ROLE OF OXIDATIVE STRESS IN THE PREVENTIVE EFFECTS OF CALORIE RESTRICTION FOR BREAST CANCER DEVELOPMENT IN MMTV-TGF-α MICE

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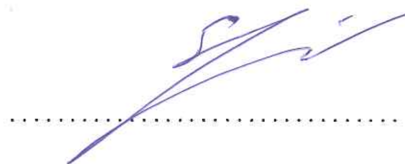

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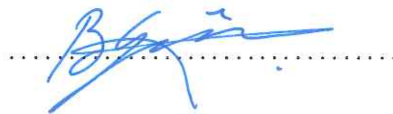

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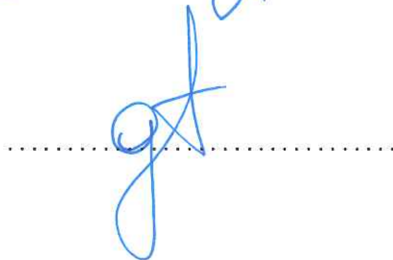

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ABSTRACT

ROLE OF OXIDATIVE STRESS IN THE PREVENTIVE EFFECTS OF CALORIE RESTRICTION FOR BREAST CANCER DEVELOPMENT IN MMTV-TGF-α MICE

Breast cancer is one of the main causes of deaths among women worldwide. Despite of countless studies on breast cancer, there are still limited numbers of successful treatments and efficient prevention strategies for it until today. On the other hand, calorie restriction (CR) is one of the most effective methods to prevent several diseases including cancer. However, the molecular mechanism of which remains unclear. The aim of this study was to understand the role of oxidative stress in the preventive effects of CR against breast cancer development in MMTV-TGF-α transgenic mouse model. Mice at 10 weeks of ages were enrolled in ad libitum (AL), Chronic Calorie Restriction (CCR, 15 per cent CR application) or Intermittent Calorie Restriction (ICR, three weeks of AL feeding and one week 60 per cent CR application in cyclic periods) groups. Blood and liver samples were collected from each dietary groups to measure malondialdehyde (MDA), glutathione (GSH) levels and catalase (CAT), glutathione peroxidase (GSH-Px) and superoxide dismutase (SOD) activity at 10, 17/18, 49/50 and 81/82 weeks of ages. Decreased body weight and lower mammary tumor (MT) incidence were observed in CCR group in the current study. In accordance with body weight and MT incidence data, CCR group had lower erythrocytes MDA level ($p>0.05$) at 17/18 and 49/50 weeks of ages. Moreover, ICR-R group had higher MDA level compared to CCR and AL at 81/82 weeks of ages. In addition, CCR group maintained its MDA level due to aging while ICR groups had gradually increasing MDA level due to aging ($p<0.05$). Moreover, CCR group had higher erythrocytes CAT and SOD activity compared to ICR and AL groups. CAT and SOD activity was also found to be positively correlated in erythrocytes samples throughout the study ($p<0.05$). Erythrocytes GSH level was higher in CCR group compared to ICR groups at week 49/50 ($p<0.05$) while there was no significant effect of different types of CR on GSH-PX activity. In the present study, the long term (82 weeks) effects and direct comparison of different types of CR on oxidative stress parameters in erythrocytes and liver samples were reported for the first time. In conclusion, CCR types of calorie restriction has better protective roles against oxidative stress than ICR types of CR.

ÖZET

KALORİ KISITLAMASININ MMTV-TGF-α FARELERDE MEME KANSERİNE KARŞI KORUYUCU ETKİSİNDEKİ OKSİDATİF STRESİN ROLÜ

Meme kanseri, tüm dünyada kadınlar arasındaki ölümlerin başlıca nedenlerinden biridir. Meme kanseri üzerine yapılan sayısız çalışma olmasına rağmen henüz kısıtlı sayıda tedavi ve etkili korunma stratejileri mevcuttur. Diğer taraftan, kalori kısıtlaması ise kanser dahil birçok hastalığı engellemekte kullanılan en etkili yöntemlerden biridir. Ancak kalori kısıtlamasının bu koruyucu etkiyi hangi moleküler mekanizma ile gösterdiği henüz belli değildir. Bu araştırmanın amacı, kalori kısıtlamasının MMTV-TGF-α transgenik farelerinde meme kanserine karşı göstermiş olduğu koruyucu etkideki oksidatif stresin rolünün anlaşılmasıdır. Araştırmada, 10 haftalık fareler ad libitum (AL), Kronik Kalori Kısıtlaması (KKK, yüzde 15 kalori kısıtlaması) ya da Aralıklı Kalori Kısıtlaması (AKK, üç hafta AL beslenme ve bir hafta yüzde 60 kalori kısıtlaması) guplarından birine yerleştirilmiştir. Kan ve karaciğer örnekleri, 10, 17/18, 49/50 ve 81/82. haftalarda malondialdehit (MDA), glutatyon (GSH) seviyelerini ve katalaz (KAT), glutatyon peroksidaz (GSH-Px) ve süperoksit dismutaz (SOD) aktivitilerini ölçmek için toplanmıştır. Araştırmada, KKK grubu diğer gruplara göre daha düşük vücut ağırlığı ve meme tümörü (MT) insidansı göstermiştir. Vücut ağırlığı ve MT insidansı verileriyle aynı doğrultuda, KKK grubu 17/18 ve 49/50. haftalarda diğer gruplara göre daha düşük eritrosit MDA seviyesi sergilemiştir ($p>0.05$). Aynı zamanda, AKK-K grubu KKK grubuna göre 81/82. haftalarda daha yüksek eritrosit MDA seviyesi göstermiştir. Bunlara ek olarak, KKK grubu MDA seviyesini yaşlanmaya beraber sabit tutarken, AKK grupları zamanla artan MDA seviyesi göstermiştir ($p<0.05$). KKK grubu, AKK ve AL gruplarına göre daha yüksek seviyede KAT ve SOD aktivitesi sergilemiştir. Ek olarak, KAT ve SOD aktiviteleri arasında pozitif bir korelasyon tespit edilmiştir ($p<0.05$). KKK grubu, 49/50. haftalarda AKK gruplarına göre daha yüksek GSH seviyesi sergilerken ($p<0.05$) farklı şekillerde uygulanan kalori kısıtlamasının GSH-Px aktivitesine herhangi bir etkisi gözlemlenmemiştir. Uzun süre uygulanan farklı kalori kısıtlaması türlerinin oksidatif stress parametreleri üzerine etkisi ve doğrudan karşılaştırılması ilk defa bu araştırmada yapılmıştır. Sonuç olarak, KKK uygulaması AKK uygulamasına göre oksidatif strese karşı daha koruyucu bir etki göstermiştir.

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LIST OF SYMBOLS/ABBREVIATIONS

ALEs	Advanced lipid peroxidation end products
AP-1	Activator protein-1
Apaf-1	Apoptotic protease activating factor-1
ATP	Adenosine triphosphate
BMI	Body mass index
CAPS	3-(cyclohexylamino)-1-propanesulfonic acid
CAT	Catalase
CR	Calorie restriction
CCR	Chronic calorie restriction
cGMP	Cyclic guanosine monophosphate
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
EGFR	Epidermal growth factor receptor
HOCl	Hypochrolous acid
GSH	Glutathione
GSH-Px	Glutathione peroxidase
ICR	Intermittent calorie restriction
IF	Intermittent fasting
IGF	Insulin-like growth factor
iNOS	Inducible nitric oxide synthase
INT	Iodonitrotetrazolum
IL-1	Interleukin-1
IL-6	Interleukin-6
IL-8	Interleukin-8
LOOH	Lipid peroxide
MAPKs	Mitogen activated protein kinases
MT	Mammary tumor
MDA	Malondialdehyde
mTOR	Mammalian target of rapamycin
MMTV-TGF-α	Mouse mammary tumor virus-transforming growth factor-alpha

NADPH	Nicotinamide adenine dinucleotide phosphate
NER	Nucleotide excision repair
NF-κβ	Nuclear factor kappa-beta
PI3K	Phosphathidyl inositol 3-kinase
PUFA	Poly unsaturated fatty acid
ROS	Reactive oxygen species
SOD	Superoxide dismutase
sGC	Soluble guanylate cyclase
TBA	Thiobarbituric acid
TNF	Tumor necrosis factor
UVA	Ultraviolet A
VCAM-1	Vascular cell adhesion molecule-1

1. INTRODUCTION

Cancer is an unignorable fact in today's world with being second leading cause of deaths and reported to be responsible for 24 per cent of total deaths [1]. The most commonly diagnosed cancer types is breast cancer among postmenopausal women [2]. Although thousands of women suffer from breast cancer, there is still no cure for breast cancer in most cases. Therefore, there is a need for more efficient and effective methods for prognostic strategies, treatment and prevention. Many risk factors were identified for breast cancer development including intrinsic factors such as gender, age, race, and having family members with breast cancer and also extrinsic factors such as smoking, excessive alcohol consumption, lack of exercise, diet and obesity [3-10]. In this context, it is reported that lifestyle related factors have more influence in the breast cancer development than that of genetic factors [11].

Obesity, a chronic metabolic disorder, is one of the vital alterable risk factors for breast cancer development [11]. According to World Health Organization (WHO), obesity incidence increased by two-fold since 1980 and approximately 600 million people were recorded as obese (BMI>30) in 2014 [12, 13]. It has been reported that compared to lean women with positive family history of breast cancer, obese women with similar family history are in higher risk for breast cancer development [14]. Also, a Meta analyses study performed by Munsell et al (2014) reported 20 to 40 per cent higher risk of breast cancer in obese women at postmenopausal stage compare to the lean ones [15]. In this context, although several factors such as estrogen, mammalian target of rapamycin (mTOR), insulin-like growth factor (IGF), leptin and adiponectin signaling pathways have been reported for the association between obesity and breast cancer development, the exact molecular mechanism of this relation remains to be elucidated [16-22]. In addition, roles of oxidative stress have been reported lately in tumor development as well as in obesity [23-28]. For example, studies have reported increased malondialdehyde (MDA), protein carbonyl and 8-hydroxy-2' –deoxyguanosine (8-OHdG) levels as biomarkers of oxidative stress in breast cancer groups compared to the healthy ones [29-31]. In addition to altered oxidative stress, reduced antioxidant defense capacitance in breast cancer groups have also been documented compared to the control groups [32, 33]. Calorie restriction (CR), is one of the most applied and most effective strategies in terms of fighting against excessive weight and obesity.

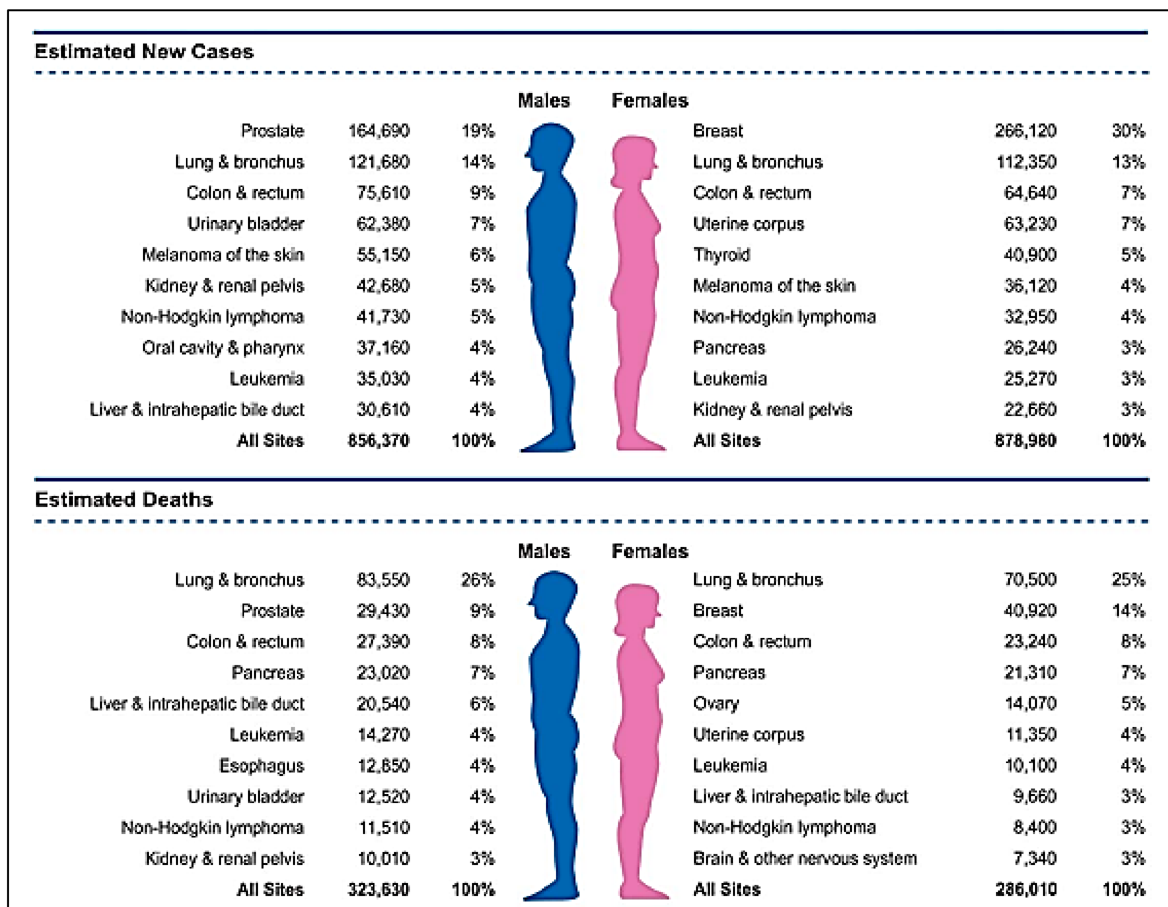
Moreover it is known to prolong lifespan and prevent several diseases including cancer in experimental tumor models [34]. According to a meta-analysis that studied the effects of CR in different types of rodent tumor models, CR has been found to reduce tumor incidence by 75.5 per cent [35]. Generally two main types of CR protocols have been followed in research for mammary tumor (MT) prevention studies: ıntermittent calorie restriction (ICR) and chronic calorie restriction (CCR) [16]. ICR has been reported to be more effective to reduce tumor occurrence rate and also significantly delay the latency of tumor development compared to CCR. Particularly in the studies performed with mouse mammary tumor virus-transforming growth factor-alpha (MMTV-TGF-α) mice, the animals fed ad-libitum (AL) had around 50-80 per cent MT incidence and the MT occurrence rate was approximately 27-44 per cent in CCR mice which applied 75 per cent intake of the calories of AL mice. In spite of high occurrence rate of MT development in AL and CCR types of diet, MT occurrence rate was only between three and 3-15 per cent in ICR mice [16, 36, 37]. Several studies have been carried out to examine the effects of CR on oxidative stress. In this context, the effects of CCR on oxidative stress have been reported in several research [38-42]. On the other hand, limited numbers of research have reported the influence of ICR on oxidative stress [43, 44]. In fact, direct comparison of the effects of both CCR and ICR has not been studied together in any studies.

The aim of this study was to understand the role of oxidative stress in the preventive effects of CR against breast cancer development. In this context, effects of different types of CR on tumor development were followed throughout the study. In order to examine the mechanism, malondialdeyde (MDA) and 8-OHdG as oxidative damage markers and superoxide dismutase (SOD), catalase (CAT), glutathione (GSH) and glutathione peroxidase (GSH-Px) as indicators of antioxidant defense system were measured in transgenic MMTV-TGF-α mice which were assigned to different CR protocols; CCR and ICR. In addition to direct comparison of oxidative stress parameters in calorie restricted groups, the differences in oxidative stress parameters due to aging in mice were reported in the present study.

1.1. BREAST CANCER

The second leading cause of deaths among women worldwide is breast cancer [2]. Breast cancer cases accounted for 30 per cent in 2018 in United States. Moreover, incidence rate of

breast cancer is increasing day by day (Figure 1.1) [45]. Similar to United States, breast cancer cases in 2008 accounted for 23 per cent of total cancer and 14 per cent of the cancer deaths in Turkey [46]. Breast cancer is caused by genetic abnormalities which are mutations in genetic material. Five to 10 per cent of breast cancer cases are caused by hereditary genetic while 85-90 per cent of breast cancer cases are caused by genetic abnormalities from environmental factors and aging.



Estimated New Cases

Males			Females		
Prostate	164,690	19%	Breast	266,120	30%
Lung & bronchus	121,680	14%	Lung & bronchus	112,350	13%
Colon & rectum	75,610	9%	Colon & rectum	64,640	7%
Urinary bladder	62,380	7%	Uterine corpus	63,230	7%
Melanoma of the skin	55,150	6%	Thyroid	40,900	5%
Kidney & renal pelvis	42,680	5%	Melanoma of the skin	36,120	4%
Non-Hodgkin lymphoma	41,730	5%	Non-Hodgkin lymphoma	32,950	4%
Oral cavity & pharynx	37,160	4%	Pancreas	26,240	3%
Leukemia	35,030	4%	Leukemia	25,270	3%
Liver & intrahepatic bile duct	30,610	4%	Kidney & renal pelvis	22,660	3%
All Sites	**856,370**	**100%**	**All Sites**	**878,980**	**100%**

Estimated Deaths

Males			Females		
Lung & bronchus	83,550	26%	Lung & bronchus	70,500	25%
Prostate	29,430	9%	Breast	40,920	14%
Colon & rectum	27,390	8%	Colon & rectum	23,240	8%
Pancreas	23,020	7%	Pancreas	21,310	7%
Liver & intrahepatic bile duct	20,540	6%	Ovary	14,070	5%
Leukemia	14,270	4%	Uterine corpus	11,350	4%
Esophagus	12,850	4%	Leukemia	10,100	4%
Urinary bladder	12,520	4%	Liver & intrahepatic bile duct	9,660	3%
Non-Hodgkin lymphoma	11,510	4%	Non-Hodgkin lymphoma	8,400	3%
Kidney & renal pelvis	10,010	3%	Brain & other nervous system	7,340	3%
All Sites	**323,630**	**100%**	**All Sites**	**286,010**	**100%**

Figure 1.1. Breast cancer statistics in United States, 2018 [45]

Several risk factors have been identified for breast cancer development including intrinsic factors such as age, gender, race, and family history and also extrinsic factors such as smoking, excessive alcohol uptake, lack of exercise, diet and obesity [3-10]. In this context, it is reported that lifestyle related factors have more influence on the breast cancer development than that of genetic influence (Figure 1.2) [11]. If these extrinsic risk factors

can be studied and characterized in details, then more efficient intervention strategies can be developed for breast cancer development.

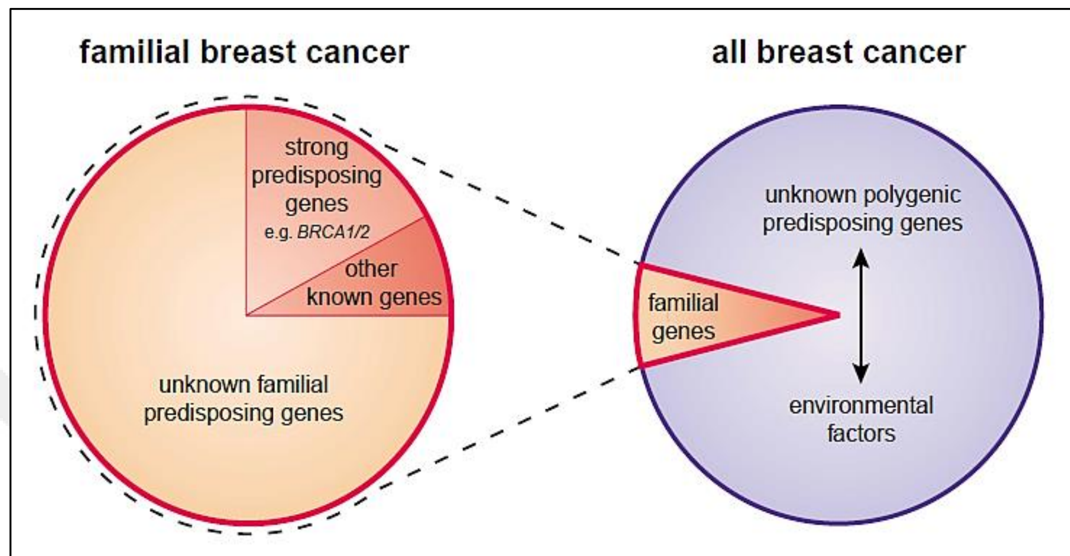


Figure 1.2. Breast cancer risk factors [47]

1.2. BREAST CANCER AND OBESITY

The World Health Organization defines obesity as a state of excess fat accumulation where body mass index (BMI) is higher than 30 k/m^2. Obesity is a serious worldwide health problem which affects millions of lives. For instance, 26 per cent in British women is recognized as obese and these incidence is predicted to increase up to 43 per cent by 2030. There are several factors that cause obesity such as genetics, unhealthy diet, physical inactivity, metabolic, environmental, socioeconomic, and psychological [48]. Obesity has been found to be responsible for 20 per cent of total deaths from cancer among women and 14 per cent deaths among men [49]. Several meta-analyses have reported the influence of overweight and obesity in breast cancer development. For instance, Bergström et al (2001) reported that overweight women ($25 \leq BMI < 30$ k/m^2) have 12 per cent and obese women ($BMI \geq 30$ k/m^2) have 25 per cent higher risk of breast cancer comparing to lenaer women [50]. Dobbins et al (2013) has also reported that obese women ($BMI > 30$ k/m^2) had significantly 25 per cent higher risk for breast cancer development comparing to leaner

women [48]. It is crucial to understand the mechanism which is responsible for the function of obesity and dietary factors in breast cancer development in order to modify the mechanism and improve new treatments. Several mechanisms have been suggested have role in this association including the effects of insulin, IGF, sex hormones, oxidative stress and adipokines [12, 51-54].

1.3. CALORIE RESTRICTION AND CANCER

Dietary intake is one of the main health-span regulators that may affect the quality and duration of life in living organism. In addition, dietary intake has been associated with several pathological conditions including cardiovascular diseases, diabetes, cancer, inflammation and neurogenesis [55]. Calorie restriction (CR) which is a dietary regimen where generally calorie intake is reduced by generally 20 to 40 per cent is one of the most applied interventions for reducing body weight and preventing against cancer development in rodent models [34]. First paper about CR is published by McCay in 1935 have reported that CR increased the lifespan of rats. Effects of CR was confirmed in several species including yeasts, fruit flies, nematodes, fishes, hamsters, and several strains of mice as well as rats. CR has been reported to prolong lifespan and retard several diseases such as type II diabetes, cardiovascular and neurodegenerative diseases. When calorie intake is reduced, metabolic alterations promote several characteristics such as improved insulin sensitivity and diminished blood glucose, GF signaling, inflammation and angiogenesis (Figure 1.3) [34].

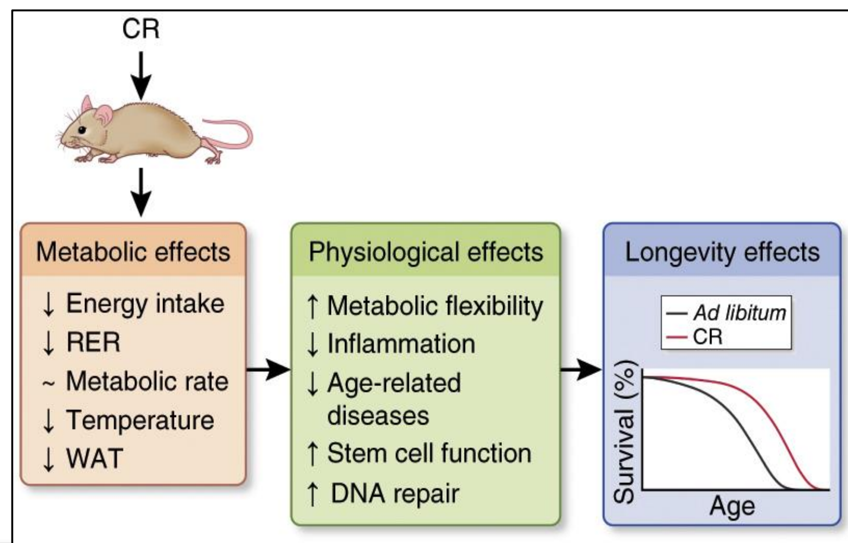


Figure 1.3. Influence of calorie restriction [56]

CR is also known to protect against cancer. However, the exact mechanism how CR performs this association remains elucidated [34, 57]. Several potential factors have been suggested to be responsible for anticancer roles of CR such as energy-balance-related hormones, steroid hormones, growth factors, insulin, IGF, leptin, adiponectin, inflammation and sirtuins [58]. A meta-analysis that evaluate the effects of calorie restriction have indicated that CR prevents tumor incidence by 75 per cent [35]. Generally, two main types of CR protocols have been followed in research for MT prevention studies: Intermittent calorie restriction (ICR) and chronic calorie restriction (CCR) [16]. CCR protocol application in healthy individuals results in toleration while cancer patients are not suitable for CCR protocol due to risk of body weight loss in higher amounts. Furthermore, because of the anti-inflammation effects of CCR application, it is with immunodeficiency or following surgery. However, ICR protocol where food consumption is deprived for certain time period causes various metabolic alterations similar to CCR application. Furthermore, even short term application of ICR protocol has resulted in higher changes [59]. ICR has also been reported to ameliorate the radio sensitivity of MT's in mice [60, 61] caused by increased oxidative stress [62]. ICR protocol has been suggested to show its beneficial effects while total energy intake is maintained. ICR has also been reported to be more effective to reduce tumor occurrence rate and also significantly delay the latency of tumor progression compared to CCR types of dietary regimens. Particularly in the studies

performed with MMTV-TGF-α mice, the animals fed ad-libitum (AL) had around 50-80 per cent MT occurrence rate and the MT occurrence was approximately 27-44 per cent in CCR which were provided 75 per cent intake of the calories of AL mice. In spite of high occurrence rate of MT development in AL and CCR types of diet, MT occurrence rate was only between three and 3-15 per cent in ICR mice [16, 36, 37].

1.4. FREE RADICALS

Free radicals are molecules that comprise unpaired electrons in their orbitals (Figure 1.4) [63]. Free radicals are highly reactive, short-lived and unstable molecules due to their unpaired electrons. Free radicals are unavoidably generated consequences in normal physiology. Discovery of free radicals in biological materials was done in 1954 [64].

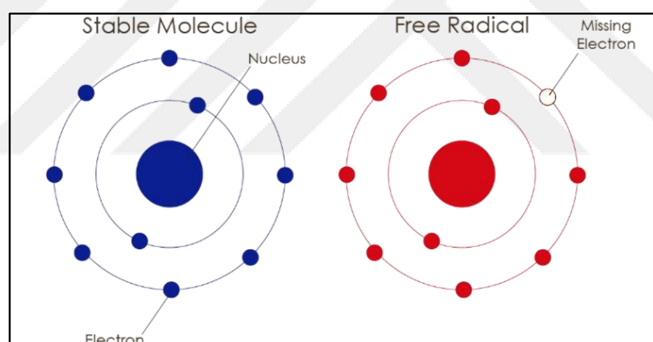


Figure 1.4. Illustration of free radicals

The most vital form of radical species are oxygen derived radicals in living systems [65]. With respect to their high reactivity, free radicals can attack other molecules to abstract electrons in order to sustain their stability. This reaction causes other molecule to become a free radical by losing its own electron thus a chain reaction begins. These reactions may damage the living cell. Hydroxyl (OH•), superoxide ($O_2{•}^-$), nitric oxide (NO), nitrogen dioxide (NO_2), peroxyl (ROO•) and lipid peroxyl (LOO•) are well known free radicals. Some molecules such as hydrogen peroxide (H_2O_2), ozone (O_3), singlet oxygen (O_2), hypochlorous acid (HOCl), nitrous acid (HNO_2), peroxynitrite ($ONOO^-$), dinitrogen trioxide (N_2O_3), lipid peroxide (LOOH) are not free radicals however, they have the ability to trigger free radical production in living organisms [66]. Because of their highly reactivity, free radicals act on

several vital biomolecules including lipids, proteins and deoxyribonucleic acids (DNA) [66, 67].

1.5. REACTIVE OXYGEN SPECIES

Reactive oxygen species (ROS) is a general concept which comprises free radicals and non-radical molecules which have the ability of generating free radicals in living organism [68]. Normally, more than 90 per cent of O_2 consumption is converted to water in electron-transport chain without any ROS production [69]. However, 10 per cent of O_2 consumption is reduced by one electron reduction in order to convert O_2 to $O_2 \bullet^-$ radical which is then converted into H_2O_2 by superoxide dismutase (SOD). Following this conversion, H_2O_2 can generate OH• radical by Fenton reaction via abstracting one electron from Fe^{+2}. OH• radical is then neutralized by abstracting one electron from biomolecules such as proteins and lipids [70]. Therefore, ROS are generated as a result of normal aerobic metabolism (Figure 1.5). ROS have several vital roles in several physiological conditions when they are at low concentration. However, they become harmful to biological molecules in living organisms when they are at high concentration [71]. ROS are summarized in Table 2.1.

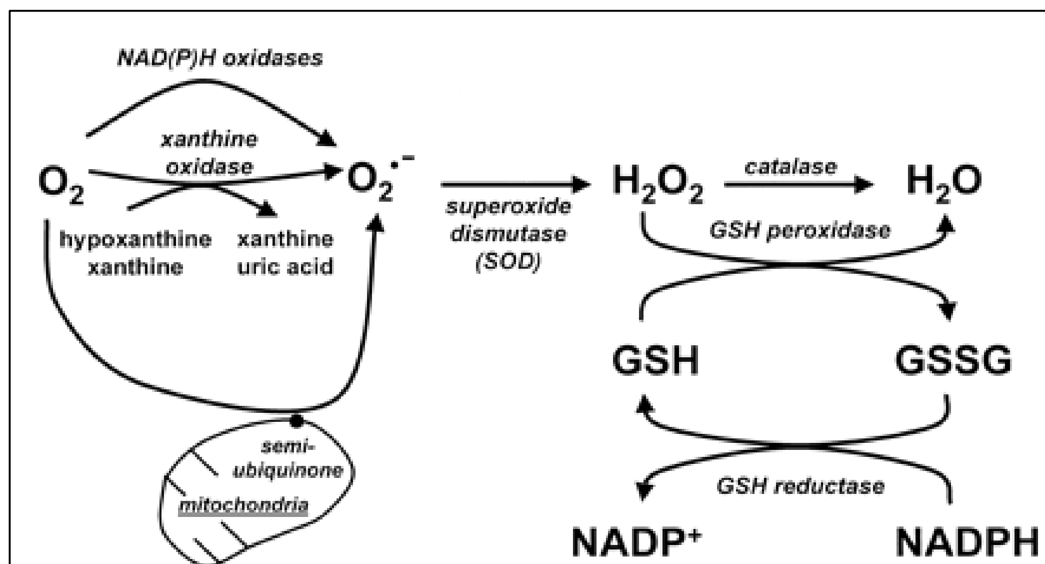


Figure 1.5. ROS signaling pathway [72]

Table 1.1. List of ROS and RNS

Name	Formula	Characteristics
Superoxide	$O_2\bullet^-$	Free radical, highly unstable, signaling function
Hydrogen peroxide	H_2O_2	Cell toxicity, signaling function, generation of other ROS
Hydroxyl radical	$OH\bullet$	Free radical, highly unstable, very reactive agent
Peroxyl radical	$ROO\bullet$	Free radical, product of lipid peroxidation
Singlet Oxygen	O_2	Induced/excited oxygen molecule, radical and non-radical form
Ozone	O_3	Environmental toxin
Nitric oxide	NO	Environmental toxin, endogenous signal molecule
Peroxynitrite	$ONOO^-$	Highly reactive reaction intermediate of superoxide and nitric oxide
Nitrogen dioxide	NO_2	Highly reactive radical, environmental toxin

1.5.1. Sources of ROS

1.5.1.1. Endogenous Sources of ROS

ROS can be generated by both enzymatic and non-enzymatic reactions. Endogenous ROS production are mainly performed in the mitochondria, plasma membrane, endoplasmic reticulum, and peroxisomes [73]. In addition, mitochondria are the main organelle where the highest amount of the ROS production is performed. In this manner, it is suggested that as much as one per cent of the mitochondrial O_2 consumption is used to generate $O_2\bullet^-$ radical

from Complex I and III in the electron transport chain [74]. The generation of $O_2\bullet^-$ radical is from mitochondria is non-enzymatic [75] while enzymatic ways such as xanthine oxidase, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, peroxidases, p66shc and monoamino oxidase act on generation of ROS in mitochondria [76]. For instance, when oxidative stress occurs, p66Shc translocate to inner space of mitochondria in order interact with cytochrome-c to generate ROS [77]. Peroxisomes are another organelle that can generate ROS. The electrons are transferred from several metabolites to O_2 to form H_2O_2 in peroxisomes. $O_2\bullet$-, OH• and NO• are also generated in peroxisomes [78]. p-450 and diamine oxidase in endoplasmic reticulum are also able to generate ROS [79].

1.5.1.2. Exogenous Sources of ROS

Cigarette smoke, ozone exposure, hyperoxia, ionizing radiation and metal ions are among exogenous sources of ROS. Cigarette smoke comprises several oxidants and free radicals including $O_2\bullet$- and NO• [80]. Lipid peroxidation can be provoked by ozone exposure [81]. Ultraviolet A (UVA) photons lead oxidative damage by excitement of porphyrins and NADPH oxidase. 8-OHdG the main products of UVA-related oxidative damage to DNA by the attack of OH• radical to guanine [82]. Heavy metal ions including iron, copper, and arsenic may trigger the production of ROS and cause harmful effects to lipids, proteins and DNA [83].

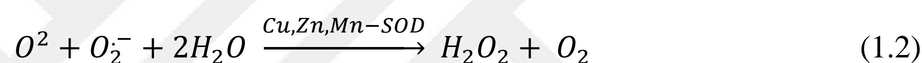
1.5.2. Properties of ROS

1.5.2.1. Superoxide Radical

$O_2\bullet^-$ radical which is the most potent ROS that is produced by both enzymatic and non-enzymatic process [84]. $O_2\bullet^-$ radical is generated by adding up one electron to O_2 [65]. Mitochondria is the primary source of $O_2\bullet^-$ generation [85].

$$O_2 + e^- \rightarrow O_2^{\cdot -} \qquad (1.1)$$

$O_2\bullet^-$ production usually happens in the inner membrane of mitochondria where electrons are found abundantly [86]. As a consequence of aerobic life, electrons are transported through electron transport chain in mitochondria which results in reduction of O_2 to water. However, around one to three per cent of all electrons escape from electron transport chain and as a result $O_2\bullet^-$ is produced [87]. In addition, there are several enzymes that can generate $O_2\bullet^-$ such as xanthine oxidase and NADPH dependent oxidase. Compare to other free radicals, $O_2\bullet^-$ radical is not as much as highly reactive and does not have the ability of penetration through membranes [23]. Two $O_2\bullet^-$ radical can react with each other to produce H_2O_2. In this dismutation reaction, one radical is oxidized to O_2 while other one is reduced to H_2O_2 [88]. H_2O_2 is more stable compared to superoxide and can penetrate through membranes to show its effect largely.



$$O^2 + O_2^{\cdot -} + 2H_2O \xrightarrow{Cu,Zn,Mn-SOD} H_2O_2 + O_2 \qquad (1.2)$$

1.5.2.2. Hydrogen Peroxide

H_2O_2 does not contain unpaired electrons, however it is included in ROS term due its ability to trigger the production of more reactive radicals such as HOCl and OH• [86]. H_2O_2 is formed from the catalysation of $O_2\bullet^-$ by the enzyme SOD. It is also important for cells due to their ability to pass through the biological membranes. H_2O_2 does not react with DNA itself however, it may have a damage on DNA by generating hydroxyl radical through Fenton Reaction [89]. Superoxide radical can also react with H_2O_2 in order to generate hydroxyl radical (Haber F, 1934). H_2O_2 is also generated by various enzymes such as xanthine oxidase and NADPH oxidase in peroxisomes [90]. H_2O_2 not only has harmful effects but also has an important role in intracellular signaling pathways [91]. H_2O_2 is catalyzed by three major antioxidant which are catalase, glutathione peroxidase and peroxiredoxins into oxygen and two molecules of water [92].

1.5.2.3. Hydroxyl Radical

The most reactive radical is OH• radical among all ROS which is the most harmful one to biomolecules It has also very short half-life of approximately 10 seconds. [93, 94]. OH• radical is generated in Fenton reaction where H_2O_2 is catalyzed in the presence of Fe and Cu. In this context, $O_2•^-$ radical recycles the metal ions and the complex of two reactions is called Haber-Weiss reaction. Therefore, transition metals are vital in the concept of OH• radical generation [95].

$$Fe^{+2} + H_2O_2 \rightarrow Fe^{+3} + OH^{\cdot} + OH^{-} \; (Fenton\ reaction) \quad (1.3)$$

$$O_2^{\cdot -} + H_2O_2 \rightarrow O_2 + OH^{\cdot} + OH^{-} \; (Haber - Weiss\ reaction) \quad (1.4)$$

Transition metals are often present in complex with proteins; ferritin which stores Fe and ceruloplasmin which carries Cu. Excessive amount of superoxide radical leads the release of transition metals to take part in Fenton reaction. OH• radical may react with every biomolecule present in cells such as DNA, lipids, proteins and carbohydrates [96].

1.5.3. Physiological Roles of ROS

ROS is necessary at low concentration for several physiological processes such as defense system, cellular signaling, redox regulation of transcription factor activity, NO production and vascular tone regulation, as a sensor for status of oxygen concentration, cell adhesion and apoptosis [71, 72, 97].

1.5.3.1. Defense System

ROS is produced from phagocytes when they are activated in order to kill bacteria [98]. NADPH complex is responsible for producing ROS in phagocytes where oxygen is converted to $O_2•^-$ radical[99]. Following production, $O_2•^-$ radical is dismutased by SOD to

H_2O_2. Afterwards, H_2O_2 is then converted to HOCl by myeloperoxidase [100]. OH• radical is then formed from HOCl. OH• radical and HOCl are highly toxic to bacteria thus shows the antimicrobial effects of ROS directly. HOCl has also a vital function in the defense system by inhibiting DNA replication of bacteria [101].

1.5.3.2. Redox Regulation

ROS have the ability to oxidize the thiol groups of proteins and glutathione. Therefore, ROS affect the configuration and function of these proteins. Several proteins which are important in signaling and carcinogenesis such as protein kinase C, collagenase, and tyrosine kinases may be affected by this oxidation caused by ROS [102]. ROS also act several transcription factors such as Nuclear Factor kappa-β (NF-κβ) and Activator Protein-1 (AP-1) [103-105]. Expression of AP-1 is altered by various pro-oxidant circumstance such as irradiation [106]. In addition, ROS can decrease the interaction of AP-1 and DNA by oxidizing the cysteine residues [107]. NF-κβ is activated following degradation of inhibitory protein B by ROS. NF-κβ acts as transcription factors for several genes related with cytokines (including interleukin-1 (IL-1), interleukin-6 (IL-6), interleukin-8 (IL-8) and tumor necrosis factor(TNF)), inducible nitric oxide synthase (iNOS), and E-selectin [102, 108, 109]. Therefore, ROS may induce the expression level of these genes through NF-κβ.

1.5.3.3. Aging Process

Aging is a physiological process where various time-dependent changes that abbreviate physiological and functional capacity occur [110]. Several factors such as mTOR and AMP-activated kinase (AMPK) have roles in the regulation of aging process [111]. mTOR and AMPK are regulated by ROS both in physiological and disease conditions [112, 113]. ROS has been suggested to modulate the aging process mediating the stress response to age-dependent damage [114].

1.6. ANTIOXIDANTS

ROS homeostasis is crucial in aerobic life. Therefore, a defense mechanism comprising prevention, interception and repair mechanisms have been evolved [115]. Antioxidants are termed as substances which may delay and prevent oxidative damage to biological molecules. At prevention level antioxidants keep ROS production at low concentrations, at interception level antioxidants scavenges ROS and finally at repair level antioxidants help repairing the damaged molecules caused by ROS [116]. In details antioxidant defense mechanism comprises following principles. First they can be enzymes such as SOD or catalase to scavenge ROS. They can control the ROS generation by electron escape the electron from electron transport chain. Oxidants such as iron and copper ions are kept at minimum levels by ferrins, albumin and haem oxygenases. Antioxidant defense mechanisms also include chaperone proteins that protect DNA. There are also some agents such as glutathione, urate, albumin, α-tocopherol and ascorbate that react with ROS itself to stop their attack against biomolecules [63]. To summarize, antioxidant mechanism work in two different ways. One of them is chain breaking where ROS is stabilized by an antioxidant by donating one electron to free radical (vitamin C, E and carotenoids), or it is degraded into a harmless product. Antioxidants also work in a preventive way by stabilizing transition metal ions or clearance of free radicals [117]. Antioxidant defense system is dependent on tissue type, cell type and organelles [63]. Antioxidants are divided into two groups: enzymatic and non-enzymatic antioxidants.

1.6.1. Enzymatic Antioxidants

1.6.1.1. Superoxide Dismutase

SOD is the first enzyme described to have an impact on a free radical [118-120]. SOD catalysis $O_2^{\bullet -}$ radical into H_2O_2 and oxygen [121]. In mammalian cells, there are different types of SOD according to their location in the cell and the types of cofactor they use. Copper–zinc SOD (Cu/Zn-SOD) which is 32-kDa dimer is located in the cytosol and in the space between inner and outer mitochondrial membranes and 80-kDa tetrameric manganese-containing SOD (Mn-SOD) is located in the matrix [122]. Other types of SOD is EC-SOD

which is located in the extracellular matrix and also contain Cu/Zn active site [123]. Cu/Zn-SOD is comprised by two subunits that have active site containing copper-zinc and di-sulphide bond. The copper ions within Cu/Zn-SOD enzyme catalysis the dismutation of $O_2•^-$ by undergoing oxidation and reduction while zinc is responsible for stabilizing the enzyme [84].

$$2O_2^{•-} + 2H^+ \rightarrow H_2O_2 + O_2 \tag{1.5}$$

$O_2•^-$ radical is the primary ROS that is produced in high concentration because of the electron escape in electron transport chain [124]. Therefore, SOD is primarily important in mitochondria for the antioxidant mechanism. Mn-SOD has been suggested to be essential since knockout mice lacking Mn-SOD do not live long after birth [125]. However, knockout mice lacking cytosolic Cu/Zn-SOD have been reported to manage to live after birth [118].

1.6.1.2. Catalase

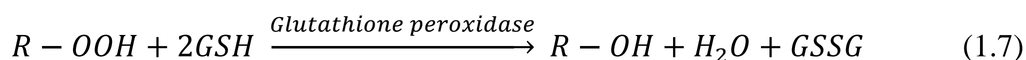
CAT is a tetramer that contains heme active sites [126]. CAT is mainly located in peroxisomes and catalysis the H_2O_2 into water and oxygen by the transition between two forms of catalase; ferricatalase and compound I (iron complex with oxygen) [86, 127].

$$2H_2O_2 \rightarrow O_2 + 2H_2O \tag{1.6}$$

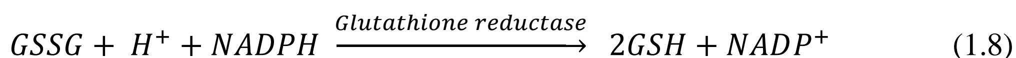
Moreover, CAT can also bind to NADPH in order to prevent inactivation caused by H_2O_2 and to increase its efficiency. In this process NADPH is used as a reducing agent and H_2O_2 is converted to water while NADPH is oxidized [128, 129]. In addition, CAT has also a role in detoxicate different substrates such as phenols and alcohols by the reaction of coupled reduction of H_2O_2 [86]. Another role of CAT is to prevent OH• radical production from Fenton reaction by H_2O_2 and transition metals [130].

1.6.1.3. Glutathione Peroxidase

GSH-Px is part of the major antioxidant system that can catalyze H_2O_2 [92]. The glutathione peroxidase system comprises GSH-Px, glutathione reductase, GSH and reduced NADPH [131]. There are four types of GSH-Px that each of them contain selenocysteine (GSH-Px 1-4) [132]. GSH-Px 1 and GSH-Px 4 are cytosolic enzymes and found in most of the tissues while GSH-Px 2 and GSH-Px 3 are found in gastrointestinal and kidney [133, 134]. GSH-Px 1 is known to catalyze H_2O_2 and fatty acid peroxides [135]. GSH-Px 2 is responsible for catalyzing dietetic peroxides [136]. GSH-Px 3 which is the only extracellular enzyme and considered to be the most important one in the family. GSH-Px 4 uses thiols as reducing agents to catalyze the esterified lipids [87, 137]. Glutathione peroxides shows its catalytic activity by using H_2O_2 as an electron donor to oxidize active site selenolate to become selenic acid. In this process, GSH work as a cofactor and make an adduct with selenic acid to form selenylsulfide. After addition of one more GSH to this process, active selenolate is regenerated and glutathione disulfide (GSSG) is formed as well. GSSG is reduced with glutathione reductase to regenerate GSH [86, 138].



$$R - OOH + 2GSH \xrightarrow{Glutathione\ peroxidase} R - OH + H_2O + GSSG \quad (1.7)$$



$$GSSG + H^+ + NADPH \xrightarrow{Glutathione\ reductase} 2GSH + NADP^+ \quad (1.8)$$

1.6.2. Non-enzymatic Antioxidants

1.6.2.1. Glutathione

GSH is a soluble antioxidant and is plentiful in cytosol, nuclei and mitochondria. Glutathione disulphid (GSSG) is the oxidised form of GSH [71, 139]. GSH is produced in the cytosol by glutamate–cysteine ligase and glutathione synthetase. Three amino acids which are glutamate, cysteine and glycine are found in the GSH structure. GSH performs its antioxidant effects in various manners. It functions as an electron donor in the dismutation

of H_2O_2 and lipid peroxides by the action of GSH-Px. Reduced form of GSH donates protons to membrane lipids in order to function against ROS attacks [140]. GSH works in relation with apoptotic signaling pathways to defense cells against apoptosis [139]. Moreover, GSH has also known to activate several transcription factors, such as AP-1 and NF-kB. In addition, GSH balance the redox status of crucial protein sulphydryls in nucleus which are essential for DNA expression and repair mechanisms. Determination of GSH/GSSG ratio is a biomarker of oxidative stress [141, 142].

1.7. OXIDATIVE STRESS

ROS level is balanced at low concentrations by antioxidant defense system in normal physiology. However, sometimes the balance between ROS and antioxidant defense system is disturbed. Imbalance between ROS and antioxidants may be caused by various reasons: (i) increased ROS production caused by endogenous and exogenous compounds; (ii) consumption of low molecular mass antioxidants; (iii) inactive antioxidant enzymes; (iv) decrease level of antioxidant. Oxidative stress is a state where the disrupted balance between ROS and antioxidants become harmful to important biomolecules (Figure 1.6) [143, 144]. The consequences of the increased ROS level varies and depended on the level and the location of ROS production, effectiveness of antioxidant systems and biomolecules they react with [70]. Excessive ROS production can be harmful to lipids, proteins and DNA that causes abnormal functions. Therefore, oxidative stress is pointed to be involved in numerous pathophysiological conditions such as cancer and aging [145]. It is crucial for living organisms to balance the level of ROS for utilizing the beneficial roles of ROS. This balance is maintained by mechanisms called “redox regulation” [72]. Considering the hallmarks of cancer, mutations in several genes such as RAS, PI3K, AKT/PKB, and HER2 as well as mutations in genes such as p53, RB, and ATM causes the distribution in redox regulation. Therefore, consisted redox state inhibits apoptosis and leads to proliferation, angiogenesis and metastasis. Taken all together, redox homeostasis and signaling are necessary for tumorigenesis [146].

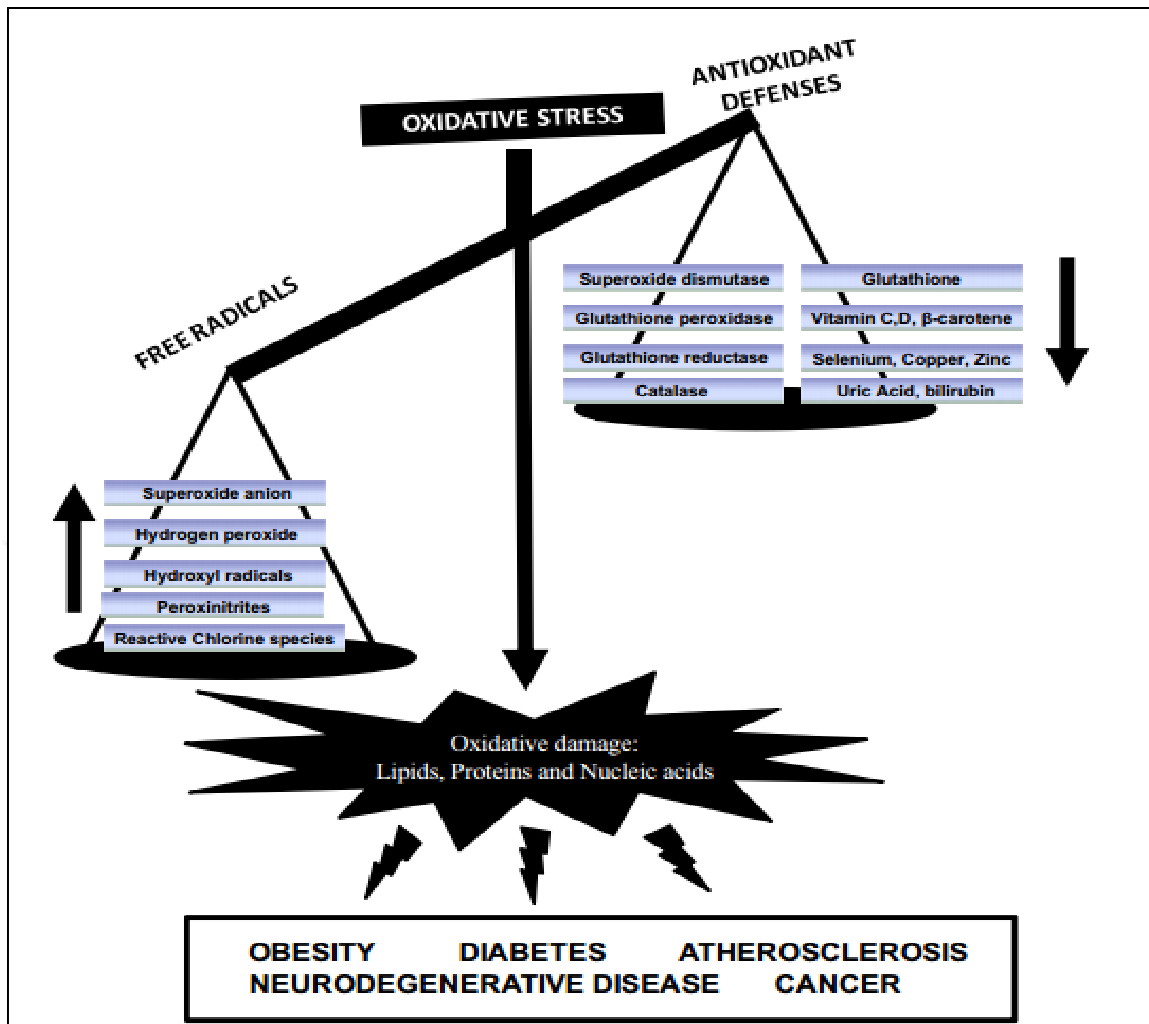


Figure 1.6. Schematic presentation of oxidative stress [25]

1.7.1. Lipid Damage

The most susceptible part of membrane lipids to ROS attack is the polyunsaturated fatty acid where plenty of double bonds are present [147]. Lipid peroxidation is a multi-step process where ROS attack polyunsaturated fatty acids within lipids [148]. Lipid peroxidation is comprised of three steps: initiation, propagation and termination (Figure 1.7) [149]. In the initiation step, ROS such as OH• radicals abstract the hydrogen that forms carbon-centered lipid radical (L·). In the propagation step, L· reacts with O_2 that forms a lipid peroxyl radical (LOO·) further abstracts a hydrogen from another lipid that causes a generation of a new L· and lipid hydroperoxide (LOOH). Generation of a new L· proceeds the chain reaction where

another L· is generated. In the termination step, antioxidants such as vitamin E donate a hydrogen atom to the LOO· and then a vitamin E radical is formed which can react with another LOO· that forms non-radical products [150]. Lipid hydroperoxides (LOOH) are the primary products of lipid peroxidation while malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE) are the secondary products of lipid peroxidation [151].

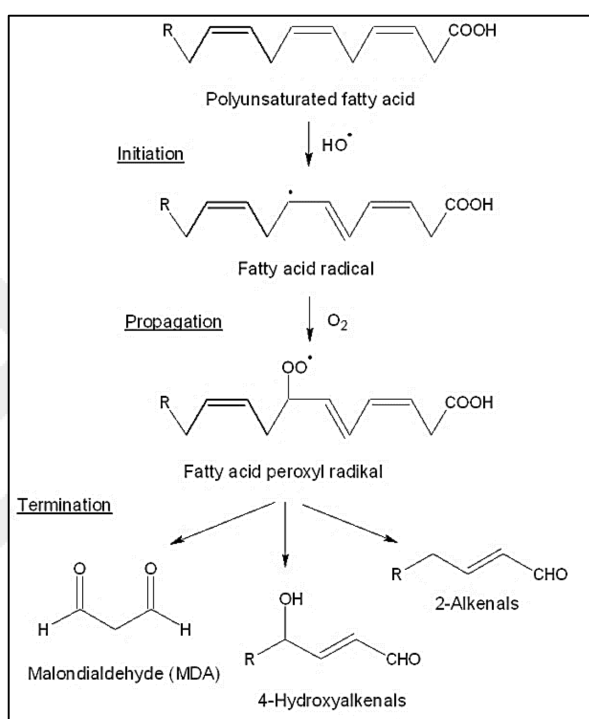


Figure 1.7. Lipid peroxidation process

MDA is generated as a secondary by-product from arachidonic acid and poly unsaturated fatty acids (PUFAs) through enzymatic or non-enzymatic processes. MDA can be enzymatically metabolized. However, in some cases, MDA make adducts with proteins or DNA which result in biological damages. Basic amino acids such as histidine, lysine and arginine are vulnerable to MDA because of the electrophilicity of MDA. Schiff-base adducts are generated from these reactions between MDA and free amino acids [152] which are also called advanced lipid peroxidation end-products (ALEs). MDA can also react with nucleosides such as deoxyguanosine and cytidine in order to form adducts to deoxyguanosine and deoxyadenosine [153]. MDA is one of the most vital cause of DNA damage and mutation. MDA-DNA adducts are repaired by nucleotide excision repair (NER)

pathway. However, MDA-DNA adducts cause strand breaks, point and frameshift mutations, cell cycle arrest and induction of apoptosis when the repair system is not working. For instance, M1dG is an exocyclic adduct to guanine which is present in human and rodent genomic DNA. M1dG is miscoded during DNA replication and it causes base pair exchange and frameshift mutations [154].These alterations caused by MDA-DNA adducts may lead to cancer and other genetic diseases [154-157]. MDA is used as a biomarker of lipid peroxidation due to its ability of reacting with thiobarbituric acid (TBA) that makes it possible to visualize [158].

1.7.2. Protein Damage

ROS may lead to amino acid oxidation, peptide chain fragmentation and cross-linking of proteins. Therefore, these alterations may cause the degradation of proteins by proteases [159, 160]. Amino acid side chains, especially cysteine and methionine residues of proteins are more vulnerable to ROS such as $O_2{\bullet}^-$, OH• and H_2O_2 [161]. As a result of the ROS attack to these residues may lead to conformational changes, protein unfolding and degradation [162]. Metal containing enzymes in their active sites are more sensitive to oxidation. When oxidized, enzymes cannot function anymore and their activities are inhibited [163]. When ROS attacks to amino acids, carbonyl groups are generated. Carbonyl groups in proteins are indicated as a marker of ROS mediated protein oxidation [164]. Increased level of protein carbonyls has been evaluated in numerous diseases such as Alzheimer's disease [165], diabetes [166] and aging [167].

1.7.3. DNA Damage

Excessive ROS production may result in damage to nucleic acids. ROS have been known as mutagenic because of its attack to DNA that causes various changes. When DNA repair systems are not able to renew DNA, a mutation results from incorrect base pairing during replication. One of the functions of ROS is apoptosis that is caused by DNA damage. ROS has also been suggested to contribute to aging process due to mitochondrial DNA damage [86, 168]. In contrast, SOD and CAT mimetics have been reported to prolong the lifespan of *C. elegans* [169]. ROS is generated mostly in mitochondria. Therefore, the mitochondrial

DNA is exposed to alterations caused by ROS in more extent compared to the nuclear DNA. OH• radical have the ability of reacting with all components of DNA such as deoxyribose sugar backbone, purine and pyrimidine bases. In case of ROS attack, modified purine and pyrimidine base by-products and DNA-protein cross-links are produced. OH• radical attack to pyrimidine causes pyrimidine adducts such as thymine glycol, uracil glycol and 5-hydroxydeoxy uridine. When OH• radical attacks to purine, 8-OHdG and 2,6-diamino-4-hydroxy-5-formamidopyrimidine are formed. These adducts cause mismatched pairing and nucleotide substitutions in the genome. For instance, 8-OHdG is involved in mutagenesis, carcinogenesis and aging due to its properties and known to be a biomarker of oxidative DNA damage.

1.8. OXIDATIVE STRESS AND CANCER

Oxidative stress takes part in numerous pathological conditions including cancer, neurogenesis, cardiovascular diseases, cataracts, rheumatoid arthritis and aging [170].

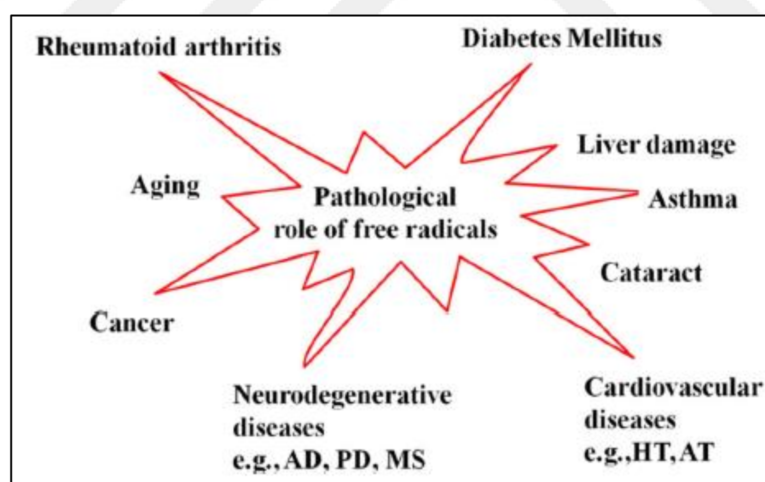


Figure 1.8. Pathological roles of free radicals [170]

Oxidative stress is known to be involved in carcinogenesis and the incidence of cancer. Chemical modifications in DNA induced by free radicals is considered mutagenic and involved in the progression of cancer [171, 172]. Oxidative stress has been reported to affect various signaling pathways in the context of cell proliferation [173]. For instance, the epidermal growth factor receptor signaling pathway (EGFR), the mitogen activated protein

kinases (MAPKs), phosphatidyl inositol 3-kinase (PI3K) and protein kinase C are known to be affected by oxidative stress signaling pathways [71]. ROS also induces the p53 expression which is an essential in apoptosis. Therefore, various alterations in gene expression, cell proliferation and apoptosis caused by oxidative stress have a function in tumor initiation and progression [174, 175]. Thymidine phosphorylase enzyme, lactoperoxidase enzyme, inflammation and matrix metalloproteinase are some of the mechanisms for inducing oxidative stress in breast carcinoma. Besides increase ROS level, breast cancer is also associated with changes in antioxidant status.

1.9. OXIDATIVE STRESS AND CALORIE RESTRICTION

Oxidative stress is implicated in numerous pathological conditions including aging and age related diseases such as neurogenesis, cancer, cardiovascular diseases and diabetes. In addition, numerous researches have suggested that CR might be a method to protect against these oxidative stress related diseases including cancer. There are three mechanisms which have been proposed to be associated with the preventive effects of CR; CR might reduce ROS generation, induce antioxidant defense system or increase the turnover of oxidized macromolecules. Although CR is usually known to inhibit oxidative stress and increase antioxidant capacity, these mechanisms are complicated and interrelated. For instance, CR might decrease the activity of an antioxidant enzyme. However, this decrease might be related to decreased level of ROS. Moreover, effects of CR on the status of oxidative stress dependent on several factors such as gender, species, types of tissue used, types of ROS and antioxidant examined, as well as duration of CR.

MDA is generated as a secondary by-product of peroxidation following the radical attack to lipids and commonly used as a biomarker of lipid peroxidation due to its ability to react with TBA [151]. Numerous studies have reported the influence of different types and amounts of CR on MDA level (Table 1.3). The data from different studies is not consistent due to variety of restriction protocols. Moreover, studies that have evaluated the same amount of CR but different in application period had different outcomes in this manner. For instance, study by Doguc et al (2013) showed decreased level of MDA in rats applied 60 per cent CR for 10 weeks [176] while Stankovic et al (2013) showed increased level of MDA in rats applied 50-60 per cent CR for 5 weeks. Similarly, Stankovic et al (2013) showed no change in the

level of MDA in rats applied 30-40 per cent CR for 5 weeks [38] while another study reported decreased TBARS level in mice applied 40 per cent CR for two months [179]. Al Safaei et al (2014) showed decreased level MDA in humans who performed Ramadan fasting [178] while BaHammam et al (2016) showed no change in MDA level in human who performed Ramadan fasting [43].

Antioxidants act as a detoxification system against free radicals and ROS. Catalase is one of the primary lines of defense against oxidative damage. Numerous studies have reported the influence of different types and amounts of CR on CAT activity level (Table 1.4). Similar to MDA level, the data for CAT activity to show the effects of CR is not consistent as well due to variety of restriction protocols. These studies were also differed in tissue examined and species used. For example, 30 per cent CR application for 6 months was reported to have no significant effect on liver CAT activity [186]. Likewise, another study reported no significant effect on CAT activity when rats were applied by 30 per cent CR for 17 weeks while another increased CAT activity was reported in the same study [188].

SOD is another enzyme that is part of the antioxidants defense system. Numerous studies have reported the influence of different types and amounts of CR on SOD activity level (Table 1.5). The data for SOD activity to show the effects of CR is not consistent as well due to variety of restriction protocols. For instance, study done by Doguc et al (2013) showed increased level of SOD activity in erythrocytes of rats applied 60 per cent CR for 10 weeks [176] while Stankovic et al (2013) have reported decreased level of SOD activity in liver of rats applied 50-60 per cent CR for five weeks [38]. Also, effects of IF where alternate day fasting was applied to mice have been reported to increase the SOD activity in kidney compared to AL fed mice. CR have variable effects on SOD activity. These differences are caused by differences in tissue examined and species used.

Glutathione is a tripeptide and one of the most vital antioxidants. Numerous studies have reported the influence of different types and amounts of CR on GSH level (Table 1.6). The data for GSH to show the effects of CR is not consistent as well due to variety of restriction protocols. However, most of the studies represent no significant changes due to CR application. For example, study by Doguc et al (2013) showed no significant change in GSH levelin erythrocytes of rats applied 60 per cent CR for 10 weeks [176]. Likewise, Stankovic et al (2013) have also reported no significant effects of 50-60 per cent CR for five weeks in liver samples [38].

Table 1.2. Effects of CR on MDA and TBARS level. MDA: Malondialdehyde, TBARS: thiobarbituric acid reactive substances, CR: Calorie restriction, IF: Intermittent fasting, F: Female, M: Male

Parameter	Species	Age	Gender	Change	Source	Factor	Reference
MDA	Rat	11 weeks	M	↓	Hippocampal	IF(7 weeks)	[177]
MDA	Human	26.6±5 years	M	↔	Blood	Ramadan Fasting	[43]
TBARS	Human	45-55 years	F	↑	Erythrocytes	Breast Cancer	[31]
MDA	Human	55±5 years	F/M	↓	Serum	Ramadan Fasting	[178]
TBARS	Mouse	4 months	M	↓	Mitochondria	40% CR (2 months)	[179]
MDA	Rat	5 months	M	↓	Erythrocytes	60% CR (10 weeks)	[176]
MDA	Rat	15 weeks	M	↔	Liver	10-20% CR (5 weeks)	[38]
MDA	Rat	15 weeks	M	↔	Liver	30-40% CR (5 weeks)	[38]
MDA	Rat	15 weeks	M	↑	Liver	50-60% CR (5 weeks)	[38]
MDA	Human	50-70 years	M	↓	Blood	IF (12 weeks)	[180]
TBARS	Rat	24 months	M	↓	Plasma	26% CR (3 weeks)	[181]
MDA	Rat	21-23 weeks	M	↓	Liver	40% CR (8-10 weeks)	[182]
MDA	Rat	10 weeks	M	↑	Liver	50-60% CR (5 weeks)	[183]

Table 1.3. Effects of CR on CAT activity level. CAT: Catalase, CR: Calorie restriction, IF: Intermittent fasting, F: Female, M: Male

Parameter	Species	Age	Gender	Change	Source	Factor	Reference
CAT	Mice	6-8 weeks	M	↔	Heart	40% CR (2 weeks)	[184]
CAT	Mice	4 months	M	↓	Aorta	40% CR (22 months)	[185]
CAT	Rat	5 months	M	↑	Erythrocytes	60% CR (10 weeks)	[176]
CAT	Mice	5 months	M	↓	Liver	40% CR (3 months)	[138]
CAT	Human	45-55 years	F	↔	Erythrocytes	Breast cancer	[31]
CAT	Mice	6-8 weeks	M	↔	Liver	30% CR (6 months)	[186]
CAT	Mice	8 months	F	↔	Liver	IF	[187]
CAT	Rat	4 months	M	↑	Kidney	30% CR (17 weeks)	[188]
CAT	Rat	4 moths	M	↔	Heart	30% CR (17 weeks)	[188]

Table 1.4. Effects of CR on SOD activity level. SOD: Superoxide dismutase, CR: Calorie restriction, F: Female, M: Male

Parameter	Species	Age	Gender	Change	Source	Factor	Reference
SOD	Mice	6-8 weeks	M	↔	Heart	40% CR (2 weeks)	[184]
SOD	Mice	4 months	M	↓	Aorta	40% CR (22 months)	[185]

SOD	Rat	5 months	M	↑	Erythrocytes	60% CR (10 weeks)	[176]
SOD	Rat	15 weeks	M	↓	Liver	50-60% CR (5 weeks)	[38]
SOD	Rat	15 weeks	M	↔	Liver	10-20% CR (5 weeks)	[38]
SOD	Rat	5 months	M	↓	Liver	20% CR (3 months)	[39]
SOD	Human	45-55 years	F	↑	Erythrocytes	Breast cancer	[31]
SOD	Rat	24 months	M	↑	Aorta	26% CR (3 weeks)	[181]

Table 1.5. Effects of CR on GSH and GSH-Px level.
GSH: Glutathione, GSH-Px: Glutathione peroxidase, CR: Calorie restriction,
IF: Intermittent fasting, F: Female, M: Male

Parameter	**Species**	**Age**	**Gender**	**Change**	**Source**	**Factor**	**Reference**
GSH	Rat	11 weeks	M	↔	Hippocampal	IF	[177]
GSH	Rat	5 months	M	↔	Erythrocytes	60% CR (10 weeks)	[176]
GSH	Mice	6-8 weeks	M	↔	Heart	40% CR (2 weeks)	[184]
GSH	Human	55±5 years	F/M	↑	Erythrocytes	Ramadan fasting	[178]
GSH	Rat	15 weeks	M	↔	Liver	10-20% CR (5 weeks)	[38]
GSH	Rat	15 weeks	M	↔	Liver	50-60% CR (5 weeks)	[38]
GSH-Px	Mice	8 months	F	↔	Liver	IF	[187]
GSH-Px	Mice	4 months	M	↓	Aorta	40% CR (22 months)	[188]

2. MATERIALS AND METHODS

2.1. INSTRUMENTS

- Spectrophotometer (Thermo Fisher Scientific, Evolution 300 UV-VIS)
- Elisa Plate Reader (Thermo Labsystems, Multiskan Ascent)
- Centrifuge
- pH meter
- Vortex
- -80º C freezer
- Sonicator
- Incubator

2.2. EQUIPMENTS

- Micro pipettes 1000- 200- 10 µl
- Centrifuge tubes 50-15 ml
- Centrifuge Eppendorf 2- 1.5 ml
- Serological Pipettes 25-10 ml
- 96- well plate
- Balloon jojes 250- 200- 100- 50- 25- 10- 5 ml
- Quartz cuvette

2.3. CHEMICALS

- Potassium chloride (KCl, Sigma Aldrich, 12636)
- Copper(II) sulfate-5-hydrate ($CuSO_4$, Riedel-de Haen, 12849)
- Potassium sodium tartrate tetrahydrate (NaK, Riedel-de Haen, 32312)
- Folin reagent (Sigma Aldrich, F9252-500ML)
- 1, 1, 3, 3- Tetramethoxy-propan, 99 per cent (Sigma Aldrich, MKBB0326)
- Tricholoroacetic acid (Sigma Aldrich, 27242-100G-R)

- Ethylenediaminetetraacetic acid disodium salt dihydrate, 99+ per cent (Sigma Aldrich, E5134-1KG)
- 2- Thiobarbituric acid, ≥98 per cent (Sigma Aldrich, T5500-25G)
- Potassium phosphate monobasic (KH_2PO_4, Sigma Aldrich, 04243-500G)
- Dodecyl sulfate sodium salt (SDS, Merck, 8.22050.1000)
- Hydrogen peroxide solution (Sigma Aldrich, 18304-1L)
- β- Nicotinamide adenine dinucleotide 2'- phosphate reduced tetrasodium salt hydrate (NADPH, Sigma Aldrich, N1630-100MG)
- Catalase from bovine liver (Sigma Aldrich, C1345-1G)
- CAPS (Sigma Aldrich, C2632-250G)
- Superoxide dismutase from bovine erythrocytes (Sigma Aldrich, S5395-15KU)
- Xantine sodium salt, ≥99 per cent (Sigma Aldrich, X2502-5G)
- Icdonitrotetrazolium chloride (INT, Sigma Aldrich, I10406-1G)
- Trizma base (Sigma Aldrich, T1503-1KG)
- Sodium azide (Sigma Aldrich, S8032-25G)
- tert-Butyl hydrogenperoxide solution (Sigma Aldrich, 416665-25ML)
- L-Glutathione reduced (Sigma Aldrich, G4251-5G)
- Glutathione reductase from baker's yeast (Sigma Aldrich, G3664-500UN)
- 5, 5' Dithiobis (2- nitrobenzoic acid) (DTNB, Sigma Aldrich, D8130-500MG)
- Tri-sodium citrate dihydrate (VWR, 27831.297)
- Sodium chloride (NaCl, Sigma Aldrich, 13423)
- Isotonic saline
- Bovine serum albumin (BSA, Sigma Aldrich, 05470)
- Sodium carbonate
- Sodium hydroxide

2.4. KIT

- 8-OHdG (8-Hydroxydeoxyguanosine) ELISA Kit (E-Lab Science, E-EL-0028)

2.5. ANIMALS

Four MMTV-TGF-α (C57BL/6) male mice were kindly given by Dr. Margot Cleary, Hormel Institute Medical Research Center, University of Minnesota to establish a breeding colony at Yeditepe University Animal Facility. Experiment was carried out on 10-weeks old female MMTV-TGF-α mice (n = 202)[189]. MMTV-TGF-α mice are well known transgenic mouse models which develop breast cancer in second-half of their life and have several similarities with breast cancer development in humans. These mice over-express human TGF-α which is a part of the epidermal growth factor receptor (EGFR)/ErbB cascade that have a significant role in breast cancer development. [190, 191]. All animals were provided with unlimited water. All of mice were housed individually under standard conditions in a room at temperature of 21–24° C and 12 h light/dark cycle. Animals were observed for any health problems on a daily basis. All procedures were performed under the guidelines and with the approval of Yeditepe University Animal Care and Use Committee.

2.6. STUDY DESIGN

MMTV-TGF-α C57BL6 female mice were randomly enrolled in the study at 10 weeks of age into three different CR groups: ad-libitum (AL), Chronic Calorie Restriction (CCR) or Intermittent Calorie Restriction (ICR). All mice were fed with Altromin TPF1414 diets that were purchased from Kobay AS (Ankara, Turkey). Mice in AL group had free access to food throughout the study. Mice in CCR group provided 85 per cent of the daily food consumption of age-matched AL group in other words, CCR group was implemented 15 per cent CR. Mice in ICR group were provided AL feeding for three weeks and one week of 60 per cent of age-matched AL consumption. These process have been applied to ICR mice in a cyclic period until they were sacrified at specific time points. Body weights of the animals as well as food consumption were measured and recorded every week.

Blood collection and sacrification were done at designated time points starting from 10 weeks old and up to 82 weeks old. Mice were applied to 8 hour fasting prior to blood collection and sacrification. Blood collection by retro orbital puncturing and organ collection were done from each mouse of all three groups at 10 week of age (AL, CCR and ICR) and from half of the mice in each of the three groups at 17, 49 and 81 week of age also the other

half of the mice in each group at 18, 50 and 82 week of age. The subgrouping was required because sequential weekly orbital bleedings are considered inappropriate and not allowed by the Committee of Animal Research Ethics. Furthermore, no statistical difference has been found in AL and CCR groups according to subgrouping samples in previous studies. For the ICR group, blood collection and sacrification at 17, 49 and 81 weeks of age referred as ICR-refeed group (ICR-RF) which happens at the end of three weeks of AL feeding. Blood collection and sacrification at 18, 50 and 82 weeks of age referred as ICR-restriction group (ICR-R) which happens at the end of one week 60 per cent CR. Tissue samples were collected during scarification and part of each tissue was separated for histopathological analysis. Remaining part of each tissue was snap-frozen and kept at -80° C freezer until it is used.

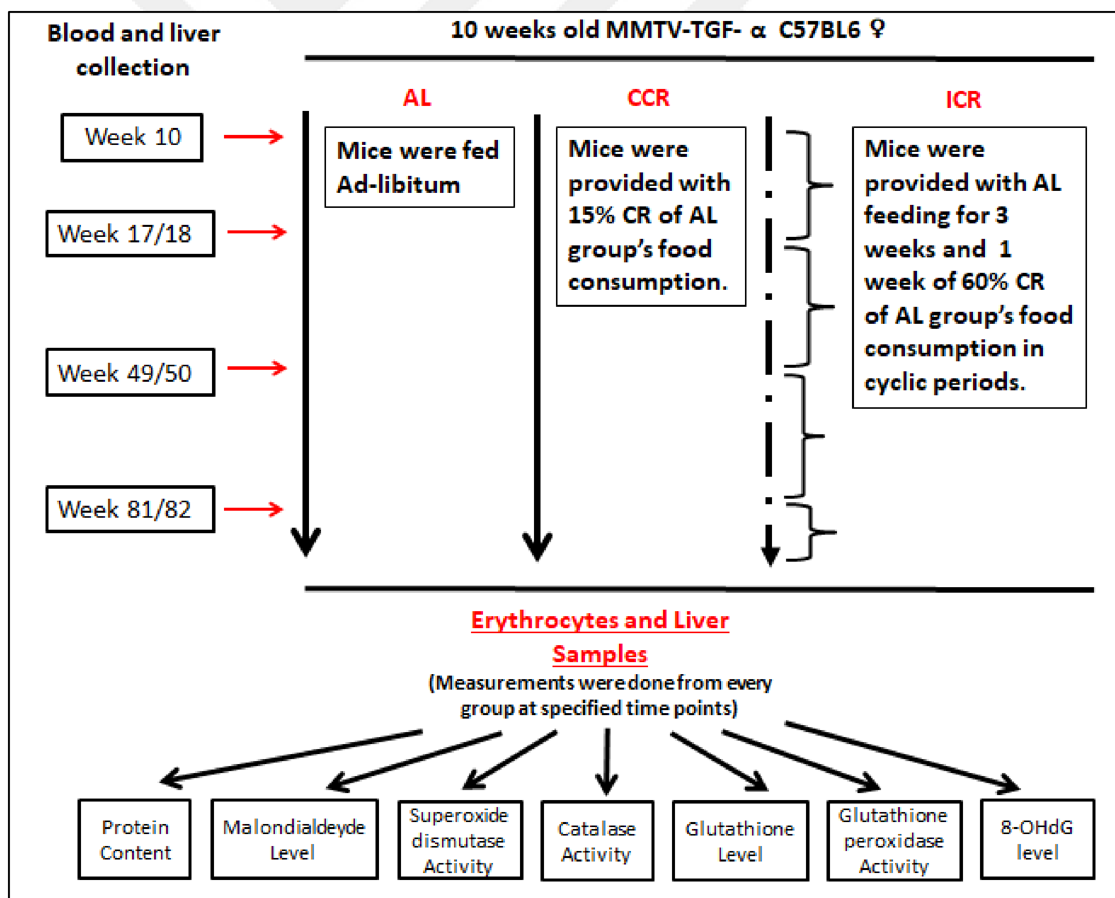


Figure 2.1. Schematic illustration of study design

2.7. SAMPLE PREPARATION

Following collection, blood was incubated at room temperature for 30 minutes and centrifuged at 4^{0} C 4000 rpm for 10 minutes. The supernatants were put into clean tubes and stored at -80^{0} C freezer. 600 µl of isotonic saline was added to remaining part and centrifuged at 4^{0} C 4000 rpm for 10 minutes. After centrifugation, supernatant was discarded and 600 µl of isotonic saline was added to pellet for further washing for three times. After centrifugation, supernatant was discarded. Afterwards, a known volume of erythrocytes was lysed with four volume of cold distilled water and the cell debris was removed by centrifugation at 2500 rpm for 10 minutes. After centrifugation, supernatant which was erythrocytes suspension was stored at -80^{0} C freezer.

Liver samples were collected and snap frozen at scarification. Prior to experiments, liver samples were sliced into pieces and 100 mg of each liver sample were placed into 1.5 ml Eppendorf tubes. One measure of 0.05 mm zirconium magnetic beads were added to each sample. Following that, 9 volumes of 1.15 per cent Potassium chloride (KCl) solution was added to liver samples. Samples were homogenized by Bullet Blender homogenizer. After homogenization, samples were centrifuged at 1000 g for 10 minutes and supernatant which was the tissue homogenate was removed. Supernatant was divided into aliquotes and kept at -80^{0} C freezer until it is used.

2.8. MEASUREMENTS OF OXIDATIVE STRESS PARAMETERS

2.8.1. Measurement of Protein Content by Lowry Method in Erythrocytes and Liver Samples

100 mg Bovine Serum Albumin (BSA) was weighed and reconstituted with 5 ml of dH_2O. This reconstitution was produced a stock solution of 20 mg/ml. BSA standards were prepared by serial dilution in the 0-20 mg/ml range.

10 µl of samples and standards were diluted with 1 ml of dH_2O in falcon tubes. Reaction mixture that contains Solution A (two per cent Na_2CO_3/ 0,1 N NaOH), Solution B (0.5 per cent $CuSO_4$/ dH_2O) and Solution C (onw per cent NaK /dH_2O in a 100:1:1 ratio were

prepared and 4.5 ml of reaction mixture was added to each sample. This mixture was incubated for 10 minutes at dark. Following incubation, 0.5 ml of Folin was added to mixture and incubated for 20 minutes at dark. 300 µl of final mixtures of erythrocytes, tissue homogenates, standards and blank were put into 96-well plate and the color change was measured at 640 nm by spectrophotometer. Standard curve was plotted and protein concentration was assessed in each sample in cooperation with the standard curve.

2.8.2. Measurement of Malondialdehyde Level in Erythrocytes Samples

50 µl of 1,1,3,3- tetraetoksipropan tetrametoksi was adjusted to 100 ml of dH_2O in order to prepare a stock standard solution. Standards were prepared in the range of 0.076-0.76 nmol/ml.

0.5 ml of erythrocyte samples and standards were taken into sterilized Eppendorf tubes. 0.5 ml of Phosphate Buffer were also taken as Blank. 0.5 ml of 0.33M Phosphate Buffer and 0.5 ml 15 per cent TCA solution were added to Eppendorf tubes. Mixtures were vortexed and incubated at 4º C for 2 hours. Following incubation period prepared mixtures were centrifuged at 4000 rpm 4º C for 10 minutes. Afterwards, 1 ml of each supernatant was transferred into new sterilized Eppendorf tubes. 75 µl of 0.1 M EDTA and 250 µl of 1 per cent TBA were added and placed into a boiling water bath for 15 minutes. 300 µl of each sample and standards were put into 96-well plate and the color change was measured at 532 nm by spectrophotometer. Standard curve was plotted and MDA level was assessed in each sample in cooperation with the standard curve.

2.8.3. Measurement of Malondialdehyde Level in Liver Samples

50 µl of 1,1,3,3- tetraetoksipropan tetrametoksi was adjusted to 100 ml of dH_2O in order to prepare a stock standard solution. Standards were prepared in 0.076-0.76 nmol/ml range.

0.2 ml of liver homogenates were taken into sterilized Eppendorf tubes. 0.2 ml of dH_2O as Blank and 0.2 ml of each standard were also taken into new Eppendorf tubes. 0.2 ml of 8.1 per cent sodium dodecyl sulfate (SDS) solution, 1.5 ml of 20 per cent acetic acid solution, 1.5 ml of 0.8 per cent 2-thiobarbituric acid (TBA) solution and 0.6 ml of dH_2O were added

to each sample and mixture was vortexed. Following that, mixtures were placed into 95° C water bath and incubated for one hour. Then, two ml of TCA solution was added to two ml of each mixture and centrifuged again. 300 µl of each supernatant were put into 96-well plate and the color change was measured at 532 nm. Standard curve was plotted and MDA level was assessed in each sample in cooperation with the standard curve.

2.8.4. Measurement of Catalase Activity in Erythrocytes and Liver Samples

24 mg of catalase was reconstituted with five ml of 50 mM phosphate buffer. 0.4 ml of this stock standard solution was adjusted to 10 ml with 50mM phosphate buffer. Then catalase standards were prepared in the range of 0.01-0.035 ku/ml.

Each sample was diluted with 50 mM phosphate buffer in 1:250 ratios. Then, two ml of each standard, diluted samples or blank were taken into Quartz cuvette and 1 ml of 30 mM H_2O_2 was added. Quartz cuvette was immediately placed in the spectrophotometer and measurement of catalase activity was done at 240 nm every 15 minutes for 45 seconds. Standard curve was plotted and CAT activity was assessed in each sample in cooperation with the standard curve.

2.8.5. Measurement of Superoxide Dismutase Activity in Erythrocytes and Liver Samples

One mg of Superoxide dismutase was reconstituted with 10 ml of isotonic saline solution in order to prepare 100 mg /ml stock standard solution. Then, one ml of stock standard solution was adjusted to 100 ml with dH_2O. Standards were prepared in the range of 60-540 ng/ml.

Each sample was diluted with dH_2O (1:400 ratio). Then, 25 µl of each diluted sample and standard was mixed with 850 µl of substrate solution (pH 10.2) containing 0.05 mmol/l xanthine sodium, 0.025 mmol/l Iodonitrotetrazolum (INT), 50 mmol/l N-cyclohexyl-3-aminopropanesulfonic acid (CAPS) and 0.94 mmol/l EDTA disodium dihydrate. Afterwards, 125 µl of xanthine oxidase (80 U/L) was added to the mixture. Quartz cuvette was immediately placed in spectrophotometer and measurement was done at 505 nm every 30 minutes for three minutes. Following measurement of SOD activity, standard curve was

plotted and superoxide dismutase activity was assessed in each sample in cooperation with the standard curve.

2.8.6. Measurement of Glutathione Peroxidase Activity in Erythrocytes and Liver Samples

Samples were diluted with dH_2O in order to measure glutathione peroxidase activity in 1:5 dilutions for erythrocytes and in a 1:17 ratio for tissue homogenates. Then, 10 µl of each sample were mixed with 990 µl of reaction mixture that contains one mmol/l of EDTA disodium dihydrate, two mmol/l of reduced glutathione, 0.2 mmol/l of NADPH, 4 mmol/L of sodium azide, and 1000 U of glutathione reductase in 50 mmol/l tris buffer. The mixture was incubated for five minutes at room temperature and 10 µl of tert-butil hydroperoxide (1:1000 dilution) was added. Quartz cuvette was immediately placed in spectrophotometer and measurement was done at 340 nm every 30 minutes for three minutes. Following measurement, Glutathione peroxidase activity was calculated according to a formula which is given below.

$$Activity\ U/L = \frac{\Delta Abs}{0.00622} \times 100 \qquad (2.1)$$

2.8.7. Measurement of Glutathione Level in Erythrocytes and Liver Samples

200 µl of each sample or dH_2O as blank was mixed with 300 µl of precipitant solution that contains meta phosphoric acid, disodium EDTA and sodium chloride (NaCl). This mixture was centrifuged at 3000 rpm for 30 minutes. Then, 100 µl of supernatant was taken into new Eppendorf tubes. 400 µl of 0.3 M Na_2PO_4 solution and 0.02 per cent 50 µl of Ellman's solution (5,5′- Dithiobis (2-nitrobenzoic acid)) was added to supernatant. Afterwards, 300 µl of each mixture was put into 96-well plate and the color change was measured at 412 nm by spectrophotometer. Following assay measurement, glutathione level in each sample was calculated according to a formula given below.

$$Glutathione\ (\mu mol/ml) = \frac{2 \times absorbance}{0.175 \times 0.307} \quad (2.2)$$

2.8.8. Measurement of 8-OHdG Level

8-OHdG level in liver lysate was measured by using E-Lab Science 8-OHdG (8-Hydroxydeoxyguanosine) ELISA Kit (Catalog No: E-EL-0028). All reagents were brought to room temperature (18~25°C) before starting. 50 µl of standard working solution in the concentration range of 100 and 0 ng/ml and sample diluent reference as blank were added to the first two columns of plate. Samples were also added to plate as duplicate. 50 µl of Biotinylated Detection Ab (1:100 dilution) working solution was immediately added to each well. The plate was covered with sealer provided in the kit and incubated for 45 min at 37 °C. Following that, the solution was aspirated from each well and 350 µl of wash buffer (1:25 diluted) was added to each well. It was soaked for one min and the solution was aspirated from each well and was pat against clean absorbent paper. This washing step was repeated 3 times. 100 µl of HRP Conjugate working solution (1:100 dilution) was added to each well. The plate was covered with sealer. The plate was incubated for 30 min at 37 °C.

At the end of the incubation period, the solution was aspirated from each well. The washing step was repeated for five times. 90 µl of Substrate Reagent was added to each well. The plate was covered with a new plate sealer. It was incubated for 15 min at 37 °C. The plate was protected from light. 50 µl of Stop Solution was added to each well. 8-OHdG in each sample were measured at 450 nm by using ELISA plate reader. Following assay measurement, standard curve was plotted and 8-OHdG level was assessed in each sample in cooperation with the standard curve.

2.9. STATISTICAL ANALYSIS

Statistical analyses were performed by using GraphPad Prism 7. Data were analyzed for outliers. D'Agostino & Pearson normality test was performed in order to determine whether data were parametric or non-parametric. All data were parametric and comparisons between different groups were made by one-way ANOVA followed by the Bonferroni Multiple

Comparison test to determine differences between specific groups. Results are presented as mean ± standard error of the mean (SEM). MT incidence was analyzed by the Chi-square test and two-group log-rank test. Kaplan-Meir test were used for survival analysis. Correlation analysis were performed by Pearson correlation test and linear regression. 'n value' represents samples from different animals. $p < 0.05$ were statistically significant.

3. RESULTS

3.1. EFFECTS OF DIFFERENT TYPES OF CALORIE RESTRICTION ON BODY WEIGHT

Body weights were 28.9 g and 20.3 g in AL and CCR, respectively, at 81/82 weeks of ages. ICR group's body weights were around 26.5 g in the refeed period and 22.2 g in the restriction week at 81/82 weeks of age. There was an increase in AL group's body weight throughout the study. CCR group decreased body weight compared to AL group ($p<0.0001$). However, no change was determined between ICR and AL group's body weight. ICR group have shown a dramatic decrease in body weight at restriction weeks and gained weight during refeed period at every cycle (Figure 3.1).

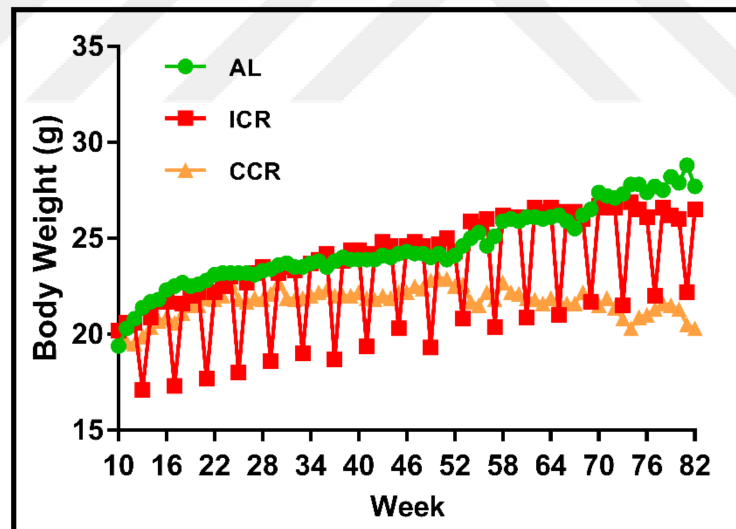


Figure 3.1. Effects of different types of CR on body weight. AL= ad-libitum, CCR= Chronic calorie restriction, ICR= Intermittent calorie restriction

3.2. EFFECTS OF DIFFERENT TYPES OF CALORIE RESTRICTION ON MAMMARY TUMOR DEVELOPMENT

12 mice which equals to 21.4 per cent of AL group developed mammary tumor. 18 mice which equals to 20.4 per cent of ICR group developed mammary tumor. However, only 4 mice which equals to 8.7 per cent of CCR group developed mammary tumor. CCR group had less MT incidence compared to AL and ICR group (p=0.12, Figure 3.2).

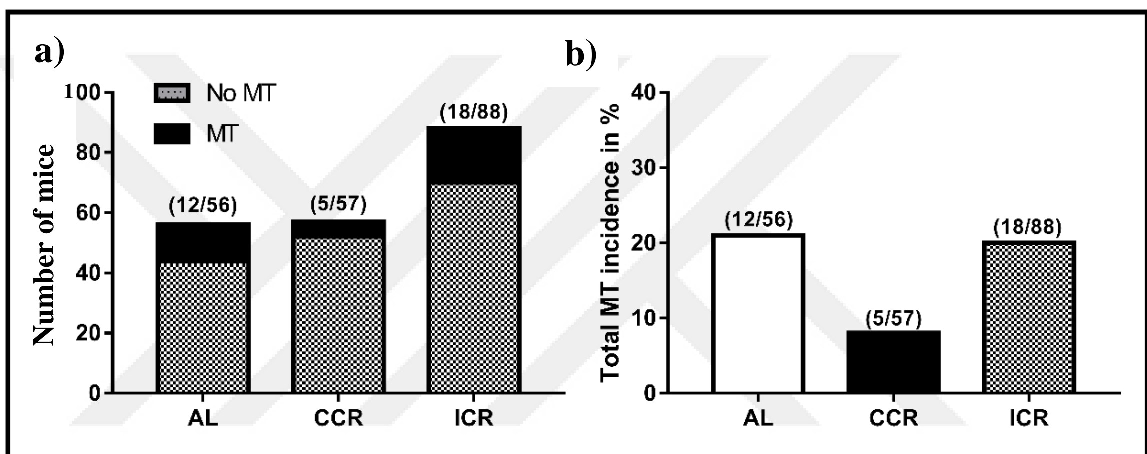


Figure 3.2. Effects of different types of CR on fraction of MT development (a) and total MT incidence in each group (b). AL= ad-libitum, CCR= Chronic calorie restriction, ICR= Intermittent calorie restriction, MT= Mammary tumor.

ICR group started to develop mammary tumor at 20 weeks of ages while AL group started to develop mammary tumor at between 40 and 50 weeks of ages. However, CCR group started to develop mammary tumor at only 60 weeks of ages. In addition, 4 mice developed mammary tumor in baseline group, at 10 weeks of age. The difference at the time of tumor detection (Figure 3.3) was not statistically changed among AL, CCR and ICR groups (log rank $\chi2$ with 2 df = 3.937; p=0.14).

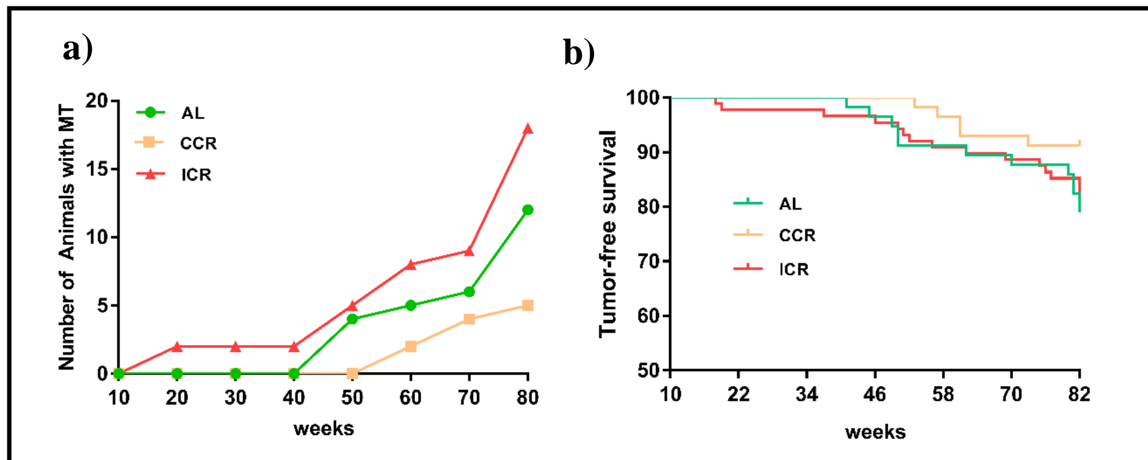


Figure 3.3. Effects of different types of CR on time of tumor detection (a) and tumor free survival (b). AL= ad-libitum, CCR= Chronic calorie restriction, MT= Mammary tumor, ICR= Intermittent calorie restriction

In AL group, 3 of MT's were Grade I, 6 of MT's were Grade II and 3 of MT's were Grade III. In CCR group, 3 of MT's were Grade I, 1 of MT was Grade II and 1 of MT's was Grade III. In ICR group 10 of MT's were Grade I, 5 of MT's were Grade II and 3 of MT's were Grade II (Figure 3.4).

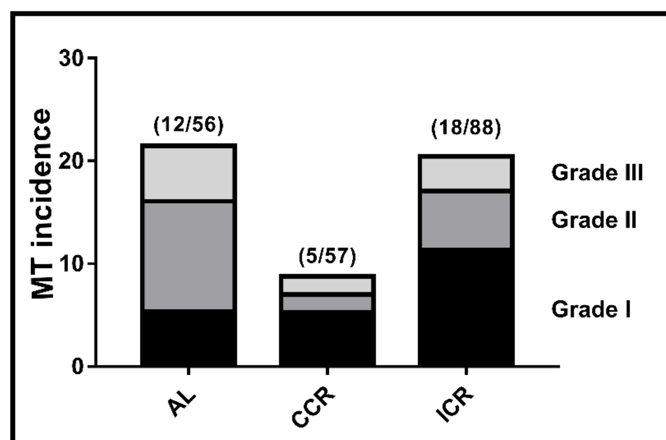


Figure 3. 4. Effects of different types of CR on MT tumor state. AL= ad-libitum, CCR= Chronic calorie restriction, ICR= Intermittent calorie restriction, MT= Mammary tumor.

3.3. EFFECTS OF DIFFERENT TYPES OF CALORIE RESTRICTION ON SURVIVAL RATE

Survival rate of AL, CCR and ICR groups were determined by using A Kaplan-Meier plot (Figure 3.5). Different types of diet did not have any statistical influence on survival rate (p>0.05).

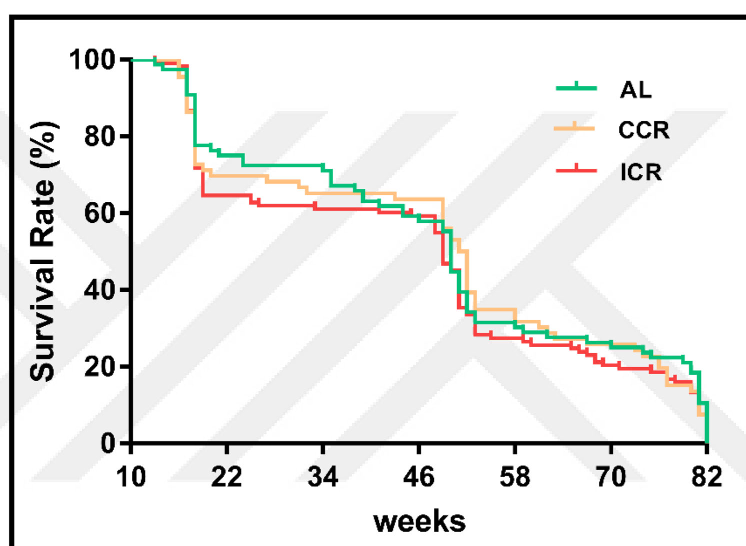


Figure 3. 5. Effects of different types of CR on survival rate

3.4. EFFECTS OF DIFFERENT TYPES OF CALORIE RESTRICTION ON OXIDATIVE STRESS PARAMETERS IN ERYTHROCYTES SAMPLES

3.4.1. Effects of Different Types of Calorie Restriction on Erythrocytes MDA Level

Erythrocytes MDA levels for each dietary groups were 5.05, 4.34, 5.35, 5.85 nmol/g protein in AL, CCR, ICR-R and ICR-RF groups, respectively at 17/18 weeks of age (Figure 3.6.a). Although MDA level in CCR group was 14.1, 18.9, and 25.8 per cent lower compared to AL, ICR-R and ICR-RF, respectively (p> 0.05). MDA levels for each dietary groups were 5.30, 4.38, 4.97, 6.11 nmol/g protein, in AL, CCR, ICR-R and ICR-RF, respectively at 49/50

weeks of age (Figure 3.6.b). Although MDA level in CCR group was 17.4, 11.9, and 28.3 per cent lower compared to AL, ICR-R and ICR-RF groups, respectively (p> 0.05). On the other hand, erythrocytes MDA levels in ICR-R group was significantly higher compared to AL group at 81/82 weeks of age (p<0.05, Figure 3.6.c). MDA levels for each dietary groups were 3.62, 4.69, 12.05, 7.59 nmol/g protein, in AL, CCR, ICR-R and ICR-RF groups, respectively 81/82 weeks of age (Figure 3.6.c). MDA level in ICR-R group was 232 and 156 per cent higher compared to both AL and CCR groups, respectively (p < 0.05).

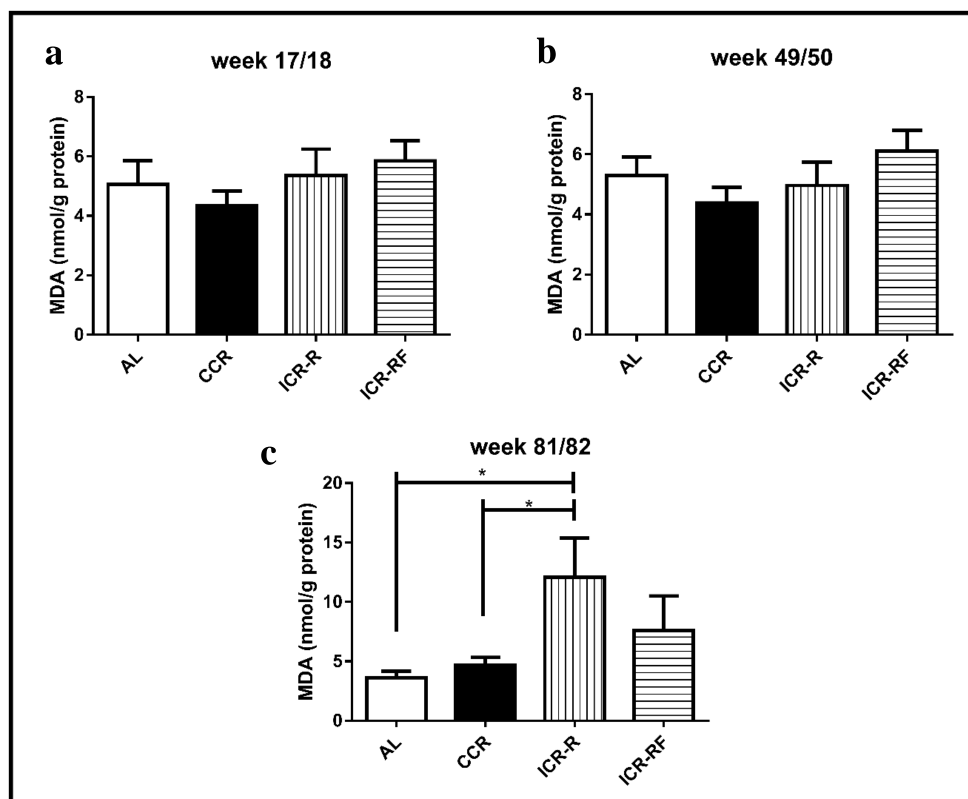


Figure 3.6. Effects of different types of CR on erythrocytes MDA level at 17/18 (a), at 49/50 (b) and at 81/82 (c) weeks of age. * represents significant differences (p <0.05). “n” values = 5-18.

3.4.2. Effects of Aging on Erythrocytes MDA Level

Erythrocytes MDA levels were significantly increased by aging in both ICR-R and ICR-RF groups (p<0.05). MDA level in ICR-R group was gradually increased with aging starting at

baseline until 81/82 weeks of age. However, this increase was significant only at 81/82 weeks of age compared to the other time points (p<0.05, Figure 3.7.c). However, in ICR-RF group changes in erythrocytes MDA levels were statistically significant only from week 10 to 81 weeks of age (p<0.05, Figure 3.7.d). On the other hand, there was about 50 per cent increase in MDA level from week 10 to week 17 and 49 in AL group (Figure 3.7.a). In CCR group, there was an increase in MDA level from week 10 to week 17/8 then maintained its MDA level rest of the study (Figure 3.7.b).

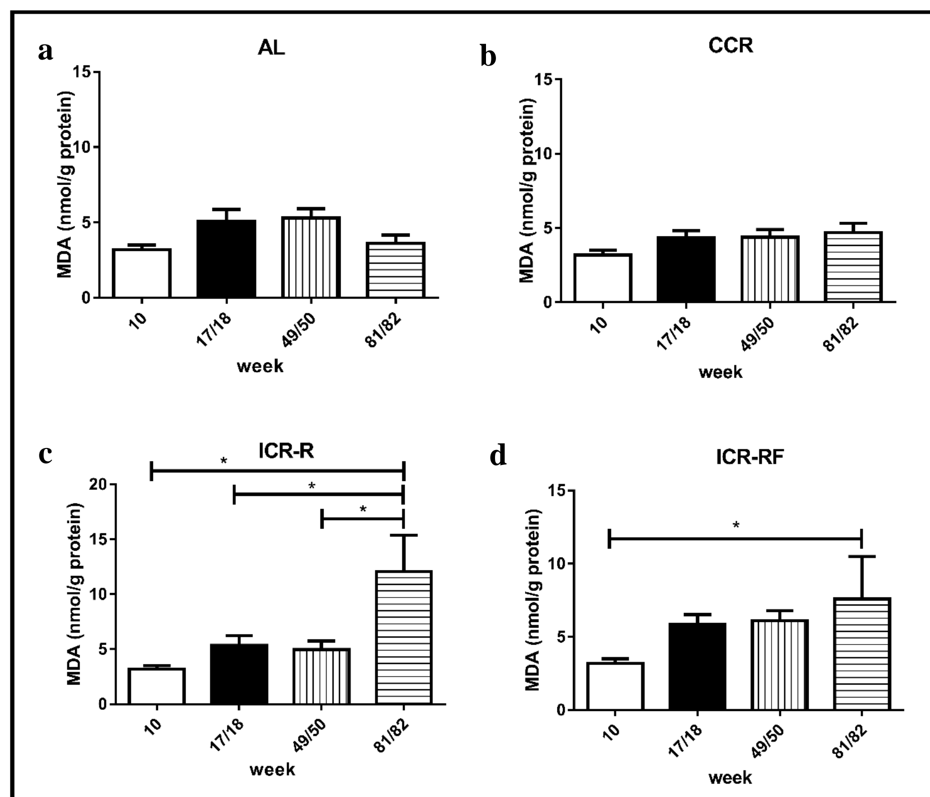


Figure 3.7. Effects of aging on erythrocytes MDA level in AL (a), CCR (b), ICR-R (c) and ICR-F (d) groups. * represents significant differences (p <0.05). “n” values = 5-18.

3.4.3. Effects of Different Types of Calorie Restriction on Erythrocytes CAT Activity

Erythrocytes CAT activity for each dietary group was 26.48, 29.15, 24.35 and 22.51 kU/g protein in AL, CCR, ICR-R and ICR-RF, respectively at 17/18 weeks of age. Although it

was not significant, CAT activity in CCR group was 10, 16.46 and 23 per cent higher compared to AL, ICR-R and ICR-RF (Figure 3.8.a). CAT activity levels for each dietary groups were 27.43, 32.85, 15.61, 21.38 kU/g protein in AL, CCR, ICR-R and ICR-RF, respectively at 49/50 weeks of age. Likewise, week 17/18, CAT activity level was 16.4, 52.5 and 34.9 per cent higher in CCR group compared to AL, ICR-R and ICR-RF (Figure 3.8.b). CAT activity levels for each dietary groups were 17.49, 25.76, 17.71, 15.9 kU/g protein in AL, CCR, ICR-R and ICR-RF, respectively at 81/82 weeks of age. Although it was not significant, CAT activity in CCR group was 32.1, 31.2 and 38.3 per cent higher compared to AL, ICR-R and ICR-RF (Figure 3.8.c).

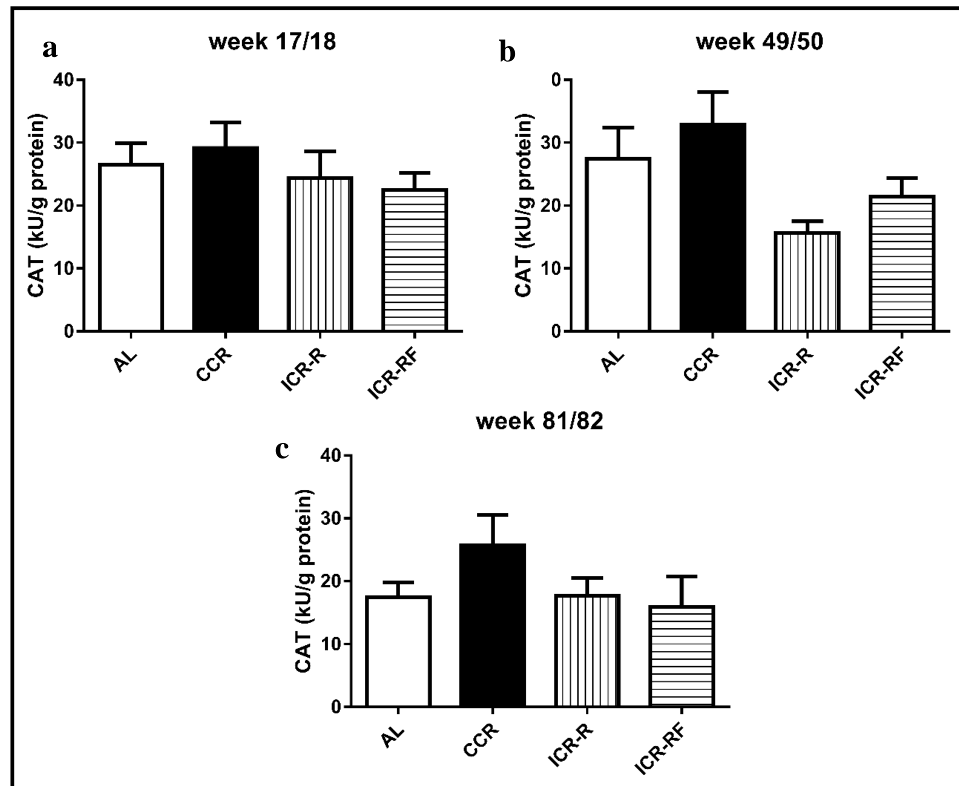


Figure 3.8. Effects of different types of CR on erythrocytes CAT activity at 17/18 (a), at 49/50 (b) and at 81/82 (c) weeks of age. * represents significant differences (p <0.05). “n” values = 5-17

3.4.4. Effects of Aging on Erythrocytes CAT Activity

CAT activity was increased by aging in all of the diet groups (Figure 3.9). AL group increased its CAT activity by approximately 3.5 fold at 17/18 and 49/50 weeks of age when it is compared with baseline ($p<0.05$, Figure 3.9.a). CAT activity was increased in CCR group until the end of the experiment. In details, CCR group had higher CAT activity by approximately 4 fold compared to baseline at 17/18, 49/50 and 81/82 weeks of ages ($p<0.05$) (Figure 3.9.b). ICR-R group increased its CAT activity by approximately 3 fold at 17/18 weeks of age when it is compared to baseline ($p<0.05$, Figure 3.9.c). ICR-RF group increased its CAT activity ($p<0.05$) by approximately 3 fold at 17/18 and 49/50 weeks of age compared to baseline (Figure 3.9.d).

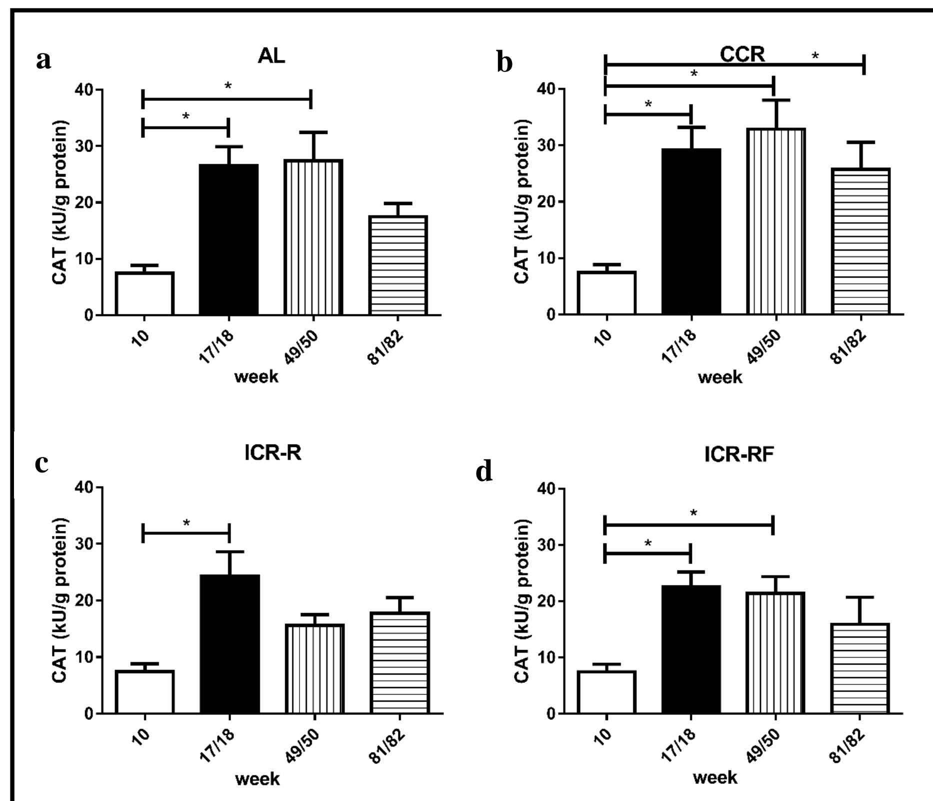


Figure 3.9. Effects of aging on erythrocytes CAT activity in AL (a), CCR (b), ICR-R (c) and ICR-F (d) groups. * represents significant differences ($p <0.05$). “n” values = 5-18.

3.4.5. Effects of Different Types of Calorie Restriction on Erythrocytes SOD Activity

Erythrocytes SOD activity for each dietary groups were 1.31, 1.92, 1.49 and 1.52 U/mg protein in AL, CCR, ICR-R and ICR-RF, respectively at 17/18 weeks of ages ($p>0.05$, Figure 3.10.a). Although it was not significant, SOD activity in CCR group was 31.3, 23.7 and 20.6 per cent higher compared to AL, ICR-R and ICR-RF groups at 17/18 weeks of age. Erythrocytes SOD activity for each dietary groups were 1.68, 2.14, 0.88 and 1.34 U/mg protein in AL, CCR, ICR-R and ICR-RF, respectively at 49/50 weeks of ages (Figure 3.10.b). CCR group had significantly 58.8 and 37.7 per cent higher SOD activity compared to ICR-R and ICR-RF groups at 49/50 weeks of age ($p<0.05$). Erythrocytes SOD activity for each dietary groups were 1.49, 1.80, 1.80 and 1.92 U/mg protein in AL, CCR, ICR-R and ICR-RF, respectively at 81/82 weeks of ages (Figure 3.10.c).

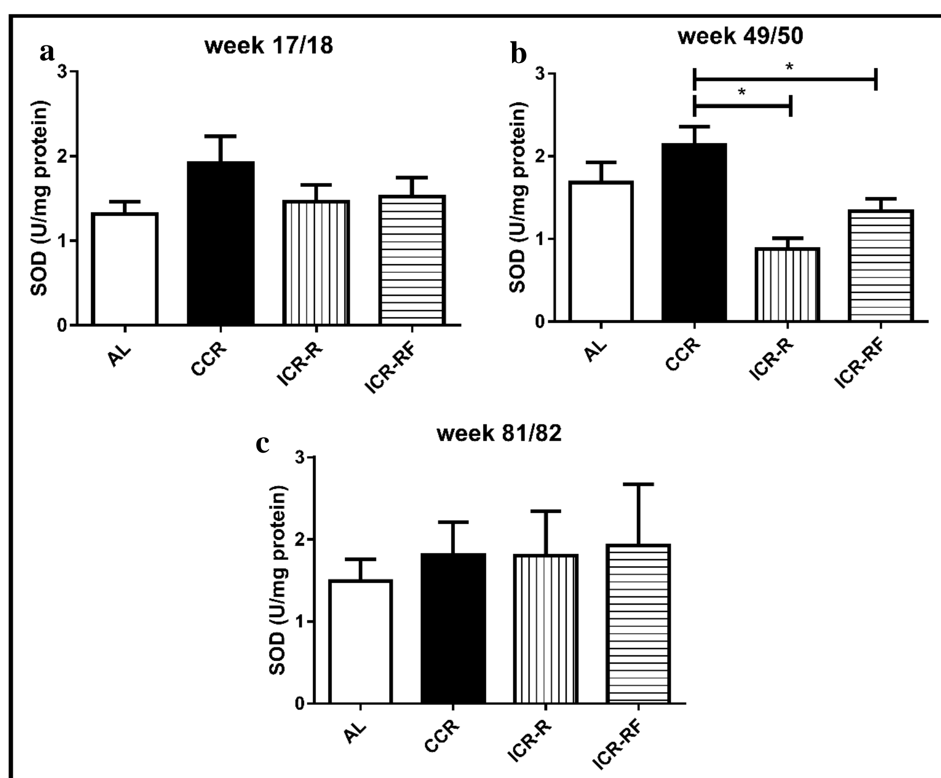


Figure 3.10. Effects of different types of CR on erythrocytes SOD activity at 17/18 (a), at 49/50 (b) and at 81/82 (c) weeks of age. * represents significant differences ($p <0.05$). "n" values = 4-15

3.4.6. Effects of Aging on Erythrocytes SOD Activity

Erythrocytes SOD activity was found to be decreased in AL, ICR-R and ICR-RF groups due to aging. In particular, AL group had lower SOD activity at 17/18 weeks of age compared to baseline ($p<0.05$, Figure 3.11. a). SOD activity was also decreased at 49/50 weeks of ages compared to baseline in both ICR-R and ICR-RF groups ($p<0.05$, Figure 11.c and d). Although SOD activity was decreased by aging in CCR group, this decrease was not significant ($p>0.05$, Figure 3.11.b).

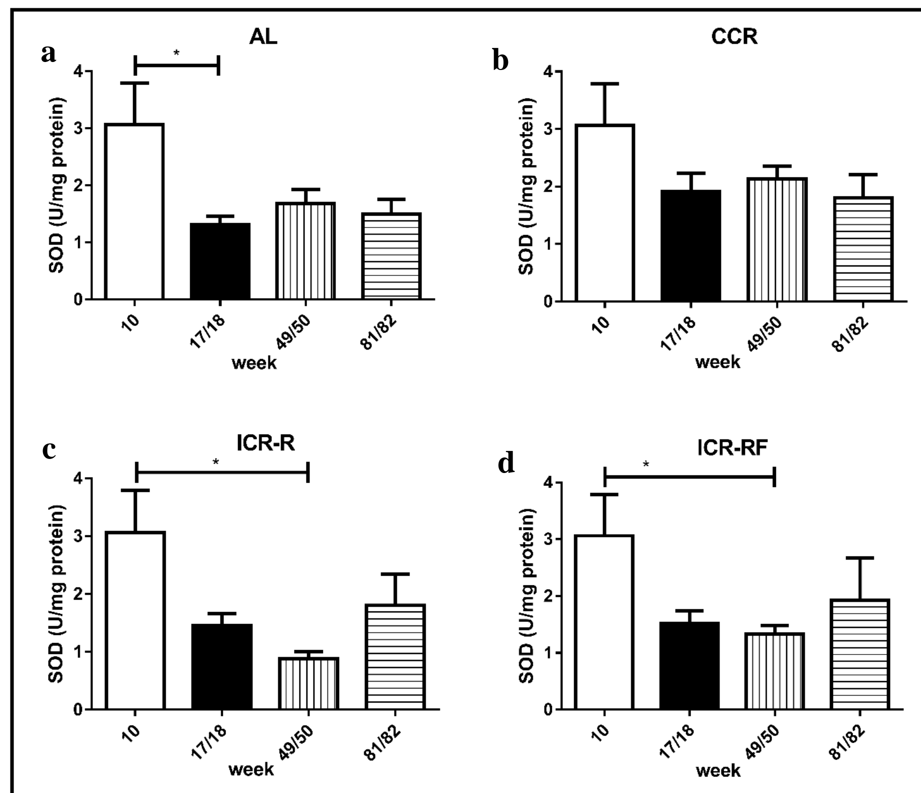


Figure 3.11. Effects of aging on erythrocytes SOD activity in AL (a), CCR (b), ICR-R (c) and ICR-F (d) groups. * represents significant differences ($p <0.05$). "n" values = 4-15.

3.4.7. Effects of Different Types of Calorie Restriction on Erythrocytes GSH Level

Different types of CR did not affect erythrocytes GSH level at 17/18 weeks of age ($p>0.05$, Figure 3.12.a). Erythrocytes GSH level for each dietary groups were 15.15, 15.84, 18.43 and 15.15 µmol/ml in AL, CCR, ICR-R and ICR-RF, respectively at 17/18 weeks of age. Erythrocytes GSH level for each dietary groups were 10.1, 17.68, 7.48 and 15.25 µmol/ml in AL, CCR, ICR-R and ICR-RF, respectively at 49/50 weeks of age. CCR group had significantly 57.7 per cent higher GSH level compared to ICR–R groups ($p<0.05$) (Figure 3.12.b). There was no significant effect of different types of diet on erythrocytes GSH level at 81/82 weeks of age. Erythrocytes GSH level for each dietary groups were 12.92, 13.49, 29.45 and 8.12 µmol/ml in AL, CCR, ICR-R and ICR-RF, respectively at 81/82 weeks of age (Figure 3.12.c).

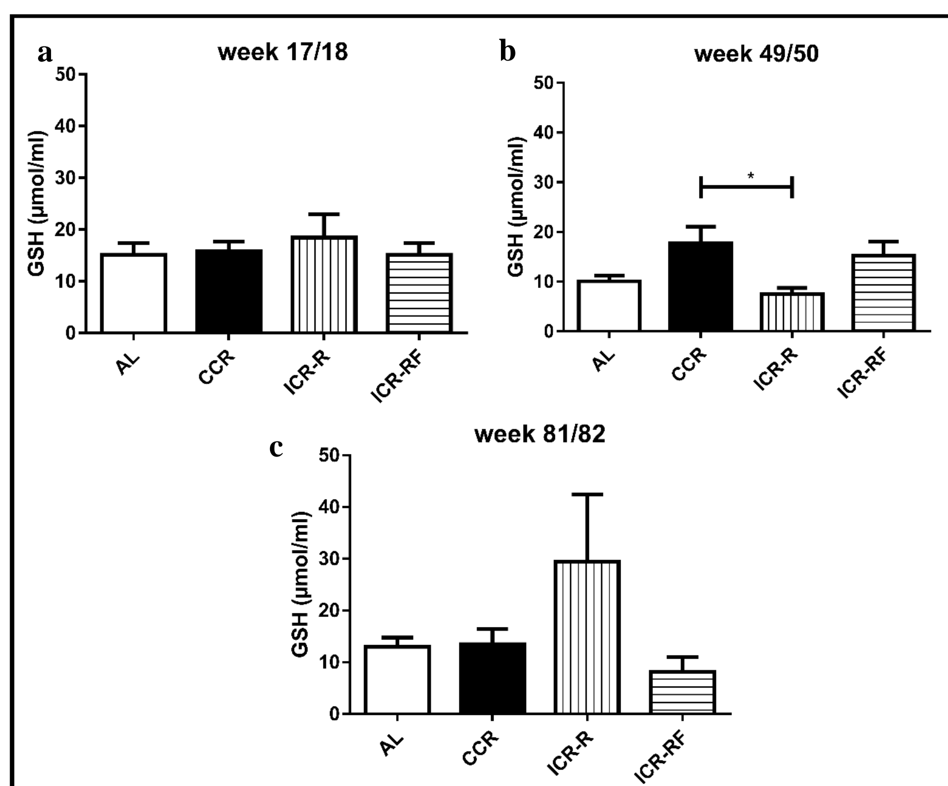


Figure 3.12. Effects of different types of CR on erythrocytes GSH level at 17/18 (a), at 49/50 (b) and at 81/82 (c) weeks of age. * represents significant differences ($p<0.05$). “n” values = 4-14

3.4.8. Effects of Aging on Erythrocytes GSH Level

GSH level was increased in AL group at 17/18 weeks of age compared to 10 and 49/50 weeks of age ($p<0.05$, Figure 3.13.a). CCR group had increasing level of GSH from baseline to 49/50 weeks of age ($p<0.05$, Figure 3.13.b). ICR-R group had increased GSH level at 81/82 weeks of age when it is compared to earlier weeks ($p<0.05$, Figure 3.13.c). Aging did not have any significant difference on ICR-RF group (Figure 3.13.d).

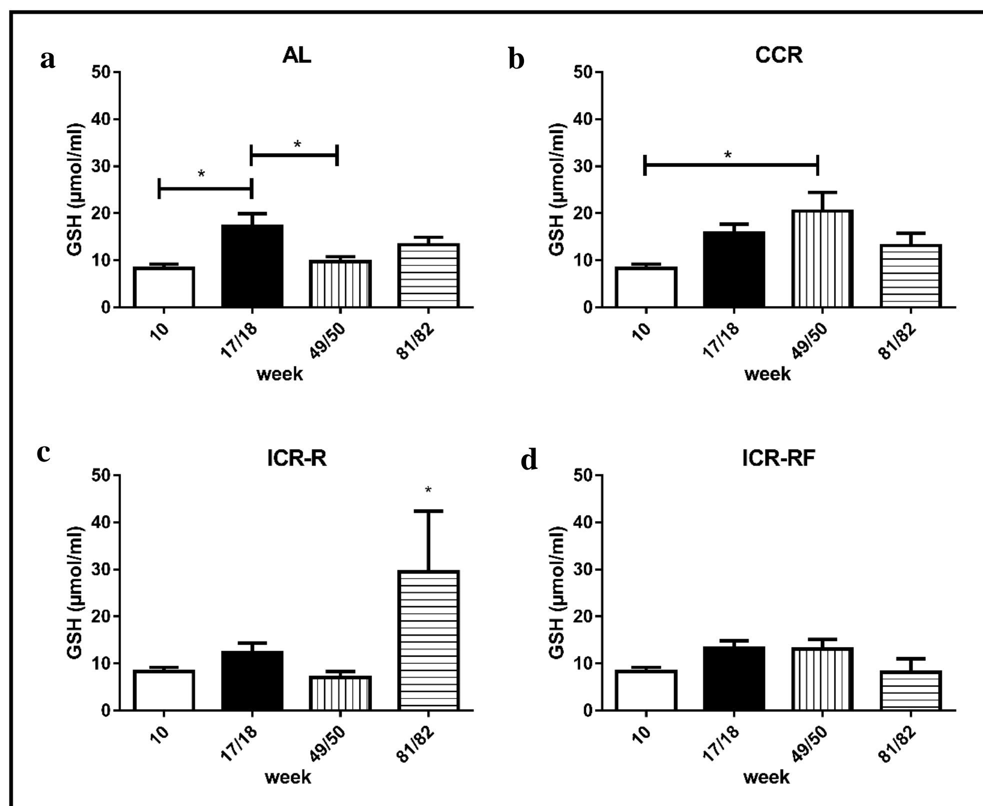


Figure 3.13. Effects of aging on erythrocytes GSH level in AL (a), CCR (b), ICR-R (c) and ICR-F (d) groups. * represents significant differences ($p <0.05$). “n” values = 4-15.

3.4.9. Effects of Different Types of Calorie Restriction on Erythrocytes GSH-Px Activity

Erythrocytes GSH-Px activity for each dietary groups were 64.03, 58.2, 62.56, 66.58 U/g protein in AL, CCR, ICR-R and ICR-RF groups, respectively at 17/18 weeks of age (Figure 3.14.a). GSH-Px activity for each dietary groups were 93.24, 61.67, 61.2, 66.29 U/g protein in AL, CCR, ICR-R and ICR-RF groups, respectively at 49/50 weeks of age (Figure 3.14.b). AL group had 34, 34 and 29 per cent higher GSH-Px activity level compared to CCR, ICR-R and ICR-RF groups, respectively at 49/50 weeks of age ($p<0.05$). Erythrocytes GSH-Px activity for each dietary groups were 60.36, 64.7, 92.8, 99.85 U/g protein in AL, CCR, ICR-R and ICR-RF groups, respectively at 81/82 weeks of age (Figure 3.14.c).

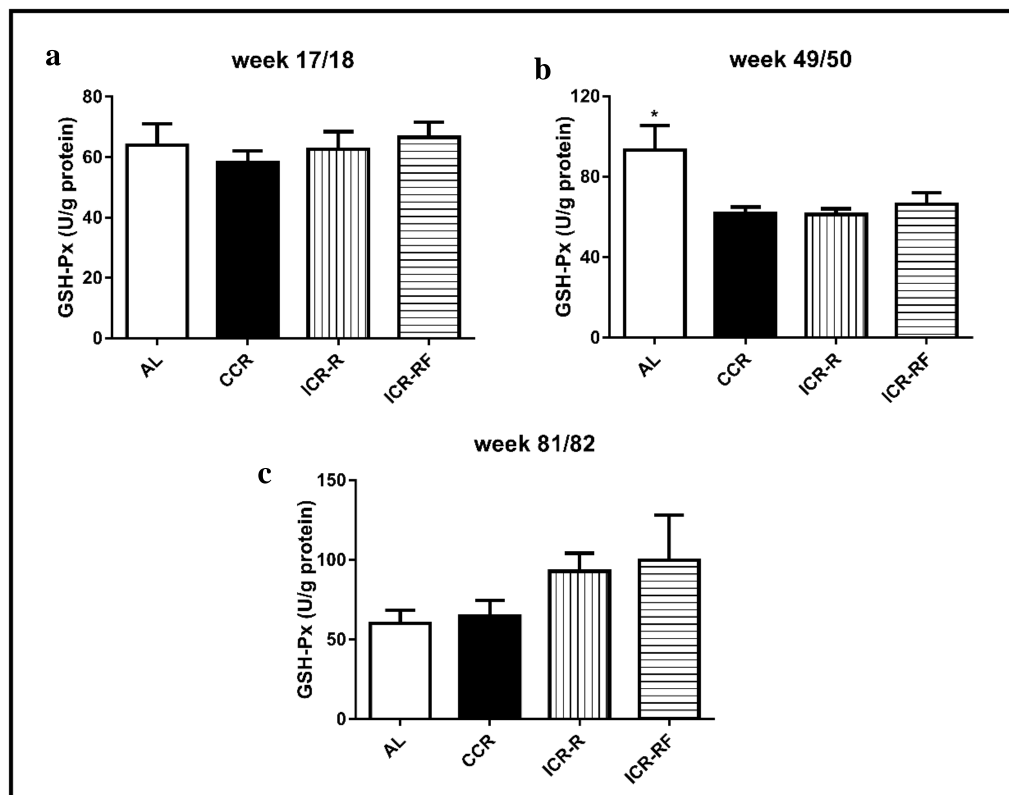


Figure 3.14. Effects of different types of CR on erythrocytes GSH-Px activity at 17/18 (a), at 49/50 (b) and at 81/82 (c) weeks of age. * represents significant differences ($p <0.05$). “n” values = 5-15.

3.4.10. Effects of Aging on Erythrocytes GSH-Px Activity

Aging did not have any statistically significant effect on GSH-Px activity in AL and CCR groups (Figure 3.15.a and b). However, ICR-R group had increased GSH-Px activity by 1.8 and 2.6 fold at 81/82 weeks of age compared to 17/18 and 49/50 ages, respectively ($p<0.05$, Figure 3.15.c).

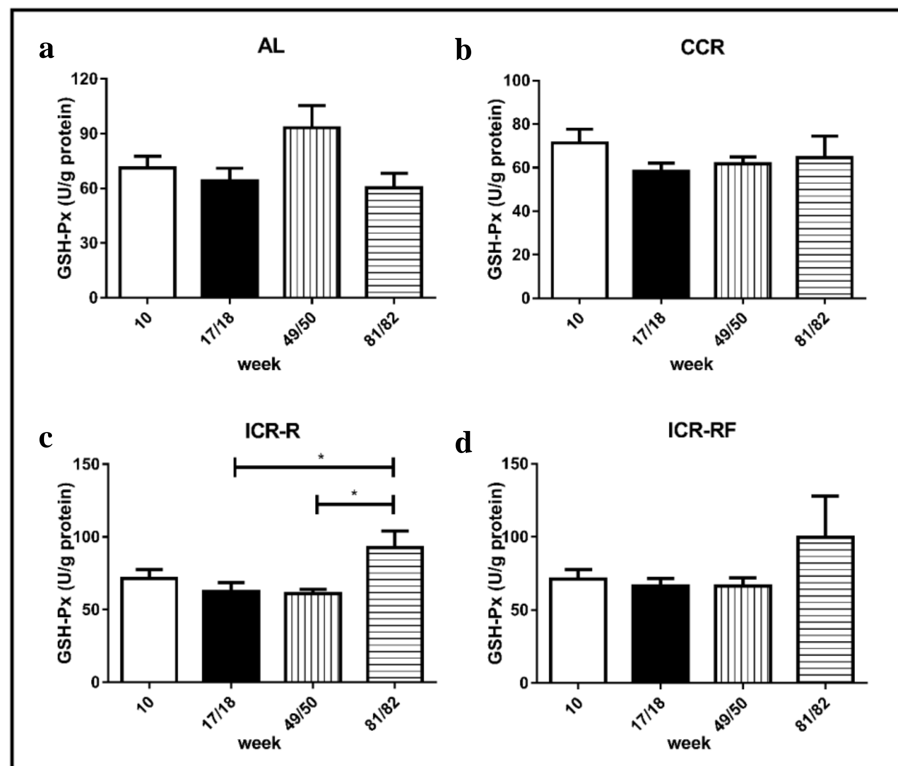


Figure 3.15. Effects of aging on erythrocytes GSH level in AL (a), CCR (b), ICR-R (c) and ICR-F (d) groups. * represents significant differences ($p < 0.05$). “n” values = 5-15.

3.5. EFFECTS OF DIFFERENT TYPES OF CALORIE RESTRICTION ON OXIDATIVE STRESS PARAMETERS IN LIVER SAMPLES

3.5.1. Effects of Different Types of Calorie Restriction on Liver MDA Level

Liver MDA levels for each dietary groups were 51.21, 52.89, 42.3, 30.23 nmol/g protein in AL, CCR, ICR-R and ICR-RF groups, respectively at 17/18 weeks of age (Figure 3.16.a). Liver MDA levels for each dietary groups were 51.35, 54.15, 81.42, 72.94 nmol/g protein, in AL, CCR, ICR-R and ICR-RF, respectively at 49/50 weeks of age (Figure 3.16.b). MDA levels for each dietary groups were 47.11, 51.34, 40.58, 64.38 nmol/g protein, in AL, CCR, ICR-R and ICR-RF groups, respectively 81/82 weeks of age (Figure 3.16.c).

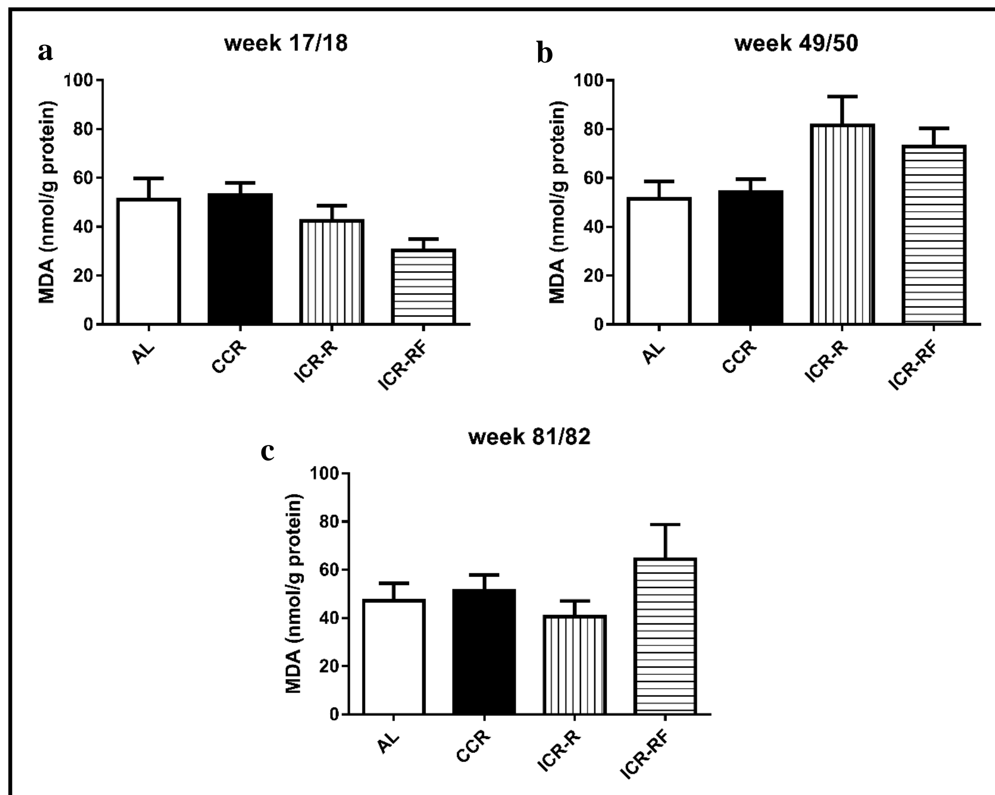


Figure 3.16. Effects of different types of CR on liver MDA level at 17/18 (a), at 49/50 (b) and at 81/82 (c) weeks of age. * represents significant differences ($p < 0.05$). “n” values = 5-17.

3.5.2. Effects of Aging on Liver MDA Level

Liver MDA level was not changed due to aging in both AL and CCR groups (p>0.05, Figure 3.17.a and b). However, ICR groups had increased MDA level at some stages. Liver MDA level was increased at 49/50 weeks of age compared to 10, 17/18 and 81/82 weeks of ages by 80 to 90 per cent in ICR-R group (p<0.05, Figure 3.17.c). Liver MDA level was increased at 49/50 weeks of ages compared to 17/18 weeks of ages in ICR-RF group (p<0.05, Figure 3.17.d).

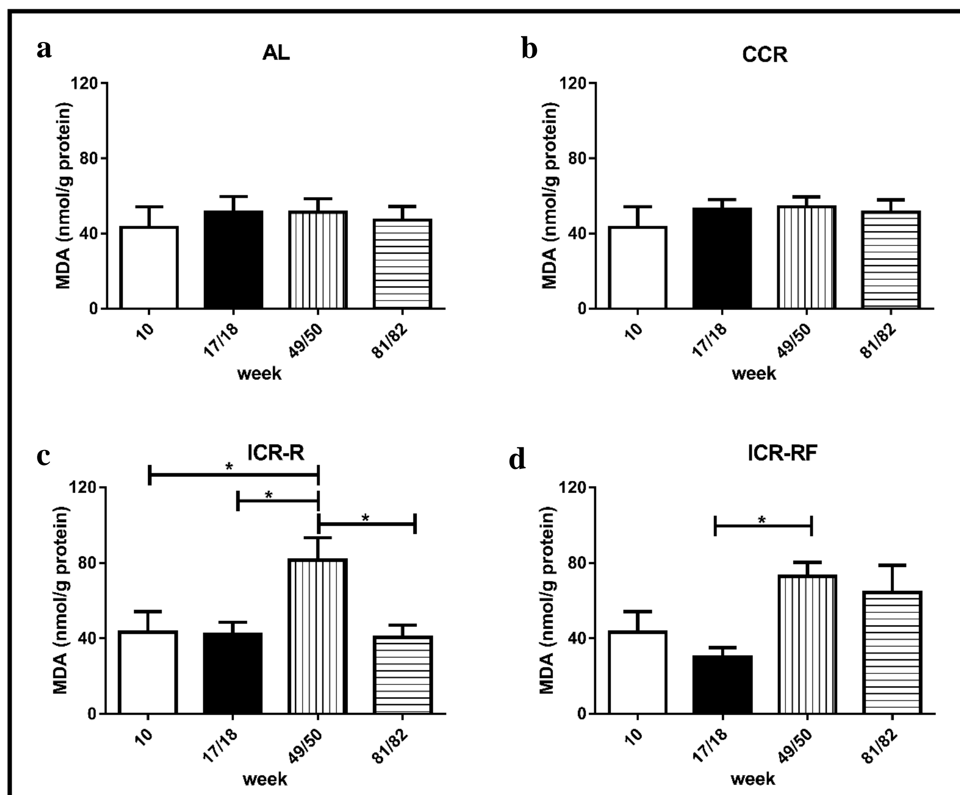


Figure 3.17. Effects of aging on liver MDA level in AL (a), CCR (b), ICR-R (c) and ICR-F (d) groups. * represents significant differences (p <0.05). “n” values = 5-17.

3.5.3. Effects of Different Types of Calorie Restriction on Liver CAT Activity

Liver CAT activity level was not affected from any type of dietary groups at any designated time points (p>0.05). Liver CAT activity for each dietary group was 282.6, 266, 305.4 and 310.2 kU/g protein in AL, CCR, ICR-R and ICR-RF, respectively at 17/18 weeks of age (Figure 3.18.a). CAT activity levels for each dietary groups were 341.2, 292.3, 333.7, 297.3 kU/g protein in AL, CCR, ICR-R and ICR-RF, respectively at 49/50 weeks of age (Figure 3.18.b). CAT activity levels for each dietary groups were 294.7, 216.1, 240.4, 282.2 kU/g protein in AL, CCR, ICR-R and ICR-RF, respectively at 81/82 weeks of age (Figure 3.18.c).

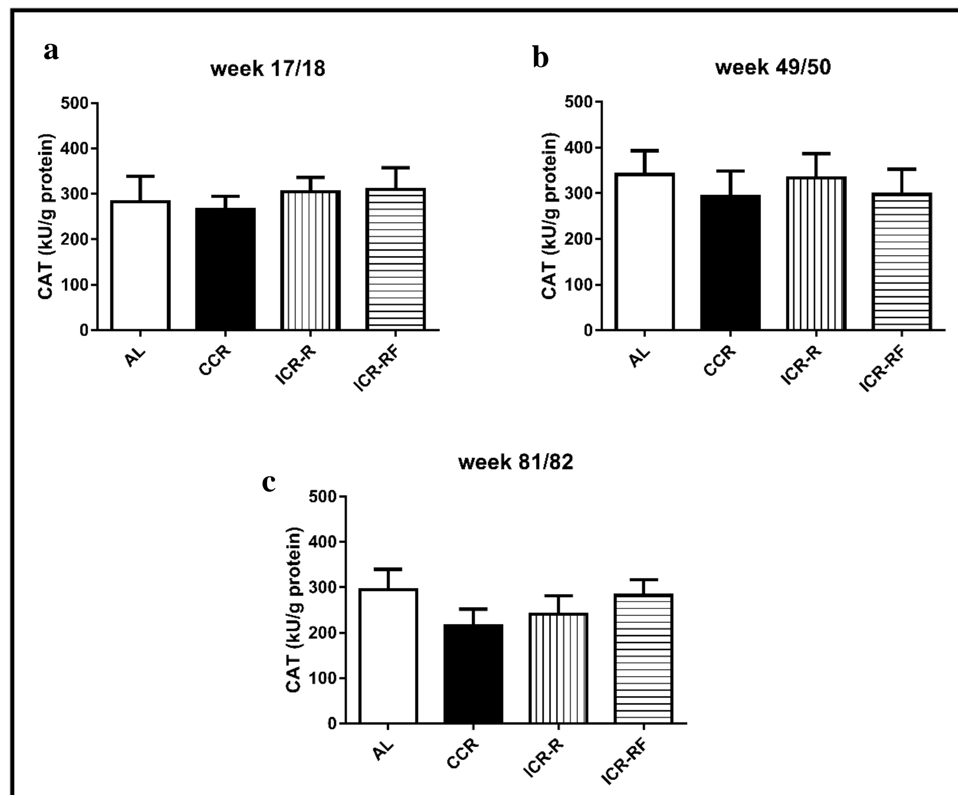


Figure 3.18. Effects of different types of CR on liver CAT activity at 17/18 (a), at 49/50 (b) and at 81/82 (c) weeks of age. * represents significant differences (p <0.05). "n" values = 5-15

3.5.4. Effects of Aging on Liver CAT Activity

CAT activity was decreased by aging in all diet groups. However, significant decrease in CAT activity due to aging was observed only in CCR group ($p<0.05$, Figure 3.19.b). Liver CAT activity was decreased by approximately 2 fold in later ages compared to baseline in all groups (Figure 3.19).

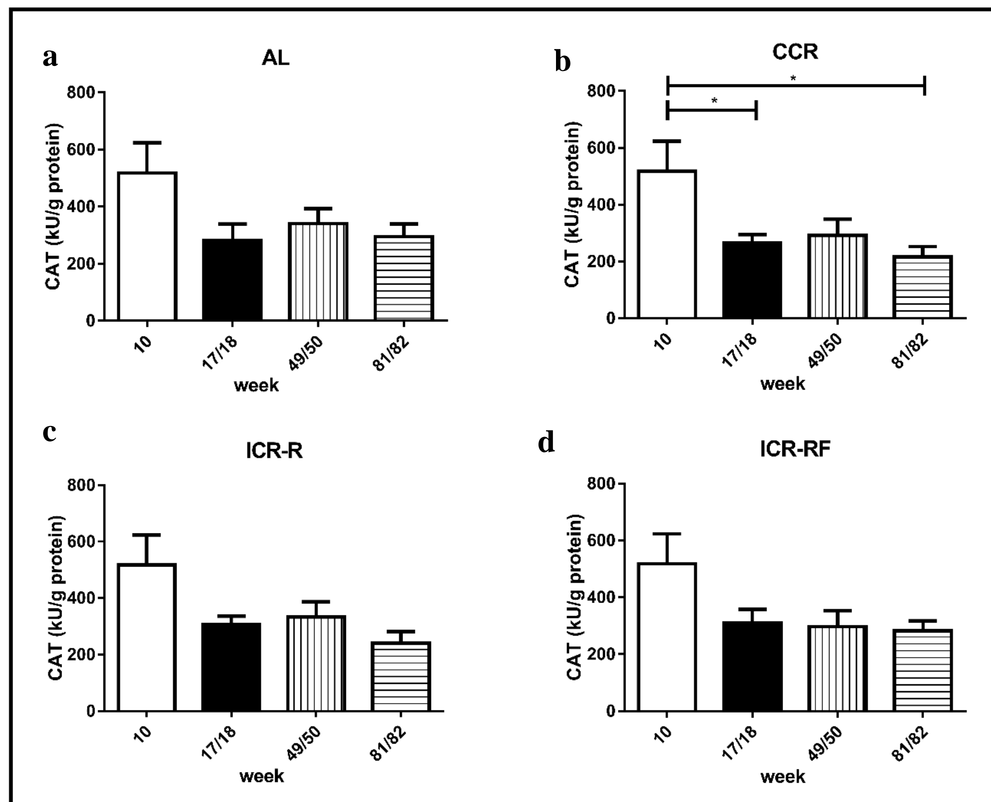


Figure 3.19. Effects of aging on liver CAT activity in AL (a), CCR (b), ICR-R (c) and ICR-F (d) groups. * represents significant differences ($p <0.05$). “n” values = 5-15.

3.5.5. Effects of Different Types of Calorie Restriction on Liver SOD Activity

Liver SOD activity level was not affected by different types of CR at any designated time points ($p>0.05$, Figure 3.20). Liver SOD activity for each dietary groups were 3.03, 2.22, 2.72 and 0.89 U/mg protein in AL, CCR, ICR-R and ICR-RF, respectively at 17/18 weeks

of ages (p>0.05, Figure 3.20.a). Although it was not significant, SOD activity was 3.4, 2.5 and 3.1 fold higher in AL, CCR and ICR-R group compared to ICR-RF group (p>0.05) at 17/18 weeks of age (Figure 3.20.a). Liver SOD activity for each dietary groups were 1.6, 1.59, 2.85 and 2.33 U/mg protein in AL, CCR, ICR-R and ICR-RF, respectively at week 49/50 (Figure 3.20.b). Liver SOD activity for each dietary groups were 2.9, 3.6, 2.7 and 2.1 U/mg protein in AL, CCR, ICR-R and ICR-RF, respectively at week 81/82 (Figure 3.20.c).

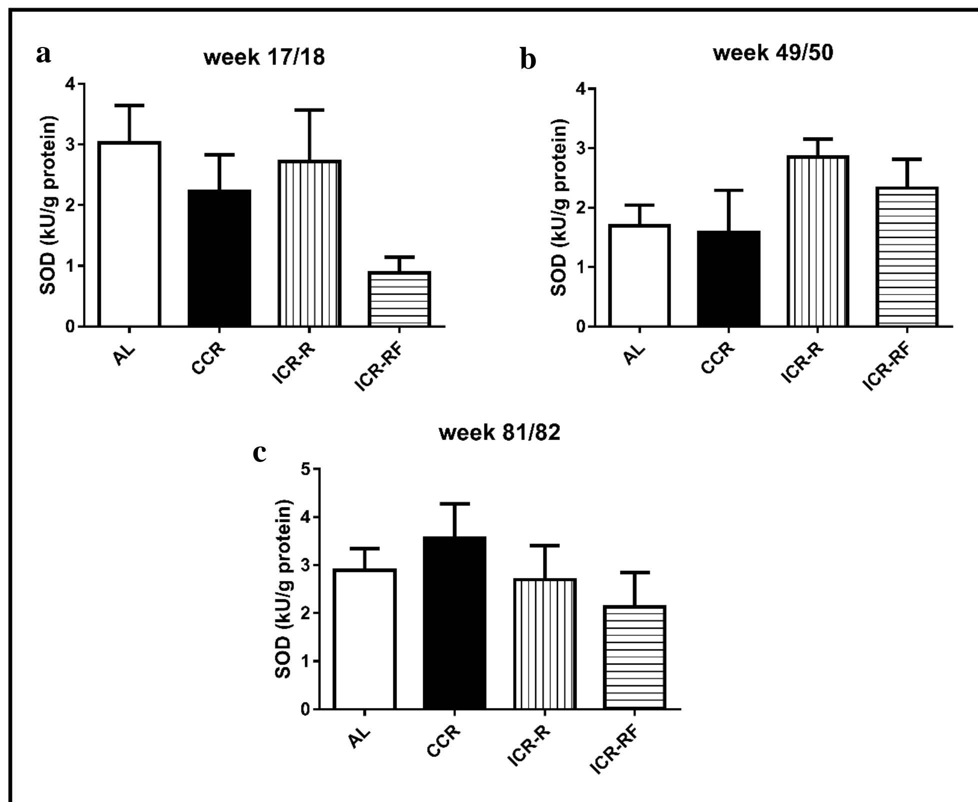


Figure 3.20. Effects of different types of CR on liver SOD activity at 17/18 (a), at 49/50 (b) and at 81/82 (c) weeks of age. * represents significant differences (p <0.05). “n” values = 4-13.

3.5.6. Effects of Aging on Liver SOD Activity

Liver SOD activity was decreased by aging in all of the diet groups (Figure 3.21). SOD activity was decreased by 48, 71 and 51 per cent in 17/18, 49/50 and 81/82 weeks of ages

compared to baseline in AL group ($p<0.05$, Figure 3.21.a). Liver SOD activity was decreased by 62 and 73 per cent in CCR group at 17/18 and 49/50 weeks of ages compared to baseline ($p<0.05$, Figure 3.21.b). SOD activity was decreased by 54, 52 and 54 per cent in 17/18, 49/50 and 81/82 weeks of ages compared to baseline in ICR-R group ($p<0.05$, Figure 3.21.c). SOD activity was decreased by 85, 61 and 64 per cent in 17/18, 49/50 and 81/82 weeks of ages compared to baseline in ICR-RF group ($p<0.05$, Figure 3.21.d).

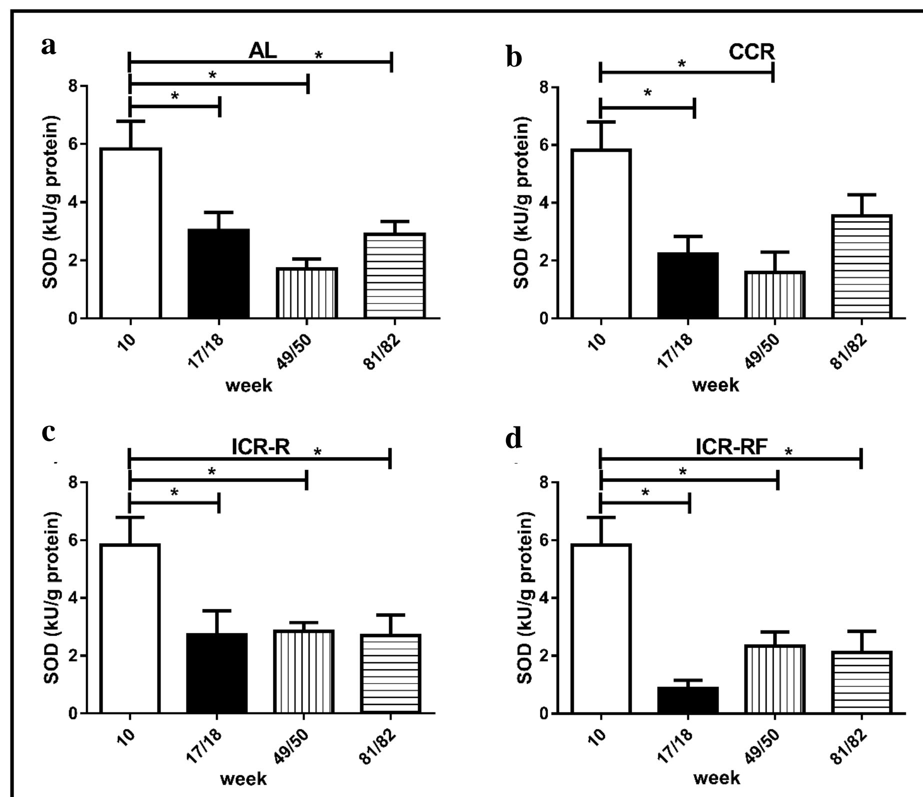


Figure 3.21. Effects of aging on liver SOD activity in AL (a), CCR (b), ICR-R (c) and ICR-F (d) groups. * represents significant differences among the groups ($p <0.05$). “n” values = 4-13.

3.5.7. Effects of Different Types of Calorie Restriction on Liver GSH Level

Liver GSH level for each dietary groups were 4.59, 2.41, 4.08, 9.69 µmol/ml in AL, CCR, ICR-R and ICR-RF groups, respectively at 17/18 weeks of ages. ICR-RF group had

significantly 75 and 58 per cent higher liver GSH level compared to CCR and ICR-R groups at 17/18 weeks of ages ($p<0.05$, Figure 3.22.a). Liver GSH level for each dietary groups were 3.14, 7.72, 2.26, 4.95 µmol/ml in AL, CCR, ICR-R and ICR-RF groups, respectively at 49/50 weeks of ages. CCR group had significantly 60 and 71 per cent higher liver GSH level compared to AL and ICR-R groups at 49/50 weeks of ages ($p<0.05$, Figure 3.22.b). Liver GSH level for each dietary groups were 2.75, 2.21, 1.86, 3.92 µmol/ml in AL, CCR, ICR-R and ICR-RF groups, respectively at 81/82 weeks of ages. GSH level was not changed by different types of dietary groups at 81/82 weeks of age (Figure 3.22.c).

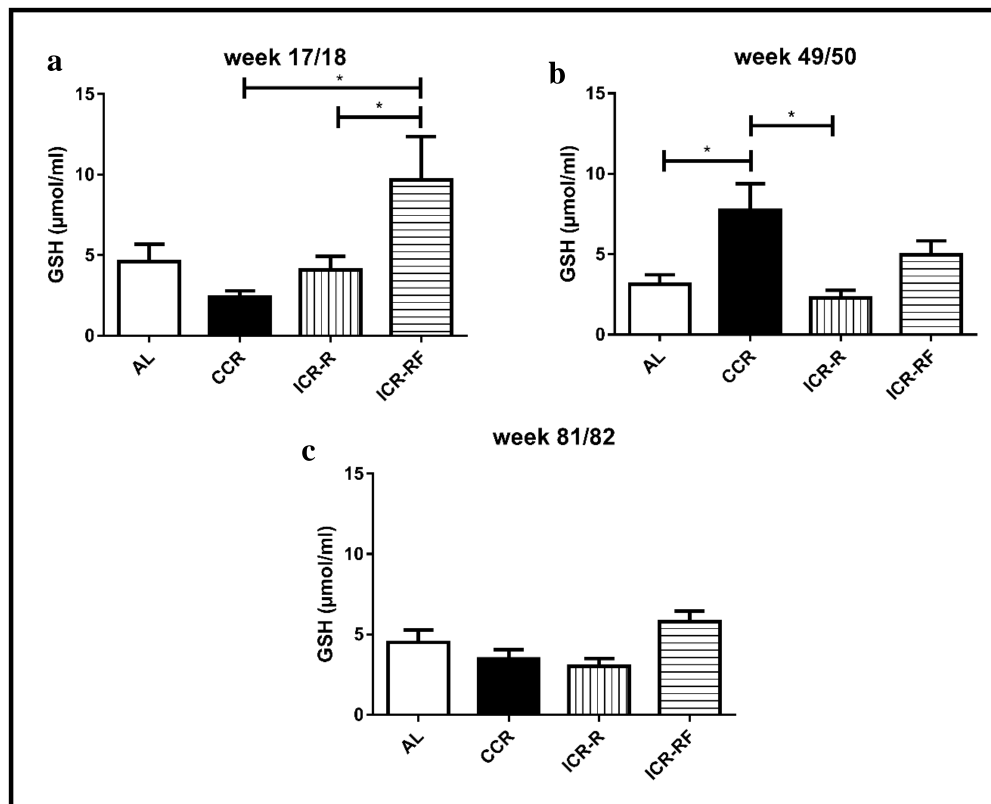


Figure 3.22. Effects of different types of CR on liver GSH level at 17/18 (a), at 49/50 (b) and at 81/82 (c) weeks of age. * represents significant differences ($p <0.05$).
"n" values = 5-12.

3.5.8. Effects of Aging on Liver GSH Level

Liver GSH level was not changed by aging in AL and ICR-R groups (p>0.05, Figure 3.23.a and c). However, liver GSH level was 69 and 56 per cent higher compared to 17/18 and 81/82 weeks of ages (p<0.05) in CCR group (Figure 3.23.b). Although, liver GSH level was approximately two fold higher at 17/18 weeks compared to baseline and 49/50 weeks of ages in ICR-RF group, this was not significant (Figure 3.23.d).

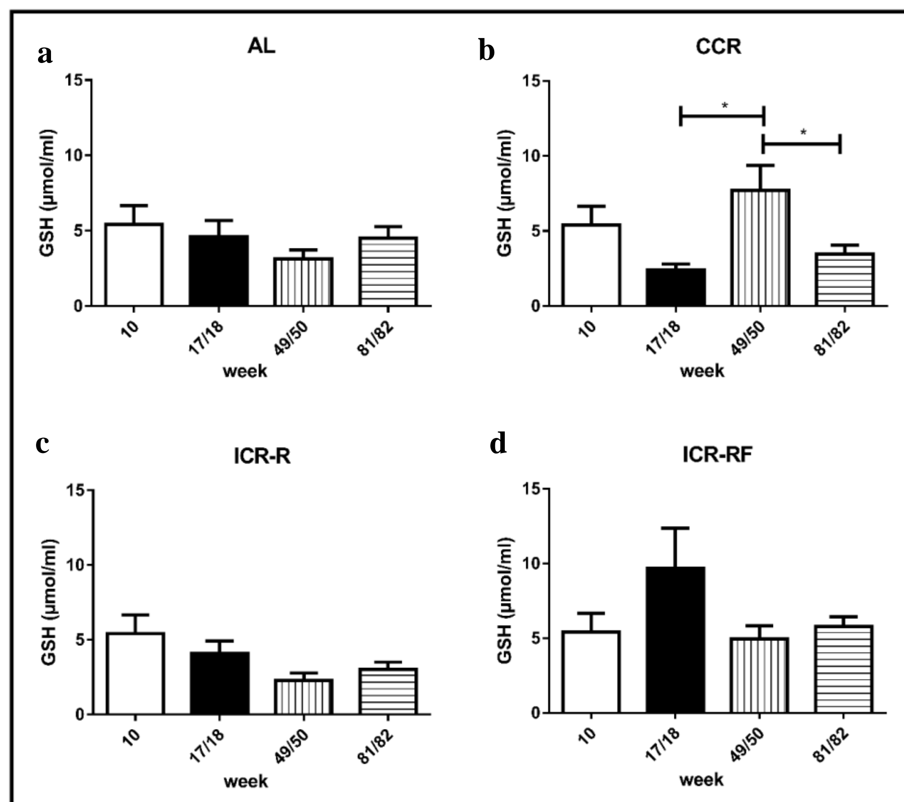


Figure 3.23. Effects of aging on liver GSH level in AL (a), CCR (b), ICR-R (c) and ICR-F (d) groups. * represents significant differences (p <0.05). “n” values = 5-12.

3.5.9. Effects of Different Types of Calorie Restriction on Liver GSH-Px Activity

Liver GSH-Px activity level was not affected by different types of calorie restriction groups (Figure 3.24). Liver GSH-Px activity level for each dietary group were 1776, 1737,1507,

1346 U/g protein in AL, CCR, ICR-R and ICR-RF groups, respectively at 17/18 weeks of ages (Figure 3.24.a). Liver GSH-Px activity level for each dietary group were 1545, 1287, 1251, 2229 U/g protein in AL, CCR, ICR-R and ICR-RF groups, respectively at 49/50 weeks of ages (Figure 3.24.b). Liver GSH-Px activity level for each dietary group were 1426, 1403, 1266, 1527 U/g protein in AL, CCR, ICR-R and ICR-RF groups, respectively at 49/50 weeks of ages (Figure 3.24.c).

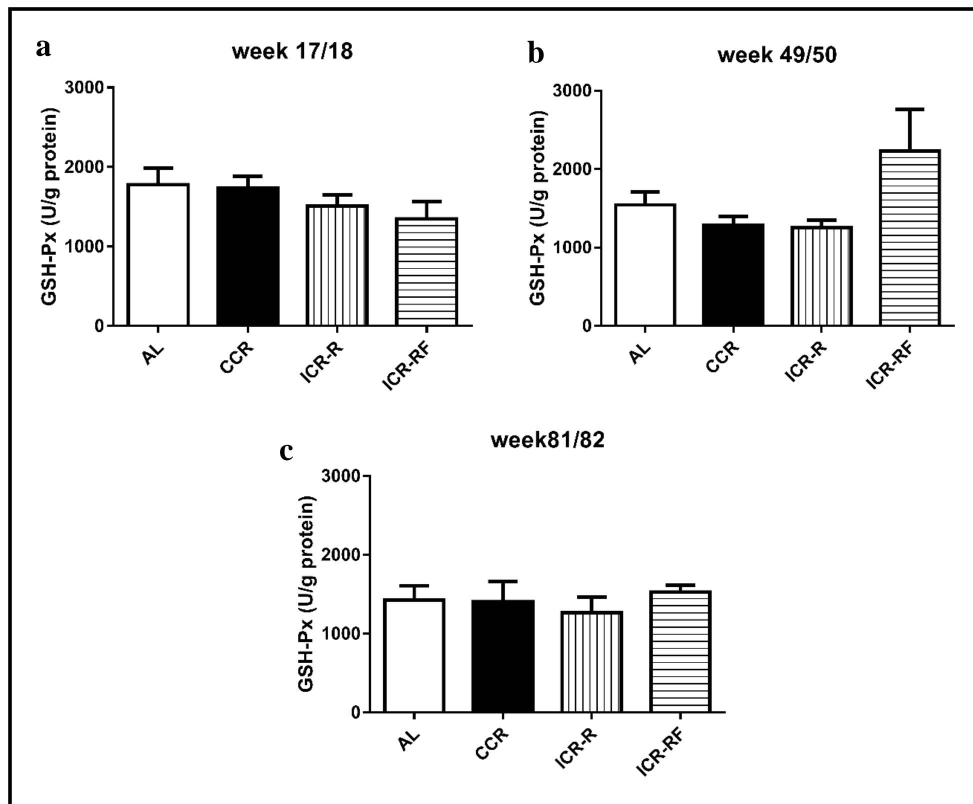


Figure 3.24. Effects of different types of CR on liver GSH-Px activity at 17/18 (a), at 49/50 (b) and at 81/82 (c) weeks of age. * represents significant differences ($p < 0.05$). "n" values = 2-16

3.5.10. Effects of Aging on Liver GSH-Px Activity

There was significant effect of aging on liver GHS-Px activity in any of the diet groups (Figure 3.25).

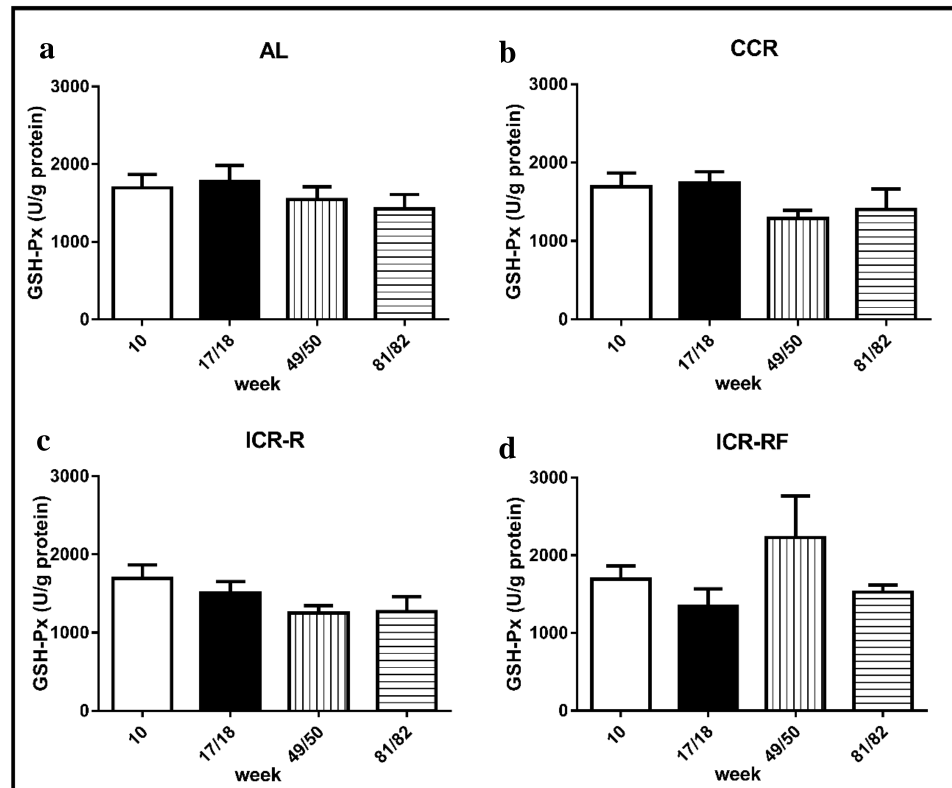


Figure 3.25. Effects of aging on liver GSH-Px activity in AL (a), CCR (b), ICR-R (c) and ICR-F (d) groups. * represents significant differences (p <0.05). "n" values = 2-16.

3.5.11. Effects of Different Types of Calorie Restriction on Liver 8-OHdG Level

Liver 8-OHDg level was not affected by any types of dietary groups in the current study (Figure 3.26).

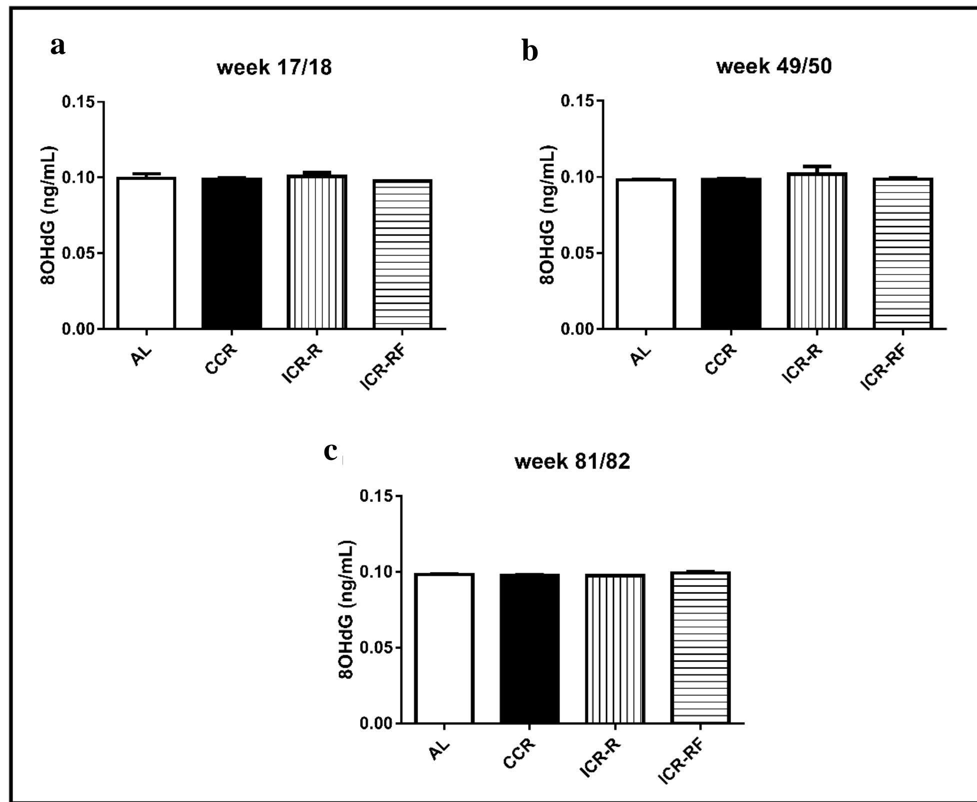


Figure 3.26. Effects of different types of CR on liver 8-OHdG level at 17/18 (a), at 49/50 (b) and at 81/82 (c) weeks of age. * represents significant differences ($p < 0.05$). "n" values = 3-4

3.5.12. Correlation Between Oxidative Stress Parameters in Erythrocytes Samples

Correlation analyses were performed with oxidative stress parameters from erythrocytes showed a positive correlation between SOD and CAT (r=0.265, p=0.04), MDA and GSH-Px (r=0.29, p=0.017) at 17/18 weeks of ages. A negative correlation between MDA and GSH (r=-0.308, p=0.02) and positive correlation between SOD and CAT (r=0.41, p=0.002), SOD and GSH (r=0.284, p=0.046) were found at 49/50 weeks of ages. A positive correlation between SOD and CAT (r=0.418, p=0.012), SOD and GSH-Px (r=0.354, p=0.031) were found at 81/82 weeks of ages (Table 3.1).

Table 3.1. Correlation between oxidative stress parameters in erythrocytes at 17/18, 49/50 and 81/82 weeks of age. All data from different calorie restricted groups were pooled together for each specified time points. * represents significant differences between the groups ($p < 0.05$).

Parameters	week 17/18		week 49/50		week 81/82	
	r	p value	r	p value	r	p value
MDA vs SOD	0.258	0.052	-0.019	0.888	0.336	0.045*
MDA vs CAT	0.022	0.861	0.080	0.540	0.090	0.612
MDA vs GSH	-0.020	0.885	-0.308	0.020*	-0.055	0.781
SOD vs CAT	0.265	0.040*	0.410	0.002*	0.418	0.012*
SOD vs GSH	0.003	0.984	0.284	0.046*	0.249	0.177
CAT vs GSH	0.140	0.284	0.252	0.066	0.292	0.131
MDA vs GSH-Px	0.290	0.017*	0.220	0.107	0.230	0.164
SOD vs GSH-Px	0.145	0.241	-0.116	0.408	0.354	0.031*
CAT vs GSH-Px	-0.047	0.698	0.062	0.658	0.021	0.896
GSH vs GSH-Px	0.031	0.810	0.012	0.934	0.154	0.363

Linear regression graphics for SOD and CAT activity level correlation at 17/18, 49/50 and 81/82 weeks of age are given as a representative of correlation analysis (Figure 3.27).

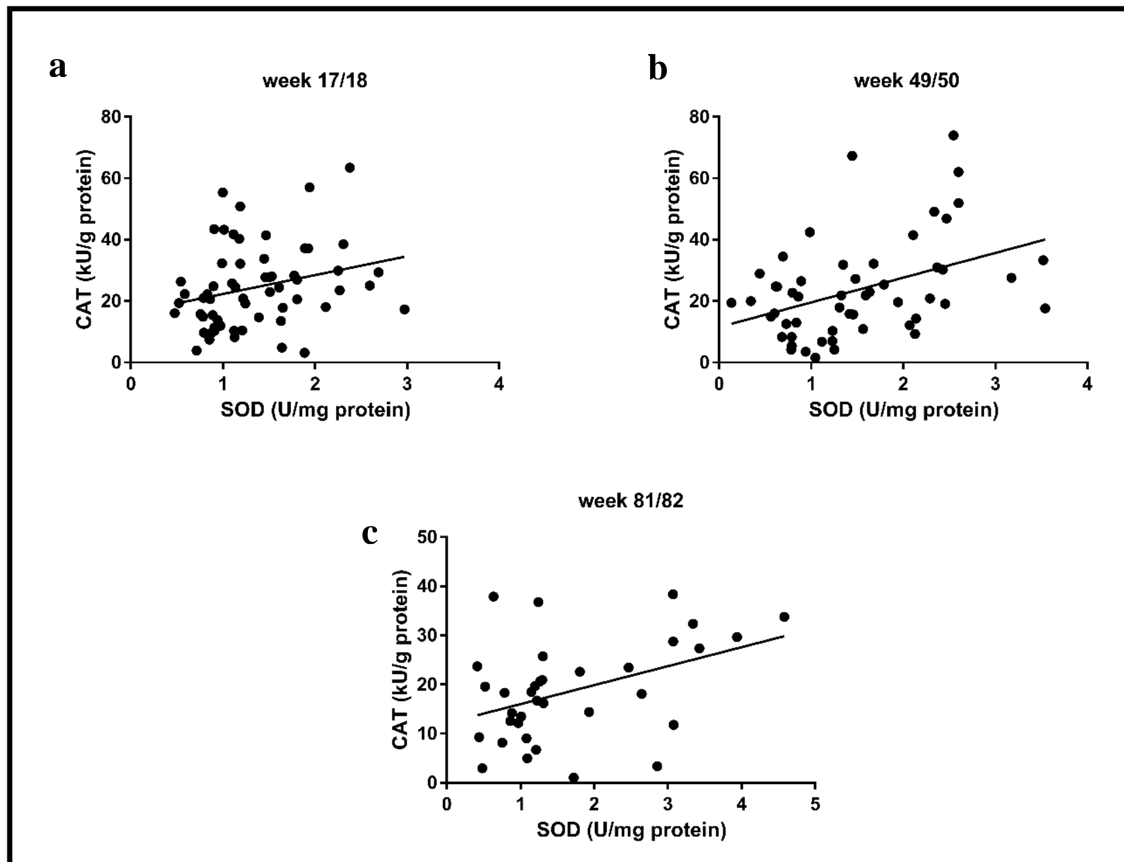


Figure 3.27. Correlation figures between SOD and CAT activity at 17/18 (a), 49/50 (b) and 81/82 (c) weeks of ages.

3.5.13. Correlation Between Oxidative Stress Parameters in Liver Samples

Correlation analyses were performed with oxidative stress parameters in liver samples showed a positive correlation between MDA and CAT (r=0.413, p=0.002), MDA and GSH (r=0.338, p=0.019), SOD and CAT (r=0.35, p=0.046), CAT and GSH (r= 0454, p=0.002) at 17/18 weeks of ages. activity. There was no significant correlation between any oxidative stress parameter at 49/50 weeks of ages. A positive correlation was found between MDA and GSH-Px (r=0.397, p=0.024) at 81/82 weeks of ages (Table 3.2).

Table 3.2. Correlation between oxidative stress parameters in liver at 17/18, 49/50 and 81/82 weeks of age. All data from different calorie restricted groups were pooled together for each specified time points. * represents significant differences between the groups ($p < 0.05$).

Parameters	week 17/18		week 49/50		week 81/82	
	r	p value	r	p value	r	p value
MDA vs SOD	-0.103	0.545	0.076	0.667	0.172	0.310
MDA vs CAT	0.413	0.002*	0.246	0.143	0.078	0.656
MDA vs GSH	0.338	0.019*	0.004	0.978	-0.037	0.826
SOD vs CAT	0.350	0.046	0.300	0.120	-0.061	0.746
SOD vs GSH	-0.114	0.540	0.120	0.522	-0.0008	0.997
CAT vs GSH	0.454	0.002	-0.070	0.690	0.311	0.079
MDA vs GSH-Px	0.256	0.089	-0.060	0.790	0.397	0.024*
SOD vs GSH-Px	0.013	0.939	-0.237	0.300	0.224	0.242
CAT vs GSH-Px	-0.013	0.932	-0.182	0.470	0.217	0.320
GSH vs GSH-Px	-0.170	0.320	-0.0003	0.998	-0.139	0.518

4. DISCUSSION

Dietary intake is one of the main health-span regulators that may affect the quality and duration of life in living organisms. Diet has been associated with several pathological conditions including cardiovascular diseases, diabetes, cancer, inflammation and neurodegeneration. Therefore, dietary interventions are very important in the disease processes [55]. CR is one of the most applied interventions for reducing body weight and preventing against cancer development in rodent models [34]. Several potential factors have been suggested to be responsible for anticancer effects of calorie restriction such as energy-balance-related hormones, growth factors, insulin, IGF, leptin, adiponectin, steroid hormones, inflammation and sirtuins [58]. However, the exact mechanism(s) how CR performs its preventive effects remain to be elucidated [34, 57]. Understanding the molecular mechanism of calorie restriction is crucial for modifying the disease processes. Moreover, possible targets may be identified by revealing the molecular mechanisms in order to simulate the effects of CR for prevention and treatment of several diseases including cancer. Generally, two main types of CR protocols are applied in experiments: Chronic CR (CCR) and Intermittent CR (ICR). Food intake is restricted on a daily basis compared to age-matched AL fed groups in CCR protocols while food intake is applied by either alternate-day fasting or restriction of food intake for certain time periods for example two weeks AL feeding and two weeks of 50 per cent CR, three weeks of AL feeding and one week of 60 per cent CR. The main aim of the ICR protocol is applying same amount of restriction with CCR protocol in a different design. Alternative protocols for CCR kind of protocols such as ICR is necessary for finding more applicable protocols for human. Moreover, ICR has been reported to be more effective to reduce tumor occurrence and also significantly delay the latency of tumor progression compared to CCR. Particularly in the studies performed with MMTV-TGF-α mice, the animals fed AL had around 50-80 per cent MT occurrence rate and the MT occurrence rate was approximately 27-44 per cent in CCR mice with 75 per cent intake of the calories of AL mice. In spite of high occurrence rate of MT development in AL and CCR types of diet, MT occurrence rate was only between three and 3-15 per cent in ICR mice [16, 18].

Oxidative stress is pointed to be related with aging, cancer [192], neurodegeneration [193], cardiovascular disease [194], and diabetes [145, 195]. It is crucial for living organisms to

balance the level of ROS to utilize the beneficial roles of ROS. Several studies have suggested that calorie restriction may be protective against oxidative stress related diseases including cancer [196], neurodegeneration [197] and cardiovascular disease [198]. Moreover, effects of CCR on oxidative parameters were examined in several studies while effects of ICR on oxidative has not been studied extensively. In addition, there is no specific study for long term period to show the comparison of the action of CCR and ICR on oxidative stress. Therefore, the aim of the present study was to understand the role of oxidative stress in the preventive effects of different types of CR against breast cancer in transgenic mouse model, MMTV-TGF-α.

In this study, 10 weeks old MMTV-TGF-α mice were enrolled in different dietary groups: Ad-libitum (AL), Chronic Calorie Restriction (CCR) and Intermittent Calorie Restriction. CCR group was provided with 85 per cent of age-matched AL group's food consumption while ICR group was provided three weeks of AL feeding following with 40 per cent of age-matched AL group's food consumption. In order to profile the oxidative damage and antioxidant capacity, several oxidative stress parameters were examined in erythrocytes and liver samples of calorie restricted mice at designated time points of experiment. In the present study, sample collection was done in designated time points, 10, 17 and 18, 49 and 50, 81 and 82 weeks of ages. These time points are representing time points before the MT detection and after MT occurred. MDA level was evaluated in order to show the lipid peroxidation level in erythrocytes and liver samples. 8-OHdG level was evaluated in order to show the DNA damage level in liver samples. CAT, SOD, GSH and GSH-Px levels were determined in order to show the antioxidant capacity in erythrocytes and liver samples.

Calorie restriction is known to decrease body weight [58]. In this study, CCR group was found to decrease body weight compare to AL group while body weight was not changed between AL and ICR groups. Moreover, ICR group had around 20 per cent decrease in body weight during restriction weeks. However, previous studies using the same type of mice, MMTV-TGF-α and similar study design have reported reducing effects of ICR on body weight compared to AL group [16, 22]. One of the reasons for different outcomes could be due to the difference in the amount of restriction in dietary groups. 25 per cent CR for CCR and three weeks of AL feeding followed by three weeks of 50 per cent of CR for ICR were applied in previous studies while 15 per cent of CR for CCR and three weeks of AL feeding followed by one week of 60 per cent CR were applied in present study [16, 18]. Another

reason could be due to the difference in diet type used in studies. AIN-93M was used in previous studies while Altromin TPF1414 was used in the present study.

CR is suggested to be protective against cancer development. In the current study, 8 per cent of animals in CCR group developed mammary tumor while 21.4 per cent of animals in AL group developed mammary tumor. Moreover, 20.4 per cent of animals of ICR group developed mammary tumor. However, previous studies performed with MMTV-TGF-α mice have reported greater degree of protection in ICR groups against tumor development compared to CCR groups [17, 37, 199, 200]. For instance, ICR protocol with 3 weeks of 50 per cent of CR followed by 3 weeks of AL feeding have been reported to be more protective compared to CCR protocol with 25 per cent of CR in MMTV-TGF-α mice [36]. The reason for the contradictory outcomes of the current study compared to previous research could be due to the difference in the amount and duration of restriction in dietary groups. 25 per cent CR for CCR and three weeks of AL feeding followed by three weeks of 50 per cent of CR for ICR were applied in previous studies while 15 per cent of CR for CCR and three weeks of AL feeding followed by one week of 60 per cent CR were applied in the present study. Moreover, in the current study, ICR group started to develop MT's at 20 weeks of ages in the current study while CCR group started to develop MT's only at 60 weeks of ages. In this context, two possible mechanisms for the effects of ICR protocol are suggested in the current study. Firstly, 60 per cent of CR for one week in every month in ICR group might act as a severe CR. Therefore, ICR protocol might be exceeding the critical amount of restriction to show the hormesis effect of CR and became harmful to organism. Secondly, 60 per cent of CR for one week in every month in ICR group might not be enough compared to three weeks of CR in six weeks in previous studies. Therefore, ICR protocol group might not have any significant effect to protect against MT development and act as AL feeding.

MDA is a secondary by-product of peroxidation as a result of radical attack to lipids and commonly used as a biomarker of lipid peroxidation due to its ability to react with TBA [151]. Several studies have reported elevated levels of MDA in breast cancer conditions [32, 201-203]. Furthermore, MDA has been reported to contribute cancer in association with diet and lifestyle [204]. In this study, the changes in erythrocytes and liver MDA level are reported at different ages of mice which were applied different dietary regimens. Erythrocytes MDA level in AL and ICR group were found to be 15-25 per cent higher compared to CCR group at 17/18 and 49/50 weeks of ages ($p>0.05$). Also, ICR-R group had

around two fold higher erythrocytes MDA level when it is compared with AL and CCR group at the end of study, 81/82 weeks of ages ($p<0.05$). Moreover, ICR groups increased their erythrocytes MDA level due to aging ($p<0.05$). AL group did not have any significant difference due to aging when analyzed by using ANOVA test. However, erythrocytes MDA level was higher at 49/50 weeks of ages compared to baseline in AL group when analyzed by using student t-test ($p<0.05$). CCR group maintained its erythrocytes MDA level stable throughout the experiment. Liver MDA level was affected by any types of CR protocol in the present study. In addition, AL and CCR group did not have any change due to aging in liver MDA level. However, ICR-R and ICR-RF group had increased liver MDA level due to aging. Taken together, ICR protocol where three weeks of AL feeding and one week of 60 per cent CR were applied to mice caused an increase in MDA level in the current study. There are several studies which support the current study's findings. For instance, Stankovic et al (2013) has reported higher level of liver MDA level when male rats received 50 per cent and less of recommended daily caloric intake. Mladenovic et al (2013) has also reported that 50-60 per cent CR group had around five-fold higher liver MDA level compared to control group in male rats [183]. These data are supportive for the increased MDA level in the current study for the ICR group where one week of 60 per cent CR was applied to mice every month. However, the data in the current study is not significant. In addition, Stankovic et al (2013) have also reported that liver MDA was not changed in 10-20 per cent CR group compared to control group in male rats [38]. This results are similar to the current study's findings in CCR group where 15 per cent of CR was applied to mice throughout the study. However, there are also several studies that shows the reducing effects of CR on MDA levels. For instance, Zanetti et al (2010) has reported increased level of plasma MDA level in 24 months old rats compared to six months old rats and reduced level of MDA after three weeks of CR (26 per cent compared to control) [181]. Likewise, Park et al (2012) has reported reducing effects of 40 per cent CR on liver MDA level in rats [182]. Hagopian et al (2013) have reported reduced mitochondrial TBARS level in 6 months old male mice which were applied to 40 per cent CR for two months [179]. However, there are also studies showing no effects of CR on MDA level. For instance, Ling and Bistrian (2009) did not find any alteration in hepatic MDA level where they studied the effects 25 and 50 per cent of CR for 14 days in male rats [205]. Effects of intermittent fasting on oxidative stress is less studied. In addition, there is no study to use an experimental design similar to current study. For instance, Chausse et al (2015) have reported decrease MDA level in only heart tissue

compared to AL feeding while there was no significant effect in brain, muscle and liver tissue when eight weeks old male rats were provided with alternate day fasting for one month [206]. Hu et al (2018) have also reported the reducing effects of IF on MDA level in hippocampal of 18 weeks old male rats provided with alternate day fasting for seven weeks [177]. To study the effects of IF dietary regimen in humans, oxidative stress parameters were examined in human who does Ramadan fasting. For instance, Al-Shafei (2014) have reported significantly 54.3 per cent decrease in serum MDA levels in humans who performed Ramadan for one month. Furthermore, serum MDA level was found to be remained significantly lower by 25.7 per cent at six weeks after fasting [178]. On the other hand, BaHammam et al (2016) have also reported no effects of Ramadan fasting on MDA level in 8 male volunteers [43]. In summary, CCR group in the current study was more robust against increasing lipid peroxidation level that comes with aging process compared to AL and ICR groups.

CAT is one of the most vital enzymes in the antioxidant system. Therefore, the effects of CCR and ICR protocols on erythrocytes and liver CAT activity were examined at different ages of mice. Erythrocytes CAT activity in CCR group was found to be 10-50 per cent higher compared to AL, ICR-R and ICR-RF groups at 17/18, 49/50 and 81/82 weeks of ages in the current study. In addition, CAT activity level was found to increase due to aging in all of the dietary groups. CAT activity was increased due to aging at 17/18 and 49/50 weeks of ages by approximately four fold compared to baseline ($p<0.05$). However, CAT activity was decreased in AL group at 81/82 weeks of ages. Similarly, ICR-RF group was increased its CAT activity at 17/18 and 49/50 weeks of ages by approximately three fold and ICR-R group increased its CAT activity at only 17/18 weeks of ages by approximately three fold compared to baseline and decreased afterwards. For CCR group, CAT activity was increased at 17/18, 49/50 and 81/82 weeks of ages by approximately four fold when it is compared to 10 weeks of ages ($p<0.05$). In addition, there was no significant change by different types of calorie restriction on liver CAT activity at any time points. In contrast to erythrocytes data, CAT activity was decreased due to aging in all of the dietary groups. However, this difference was significant in only CCR group when analyzed by using one-way ANOVA. In details, CCR group had approximately two-fold decrease at 17/18 and 81/82 weeks of ages compared to baseline ($p<0.05$). Taken together, erythrocytes and liver CAT activity was not significantly affected by any type of dietary regimen in the current study. Moreover, erythrocytes CAT

activity was found to be increased due to aging while liver CAT activity was negatively affected due to aging. There are several studies that support the current study's findings. For instance, Schloesser et al (2015) have reported no significant effect of six months 30 per cent CR application on liver catalase activity in six to eight weeks old male C57BL/6J mice [186]. Another study which is performed by Dutra et al (2012) have reported that 30 per cent CR had no effect on CAT activity in heart while CAT activity was increased by 33 per cent in kidney in the same study [188]. However, there are other studies that show different findings of CR on CAT activity. For instance, Doguc et al (2013) have reported increased erythrocytes CAT activity in mice applied to 60 per cent CR for 10 weeks [176]. In contrast, Mitchell et al (2015) have reported lower level of CAT activity in liver of 40 per cent calorie restricted mice comparing to AL mice [39]. Another study which is performed by Guo et al (2001) have reported reduced aortic CAT activity after 40 per cent CR application at 26 months old mice compared to 26 months old mice fed AL [185]. Since the effects of IF on oxidative stress is less studied, there are not much studies to show the effects of IF on CAT activity. Moreover, there is no study to compare the influence of normal CR and IF on CAT activity in one study. For instance, Chausse et al (2015) have reported decrease CAT activity in brain and no change in heart, muscle and liver tissue in eight weeks old male rats after one month of alternate fasting [206]. In addition, Descamps et al (2005) have reported no difference on CAT activity in spleen and liver of female mice after IF application for 4 months while CAT activity in brain was increased by 19 per cent in IF group compared to AL [187]. These results are similar to present study's results where ICR groups decreased catalase activity. In summary, effects of different types of CR on CAT activity was not significant although erythrocytes CAT activity was higher in erythrocytes. On the other hand, CCR group had significantly increased CAT activity until the end of the study due to aging while AL and ICR groups were found to decrease their CAT activity after 49/50 weeks of ages in the current study.

SOD is a primary antioxidant that catalyze the dismutation of O_2^- radicals to O_2 and H_2O_2 which provides cellular defense against ROS. In the current study, the changes in erythrocytes and liver SOD activity level are determined at different ages of mice which were applied different dietary regimens. Erythrocytes SOD activity was not changed by different types of CR at 17/18 and 81/82 weeks of ages. Moreover, CCR group had significantly 58.8 and 37.7 per cent higher erythrocytes SOD activity compared to ICR-R

and ICR-RF groups at 49/50 weeks of age ($p<0.05$). CCR group did not have any significant change due to aging in erythrocytes SOD however AL, ICR-R and ICR-RF had decreasing level of erythrocytes SOD activity due to aging. In addition, liver SOD activity was not affected by any types of dietary regimens in present study. Moreover, liver SOD activity was decreased due to aging in all dietary groups. In details, liver SOD activity was decreased in AL, ICR-R and ICR-RF groups by approximately three fold due to aging at 17/18, 49/50 and 81/82 weeks of ages compared to baseline while CCR group had decreased liver SOD activity at only 17/18 and 49/50 weeks of ages compared to baseline ($p<0.05$). There are several studies that support the current study's findings. For instance, Kabora et al (2015) have reported that SOD activity was not affected in mouse heart after 40 per cent CR application for two weeks [184]. Another study which is performed by Stankovic et al (2013) have reported supporting data for the current study's ICR group. Stankovic et al (2013) have reported the reducing effects of 50-60 per cent CR application for five weeks in rat liver [38]. In this study, SOD activity was also decreased in ICR groups compared to CCR group at 49/50 weeks of ages. Moreover, Stankovic et al (2013) have also reported the non-significant effects of 10-20 per cent CR application for five weeks in rat liver which can be equivalent to CCR group in the current study [38]. Contrary to these results, another study performed by Mitchell et al (2015) have reported decreased liver SOD activity in mice applied to 20 and 40 per cent CR protocol for three months [39]. However, there also other studies to show that CR application might increase the SOD activity. For instance, Zanetti et al (2010) have reported increased SOD activity in 26 per cent CR application in rats for three weeks compared to AL feeding at 24 months old [181]. Moreover, Zanetti et al (2010) have reported decreased level of SOD activity at 24 months old compared to six months old rats [181]. In addition to these, there are also other studies where no difference was found between CR and control groups. Since the effects of IF on oxidative stress is less studied, there are not much studies to show the effects of IF on CAT activity. Moreover, there is no study to compare the action of CCR and IF in the same study. Descamps et al (2005) have reported that IF application for four months significantly increase SOD activity by 27 per cent in brain compared to AL feeding while SOD activity in liver was decreased by 29 per cent in IF group compared to AL [187]. Taken together, SOD activity was found to be reduced due to aging and CCR group was more prone to stable its SOD activity with aging. SOD and CAT enzymes are vital antioxidants in the defense system against oxidative stress. In accordance with current study's findings, a positive correlation between erythrocytes SOD

and CAT activity were observed at 17/18, 49/50 and 81/82 weeks of ages ($p<0.05$). It can be concluded that CAT and SOD enzymes were more altered by CCR application.

GSH is one of the most vital antioxidants that is generated in the body. In this study, the changes in erythrocytes and liver GSH level are determined at different ages of mice which were applied different dietary regimens. Erythrocytes GSH level did not differ among different dietary groups at 17/18 and 81/82 weeks of ages. However, CCR group had significantly higher erythrocytes GSH level compared to ICR-R group at 49/50 weeks of age. Moreover, AL group had increased erythrocytes GSH level at 17/18 weeks of ages compared to baseline. Then this increase was replaced with a decrease at 49/50 weeks of ages. However, CCR group had increased GSH level until 49/50 weeks of ages compared to baseline. ICR-R group had higher GSH level at 81/82 weeks of age compared to earlier time points while ICR-RF group did not affected by aging at any time points. Liver GSH level was higher in ICR-RF group at 17/18 weeks of ages compared to CCR and ICR-R group. However, CCR group had higher liver GSH level compared to AL and ICR-R group at 49/50 weeks of ages. Liver GSH level was not changed by aging in AL, ICR-R and ICR-RF group. CCR group had higher liver GSH level at 49/50 weeks of ages compared to 17/18 weeks of ages however this increase was replaced with a decrease at 81/82 weeks of ages compared to 49/50 weeks of ages. There are several studies which shows the influence of CR on GSH level. For instance, Doguc et al (2013) have reported no significant effects of 60 per cent CR application for 10 weeks on erythrocytes GSH level in rats [176]. Stankovic et al (2013) have also reported that there was no significant effect of 10-20 per cent and 50-60 per cent CR application for five weeks on liver GSH [38]. However, they also have reported that 30-40 per cent CR application increased liver GSH level in rats compared to AL fed rats [38]. In contrast, Laganiere and Yu (1989) reported increase in hepatic GSH concentration in rats fed with 60 per cent of the mean calorie intake of ad libitum fed group for 24 months. In addition to contradictory results regarding the effects of CR on GSH level, effects of intermittent fasting on oxidative stress is less studied. Moreover, there is no study to use an experimental design similar to current study. In this context, Hu et al (2018) have reported the increasing roles of IF on GSH level in hippocampal of 18 weeks old male rats [177]. Chausse et al (2015) have reported increased GSH level only liver tissue in eight weeks old male rats applied alternate day fasting for one month compared to AL feeding while there was no significant effect in brain, muscle and heart tissue [206]. In contrast to these two

studies, ICR group in the current study did not represent an increasing factor for GSH level. Only ICR-R group had increased erythrocytes GSH level at 81/82 weeks of ages compared to earlier time points. However, this situation was not preserved for the ICR-RF group. Therefore, the increase in ICR-R group might be due to lower sample size and higher standard deviation at 81/82 weeks of ages. In addition, similar to other studies ICR-RF group had higher liver GSH level compared to CCR and ICR-R groups at 17/18 weeks of ages where seven to eight weeks of CR protocol was applied. However, ICR-RF group decreased its GSH level at later ages. These differences might be due to the variety of the protocol that is used by researches. In contrary to present study, alternate-day fasting was applied in other studies.

In this study, the influence of different dietary regimens was determined in erythrocytes and liver GSH-Px activity levels. Erythrocytes GSH-Px activity was not affected with the application of any types of CR at 17/18 and 81/82 weeks of ages. However, AL group had approximately 30 per cent higher erythrocytes GSH-Px activity level at 49/50 weeks of ages ($p<0.05$). Erythrocytes GSH-Px activity level was not influenced by aging in AL, CCR and ICR-RF group while ICR-R group had significantly increased erythrocytes GSH-Px activity level at 81/82 weeks of ages compared to earlier time points ($p<0.05$). In addition, different types of calorie restriction groups did not affect liver GSH-Px activity level. Likewise, liver GSH-Px activity level was not affected by aging. Similar to the current study's findings, Descamps et al (2005) have reported that liver GSH-Px activity was not affected by IF application for four months [187]. In contrast, another study which is performed by Guo et al (2001) have reported the reducing effects of 40 per cent CR application on aortic GSH-Px activity at 26 months old mice comparing to 26 months old mice fed AL. Moreover, Guo et al (2001) have also reported the increased GSH-Px activity in 26 months of aged mice fed AL compared to six months of aged mice fed AL [185]. Similar to the current study's findings, Kabora et al (2015) have indicated no significant effect of 40 per cent CR on GSH-Px activity in mouse heart after two weeks of application [184]. Effects of IF on oxidative stress is less studied. In addition, there is no study to use an experimental design similar to current study. Chausse et al (2015) have reported that GSH-Px activity level was not affected by IF application for one month in heart, brain and liver tissue of eight weeks old male rats [206].

Correlation analyses were carried out in order to better understand the relationship between oxidative stress parameters in erythrocytes and liver samples. There was a significantly positive correlation between SOD and CAT activities in erythrocytes samples at 17/18, 49/50 and 81/82 weeks of ages ($p<0.05$). The positive correlation supports that SOD and CAT enzymes work synergistically. In this context, Mitchell et al (2015) have also reported positive correlation between antioxidant levels [39] while another study suggested that antioxidants might work compensatory [207]. However, the positive correlation in erythrocytes samples was not detected in liver samples. Liver samples in the current study was not affected from different types of calorie restriction regimens. Different results from erythrocytes and liver samples in present study support the diversity of oxidative stress parameters depending on the tissue examined.

In the current study, CCR and ICR groups had different patterns on oxidative stress parameters. CCR group was more likely to be protective against oxidative stress compared to ICR groups. These results for oxidative stress were in accordance with the MT incidence and body weight change throughout the experiment where higher MT incidence and increase body weight were determined in ICR groups. Therefore, higher degree of restriction in ICR group resulted in higher oxidative stress level and lower antioxidant potential. In addition, higher oxidative stress level might be associated with higher MT incidence observed in ICR group as well [25]. Moreover, in a meta-analysis study which is performed by Walsh et al (2014), it has been reported that most of the research studying the oxidative stress parameters likely to have none significant effects of CR [42]. Furthermore, influence of CR on oxidative stress parameters are known to be complex and dependent on several varieties such as gender, species, tissue examined and duration of CR. Non-significant results in the current study might rely on these facts.

5. CONCLUSION AND FUTURE PERSPECTIVES

In present study, the direct comparison of oxidative stress parameters between ICR and CCR groups in a long-term period is reported for the first time. Therefore, this study is promising in order to elucidate the mechanism of which CR shows its protective effects through oxidative stress. In summary, oxidative stress can be modulated by both CCR and ICR protocols. Moreover, the present study indicates that the amount of CR and duration of application is a crucial factor in the preventive actions of CR against breast cancer development. Since there is a striking parallelism between oxidative stress parameters and MT occurrence, oxidative stress might be the possible mechanism for the connection between CR and breast cancer development. In addition, CCR protocol is reported to be more protective against breast cancer development and oxidative stress in the present study.

To summarize the action of ICR protocol, Figure 5.1 illustrates the decreased antioxidant capacity and increased lipid peroxidation level which might result in DNA and lipid damage to contribute breast cancer development.

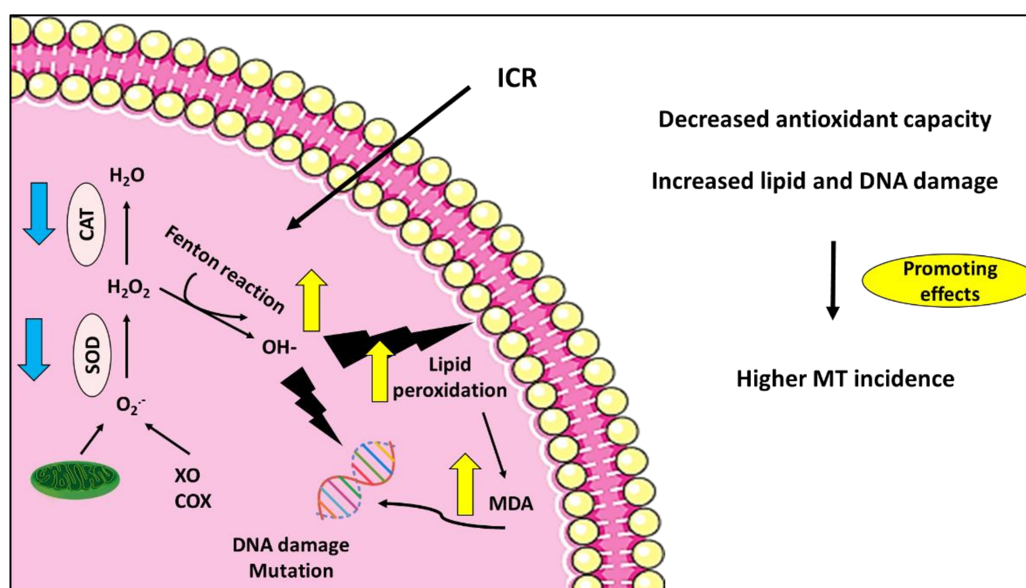


Figure 5.1. The summary mechanism of effects of ICR protocol

To summarize the action of CCR protocol, Figure 5.2 illustrates the increased antioxidant capacity and decreased lipid peroxidation level which provide preventive effects against further DNA and lipid damage to protect against breast cancer development.

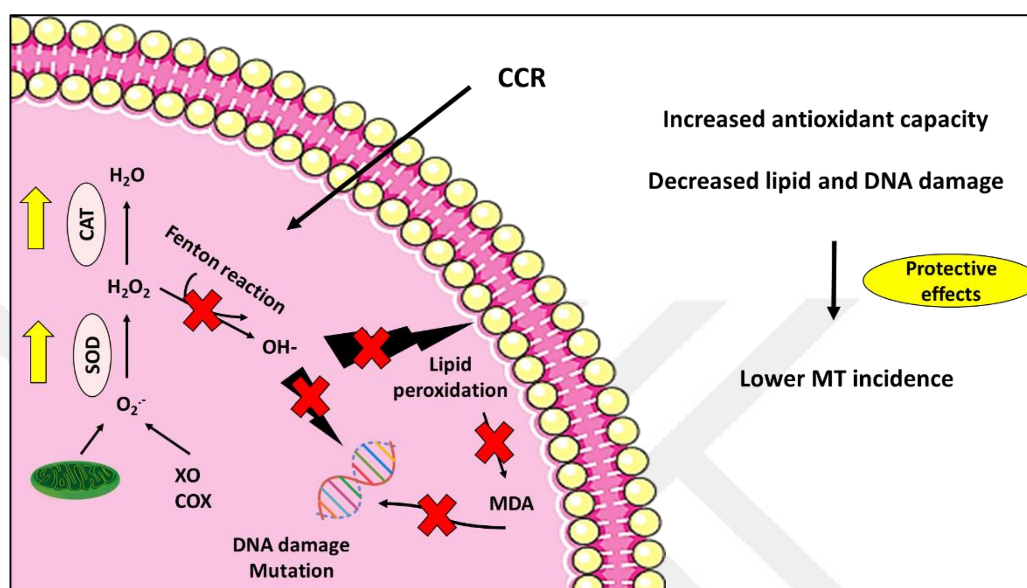


Figure 5.2. The summary mechanism of effects of CCR protocol

CR is one of the most applied and impressive methods for prolonging lifespan and protecting against cancer development [208]. Understanding the underlying factors of the protective roles of CR against cancer is crucial in order to develop more efficient drugs and therapies as well as prevention strategies. Therefore, further studies are necessary to determine the critical border of CR application amount to be protective rather than being harmful and to further interpret the action of oxidative stress in this manner. Since the current study indicates the different patterns of oxidative stress parameters in tissue specific manner, different tissue types especially mammary fat pad may be used in further studies. Therefore, the status of oxidative stress parameters influenced by CCR and ICR can be better profiled. In addition, other experimental techniques such as gene expression analysis by Real-Time PCR and protein expression analysis by Western Blotting may be used to validate the results obtained from spectrophotometric analysis.

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