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The effect of 8 weeks of quercetin supplementation and intermittent exercise on gene expression of Muc5Ac, Muc4 and polyphosphate in rats with colon cancer

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Abstract

Background: Expression of mucosal levels would affect the function of internal organs of the body and the digestive system, such as by creating a blockage for the progression of cancerous tumors and the failure of the target tissue, especially the large intestine.

Aim: The purpose of this sudy was to investigate the effect of 8 weeks of quercetin supplementation and intermittent exercise on protein levels of intestinal Muc5Ac, Muc4 and polyphosphate in rats with colon cancer.

Material and Methods: Tewenty-four rats were randomly assigned into four groups including quercetin (n=6), exercise (n=6), quercetin + exercise (n=6) and control group (n=6). Colon cancer induction was provided with the use of 1,2-dimethylhydrazine for 8 weeks and daily quercetin supplementation of 50 mg/kg body weight of mice by Gavagene method. Exercise protocol was performed 5 sessions per week with intensity of 60-70%, maximum speed of 23 m/min with 2-min rest in 8 weeks. ANOVA was used to analyze data. The level of significance was set at $P<0.05$.

Results: It was suggested that there was a significant difference in protein levels of intestinal Muc5Ac, Muc4 and polyphosphate in all groups ($P<0.05$). Furthermore, it was also indicated that Muc5Ac levels was significantly higher in the quercetin+ exercise group other than pther groups ($P<0.05$).

Conclusions: It was concluded that intermittent exercise and quercetin supplementation would increase the levels of Muc5Ac and Muc4 proteins in the large intestine of mice with colon cancer.

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1. Introduction

The global rise of chronic diseases such as cancer has caused serious health concens worldwide. In this resoect, with the increase of health risk factors, the incidence of cancer rises dramatically. One of the most common cancers in men is colorectal cancer (CRC), also known as bowel cancer, colon cancer, or rectal cancer which is the development of cancer from the colon or rectum [1]. One-million people in the world are diagnosed with CRC annually. The change in cellular energy metabolism is one of the most important symptoms of cancer cells [1]. Hereditary and enviornmental factors are both influential factors for the cancer [2].

Based on the last report in Iran, CRC is the third most common cancer among men and the fourth most common cancer among Iranian women. It should be noted that young people in Iran are more susceptible to this cancer than older people [3]. CRC would alter the digestive system's ability to make mucins which are known as genes that can be strongly associated with inflammation and cancer and a variety of viral diseases. However, it is not clearly understood whether the amount or type of mucin produced by colon cancers indicates their biological behavior [4, 5].

Recently, researchers have shown that the development of tumors caused by Aberrant crypt foci (ACF) is associated with MUC5AC expression in rat colon carcinogenesis [6]. Muc4 is mostly observed in lower levels in colon tumors, but in some cases, an increase in expression is found. Its tumorigenic potential is due to the short glycan epitopes in it and the change in binding affinity of MUC4 antibodies, and through Wnt/β-catenin signaling. It is negatively regulated in CRC cell lines and Muc4 expression has decreased during the progression of CRC [7].

The treatment of colon cancer usually involves surgery [8]. Polyphosphate (polyp), which is formed by chains of up to 1000 phosphate, has been shown to increases mucin levels, especially the MUC5AC protein [9]. It has been reported that simultaneous intake of polyphosphate and quercetin causes significant effects [10]. Chemotherapy is one of the main ways of changing cancer tumors; however, it always has relatively serious or minor side effects for other unaffected organs. Therefore, it is necessary to improve treatment noninvasive method with least or non-side effects. In this regard, natural plant chemical applications have been studied for their possible effects on tumor. Various plant polyphenolic compounds have attracted more attention due to their multiple medicinal activities. Flavonoids have potential anti-proliferative, anti-tumor and antioxidant activities which have recommended for the treatment of various diseases including tumors [10, 11, 12]. It has been reported that onions, lettuce, broccoli, tomatoes, tea, berries, olive oil, red grape juice, and apple peel are enriched with flavonoids [13]. Quercetin supplementation would probably increase the expression of mucin protein by affecting the cells of the intestinal goblet [14]. Physical inactivity is associated with a wide range of cancer types.

Based on research evidences, a strong relationship between exercise and the development of this cancer in patients with CRC is found [15, 16]. Given the increasing rate of CRC and importance of preventive strategies along with non-invasive methods, the aim of this study was to determine the effect of 8 weeks of quercetin supplementation and intermittent exercise on protein levels of Muc5Ac, Muc4 and polyphosphate in rats with colon cancer.

2. Materials and Method

Twenty-four healthy male Wistar rats (225-300 gr) were purchased from Pasteur Institute (Tehran, Iran). They were kept at a temperature of 20-23°C with 50% humidity, low noise conditions and a cycle of 12 h light and 12 h dark. Ethical issues declared by laboratory animals protocols was also considered. The research work has been approved by local ethical committee of Imma Khomeini International University as master thesis. Exclusion criteria included getting sick during the research, not being able to continue the protocol. They were randomly assigned into four groups including quercetin (n=6), exercise (n=6), quercetin+ exercise (n=6) and control group (n=6).

2.1. Colon cancer induction

After adaptation phase (2 weeks), colorectal cancer was induced using 1,2-dimethylhydrazine (DMH; Sigma-Aldrich; St. Louis, MO, United States) at a dose of 20 mg/kg body weight once a week by injection. It was administered subcutaneously for 10 consecutive weeks [7].

2.2. Quercetin supplementation

Quercetin is a well-known flavonoid with a broad range of biological functions, including antioxidant, anti-inflammatory, anti-apoptotic, neuroprotective, cardio protective and anti-cancer functions. The preventive and therapeutic effects of quercetin have been extensively evaluated in colorectal cancer, and results from clinical trials are promising. In the quercetin supplementation program, the groups were given the supplement in the form of gavage method, 1 ml per mouse with a dose of 50 mg/kg daily for 8 weeks. They received melioration of 1,2 Dimethylhydrazine (DMH) after the last injection.

2.3. Exercise protocol

During the experiment in the animal laboratory, they were placed on a silent treadmill every day to reduce their stress level and it was tried to eliminate their stress gradually to avoid the negative effect of stress on obtained results. Then, familiarization with exercise protocol was done. This protocol was performed 5 sessions per week for 8 weeks on the treadmill (25 degree incline, each session consisted of 4-10 min bouts of 85-90% VO_2max intensity along with 2-min active rest periods). The treadmill speed was increased until the sixth week (17 m/min), while maintained constantly in the last two weeks (seventh and eighth week; 26 m/min). Also, the periods of active rest increased from the speed of 8 m/min in the first week to 13 m/min in the sixth week and this speed was maintained in the last two weeks. It should be noted that 10 min of warming up and 5 min of cooling down were performed at the beginning and end of each training session [1].

2.4. Protein levels measurement

After the last training session and also implementation of surgical instructions and easy killing processes, blood sampling of rats of all groups was done through intratracheal injection and they were anesthetized with the combination of ketamine 90 mg/kg and zaplan 10 mg/kg. Blood was collected through a direct puncture of the heart and the colon tissue was surgically removed. When the rats were ready for sampling, the whole intestine was removed and spread on filter paper with the lumen side up and fixed in 10% buffered formalin [14].

2.4.1. Measurement of polyphosphate level by fluorometric method

First the sample tissue was homogenized and exposed to colors according to the

laboratory tables according to the kit protocol; In the second step it was incubated and at the end it was kept in the refrigerator for 24 hours. The amount of fluorescent was measured with flowmeter at certain standard wavelengths (20-480-949-1276-2175-2451) and was read with the laboratory formula Ex/Em= 550/415 nm and based on the standard curve. The fluorescent amount was translated into Pico mole,

2.4.2. Measuring the protein levels of Muc5Ac and Muc4

Tissue or cells are lysed in RIPA buffer containing protease and phosphatase inhibitors and centrifuged (13,000 min at a concentration of 5 x 1°C) and protein at a concentration of 4 x 1°C and with Protein Assay Kit Protein lysates (30 µg) were subjected to western blotting and immunoreactivity bands were evaluated using an imaging system,

2.4.3. Measuring the level of Muc5Ac and Muc4 protein levels by immunohistochemistry

Tissue sections were deparaffinized twice in xylene at 30-min intervals, then twice in 100% ethanol for 3 min and in 95% ethanol and 80% ethanol for 1 min. Then, it was hydrated and washed with distilled water and the tissue slices was then placed inside plastic containers filled with citrate buffer 4.7 and covered the containers with perforated film to minimize evaporation, then placed them inside the microwave. Microwave was turned on medium power (600w) for 5 min, in case of evaporative lost liquids; We replaced it with fresh buffer cycle (three repetitions), brought the staining container to room temperature and allowed the slides to cool for 20 min. Slides were washed twice for 5 min in TBST with gentle shaking, then blocked in TBS containing of 10% normal serum and 1% BSA for 2 hours at room temperature. Then, the slides were rinsed for a few seconds.

It should be noted that we did not wash at this stage. We dried around the tissue sections with paper towels and probed the sections with the primary antibody diluted in TBS-BSA for 24 hours at 4°C. The slides were washed 2 times for 5 min in TBS with gentle shaking and then we incubated the slides in H_2O_2-TBS for 15 min, added enzyme-conjugated secondary antibody (diluted in TBS-BSA) to the slides and were incubated for 1 hour at room temperature. We added chromogen (DAB) at room temperature for 10 min and washed with tap water for 10 min for 5 min. Next, we painted the sides (of course, if needed, you can use other instructions in this section. ANOVA tst was used to analyze data. The level of significance was set at alpha $P < 0.05$.

3. Results

In this research, 24 Wistar rats from the Pasteur Institute, weighing 225 to 300 gr and aged nearly 6 months, were tested in four homogeneous test groups. The results are shown in Table 1 for Muc5Ac and Muc4 protein levels by immunohistochemically and western blot methods and polyphosphate is expressed by flowmetry method.

Figure 1 shows the averages of Muc5Ac protein levels and Muc4 gene measured by Western blot and immunohistochemistry methods. Figure 2 shows MUC5ac and Muc4 protein levels for the third sample.

To compare the groups, one-way analysis of variance was used at a significance level of 0.05. Presumption of this test include homogeneity of variances and normal distribution of samples (Shapiro-Wilk test was used and it was $P<0.05$ for all variables). Table 2 shows the results of one-way analysis of variance for the groupsl

Table 1. Descriptive statistics of Muc5Ac and Muc4 protein levels (mean ± standard deviation)

Method	Variables/ Group	Quercetin supplement	Intermittent exercise	Quercetin and Intermittent exercise	Control
Immunohistochemistry	Promoter Muc5Ac (unit)	0.16±1.35	0.24±1.80	0.18±2.13	0.110±1
Western blot		0.49±1.16	0.69±2.04	0.64±2.02	
Immunohistochemistry	Promoter Muc4 (unit)	0.19±1.03	0.19±1.77	0.29±2.02	0.16±0.98

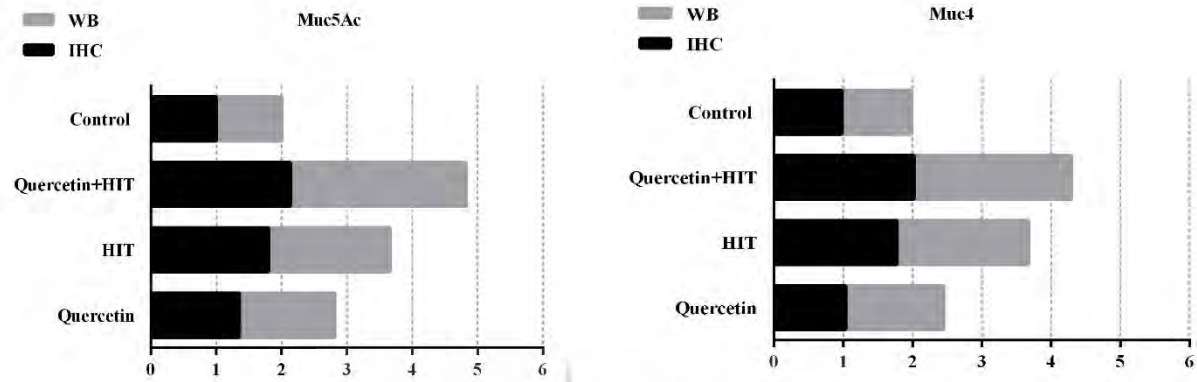


Figure 1. Average of Muc5Ac and Muc4 protein levels measured by western blot and immunohistochemistry

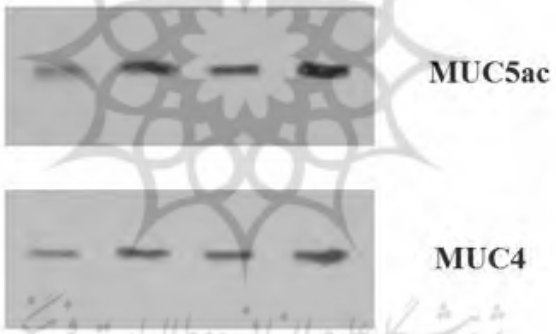


Figure 2a. Image of MUC5ac and Muc4 protein levels (From left to right: the groups compared with the control; The first group is the control. The second is intermittent exercise. The third group is quercetin. The fourth group is intermittent exercise and quercetin.)

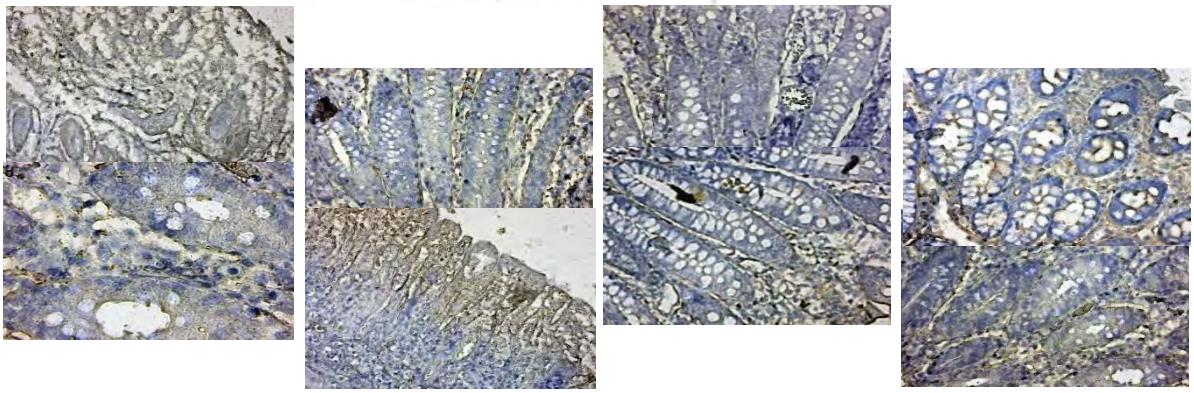
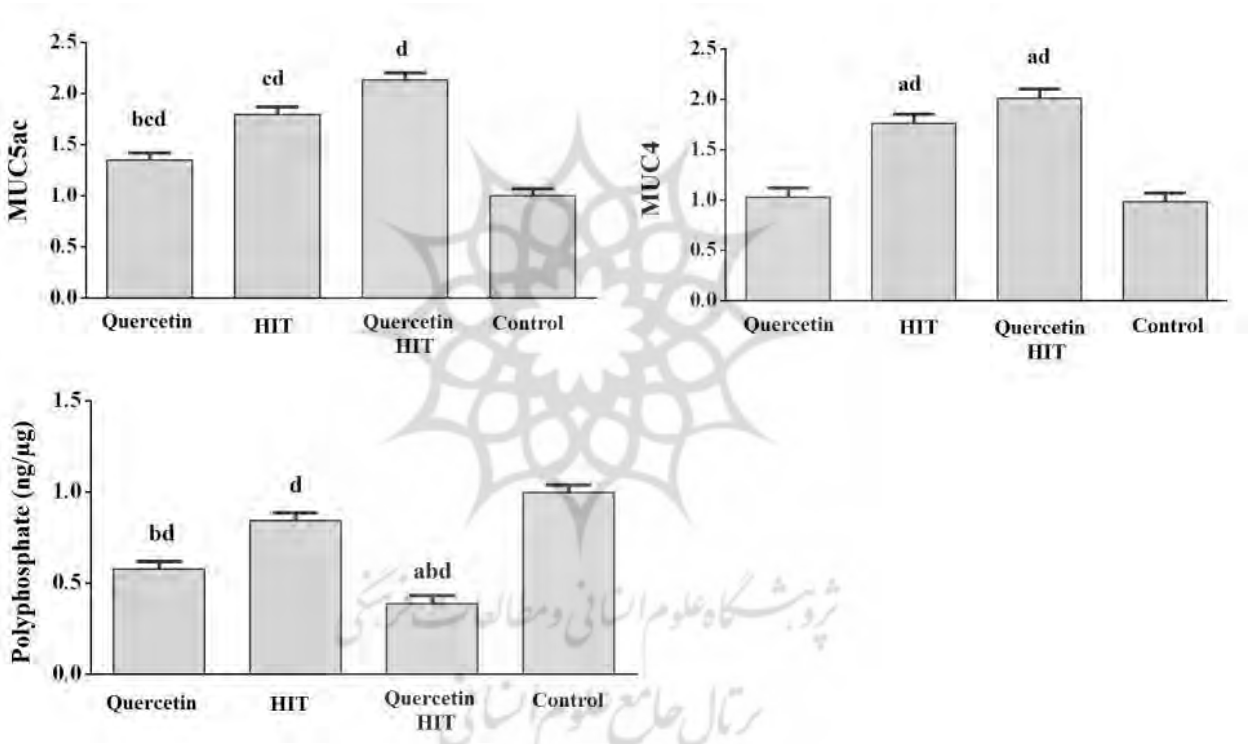


Figure 2b. The surface of mucin proteins is observed to be brown, from left to right in the control group, intermittent exercise, quercetin, intermittent exercise and quercetin.

Table 2. Results of one-way analysis of variance for the test variables of four research groups

Sub scale	Source	Sum of squares	df	Mean square	F	Sig	Effect size
Muc5Ac	Groups	4.46	3	1.49	47.33	<0.001	0.88
	Error	0.63	20	0.03	-	-	-
Muc4	Groups	4.88	3	1.63	35.99	<0.001	0.84
	Error	0.90	20	0.05	-	-	-
Polyphosphate	Groups	1.33	3	0.45	41.82	<0.001	0.86
	Error	0.21	20	0.01	-	-	-

According to the results of Bonferroni test in Figure 3, it was found that the protein levels were significantly different in the groups (P<0.001) and the MUC5ac gene in the intermittent exercise and quercetin group is significantly higher than the quercetin group (P< 0.001), intermittent exercise (P= 0.004) and control (P< 0.001). Also, the MUC5ac levels in the intermittent exercise group was significantly higher than the quercetin group (P<0.001) and the control group (P<0.001).



[a] Significant difference with quercetin group (P<0.05)
[b] Significant difference with intermittent exercise group (P<0.05)
[c] Significant difference with intermittent exercise and quercetin group (P<0.05)
[d] Significant difference with the control group (P<0.05)

Figure 3. Post-hoc Bonferroni test results to compare groups for means in the groups

Finally, the MUC5ac levels in the quercetin group was significantly higher than the control group (P=0.003). Further, the MUC4 levels was significantly different in the four groups (P<0.001). the MUC4 levels in the intermittent exercise group was significantly higher than the quercetin group (P<0.001) and the control group (P<0.001). Also, the MUC4 levels in the intermittent exercise and quercetin group was significantly higher than the quercetin group (P<0.001) and the control group (P<0.001).

Polyphosphate was significantly different in the four groups (P<0.001). Polyphosphate in the intermittent exercise

and quercetin group was significantly lower than the quercetin group (P=0.005), intermittent exercise (P<0.001) and control groups (P<0.001). Polyphosphate in the quercetin group was significantly lower than the intermittent exercise (P<0.001) and control groups (P<0.001). Finally, polyphosphate in the intermittent exercise group was significantly lower than the control group (P=0.018).

4. Discussion

The present study suggested that 8 weeks of quercetin supplementation and intermittent exercise had a positive effect on the expression of Muc5Ac and Muc4, polyphosphate protein levels in the colon of rats with colon cancer, which was positively observed due to the significant difference between the groups in the expression of the mucosal levels, but in the polyphosphate assay. There was no significant difference between the groups. The highest Muc5Ac levels was seen in the intermittent exercise and quercetin group than the other three groups, and respectively, the intermittent exercise and quercetin groups had significant positive expression compared to the control group, Given the second method in relation to western blot protein expression, it was also shown that the group of intermittent exercise and quercetin is more than the two groups of quercetin and control, and the group of intermittent exercise was at the same level of Muc5Ac protein expression.

It can be stated that in the immunohistochemically method, the method of measuring levels by colorimetry from the surface of cells colorectal cancer versus western blot method in measuring protein expression, differences in cell activity steps or errors in the kits and devices are effective. Muc4 levels in the immunohistochemically method in the group of intermittent exercise and quercetin compared to the group other levels were higher and the intermittent exercise group was higher than quercetin. In the western blot method, Muc5Ac protein expression in the intermittent exercise group and quercetin was higher than the intermittent exercise group, but there was no significant difference between the quercetin group and the control group.

According to the obtained results, we come to the point that the consumption of quercetin supplement and intermittent exercise, together or individually, positively induces the expression of Muc5Ac and Muc4 mucus levels. There is a significant difference in the obtained results of the experimental groups compared to the control group. The expression of Muc5Ac mucin levels increased compared to the control group, which was in line with the study of Damiano et al. (2018) [14].

In contrast, another study indicated that polyP-based NPs, both "Mg-polyP-NP" and "Mg-polyP" significantly increased the expression of MUC5AC during the encapsulation period of QCT-NP [10].

Kim et al. (2018) showed that quercetin supplementation with three levels of low, medium and high, respectively, had an effect on the expression of mucin levels [17].

Several studies have reported that quercetin has several medicinal properties, including anti-proliferative activity by stopping at different stages of the cell cycle in a number of colon cancer cells.

Shree et al. (2021) examined 32 female Wistar rats divided into four random groups with the quercetin supplementation program with doses of 25 and 50 mg/kg/body weight. The results showed a decrease in the mucous layer and disintegration of goblet cells (mucus producer) [18].

Consistent with our study, Volstatova

et al. (2019) applied quercetin supplement and came to the conclusion that this supplement can regulate membrane model mucin and increase its expression [19].

In a heterogeneous study conducted by Barcelo et al. (2018), it was shown that concentrations up to 20 mm were not able to increase the mucosa of the colon tissue [20]. The expression of the Muc5Ac levels e is the most preferred mucosal levels e due to its association with colon polyps and cells. This protein is expressed by the goblet cells of the intestine [18].

There is evidence that colon cancer causes a change in the intestinal state of the goblet cells of the large intestine, which can cause mucus levels to flow in the large intestine [15]. In explaining the mechanisms involved in obtained results, it would be stated that one of the mechanisms of the antioxidant activity of quercetin is the inhibition of the enzymes that activate carcinogens, and the regulation of intracellular signal transmission pathways. It is the second most prominent flavonoid in plant species, which is found in fruits and most vegetables, and is considered as a cancer prevention compound [16].

It should be noted that the relationship between dietary fibers and quercetin and the expression of mucus genes needs more studies, and normal tissue homeostasis requires the precise regulation of mucin expression. Therefore, understanding the molecular mechanisms of mucin deregulation is necessary to devise appropriate therapeutic methods to combat mucin-related pathologies.

5. Conclusion

According to the obtained results, the use of quercetin supplement alone increased the levels of Muc5Ac and Muc4 significantly in the cancerous colon, as a result, quercetin supplement can be used to increase the expression of mucosal levels, and intermittent exercise also increased the levels of Muc5Ac and Muc4 significantly. Therefore, intermittent exercise is one of the most effective treatments available to improve the levels of these proteins in cases of drug contraindications. Also, the results of this research showed that the use of quercetin supplement and intermittent exercise together would have a more significant improvement on the levels of Muc5Ac and Muc4 proteins.

Conflict of interest

The authors declared no conflicts of interest.

Authors' contributions

All authors contributed to the original idea, study design.

Ethical considerations

The authors have completely considered ethical issues, including informed consent, plagiarism, data fabrication, misconduct, and/or falsification, double publication and/or redundancy, submission, etc.

Data availability

The dataset generated and analyzed during the current study is available from the corresponding author on reasonable request.

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