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Original Article

In Vitro and In Vivo Antitumorigenic Activity of a Mixture of Lysine, Proline, Ascorbic Acid, and Green Tea Extract on Human Breast Cancer Lines MDA-MB-231 and MCF-7

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Abstract

Current treatments are generally ineffective once breast cancer has metastasized; median survival is reduced to 2–3 yr. Previous research studies demonstrating potent synergistic antitumor activity of lysine, proline, ascorbic acid, and epigallocatechin gallate prompted us to investigate the in vivo inhibitory effect of a nutrient mixture containing lysine, proline, arginine, ascorbic acid, and epigallocatechin gallate (NM) on the growth of human cancer xenografts in female athymic nude mice. Five to six week old female mice were inoculated with 3×10^6 breast cancer cells MDA-MB-231. After injection, the mice were randomly divided into two groups A and B; group A was fed a regular diet and group B with the regular diet supplemented with 0.5% of the nutrient mixture (NM). Four weeks later, the mice were sacrificed, and their tumors were excised, weighed, and processed for histology. We also tested the effect of NM in vitro on estrogen-receptor positive (ER^+) MCF-7 and estrogen-receptor negative (ER^-) MDA-MB-231 breast cancer cell lines by measuring: cell proliferation by MTT assay, expression of MMPs by gelatinase zymography, invasion through Matrigel, and VEGF by ELISA. MCF-7 cells were also treated with estradiol to study enhanced invasion and expression of MMPs and VEGF. Results showed that NM inhibited the growth and reduced the size of tumors in female nude mice by 27%. Furthermore, histological evaluation revealed increased mitotic index, MMP-9 and VEGF secretion, and PAS material (mucin) in the control group tissues. In vitro studies showed NM inhibited MDA-MB-231 cell growth by 34% at 500 µg/mL and MCF-7 cell growth by 18% at 1000 µg/mL. Invasion of MDA-MB-231 through Matrigel was inhibited by 50%, 60%, and 95% by 10, 50, and 100 µg/mL of NM, respectively. The results of this study demonstrated that the nutrient mixture tested significantly suppressed tumor growth of breast cancer cells in female athymic nude mice and significantly inhibited MMP expression, angiogenesis, and invasion in breast cancer cells, in vitro, offering promise for therapeutic use in the treatment of breast cancer.

Key Words: Breast cancer; MCF-7; MDA-MB-231; MMP; VEGF; athymic nude mice; xenograft; Matrigel invasion; antitumor.

Introduction

Surgery, radiation, chemotherapy, and hormone therapy are the standards of treatment, the regimen primarily dependent on the breast cancer stage. Once the patient tumor has metastasized, the survival rate is drastically reduced to a median of 2–3 yr. At this stage, current therapy is aimed at controlling symptoms, prolonging survival, and improving quality of life *(1)*. Unfortunately, current diagnostic criteria used to stage breast cancer is often inaccurate in regard to metastasis. Analyses of bone marrow samples (not a

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routine procedure) have shown presence of disseminated cells in up to 40% of primary breast cancer patients without any clinical or histopathological signs of metastasis. Circulating breast cancer cells in bone marrow are indicative of metastasis to such sites as bone, lung, or liver *(2)*.

One key mechanism cancer cells use to spread and metastasize in the body involves enzymatic destruction of the surrounding connective tissue. Therapeutic approaches for controlling this process with specific drugs have not been successful, and currently there are no means available to control cancer metastasis. Radiation and chemotherapy have not only been ineffective in providing a cure, but also indiscriminately attack all cells—causing cellular damage and destruction of the body's connective tissue, facilitating cancer metastasis.

Metastasis potential and invasiveness of breast cancer are attributed to up-regulation of matrix metalloproteinases (MMPs). Cancer cells form tumors and spread by degrading the extracellular matrix (ECM) through various MMPS. The activity of these enzymes correlates with the aggressiveness of tumor growth and invasiveness of the cancer. Rath and Pauling *(3)* postulated that nutrients such as lysine and ascorbic acid could act as natural inhibitors of ECM proteolysis and, as such, have the potential to control tumor growth and expansion. These nutrients exercise their anti-tumor effect through the inhibition of MMPs and strengthening of connective tissue surrounding cancer cells (tumor-encapsulating effect). In a previous study, we demonstrated the anti-proliferative and anti-invasive potential of lysine, ascorbic acid, proline, and epigallocatechin gallate (EGCG) on several human cancer cell lines *(4)*. These nutrients manifested markedly higher anti-proliferative and anti-invasive when used synergistically than individually. Our results also showed that the nutrient mixture suppressed the growth of these tumors, without any adverse effects, in nude mice.

In the current study, we investigated the anti-tumor potential of a nutrient mixture containing lysine, proline, arginine, ascorbic acid, and epigallocatechin gallate (NM) on breast cancer cell MDA MB-231 xenografts in nude mice. We also studied the effect of NM in vitro on human estrogen-sensitive breast cancer (MCF-7) cells in the presence and absence of estrogen as well as in estrogen-insensitive breast cancer (MDA MB-231) cells.

Materials and Methods

In vivo Study

Cell Culture

MDA-MB-231 cells were cultured in Leibovitz medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 mg/mL streptomycin. The media and sera used were obtained from ATCC, and antibiotics (penicillin and streptomycin) were from Gibco BRL, Grand Island, NY. At near confluence, the cultured cells were detached by trypsinizing, washed with PBS, and diluted and emulsified to a concentration of 3×10^6 cells in 0.2 mL PBS and 0.1 mL Matrigel (BD Bioscience, Bedford, MA) for inoculation.

Animals

Female athymic nude mice (NCr-nu/nu), approx 6 wk of age on arrival, were purchased from Simonsen Laboratories, Gilroy, CA and maintained in microinsulator cages under pathogen-free conditions on a 12-h light/12-h dark schedule for a week. All animals were cared for in accordance with institutional guidelines for the care and use of experimental animals.

After housing for a week, the mice were inoculated with 3×10^6 breast cancer MDA-MB-231 cells in 0.2 mL of PBS and 0.1 mL of Matrigel in the right flank. After injection, the mice were randomly divided into two groups, A and B. Six mice were allocated to each group. From d 1, Group A was fed a regular diet and Group B was fed a regular diet supplemented with 0.5% of the nutrient formulation (NM). The growth of tumors was monitored, by measuring the length and width of the tumors with a caliper every 3 d. Tumor volume was calculated using the following equation: Volume (mm^3) = Length $\times$ Width^2 $\times$ $^1/_2$. After 4 wk, mice were sacrificed, tumors were excised, weighed, fixed in 10% (v/v) buffered formalin, and processed for histology.

Cytochemistry and Immunohistochemistry

Tissue samples were fixed in 10% buffered formalin. All tissues were embedded in paraffin and cut at 4–5 μm. Sections were deparaffinized through xylene and graduated alcohol series to water, and

incubated for 5 min in aqueous 3% hydrogen peroxide to block endogenous peroxidase. Histological sections were stained with hematoxylin and eosin (H & E) and periodic acid-Schiff (PAS) stains for evaluation using a standard light microscope.

Immunochemical studies were performed on formalin-fixed, paraffin-embedded sections. We used standard immunohistochemical procedures for staining antibodies. After deparaffinization and appropriate epitope retrieval, the sections were incubated with primary antibody. Detection was by biotinylated goat anti-mouse antibodies followed by streptavidin conjugated to horseradish peroxidase with the use of diaminobenzidine as the chromogen. Polyclonal rabbit anti-human antibodies used for MMP-9, VEGF, and Ki-67 were obtained from Santa Cruz Biotechnology, Inc., CA, and from Sigma.

In Vitro Studies

Cell Culture

Human breast cancer cells MDA MB-231 and MCF-7 were obtained from ATCC (American Type Culture Collection, Rockville, MD) and grown in MEM (modified Eagle's medium) supplemented with 10% fetal bovine serum, penicillin (100 U/mL), and streptomycin (100 mg/mL) in 24-well tissue culture plates (Costar, Cambridge, MA). Cells were incubated with 1 mL of media at 37°C in a tissue culture incubator equilibrated with 95% air and 5% CO_2. At near confluence, the cells were divided into several groups. Group 1A (MDA MB-231) and Group 1B (MCF-7) were treated with NM dissolved in media and tested at 0, 10, 100, and 1000 µg/mL in triplicate at each dose. Group 2A (MDA MB-231) and Group 2B (MCF-7) were treated with PMA 200 ng/mL and NM dissolved in media and tested at 0, 10, 100, and 1000 µg/mL in triplicate at each dose. Group 3 (MCF-7) was incubated with estradiol 25 ng/mL and NS dissolved in media and tested at 0, 10, 100, and 1000 µg/mL in triplicate at each dose. The plates were then returned to the incubator. Cell proliferation was evaluated after 24 h following incubation with test reagents.

MTT Assay

Nutrient effects on cell proliferation were evaluated by MTT assay, a colorimetric assay based on the ability of viable cells to reduce a soluble tetrazolium salt [3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyl tetrazolium bromide] (MTT) to a blue formazan crystal by mitochondrial succinate dehydrogenase activity of viable cells. After MTT addition (0.5 mg/mL), the plates were covered and returned to the 37°C incubator for 2 h, the optimal time for formazan product formation. Following incubation, the supernatant was carefully removed from the wells, the formazan product was dissolved in 1 mL DMSO, and absorbance was measured at 570 nm in a Bio Spec 1601, Shimadzu spectrometer. The OD_{570} of the DMSO solution in each well was considered to be proportional to the number of cells. The OD_{570} of the control (treatment without supplement) was considered to be 100%.

Gelatinase Zymography

MMP expression in condition media was determined by gelatinase zymography. Gelatinase zymography was performed in 10% polyacrylamide precast Novex gel (Invitrogen Corporation) in the presence of 0.1% gelatin. Culture media (20 µL) was loaded and SDS-PAGE was performed with a Tris-glycine SDS buffer. After electrophoresis, the gels were washed with 5% Triton X-100 for 30 min. The gels were then incubated for 24 h at 37°C in the presence of 50 m*M* Tris-HCl, 5 m*M* $CaCl_2$, 5 µ*M* $ZnCl_2$, pH 7.5 and stained with Coomassie Blue R 0.5% for 30 min and destained. Protein standards were run concurrently and approximate molecular weights were determined.

Matrigel Invasion Studies

Invasion studies were conducted using Matrigel™ (Becton Dickinson) inserts in 24-well plates. Suspended in medium, breast cancer cells were supplemented with nutrients, as specified in the design of the experiment and seeded on the insert in the well. Thus both the medium on the insert and in the well contained the same supplements. The plates with the inserts were then incubated in a culture incubator equilibrated with 95% air and 5% CO2 for 24 h. After incubation, the media from the wells were withdrawn. The cells on the upper surface of the inserts were gently scrubbed away with cotton swabs. The cells that had penetrated the Matrigel membrane and migrated onto the lower surface of the Matrigel were stained with hematoxylin and eosin and visually counted under the microscope.

VEGF Assay

Conditioned media were collected after confluent cell culture incubation for 24 h in serum-free medium with the indicated supplements. Triplicate samples were pooled and the level of VEGF was measured in duplicate using immunoassay kit (BioSource International) according to manufacturer's protocol. Values are expressed as mean ± range of two replicates in percentage units of unstimulated control.

Nutrient Mixture (NM) Composition

In the design of the experiment, a stock solution of a nutrient mixture (NM), weighing 4.4 g, was prepared daily to treat the cells, composed of the following nutrients: vitamin C (as ascorbic acid and as Mg, Ca, and palmitate ascorbate) 700 mg; L-lysine 1000 mg; L-proline 750 mg; L-arginine 500 mg; *N*-acetyl cysteine 200 mg; standardized green tea extract (80% polyphenol) 1000 mg; selenium 30 mg; copper 2 µg; manganese 1 mg.

Statistical Analysis

The results were expressed as means ± SD for the groups. Data was analyzed by independent sample "*t*" test.

Results

In Vivo Studies

Tumor Growth Study

Results showed that the nutrient mixture inhibited the growth and reduced the size of the tumors in nude mice supplemented with 0.5% nutrient mixture in breast cancer cells MBA-MD-231 by 27% ($p = 0.0082$). (See Fig. 1) No significant change in weight was observed in either group of mice over the study period.

Cytochemistry and Immunohistochemistry

Ki-67 level was higher in the tumor tissue of the control mice—80–90% mitotic figures in contrast to the supplemented 70–80% (Figs. 2A,B). There is a strong positive correlation between the high Ki-67 index and high-grade histopathology of neoplasms. The control tissue cytoplasmic staining for MMP-9 was greater than in the supplemented mouse tissue (Figs. 2C,D). PAS staining, a measure of mucin, showed increased PAS material in the tumor tissue of the control group of mice (Figs. 2 E,F). VEGF staining, an indicator of angiogenesis, was higher in the control than in the supplemented group (Figs. 2G,H).

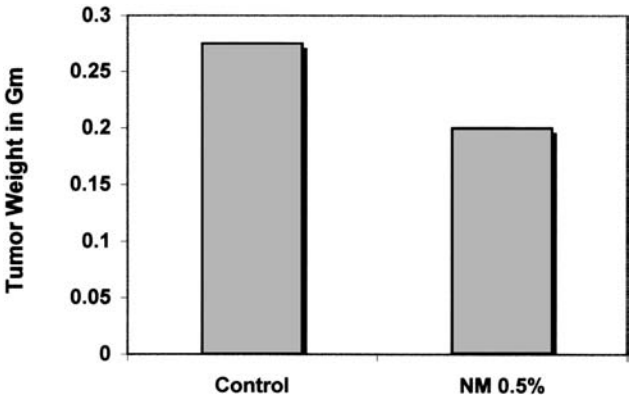

Fig. 1. Effect of nutrient mixture (NM) on tumor weight of breast cancer MDA MB-231 cell xenografts in female nude mice

In Vitro Studies

Cell Proliferation Study

The nutrient mixture (NM) did not show significant anti-proliferative effects on MDA MB-231 cells at 100 µg/mL but, at 500 µg/mL, inhibited cell growth by 34% ($p < 0.0001$, Fig. 3A) in regular medium and by 18% ($p = 0.0022$, Fig. 3B) in medium containing PMA 200 ng/mL. Cell proliferation of estrogen-sensitive MCF-7 cells was not significantly affected by NM concentrations up to 500 µg/mL. At 1000 µg/mL NM, cell proliferation decreased by 18% ($p = 0.0039$, Fig. 3C) in regular medium, 21% ($p = 0.0037$; Fig. 3D) in media with PMA, and 25% ($p = 0.0040$; Fig. 3E) in media with estradiol 25 ng/mL.

Gelatinase Zymography Study

For both cell lines, neither MMP-2 nor MMP-9 expression was detected by gelatinase zymography (Figs. 4A,C). PMA (200 ng/mL) induced enhanced MMP-9 expression by MCF-7 cell line. This induction was higher than observed in MDA MB-231 cells exposed to the same PMA concentrations. In both cancer cell lines the nutrient mixture inhibited MMP-9 expression in a dose-dependent fashion (Figs. 4B,D). Estradiol induced MMP-9 expression by MCF-7 cells, but to a lesser extent than PMA (Fig. 4E). NM also inhibited estradiol-induced MMP-9 expression at 50 µg/mL.

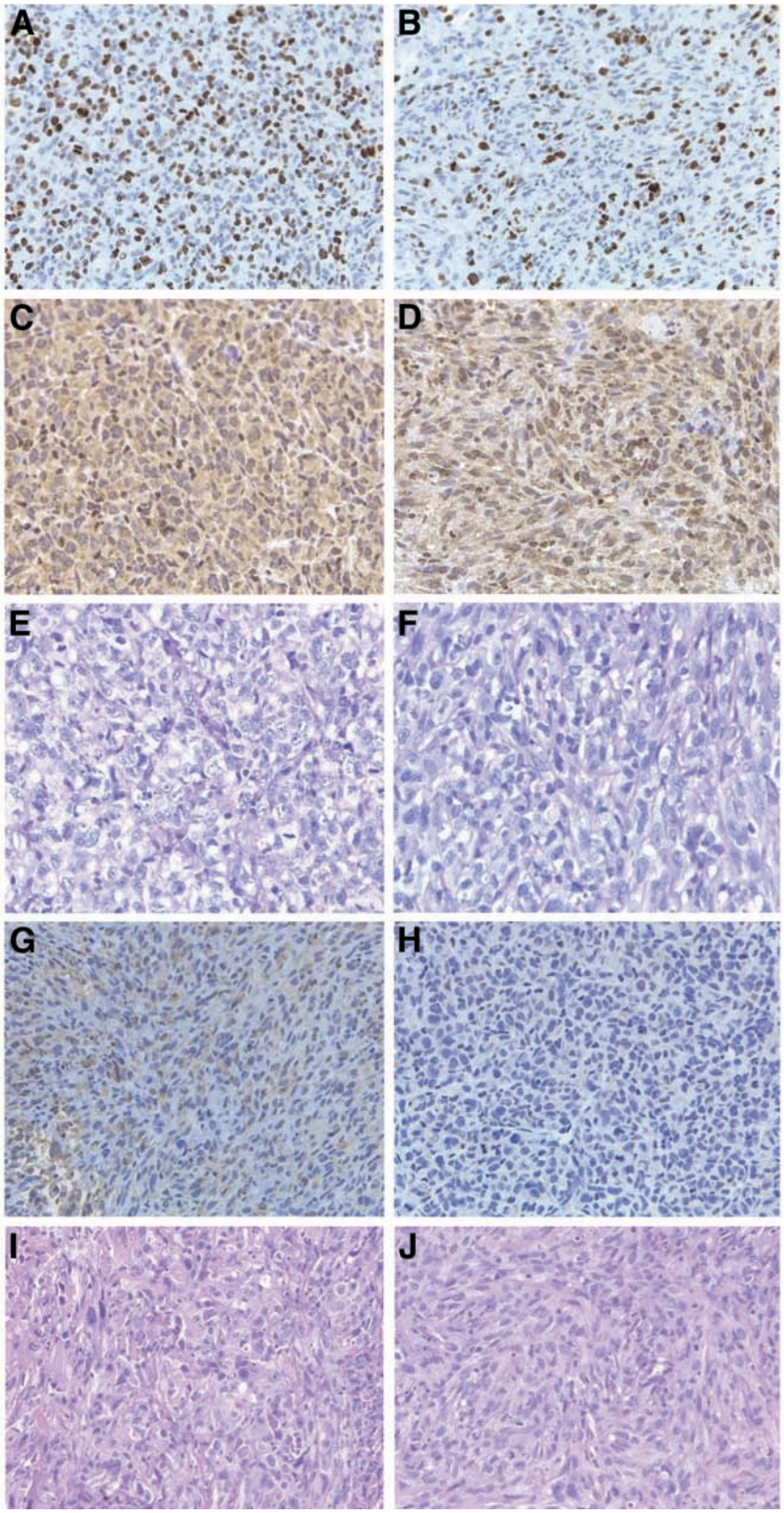

Fig. 2. Effect of nutrient supplementation (NM) on tumor tissues from nude mice with breast cancer cell MDA MB-231 xenografts (×100 magnification). **A** Ki-67 control; **B,** Ki-67 NM 0.5%; **C,** MMP-9 control; **D,** MMP-9 NM 0.5%; **E,** PAS control; **F,** PAS NM 0.5%; **G,** VEGF control; **H,** VEGF NM 0.5%; **I,** H&E control; **J,** H&E NM 0.5%.

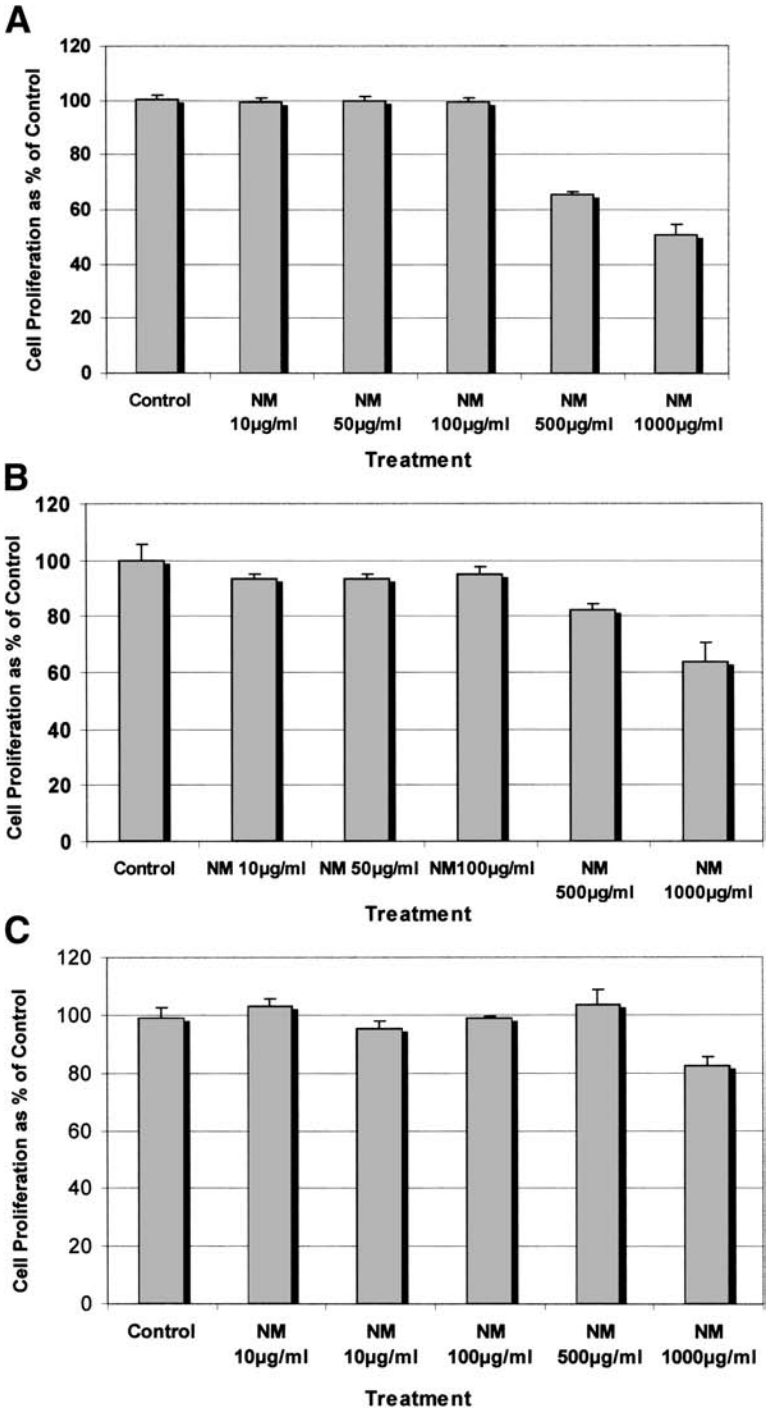

Fig. 3. (A) Effect of nutrient mixture (NM) on breast cancer MDA MB-231 cell proliferation (MTT Assay). **(B)** Effect of nutrient mixture (NM) on cell proliferation of breast cancer MDA MB-231 cells treated with PMA 200 ng/mL (MTT assay). **(C)** Effect of nutrient mixture (NM) on cell proliferation of breast cancer MCF-7 (MTT assay). (*Figure continues)*

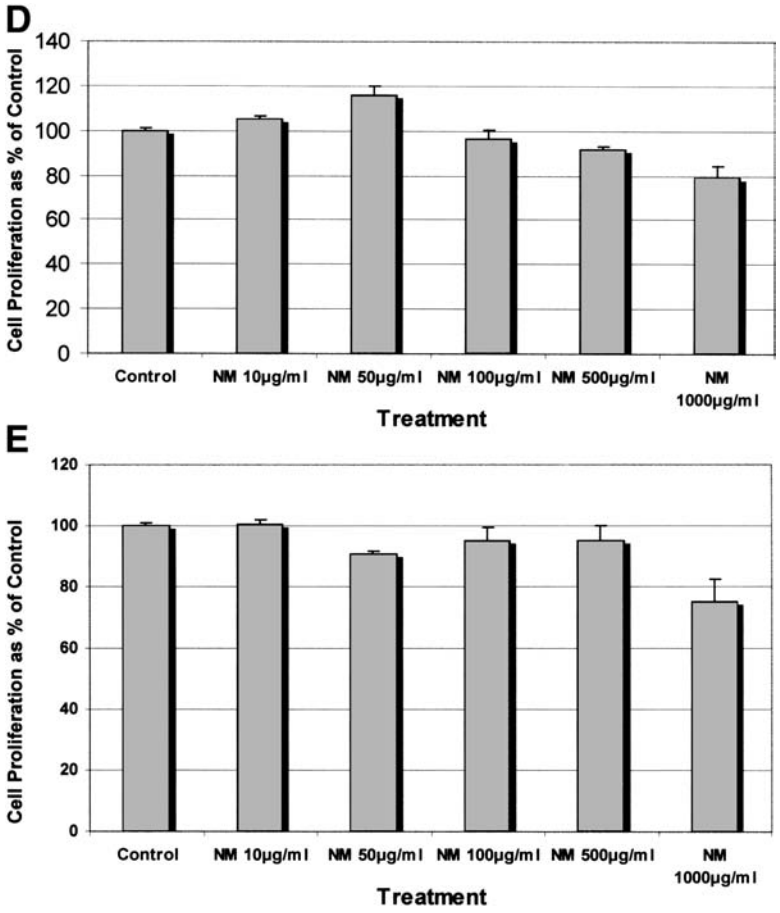

Fig. 3. ***(continued)*** **(D)** Effect of nutrient mixture (NM) on cell proliferation of breast cancer MCF-7 cells treated with PMA 200 ng/mL (MTT assay). **(E)** Effect of nutrient mixture (NM) on cell proliferation of breast cancer MCF-7 cells treated with estradiol 25 ng/mL (MTT assay).

Matrigel Invasion Study

Invasion of MDA MB-231 (ER^-) cells through Matrigel was inhibited by 26%, 73%, and 97% by 10, 50, and 100 µg/mL of NM, respectively ($p < 0.0001$; Fig. 5A). MCF-7 (ER^+) cells were not invasive and did not migrate through Matrigel. However, exposure to estradiol (25 ng/mL) increased MCF-7 invasive potential and NM significantly inhibited invasion through Matrigel by 75%, 94%, and 100% by 10, 50, and 100 µg/mL of NM, respectively ($p < 0.0001$; Fig. 5B).

VEGF Expression

Estradiol increased breast cancer cell VEGF expression to 41% above the control level. NM not only reversed the stimulatory effect of estradiol, but also reduced VEGF expression of the breast cancer cells not treated with estradiol (control) by 16% ($p = 0.0001$; Fig. 6).

Discussion

This study demonstrated significant inhibition of tumor growth of human breast xenografts in nude mice by supplementation with the 0.5% nutrient formulation. Furthermore, histological analysis revealed decreased Ki-67, MMP-9, and VEGF material in tissues of supplemented mice when compared to mice fed control diets. These results suggest that nutrient inhibition of tumor growth was associated

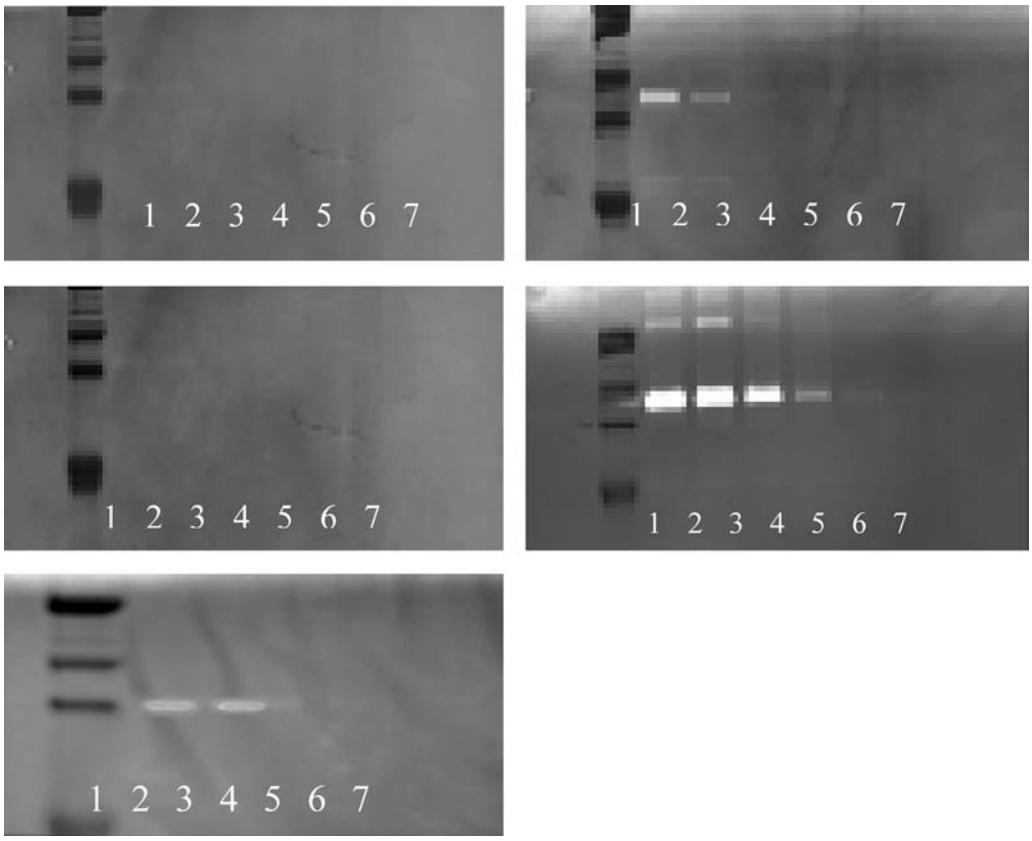

Fig. 4. Effect of nutrient mixture (NM) on MMP-9 expression of human breast cancer cells MDA MB-231 control **(A),** MDA MB-231 + PMA 200 ng/mL **(B),** MCF-7 control **(C),** MCF-7 + PMA 200 ng/mL **(D),** and MCF-7 with estradiol 25 ng/mL **(E)** was measured by gelatinase zymography in condition media. Lanes correspond as follows: 1, markers, 2, control, and 3–7 NM 10, 50, 100, 500, 1000 μg/mL.

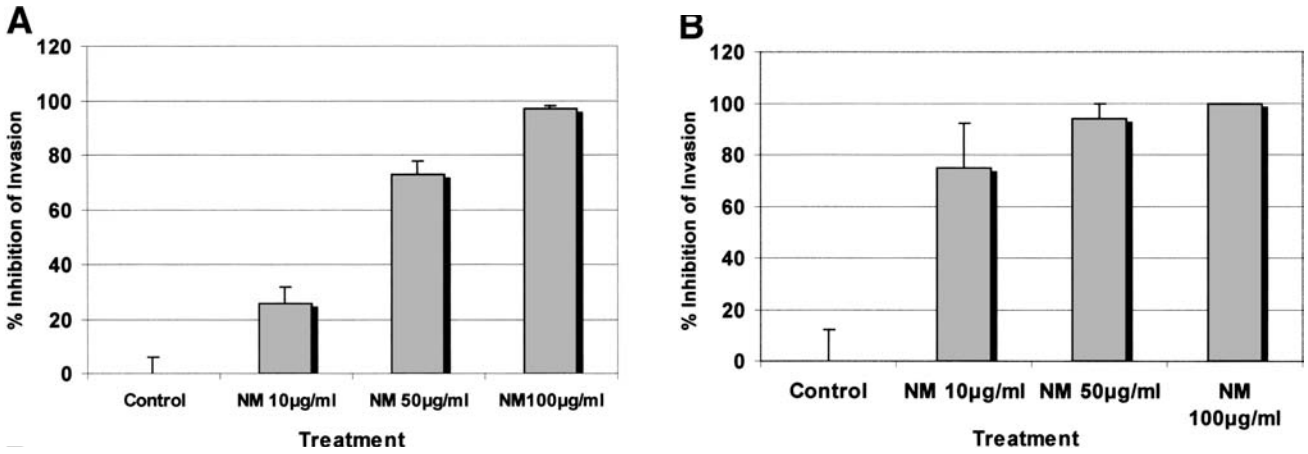

Fig. 5. Effect of NM on Matrigel invasion of MDA MB-231 breast cancer cells **(A).** Effect of NM on Matrigel invasion of MCF-7 breast cancer cells exposed to estradiol 25 ng/mL **(B).**

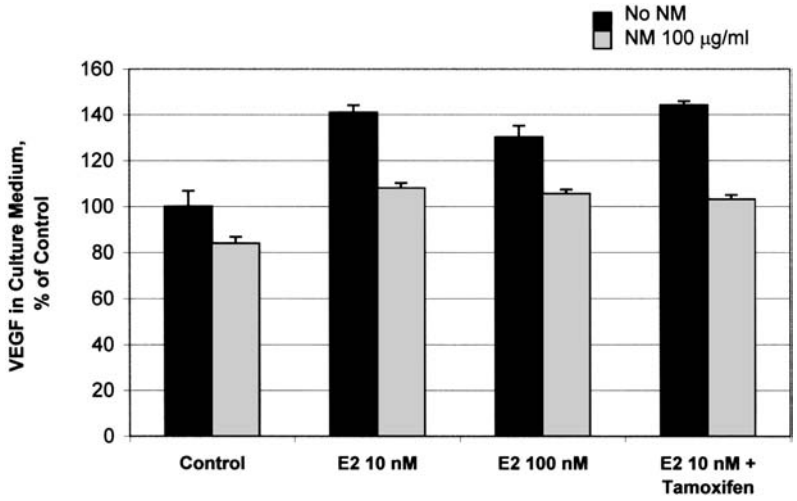

Fig. 6. NM inhibited VEGF secretion by breast cancer MCF-7 cells stimulated by 17-beta estradiol (E2).

with decreased cell proliferation and decreased angiogensis and were supported by the in vitro results for these parameters.

The in vitro results showed VEGF expression by MCF-7 breast cancer cells increased by 40% above the control with addition of 10 µg/mL of estradiol in the presence and absence of tamoxifen, but was completely reversed when treated with 100 µg/mL of the nutrient mixture, implying significant inhibition of angiogenesis potential. High tissue VEGF levels appear to correlate with poor prognosis and decreased overall survival in node-positive and node-negative breast cancer patients. In an in vivo study, vascular endothelial growth factor (VEGF) overexpression in breast cancer cells was reported to significantly increase intratumoral lymphangiogenesis and promote metastasis to regional lymph nodes and to the lungs. The degree of tumor lymphangiogenesis was highly correlated with the extent of lymph node and lung metastases *(5)*. Hormonal regulation of VEGF expression has been demonstrated with estrogen receptor agonists, as tamoxifen, which was reported to decrease extracellular levels of VEGF in vivo in solid MCF-7 tumors in nude mice *(6)*.

Secretion of various MMPs in breast cancer cell lines of different tumorigenicity correlates with the biological behaviors of these cells—more malignant cells synthesize more MMPs than less malignant ones *(7)*. In our in vitro study, the nutrient mixture demonstrated significant anti-invasive effects (73% at 50 µg/mL and 97% at 100 µg/mL) on estrogen-receptor-negative human breast cancer cell MDA MB-231, without morphological changes. Matrigel invasion by MCF-7 cells was not detectable except in the presence of PMA or estradiol, but was inhibited significantly by NM in a dose-dependent manner. MMP-9 expression by both MCF-7 and MDA-MB-231 cancer cells decreased in a dose-dependent fashion with complete inhibition of invasion and MMP expression at 50 µg/mL and 500 µg/mL, respectively.

Matrix invasion can be controlled by inhibition of MMP expression, as well as by increasing connective tissue strength and stability, contributing to the "encapsulation" of the tumor. In this study, the dose-dependent inhibitory effect of the nutrient mixture on MMP-9 expression of both breast cancer cell lines was consistent with the respective inhibition of matrix invasion. In addition, matrix invasion was also restricted by enhanced stability and strength of the connective tissue secondary to the synergistic activity of the nutrients. Optimization of synthesis and structure of collagen fibrils depends on hydroxylation of proline and lysine residues in collagen fibers. It is well known that ascorbic acid is essential for the hydroxylation of these amino acids and it regulates collagen synthesis at the transcriptional level. Ascorbic acid and lysine are not produced in the

human body; therefore, sub-optimal levels of these nutrients are possible in various pathological stages and through deficient diets.

The inhibitory effects of the individual nutrients in the mixture have been reported in both clinical and experimental studies. Ascorbic acid has been reported to have cytotoxic and antimetastatic actions on malignant cell lines *(8–10);* in addition, low levels of ascorbic acid have been reported in cancer patients *(11–13).* EGCG is a potent anticancer agent proven to have a growth inhibitory effect against certain human cancer cell lines, especially breast cancer *(14–16).* For example, both in vitro and animal studies conducted on the effect of green tea extract on breast cancer revealed suppressed xenograft size and tumor vessel density and suppression of cell proliferation *(17).* Furthermore, clinical studies conducted prior to clinical onset of breast cancer showed increased green tea consumption was associated with improved prognosis of stage I and II breast cancer, as well as decreased numbers of axillary lymph node metastases in premenopausal women, suggesting significant chemopreventative potential *(18).*

However, our previous studies demonstrated that the synergistic anticancer effect of ascorbic acid, proline, lysine, and EGCG on several cancer cell lines in tissue culture studies was greater than that of the individual nutrients *(4).* Furthermore, in contrast to chemotherapy, which causes indiscriminate cellular and ECM damage, morphological studies showed that even at the highest concentrations of the nutrient mixture, the breast cancer cells were not affected, demonstrating that this formulation is safe to cells.

While clinical studies are necessary to evaluate the effect of nutrient supplementation on breast cancer, our results suggest that the specific mixture of lysine, proline, ascorbic acid, and green tea extract studied is an excellent candidate for preventative and therapeutic use in the treatment of breast cancer, by inhibiting tumor growth, MMP expression, invasion, and angiogenesis.

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