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Anti-Tumor Effect of the Ketogenic Diet against DMH-Induced Colon Cancer in Rats

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ABSTRACT

Colon cancer is one of the most common malignancies with significant importance. Recent theories believe that cancers are metabolic diseases. Therefore, the role of metabolism in the prevention and treatment of cancer has been considered and the ketogenic diet is one example. In the present study, we evaluated the effect of the ketogenic diet and a high carbohydrate diet on tumor size and number, histopathology, and insulin level as well as VEGF level in 1, 2 dymethylhydrazine (DMH)-induced colon cancer in rats. Forty adult male Wistar rats were divided into four groups as follows: control, colon cancer, ketogenic diet, and high carbohydrate diet groups. For induction of colon cancer, 30 mg/kg of 1,2 DMH solution was injected subcutaneously twice a week for 24 weeks. The results showed that the ketogenic diet reduced tumor size, number, and histopathological changes as well as VEGF level ($P<0.01$) compared to the colon cancer group. The ketogenic diet also increased the levels of beta hydroxyl butyrate ($P<0.001$) and decreased those of glucose, insulin and HbA_{1c} ($P<0.001$). Furthermore, a high carbohydrate diet did not show any protective effects on colon cancer prevention. In conclusion, the ketogenic diet demonstrated prophylactic effects on colon cancer, and this anti-cancer effect could be partially attributed to the reduction in VEGF and insulin levels.

Keywords: ketogenic diet, colon cancer, VEGF, high carbohydrate diet, insulin.

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INTRODUCTION

Colon cancer is the third leading cause of death in the world, with an increasing incidence in developing countries. Colon cancer is caused by genetic or epigenetic alterations of colon epithelial cells. With an abnormal increase in the proliferation and growth of these cells, a benign adenoma develops that over time turns into cancer and metastasis occurs (1, 2). Various therapies have been used to treat or inhibit colon cancer, including non-steroidal anti-inflammatory drugs (NSAIDs) such as solindac (3), bisphosphonates (4), and angiotensin converting enzyme inhibitors (5). However, due to drug resistance and the recurrence of colon cancer, as well as the numerous side effects and interactions of these drugs, there is an urgent need for adjuvant therapies (1).

Diet can exacerbate or prevent colon cancer because it directly affects the diversity and number of intestinal microbiota as well as inflammation of intestinal cells (6). The ketogenic diet is one of the most important diets used in colon cancer (7). A ketogenic diet is a process that stimulates hunger and, by restricting carbohydrates, forces fat to be burned as the main source of energy, which leads to the production of ketones (8). Cancer cells are not able to use ketones to produce energy. In fact, the ketogenic diet affects glucose metabolism, which due to the high need for glucose in cancer cells, causes metabolic disorders in these cells and inhibits their growth and proliferation (9). On the other hand, lowering glucose levels and inhibiting the lactate-pyruvate cycle reduces the level of vascular growth factor and angiogenesis, which leads to necrosis of colon cancer cells (10). It was also demonstrated that some ketone bodies, such as beta-hydroxybutyrate prevents the development of cancerous tumors by reducing inflammation and oxidative stress (9, 10). It was reported that hyperglycemia increases the production of reactive oxygen species, which increases inflammation and oxidative stress and thus damages the DNA of intestinal cells. (11, 12). On the other hand, hyperinsulinemia induces cancer by affecting the growth of cancer cells, stimulating their proliferation, and reducing apoptosis (13, 14). However, the present *in vivo* study was aimed to clarify the effects of ketogenic diet on insulin level, histology, tumor size, and number as well as VEGF level in DMH-induced colon cancer in rats. ❑

MATERIALS AND METHODS

Animals

Forty healthy adult male Wistar rats (150 g ± 30 g) were used in this study (n=10 in each group). All animals were housed in Animal House of Urmia University of Medical Sciences, under standard condition (temperature of 22 ± 2, humidity of 50 ± 10%, and 12 hours light/12 hours dark). This study was approved by the ethics committee of Urmia University of Medical Sciences (Ethics code: IR.UMSU.REC.1398.183).

Experimental protocol

All rats were randomly divided into four groups (n=10 in each group). Rats in group 1 (control) were left untreated for the whole period of the experiment. Rats in group 2 (colon cancer) received 30 mg/kg of DMH solution subcutaneously (*sc*) twice a week, for 24 weeks, and were fed with standard rodent diet. Rats in group 3 (ketogenic diet group) received 30 mg/kg of DMH solution *sc* twice a week, for 24 weeks, and were fed with the ketogenic diet, starting two weeks before the DMH injection. Rats in group 4 (high carbohydrate diet group) received 30 mg/kg of DMH solution *sc* twice a week, for 24 weeks, and were fed with high carbohydrate diet, starting two weeks before the DMH injection (Table 1).

Induction of colon cancer

DMH-induced colon cancer model was used for induction of cancer. At first, the DMH powder was dissolved in 5% DMSO and the pH was ad-

TABLE 1. Composition of each 100 grams of the standard, ketogenic and high carbohydrate diets used in this study

	Standard diet	Ketogenic diet	High carbohydrate diet
Starch	47.3	0	23.7
Coconut flour	0	20	0
Sucrose	11.5	0	56
Caseinate*	22.7	32	11.3
Whey*	0	8	0
Lard	10.2	0	5.1
Corn oil	2.3	0	1.1
Ghee	0	30	0

*We used milk protein concentrate (MPC) powder with 65 % protein (80% casein and 20% whey) in the composition of the ketogenic diet.

justed to 6.5. After that, dymethyl sulfoxide (DMSO) was injected *sc* under the abdominal part of the body at a dose of 30 mg/kg/day. The injection was performed twice a week for 24 weeks (15).

Tumor size and number in the colon

Anesthetization of rats was performed with a mixture of xylazine (10 mg/kg) and ketamine (60 mg/kg); the abdominal part was opened and the colon was removed from the body of the rats. The colon was irrigated with normal saline. In the next step, DMH-induced polyps were counted and their sizes were measured by a digital caliper (mm).

Histopathological studies

After isolation in 10% formalin buffer, colon tissues were then embedded in paraffin and sectioned at 5 µm thick sections. Hematoxylin and eosin (H & E) staining was performed for histopathological examinations.

VEGF assay

The level of serum VEGF was measured according to the instructions of the DuoSet ELISA kit (CA no: DY564).

Enzymatic measurements

Glucose and HbA_{1c}, β-hydroxybutyrate, and insulin levels were assessed in serum by the corresponding kit using the manufacturer's instructions.

Statistical analysis

Data were expressed as mean ± S.E.M. Statistical significance was determined by One-way ANOVA with Tukey post-test. Values of $P<0.05$ were considered statistically significant. ❑

RESULTS

Body weight changes as well as number and size of polyps in rats with colon cancer in different groups

Our study demonstrated that the final weights of animals at the end of the study increased by 89% in the normal control group, 113% in the colon cancer group, 88% in the ketogenic diet group, and 113% in the high carbohydrate diet group. As seen in Figure 1A, a ketogenic diet prevented weight gain compared to the colon cancer group ($P<0.01$). Our results showed that the number and size of polyps significantly increased in the colon cancer group as compared to the normal control group ($P<0.01$). The ketogenic diet demonstrated protective effects against DMH-induced colon cancer. As shown in Figure 1B and 1C, animals in the ketogenic diet group had a reduced number ($P<0.05$) and size ($P<0.05$) of polyps compared to the colon cancer group. The high carbohydrate group had

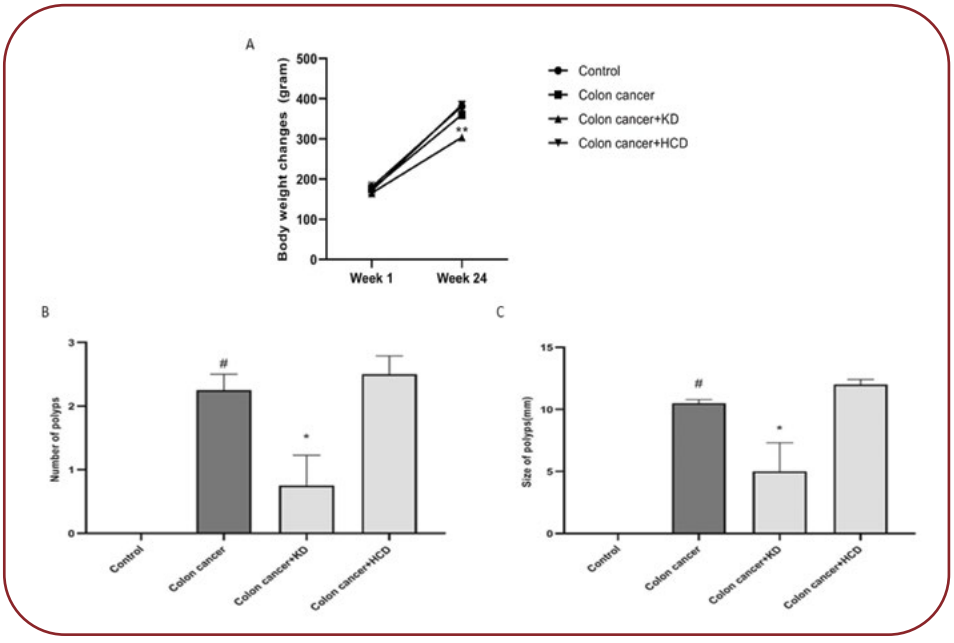

FIGURE 1. A: Body weight changes of rats at the end of the study compared to initial weights. **B, C:** Number and size of polyps in normal control, 1,2 dimethylhydrazine-induced colon cancer, ketogenic diet and high carbohydr *vs.* ate diet groups. Values are mean ± SEM; #P< 0.01 *vs.* the control group. *P< 0.05; **P<0.01 *vs.* The colon cancer group using one-way ANOVA with Tukey post-test. KD=ketogenic diet; HCD=high carbohydrate diet.

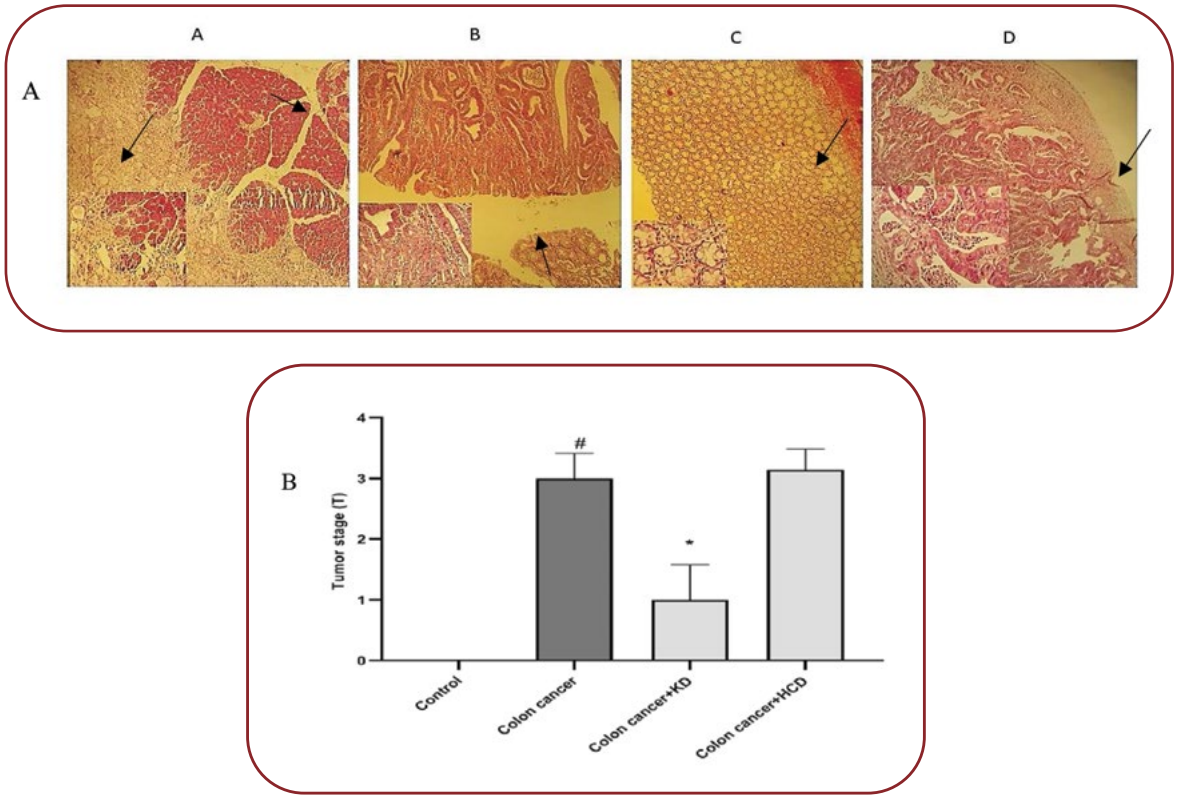

FIGURE 2. A. Photomicrographs of histopathological changes in colon tissue in different groups. (H&E staining). **A:** right arrow: normal pancreas/left arrow: pancreas tissue involved by tumoral omentum (locally advanced colon carcinoma), **B:** polyp involved by carcinoma, **C:** normal colonic tissue, **D:** polyp involved by carcinoma (reduction of size and number of polyps in HCD group). Magnification 10×; lower left box shows magnified tissue; magnification 40×. **B:** Graph of tumor stage. Values are mean ± SEM; #P< 0.001 *vs.* control group. *P< 0.05 *vs.* the colon cancer group using one-way ANOVA with Tukey post-test.

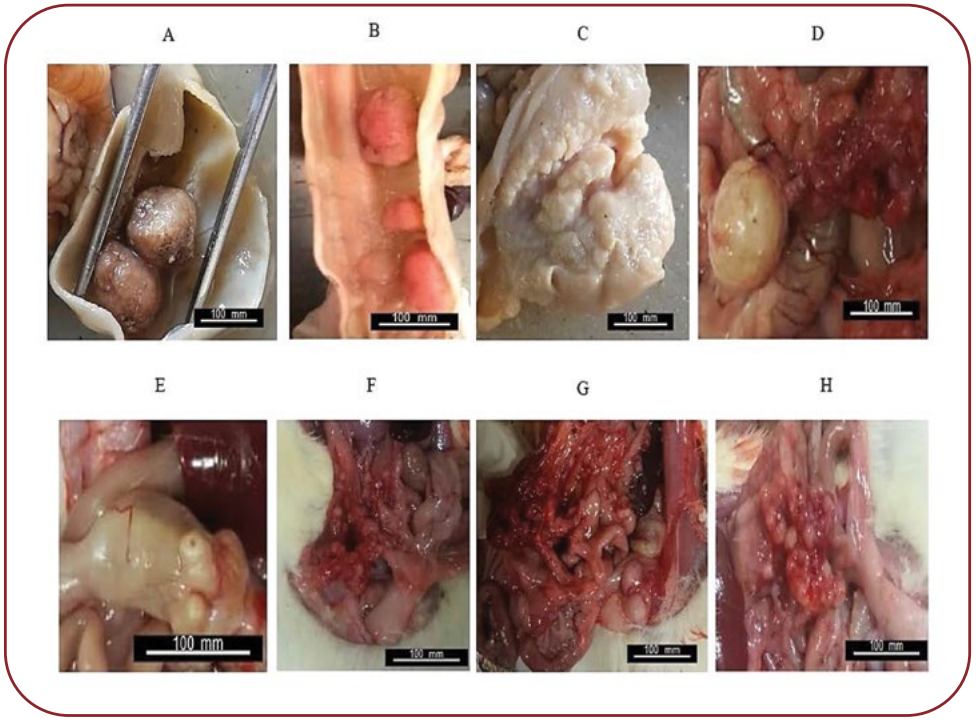

FIGURE 3. Gross images of intact colons. **A, B:** colon polyps; **C:** small intestine adenocarcinoma; **D:** colon tumor with omentum carcinomatosis; E: colon mass (colon adenocarcinoma without polyps); **F, G, H:** carcinomatosis of colonic adenocarcinoma origin. Scale bar denotes 100 mm (macro).

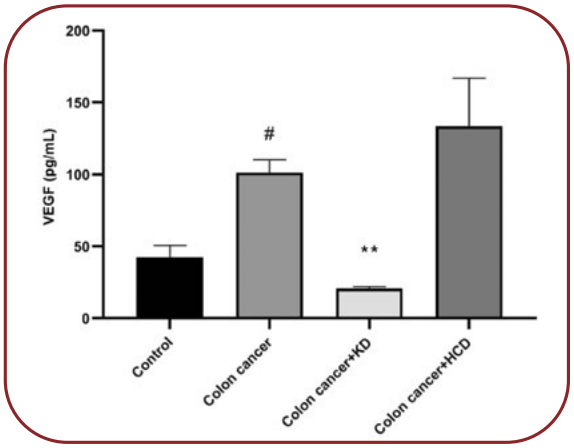

FIGURE 4. VEGF level in the normal control, DMH-induced colon cancer, ketogenic diet and high carbohydrate diet groups (n=10). Values are mean ± SEM; #P< 0.05 *vs.* the control group. **P< 0.01 *vs.* the colon cancer group using one-way ANOVA with Tukey post-test. KD=ketogenic diet; HCD=high carbohydrate diet; VEGF=vascular endothelial growth factor.

no effect on the number and size of tumors, although it did increase them to some extent.

Histopathological examination of the colon tissues

In the histopathological examination, no signs of polyps were observed in samples of the control group, whereas in the group of colon cancer, abundant polyps and adenocarcinoma with metastasis to pancreatic tissue were seen. H&E staining of colon tissues showed that ketogenic diet decreased tumor stage (T) (P<0.05) compared to the colon cancer group (Figure 2). The gross images of colon polyps are shown in Figure 3.

Ketogenic diet reduces VEGF level in DMH-induced colon cancer

In our study, the level of serum VEGF was measured as a potent angiogenic factor with an important role in colon cancer progression. As shown in Figure 4, the level of VEGF increased significantly (P<0.05) in the colon cancer group compared to the control group. Rats with a ketogenic diet showed a decreased level of VEGF (P<0.01) compared to the colon cancer group (Figure 4).

The effects of the ketogenic diet and high carbohydrate diet on glucose, HbA_{1c} and insulin levels in DMH-induced colon cancer

Our results show that the glucose (Figure 5A) and HbA_{1c} (Figure 6B) levels in the DMH-induced colon cancer group were significantly higher than in the control group (P<0.001). A high carbohydrate diet increased both glucose (P<0.001) and HbA_{1c} (P<0.001) levels compared to the colon cancer group. Interestingly, the ketogenic diet reduced glucose levels from 137.6±4.6 mg/dL

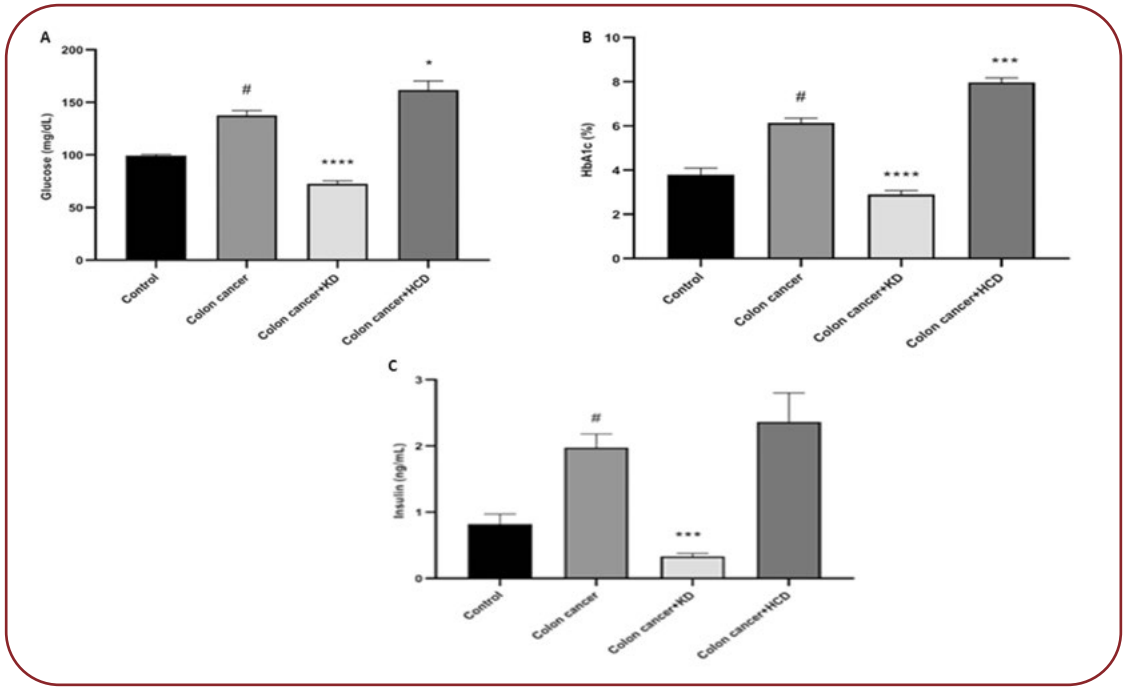

FIGURE 5. Glucose (A), HbA_{1c} (B), and insulin (C) levels in the normal control, DMH-induced colon cancer, ketogenic diet and high carbohydrate diet groups (n=10). Values are mean ± SEM; #P< 0.001 *vs.* the control group. ***P<0.001 and ****P<0.0001 *vs.* the colon cancer group using one-way ANOVA with Tukey post-test. KD=ketogenic diet; HCD=high carbohydrate diet.

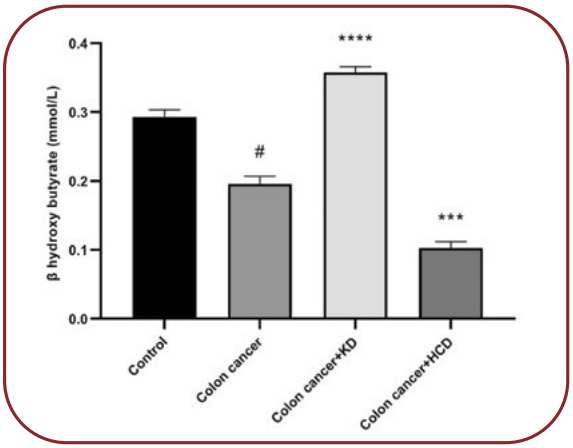

FIGURE 6. β-hydroxybutyrate level of normal control, DMH-induced colon cancer, ketogenic diet and high carbohydrate diet groups (n=10). Values are mean ± SEM; #P< 0.0001 *vs.* the control group. ***P< 0.001 *vs.* the colon cancer group using one-way ANOVA with Tukey post-test. KD=ketogenic diet; HCD=high carbohydrate diet.

in the colon cancer group to 72.5±3 mg/dL (P<0.0001) as well as HbA_{1c} levels from 6.1±0.2 in the colon cancer group to 2.9±0.17% (P<0.0001). In the colon cancer group, the level of insulin was significantly increased to 1.97±0.2 ng/mL from 0.82±0.15 ng/mL in the normal control group (P<0.01). Giving a ketogenic diet to rats lead to a significant (0.33±0.04; P<0.001) reduction in insulin levels compared to the DMH-induced colon cancer group (Figure 5C).

The effects of the ketogenic diet and high carbohydrate diet on β-hydroxybutyrate level in DMH-induced colon cancer

In this study, the level of β-hydroxybutyrate was measured as an important ketone body. As shown in Figure 6, the β-hydroxybutyrate level decreased from 0.29±0.01 mmol/L in the control group to 0.19±0.01 mmol/L in the colon cancer group (P<0.0001). As expected, the ketogenic diet increased the β-hydroxybutyrate level to 0.35±0.008 mmol/L (P<0.0001), whereas the high carbohydrate group reduced it to 0.1±0.009 mmol/L (P<0.001) compared to the colon cancer group (Figure 6). ❑

DISCUSSION

In this study, we investigated the effects of the ketogenic diet and high carbohydrate diet on colorectal cancer in rats. The results showed that a ketogenic diet reduced the incidence of colon cancer and also caused decrement in the size and number of tumors. The protective effect of a ketogenic diet could be attributed to the reduction of VEGF, glucose, HbA_{1c} and insulin level. The high carbohydrate diet not only showed no protective effect, but also increased some of these factors.

Among various risk factors affecting the growth and development of cancer cells, particularly colon cancer, diet and nutrition seem to play critical functions in this process. Ketogenic and low carbohydrate diet is one of the most popular dietary approaches for preventing cancer (7, 9). The ketogenic diet has previously been used as adjunctive therapy in the treatment of glioblastoma as well as for the prevention of lung, pancreatic, prostate, and colon cancers (10). Cancer cells need very high amounts of glucose to grow and multiply. Therefore, using a ketogenic diet reduces the energy level required by cells and targets glucose metabolism thus inhibiting their growth and proliferation. On the other hand, the ketogenic diet can reduce the incidence of tumors by producing ketone bodies in the liver, due to abnormal mitochondrial function and decreased enzyme activity for ketone consumption. Cancer cells, unlike normal cells, are not able to use ketone bodies to produce energy. In addition, it was demonstrated that some ketone bodies, such as beta-hydroxybutyrate, prevents the development of cancerous tumors by reducing inflammation and oxidative stress (9, 10, 17). There are an accumulating number of evidence demonstrating the positive effects of ketogenic diet in preventing cancer progression. For example, in a study by Hagihara *et al* it was reported that the use of a ketogenic diet in patients with advanced cancer for three months caused an increase in total ketone body levels and decreased fasting blood sugar, insulin, and C-reactive protein (CRP) levels, which significantly elevated the survival rate of those patients (18). The ketogenic diet was reported to significantly reduce prostate-specific antigen (PSA) levels as a tumor marker and also enhanced lipid profile; therefore, it can be mentioned as one of the most important supportive treatments (8, 18, 19). A study showed that the use of ketogenic diet in patients with uterine and breast cancer reduced insulin levels, increased beta-hydroxybutyrate levels and maintained patients' muscle mass, which could be associated

with increased insulin sensitivity. Elevated levels of beta-hydroxybutyrate also provides an unusable metabolic environment for cancer cells and inhibits their growth (20). It has been demonstrated that concomitant use of ketogenic diet and rapamycin significantly reduced glucose and insulin levels and significantly increased beta-hydroxybutyrate in mice with breast cancer. Decreased tumor growth and metastasis occurred in rapamycin alone and in combination with a ketogenic regimen, but the decrease was more perceptible in the combination group (21). It has already been observed that increasing fiber intake was inversely related to the incidence of colorectal cancer. High fiber intake increases the butyrogenic activity of gut microbiota; as a result, high amounts of butyrate production are stimulated, which has widespread anti-cancer effects. (16). The results of our study inconsistent with previous studies showed that the use of a ketogenic diet could reduce the incidence of colon tumors through the elevation in beta-hydroxybutyrate levels.

A recent study on the effects of a ketogenic diet on colon cancer, inconsistent with our findings, demonstrated that the use of a ketogenic diet in mice increased the diversity of intestinal microbiota and decreased the level of inflammatory cytokines, which ultimately reduced the size and number of small intestinal and colon polyps (22, 23). Systemic insulin resistance is associated with elevated insulin levels and eventually, hyperglycemia. The occurrence of hyperglycemia stimulates tumor growth through cellular apoptosis and epigenetic changes in DNA. On the other hand, insulin resistance and the development of hyperinsulinemia lead to increased growth factors, which stimulate intracellular signaling and lead to various cancers, including breast, prostate, liver, pancreas, and colon cancers (18). In the present study, measurement of insulin and blood glucose levels in rats demonstrated that in the colon cancer and high carbohydrate groups there was a significant rise in insulin and glucose levels compared to the control group, but in the ketogenic diet group, both levels were significantly lower than other groups. The ketogenic diet also improves the symptoms of cancer by helping to maintain balanced body weight and muscle strength. Despite the insufficient level of carbohydrates in cancer cells due to the consumption of ketogenic diets, healthy cells such as muscles use ketone bodies to produce more energy and higher levels of ATP through mitochondria (10). Ketone bodies may also modulate signaling molecules and reduce inflammation, which may have cancer benefits (24). The anti-inflammatory profile of the ketogenic diet, especially beta-hydroxybutyrate, also prevents proteolysis and muscle breakdown (10). It has also been proven that weight gain by 4 kg can increase the risk of colon cancer by up to five times, even in people over the age of 50. Gaining every kilogram of body weight increases the person's risk of colon cancer by 60%. On the other hand, a direct relationship has been found between the increasing waist size and increment of the colon cancer incidence, which emphasizes the importance of weight management to prevent colon cancer (25, 26). In the present study, the weight gain of rats in the ketogenic diet group was much less than other groups, showing the usefulness of this diet in the management of weight gain, which could be one of the possible mechanisms of reducing colon cancer risk.

Attenuation of VEGF expression levels is another potential mechanism suggested by various studies for anti-cancer effects of a ketogenic diet. It leads to a reduction of vascular permeability, angiogenesis, and metastasis of cancer cells. The use of a ketogenic diet (high fat, moderate protein, and low carbohydrate) shifts the energy source from glucose to ketone bodies and reduces glucose supply to cancer cells, thereby resulting in a reduced level of peritoneal dissemination (colorectal cancer metastasis) and intestinal ascites (27). A study by Nakamura *et al* (2018) reported the protective effects of the ketogenic diet on colon cancer. The use of a high fat, low carbohydrate diet resulted in no weight loss or muscle tone in cancer in mice. Additionally, the level of plasma inflammatory factors, ketone bodies level, and tumor weight in the diet group was much lower than in the control group (28). In agreement with these studies, our results showed a significant decrease in VEGF levels when using a ketogenic diet, which could be one of the potential mechanisms of the protective effect of this diet in colon cancer. ❑

CONCLUSION

Our results suggest that a ketogenic diet may be effective as adjunctive therapy in colon

cancer, and these effects are partly attributed to elevation in beta-hydroxybutyrate levels and lower insulin and VEGF levels. ❑

Conflicts of interest: none declared.

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