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YEDİTEPE UNIVERSITY
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The Investigation of High-Fat Diet Exposure and Effects in Colon During Maternal and Maturation Periods in Sprague Dawley Rats

MASTER THESIS

Meyli Ezgi KARAGÖZ

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APPROVAL

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

Prof. Dr. Bayram YILMAZ
Director of Institute of Health Sciences

DECLARATION

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

Date: 29.07.2021
Signature:
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LIST OF SYMBOLS AND ABBREVIATIONS

ACF	Aberrant Crypt Foci
APC	Adenomatous Polyposis Coli
ATP	Adenosine Triphosphate
BA	Bile Acid
CA	Cholic Acid
CDCA	Chenodeoxycholic Acid
CRC	Colorectal Cancer
CytC	Cytochrome C
CYP7A1	Cholestrol 7 alpha Hydroxlase
DCA	Deoxycholic Acid
DNA	Deoxyribonucleic Acid
DSB	Double Strand Break
HCA	Heterocyclic Amines
HCD	High Carbohydrate Diet
HFD	High Fat Diet
HR	Homolog Recombination
LCA	Lithocholic Acid
NHEJ	Non-Homolog End Joining
OSI	Oxidative Stress Index
PAH	Polycyclicaromatic Hydrocarbons
ROS	Reactive Oxygen Species
SCFA	Short Chain Fatty Acid
SFA	Saturated Fat Acid
SFD	Standart Fat Diet
TAS	Total Antioxidant Status
TOS	Total Oxidant Status
UC	Ulcerative Colitis
UDCA	Ursodeoxycholic Acid

ABSTRACT

KARAGÖZ, M. E (2021). The Investigation of High-Fat Diet Exposure and Effects in Colon During Maternal and Maturation Periods in Sprague Dawley Rats, Yeditepe University, Institute of Health Science, Department of Physiology, MSc Thesis, İstanbul.

Western-style diet has become an important danger in societies with the developing industrialization and the increase of fast food culture. Consumption of a high-fat diet is effective on the initiation and progression of the carcinogenesis process in the colon, which is one of the most effective organs in the composition of feces, which provides the absorption and release of water and electrolytes during the nutrition and digestion process, contains a large variety of important bacteria and is one of the most effective organs in the composition of feces. For all the type of diet consumed, it has been proven that the nutrition of the individual both in the maturation period and in the maternal period affects the epithelial tissue and microbiota in the colon. As a result of high fat diet, colonic mucosal barrier dysfunction occurs it causes luminal antigens and bacteria to enter the lamina propria, triggering immune cell-mediated mucosal inflammation. Consequently, the production of reactive oxygen species increases in colon epithelial cells. Carcinogenesis occurs as a result of genomic instability in the colon with the effect of genetic and environmental factors. In the literature, there are studies to explain the effects of mutations in the DNA repair mechanism and various signaling pathways on the development of colon carcinogenesis. Gamma-H2AX, a DNA damage marker, and RAD51, a double-stranded DNA repair marker, are prominent markers in determining the process of carcinogenesis. In this study, maternal (21 days of gestation+21 days of lactation) and maturation (100 days) rats were fed a low fiber and high fat western diet. We aimed to show DNA damage and repair with immunofluorescent staining method, morphological changes with hematoxylin-eosin staining and oxidative stress change with a commercial kit, which are the parameters that are indicative of carcinogenesis formation in the colon of exposure to this diet. In our results, no statistically significant results were observed in RAD51 and H2AX expressions in total oxidant and antioxidant stress conditions, while a tendency to the formation of abnormal crypt foci was observed in histological sections taken from the colon tissue of the groups fed with high-fat maturation period. In line with these results, it is predicted that the possible negative

effects of high-fat diet on the colon at different periods of development are observed and may contribute to future studies.
Keywords: High Fat Diet, Maternal/Maturation Period, Gamma-H2AX, RAD51, TAS, TOS.

ÖZET

KARAGÖZ, M.E (2021). Sprague Dawley Sıçanlarda Maternal ve Maturasyon Dönemlerinde Yüksek Yağlı Diyete Maruz Kalmanın Kolona Etkisinin İncelenmesi, Yeditepe Üniversitesi Sağlık Bilimleri Enstitüsü, Fizyoloji ABD. Master Tezi. İstanbul.

Batı tarzı diyet, gelişen sanayileşme ve hazır yemek kültürünün artışıyla birlikte toplumlarda önemli bir tehlike haline gelmiştir. Yüksek miktarda yağ içeren diyetin tüketilmesi, beslenme ve sindirim sürecinde su ve elektrolitlerin emilimi ve salımını sağlayan, çok sayıda önemli bakteri çeşitliliğini barındıran ve dışkının bileşiminde en etkin organlardan biri olan kolonda, karsinogenez sürecinin başlaması ve ilerlemesi üzerinde etkilidir. Tüketilen diyetin türüne göre, bireyin hem maturasyon döneminde hem de maternal dönemindeki beslenmesinin kolondaki epitel dokuyu ve mikrobiotasını etkilediği kanıtlanmıştır. Yağlı beslenme sonucunda kolon mukozal bariyeri disfonksiyonu oluşarak luminal antijenlerin ve bakterilerin lamina propriaya girmesine neden olmaktadır ve immün hücre aracılı mukozal inflamasyon tetiklenmektedir. Sonuçta kolon epitel hücrelerinde reaktif oksijen türlerinin üretimini artmaktadır. Karsinogenez genetik ve çevresel faktörlerin etkisi ile kolonda meydana gelen genomik dayanıksızlık (instabilite) sonucu ortaya çıkmaktadır. Literatürde DNA onarım mekanizmasında meydana gelen mutasyonların ve çeşitli sinyal yolaklarının kolon karsinogenezi gelişimine etkisini açıklamaya yönelik çalışmalar bulunmaktadır. DNA hasar belirteci olan Gamma-H2aX ve çift iplik DNA onarım belirteci olan RAD51 proteinleri, karsiyogenez sürecinin belirlenmeside ön plana çıkan belirteçlerdendir. Bu çalışmada, maternal (21 gün gestasyon+21 gün laktasyon) ve maturasyon (100 gün) dönemindeki sıçanlarda düşük lif ve yüksek yağ içeriğine sahip batı diyeti ile beslendi. Bu diyete maruz kalmanın kolonda karsinogenez oluşumunun belirteci olan paremetrelerden immünfloresan boyama yöntemi ile DNA hasarı ve onarımı, hematoksilen eozin boyama ile morfolojik değişimleri ve ticari bir kit aracılığıyla oksidatif stres değişimini göstermeyi hedefledik. Sonuçlarımızda, toplam oksidan ve antioksidan stres durumlarında, RAD51 ve H2AX ekspresyonlarında istatistiksel olarak anlamlı sonuç gözlenmezken, maturasyon dönemi yüksek yağlı beslenen grupların kolon dokusundan alınan histolojik kesitlerinde anormal kript odaklarının oluşumuna yatkınlık gözlenmiştir. Bu sonuçların doğrultusunda, gelişimin farklı dönemlerdeki yüksek yağlı diyetin kolon

üzerindeki olası negatif etkilerin gözlenlendiği ve gelecek çalışmalar için katkıda bulunabileceği ön görülmektedir.

Anahtar Kelimeler: Yüksek yağlı diyet, maternal/maturasyon dönemi, Gamma-H2AX, RAD51, TAS, TOS.

1. INTRODUCTION and PURPOSE

Western diet style has become an effective danger in societies with the developing industrialization and the increase of fast food culture[1]. In human physiology, it is known that the colon is one of the organs that provide both absorption and release of water and electrolyte in the process of nutrition and digestion, hosting a large number of important bacterial diversity and being the most effective in the composition of the faeces[2]. The consumption of a high-fat diet has been shown to have an effective role in initiating and promoting the carcinogenesis process in the colon[3]. According to the researches, it has been proven that not only the diet in the developmental process but also the nutrition during the maternal period affects the epithelial tissue and microbiota of the individual in the colon, depending on the type of diet consumed[4,5]. It has been reported in many studies that dysfunction of the colon mucosal barrier occurs as a result of fatty nutrition and an increase in barrier permeability[5,6]. Moreover, a high-fat diet causes luminal antigens and bacteria to enter the lamina propria and immune cell-mediated mucosal inflammation is triggered[7]. As a result, it increases the production of Reactive Oxygen Species (ROS) in both colonic epithelial cells and lamina propria cells[8]. It has been emphasized that fatty acids and proinflammatory cytokines cause an increase in oxidative stress in colonic epithelial cells[9]. It has been shown to occur as a result of genomic instability in the colon due to carcinogenesis, genetic and environmental factors[10]. There are studies in the literature to explain the effects of mutations in the DNA repair mechanism and various signal pathways on the development of colon complexity[11]. Chromosomal Instability (CIN), Microsatellite Instability (MSI) and CpG Island Methylator Phenotype (CIMP) are three important mechanisms that are stated to be effective in colon carcinogenesis.[12]. DNA damage marker Gamma-H2AX[13] and double-stranded DNA repair marker RAD51[14,15] proteins are prominent markers in determining the carcinogenesis process. The aim of this study is to express the relationship between the western diet with low fiber and high fat content, the diet type in the maternal and lactation period of the individual, and the possible DNA damage, morphological changes and oxidatic stress that are effective in the formation of carcinogenesis on the colon.

2. GENERAL INFORMATION

2. 1. The Overview to The Colon

2. 1. 1. Anatomy and Histology of Colon

The colon is region of the large intestine. It is located distal to the ileum and proximal to the anus which is avarage 150 cm long. It is one-fifth of the complete length of the intestinal canal. It can be dissected to four section. These are respectively, proximal to distal, ascending, transverse, descending, and sigmoid. On the surface of the intestine are three longitudinal bands of muscle fibers known as Taeniae coli. Each is an average of 0.2 inches wide. The orginate point is the base of the appendix and they expand from the cecum to the rectum. It initiates as the ascending colon, behind to peritoneum form that goes up superiorly from the cecum. When it joins liver's right part, it rotates 90 degrees and moves straightly. This turn is known as right colic flection and marks the transverse colon's initiation point. The transverse colon extends from the right colic bend to the spleen, where it moves another 90 degrees to point down. This turn is named as the left colic twist. Here the colon is attached to the diaphragm via the phrenicocolic ligament. Different from the descending and ascending colon, the transverse colon is within the peritoneal cavity and surrounded by a transverse mesocolon. After the left colic flexion, the colon turns downward into the pelvis and this is identified the descending colon. It is located behind the peritoneal in most particular, but is placed anterior to the left kidney and crosses its lateral border. When the colon begins to revolve medially it becomes the sigmoid colon. The sigmoid colon, (avarage 40 cm long), is laid in the left lower quadrant extending from the left iliac fossa to the S3 vertebral level. This travel gives the sigmoid column its feature "S" shape. The sigmoid colon is attached to the posterior pelvic wall via a mesentery- sigmoidmesocolon. The long dimension of the mesentery allows this section of the colon to be particularly mobile[16].

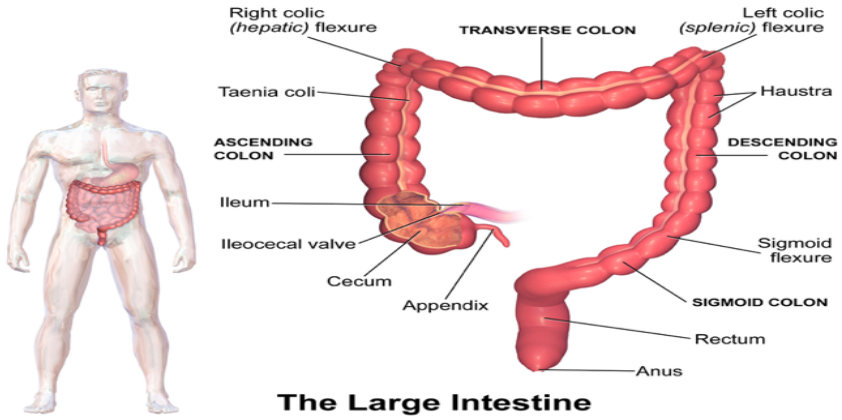

Figure 2.1. Overview to the large intestine and four principal part of colon[17]

There are two spaces called Paracolic gutters in the medial of the descending-ascending colon and the posterolateral abdominal wall. These systems allow material released from infected or inflamed abdominal organs to collect elsewhere in the abdomen. Therefore, it has a critical role in clinical practice. The large intestine is physically separated by being much larger diameter than the small intestine. Three muscle strips known as teniae coli that run longwise the surface of the large intestine each about 1/5 in wide. Omental Appendices are linked to the large intestine exteriority area. They are short sacs of peritoneum, covered with fat. They are called free, omental coli and mesocolic. Teniae coli shorten the intestinal wall to produce pouches known as haustra. These attributes end at the rectosigmoid connection where the smooth muscle of teniae coli expands to form a complete layer in the rectum[18].

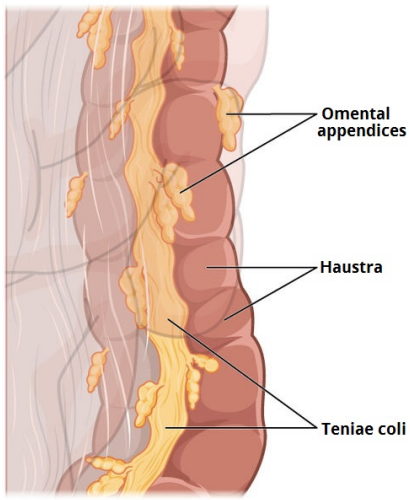

Figure 2.2. The macroscopic characteristic of the large intestine

The neurovascular source of the colon is connected to its embryological origin. Proximal 2/3 of the ascending colon and transverse colon-derived from the midgut. The transverse colon, descending colon and distal 1/3 of the sigmoid colon-derived from the hind gut. Arterial supplies have a general rule, structures derived from the midgut are provided by the superior mesenteric artery and structures derived from the hindgut by the inferior mesenteric artery. The ascending colon originates from two branches of the superior mesenteric artery; ileocolic and right colic arteries. The ileocolic artery leads to colic, anterior cecal and posterior cecal branches-all of which feed the ascending colon. The transverse colon is derived from both the midgut and hindgut and is therefore supplied by the upper mesenteric artery and branches of the lower mesenteric artery: the right colic artery. Middle colic artery. Left colic artery. A single branch of the inferior mesenteric artery supplies the descending colon; left colic artery. The sigmoid colon is supplied arterially through the sigmoid arteries (branches of the inferior mesenteric artery). The venous drainage of the colon is similar to the arterial supply. Transverse colon-middle colic vein flowing into the upper mesenteric vein. Descending colon-left colic vein flowing into the lower mesenteric vein. Sigmoid colon- empties into the inferior mesenteric vein by sigmoid vessels. The upper mesenteric and inferior mesenteric vessels eventually flow into the hepatic portal vein. The superior mesenteric and inferior mesenteric veins eventually flow into the hepatic portal vein. This accomplishes the detoxification of the toxins absorbed from the colon in the liver[16,18].

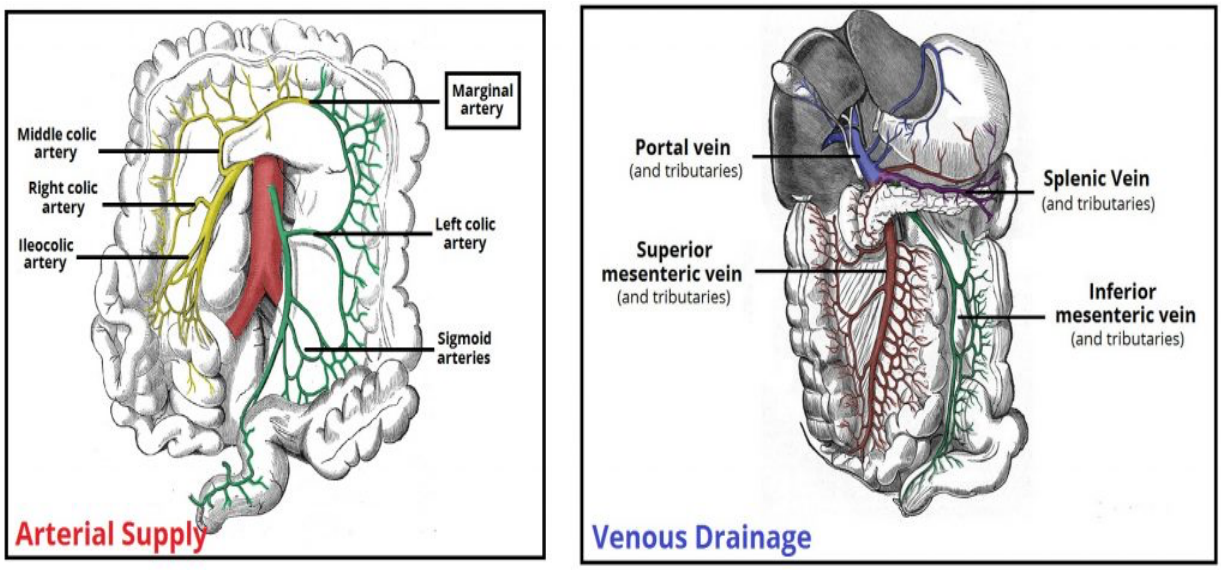

Figure 2.3. The main arteries and veins supplying the colon

The origin of the innervation of the colon is embryological base. The ascending colon, derived from the midgut, and the proximal 2/3 of the transverse colon obtain their parasympathetic, sympathetic, and sensory needs via nerves from the superior mesenteric plexus. The hindgut-derived structures receive their parasympathetic, sympathetic and sensory needs from the inferior mesenteric plexus by way of nerves. Parasympathetic excitation is supplied by the pelvic splanchnic nerves. Sympathetic excitation is supplied by the lumbar splanchnic nerves. The superior mesenteric nodes provide lymphatic drainage of the ascending and transverse colon. The descending colon and sigmoid drain into the lower mesenteric nodes[18].

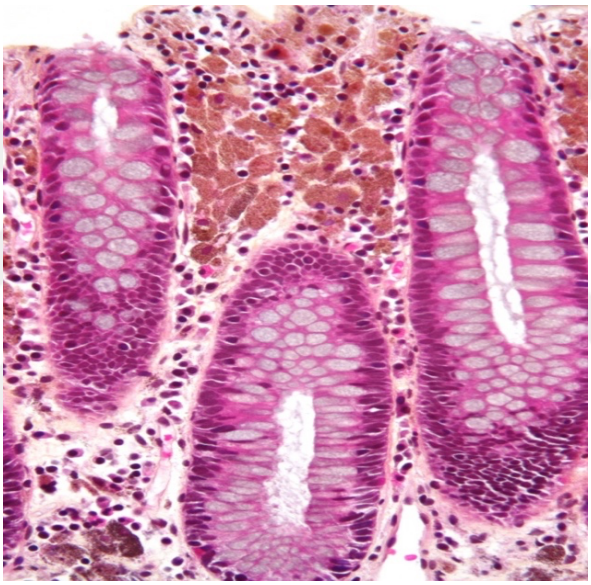

Figure 2.4. Micrograph of a colon biopsy[19].

The colon wall is lined with basic columnar epithelium and it consists of four layers; from outside to inside serosa, muscularis externa, submucosa and mucosa. The luminal aspect of mucosa is orginated mainly attached columnar epithelial cells that are enfolded into finger-like projections assisted by the lamina propria[20]. The apparent crypt-like systems, called Liberkuhn crypts represent the functional unit of the colomn[19]. They provide the maximum absorbent surface area while prolonging the continuous renewal of the barrier-resistant epithelial layer[21]. The normal colon contains millions of crypts, each having average 2000 cells[20]. In general, three main specialized epithelial cell lines include a crypt: primary absorbent cells called colonocytes or columnar cells, hormone-secreting enteroendocrine cells and mucus-secreting goblet cells[21].

There are multipotent stem cells in the basal part of crypt further away from the intestinal lumen. One of the two daughter cells that eventuate with mitosis remains in the crypta as a stem cell, while the other differentiates and migrates to the side of the crypt and eventually to the villi. Among the cells produced in this way are Goblet cells. It is known that many genes are effective in differentiation of intestinal stem cells. The loss of proliferation control in the crypts is thought to lead to colorectal cancer[22].

2. 1. 2. General Functions of The Colon

The competence of the large intestine is to provide absorbing water from remaining indigestible food and then to compact the ineffective waste products prior to defecation. The development of the colon is to fulfill four main functions. (1) Mobilization of colonic components towards the rectum and anus. The term colonic motion is used to represent the mixing and pushing movements of the colon that provide digestion, absorption and passage of intraluminal components. The mechanisms executive for absorption in the colon are gradual, and the colon microbiota is facilitated by the speed and direction of mixing motility. Therefore, the distal pushing of the contents is slow to allow mixing and homogeneous contact with the colonic mucosa. (2) Absorption of water and electrolytes from intraluminal contents. Absorption of water and electrolytes is one of the main functions of the colon. Approximately 1500 mL of wastewater reaches the ileocecal valve each day, and the healthy colon usually absorbs 90% of the fluid, resulting in 200g of solid stool. (3) absorption of short-chain fatty acids genereted by resident microbiota. The colon has a role in nutrient recovery during the fermentation process. The colon contains about 10^{11}–10^{12} bacteria per gram content and these bacteria are capable of breaking down carbohydrates and proteins into short chain fatty acids (SCFAs)[23]. If needed, this could provide up to 15% of an individual's total energy needs[24]. (4) Defecation. The defecation procces consists four main phases: the basal phase (characterized by a change in colonic motor activity), the pre-descriptive phase (during the gradual rectal distension becoming aware of rectal filling), removal (following a conscious desire to evacuate) and termination (characterized by contraction of the external anal sphincter and closure of the anal canal)[25]. In the 16-hour period, the remaining processes of the large intestine in the digestive system are completed. At this stage of digestion, food does not break down. Bacterial Flora is the largest bacterial

ecosystem in the human body, with more than 700 bacterial species found in the large intestine and playing various important roles. One of these tasks of the colon is to absorb the vitamins created by colonic bacteria-vitamin K, vitamin B12, thiamine and riboflavin. It also compresses stool and stores fecal matter in the rectum until excretion.

The large intestine absorbs some of the outcomes created by the bacteria living in this area, for instance, undigested polysaccharides (fiber) are metabolized by the bacteria in the large intestine into short-chain fatty acids and then absorbed by passive diffusion. As a result of the formation of these fatty acids, the acidity increases and the bicarbonate secreted from the large intestine enables the acidity to be neutralized. Other bacterial products of undigested polysaccharide fermentation include gas, which is mainly nitrogen and carbon dioxide with small amounts of hydrogen, methane and hydrogen sulfide. These are produced as a result of bacterial fermentation of undigested polysaccharides. It is also requirement for the development of certain tissues, including normal flora, cecum and lymphatics. In the digestive processes of the colon, a number of microorganisms known as intestinal flora help digest the remaining nutrients and create vitamins. The gut flora has an significant role in the fermentation of unused energy substrates, maintaining the development of the immune system, preventing the growth of pathogenic bacteria, regulating intestinal development, producing vitamins for the host, and producing hormones to direct fat to storage. Intestinal flora consists of microorganisms that live in the digestive systems of animals. The human body consists of about 10 trillion cells, and the intestines contain about ten times as many microorganisms. There are 300 to 1000 varied species living in the gut, and ninety-nine percent of the bacteria probably come from about 30 or 40 species. This intestinal flora is estimated to have about a hundred times more genes than in the human genome. Bacteria make up most of the flora in the colon and about 60% of dry stool weight. Without intestinal flora, the human body cannot use some of the undigested carbohydrates it consumes. Some types of gut flora have enzymes that are missing in human cells to break down certain polysaccharides. Certain starches, fiber, oligosaccharides and sugars like lactose (in the case of lactose intolerance) and sugar alcohols, mucus produced by the gut, various proteins need bacterial assistance for digestion. The large intestine absorbs water from the chyme and stores stool until it is excreted. Food products that cannot pass through villi, such as cellulose (dietary fiber), are mixed with other waste products in the body to become hard and concentrated stools.

Stool is stored in the rectum for a certain period of time, and then the stored stool is excreted from the body due to the contraction and relaxation of the anus. The anal sphincter modulates the output of this waste material.

The defecation process is the whole of the voluntary and involuntary processes that provide the necessary force for the excretion of wastes from the body. The rectal ampulla is where the unnecessary digestive components are stored. As a result of the increase in the fecal component in the rectum, the receptors on the rectal walls that trigger the contraction of the rectal muscles from the nervous system are stretched. The internal anal sphincter relaxes, while the skeletal muscle of the external sphincter contracts.

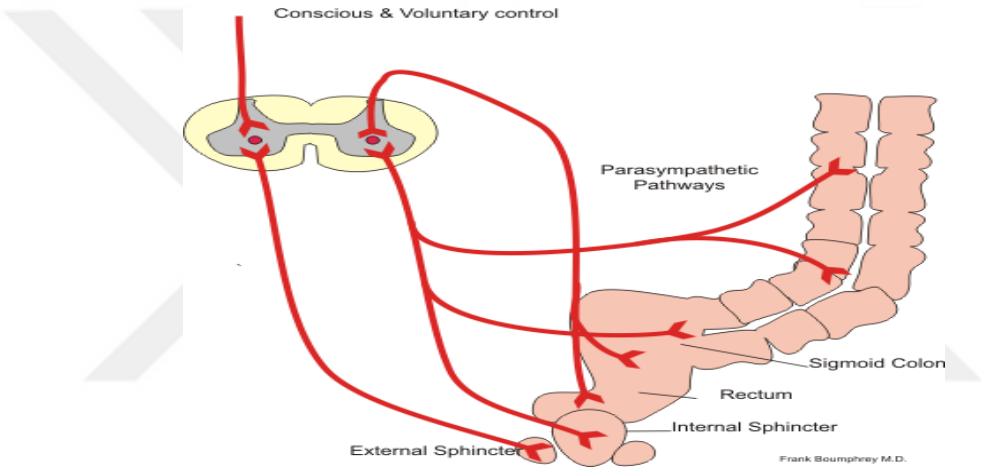

Figure 2.5. The defecation reflex.

In consequence of the relaxation of the internal anal sphincter, a stimulus is sent to the brain that gives the urge to defecate. With prolonged defecation delay, the stool component may solidify, resulting in constipation. As a consequence of the voluntary defecation signal, the anorectal angle decreases and the external anal sphincter relaxes. Peristaltic movements occur in the rectum, shrinking and shortening. The fecal component is forced out of the rectum and down the anal canal.[18,25].

2. 2. The Overview to Relationship Between Diet and Colon

The current research in many areas focus on the impact and effects of dietary habits on human health. It may affect the host directly or lead to change in the microbial community. Food is a complicated structure of thousands of bioactive molecules, many

of which are modified by conservation, cooking technics, digestion, metabolism by the host and the luminal intestine microflora[26].Even though verification suggests that an over abundance nutrients such as fat and protein might be inflammatory and therefore might potentiate neoplastic modulate[27] the interaction between dietary factors and genes may have protective mechanism for mucosal health[26]. Undigested dietary residues are generally complex carbohydrate fibers that enter the colon and are the energy source for the microbiota and mutualism will be achieved with a diet containing sufficient fiber. As a result of the fermentation of dietary fiber, short-chain fatty acids such as butyrate, acetate, propionate which are essential for mucosal health of the colonic lumen, are released and cannot otherwise be obtained from the diet. They are only taken in very small amounts and are absorbed by the small intestine[28]. Dietary fibers increase the formation of SCFAs and provide protective effects on health by selectively affecting a limited number of bacteria (Lactobacillus and Bifidobacteria) in the intestine[29]. A recent meta-analysis including 185 prospective studies and 58 clinical trials provided compelling evidence of an reverse correlation between fiber intake and colon carcinogenes[30]. Focusing on the meta-analysis, the highest fiber consumption was associated with a crucial reduction in the incidence of colorectal cancer (CRC) compared with lowest fiber intake. Since a linear dose-response relationship was defined for dietary fiber and the incidence of CRC, the authors suggested that adults' daily fiber intake should not be less than 25-29 g, and estimated that higher amounts would have a greater protective effect[30]. Also, according to one study, in a simplified microbiota, a low-fiber diet stimulates the activity and growth of mucus-impairing bacteria (eg Akkermansia muciniphila). Infection with a murine pathogen in wild type mice resulted in a penetrable mucus barrier and colitis[31]. Excessive body mass, sedentary behaviors and specially high-fat diets, which are common in the Western lifestyle, have a strong relationship with the prominence of developing colon cancer[1,32].

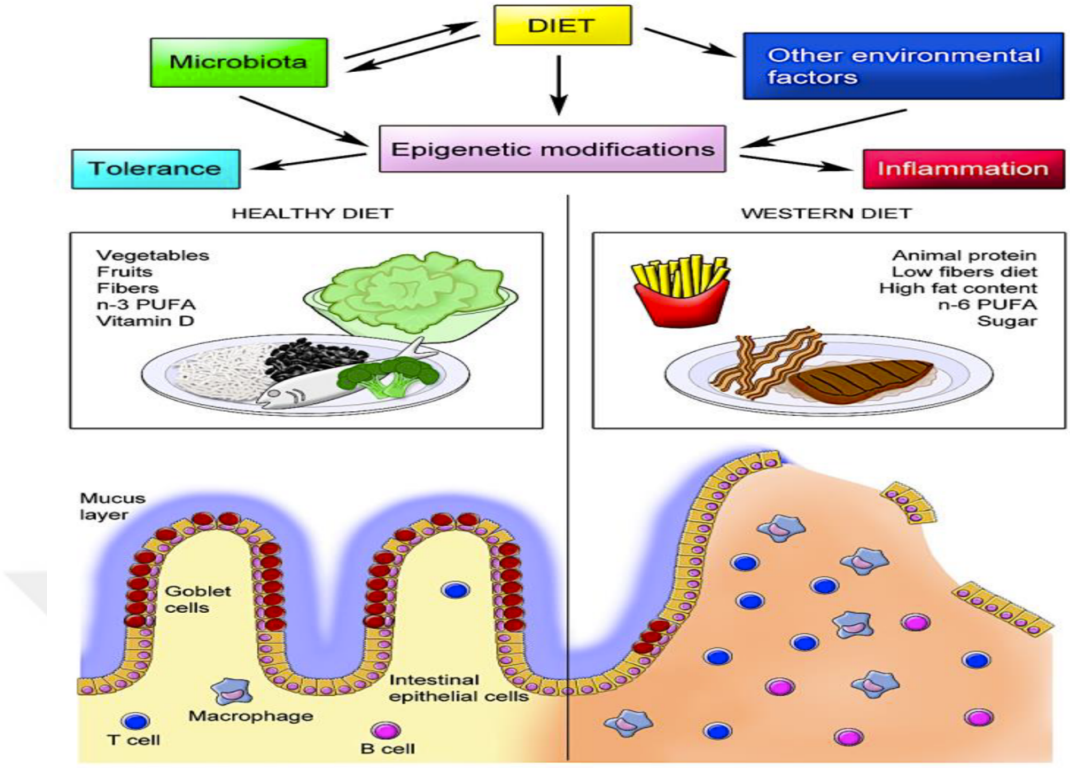

Figure 2.6. Schematic model of diet–host interactions in the intestine[33].

2. 2. 1. Effects of High Fat Diet on Colon (Western Diet)

The Western diet consists of excess energy from fat (35-45% kcal from fat). It is rich in red and processed meats, sugar and saturated, refined starches and trans fatty acids, but also low in vegetables, fruits, fiber, calcium, vitamin D, omega-3 fatty acids and whole grains. This diet is closely related to an increased risk of colon cancer[3].

The ingestion of a Westernized diet that includes extreme amounts of refined and processed foods, red meat and sugary beverages, along with the consumption of low amounts of fiber, fruits and vegetables is associated with increased metabolic diseases such as diabetes and obesity[34] . The total amount of fat taken with the western style diet is high. With the entry of fat molecules into the body, bile acids are released to facilitate the absorption of detergent-like amphipathic molecules, dietary lipps and fat-soluble vitamins. Bile acids (BAs) are synthesized in cholesterol in the liver through several enzymatic reaction chains with the rate limiting enzyme cholesterol 7 alpha hydroxylase (CYP7A1)[35]. As a consequence, it forms primary bile acids, cholic acid (CA) and chenoxycholic acid (CDCA), and are stored in the gallbladder. BAs motion through the intestine and ninety-five percent of them are actively absorbed in the distal ileum and in

the enterohepatic circulation, their return to the liver occurs by way of the portal vein. 5% escapes from the recycling process, reaches the colon and disappears through the faeces[35]. Conjugated bile acids reaching the colon form secondary bile acids, deoxycholic acid (DCA) and lithocholic acid (LCA), and another form, ursodeoxycholic acid (UDCA), as deconjugated by enteric bacteria[36]. Of the basic fecal bile acids, DCA and LCA are the most hydrophobic and consequently the most cytotic. UDCA as the highest hydrophilic BA and LCA as the highest hydrophobic BA. is a distinctive bile acid toxicity rating (BA hydrophobicity scale: LCA >DCA>CDCA>CA>UDCA)[37]. Trigger role of western (high-fat) diet on tumor development has been shown to be associated with increased levels of BA in the colon and faeces in patients diagnosed with CRC and colon cancer-induced rodents. Since the associate between excessive colonic BA levels and increased change of CRC, the consequences of prolonged exposure of the intestinal mucosa to hydrophobic BAs (like LCA and DCA) have been comprehensively studied[38]. Bile acids induce cellular responses during and after colon tumor formation. These responses have been shown to involve, directly or indirectly, b-catenin signaling, deoxyribonucleic acid (DNA) oxidative damage, mitotic activities, p53 degradation and cell death. It causes colonic cell hyperproliferation and aggression[39]. In particular, DCA and LCA can raise tumor formation in the colon with a direct proliferative capacity on undifferentiated mucosal epithelial cells[40]. The hyperproliferative effect of DCA has been demonstrated in various colon cancer cell lines. Moreover, the prolonged persistence of non-lethal BA levels in colonic cells affects apoptosis resistance. This has been suggested to potentially contribute to the progression of colon carcinogenesis[41] . As a result of a high-fat diet, increased levels of DCA and LCA mainly promote induction of mitochondrial oxidative stress, cytochrome C (cytC) release, formation of reactive oxygen species (ROS), and apoptosis through activation of cytotic caspases[42]. In fact, BA levels were compared between ulcerative colitis (UC) patients and correlated colon carcinoma or dysplasia versus UC patients without neoplasia[43]. Higher fecal BA concentrations have been observed in individuals with these diseases. However, it is notable to report that BAs are considered tumor promoters, not mutagens, as they cannot lead to tumor formation in animal models without carcinogenic or genetic modification. In fact, it has been observed in different CRC rodent models that both DCA and LCA represent as tumor promoters in the early post-initial stages of colon carcinogenesis[44]. Another important factor in which

the western diet plays an active role in colon health is the intestinal microbiota and its relationship with BAs. It contains about 10^{12} microbial organisms packed together per gram of lumen that make up its ecosystem in the gastrointestinal tract. These organisms establish a symbiotic relationship with the host[45]. Apart from facilitating the absorption of dietary lipophilic nutrients and their effect on intestinal permeability and motility, BAs also have an interrelated relationship with the host microbiota[46]. While BAs are known to regulate bacterial intestinal growth, the microbiota has been reported to activate signal receptors by metabolizing intestinal BAs into unconjugated and secondary forms[47]. Carbohydrates undergo saccharolytic fermentation by the microbiota. It produces short-chain fatty acids, predominantly butyrate, which have anti-inflammatory and antineoplastic properties[48]. In contrast, higher levels of colonic BA and protein fermentation predominate with an unbalanced Western diet containing high fat, low fiber. As a result, it leads to a proinflammatory[49]. Many aspects that reflect the Western lifestyle, including obesity and reduced physical activity, are known some risks for gastrointestinal cancer[50]. There are important cohort studies showing that the type of diet effectively alters the diversity of gut microbiota[1,51]. Moreover, it is now known for certain that dysbiosis is associated with cancer development[52]. However, the mechanisms by which changes in the microbiota composition caused by a high-fat diet change the intensity of tumorigenesis in the intestine have not been fully elucidated[53]. Various factors undoubtedly contribute to the etiology of colorectal cancer, but there are convincing arguments to especially high-fat diet (HFD) and the combination of gut microbiota as key risk factors and recent evidence for a more efficient nutrient in obese individuals' microbiota[54]. The role of intestinal microbiota in cancer pathogenesis is very important[53]. It has been shown that increased Fusobacterium is associated with colonic tumorigenesis[55]. The diets of rural Africans normally contain high fiber and low fat, in a controlled study, increased F. nucleatum in stool was observed when they consumed a low-fiber, high-fat diet for 2 weeks[1]. Fat modulates the composition of the microbiota in the intestine mainly by stimulating the secretion of bile acids. One of the critical tasks of the microbiome is the conversion of primary bile acids entering the colon into secondary pure acids[56].

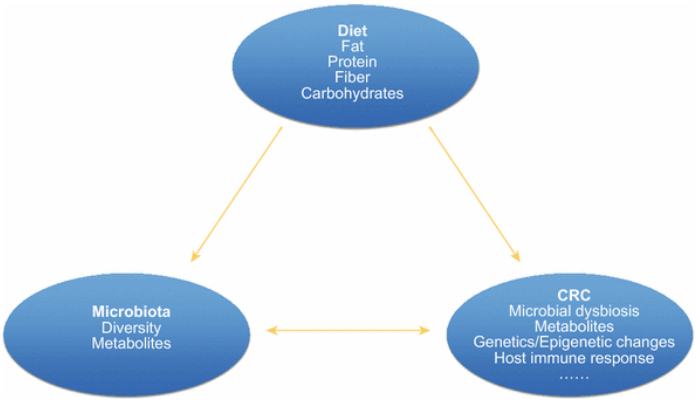

Figure 2.7. The relationship of diet, microbiota and carcinogenesis [29].

2. 2. 2. Maternal Nutrition and Colon

Maternal period means including both gestation and lactation periods. A balanced maternal diet has a great effect on the health and growing of the offspring. As stated in the fetal origin hypothesis, intrauterine feeding has important effects on the long-term health of the offspring[57].Recent clinical and preclinical studies have strongly demonstrated that maternal exposure to a high-fat diet (HFD) or/and high-carbohydrate diet (HCD) can have an irreversible effect on the structure and function of many parts of the offspring's body. In particular, these impacts often indicated in altering gut morphology, microbiota, immunity, and inflammation, etc[4,5]. High fat exposure during the maternal period may cause changes in the physiology of the offspring by causing effects such as increase in body weight, metabolic syndrome, decrease in leptin levels, and insulin resistance[6]. The development of the fetal intestine accelerates at the end of pregnancy, then, the transition from the uterus to the external environment is prepared in the perinatal period. It is known that the primary task of the colon is food digestion and nutrient absorption, also has functions such as barrier and immunity[58]. The microbial composition of the offspring is known to be largely affected by the variety of the maternal diet[59] According to both human and animal research, it was found that the balanced nutrition in early life and the maternal process was benefical in the regulation of microbiotes and the newborn to the gastrointestinal systemt[60]. In contrast, both epidemiological evidence and experimental information show that the exposure to the maternal high fat diet during pregnancy and lactation period, resulting in specific pathogenic bacteria may lead to the increase in the column and the risk of progenia is further increased[61].

2. 3. Colonic Carcinogenesis

The pathogenesis of colorectal carcinoma is heterogeneous process. It include composite multistage molecular pathways originated by epigenetic and genetic occasions[11]. Molecular classification of colorectal carcinoma contributes the main for utilizing prognostic markers. Colorectal carcinogenesis; It is a multi-step process in which a series of genetic and epigenetic changes occur as a result of the activation of oncogenes and loss of tumor repressive genes in the transformation of normal cells into pernicious cells. Genetic and epigenetic changes cause impairment of the signal transduction necessary for cellular metabolism, proliferation, differentiation, life, and apoptosis.

Genomic instability is the basis of the transformation process of a normal cell into a malignant cell. Experimental observations claim that carcinogenesis is expressed in three different phases as initiation, promotion and progression[62]. The initiation step in colonic carcinogenesis is indicated as the process by which it is exposed to environmental carcinogens and consequence in insufficent or no noticeable transform in the cells or the mucosa morphology. However, it causes a constant raise in cancer susceptibility[63]. Tumor elevation appears as a result of damage to the mucosa by inflammation or/and injury, resulting in the development of a non-malignant tumor (adenoma) that can regress without further stimulation [63]. When defining an adenomatous polyp (or adenoma) it is expressed as a well-defined mass of epithelial dysplasia with unrestrained crypt cell proliferation. In the promotion stage of colon carcinogenesis, initial onset lesions, aberrant crypt foci (ACF) on the lumen surface of the colon can be noticed in both rodents and humans[22,64]. ACF can be described as precursors of adenoma and carcinoma in the colon. ACF formation, one of the first steps in colon carcinogenesis is the occurrence of change in the proliferative pattern, as well as imperfection of apoptosis in the epithelial cells of the colon crypts[64].While the transition from the promotion stage to the progression phase, ACF can transform into hyperplastic polyps into mixed polyps, serrated adenomas, or traditional adenomas[65]. Consequently, tumor progression and malignant transformation occur with additional tissue degradation, but the vast generality of cellular improvement in this stage appears to necessitate no external stimulation [66]. Therefore, neoplastic cells are malignant when they pass through the muscularis mucosa and infiltrate the submucosa[66]. It involves the progressive acquisition of activation or loss of function mutations in a number of key oncogene and tumor suppressor genes, with the

progressive transition of dysplastic adenoma in the healthy colonic epithelium to malignant cancer. It includes DNA mismatch repair genes and adenomatous polyposis coli (APC) that are identical to those mutated in hereditary colon cancer [67]. Hepatic bile acid synthesis stimulated by a high-fat diet has an indirect effect in carcinogenesis mechanism in the colon[68]. At the end of this process, large amounts of bile acids escape from the enterohepatic circulation and enter the colon. Where it causes an increase in microorganisms containing the deconjugate enzyme (bile acid 7α dehydratase), which is responsible for the transition of bile acids into secondary bile acids[68]. The first organ to respond to HFD is the colon [69]. As reported prolonged inflammation, stem cell activity, and alteration of gut microbiota, all of which may contribute to colon carcinogenesis initiation[70]. Studies have shown that mice exposed to the western diet for a long time do not develop hyperplasia and single crypt dysplastic lesions, which are indicative of tumorigenesis[44,71].

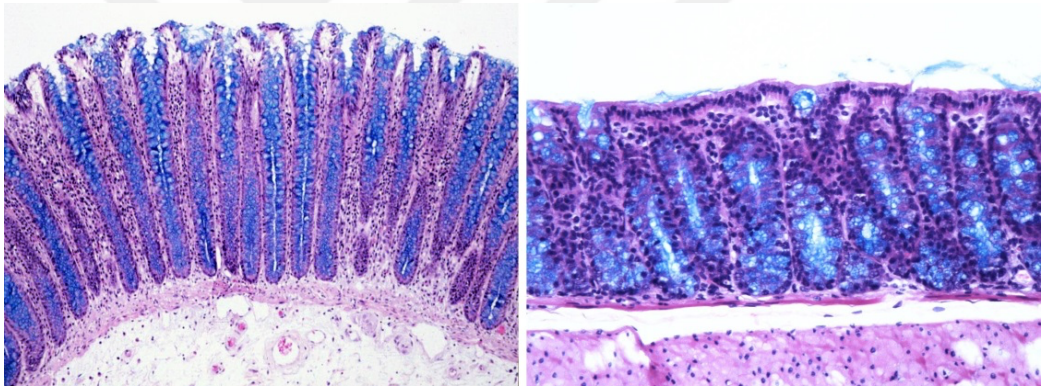

Figure 2.8. A normal human (left) and rat (right) colorectal mucosa. Crypts are parallel. (Kreyberg trichrom stain)[72].

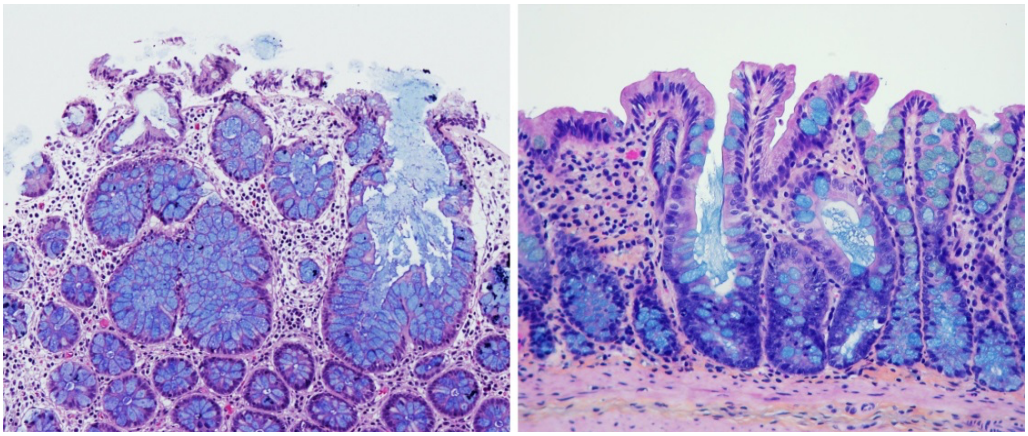

Figure 2.9. Hyperplastic aberrant crypts of human (left). Hyperplastic aberrant crypts of rat(right). (Kreyberg trichrom stain)[72].

2. 3. 1. DNA Damage and Repair-Gamma H2AX and Rad51

Through recent developments in cancer, have specified the crucial pathways affected in tumorigenesis[15]. The linkage of DNA disfiguration with premalignant phases of tumor advance, genome unbalance and further oncogenic transfiguration initiates the resourse of using prevalent DNA damage markers for early cancer prognosis, detection, therapeutics and conceivable for cancer prevention. There are studies in the literature to explain the effects of mutations in the DNA repair mechanism and various signal pathways on the development of colon complexity[11]. It has been stated that these three mechanisms, Microsatellite Instability (MSI), CpG Island Methylator Phenotype (CIMP) mechanism and Chromosal Instability (CIN), and, are effective in colon carcinogenesis[12]. DNA double-strand breaks (DSBs) are expectative to be the most detrimental type of DNA lesions. Environmental stress, chemicals or the failling of the DNA replication fork can be cause them[73].

γH2AX is one of the the most sensitive DNA damage marker generation in the chromatin flanking the free DNA double-stranded ends in DSBs and abraded telomeres, both exhibit during carcinogenesis transformation[74]. In eukaryotes, repair of DSBs occur either by homologous recombination (HR) or by non-homologous end-joining (NHEJ) DNA pathways. Essentially, NHEJ binds broken DNA ends directly, but HR needs the genetic information of an unimpaired sister chromatid or chromosomal homologue to proceed and it is requisite for the sustentation of genome integrity[75]. RAD51 protein was identified as essential in HR. Single nucleotide polymorphisms are the main source of genetic diversity where avability in the RAD51 promoter region can affect on its expression and ultimately regulate HR efficiency[76].

For about 10 years, it has been noted that in mammalian cells, a subtype of histone H2A, designated H2AX, is expressly phosphorylated via Ser 139 (g-H2AX) in response to DBSs[77]. When DSBs are incorporated into the DNA of eukaryotic cells, one of the initial responses is the sudden and intense phosphorylation of serine 139 at the COOH terminus of histone H2AX, concending it a specific modified form called yH2AX in mammals[74]. DNA damage triggers a signaling cascade aimed at cell cycle arrest through checkpoint activation. Many publications have shown that H2AX phosphorylation functions as a checkpoint maintenance component and its phosphorylation is a signal for cell cycle resumption. Thus suggesting that H2AX is essential for genome stability[78].

According to recent studies, increased spontaneous γH2AX levels have been published in human malignant tumors such as colon, ovarian, lung and breast carcinomas, and a few corresponding premalignant lesions[79]. RAD51 has an important capacity in the repair of DSBs via HrR; subsequently particular DNA damage, RAD51 places to nuclear foci for DNA repair. Within the context of HR, RAD51 assists in chain transfer between interrupted progression and their undamaged homologies[80]. The biological and biochemical functions of RAD51 can be related to the filaments they form and the structure of individual promoters. It has long been noted that certain structural filaments features originated by recombinase proteins such as RAD51 reflect its function[81]. Similar to many other proteins, RAD51 is transformed by phosphorylation in reaction to genotoxic stress[14]. RAD51 nucleoprotein filament generation and dynamics are principal components of DNA chain exchange function, little changes in protomers could have expressive effects[14]. According to many studies, mostly the RAD51 expression has raised cellular resistance against radiotherapy and chemotherapy similar to the crosslinking agents or topoisomerase inhibitors[82]. RAD51 connects to DNA and demonstrate an adenosine-dependent tryphosphatase and recombination. It develops helical nucleoprotein filaments inducing homologous pairing and triphosphatase (ATPase) activity and homologous strand switch between DNA molecules. The mutations in RAD51 cause a deficiency for recombination, ionizing radiation and hyphenate sensitivity for recombination and a deficiency in DNA recombination and hyphenship for ionizing radiation and methylmethanphonate and a lacking for a DSBs repair[83].

2. 3. 2. Effects of Oxidative Stress

Oxidative stress is expressed as the deformation of oxidative balance as in consequence of the increase of reactive oxygen species (ROS) like hydroxyl radical (OH•), superoxide radical (O2−) and hydrogen peroxide (H2O2) formed during cellular metabolism. Also, it defined as the inadequacy of antioxidants that detoxify them. Oxidative stress has a significant function in the occurrence of different diseases[84]. Reactive nitrogen species (RNS) and Reactive oxygen species (ROS) are incessantly generated via mitochondrial bioenergetics oxidative metabolism and immunity in the body[85]. The most common construct of ROS are superoxide anion, singlet oxygen, hypochlorous acid, lipid peroxides, hydrogen peroxide, hypochlorite and hydroxyl radical. These are concerned in triggering cell damage, growth, death and distinction.

They can interact with enzymes, nucleic acids, proteins, membrane lipids, and other small molecules[84]. As a harmful effect, free RNS causes nitrosative stress, ROS radicals cause oxidative stress, as a result, they can cause potential biological damage[86]. ROS are produced as a byproduct of standard aerobic metabolism; nevertheless, when the level rises under stress, it can cause a major health risk[87]. Mitochondria play an essential function in the production of ROS and it has an essential function in cell signalling and homeostasis[88]. It produces adenosine triphosphate (ATP) through a series of oxidative phosphorylation periods. During this period, instead of the four-electron reduction of oxygen, one or two electron reduction occurs, resulting in its subsequent the formation of H_2O_2 or $O_2\cdot$ and to modify other ROS[89]. The main form of RNS includes nitric oxide (NO) and peroxynitrite (ONOO−). The main form of RNS includes nitric oxide (NO) and peroxynitrite (ONOO−). In the presence of excess NO, nitrogen dioxide radical is formed as a result of this reaction. A raised NO density causes to the composition of N_2O_3, which often results in nitrosation[86]. Oxygen free radicals, such as alkyl peroxyl radical (·OOCR), hydroxyl radical (OH·−), and superoxide anion radical (O2·−), are competent initiators of lipid peroxidation, whose roles are well defined in the pathogenesis of diseases[87]. Once lipid peroxidation is given rise to, a propagation of chain reactions occur until termination outputs are generated. DNA bases are sensitive to ROS oxidation and 8-hydroxy-2-deoxyguanosine is the main noticeable oxidation product of DNA bases[87]. DNA bases oxidation may stimulate mutations and excisions in both mitochondrial and nuclear DNA. Moreover, mitochondrial DNA is more prone to oxidative damage because it is close to ROS and has insufficient repair ability compared to nuclear DNA[87]. These oxidative alteration cause important modulation in enzymatic and structural proteins that can have important physiological effects. Furthermore, redox transition of transcription components raise or reduce their specific DNA binding functions and thus change gene expression[87].

It can express nutrition as one of the principal modulators of oxidative stress in the human body[90]. The Western diet is excessive in dietary protein and saturated fatty acids (SFA). Fermentation of high proteins in the gut produces metabolites such as hydrogen sulfide (H2S) and ammonia (NH3), combines known to induce colon mucosal toxicity[91]. Research has established that high oxidative stress, especially ROS leads to cancer development, including colorectal cancer[92]. An increase in the amount of ROS

leads to increased mutation rates or susceptibility to mutagenic factors, and consequently imparts to DNA impairment in the initially steps of carcinogenesis[93].

3. MATERIALS & METHODS

3. 1. EXPERIMENTAL PROCEDURE

Laboratory animals were used and maintained in accordance with the Yeditepe University Animal Research Ethics Committee (Decision No: 2019/11-11). Housing, feeding and euthanasia of Sprague Dawley rats were carried out at Yeditepe University Experimental Animal Research Center. Sprague Dawley rats were allowed to access ad libitum chow and tap water. Their accommondation was at 22C temperature and 12 h light: 12 h dark cycle.

The study is planned in 2 stages. In the first stage, adult female sprague-dawley rats (mothers) have been exposed to high-fat diet or standard diet during pregnancy (21days) and lactation (21 days) periods. Then, in the second part of the study, male offspring from these two main groups were exposed to HFD and SFD during 120 days. HFD means 60 percent of the total energy from the fat, SFD has 13 percent of the total energy from the fat. Experimental Design given Figure 3.1. The macronutrient percentages of the diets used in the experiment are given in Table3.1.

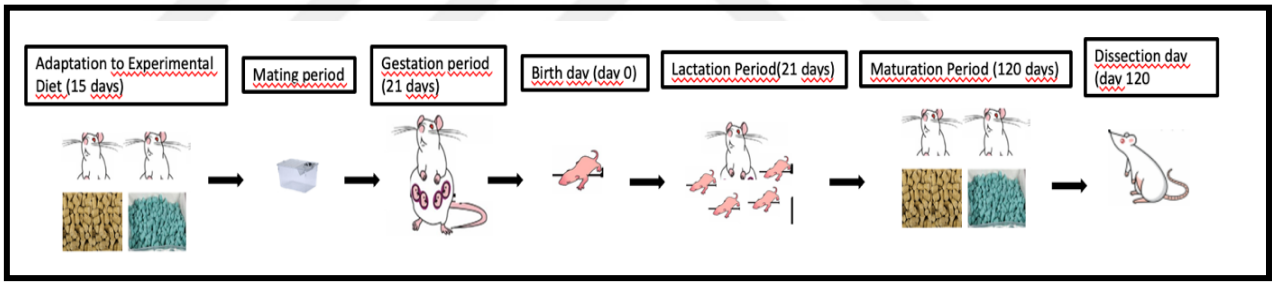

Figure 3.1. Experimental Design

Table 3.1 The macronutrient percentages of experimental diets.

Facts of Diets	**Standard Fat Diet (SFD)**	**High Fat Diet (HFD)**
Total Calorie (kcal/gr)	3,3	5,2
Fat (% kJ)	**13**	**60**
Protein(%kJ)	23	20
Carbohydrat(%kJ)	64	20

In this study, a total of 20 male rats were divided in 4 groups. After dissection of the experimental groups, a part of the colon tissues were stored at -80 ° C to use in determining the oxidative stress parameters. Experimental groups are given in Table 2.

Briefly; 250-300 gr female rats were allowed to access the standard fat diet (SFD) and high-fat diet (HFD) during two weeks as an adaptation period for the diets. After food adaptation period one male rat and two female rats were placed together in a cage for the mating period. This period was approximately for 8 days which means two oestrous cycles and at the end of the mating period, male rats were removed from the cage. During of mating period, vaginal smears were checked to determine the mating day and smear-positive animals were assumed as the first day of gestation. Approximately the 21st day of gestation the offsprings were born and mother animals continued feeding with same experimental diet in groups during the lactation period. After the lactation period (21st day), the mothers and female pups were taken from cages and remaining animals of each main group separated into two different groups which were feeding with SFD and HFD for maturation period. At the 120th day the experiment was terminated., Tissues were collected.

Table 3.2. Experimental groups.

Groups	**MATERNAL PERIOD [GESTATION & LACTATION] [21days & 21 days]**	**MATURATION PERIOD [120 Days]**
Group 1 (n=5)	SFD	SFD
Group 2 (n=5)	SFD	HFD
Group 3 (n=5)	HFD	SFD
Group 4 (n=5)	HFD	HFD

3. 2. PARAMETERS

3. 2. 1. Collection of Colon Tissue

3. 2. 2. Determination of Colon Total Antioxidant Status (TAS), Total Oxidant Status (TOS) and Oxidative Stress Index (OSI)

Colon oxidative stress status has been determined through commercial total oxidant status (TOS) and total antioxidant status (TAS) (Rel Assay, Turkey) kits. Oxidative stress index is calculated through the formulation given in the manufacturer's protochol.

3. 2. 2. 1. Homogenization of the Colon Tissues

50 mg of all experimental groups of frozen colon tissues are weighed and transferred to the tubes. 500 µL Phosphate Buffered Saline (PBS) solution is added to the tubes for the each colon tissue. The tissues were homogenised with homogenizer for 3-5 minutes. (Dia x 900 Heidolph, Germany). Then, the tubes were centrifuged at 3000 rpm for 5 minutes. The supernatants were taken into the new tubes and used as an example for TAS and TOS assay analysis.

3. 2. 2. 2. Measurement of TAS Level on the Colon

Principle of Assay: While the individual measurement of various antioxidant molecules are made, it may require materially high budget, long term and complex methods and their antioxidant actions are additive. Therefore, the total antioxidant situation (TAS) is defined as the total antioxidant capacity of a sample. With the antioxidants found in the sample, the dark-colored ABST radical drops in colorless reduced ABST form. With the change of absorbance in the sample at 660 NM, there are related to the total antioxidant level of the sample. The test is conventionally calibrated with a constant antioxidant standard solution called TROLOX equivalent, traditionally vitamin E analogue.

Method: As a first step, the amount of 18 µL samples, standard and deionized water (dH_2O) was included in the 96-well plate. On top of each well (all samples, standard and water) 300 µL of reactive 1 (R-1) solusion was added and mixed with the pipette. In the 660 nm absorbance of the plate was measured with Microplate Spectrophotometer (BioTek Instruments, USA) [First measurement: A1]. Then, the Reactive 2 solution in the amount of 45 µL was added to each well and mixed with the reactive 1 solution in the plate. The plate was incubated at room temperature for 10 minutes. As the next step, the absorbance of the plate was re-measured in 660 nm [second measurement: A2]. ΔAbs was calculated for each well with A2 minus from A1. The TAS formulation is available below.

TAS (mmol/L) = [ΔAbs H_2O-ΔAbs Sample] / [ΔAbs H_2O-ΔAbs Standard]

3. 2. 2. 3. Measurement of TOS Level on the Colon

Principle: The oxidants contained in the samples oxidize the ferrous ion-chelator complex to the iron ion. There are plenty of enhancer molecules in the reaction medium, these molecules are extended by the oxidation reaction. With the chromogen molecule in acidic solution, ferric ion gives a colored complex. There is a correlation between the total amount of oxidant ions in the sample and the color intensity. Meanwhile the change in colors can be measured spectrophotometrically. H_2O_2 was used while performing the calibration of this test. Results are expressed in nM H_2O_2 equivalent per liter (μmol H_2O_2 Equiv./L).

Method: First of all, the amount of 45 μL samples, standard and deionized water (dH_2O) was included in the 96-well plate. On top of each well (all samples, standard and water) 300 μL of reactive 1 (R-1) solusion was added and mixed with the pipette. In the 530 nm absorbance of the plate was measured with Microplate Spectrophotometer (BioTek Instruments, USA) [First measurement: A1]. Then, the Reactive 2 solution in the amount of 45 μL was added to each well and mixed with the reactive 1 solution in the plate. The plate was incubated at room temperature for 10 minutes. As the next step, the absorbance of the plate was re-measured in 530 nm [second measurement: A2]. ΔAbs was calculated for each well with A2 minus from A1. The TAS formulation is available below.

TOS (μmol/L) = [ΔAbs Sample] / [ΔAbs Standard] * 10

3. 2. 3. 4. Determination of OSI Level on the Colon

The oxidative stress index is accepted as a new marker in determining the oxidative stress status. Its equition is given below. As stated in the formulation, OSI is calculated by dividing the TOS value by the TAS value. However, the units of TAS and TOS values must be the same in the equation.

OSI = [(TOS, μmol H_2C_2 equivalent/L)/(TAS, μmol Trolcx equivalent/L)] x 100

3. 2. 3. Microtomy of Paraffin Embedded Section

Microtomy allows tissue to be sectioned and fixed to a surface for further histological examinations. Tissue blocks embedded with paraffin wax are effective in maintaining tissue integrity and allow cutting very thin sections. Following the procedure given in Table 3.2 to perform paraffin section microtomy, the colon was excised and

applied to the tissue. All procedures except fixation were performed on a tissue following device. The column tissues were embedded in paraffin and the paraffin blocks were kept at room temperature until cutting part. Paraffin blocks were placed at the appropriate location for serial sectioning and 5 µm thick sections were obtained from the tissues with a microtome (Leica RM2245, Germany). The sections obtained were then transferred to the float bath (Leica RM2245, Germany) at 37oC with forceps and brush to prevent tissue folding. After 60-90 seconds, tissues were transferred from water onto slides. The slides were allowed to dry on filter paper and placed in the slide box for storage at room temperature.

Materials:

- Floatation (water) bath (Leica RM2245, Germany)
- Microtome (Leica RM2245, Germany)
- Tissue following system (Leica 1020, Germany)
- Parafin embedding system (Leica EG1160, Germany)
- %10 Neutral Formaline (Sigma Aldrich #, USA)
- Clean Slides (ISOLAB)

Method

Table 3. 3. Tissue following protocole.

Stations	Number of Stations	Duration
Tap Water	3	3x10 minutes
70% Alcohol	1	3 hours
80% Alcohol	1	3 hours
90% Alcohol	1	3 hours
100% Alcohol	2	2+3 hours
Xylene	2	1 +2 hours
Paraffin	2	> 10 hours

3. 2. 4. Hematoxylin-Eosin (H&E) Staining

Principle: Hematoxylin-Eosin (HE) combination is one of the most frequently used staining methods in routine histopathological analysis. HE dye provides us with a lot of basic information about cells and tissues. hematoxylin is dark blue-purple in color and binds to basophilic parts of tissues containing nucleic acid fragments as an acidic dye.

At the end of the reaction, hematoxylin gives a blue-black color in the nuclei of the cell. Also, Eosin is pink and stains proteins in a non-specific way. The cytoplasm, erythrocytes, muscle and collagen in the basic elements of the regions are dyed pink, orange and red with an acidic eosin.

Reagents:

1. Hematoxylin (Merck # HX68297074, USA)
2. Eosin Y Alcoholic Solution (Bio Optica # 05-10003/L)
3. Absolute Ethanol (Sigma Aldrich #24102, USA)
4. Mounting Medium (Bio Mount HM) (Bio Optica #05-BMHM500)
5. Xylene (Sigma Aldrich # 16446, USA)
6. Slide Staining System (Leica Autostainer XL, Germany)
7. Light Microscope (Leica CTR6000, Germany)

Method:

Table 3. 4. Hematoxylin-Eosin staining protocole.

Stations	Number of Station	Duration
Oven	1	3 minutes
Xylene	3	3+3+5 minutes
%100 Alcohol	1	2 minutes
%96 Alcohol	2	2+2 minutes
Tap Water	1	2 minutes
dH_2O	1	1 minutes
Hematoxylin	1	2,1 minutes
Tap Water	5	1x5 minutes
%96 Alcohol	1	30 seconds
Eosin Y	1	4,1 minutes
%96 Alcohol	3	30+30+60 seconds
%100 Alcohol	1	1 minutes
Xylene	3	1x2 minutes

3. 2. 5. Immunofluorescent (IF) Staining

Immunostaining is conventionally identified as the methodology for detecting certain antigens in cellular structures using certain fluorescently labeled antibodies in

tissues or cells. Immunofluorescence authorize researchers to determine whether in a particular sample represent the antigen in question.

In this procedure, to decide which slides will be applied to the IFC procedure, firstly the sections will be examined under the microscope and the selected slides will be kept in the etuve at 37 ° C for 1 night. The next day the slides will be incubated at 60 ° C for 1 hour for deparaffinization and then the slides will be kept in xylene twice for 20 minutes. Then, for the dehydration of the colon, the slides will be passed through an alcohol (ethanol) bath (100%, 90%, 80%, 70%, respectively) in decreasing concentration for 10 minutes. Then the sections will be kept in dH20 for 2 times, 5 minutes each. Then the sections will be washed 2 times in PBS for 5 minutes, placed in EDTA buffer, boiled for 4 minutes at the "med-high" setting of the microwave oven, cooled at room temperature for 20 minutes in order to eliminate antigenic masking in the tissue. Then again, PBS-T will be washed 3 times for 5 minutes and the periphery of the samples will be drawn with a hydrophobic pen. From the sections blocked in 5% goat serum at room temperature for 1 hour, primary rabbit Rad51 antibody (1: 500 dilution) to positive groups and PBS to negative groups will be dropped and the sections will be incubated overnight in a refrigerator at +4 C. The sections washed 3 times in PBS-T for 5 minutes the next morning, kept in the secondary anti-rabbit antibody diluted 1: 1000 for 90 minutes, washed 3 times in PBS-T for 5 minutes, and determination of cell nuclei with DAPI capping solution will be closed for. The samples whose staining has been completed will be displayed under the confocal microscope and the expression level and localization of Rad51 will be shown. The same procedure will be used to image the Gamma-H2AX protein.

Figure 3.2. IF staining, wet chamber

3.2.4. Statistical Analysis

The power analysis of our study, which was planned to use a total of 20 animals in 4 groups, was performed with the G * Power software program, and it was determined as Type 1 Error: 0.05, Test Power: 0.8 and Effect Size: 0.52. The results obtained at the end of the thesis will be evaluated using the SPSS-18 software statistics program. If the data does not conform to normal distribution after testing, the non-parametric Kruskal Wallis and Mann-Whitney U tests will be applied to determine the differences between groups and paired groups, respectively. In the evaluation of the results, $p < 0.05$ will be considered statistically significant.

4. RESULTS

4.1. TAS, TOS and OSI Levels of Proximal Colon

Table 4. 1. TAS, TOS and OSI values of proximal colon of experimental groups (means ± standard deviation)

Groups	TAS (mmol/L)	TOS (**µmol/L**)	**OSI**
Group 1 (SFD-SFD)	1,04 ± 0,38	0,09 ± 0,01	0,009 ± 0,003
Group 2 (SFD-HFD)	1,03 ± 0,14	0,12 ± 0,16	0,012 ± 0,013
Group 3 (HFD-SFD)	1,09 ± 0,14	0,09 ± 0,05	0,009 ± 0,003
Group 4 (HFD-HFD)	1,16 ± 0,43	0,10 ± 0,01	0,009 ± 0,0500

4.1.1. Total Antioxidant Status of Proximal Colon Tissues

As shown in table 4.1 TAS of proximal colon was measured 1,04 ± 0,38 mmol/L in SFD-SFD group. SFD-HFD group was measured 1,03 ± 0,14 mmol/L, HFD-SFD group measured 1,09 ± 0,14 mmol/L and HFD-HFD group measured 1,16 ± 0,43 mmol/L. There is no significant difference in TAS level of any groups. (Figure 4.1.)

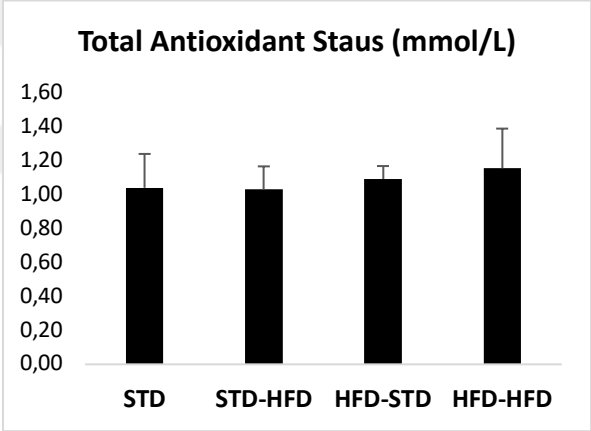

Figure 4.1. The effect of SFD and HFD during the maternal/maturation period on TAS of proximal colon (mean ± standard deviation) (n=5)

4.1.2. Total Oxidant Status of Proximal Colon Tissues

As shown in table 4.1 TOS of proximal colon was measured 0,09 ± 0,01µmol/L in SFD-SFD group. SFD-HFD group was measured 0,12 ± 0,16 µmol/L, HFD-SFD group measured 0,09 ± 0,05 µmol/L and HFD-HFD group measured 0,10 ± 0,01 µmol/L. There is no significant difference in TOS level of any groups. (Figure 4.2.)

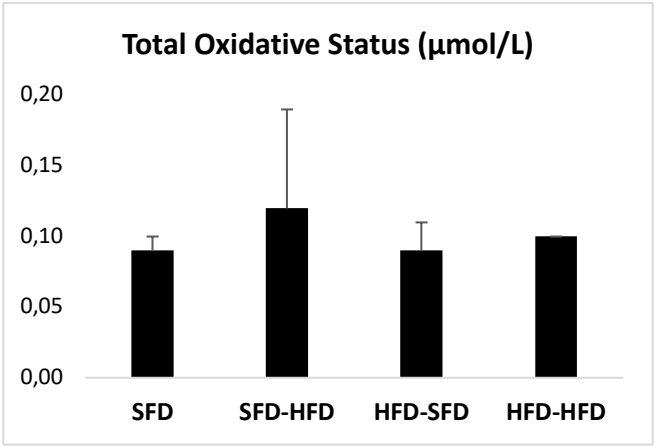

Figure 4.2. The effect of SFD and HFD during the maternal/maturation period on TOS of proximal colon (mean ± standard deviation) (n=5)

4.1.3. Oxidative Stress Index of Proximal Colon Tissues

As shown in table 4.1 OSI of proximal colon was measured 0,009 ± 0,003 in SFD-SFD group. SFD-HFD group was measured 0,012 ± 0,013, HFD-SFD group measured 0,009 ± 0,003 and HFD-HFD group measured 0,009 ± 0,0500. There is no significant difference in OSI level of any groups. (Figure 4.3.)

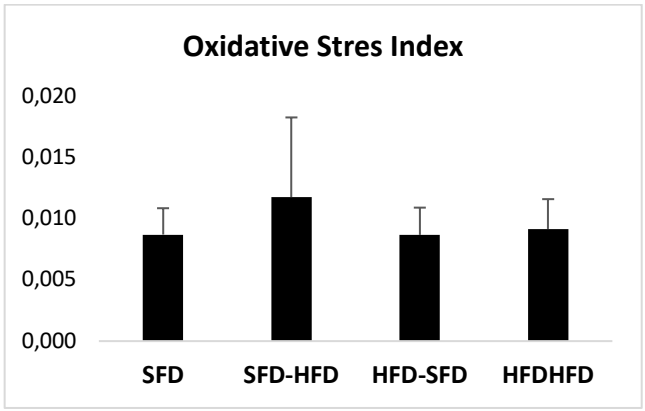

Figure 4.3. The effect of SFD and HFD during the maternal/maturation period on OSI of proximal colon (mean ± standard deviation) (n=5)

4. 2. Morphologic Assessment of Proximal Colon Tissues with HE Staining

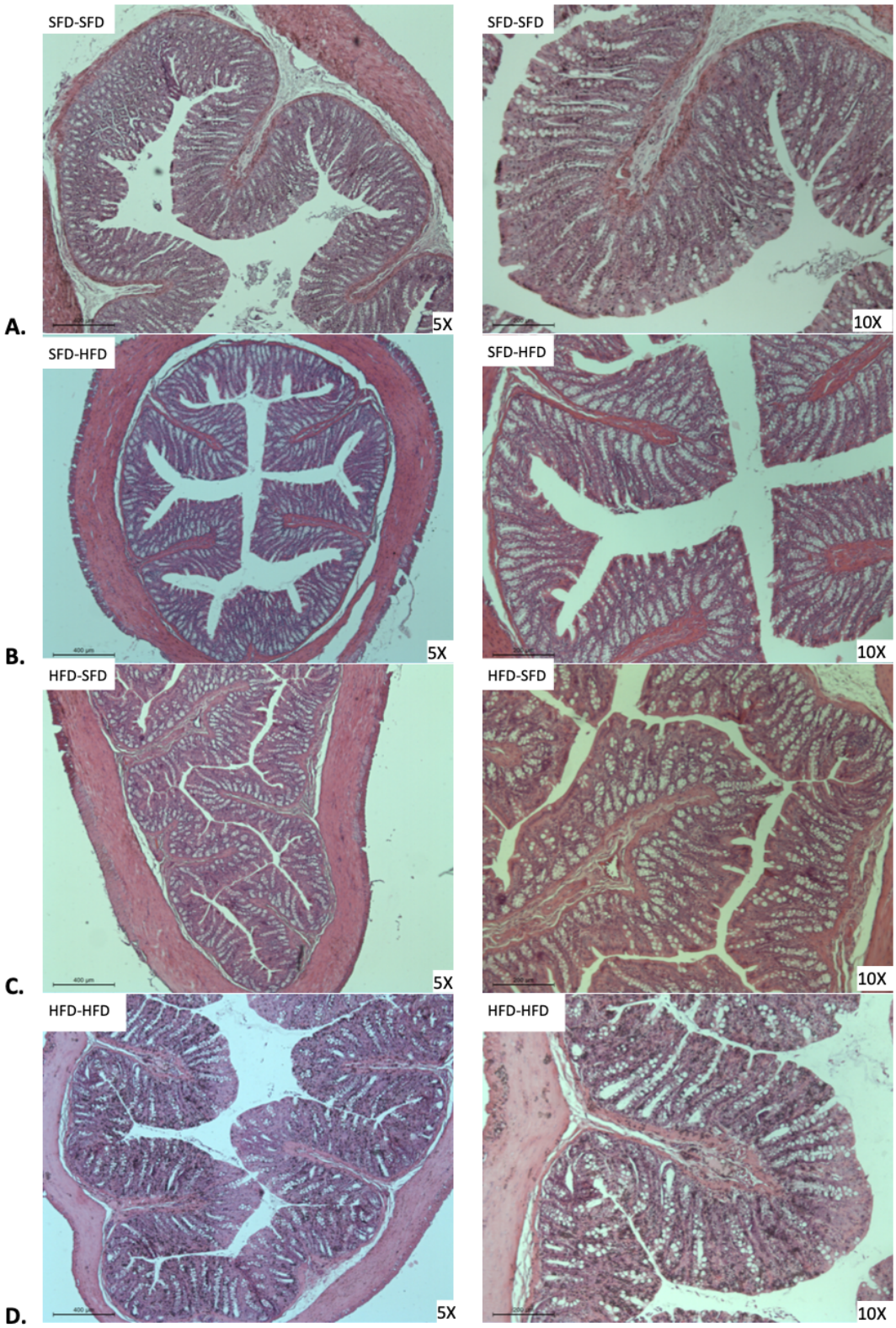

Figure 4. 4. HE staining of proximal colon tissues. taken 5x magni (left) / 10x magni.(right). **A.**SFD-SFD, **B.**SFD-HFD, **C.**HFD-SFD, **D.**HFD-HFD

4.3. H2AX and Rad51 IF Imaging With Confocal Laser Scanning Microscope

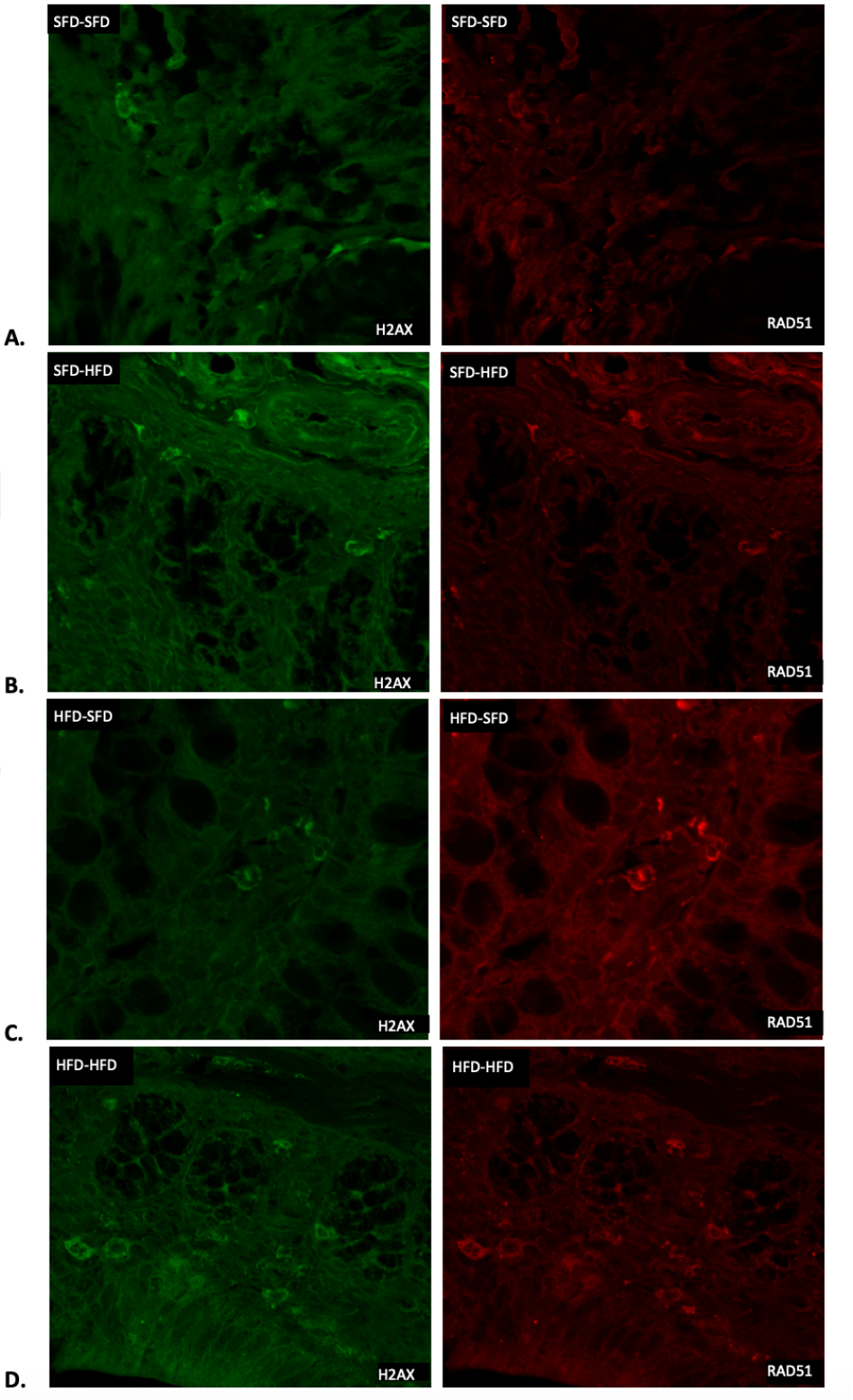

Figure 4. 5. IF staining of proximal colon tissues. taken 63x. H2AX (left) / Rad51(right). **A.**SFD-SFD, **B.**SFD-HFD, **C.**HFD-SFD, **D.**HFD-HFD

4.3.1. H2AX and RAD51 Expressions

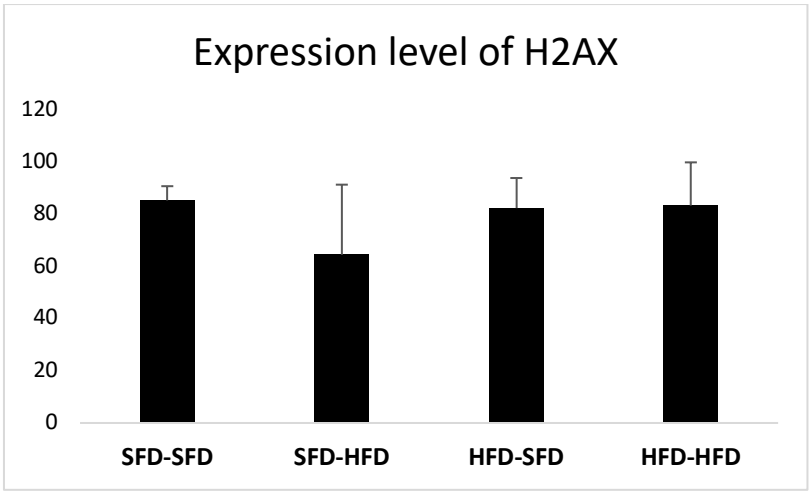

Figure 4. 6. Expression level of H2aX in experimental groups

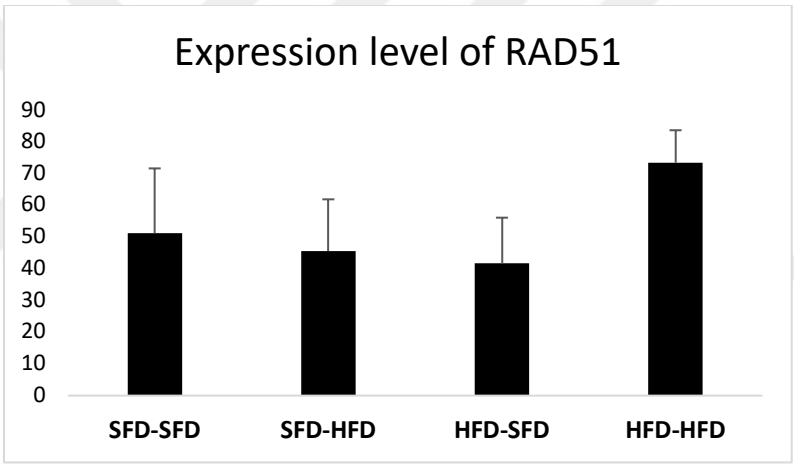

Figure 4. 7. Expression level of RAD51 in experimental groups

5. DISCUSSION and CONCLUSION

In many countries, the consumption of western-style diet has increased significantly in societies with high fat and low fiber intake[1]. According to studies, high-fat food consumption significantly increases the risk of developing colon cancer[3,94]. Colon cancer ranks third among cancers in mortality for women and second for men.

Another critical phenomenon that has been emphasized in recent years is the knowledge that nutrition in the maternal period affects the health of the individual during the maturation period in many respects[57,95]. It is known that especially the lactation period is an important process in terms of the diversity in the colon microbiota of the child and the formation of beneficial bacteria. In colonic carciogenesis, secondary bile acids formed as a result of high-fat food consumption have negative effects on both the microbiota and morphology of the colon in a long time and as a result of many stages[59,96]. At the same time, it has been proven that oxidative stress indicators increase during the carcinogenesis process and both the damage mechanism and the repair mechanism of DNA are negatively affected. The H2AX protein is a widely used DNA damage marker. RAD51 is an important DNA repair marker[97,98].

The formation of pathologies is associated with unbalanced metabolisms. Oxidative stress as a result of high-fat diet has an active role in pathophysiological formations[87]. In this study, TAS, TOS and OSI values measured from the proximal colon in rats fed standard diet and/or high-fat diet during gestation-lactation and maturation. We could not detect any significant difference between the experimental groups.

Abberant crypt focus is a primitive determinable lesion in colon carcinogenesis. The underlying neoplastic potential of this lesion is not clear. However, it has been stated that some of these lesions trigger mutations in important genes in the colon and may cause adenocarcinoma[11]. The microscopic view of the proximal colon of sprague dawley rats fed with high-fat diets at different maternal and maturation periods is given in figure 4.4. Figure 4.4. A and C: normal colon showing vertically oriented epithelial glands with tubular morphology. The epithelial cells in the upper segment of the glands contain abundant mucin and a small basal nucleus in their cytoplasm. A predisposition to the formation of abnormal crypt foci was observed in figure 4.4. B and D: epithelial cells lack strong mucin staining and show enlarged nuclei. In a high-grade dysplasia, the lesions consist of distinctly crowded and irregular cryptic cells with depleted mucin and

pleomorphic nuclei, and this was not observed in any experimental group. IF images of H2AX and RAD51 proteins taken (63X) by confocal microscopy are shown in figure 4.5. Expression levels are given in Figures 4.6 and Figuer 4.7. In Figure 4.7. there is an increase tendency in RAD51 levels of the group HFD-HFD compared to the SFD-SFD. However, there is no significant difference was observed between the groups for both H2AX and RAD51 proteins.

In this study, we aimed to examine the effects of high-fat diet on the colon during the maternal and/or maturation period using different techniques. As a result of the methodologies we used and the analyzes we made, we could not obtain any statistical results. The feeding process of rats may not long enough or the diet content may not be sufficient for colon damage to occur. As a future direction, the feeding period can be extended and the diet content can be changed. In addition to total oxidative/antioxidative status, specific oxidative/antioxidative stress marker levels can be measured. The amount of bile acid can be determined from stool, which is a confirmation of a fatty diet and known to cause damage to the colon. At the same time, mRNA levels of some genes known to be affected by fatty diet in the colon can be evaluated.

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7. APPENDICES

7.1.Ethical Approval

T.C. YEDİTEPE ÜNİVERSİTESİ
Hayvan Deneyleri Yerel Etik Kurulu (HADYEK)

ETİK KURUL KARARI

Protokol No	Toplantı Tarihi	Toplantı Sayısı	Karar No	Proje Yürütücüsü
2019-811	08.11.2019	2019/11	2019/11-11	Doç. Dr. Burcu Gemici Başol

'Sprague Dawley Sıçanlarda Maternal ve Maturasyon Dönemlerinde Farklı Yağ Konsantrasyonlarında Diyete Maruz Kalmanın Kolorektal Kanser ile İlişkisinin İncelenmesi' isimli proje oy birliğiyle etik açıdan uygun görülmüştür.

Hayvan Türü / Irkı	Toplam Hayvan Sayısı	Hayvanın Cinsiyeti
Sıçan	56	Erkek

Görevi	Adı Soyadı	Katılım Durumu
Başkan	Prof. Dr. Bayram YILMAZ	
Başkan Vekili	Prof. Dr. Erdem YEŞİLADA	
Üye	Veteriner Hekim Engin SÜMER	
Üye	Prof. Dr. M. Ece GENÇ	
Üye	Prof. Dr. Rukset ATTAR	
Üye	Prof. Dr. Gamze TORUN KÖSE	
Üye	Doç. Dr. Ediz DENİZ	
Üye	Doç. Dr. Aylin YABA UÇAR	
Üye	Hakan GÖKSEL	
Üye	Ahmet ŞENKARDEŞLER	