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**REVIEW** / DERLEME

# Free radicals, whey proteins and colorectal cancer

## Serbest radikaller, süt serumu proteinleri ve kolorektal kanser

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**ABSTRACT**

Evidence that has accumulated for many years suggests that diet is an important environmental factor in the etiology of colorectal cancers. Epidemiological data generally support the association between total energy intake, high fat diets, red meat intake and increased colon cancer risk. The Western-style diet and cooking techniques are risk factors for developing colon cancer. Further, oxidative stress caused by reactive oxygen species plays a significant role in a number of age-specific diseases such as cancer and neurodegenerative disorders. Dietary proteins including whey proteins have been reported to have the ability to scavenge reactive oxygen species. Animal studies have also shown that whey protein protects against the development of carcinogen induced colon tumors in rats. In addition to proteins, protein hydrolyzates have been found to exhibit antioxidant activity. During protein hydrolysis, overall antioxidant activity of protein is enhanced as its tertiary structure is disrupted and the solvent accessibility of released amino acids increases. In this review, we summarize the present knowledge on the etiopathogenesis of colorectal cancers and the potential use of whey proteins in its treatment.

**Key words:** Free radicals, Whey proteins, Colorectal cancer

**ÖZET**

Kolorektal kanser etiyolojisinde diyetin önemli bir çevresel faktör olduğu yapılan çalışmalarla kanıtlanmıştır. Total enerji alımı, yüksek yağlı diyet ve kırmızı et alımı ile kolon kanseri gelişimi arasında bağlantı olduğu epidemiyolojik verilerle de desteklenmiştir. Batı tarzı diyet ve pişirme teknikleri kolon kanseri için risk faktörü olarak yer almaktadır. Öte yandan, reaktif oksijen türleri tarafından oluşturulan oksidatif stres, kanser ve nörodejeneratif bozukluklar gibi yaşa bağlı hastalıklarda önemli rol oynamaktadır. Süt serumu proteinleri ve diğer diyetsel proteinlerin reaktif oksijen radikallerini temizleme gücüne sahip olduğu bildirilmiştir. Hayvan çalışmalarında karsinojen kullanılarak oluşturulan kolon kanserine karşı süt serumu proteinlerinin koruyucu olduğu gösterilmiştir. Proteinlerin yanında protein hidrolizatlarının da antioksidan aktivite gösterdiği bulunmuştur. Protein hidrolizi sırasında proteinin tersiyer yapısının bozulması ve amino asit salınmasındaki artışa bağlı olarak proteinin antioksidan aktivitesinde artış izlenmektedir. Bu derlemede, kolorektal kanser etiyopatogenezindeki mevcut bilgileri özetlemeye ve tedavide süt serumu proteinlerinin potansiyel kullanımını irdelemeye çalıştık.

**Anahtar kelimeler:** Serbest radikaller, Süt serumu proteinleri, Kolorektal kanser

## Introduction

Colorectal cancer (CRC) is the second leading cause of cancer deaths in the Western world with a million new cases diagnosed each year. Although a small subset of these cases are well-characterized hereditary syndromes, the vast majority of CRCs are considered non-familial occurring in individuals with genetic susceptibility as a result of the interaction between environmental exposures and multiple genes with low penetrance. Advances in early detection and surgery have been largely responsible for reducing mortality and morbidity of colon cancer and our understanding of prevention is increasing. Diet is an important environmental factor in the etiology of CRC and epidemiological data generally support the association between total energy

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intake, high fat diets, red meat intake and increased colon cancer risk. Western-style diet and cooking techniques are risk factors for developing colon cancer [1,2].

Oxidative stress caused by reactive oxygen species plays a significant role in a number of age-specific diseases such as cancer and neurodegenerative disorders [3,4]. Dietary proteins including whey proteins have the ability to scavenge reactive oxygen species [5,6]. Animal studies have shown that whey protein protects against carcinogen induced colon tumors in rats [7,8]. In addition to proteins, protein hydrolyzates have also been found to exhibit antioxidant activity. During protein hydrolysis, overall antioxidant activity of protein is enhanced as its tertiary structure is disrupted and the solvent accessibility of released amino acids increases [9].

## Free radicals and cancer

Free radicals are defined as molecules which contain an unpaired electron in their outermost orbital. To stabilize the unpaired electron, free radicals will tend to donate or receive an electron by interacting with molecules in their environment. Uncontrolled generation of radicals and related reactants are deleterious to cell structure and function because they will react with macromolecules such as unsaturated lipids, proteins and nucleic acids [3,4].

Oxygen derived free radicals are: hydroxyl radicals ($OH^{\cdot}$), hydrogen peroxide ($H_2O_2$), hypochlorous acid (HOCl), singlet oxygen ($O_2$), peroxynitrite anion (ONOO) and peroxynitrous acid (OHOOH). In most cells enzymes such as superoxide dismutase, glutathione peroxidase, glutathione reductase, glutathione transferase and catalase protect cells against free radicals. In addition, agents such as vitamin E, cysteine, glutathione (GSH) and ceruloplasmin protect cells by inhibiting free radical formation or will inactivate them. The definitive effect of free radicals is related to the balance between their destruction and formation. If formation of free radicals exceeds the antioxidant defense mechanisms then a condition termed oxidative stress arises [10].

Detection of harmful effects caused by free radicals in the cell are measured as markers of lipid, protein and DNA oxidation or intermediate products. Hydroxyl radicals primarily interact with unsaturated fatty acids of membrane phospholipids, starting oxidative damage. During lipid peroxidation biologically active intermediates are formed. The well known end product of lipid peroxidation is malondialdehyde (MDA) which is itself cytotoxic. Measurement of the amount of MDA is a common method of assessing oxidative damage. When lipid peroxidation is not blocked, increased membrane permeability, cell membrane damage, decreased fluidity, imbalance of transmembrane ions, corruption of secretory functions emerge and lead to cell swelling and death [11].

GSH is a water-soluble tripeptide made up of glutamine, cysteine and glycine [12]. The potency of GSH exists in its cysteine residue (thiol group). It is the most important thiol antioxidant in the organism and protects the body against electrophilic, halogenated structures and epoxides. GSH is involved in many direct and indirect protective mechanisms [13]. It is an important antioxidant that provides a balance between oxidation and reduction, as well as protecting cells from harmful effects of endogenous and exogenous oxidants. This protection is carried out by GSH S-transferase and GSH peroxidase. In addition to detoxification, reduction of ribonucleic acids to deoxyribonucleotides in the glyoxalase system as well as gene expression of various proteins are also involved through the thiol group [12,13].

*De novo* synthesis of GSH requires the presence of cysteine, glutamic acid and glycine. Enzymes involved in the synthesis are g-glutamylcysteine synthetase and GSH synthetase. GSH is converted to glutathione disulfide (oxidized glutathione, GSSG) by a selenium-containing enzyme, GSH peroxidase. This enzyme catalyzes the reduction of hydrogen peroxide and other peroxides. GSSG, is also produced after reaction of GSH with free radicals. Reduction of GSSG back to GSH is catalyzed by GSH reductase in the presence of NADPH. In the extracellular space, GSH is converted to GSSG and this reaction requires oxygen causing the formation of hydrogen peroxide [14].

Cancer is one of the leading causes of death worldwide, and understanding the factors that contribute to cancer development may facilitate strategies for cancer prevention and control [15,16]. Cancer development involves genetic and epigenetic alterations. Nutrients can either contribute directly to cancer prevention or support the repair of genomic and epigenomic damage caused by exposure to cancer-causing agents such as toxins, free radicals, radiation, and infectious agents. One of the earliest lines of evidence for the involvement of free radicals in carcinogenesis was chromosome fragmentation induced by hydrogen peroxide in the presence of a peroxidation factor [17]. Involvement of free radicals in carcinogenesis was further supported by several *in vitro* studies describing the role of free radicals in DNA damage as well as in structural and functional modifications of proteins [18-20].

Free radical related mutagenesis that can result in cancer initiation and progression is a frequent event in normal human cells. Although free radical mediated tumor promotion has not been directly demonstrated in humans, there is convincing experimental evidence that oxidative stress can differentially induce the proliferation of tumor cells. Thus, oxygen derived free radicals should be recognized as an important class of carcinogens that stimulate cancer development at multiple stages [21].

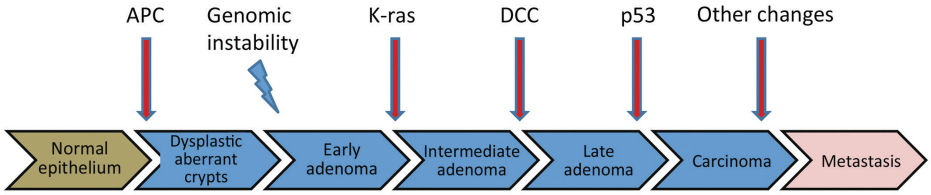


**Figure 1:** Fearon and Vogelstein's model for the development of colorectal cancer [27].

Several molecules present in the diet, including flavonoids, can inhibit the growth of cancer cells with their ability to act in chemoprevention. Recent research has revealed that these molecules possess antioxidant activities which prevent free radical damage to biological molecules like lipids, proteins and DNA, damage which can cause many cardiovascular and neurodegenerative diseases as well as cancer and diabetes [22]. The underlying mechanisms involve metal chelation, a capacity to act as a free radical scavenger and inhibition of enzymes that produce free radicals . Cancer preventive effects have also been attributed to the induction of cell-cycle arrest and/or apoptosis as well as the antioxidant functions. Since antioxidants are capable of preventing oxidative damage, the wide use of natural food derived antioxidants is receiving greater attention as potential anticarcinogens [23].

## Development of colorectal cancer

Colorectal carcinoma is one of the best-studied tumors in terms of its developmental mechanisms. Various studies have shown that large numbers of consecutive mutations finally create cancer. There is not a single mechanism in the development of colorectal cancer but several stages and multi-factorial mechanisms are effective [24-26]. Specific steps in a sequence are also required for tumor growth. Fearon and Vogelstein [27] proposed a model of colorectal carcinogenesis that correlates specific genetic changes with evolving tissue morphology (Figure 1). Over the past two decades, intense research has focused on elucidating the genetic defects and molecular abnormalities associated with the development and progression of colorectal adenomas and carcinomas. Mutations may cause activation of oncogenes (K-ras) and/or inactivation of tumor-suppressor genes [Adenomatous Plyposis Cli (APC), Deleted in Colorectal Carcinoma (DCC) p53]. Colorectal carcinoma is thought to develop from adenomatous polyps by accumulation of these mutations. Defects in the APC gene were first described in patients with familial adenomatous polyposis (FAP). By investigating these families, characteristic mutations in the APC gene were identified. They are now known to be present in 80% of sporadic colorectal cancers as well. The APC gene is a tumor-suppressor gene. Mutations in both alleles are necessary for the initiation of polyp formation. The majority of mutations are premature stop codons, which result in a truncated APC protein. In FAP, the site of the mutation correlates with the clinical severity of the disease. For example, mutations in either the 3' or 5' end of the gene result in attenuated forms of FAP, while mutations in the center of the gene result in more virulent disease. Thus, knowledge of the specific mutation in a family may help guide clinical decisions [28].

APC inactivation alone does not result in carcinoma. Instead, this mutation sets the stage for the accumulation of genetic damage that results in malignancy via mutations accumulated in the loss of heterozygosity pathway [29-31]. Additional mutations involved in this pathway include activation of the K-ras oncogene and loss of the tumor suppressor genes DCC and p53. K-ras is classified as a proto-oncogene because mutation of only one allele will perturb the cell cycle. The K-ras gene product is a G protein involved in intracellular signal transduction. When active, K-ras binds guanosine triphosphate (GTP) and hydrolysis of GTP to guanosine diphosphate, and then inactivates the G protein. Mutation of K-ras results in an inability to hydrolyze GTP, thus leaving the G protein permanently in the active form. It is thought that this activation leads to uncontrolled cell division. A mutation associated with an increased risk of colorectal cancer was found in the MutY human homologue (MYH) gene on chromosome 1p. MYH is a base excision repair gene, and biallelic deletion results in changes in other downstream molecules. Since its discovery, MYH mutations have been associated with an attenuated familial adenomatous polyposis (AFAP) phenotype in addition to sporadic cancers. Unlike APC gene mutations that are expressed in an autosomal dominant pattern, the requirement for a biallelic mutation in MYH results in an autosomal recessive pattern of inheritance. DCC is a tumor-suppressor gene and loss of both alleles is required for malignant degeneration. DCC mutations are present in more than 70 % of colorectal carcinomas and may negatively impact prognosis.

Aberrant crypt focus (ACF) is morphologically abnormal crypt in the colonic mucosa and a real intermediate biomarker for colon carcinogenesis [31]. As this lesion is an early preneoplastic event for colorectal cancer, it is used for short-term bioassays to identify carcinogenesis modulators [32-34]. Induction of ACF implies genotoxic events and primary DNA damage in colon cells. Thus it is an important step for colon cancer chemoprevention. An ACF is darker

than the adjacent crypts when stained with methylene blue and is surrounded by thickened epithelium, and also has a long and thin lumen. Crypts are 2-3 times larger than normal and macroscopically protrude from surrounding tissue [35]. Microsatellite instability can be seen. When the first mutation belongs to an oncogene (e.g. K-ras) while genes such as p53 and APC are intact, the cells gradually go to apoptosis to prevent tumor development.

Finally, the tumor-suppressor gene p53 has been well characterized in a number of malignancies. The p53 protein appears to be crucial for initiating apoptosis in cells with irreparable genetic damage. Mutations in p53 are present in 75 % of colorectal cancers [36]. The most important step in the transition from adenoma to carcinoma is a p53 gene mutation [37]. This mutation is defined as a "genome keeper" and it is one of the most important regulators of the cell cycle. It recognizes defects in DNA and in case of unrepaired DNA damage it is responsible for activation of genes that take the cell to apoptosis. With deactivation in both alleles of this gene transition of an adenoma to a carcinoma occurs.

## Genotoxic chemicals and models of colon cancer

Chemical carcinogens may be used to create a tumor in various organisms. The most widely used chemical carcinogens used in models of colon cancer are: 3,2 dimethyl-4-aminobiphenyl (DMBA), 1,2-dimethyl hydrazine (DMH) with azoxymethane (AOM), methylnitrosourea (MNU), "N-methyl-N-nitro-N-Nitroguanidine (MNNG) and 2-Amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP). In order to evaluate the effect of any diet or agent for cancer prevention, experimental tumor(s) induced in animals are used [38]. For this purpose a carcinogenic agent is given to rodents since colon cancer cannot develop spontaneously in these rodents. DMH is a commonly used agent and most of the published rat studies on tumor induction were done with DMH or its metabolite AOM [39]. Due to the difference between natural strains and similarity of the disease model to human CRC, this model is used to investigate the genetic and non-genetic predisposing factors of CRC.

The risk of developing CRC in inflammatory bowel diseases like ulcerative colitis and Crohn's is higher than in the normal population [40]. It has been reported that inflammation seen in sporadic CRC and CRC secondary to inflammatory bowel disease is associated with increased expression of nitric oxide synthase and cyclooxygenase-2 [41]. Dextran sulfate sodium (DSS) is a non-genotoxic, colonic carcinogen that is commonly used to produce a form of colitis in rodents that is similar to human ulcerative colitis [42]. Colonic inflammation due to exposure to DSS and to nitrosative stress are thought to play a role in the development of dysplasia and neoplasm of the cryptic dysplasia. Thus the inflammatory response alone may lead to colon cancer [43]. Initially giving AOM makes the DSS-induction of colon carcinogenesis faster and may increase the incidence of tumors [44].

## Anticancer activity of whey proteins

Nutritional studies, reports and trials to identify anticancer properties of foods have been extensive. It is generally agreed that diets that are high in grains, green vegetables, fresh fruit and fiber, and low in total and saturated fats are beneficial to health. Relatively less emphasis has been placed on bovine milk. Humans who consume milk are less likely to develop cancer of the colon and rectum than those who do not consume milk. [45,46]. Calcium and vitamin D were identified as protective against colorectal cancer. Bounous et al. [47, 48] were the first to report that whey proteins from bovine milk protect against chemically induced carcinogenesis in several animal models. There is now enough evidence to suggest that bovine milk contains major and minor components with anticancer properties [46].

Dietary influences on cancer risks have become an increasingly important area of research. Prevention of colon cancer by dietary whey proteins has been studied in mice and rats, but the results are contradictory. McIntosh et al. [49] reported that DMH-induced colon tumor incidence was reduced in rats fed diets made with either casein or whey protein compared with diets made with red meat or soy protein. Although there was a tendency toward a lower tumor incidence in whey-fed compared to casein-fed rats, the difference was not significant and data on tumor mass were not consistent. Others showed that whey proteins protect more effectively than red meat, soy bean and casein against carcinogen-induced colon tumor expression in male rats [7, 50]. However, a clear mechanism showing how the risk of colon cancer could be reduced by whey protein has not been deduced. GSH concentrations in a number of tissues have been reported to increase in rats fed whey protein, and this is thought to be attributable to relatively high levels of γ-glutamylcysteine groups, which serve as substrate for glutathione synthetase [48]. The protective role of whey proteins is most probably related to the high content of cystine/cysteine and γ-glutamylcysteine which are efficient substrates for glutathione synthesis [46-48].

Other mechanisms for the protective effect of whey proteins may be suggested. A protein-fatty acid complex consisting of human or bovine whey protein α-lactalbumin and the fatty acid oleic acid was reported to have potent anticancer properties [51]. In a recent study, it was shown that a similar complex may be formed under simulated gastric conditions [52]. Tsuda et al. [53] reported that a

minor whey protein component, bovine lactoferrin, reduced the incidence and multiplicity of colon carcinomas in male rats. Dietary cysteine-rich proteins had a significant effect on the immunological response and on tumor formation in a mouse model of colon carcinogenesis [54].

In a recent study a whey protein concentrate given to breast cancer patients it was suggested that this has a protective effect due to elevated antioxidant capacity [55]. Whey protein hydrolyzate is the hydrolyzed form of an 80% whey protein derived from dairy whey processed by a special cross-flow filtration process. We have recently compared the protective effect of dietary whey protein with whey protein hydrolyzate against AOM and DSS induced colon cancer in rats [8]. There was a tendency toward a lower adenoma incidence in whey-fed compared to standard diet-fed rats but the difference was not significant. However, in rats fed whey protein hydrolyzate the adenoma incidence was significantly decreased. Whey protein hydrolyzate prevented colorectal carcinogenesis by inhibiting adenoma formation which is the best and most relevant marker of CRC.

CRC begins with ACF at the earliest stage and progresses to early adenoma, then to late adenoma, then to intramucosal carcinoma and finally to invasive adenocarcinoma [33]. A small portion of colorectal carcinoma arises *de novo* starting with epithelial dysplasia, then high-grade dysplasia without creating adenoma and proceeds to carcinoma. This type of carcinoma can be seen in inflammatory diseases such as ulcerative colitis [36]. In our study the ulcerative colitis model was created by giving DSS. Microscopic examination showed active colitis in all rats receiving DSS indicating that whey protein hydrolyzate can also prevent colorectal carcinogenesis associated with ulcerative colitis and is more effective in preventing colon tumor development compared with whey protein [8]. A direct association with lipid peroxidation, protein oxidation and glutathione was not observed. Other mechanisms may also be present.

### Conclusion

Whey protein has been shown to reduce the risk of colon cancer and other cancers. The exact mechanism of action of whey protein against colon cancer is not clear. It was shown that whey protein stimulates the immune system by enhancing hepatic glutathione synthesis and can act like an antioxidant. The anticancer properties of whey proteins may be ascribed to their ability to elevate cellular levels of glutathione. Increased tissue concentrations of glutathione can be predicted to have a protective effect because elevated antioxidant capacity would favor decreased mutagenicity. However, further studies are needed to elucidate the exact mechanism of action of whey protein against colon cancer.

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