L-Arginine Reduces Cell Proliferation and Ornithine Decarboxylase Activity in Patients with Colorectal Adenoma and Adenocarcinoma

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Abstract **Purpose:** Evidence suggests that the majority of colorectal carcinomas arise from adenomas, and L-arginine suppresses colorectal tumorigenesis. We suppose that L-arginine may inhibit the process of carcinogenesis from colorectal adenoma to adenocarcinoma. The aim of this study was to investigate the effects of L-arginine on the formation and development of colorectal tumors.

Experimental Design: We selected 60 patients with colorectal cancer and 60 patients with colorectal adenoma (CRA) and divided them into four groups of 30 patients each. We gave 30 g (120 mL) of L-arginine everyday for 3 days to the test groups, whereas L-arginine was substituted by 5% glucose in the control groups. The expression of the proliferating cell nuclear antigen, survivin, and nitric oxide synthase was examined immunohistochemically, and ornithine decarboxylase (ODC) activity was examined spectrophotometrically. Serum nitric oxide (NO) was detected by the Griess assay.

Results: In patients with CRA, the proliferating cell nuclear antigen and survivin labeling indexes and ODC activity of the tumor and paratumor mucosa in the L-arginine–treated group after L-arginine treatment were significantly lower as compared with the corresponding pretreatment values ($P < 0.01$). Moreover, inducible nitric oxide synthase expression in the tumor markedly increased after L-arginine treatment ($P < 0.05$). Serum NO levels in the patients with colorectal cancer were markedly higher than those in the patients with CRA, and L-arginine treatment was responsible for this increase ($P < 0.05$).

Conclusions: Our results show that L-arginine can restrain crypt cell hyperproliferation and the expression of survivin, an inhibitor of apoptosis protein. This suggests that L-arginine can block the formation and development of colorectal tumors, and this effect might be related to the increased serum NO concentration and decreased ODC activity.

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Colorectal carcinoma (CRC) is one of the most common cancers in the world, and colorectal adenoma (CRA) is known to be a precursor of both sporadic and hereditary CRC. Recent studies have shown that the frequency of adenomas in a population with an increased risk of CRC is higher as compared with that in the general population; moreover, patients with the so-called hereditary nonpolyposis syndrome also had an earlier onset of adenomas and faster progression to cancer (1). It is widely accepted that environmental factors, particularly dietary factors, are involved in the etiology of CRC. A high intake of fat, red meat, and energy have not only been associated with an increased risk of CRC (2–5), but have also influenced the 5-year survival in the patients who underwent surgical treatment for CRC (6). Therefore, developing strategies to decrease the incidence of CRC and improve its prognosis by changing the diet or certain dietary components have currently become a hot topic of study. The focus on L-arginine supplements has been increasing in recent years.

L-Arginine, a semiessential amino acid, is required for the synthesis of proteins, creatinine, and polyamines, and it induces the active conformation of these substances *in vivo*. L-Arginine is metabolized by the oxidative deaminase pathway to form nitric oxide (NO) and by the arginase pathway to yield ornithine and polyamines. NO is generated from the terminal guanidino nitrogen atom of L-arginine by the action of the NADPH-dependent enzyme nitric oxide synthase (NOS). The availability of intracellular L-arginine is a rate-limiting factor in NO production (7). NOS has three distinct isoforms: two constitutive, i.e., endothelial NOS and neuronal NOS, and one inducible, i.e., inducible NOS (iNOS). All isoforms require the amino acid L-arginine and oxygen as substrates in addition to a variety of cofactors (8). Although NO is widely known for its physiologic roles in vasorelaxation, neurotransmission, inhibition of platelet aggregation, and immune defense, it also acts as an intracellular messenger for various cells in almost every system in the body. NO participates in many cellular metabolic processes by activating cyclic GMP, and hence, it sequentially influences the immunologic functions and cell proliferation.

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Table 1. Characteristics of the subjects

	CRA		CRC	
	Control	**L-Arginine – treated**	**Control**	**L-Arginine – treated**
Age (y)	53.1 ± 5.1	54.2 ± 3.8	56.7 ± 4.7	58.5 ± 5.5
Male/female	16:14	18:12	18:12	17:13
Rectum	3	4	13	15
Sigmoid	7	8	7	7
Descending colon	6	7	4	3
Transverse colon	6	6	3	2
Ascending colon	8	5	3	3

Recent studies have reported that the so-called immune-enhancing dietary compounds include L-arginine, ω-3 fatty acids, and nucleotides (9, 10). The significant effects of L-arginine on nitrogen metabolism and immune function were shown by animal experiments and studies in humans (11 – 14). The relationship between L-arginine and tumorigenesis has been extensively studied, and increasing evidence has shown that L-arginine can reduce tumor occurrence and development in some tumor models, including chemically induced solid tumors, transplantable solid tumors, and human tumors (15, 16). Our previous study has shown that L-arginine reduces tumor formation and tumor burden in 1,2-dimethyl hydralazine – induced CRC by inhibiting crypt cell hyperproliferation (15). Therefore, we hypothesized that L-arginine would reduce the development of CRC from CRA. However, there seems to be a lack of studies regarding the role played by L-arginine in patients with CRA and CRC in the literature.

In the present study, we investigated the effects of L-arginine on the expression of the proliferating cell nuclear antigen (PCNA) and survivin—an inhibitor of apoptosis protein—in CRA and CRC. Furthermore, we also studied the two metabolic pathways that are closely related to tumorigenesis: the L-arginine-NO pathway, which has conflicting actions on tumors at different L-arginine concentrations, and the L-arginine-polyamine pathway, which is essential for cell differentiation and proliferation.

Materials and Methods

Subjects. This randomized, double-blind, placebo-controlled protocol was approved by The Human Research Review Committee at the University Hospital. The criteria for the selection of the subjects included the following: (*a*) Karnofsky grade >70%; (*b*) no liver or renal dysfunction, severe chronic disease, or septicemia; (*c*) no history of immunosuppressant and immunointensifier therapy in the last 6 months; (*d*) not treated with chemotherapy or radiotherapy; (*e*) had not undergone a splenectomy; (*f*) no endocrine or metabolic disease; and (*g*) no distant metastasis. The study was carried out on 60 patients with CRC and 60 patients with CRA from The First Hospital of Xi'an Jiaotong University. Each group was randomly divided further into two groups of 30 patients each. There were 69 males and 51 females (male/female, 1.35:1) with an average age of 56.3 years (range, 28-71 years), and there were no significant differences between the test and control groups with regard to age, sex, location of the tumor, pathologic type, and Dukes stages (Tables 1 and 2). None of the subjects had any coexistent disease, and the hematologic, biochemical, and urinalysis profiles of all the subjects were normal.

Treatment and sampling. After overnight fasting, the subjects underwent colonoscopy. Biopsy specimens were obtained from the tumor, paratumor mucosa (within 5 cm of the tumor), and normal mucosa (>10 cm distance from the tumor) during the colonoscopy. The tumor sections were individually assessed for CRC and CRA by two pathologists. The test groups were given 30 g (120 mL) of freshly prepared L-arginine (Shanghai Biochemistry, Ltd.) orally once a day for 3 days. In the control groups, L-arginine was substituted with 5% glucose (120 mL). Peripheral blood samples obtained before and after the L-arginine treatment were placed on ice and analyzed immediately. All subjects with CRC underwent radical surgical resection 3 days after treatment, whereas those with CRA underwent tumor resection by electrocautery under colonoscopic guidance; tissue samples were obtained during the procedures.

Assessment of PCNA, NOS, and survivin. The expression of PCNA, NOS, and survivin in the tumor, paratumor mucosa, and normal mucosa in both the L-arginine – treated and control groups was examined immunohistochemically. Briefly, formalin-fixed and paraffin-embedded samples were prepared for histologic examination and immunohistochemical analysis. The primary antibodies used were polyclonal rabbit anti-human survivin and monoclonal mouse anti-human PCNA antibodies (Zhongshan Biotech, Co. Ltd.) and polyclonal mouse anti-human endothelial NOS, neuronal NOS, and iNOS antibodies (Neomarkers, Lab Vision Corporation). Several 3-μm sections were cut and placed on poly-L-lysine – coated slides. After deparaffinization, the sections were treated with 3% methanol/hydrogen peroxide for 5 min to block endogenous peroxidase; in addition, nonspecific staining was blocked by incubation for 30 min with 10% goat serum. In order to retrieve masked antigens, the slides were immersed in citrate buffer (pH 6.0) and heated in an autoclave for 15 min at 120°C. The slides were then incubated for 120 min with the primary antibody, followed by incubation with the biotinylated secondary antibody for 30 min, and subsequent incubation with peroxidase-labeled streptavidin for 20 min. Negative control slides that were not treated with the primary antibody were included for each staining. Finally, the reaction product was visualized using

Table 2. Characteristics of CRA and CRC

	Control	**L-arginine – treated**
CRA		
Tubular adenoma	23	21
Villous adenoma	3	4
Mixed type	4	5
CRC		
Papillary adenocarcinoma	5	4
Tubular adenocarcinoma	21	22
Mucinous carcinoma	4	3
Undifferentiated carcinoma	0	1
Dukes stage		
A	3	2
B	10	12
C	16	14
D	1	2

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Table 3. PCNA LIs

	PCNA LIs, $\bar{x} \pm s$ (%)	
	Pretreatment	**Posttreatment**
CRA		
Control group		
Tumor	61.5 ± 4.0	59.8 ± 4.3
Paratumor	27.8 ± 4.7*	26.7 ± 3.6*
Normal	25.9 ± 5.1*	26.2 ± 5.6*
Test group		
Tumor	60.3 ± 6.5	52.9 ± 4.2 †
Paratumor	28.2 ± 4.8*	27.0 ± 4.6*
Normal	26.5 ± 5.4*	27.3 ± 5.0*
CRC		
Control group		
Tumor	79.5 ± 4.0 ‡	78.9 ± 4.3 ‡
Paratumor	41.6 ± 4.7*	42.3 ± 5.3*
Normal	25.9 ± 5.7*	24.8 ± 5.6*
Test group		
Tumor	78.8 ± 5.6 ‡	70.5 ± 4.7 †, ‡
Paratumor	40.2 ± 4.4*	32.7 ± 4.1*, †
Normal	25.4 ± 4.6*	23.8 ± 5.5*

*$P < 0.05$ when compared with a tumor in the same group.
†$P < 0.05$ when compared pretreatment and posttreatment.
‡$P < 0.05$ when compared with a tumor in the CRA group.

diaminobenzidine hydrochloride (Sigma) primed with 100 μL of 30% hydrogen peroxide for ~5 min, and hematoxylin was used for counterstaining. The sections were individually read by two pathologists. For every section, 10 high-power fields (magnification, ×400) were selected at random and examined under a microscope. A total of 1,000 cells were read per slide, i.e., 100 cells per high-power field. The labeling index (LI) was calculated as follows and expressed as a percentage

$$\mathrm{LI} = (\text{labeled cells}/\text{total cells}) \times 100$$

Assessment of NO. NO production was measured as the amount of nitrite (NO_2^-), which is the stable end product of NO metabolism. The peripheral blood was centrifuged at 3,000 rpm for 10 min, and the supernatants were collected and measured by a colorimetric assay based on the Griess reaction (17). Briefly, the samples (100 μL) were mixed with an equal volume of Griess reagent [1% sulfanilamide, 0.1% naphthylethylenediamine dihydrochloride, and 2.5% phosphoric acid (H_3PO_4)] and incubated at room temperature for 10 min. The absorbance was measured at 550 nm by using a microplate reader. Sodium nitrite was used as the standard.

Assessment of ornithine decarboxylase. The tissue samples were cut into chips and placed in ice-cold ornithine decarboxylase (ODC) reaction buffer [10 mmol/L Tris (pH 7.4), 2.5 mmol/L DTT, 0.3 mmol/L pyridoxyl-5-phosphate, and 0.1 mmol/L EDTA]. The samples were then homogenized and centrifuged at 4,800 rpm for 20 min at 4°C. The supernatant was collected and assayed for ODC activity by the Ngo assay (18). ODC activity was determined by measuring the amount of putrescine (nmol) produced every 30 min. The protein content of the cells was determined by the Lowry assay using bovine serum albumin as the standard.

Statistical analysis. The measured values of the PCNA, NOS, and survivin LIs, and those of ODC activity and serum NO are represented as the group mean ± SE ($\bar{x} \pm s$). The intergroup differences between the values were compared by the independent sample t test by using STAT software SPSS version 11.0. Statistical comparisons between the pretreatment and posttreatment measurements were made by paired Student's t test. The numerous data was evaluated by χ^2 test. $P < 0.05$ was considered statistically significant.

Results

All the subjects completed the procedure. No adverse events were reported during the experiment.

PCNA LIs. In patients with CRA, the PCNA LIs of the tumor were significantly higher than those of the paratumor and normal mucosa. However, there was no significant difference between the PCNA LIs of the latter two tissues. In these patients, the PCNA LIs of the tumor in the test group decreased significantly after L-arginine treatment, whereas the PCNA LIs in the control group showed no significant change (Table 3). Furthermore, there was no significant difference between the pretreatment and posttreatment PCNA LIs of the paratumor and normal mucosa.

On the other hand, in patients with CRC, the PCNA LIs of the tissues increased in the order: normal mucosa, paratumor mucosa, and tumor ($P < 0.01$). In these patients, the posttreatment PCNA LIs of the tumor and paratumor mucosa were significantly lower as compared with the pretreatment PCNA LIs in the L-arginine–treated group (Table 3), whereas there was no significant change in the pretreatment and posttreatment PCNA LIs of the paratumor mucosa in the control group. The pretreatment and posttreatment (with L-arginine) PCNA LIs of the normal mucosa did not differ significantly. Furthermore, the PCNA LIs of CRC were significantly higher than those of CRA ($P < 0.05$).

Expression of survivin. Survivin expression was absent in both the normal and paratumor mucosa. Survivin is located in the cytoplasm and is only expressed in tumors. The survivin LIs of CRA were significantly higher than those of CRC ($P < 0.01$). L-Arginine significantly inhibited the expression of survivin in both CRA and CRC (Fig. 1), whereas no significant difference was detected between the two control groups.

Assessment of NOS and serum NO. Endothelial NOS was expressed with an average LI of 50.5% in the normal and paratumor mucosa; however, it was not expressed in the tumor cells. The pattern of expression of neuronal NOS was similar to that of endothelial NOS. The tumor cells showed moderate or intense staining for iNOS with an average LI of 50.6% (Fig. 2), whereas the glandular cells in the normal and paratumor mucosa did not express iNOS. The increase in the expression of

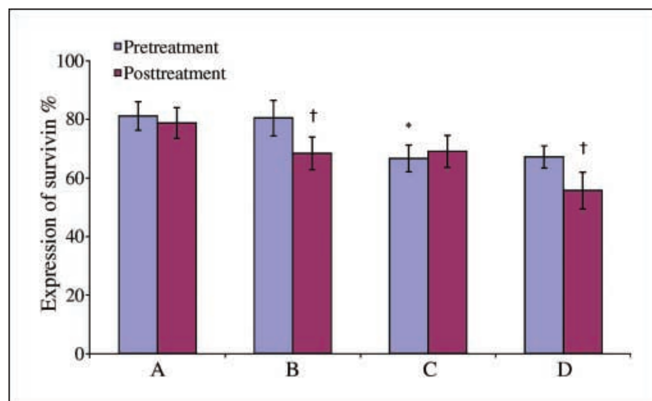

Fig. 1. Survivin expression. *A,* adenoma in the control group. *B,* adenoma in the test group. *C,* cancer in the control group. *D,* cancer in the test group. *, $P < 0.05$ as compared with the adenoma groups (*A* and *B*); †, $P < 0.05$ as compared with the pretreatment values.

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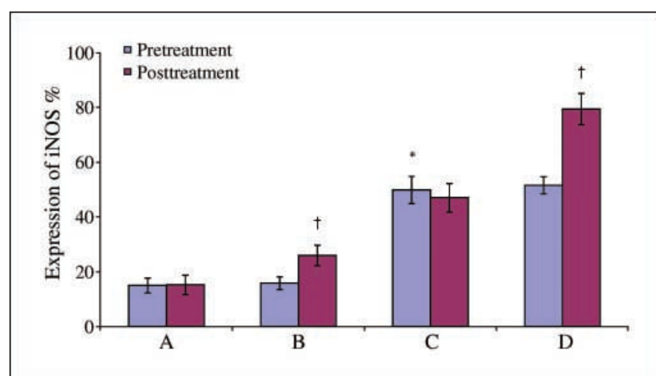

Fig. 2. iNOS expression. *A,* adenoma in the control group. *B,* adenoma in the test group. *C,* cancer in the control group. *D,* cancer in the test group. *, $P < 0.05$ as compared with the adenoma groups (*A* and *B*); †, $P < 0.05$ as compared with the pretreatment values.

iNOS in the tumor tissue posttreatment was obvious ($P < 0.01$). The serum NO levels in the patients with CRC (32.4 ± 3.98 μmol/L) were similar to those in the patients with CRA (30.54 ± 4.48 μmol/L), and the values significantly increased (54.3 ± 3.35 μmol/L) after the short-term administration of L-arginine to the test group with CRC; however, in the test group with CRA, there was no significant change in the posttreatment serum NO levels (30.4 ± 4.43 μmol/L).

Assessment of ODC activity. In patients with CRA, the ODC activity in the tumor was significantly higher than that in the paratumor and normal mucosa; however, in the patients with CRC, the ODC activity increased in the order: normal mucosa, paratumor mucosa, and tumor. There was a marked difference between the ODC activity in the tumor tissues of CRA and CRC. In the test groups, after the administration of L-arginine, the ODC activity in the tumor tissues of CRA and CRC and in the paratumor mucosa of CRC was significantly lower as compared with the corresponding pretreatment values; however, there was no significant difference between the pretreatment and posttreatment values in the two control groups. There ware no significant differences between the pretreatment and posttreatment ODC activities in the paraadenoma and normal musoca (Table 4).

Discussion

Colonic crypt cell hyperproliferation has been suggested to play a significant role in the multistep processes involved in the formation and development of CRC. PCNA is an index of cellular proliferative status and functions as a cofactor for DNA polymerase. It is associated with DNA repair in both the S phase and the DNA synthesis phase and has been detected in various lesions. A high frequency of PCNA overexpression is generally used as a reliable marker for the assessment of tumor progression, premalignant evolution, and clinical prognosis of patients with various malignancies (19–23).

Previously, we have reported that when Wistar rats were given L-arginine during the initiation phase of chemically induced carcinogenesis, the L-arginine significantly reduced colorectal tumor production and crypt cell hyperproliferation (24). The present study indicated that in patients with CRC, PCNA expression in the tissues increased in the order: normal mucosa, paratumor mucosa, and tumor, and was higher than that in the patients with CRA. Previous studies have also shown that PCNA expression increased with Dukes stage (25). Therefore, it can be used to predict the degree of malignancy and the prognosis of patients with colorectal tumors. The evidence that L-arginine inhibits PCNA expression suggests that L-arginine can inhibit the hyperproliferation of the colorectal tumor cells and thus improve the prognosis of patients with colorectal tumors.

The progression of the cell cycle and the control of apoptosis (programmed cell death) are intimately linked with carcinogenesis. Survivin, a new inhibitor of apoptosis protein, is expressed in the G_2-M phase of the cell cycle in a cycle-regulated manner (26). At the beginning of mitosis, survivin associates with microtubules of the mitotic spindle in a specific and saturable reaction that is regulated by microtubule dynamics (25). The disruption of survivin-microtubule interactions results in the loss of the antiapoptosis function of survivin and increased caspase-3 activity—a mechanism involved in cell death—during mitosis. These results indicated that survivin might counteract a default induction of apoptosis in the G_2-M phase. The overexpression of survivin in cancer may overcome this apoptotic checkpoint (27) and favor aberrant progression of the transformed cells through mitosis.

Although survivin is undetectable in quiescent tissues, it is abundantly expressed in proliferating cells and is rapidly down-regulated by cell cycle arrest in the G_1 phase (28). In contrast to BCL-2, survivin does not seem to be involved in the physiologic regulation of apoptosis in adult colonic epithelium; however, it is prominently expressed in CRC (29) and several other malignancies (30, 31). The overexpression of survivin in CRA was equal to (32), and in some cases even higher than, that in CRC (33). This evidence suggests that the overexpression of survivin occurs in the early stage of tumor formation and implies that it is closely related to colorectal tumorigenesis. Additionally, studies have indicated that the expression of

Table 4. ODC activity

	ODC activity ($\bar{x}$ ± s)	
	Pretreatment	Posttreatment
CRA		
Control group		
Tumor	9.46 ± 1.78	9.50 ± 1.65
Paratumor	7.67 ± 1.18*	7.42 ± 1.45*
Normal	7.05 ± 1.42*	6.95 ± 1.30*
Test group		
Tumor	9.58 ± 1.12	8.33 ± 1.33 †
Paratumor	7.31 ± 1.32*	7.25 ± 1.40*
Normal	6.83 ± 1.25*	6.75 ± 1.21*
CRC		
Control group		
Tumor	11.97 ± 2.55 ‡	12.04 ± 2.78 ‡
Paratumor	8.08 ± 1.71*	8.01 ± 1.56*
Normal	7.19 ± 1.33*	7.31 ± 1.39*
Test group		
Tumor	12.35 ± 2.47 ‡	10.55 ± 1.93 †,‡
Paratumor	8.16 ± 1.53*	7.31 ± 1.42*,†
Normal	6.90 ± 0.95*	6.96 ± 1.11*

*$P < 0.05$ when compared with a tumor in the same group.
†$P < 0.05$ when compared pretreatment and posttreatment.
‡$P < 0.05$ when compared with a tumor in the CRA group.

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survivin may be used as an index for the prognosis of patients with CRC (29). Accordingly, survivin has been validated as an ideal cancer therapeutic target (34). In the present study, survivin overexpression was markedly inhibited after L-arginine treatment.

The role of NO in tumor cell apoptosis and survival depends on the cell type, the concentration of NO in the cellular microenvironment, the duration of cellular exposure to NO, and possibly other factors. High NO concentration induces cytostasis and cytotoxicity in tumor cells both *in vitro* and *in vivo* (35–37); however, a low NO concentration plays an active role in the carcinogenesis (38, 39) and angiogenesis of solid tumors.

Polyamines have long been known to be associated with rapid cell proliferation in both normal and neoplastic cells and tissues. ODC is the initial rate-limiting enzyme involved in the biosynthesis of polyamines and is responsible for converting L-ornithine to putrescine. ODC is aberrantly regulated in tumor cells and results in high basal levels of polyamines in many epithelial tumors (40–44). Experimental data indicated that tumor development and progression to a malignant phenotype were dependent on the elevated levels of ODC and polyamines (45). Hence, assessment of mucosal ODC activity may be of value as a marker for the potential risk of malignancy in the human large bowel and upper gastrointestinal tract (46), and as a molecular target for the chemoprevention of some carcinomas (47).

Georgette et al. (48) showed that in a culture of the Caco-2 human colon carcinoma cell line, NO caused cytostasis by mechanisms that might involve the inhibition of ODC activity. In the present study, we found that after L-arginine treatment, the serum NO level considerably increased, which might be related to the increase in iNOS expression, and ODC activity markedly decreased. This suggests that L-arginine inhibits crypt cell hyperproliferation by producing high amounts of NO in the cellular microenvironment, thereby inhibiting ODC activity. However, the concentration of NO in CRA remains unchanged even with high iNOS expression. Although the mechanism behind this phenomenon is still unclear and needs further investigation, it may be associated with the local consumption of NO.

When cocultures of normal and tumor cells were deprived of L-arginine, the normal cells survived and the tumor cells died (49, 50). Similarly, we found that the L-arginine supplement selectively inhibited the hyperproliferation of tumor cells; however, it had no such effect on the normal glandular cells. This fact might result from the high metabolic activity of the hyperproliferating cells. High metabolic activity required more L-arginine and consequently produced more NO. The increased concentration of NO in the cellular microenvironment induced cytostasis and cytotoxicity in the tumor cells (37).

In summary, our results show that L-arginine can inhibit the hyperproliferation of tumor cells and the expression of survivin in them. Furthermore, our results suggest that L-arginine may block the formation and development of CRC. These results might be related to the increase in iNOS expression and NO production as well as the decrease in ODC activity. The beneficial effects of L-arginine on the variables that we measured in patients with CRC need to be studied further in greater detail.

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