl-2 levels of 16 BPA+DEHP+20CA groups significantly decreased as compared to 16 BPA+DEHP groups ($p < 0.05$).

4.3.1.2. Analysis of Bax Protein

Mice treated with BPA, DEHP and CA were given in different doses and different combinations during 28 days. Later on, Bax protein level was calculated by Western Blot technique with tissues taken from the cortex of mice. The results were represented in Figure 4.10.

Measurements of Bax protein were calculated by proportioning them with the control group. According to this, control group 100 ± 23.03, 1.6 BPA group 70 ± 13.28, 5 BPA group 82±14, 16 BPA group 75±12.03, 1.6 DEHP group 88 ± 16.32, 5 DEHP 134 ±14.95, 16 DEHP group 174±26.06, 20 CA group 139±12.30, 1.6 BPA+DEHP group 326 ±69.06, 5 BPA+DEHP group 405±29.39, 16 BPA+DEHP group 220±27, 1.6 BPA+DEHP+20CA group 138±21.80, 5 BPA+DEHP+20CA group 157±25.71 and finally 16 BPA+DEHP+20CA group 183±25.45 were calculated. Bax level significantly increased in all combined groups of BPA + DEHP and BPA + DEHP + 20CA compared to control ($p < 0.05$). Bax level of 5 BPA + DEHP group significantly increased as compared to 5 BPA and 5 DEHP groups ($p < 0.05$). Bax level of 1.6 BPA + DEHP group significantly increased as compared to 1.6 BPA and 1.6 DEHP groups ($p < 0.05$). Additionally, Bax level of 16 BPA + DEHP group significantly increased as compared to 16 BPA and 16 DEHP groups ($p < 0.05$). Bax level of 1.6 BPA + DEHP + 20CA group significantly decreased as compared to 1.6 BPA+DEHP group ($p < 0.05$).

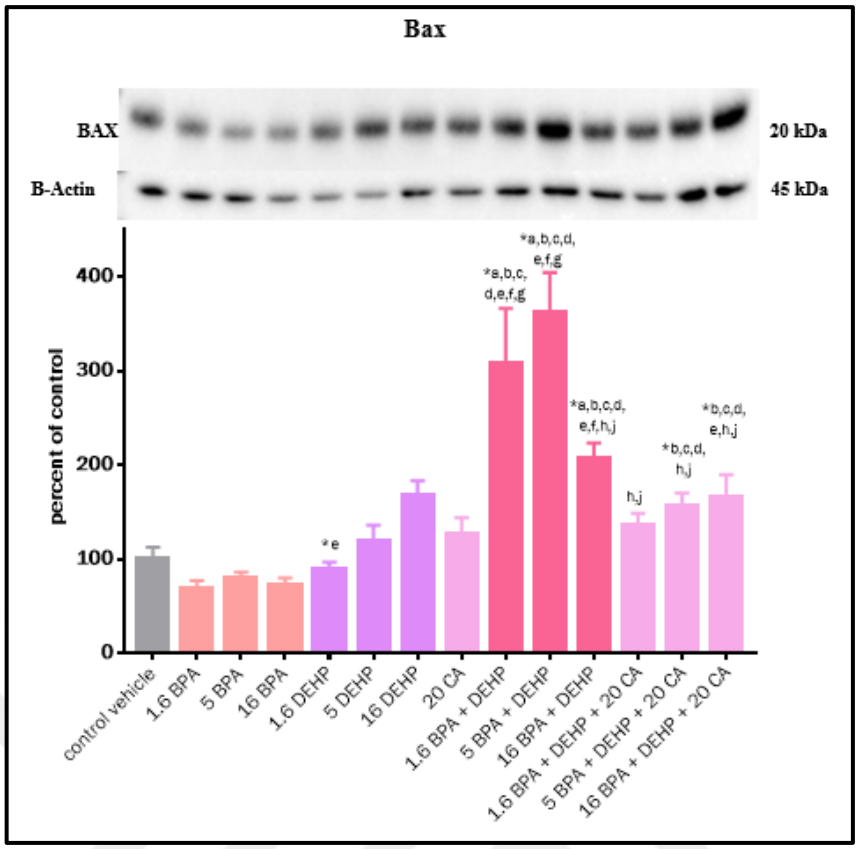

Figure 4.10: The results of Bax protein expression level

The results were expressed as the mean ± SD from three independent experiments. Protein expression levels were normalized relative to β-actin control. ($p \leq 0.05$) *a significantly different from control, *b significantly different from 1.6 BPA, *c significantly different from 5 BPA, *d significantly different from 16 BPA, *e significantly different from 1.6 DEHP, *f significantly different from 5 DEHP, *g significantly different from 16 DEHP, *h significantly different from 20 CA, *j significantly different from 1.6 BPA+DEHP. Protein measurements were made in three replicates.

4.3.2. Analysis of BACE Protein

Mice treated with BPA, DEHP and CA were given in different doses and different combinations during 28 days. Later on, BACE protein level was calculated by Western Blot technique with tissues taken from the cortex of mice. The results were represented in Figure 4.11.

Measurements of BACE protein were calculated by proportioning them with the control group. According to this, control group 100 ± 15.61, 1.6 BPA group 77 ±14.28, 5 BPA group 127±25.56, 16 BPA group 985±20.91, 1.6 DEHP group 97 ± 13.56, 5 DEHP 111 ±11.41, 16 DEHP group 160±19.81, 20 CA group 127±13.98, 1.6 BPA+DEHP group 260±39.27, 5 BPA+DEHP group 251±18.34, 16 BPA+DEHP group 197±21.40, 1.6 BPA+DEHP+20CA group 125±12.50, 5 BPA+DEHP+20CA group 114±11.21 and finally 16 BPA+DEHP+20CA group 97±11.52 were calculated.

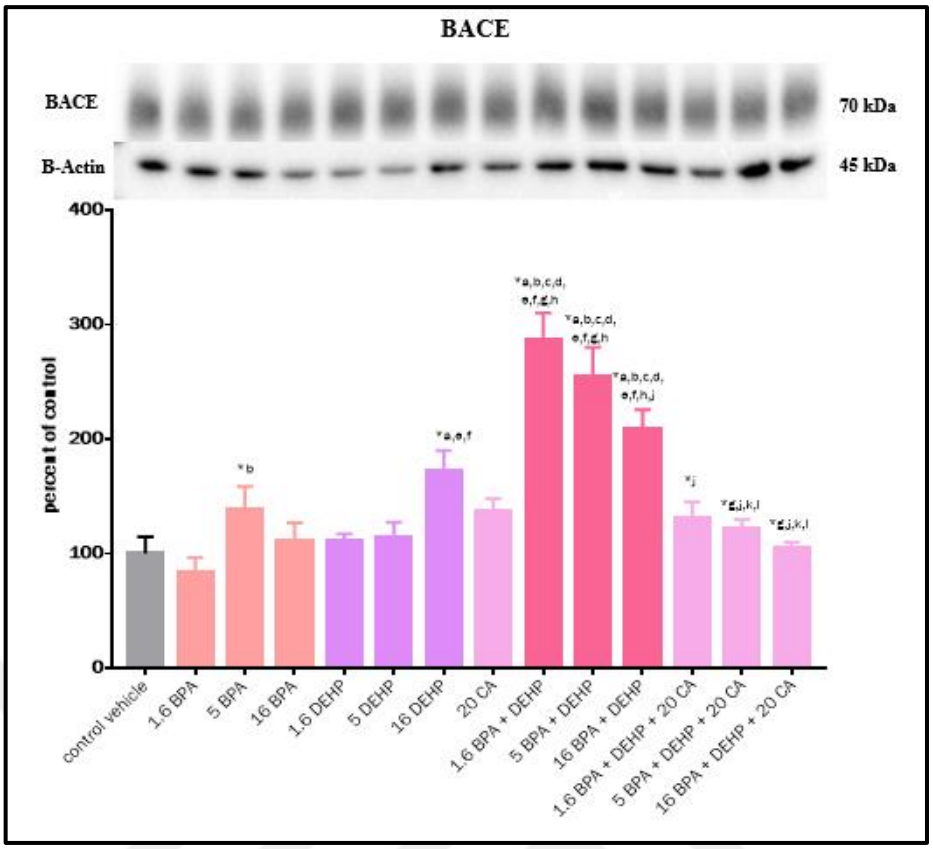

Figure 4.11: The results of BACE protein expression level

The results were expressed as the mean ± SD from three independent experiments. Protein expression levels were normalized relative to β-actin control. ($p \leq 0.05$) *a significantly different from control, *b significantly different from 1.6 BPA, *c significantly different from 5 BPA, *d significantly different from 16 BPA, *e significantly different from 1.6 DEHP, *f significantly different from 5 DEHP, *g significantly different from 16 DEHP, *h significantly different from 20 CA, *j significantly different from 1.6 BPA+DEHP, *k significantly different from 5 BPA+DEHP, *l significantly different from 16 BPA+DEHP. Protein measurements were made in three replicates.

BACE level significantly increased in 16 DEHP and all combined groups of BPA + DEHP compared to control ($p < 0.05$). BACE level of 1.6 BPA + DEHP group significantly increased as compared to 1.6 BPA and 1.6 DEHP groups ($p < 0.05$). BACE level of 5 BPA + DEHP group significantly increased as compared to 5 BPA and 5 DEHP groups ($p < 0.05$). Additionally, BACE level of 16 BPA + DEHP group significantly increased as compared to 16 BPA and 16 DEHP groups ($p < 0.05$). Additionally, BACE level of 16 BPA + DEHP group significantly decreased as compared to 1.6 BPA + DEHP group ($p < 0.05$). BACE level of 1.6 BPA + DEHP+ 20 CA group significantly decreased as compared to 1.6 BPA + DEHP group ($p < 0.05$). BACE level of 5 BPA + DEHP+ 20 CA group significantly decreased as compared to 5 BPA + DEHP group ($p < 0.05$). BACE level of 16 BPA + DEHP+ 20 CA group significantly decreased as compared to 16 BPA + DEHP group ($p < 0.05$).

4.3.3. Analysis of Phosphorylated Tau Protein

Mice treated with BPA, DEHP and CA were given in different doses and different combinations during 28 days. Later on, phosphorylated tau (p-tau) protein level was calculated by Western Blot technique with tissues taken from the cortex of mice. The results were represented in Figure 4.12.

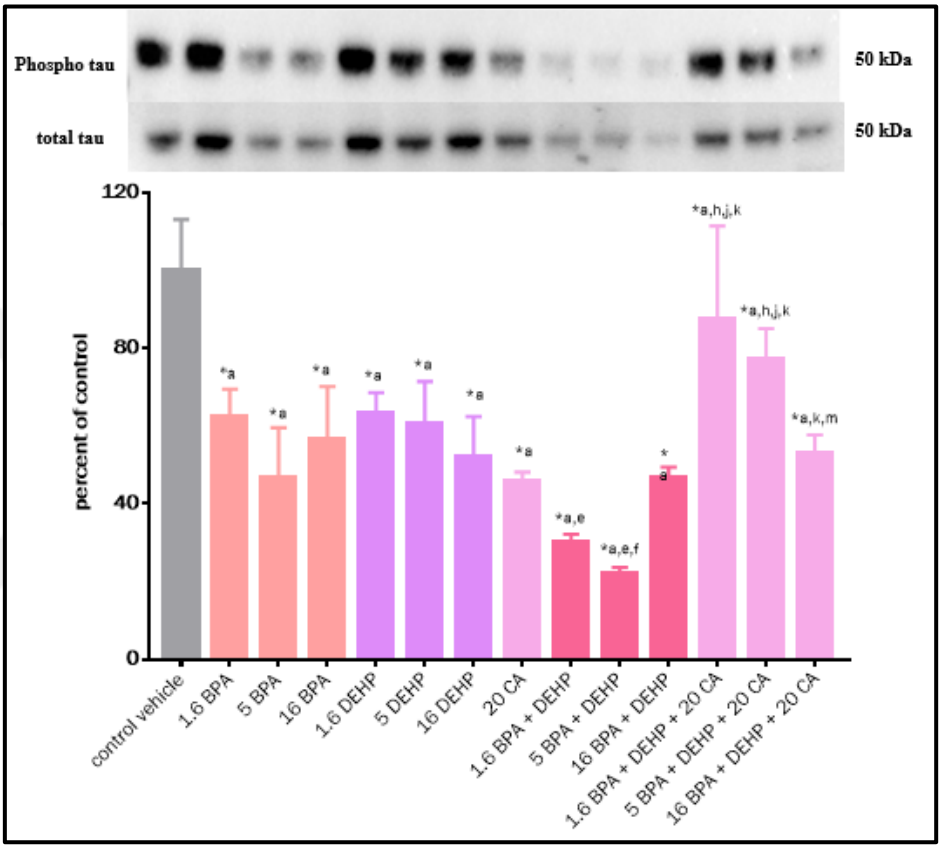

Figure 4.12: The results of phosphorylated tau protein expression level

The results were expressed as the mean ± SD from three independent experiments with a protein of six mice. Protein expression levels were normalized relative t-tau. ($p \leq 0.05$) *a significantly different from control, *e significantly different from 1.6 DEHP, *f significantly different from 5 DEHP, *h significantly different from 20 CA, *j significantly different from 1.6 BPA+DEHP, *k significantly different from 5 BPA+DEHP, *m significantly different from 1.6 BPA+DEHP+ 20 CA. Protein measurements were made in three replicates.

Measurements of phosphorylated tau protein were calculated by proportioning them with the control group. According to this, control group 100 ± 13.03, 1.6 BPA group 62 ± 7.26, 5 BPA group 47±12.74, 16 BPA group 56±13.70, 1.6 DEHP group 63 ± 5.30, 5 DEHP 60 ±11.01, 16 DEHP group 52±10.39, 20 CA group 46±2.55, 1.6 BPA+DEHP group 30 ± 2.06, 5 BPA+DEHP group 22±1.93, 16 BPA+DEHP group 46±3.02, 1.6 BPA+DEHP+20CA group 87±24.07, 5 BPA+DEHP+20CA group 77±8.11 and finally

16 BPA+DEHP+20CA group 53±4.76 were calculated. P-tau level significantly decreased in all groups as compared to control ($p < 0.05$). P-tau levels of all combined groups of BPA + DEHP groups significantly decreased as compared to 1.6 DEHP, 5 DEHP and control groups ($p < 0.05$). P-tau level of 1.6 BPA + DEHP+ 20 CA group significantly increased as compared to 1.6 BPA + DEHP group ($p < 0.05$). Additionally, p-tau level of 5BPA + DEHP+ 20 CA group significantly increased as compared to 5 BPA + DEHP group ($p < 0.05$).

5. DISCUSSION

Free radicals are found in the cell under normal conditions. The oxidant and antioxidant mechanisms are in dynamic equilibrium. However, the increase of free radicals in the cell may cause oxidative stress which also increase the amount of ROS in the cell by breaking this balance. However, elevated levels of free radicals can cause oxidative stress in cells by disrupting this balance. Antioxidant enzymes such as SOD, CAT and GSH-Px present in the cell to prevent this effect. SOD enzyme neutralizes superoxide radical by converting to hydrogen peroxide. CAT and GSH-Px provide protection by converting hydrogen peroxide to water. If this protection mechanism does not occur, free radicals bind to macromolecules such as DNA, RNA, protein and can cause cell damage. Free radicals are the major cause of neurotoxicity. Due to the brain consumes large amounts of oxygen, it makes them particularly susceptible. Many studies on both DEHP and BPA have suggested that antioxidant enzyme levels are reduced[239–246]. In our study, we looked at the effects of BPA and DEHP and their combined administration, BPA + DEHP and BPA + DEHP + 20CA, in the adult mice cortex on oxidative stress. The SOD activity significantly decreased upon treatment of all dosage of BPA+ DEHP and BPA+DEHP +20 CA compared to control. Interestingly, SOD activity of 5 and 16 DEHP significantly increased in contrast to 1.6 DEHP. Despite the fact that insignificant change was observed between BPA groups, there was a decrease in a dose-dependent manner. While BPA and DEHP did not have an adverse effect on SOD activity when administrated alone; their combined administration led to a significant decrease in SOD activity. This decrease in SOD activity may be due to increased oxidative stress or insufficient radical scavenging activity of CA. Although no significant change was observed in GSH-Px activity in DEHP administrated groups, there was a decrease between BPA groups in a dose-dependent manner. Interestingly, meanwhile the combination of BPA+DEHP was observed increase GSH-Px activity, the opposite effect was observed in the combined administration of BPA+DEHP+20 CA. The production of GSH-Px enzyme increased due to adaptation mechanism of body to compensate damage.

An insignificant decrease in CAT activity was observed when BPA was used alone, however CAT activity was observed an increase in the combined administration of BPA+DEHP as similar to GSH-Px. Also, we found that CAT activity insignificantly decreased upon combined administration of BPA+DEHP+20 CA. This is because of BPA+DEHP or BPA+DEHP+20 CA may show its toxicity by increasing hydrogen

peroxide. In this case, formed hydrogen peroxide was removed from the medium by the CAT and GSH-Px enzymes and thus lead to increase the amount of these enzymes by adaptation mechanism of the body. Also, addition of carnosic acid decreased production of these enzymes by radical scavenging property.

At the same time, there are a lot of phospholipid side chains in the brain which can cause peroxidation of free radicals. Because these free radicals firstly initiate a chain reaction from the lipid membrane by taking an electron, they may cause deterioration of the integrity of the cell. Many studies on both BPA and DEHP have shown that increased MDA levels are a product of lipid peroxidation[86,239,243,246,247]. However, in our study, there was no significant change in MDA level compared to the control. While MDA levels were significantly decreased in BPA+DEHP groups, there was no increase in BPA + DEHP + 20CA groups.

The other parameter was investigation of apoptosis which may occur in two ways in the cell. One of them is an intrinsic pathway or mitochondrial apoptosis and the other is an extrinsic pathway or death receptor. These two pathways use their own initiators and the intersection of both is caspase-3. Bcl-2 family proteins control and regulate mitochondria-dependent apoptosis. Bcl-2 family proteins control mitochondrial membrane potential by regulating cytochrome c release. These proteins can be anti-apoptotic or pro-apoptotic. Bcl-2 is an anti-apoptotic protein and is found outside of mitochondria. It regulates ion transport. Therefore, overexpression of Bcl-2 prevents apoptosis or inhibit cell death response. That is, increased Bcl-2 level shows a stress condition and this can trigger cancer. While Bcl-2 level increases in neuronal damage without apoptosis. There are different studies about the effect of both BPA and DEHP on Bcl-2 protein. While a study reported that BPA caused a decrease in Bcl-2 level, in another study, it was reported an insignificant increase in Bcl-2 level[248,249]. A study of DEHP found that Bcl-2 levels did not change[5]. In a study of mono and di (n-butyl) phthalates there was no change in Bcl-2 and Bax levels. In our results, we showed that Bcl-2 levels of BPA+DEHP groups were significantly higher than BPA and DEHP groups. Interestingly, addition of CA to the combination of BPA+DEHP groups caused a significantly decrease in Bcl-2 protein levels. Tatton and Olanow indicated that "Increased Bcl-2 levels may reflect the induction of a survival program in neurons that have sustained damage that is not sufficient to cause apoptosis, while increased Bax levels may mean that the neuron has progressed to the early stages of apoptosis"[250].

Another parameter of apoptosis is Bax which is a pro-apoptotic protein and is mostly found in cytosol. However, Bax mostly migrates to mitochondria during apoptosis and its concentration increases during apoptosis. In our results, Bax levels of DEHP groups insignificantly increased in a dose-dependent manner. Like Bcl-2, we showed that Bax levels of 1.6 and 5 BPA+DEHP groups were significantly higher than the groups in which BPA and DEHP was administrated alone. Moreover, addition of CA to the combination of 1.6 and 5 BPA+DEHP groups caused a significant decrease in Bax protein levels. However, Bax level of 16 BPA+DEHP significantly decreased compared to 1.6 and 5 BPA+DEHP. And also, no results were found in the cleaved-caspase-3 test.

As another parameter, we investigated tau protein levels, which are known as microtubule associated proteins and they are one of the important components of cytoskeletal. They are found in neurons and abnormal phosphorylation of tau protein, which may result in neurofibrillary tangles. Accumulation of them may cause toxicity to neurons. In correspondence with that in some of *in vivo* and an *in vitro* studies with BPA, there was an increase in p-tau, APP and BACE levels[8,251,252], while in another study of BPA, there was no change in tau levels in both male and female mice [3]. And also, in a study it was reported that DEHP caused an increase in p-tau level[5]. In our results, p-tau levels of all groups were significantly decreased as compared to control. Furthermore, p-tau levels of 1.6 and 5 BPA+DEHP significantly decreased as compared to 1.6 and 5 DEHP respectively. Interestingly, p-tau levels of 1.6 and 5 BPA+DEHP+CA significantly increased as compared to 1.6 and 5 BPA+DEHP respectively.

Another test parameter was Beta-secretase (BACE) which is a protease enzyme and it cleaves amyloid precursor protein from the β-site and trigger amyloid beta $_{(1\text{-}42)}$ formation. In our studies, we found that BACE level of 16 DEHP group significantly increased in comparison to 1.6 and 5 DEHP groups. Meanwhile, BACE levels of BPA+DEHP significantly increased in comparison to all of BPA and DEHP groups, BACE levels of BPA+DEHP+20CA significantly decreased in comparison to all of BPA+DEHP groups, respectively. However, there was a dose-dependent decrease in the BACE levels of BPA+DEHP and BPA+DEHP+20CA groups. The combined administration of BPA+DEHP may be trigger Alzheimer by increasing BACE enzyme level and the addition of CA to combination showed protective effect against Alzheimer's disease. And also, in our studies no relevance was observed in the APP and amyloid beta tests.

Furthermore, we investigated the effects of BPA, DEHP and CA usage on behavior. Insignificantly, we observed mice that were given high dose of BPA, they forgot feed-shock and passed dark chamber in a shorter time. Which suggests that low dose of BPA had no effect on memory, while high dose of BPA had adverse effect on learning-memory. Insignificantly, in mice that received low dose of DEHP, they forgot feed-shock and passed dark chamber in a shorter time. Whereas opposite effect was observed for entering time to dark chamber increased in high dose received mice. In combination of BPA and DEHP, mice that received low dose, they forgot feed-shock and entered the dark chamber in a shorter time. That is, adverse effect was observed on learning-memory. However, this effect is less than alone DEHP administration. Inversely proportional to dose, an increase in entering times to the dark chamber was observed in high doses of BPA+DEHP, like DEHP. The same effect was seen in CA which was given to decrease adverse effect of BPA and DEHP on learning-memory. Furthermore, at low dose of BPA+DEHP+20CA it was observed a further reduction in entering time to dark chamber compared to mice receiving BPA and DEHP. When given low dose of DEHP adverse effect was seen, whereas when BPA and DEHP were given as combination this effect was significantly reduced, however, no protective effect was observed when CA was added.

Moreover, Rotarod test was performed as another behavior test. It was observed that in the case of 5 and 16 BPA + DEHP administration, there was an insignificant a coordination disturbance compared to BPA and DEHP administration and CA addition to BPA+DEHP administration provided a protective effect. Also, it has been observed that low dose BPA + DEHP and BPA+DEHP+20CA administration did not cause any change in motor coordination.

6. CONCLUSION

In this study, we studied combined effects of BPA+DEHP as well as their combination with CA on neurodegeneration and their underlying mechanisms as well. It has been found that only on the SOD activity, one of the oxidative stress parameters, was significantly decreased and had been found that BPA+DEHP may show its toxicity by increasing hydrogen peroxide radical formation. And also, body compensated radical formation by increasing the concentration of GSH-Px and CAT enzymes. Furthermore, CA showed antioxidant properties and the concentration of GSH-Px and CAT enzymes decreased with addition of CA to BPA+DEHP combination. When the effect on apoptosis was examined, it was seen that there was a significant increase in Bcl-2 level and this is thought to initiate neuronal damage. In support of this, the increased Bax level was observed upon the combined administration of BPA+DEHP and no results were obtained in the active-caspase-3 assay. Although there was an increase in the activity of the BACE enzyme, which is one of the Alzheimer's markers, APP protein was not formed in the cortex and therefore amyloid $beta_{(1-42)}$ protein was not formed. CA showed neuroprotective effect by decreasing the levels of Bcl-2, Bax and BACE proteins.

In conclusion, while it had been found that when BPA and DEHP are used together, they damage the cortex in terms of neurodegeneration by oxidative stress and also CA had been found showing antioxidant properties.

7. REFERENCES

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8. APPENDICES

8.1. Appendices-1

T.C.

İSTANBUL MEDİPOL ÜNİVERSİTESİ,

HAYVAN DENEYLERİ YEREL ETİK KURULU (İMÜ-HADYEK)

ETİK KURULU KARARI

Toplantı Tarihi	Karar No	İlgi	Proje Yürütücüsü
22/06/2016	60		Şükran Özdatlı

"BPA ve DEHP'ye kombine maruz kalmanın toksikolojik açıdan değerlendirilmesi ile Rosmarinik asit ve Karnosik asitin koruyucu etkisinin araştırılması" başlıklı bilimsel araştırma Etik Kurulumuzda görüşülmüş olup, çalışmanın etik kurallara uygun olduğuna **"oybirliği"** ile karar verilmiştir.
Etik Onay Geçerlilik Süresi: 24 ay

GÖREVİ	ADI SOYADI	İMZA
Başkan	Doç. Dr. Hanefi ÖZBEK	
Üye	Prof. Dr. Ülkan KILIÇ	
Üye	Prof. Dr. Mustafa ÖZTÜRK	
Üye	Yrd. Doç. Dr. Turan DEMİRCAN	
Üye	Yrd. Doç. Dr. Mehmet OZANSOY	
Üye	Yrd. Doç. Dr. Sultan Sibel ERDEM	
Üye	Taha KELEŞTEMUR	
Üye	Özge Şeyda DURGUT	
Üye	Fahriye ŞENBAHÇE	

9. CIRRICULUM VITAE

Kişisel Bilgiler

Adı	Şükran	**Soyadı**	Özdatlı Kurtuluş
Doğum Yeri	Siirt	**Doğum Tarihi**	1989
Uyruğu	T.C.	**TC Kimlik No**	19898832952
E-mail	sozdatli@gmail.com	**Tel**	+90 (531) 647 9484

Öğrenim Durumu

Derece	Alan	Mezun Olduğu Kurumun Adı	Mezuniyet Yılı
Lisans	Eczacılık	**Yeditepe Üniversitesi**	2013
Lise	Sayısal	**İbrahim Turhan Lisesi**	2007

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

Bildiği Yabancı Dilleri	Yabancı Dil Sınav Notu
İngilizce	82,5

İş Deneyimi (Sondan geçmişe doğru sıralayın)

Görevi	Kurum	Süre (Yıl - Yıl)
Öğr.Gör	İstanbul Medipol Üniversitesi	2014-
Arş.Gör	Yeditepe Üniversitesi	2013-2014

Bilgisayar Bilgisi

Program	Kullanma becerisi
Office	Çok iyi
Graphpad	Çok iyi
SPSS	İyi

*****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/β-TCP scaffold for osteochondral tissue. Int J Biol Macromol. 2015
Akan H, Gurol Y, Izbirak G, Ozdatlı S, Yilmaz G, Vitrinel A, Hayran O. Knowledge and attitudes of university students toward pandemic influenza: a cross-sectional study from Turkey. BMC Public Health. 2010

Diğer dergilerde yayınlanan makaleler

Özdatlı Ş, Sipahi H, Charehsaz M, Aydın A, Yeşilada E. Bitki Çaylarına Bal İlavesinin Total Antioksidan Kapasitesine Etkisi. Marmara Pharmaceutical Journal 18: 147-152, 2014
Özdatlı Ş, Sipahi H, Charehsaz M, Behzetoğlu Y, Duman G, Aydın A. A Pilot Study on Effects of Concomitant Usage of Acetaminophen and N-Acetylcysteine to Prevent Possible Acetaminophen Toxicity. Turk J Pharm Sci 12(1), 45-52, 2015

Uluslararası bilimsel toplantılarda sunulan ve bildiri kitabında (*Proceedings*) basılan bildiriler

D. Algul, F. Kelleci , S. Ozdatli, H. Sipahi, F.G. Yener. Development And Biocompatibility Evaluation Of Novel Multilayered Alginate-Chitosan/Tıcalcium Phosphate For Cartilage Tissue Engineering , 17th International Technology Symposium-IPTS 2014
Ş Özdatlı, F. Kelleci, M.Chareshaz, H. Sipahi, A. Aydın. Gold Nanoparticles Induce Cytotoxicity: A Review of In Vitro and In Vivo Studie, 1st International Congress and Workshop of Forensic Toxicology.
Ş Özdatlı, H. Sipahi M.Chareshaz, A. Aydın, E. Yeşilada.Effect of Honey Supplementation on Total Antioxidant Capacıty of Herbal Teas. 2nd International Congress On Food Technology.