Pathol. Oncol. Res. (2012) 18:817–822
DOI 10.1007/s12253-012-9509-9

RESEARCH

Whey Protein Versus Whey Protein Hydrolyzate for the Protection of Azoxymethane and Dextran Sodium Sulfate Induced Colonic Tumors in Rats

Wafi Attaallah · Ayşe Mine Yılmaz · Nusret Erdoğan · A. Suha Yalçın · A. Özdemir Aktan

Received: 13 September 2011 / Accepted: 20 February 2012 / Published online: 7 March 2012

Abstract Recent studies have shown that whey protein has many useful effects including its anti-cancer effect. In this study we have compared the protective effect of dietary whey protein with whey protein hydrolyzate against azoxymethane and dextran sodium sulfate induced colon cancer in rats. We used a rat model of the colon cancer induced by administration of azoxymethane followed by repeated dextran sodium sulfate ingestion which causes multiple tumor development. Colon tissues were analyzed histologically in addition to biochemical analyses performed by measuring lipid peroxidation, protein oxidation and glutathione levels in both of colon and liver tissues of rats after sacrification. Macroscopic and microscopic tumors were identified in all groups that received azoxymethane followed by repeated dextran sodium sulfate. Group fed with whey protein hydrolyzate showed significantly less macroscopic and microscopic tumor development compared with group fed with whey protein. The protocol applied to generate an appropriate model of colon cancer was successful. Whey protein hydrolyzate was found to be more effective in preventing colon tumor development compared with whey protein.

W. Attaallah (✉) · A. Ö. Aktan
Department of General Surgery, School of Medicine,
Marmara University,
İstanbul, Turkey
e-mail: drwafi2003@yahoo.com

A. M. Yılmaz · A. S. Yalçın
Department of Biochemistry, School of Medicine,
Marmara University,
İstanbul, Turkey

N. Erdoğan
Department of Pathology,
Taksim Education and Research Hospital,
İstanbul, Turkey

Keywords Colon cancer · Azoxymethane · Dextran sodium sulfate · Whey protein · Whey protein hydrolyzate

Colorectal cancer (CRC) is the second leading cause of cancer deaths in the United States, with over 150,000 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 heightened genetic susceptibility as a result of the interaction between multiple genes with low penetrance and environmental exposures [1]. 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 [2]. Accumulating evidence suggests that diet is an important environmental factor in the etiology of CRC [3]. Epidemiological data generally support the association between total energy intake, high fat diets and red meat intake and increased colon cancer risk [4]. The Western-style diet and cooking techniques are risk factors for developing colon cancer [5, 6]. Oxidative stress caused by reactive oxygen species plays a significant role in a number of age-specific diseases such as cancer and neurodegenerative disorders. Some proteins such as whey proteins have also been reported to have the ability to scavenge active oxygen species. Animal studies have shown that whey protein protects against carcinogen induced colon tumors in male rats [2, 7–9].

In addition to proteins, protein hydrolyzates have also been found to exhibit the 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 [4, 10]. Peptides generated from whey protein hydrolysis have been also found to have antioxidant properties [11].

In this study, azoxymethane (AOM) and dextran sodium sulfate (DSS) were used for induction of tumors. The discovery that 1, 2-dimethylhydrazine and its metabolite azoxymethane are specific colon carcinogens paved the way to model non-familial CRC in rodents. It has been shown that AOM-induced CRC mirrors the occurrence of non-familial CRC in humans, including a preponderance of distal colonic tumors that are pathologically similar to sporadic CRC in left-sided human colonic cancers [1].

Oral administration of DSS solution to rodents can cause acute inflammatory reaction and ulceration in the entire colon, and has been employed to recapitulate human ulcerative colitis [12]. Moreover, repeated oral DSS ingestion was shown to cause colon carcinoma in mice when the ingestion is of 7 days' duration and is repeated 9 times [13, 14]. It has also been found that a prior administration of AOM can accelerate and increase the incidence of DSS-induced colon carcinogenesis [15, 16]. In this study, we compared the protective effect of whey protein with whey protein hydrolyzate against AOM and DSS-induced colon carcinogenesis in rats.

Materials and Methods

Animals and Diets

Wistar albino male rats were obtained from Marmara University Experimental Research and Animal Laboratory. Marmara University Animal Ethical Committee approved the protocols used in this study. Rats were 13 weeks of age and their weights ranged from 150 to 340 g. They were housed in cages (five rats/cage) and maintained in an air-conditioned environment of 20 °C with 12 h light–dark cycle. Rats in all groups were fed standard commercial rat chow. All rats were given tap water ad libitum. Induction of colonic cancer in rats was achieved by injection of AOM subcutaneously at a dose of 15 mg/kg, once a week for 2 weeks. Seven days later, 2 % DSS was added to the drinking water over 5 days, followed by 15 days of regular water. This procedure was repeated for a total of 4 times to induce development of tumors in the colon. Figure 1 describes this protocol.

Whey protein and whey protein hydrolyzate were the whey products used in this study. Hilmar™ 8010 is a functional 80 % whey protein concentrate designed specifically by dry-mix applications to enhance dispersibility and quick hydration into solution. It is derived from fresh, sweet dairy whey processed by a special cross-flow filtration, agglomeration and surface treatment. Hilmar™ 8380 is the hydrolyzed form of an 80 % whey protein derived from fresh, sweet dairy whey processed by special cross-flow filtration process. The concentrate is then enzymatically hydrolyzed to produce a mixture of peptides and free amino acids for enhanced nutritional and functional benefits. For standardizing the dose given to rats and to guarantee ingestion, both whey protein and whey protein hydrolyzate solutions were given by orogastric tube under mild ether anesthesia at a dose of 2 ml/rat for 2 times weekly for 15 weeks.

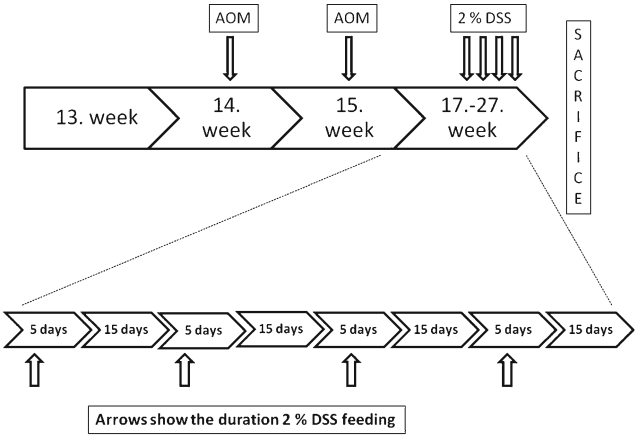

Fig. 1 Tumor induction protocol with azoxymethane (AOM) and 2 % dextran sulfate sodium (DSS)

Rats were divided into 6 groups as shown in Table 1 and were followed for weight-change, bloody diarrhea and survival until sacrification. Weights of rats were measured and recorded weekly. They were sacrified 15 weeks after the last AOM injection. The colon (from cecum to anus) was removed and opened longitudinally. It was then washed and cleaned from its contents with saline. The whole colon was examined for tumors both visually and by palpation. A small sample (400 mg) from distal colon was excised from the lateral edge of the colon and away from tumors without disturbing the integrity of the colon. This sample was used for biochemical analyses. The whole colon was fixed in 10 % neutral-buffered formalin solution for histopathological examination. The liver tissue was resected and wrapped with aluminum foil, and then kept in −70 °C liquid nitrogen for biochemical determinations.

Table 1 Treatment groups, received material and number of rats in each group

Group no.	Group name	Number of rats	Received material(s)
1	Sham	10	Saline
2	Tumor	11	AOM+DSS
3	Tumor+WP	11	AOM, DSS and WP
4	WP	10	WP
5	Tumor+WPH	10	AOM, DSS and WPH
6	WPH	10	WPH

WP whey protein

WPH whey protein hydrolyzate

AOM azoxymethane

DSS dextran sodium sulfate

Histopathological Evaluation

After macroscopic examining of tumors visually and by palpation, the location, the diameter and the multiplicity (the number of tumors per tumor-bearing rat) were recorded. Then, gross tumors were removed and the remaining colon tissue was divided into 2 cm segments for further evaluation of microscopic lesions. Later, paraffin-embedded cross-sections of the colon were examined by routine procedures. Sections of paraffin-embedded colons (5 μm) were stained with hematoxylin and eosine (H&E) for histopathological evaluation. All sections were evaluated by two different pathologists unaware of the groups.

Biochemical Analyses

Oxidative stress markers were measured in colon and liver tissues. These were malondialdehyde (MDA) for lipid peroxidation, protein carbonyls for protein oxidation (PCO) and glutathione (GSH/GSSG). Tissue lipid peroxidation was measured after reaction with thiobarbituric acid spectrophotometrically. Protein oxidation was followed through the oxidant effect caused by formation of carbonyl groups from protein structures. These carbonyl groups react with 2,4-dinitrophenylhydrazine to give the colored product which can be determined spectrophotometrically. The fluorescence intensity of the product formed by the complex of glutathione with o-phthaldialdehyde was measured as a function of time for GSH/GSSG [17–19].

Statistical Analysis

Statistical analysis was performed with SPSS 11.0.1 for Windows statistical program. Kruskal-Wallis analysis for more than two independent groups of data not normally distributed, while Mann–Whitney-U analysis was used for binary comparisons. For comparison of qualitative data between groups (tumor number, presence of adenoma, etc.), Chi-square test and Fisher's exact test were used. In all analyses, $p < 0.05$ was considered statistically significant.

Results

Weight Loss and Bloody Diarrhea

There were no differences among the groups in terms of initial weight, weight gain or loss except for WPH group. WPH group showed a significant weight gain compared with Sham, Tumor and Tumor+WP group ($p=0.0013$). Bloody diarrhea was seen only in one rat in Tumor+WP group at week 15 of the study. During the course of the study, 2 rats of WP group died after ether anesthesia at week 10 and week 12. One rat in Tumor+WPH group died at 2 weeks.

Tumor Incidence and Multiplicity

The injection of AOM which was followed by 4 rounds of 2 % DSS was effective in inducing tumor development in the distal colon. At the end of the study, macroscopic and microscopic tumor development was identified in all groups that received AOM and DSS. In groups which had not received AOM and DSS (Groups 1, 4, and 6), none of the rats developed gross tumors or demonstrated histopathological change. At least one palpable macroscopical tumor was detected in 10 of 11 rats (91.9 %) in both Tumor and Tumor+WP groups. In Tumor+WPH group, macroscopic tumor was found in only 3 of 9 rats (33 %). The difference was statistically significant ($p=0.004$). Multiplicity, which is the number of tumors per tumor-bearing rat, varied between 1 and 3. Mean tumor multiplicity in Tumor+WP group was 1.33±0.5. In Tumor group it was 1.4±0.7 and in Tumor+WPH group it was 2±1. However, there was no significant difference between the means of these groups ($p=0.40$).

Tumor Diameter

Mean tumor diameters in group 2, 3 and 5 were 0.54±0.25, 0.45 ±0.23 and 0.6 ±0.17 cm, respectively. Tumor diameters ranged between 0.1 and 1 cm. No statistical differences were noted in mean tumor diameters between groups receiving AOM and DSS ($p=0.5$).

Macroscopic and Histopathological View of Colon Tumors

Macroscopically flat, nodular, polypoid and/or caterpillar-like tumors were seen. These were mainly located in the distal parts of colon (Fig. 2). Histopathologic evaluation showed different types of lesions such as aberrant crypt foci, early adenoma, late adenoma, diffuse dysplasia, high grade dysplasia, intramucosal carcinoma, invasive adenocarcinoma and signet ring cell adenocarcinoma (Fig. 3). Table 2 lists the pathological findings observed in Tumor, Tumor+WP and Tumor+WPH groups. Adenomas were detected in 90.9 % (10 rats) of the tumor group and in 63.6 % (7 rats) of Tumor+WP group, but the difference was not statistically significant. The difference was significant in the Tumor+WPH group, where 44 % (4 rats) developed adenomas ($p=0.049$). Diffuse colonic dysplasia development was detected in 9 rats of Tumor group (82 %) and it was seen in 10/11 of Tumor+WP group (91 %). Colonic dysplasia was not seen in any rats of the Tumor+WPH group ($p<0.001$). Adenocarcinoma was detected in 2 rats of Tumor group (18 %) and in 1 rat of Tumor+WP group (9 %) and in 2 rats of Tumor+WPH group (22 %). This difference was not significant ($p=0.7$).

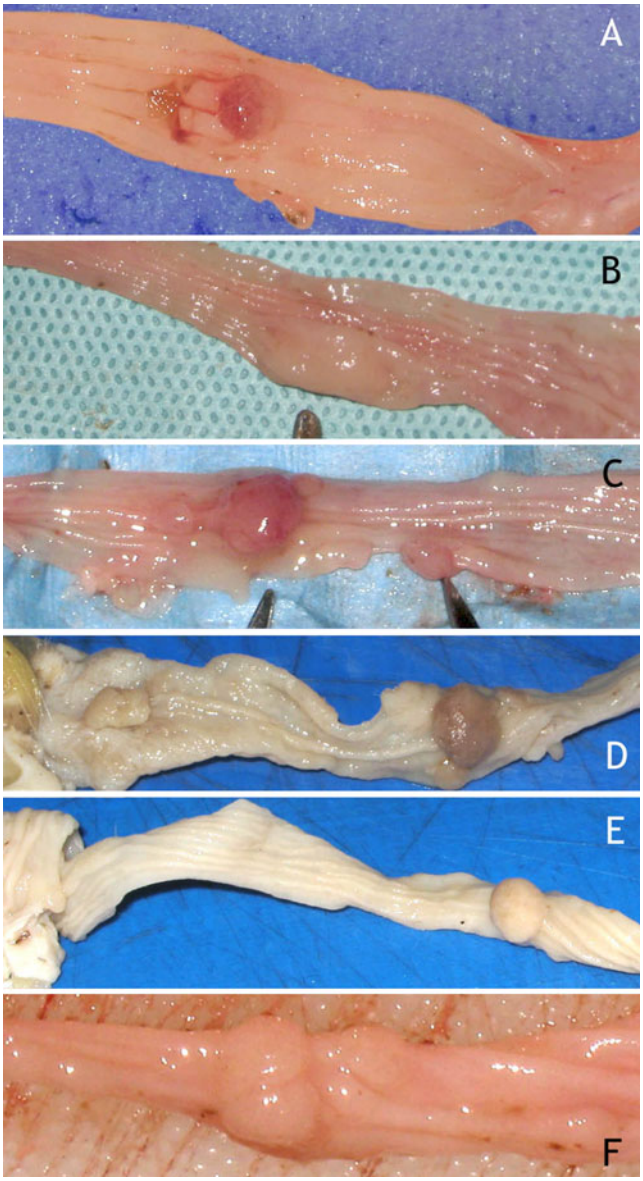

Fig. 2 Macroscopic view of colon tumors: tumors were seen mainly in the distal parts of colon. **a** Nodular **b** flat **c**, **d**, **e** Polypoid **f** Caterpillar-like tumor

Biochemical Analyses

Biochemical evaluations for colon and liver tissue showed no significant change in lipid peroxidation (MDA), protein oxidation (PCO) and GSH/GSSG levels in all groups.

Discussion

Dietary influences on cancer risks have become an increasingly important area of research. The prevention of colon cancer by dietary whey proteins has been studied in mice and rats, but the results are contradictory. McIntosh et al. [9] reported that 1,2-dimethylhydrazine-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 than casein-fed rats, the difference was not significant in these studies, and data on tumor mass were not consistent. Other animal studies showed that whey proteins protect more effectively than red meat, soy bean and casein against carcinogen-induced colon tumor expression in male rats [2, 20]. However, a clear mechanism of how the risk of colon cancer could be reduced by whey protein has not been shown. In this study, whey protein hydrolyzate was found to be more effective in preventing colon tumor development compared with whey protein.

Rats response to AOM-induced cancer exhibits the same non-familial form of CRC in humans as tumors tend to localize distally [1]. In this study, tumors also exhibit the same non-familial form of CRC in humans with localization at the mid and distal colon (0.7–15 cm from anus) and were macroscopically seen as flat, nodular or polypoid. Histopathologically, the cancer type was adenocarcinoma. All of these properties are exhibited in the same non-familial form of CRC in humans. In this study, signet ring cell carcinoma was detected in 2 rats while this was not reported in similar studies before. Colorectal carcinoma is thought to develop from adenomatous polyps by accumulation of some mutations. Today, more than 90 % of colorectal cancers are thought to develop in this way. So the best and most relevant marker of colorectal cancer is adenoma formation [21]. Colorectal carcinogenesis begins with the earliest stage aberrant crypt focus (ACF) and progresses to early adenoma, then to late adenoma, then to intramucosal carcinoma and finally to invasive adenocarcinoma.

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 [15]. In our study ulcerative colitis model was created by giving DSS. Microscopic examination showed active colitis in all rats receiving DSS. Colonic dysplasia development was detected in 9 rats of control group (82 %) and it was seen in 10 of Tumor+WP group (91 %) while it was not seen in any rat of Tumor+WPH group ($p < 0.001$). This indicates that WPH can also prevent colorectal carcinogenesis associated with chronic colitis. In this study, 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 whey protein hydrolyzate-fed rats adenoma incidence was significantly decreased. Adenocarcinoma was detected in only 2 rats of Tumor group (18 %) and in 1 rat of Tumor+WP group (9 %) and in 2 rats of Tumor+WPH group (22 %). This low incidence of adenocarcinoma in our experiments may be due to

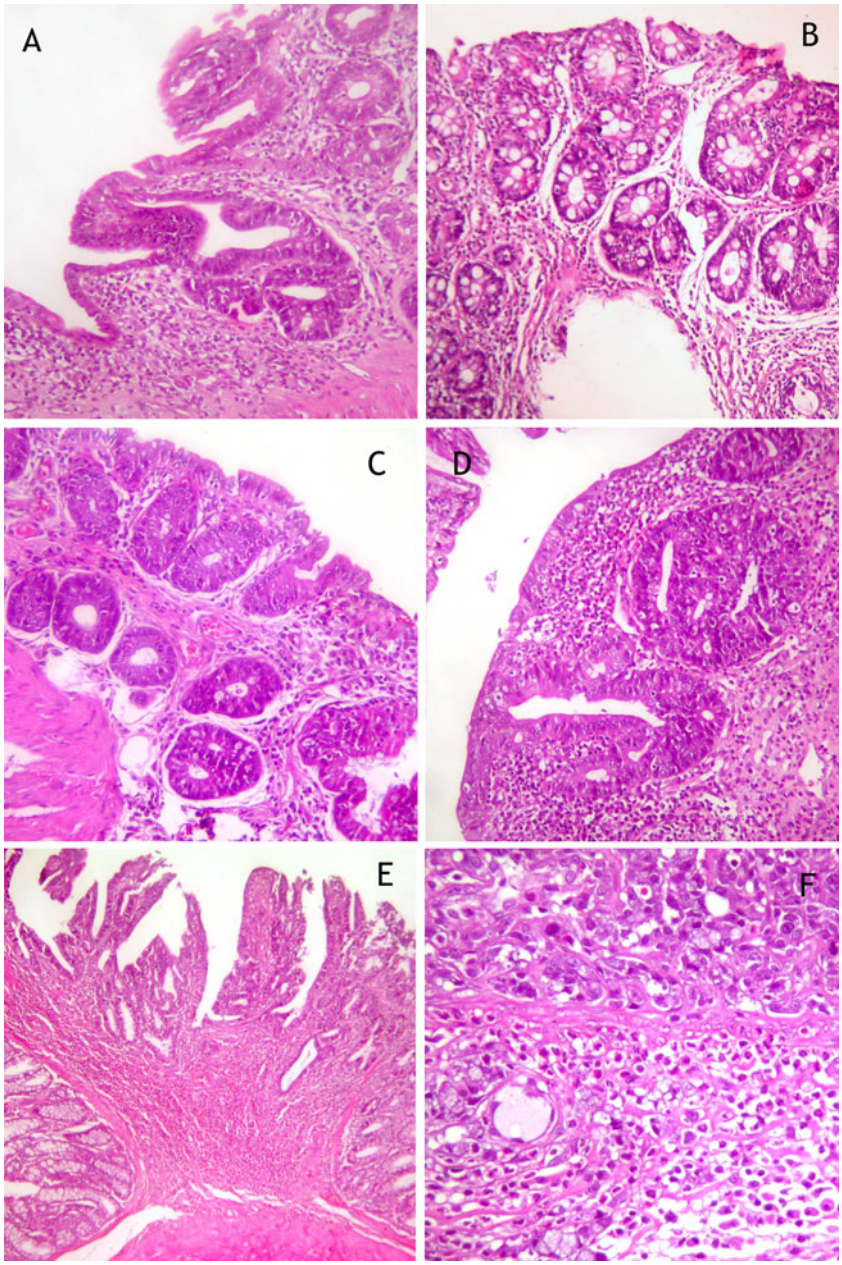

Fig. 3 Histopathological view of colon tumors: different types of tumors observed. **a** Aberrant crypt foci, **b** Early adenoma, **c** Late adenoma, **d** Intramucosal carcinoma, **e** Invasive adenocarcinoma (×100), **f** Signet ring cell adenocarcinoma (×200)

Table 2 Pathological findings and lesions observed in Tumor, Tumor+WP and Tumor+WPH groups

Type of lesion	Tumor	Tumor+WP	Tumor+WPH	P value
Macroscopic tumor	10/11 (% 91)	10/11 (% 91)	3/9 (% 30)	0.004
Aberrant crypt foci,	5/11 (% 45)	3/11 (% 27)	6/9 (% 67)	0.211
Adenoma (early and late)	10/11 (% 91)	7/11 (% 64)	4/9 (% 44)	0.08
Early adenoma	10/11 (% 91)	5/11 (% 45)	3/9 (% 30)	0.019
Late adenoma	3/11 (% 27)	3/11 (% 27)	2/9 (% 22)	0.95
High grade dysplasia	4/11 (% 36)	4/11 (% 36)	0/9 (% 0)	0.110
Dysplasia	9/11 (% 82)	10/11 (% 91)	0/9 (% 0)	< 0.001
Intramucosal carcinoma	2/11 (% 18)	3/11 (% 27)	2/9 (% 22)	0.877
Adenocarcinoma	2/11 (% 18)	1/11 (% 9)	2/9 (% 22)	0.710
Signet ring cell adenocarcinoma	1/11 (% 9)	0/11 (% 0)	1/9 (% 11)	0.546

relatively short period of time that passed from AOM treatment to sacrification. Other investigators studying this colon cancer model used two doses of AOM and sacrificed rats 52 weeks post-AOM injection compared with the 15 weeks post-AOM that we used in this experiment [22].

Whey protein has been shown to reduce the risk of colon cancer and other cancers [23]. The exact mechanism of action of whey protein against colon cancer is not clear. It was shown that whey protein stimulates the immune system [24, 25] by enhancing hepatic glutathione synthesis [26–28] and can act like an antioxidant [29, 30]. Rowlands et al. [31] reported that dietary soy and whey proteins down-regulate expression of liver and mammary gland phase I enzymes which are involved in carcinogen activation. Elevated activities of phase II detoxification enzymes were also reported in soy-fed rats. Such dietary effects may result in lower tissue concentrations of activated carcinogen. The anticancer properties of whey proteins have been ascribed to their ability to elevate cellular levels of glutathione [8]. Increased tissue concentrations of GSH would be predicted to have a protective effect because elevated antioxidant capacity would favor decreased mutagenicity [2].

In conclusion, whey protein hydrolyzate was found to be more effective in preventing colon tumor development compared with whey protein. A direct association with lipid peroxidation, protein oxidation and glutathione was not observed. Therefore, further studies are needed to elucidate the mechanisms responsible for the observed better protective effect of whey protein hydrolyzate.

References

1. Bissahoyo A, Pearsall RS, Hanlon K, Vicky A, Donna H, Virginia LG, David WT (2005) Azoxymethane is a genetic background-dependent colorectal tumor initiator and promoter in mice: effects of dose, route, and diet. Toxicol Sci 88:340–345
2. Hakkak R, Korourian S, Ronis MJJ, Johnston JM, Badger TM (2001) Dietary whey protein protect against azoxymethane-induced colon tumors in male rats. Cancer Epidem Biomar 10:555–558
3. Xiao R, Badger TM, Simmen FA (2005) Dietary exposure to soy or whey proteins alters colonic golbal gene expression profiles during rat colon tumorigenesis. Mol Cancer 4:1
4. Belobrajdic DP, McIntosh GH, Owens JA (2003) Whey proteins protect more than red meat against azoxymethane induced ACF in Wistar rats. Cancer Lett 198:43–51
5. Sandler RS (1996) Epidemiology and risk factors for colorectal cancer. Gastroenterol Clin N 25:717–735
6. Potter JD (1996) Nutrition and colorectal cancer. Cancer Cause Control 7:127–146
7. Bounous G, Papenburg R, Kongshavn PA, Gold P, Fleiszer D (1990) Dietary whey protein inhibits the development of dimethylhydrazine induced malignancy. Tumor Biol 11:129–136
8. Papenburg R, Bounous G, Fleiszer D, Gold P (1988) Dietary whey protein inhibits the development of dimethylhydrazine induced malignancy. Clin Invest Med 11:213–217
9. McIntosh GH, Regester GO, Le Leu RK, Royle PJ, Smithers GW (1995) Dietary proteins protect against dimethylhydrazine-induced intestinal cancers in rats. J Nutr 125:809–816
10. Dryakova A, Pihlanto A, Marnila P, Ladislav C, Korhone HJT (2010) Antioxidant properties of whey protein hydrolysates as measured by three methods. Eur Food Res Technol 230:865–874
11. Contreras MM, Hernández-Ledesma B, Amigo L, Martín-Alvarez PJ, Recio I (2011) Production of antioxidant hydrolyzates from a whey protein concentrate with thermolysin: optimization by response surface methodology. Food Sci Technol 44:9–15
12. Okayasu I, Hatakeyama S, Yamada M, Ohkusa T, Inagaki Y, Nakaya R (1990) A novel method in the induction of reliable experimental acute and chronic ulcerative colitis in mice. Gastroenterology 98:694–702
13. Okayasu I, Yamada M, Mikami T, Kano J, Ohkusa T (2002) Dysplasia and carcinoma development in a repeated dextran sulfate sodium induced colitis model. J Gastroen Hepatol 17:1078–1083
14. Boivin GP, Washington K, Yang K, Ward JM, Pretlow TP (2003) Pathology of mouse models of intestinal cancer: consensus report and recommendations. Gastroenterology 124:762–777
15. Okayasu I, Ohkusa T, Kajiura K, Kanno J, Sakamoto S (1996) Promotion of colorectal neoplasia in experimental murine ulcerative colitis. Gut 39:87–92
16. Popivanova BK, Kitamura K, Wu Y, Kondo T, Kagaya T, Kaneko S, Oshima M, Fujii C, Mukaida N (2008) Blocking TNF-α in mice reduces colorectal carcinogenesis associated with chronic colitis. J Clin Invest 118:560–570
17. Botsoglou NA (1994) Rapid sensitive and specific TBA method for measuring lipid peroxidation in animal tissue, food and feedstuff samples. J Agr Food Chem 42:1931–1937
18. Levine RL, Garland D, Oliver CN et al (1990) Determination of carbonyl content in oxidatively modified proteins. Method Enzymol 186:464–473
19. Browne RW, Armstrong D (1998) Reduced glutathione and glutathione disulfide. Methods Mol Biol 108:347–352
20. Korhonen H, Pihlanto-Leppala A, Rantamaki P, Tupasela T (1998) Impact of processing on bioactive proteins and peptides. Trends Food Sci Tech 9:307–319
21. Fearson ER, Vogalstein BA (1990) Genetic model for colorectal tumorigenesis. Cell 61:759–767
22. Reddy BU, Maruyama H (1986) Effects of dietary fish oil on azoxymethane induced colon carcinogenesis in male F344 rats. Cancer Res 46:3367–3370
23. Bounous G (2000) Whey protein concentrate (WPC) and glutathione modulation in cancer treatment. Anticancer Res 20:4785–4792
24. Bounous G, Papenburg R, Kongshavn PA, Gold P (1998) The immunoenhancing property of dietary whey protein concentrate. Clin Invest Med 11:271–278
25. Bounous G, Batist G, Gold P (1989) Immunoenhancing property of dietary whey protein in mice: role of glutathione. Clin Invest Med 12:154–161
26. Lothian B, Grey V, Kimoff RJ, Lands LC (2000) Treatment of obstructive airway disease with a cysteine donor protein supplement: a case report. Chest 117:914–916
27. Micke P, Beeh KM, Schlaak JF, Buhl R (2001) Oral supplemention with whey proteins increases plasma glutathione levels of HIV-infected patients. Eur J Clin Invest 31:171–178
28. Micke P, Beeh KM, Buhl R (2002) Effects of long term supplemention with whey proteins on plasma glutathione levels of HIV-infected patients. Eur J Nutr 41:12–18
29. Colbert LB, Decker EA (1991) Antioxidant activity of an ultrafiltration permeate from acid whey. J Food Sci 56:1248–1250
30. Tong LM, Sasaki S, McClements DJ, Decker EA (2000) Mechanisms of antioxidant activity of a high molecular weight fraction of whey. J Agric Food Chem 48:1473–1478
31. Rowlands JC, He L, Hakkak R, Ronis MJ, Badger TM (2001) Soy and whey proteins downregulate DMBA-induced liver and mammary gland CYP1 expression in female rats. J Nutr 131:3281–3287