Anti-tumor effect of memantine, an N-methyl-D-aspartate receptor antagonist, against DMH-induced colon cancer in rats

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ABSTRACT: Glutamate levels are significantly higher in colon cancer cells than in normal cells. Increased expression of N-methyl-D-aspartate (NMDA) receptors has been observed in tumor cell lines that cause angiogenesis. Vascular endothelial growth factor (VEGF) promotes proliferation and endothelial migration through the calcium influx. As a result, NMDA receptors may be a therapeutic target of cancer, and inhibition of these receptors may reduce tumor growth. In this study, the effects of memantine, an NMDA receptor antagonist, on histology, tumor size, and number, as well as VEGF level in 1,2 dimethylhydrazine (DMH)-induced colon cancer in rats were investigated. Thirty male Wistar rats were divided into three groups: the control group, the colon cancer group (30 mg/kg of DMH solution was injected subcutaneously twice a week for 24 weeks), and the memantine group (20 mg/kg). The results showed that the injection of DMH induced colon polyps ($P<0.001$) in the colon cancer group, but memantine 20 mg/kg showed protective effects and reduced the number and size of colon polyps ($P<0.001$). The level of VEGF also increased significantly ($P<0.05$) in the colon cancer group compared to the control group. Treatment with memantine 20 mg/kg/day reduced VEGF level significantly ($P<0.01$) in comparison to that of the colon cancer group. The present *in vivo* study, for the first time, showed the anti-cancer effects of memantine in colon cancer, which can be attributed partially to a reduction in VEGF level.

KEYWORDS: NMDA receptor; Memantine; Colon cancer; VEGF; DMH.

1. INTRODUCTION

Colorectal cancer is currently the third-leading cause of cancer death in the world, and its incidence is increasing rapidly, especially in developing countries [1]. If it is diagnosed early, surgical methods are usually used, but in case of delay in the diagnosis and progression of the disease, surgery will no longer be effective. Therefore, identifying the pathophysiological pathways of the disease and discovering new treatments is of great importance [2]. In colon cancer cells, glutamate and aspartate levels are much higher than normal and even other tumor cells, including gastric tumors [3]. Glutamate is one of the most important neurotransmitters in the central nervous system (CNS) that is involved in various physiological and pathological processes. For example, an increase in glutamate levels can lead to the death of nerve cells and various neurological diseases.[4,5]. In addition to these effects, various studies have shown its effects on the proliferation and migration of cells [6,7]. Since uncontrolled growth and proliferation are characteristic of neoplastic cells, glutamate can be considered as an important growth factor in tumor development, even in non-neuronal cancers [4,8]. N-methyl-D-aspartate (NMDA) receptors are one of the main glutamate receptors in most tumor cells outside the CNS. Increased expression of NMDA receptors has been observed in cell lines related to

gastric [9,10], esophageal [11], prostate [12], thyroid [13], breast [13,14], laryngeal [15], lung [16-18], colorectal [13,19], and hepatocellular cancer [19,20]. Therefore, NMDA receptors can be considered as one of the therapeutic targets of cancer [21]. NMDA receptors, as part of calcium channels, also play a crucial role in angiogenesis. Vascular endothelial growth factor (VEGF) usually promotes capillary formation, proliferation, and endothelial migration through the calcium influx. Therefore, an increase in intracellular calcium concentrations accelerate the capillary formation and endothelial migration [22,23]. If the expression of NMDA receptors reduces, it leads to a decrease in endothelial angiogenesis, which can be due to the reduction of the calcium influx to endothelial cells and endothelial function mediated by VEGF [24]. It has been shown that inhibiting NMDA receptor activity can decrease tumor growth. Ketamine (NMDA receptor inhibitor) prevents cell migration and inhibits VEGF expression in colorectal cancer by inhibiting receptors and lowering intracellular calcium levels [25]. In *in vitro* study, dizocilpine (MK-801) as an NMDA receptor antagonist reduces the growth of tumor cells. It inhibits metastasis and malignancy of tumors in pancreatic cancer by decreasing the amount of VEGF and intracellular calcium, respectively [26]. Another study showed that dizocilpine maleate and ifenprodil Hemi tartrate as NMDA antagonists significantly reduces the viability of tumor cells and decreases the growth of pancreatic tumor xenografts in mice [27]. *In vitro* studies with memantine as an NMDA receptor antagonist on different cell lines, including prostate, breast, and colon tumors, demonstrated that memantine (20 µg/ml) significantly reduced tumor growth in all cell lines. Memantine in lung cancer cells stimulates autophagy and apoptosis and reduces the amount of cell metabolism regulators that cause a stop in cell cycle G0/G1 and reduce cell viability [28]. The cytotoxic effects of memantine in combination with metformin have also been demonstrated in prostate cancer cell lines [29]. In breast cancer cells, this drug has a decreasing effect on cell migration and malignancy [30]. The purpose of the present *in vivo* study was to clarify the effects of memantine on histology, tumor size, and number as well as VEGF level in DMH-induced colon cancer in rats.

2. RESULTS

2.1. Body weight changes and number and size of polyps in rats with colon cancer in different groups

As shown in Fig. 1A, the final weights of rats at the end of the study compared to the initial weights increased by 88% in the control group, 112% in the colon cancer group, and 90% in the memantine-treated group. Our results showed that memantine administration significantly prevented weight gain in comparison to the colon cancer alone group (P<0.001). Our findings indicated that the number and size of polyps significantly increased in the colon cancer group in comparison to the normal control group (P<0.001). Memantine administration showed strong protective effects against DMH-induced colon cancer. Rat in memantine treated group demonstrated decreased number (P<0.001) and size (P<0.001) of polyps in comparison to colon cancer group (Fig. 1B and 1C).
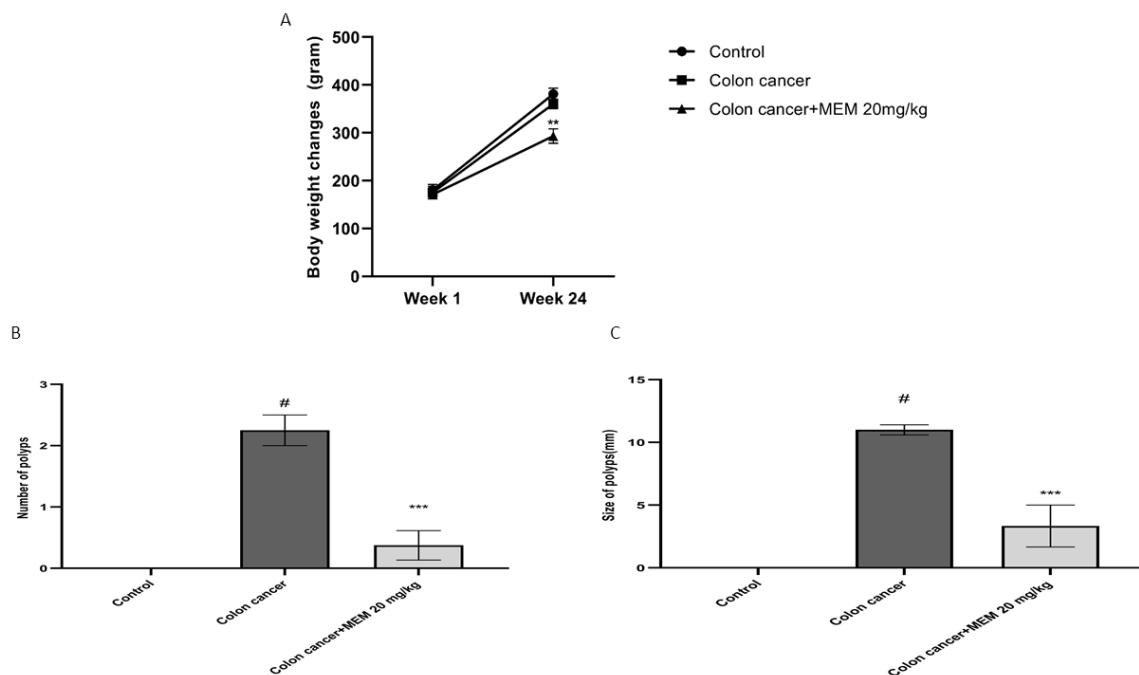
Figure 1. A: Body weight changes of rats at the end of the study compared to initial weights. B, C: Number and size of polyps in normal control, 1,2 dimethylhydrazine-induced colon cancer, and memantine treated groups. Values are mean ± SEM; # $P < 0.001$ vs. control group. *** $P < 0.001$ vs. colon cancer group using one-way ANOVA with Tukey post-test. MEM: memantine.

## 2.2. Macroscopic images of colon polyps and tumor size

In the colon cancer group, 80 percent of rats showed colon polyps with a size of 11±0.4 mm. Carcinomatosis was also observed in one of the rats in this group. In the memantine group, just 10 percent of rats had a 3.3±1 mm colon polyp. The gross images of colon polyps are shown in Fig. 2.
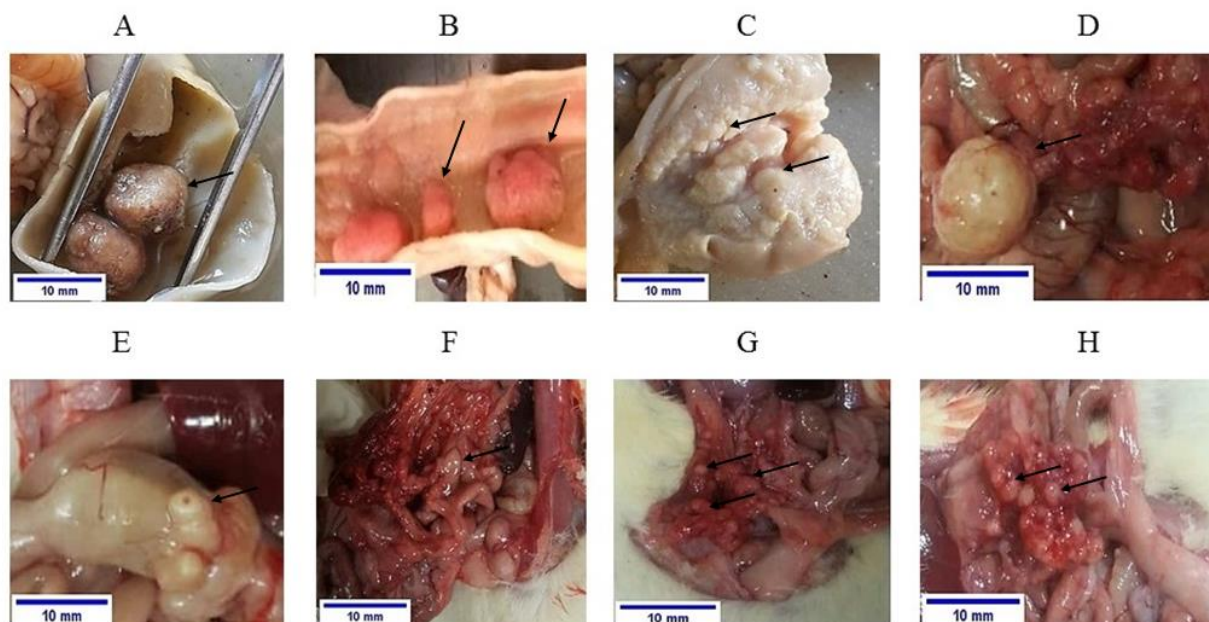
Figure 2. Gross images of intact colons (arrows indicate colon tumors). A, B: Colon polyps, C: Small intestine adenocarcinoma, D: Colon tumor with omentum carcinomatosis, E: Colon mass (Colon adenocarcinoma without polyps), F, G, H: Carcinomatosis of colonic adenocarcinoma origin. Scale bar denotes 10 mm (macro).

2.3. Histopathological examination of the colon tissues

There were no signs of cancer or polyps in the histopathological examination of the control group. Multiple polyps and adenocarcinoma with pancreatic metastasis were observed in the colon cancer group. H&E staining of colon tissues showed that memantine treated group decreased tumor stage (T) (P<0.01) compared to the colon cancer group (Fig. 3).
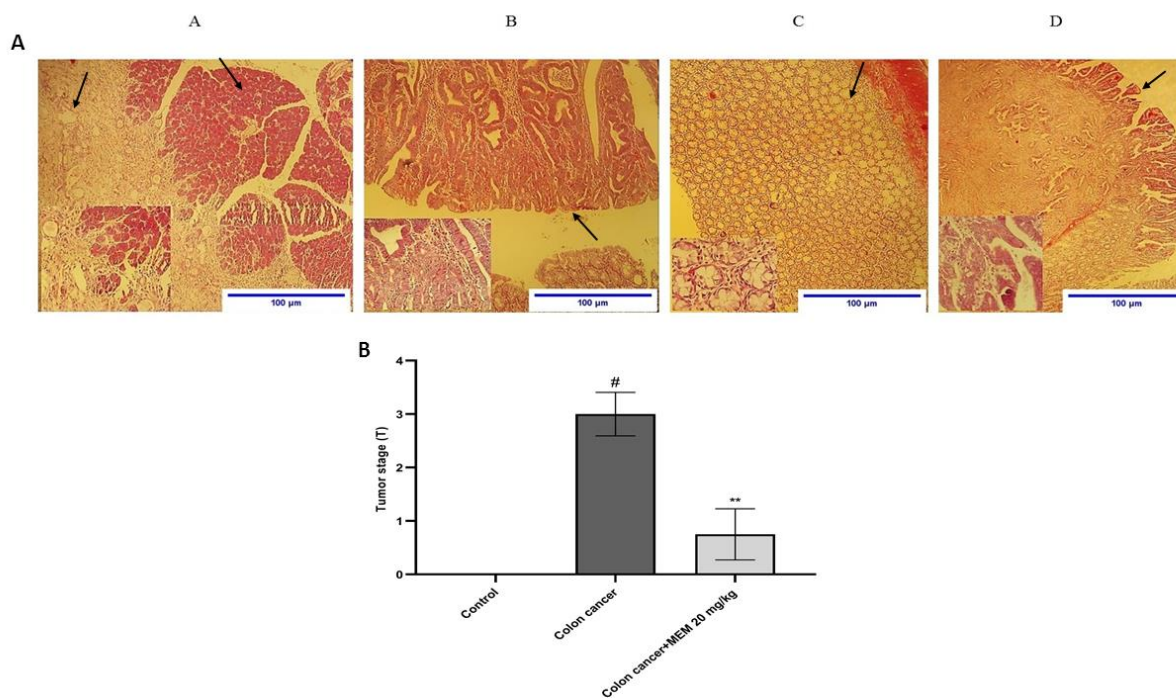

Figure 3. A: Photomicrographs of histopathological changes in colon tissue in different groups. (H&E staining). A: right arrow: normal pancreas/left arrow: pancreas tissue involved by tumoral omentum (locally advanced colon carcinoma), B: polyp involved by carcinoma, C: normal colonic tissue, D: polyp involved by carcinoma (Reduction of size and number of polyps in memantine group). Magnification 10×; lower left box shows magnified tissue; magnification 40×. Scale bar denotes 100 μm. **B**: Graph of T of tumor stage. Values are mean ± SEM; # P< 0.001 vs. control group. ** P< 0.01 vs. colon cancer group using one-way ANOVA with Tukey post-test.

2.4. Memantine reduces serum VEGF level in DMH-induced colon cancer

In this study, the level of VEGF as a potent angiogenic factor that plays a major role in colon cancer progression was measured. As expected, the serum VEGF level increased significantly (P<0.05) in the colon cancer group as compared with the normal control group. Memantine administration at a dose of 20 mg/kg reduced the level of VEGF significantly (P<0.01) in comparison to the colon cancer group (Fig. 4).
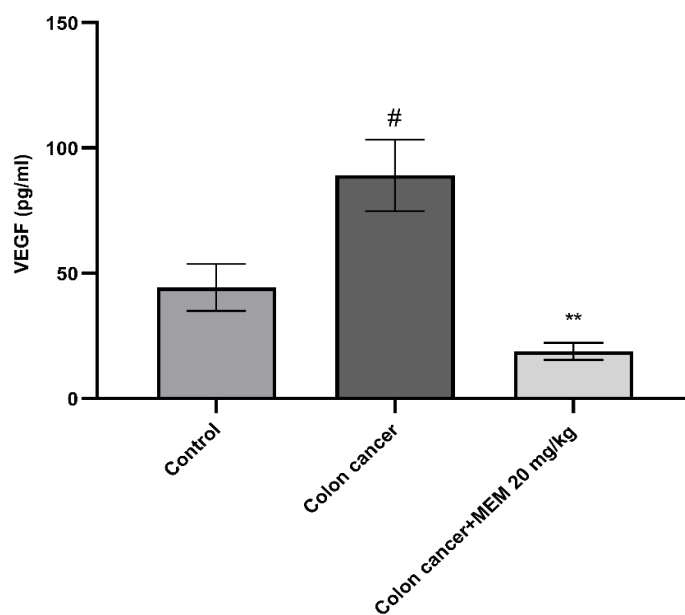
Figure 4. Serum VEGF level in normal control, DMH-induced colon cancer, and memantine treated groups (n=10). Values are mean ± SEM; # $P < 0.05$ vs. control group. ** $P < 0.01$ vs. colon cancer group using one-way ANOVA with Tukey post-test. MEM: memantine; VEGF: vascular endothelial growth factor.

3. DISCUSSION

In this study, the effects of memantine, an important NMDA receptor antagonist, on DMH-induced colon cancer in rats were investigated. Our results showed that memantine possesses strong anti-cancer effects in colon cancer. We demonstrated that memantine at a dose of 20 mg/kg reduced the size and number of colon tumors and polyps, which could be attributed in part to a reduction in VEGF level.

NMDA receptors are one of the most important glutamate receptors in the CNS [31]. Various studies have shown the overexpression of NMDA receptors in different cancer cell lines [11-14, 19] and its role in the growth and proliferation of these cells [32, 33]. It has been reported that NMDA receptors are associated with increased calcium ion influx and angiogenesis, and inhibition of these receptors reduces calcium transport and capillary formation through the reduction in cell growth and proliferation [26, 34-36].

A study reported the role of NMDA receptors in regulating the proliferation and growth of breast cancer cells. The use of memantine and MK-801 as NMDA receptor inhibitors significantly reduced the proliferation and growth of cancer cells as well as the survival of cancer cells [37]. Glutamate stimulates the proliferation of cancer cells. The use of various NMDA receptor antagonists (ketamine, memantine, and dizocilpine) inhibits the migration and division of cancer cells and increases cell death. These results suggest that NMDA receptor antagonists could be considered as new therapies for cancer [38]. The present study showed that memantine reduces the size and number of polyps in colon cancer. Consistent with our results, a study showed that inhibition of NMDA receptors with propofol reduces the growth and proliferation of tumor cells [26]. Recent studies, while confirming the overexpression of NMDA receptors in cancer cells, have shown that the use of ketamine, s-ketamine, and MK-801 reduces the proliferation of cancer cells in all cell lines. One of the most important pathways involved in cancer proliferation is a calcium ion influx. NMDA receptors play an important role in the transport of this ion. Therefore, by inhibiting these receptors and reducing intracellular calcium, cell proliferation can be decreased [39]. Inhibition of NMDA receptors also reduces the growth of lung and liver cancer cells by disrupting the G1/S cell cycle [19, 20].

Recent studies have demonstrated that one of the important factors involved in the growth and proliferation of cancer cells is vascular endothelial growth factor (VEGF) and inhibition of its cascade and angiogenesis is one of the therapeutic goals for cancer treatment [40, 41]. Decreased VEGF levels are associated with inhibition of glutamate synthesis and reduced glutamate level, which increases the antitumor effects [42]. It has also been reported that the VEGF signaling pathway plays an essential role in the antidepressant actions of ketamine [43]. Similarly, in colorectal cancer, ketamine reduces VEGF expression and intracellular calcium

ion levels by inhibiting the NMDA receptor, which leads to a reduction in cancer cell migration and malignancy [25]. Our results in the present study showed that the use of memantine significantly reduced VEGF compared to the colorectal cancer group. This finding is probably related to the reduction in tumor incidence and size and number of tumors in the memantine group. The anti-cancer effect of propofol (NMDA receptor inhibitor) is also associated with decreased expression of VEGF and intracellular Ca^{2+} ions level [26]. In pancreatic cancer, MK-801 and ifenprodil reduced the growth and survival of cancer cells by reducing the activity and expression of NMDA receptors. These findings suggest that the use of NMDA receptor inhibitors alone or in combination may effectively reduce the growth and proliferation of cancer cells [27]. In line with the results of a previous study on the antiproliferative effects of memantine in the colon, prostate, and breast cell lines, [44] the results of us *in vivo* study also indicated the anti-colon cancer effects of memantine.

4. CONCLUSION

In conclusion, the present study indicates the anti-cancer effects of memantine (an NMDA receptor antagonist) in colon cancer that is mostly characterized by decreasing in size and number of colon polyps which can be attributed partially to a reduction in VEGF level.

5. MATERIALS AND METHODS

5.1. Animals

Thirty male Wistar rats weighing 150±30 gr were used in this study. Rats were housed in the animal house of Urmia University of Medical Sciences in standard conditions with a temperature of 22±2 °C, relative humidity of 50±10%, and a cycle of 12 h light/12 h darkness and were fed with food and water ad libitum. This study was approved by the ethics committee of Urmia University of Medical Sciences (Ethics code: IR.UMSU.REC.1400.007) and was performed according to the guidelines for the care and use of laboratory animals by the US National Institutes of Health.

5.2. Test substances

Memantine was provided by Sobhan Pharmaceutical Inc, Rasht, Iran, and 1,2 dimethylhydrazine (DMH) was purchased from Sigma Aldrich Co, Germany (cat no: D161802). Memantine powder was dissolved in normal saline, and DMH was dissolved in 5% dimethyl sulfoxide (DMSO), and the pH of the solution was adjusted to 6.5.

5.3. Experimental protocol

Rats were randomly divided into three groups (n=10 in each group): In group 1 (control), 0.5 ml of saline was injected subcutaneously twice a week throughout the study period. In group 2 (colon cancer), 30 mg/kg of DMH solution was injected subcutaneously twice a week for 24 weeks. In group 3 (memantine group), 30 mg/kg of DMH solution was injected subcutaneously twice a week for 24 weeks, and simultaneously, 20 mg/kg of memantine in drinking water was consumed by rats individually in separate cages. At the end of the study, rats were anesthetized with an intraperitoneal (ip) injection of ketamine (60 mg/kg) and xylazine (10 mg/kg). After the rats did not respond to stimuli, blood samples were collected from the inferior vena cave and then were euthanized with a high dose of anesthetics. Then tissue samples were extracted rapidly.

5.4. Induction of colon cancer

For induction of colon cancer, the DMH-induced colon cancer model was used. DMH was dissolved in 5% DMSO, and the pH of the solution was adjusted to 6.5. Then the animals were given a twice-weekly subcutaneous injection of DMH solution under the abdomen part of the rat body at a dose of 30 mg/kg/day for 24 weeks [45].

5.5. Tumor size and number in the colon

For measuring tumor size and number of tumors in the colon, first, rats were anesthetized with a mixture of ketamine (60 mg/kg) and xylazine (10 mg/kg). Then, the abdominal area was opened, the colon was removed from the body, and it was washed with normal saline. Later, the size of polyps was measured by a digital caliper (mm), and the polyps created by DMH were counted.

5.6. Histopathological studies

The colon tissue was isolated for histopathological examination placed in 10% formalin buffer and embedded in paraffin. The tissue samples were then sectioned at 5 μm thick sections, and H&E staining was performed for routine histology. T of tumor was determined from pathological examination of samples, based on depth of the tumor penetration. T1: tumor invades submucosa, T2: tumor invades muscularis propria, T3: tumor invades through muscularis propria into subserosa or nonperitonealized pericolic or perirectal tissue. T4: tumor directly invades other organs or structures or perforates visceral peritoneum [46].

5.7. VEGF measuring assay

At the end of the experiment, the animals were anesthetized with an intraperitoneal injection of ketamine (60 mg/kg) and xylazine (10 mg/kg) (Alfasan, Netherlands). After deep anesthesia, blood samples were collected from the inferior vena cave and centrifuged at 3000 rpm for 5 min. Then, the level of serum VEGF was measured by the DuoSet ELISA kit (ca no: DY564) according to the manufacturer's instruction.

5.8. Statistical analysis

The results 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.

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