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In vitro anticarcinogenic effect of a nutrient mixture on human rhabdomyosarcoma cells

Research Article

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Abbreviations: Matrix metalloproteinases, (MMPs); nitric oxide, (NO); phosphate buffered saline, (PBS); Rhabdomyosarcoma, (RMS)

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Summary

Rhabdomyosarcoma, the most common pediatric soft tissue sarcoma of mesenchymal origin, has metastasized in ~25% of all patients at time of diagnosis. Though current treatment strategies have achieved some success, they are associated with severe adverse effects. We investigated the effect of a nutrient mixture (NM), which has shown antitumor effects on various cancer cell lines, on rhabdomyosarcoma cell growth, apoptosis, MMP secretion, and invasion. Human rhabdomyosarcoma cells, grown in DME, were treated at near confluence with NM at 0, 10, 50, 100, 500 and 1000 μg/ml in triplicate at each dose. MMP secretion was studied by zymography, viability by MTT assay, cell invasion through Matrigel, and morphology and apoptosis by H&E staining and live green caspase kit. Zymography demonstrated MMP-2 secretion and PMA-induced MMP-9 secretion. NM inhibited the secretion of both MMPs in a dose-dependent fashion, with virtual total inhibition at 500 μg/ml NM. Cell invasion through Matrigel was inhibited at 10, 50, 100 and 500 μg/ml by 75%, 80%, 92% and 100% (p=0.02) respectively. NM was slightly toxic at 1000 μg/ml (20% over control, p=0.016) to rhabdomyosarcoma cells. Cells exposed to NM showed dose-dependent apoptosis with 90% of cells in late apoptosis at 1000 μg/ml. These results suggest that NM has potential in the treatment of rhabdomyosarcoma by inducing cell apoptosis and inhibiting cell invasion and MMP secretion without toxic effects.

I. Introduction

Rhabdomyosarcoma (RMS), a soft tissue tumor of skeletal muscle origin is the third most common extracranial solid childhood neoplasm with approximately 250 new cases diagnosed each year in the United Sates (Kramer, 1983). While tumors can appear at numerous locations, the primary sites are: the head and neck (35%), the genitourinary tract (22%), and the extremities (18%) (Barr, 1997). There are two main histological types of pediatric RMS: embryonic RMS and alveolar RMS. Embryonal RMS is more prevalent, contributing to roughly 53% of all diagnosed cases; it generally presents in children under fifteen in either the head and neck region or the genitourinary tract (Parham, 2001). Alveolar RMS generally affects the muscles of the extremities or trunk and has been found to be more resistant to treatment and more likely to spread to regional lymph nodes than embryonal RMS and the botryoid variant commonly found in infants (Mandell et al, 1990). Pleomorphic RMS is usually seen in adults and arises in the muscles of the extremities. At diagnosis, roughly 50% of cases consist of patients aged five and younger and 25% of all patients have metastatic disease (Koscielniak et al, 1992).

Standard multimodality treatment approaches developed through such trials as the Intergroup Rhabdomyosarcoma Study Group (IRSG) include surgery, radiation therapy, and chemotherapy. The use of neoadjunctive therapy has increased survival in patients with localized disease to 60% five-year survival (Stevens et al, 2005); however, complications resulting from therapy are serious and can be life threatening. Additionally, clinical trials focused on three or five-year event-free survival does not adequately address the needs of pediatric cancer patients. Toxicities and delayed effects of treatment not present during treatment can manifest later as a result of growth and development. Patients with

primary tumor sites at the bladder or prostate treated with radiation have been found to be at an increased risk of developing bowel complications, poor bladder function, hemorrhagic cystitis, and sex hormone deficiency (Raney et al, 1993). Ifosfamide (high dose) can lead to renal Fanconi's syndrome with glycosuria, phosphaturia and aminoaciduria (Skinner, 2003). Cyclophosphamide can increase the risk of hepatic dysfunction and lead to early menopause in young women (Sklar, 2005). Anthracyclines cause myocardial cell death and have been implicated in cardiac failure and fatal arrhythmia ten to twenty years after administration (Iarussi et al, 2005). Adriamycin can increase the risk of "late" cardiomyopathy (Lipshultz et al, 1991). Cisplatin is known to cause glomerular and tubular injury (Taguchi et al, 2005). Radiation therapy contributes to tubular damage and hypertension associated with renal artery stenosis (Moulder and Cohen, 2005). Localized radiotherapy is associated with hypoplasia with asymmetry most apparent during pubertal development (Denys et al, 1998). Cranial irradiation affects the hypothalamic-pituitary axis leading to the early onset of puberty and subsequently thwarts ultimate height (Darzy and Shalet, 2005). Children under age five are particularly sensitive to central nervous system irradiation and chemotherapy, which can impair their attention, memory and motor skills, having a profound effect on their educational and occupational success (Monje and Palmer, 2003).

The most serious late effect of current treatment is the development of a second malignant neoplasm; childhood cancer survivors have an 8-10% risk within 20 years (Sung et al, 2004) and the risk of developing second malignancies is particularly high among patients who received combined modality therapy (Cohen et al, 2005). Given this data, it is clear that current treatment brings limited benefit to patients, which compels the need for new agents aimed at specific targets involved in metastatic behavior, that are not injurious to the health of the patient.

It is now well documented that the family of zinc-dependent endoproteinases, matrix metalloproteinases (MMPs), facilitate tumor cell invasion and metastasis through: removal of physical barriers to invasion, degradation of extracellular matrix (ECM) macromolecules, and modulation of cell adhesion and activation of ECM components to expose hidden biologic activities. Such has prompted researchers to design therapies that inhibit MMP activity to prevent metastasis. Rath and Pauling proposed that natural inhibitors, such as lysine and ascorbic acid, have the potential to inhibit tumor growth and expansion through the modulation of ECM proteolysis and optimization of connective tissue integrity (Rath and Pauling, 1992). Our previous studies have demonstrated significant antitumoral activity of the nutrient mixture containing lysine, proline, ascorbic acid and green tea extract against a large number of cancer cell lines *in vitro* (Roomi et al, 2005a,b,c) and *in vivo* (Roomi et al, 2005d,e,f).

In the current study, we investigated the effect of NM on human rhabdomyosarcoma cells by measuring cell proliferation, apoptosis, modulation of MMP-2 and MMP-9 secretion, and cancer cell invasive potential.

II. Materials and methods

A. Cell Culture

Embryonal rhabdomyosarcoma cells (CCL-136RD) obtained from ATCC (American Type Culture Collection, Rockville, MD), were grown in DME media, 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 treated with the nutrient mixture, dissolved in media and tested at 0, 10, 50, 100, 500, and 1000 µg/ml in triplicate at each dose. Cells were also treated with phorbol 12-myristate 13-acetate (PMA) 200ng/ml to induce MMP-9 secretion. The plates were then returned to the incubator.

B. MTT Assay

Cell viability was evaluated by [3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyl tetrazolium bromide] (MTT) assay, a colorimetric assay based on the ability of viable cells to reduce a soluble yellow tetrazolium salt MTT to a blue formazan crystal by mitochondrial succinate dehydrogenase activity of viable cells. This test is a good index of mitochondrial activity and thus of cell viability. After 24 h incubation, the cells were washed with phosphate buffered saline (PBS) and 500 µl of MTT (Sigma #M-2128) 0.5 mg/ml in media was added to each well. After MTT addition (0.5mg/ml) the plates were covered and returned to the 37°C incubator for 2h, the optimal time for formazan product formation. Following incubation, the supernatant was carefully removed from the wells, the formazan product was dissolved in 1ml DMSO, and absorbance was measured at 570 nm in 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 100%.

C. Gelatinase zymography

MMP activity in conditioned media was determined by gelatinase zymography. Gelatinase zymography was performed in 10% Novex precast SDS-polyacrylamide gel (Invitrogen Corporation) in the presence of 0.1% gelatin under non-reduced conditions. Culture media (20 µl) mixed with sample buffer was loaded and SDS-PAGE was performed with tris glycine SDS buffer as described by the manufacturer (Novex). Samples were not boiled before electrophoresis. Following electrophoresis the gels were washed twice in 2.5% Triton X-100 for 30 minutes at room temperature to remove SDS. The gels were then incubated at 37° C overnight in substrate buffer containing 50 mM Tris-HCl and 10 mM $CaCl_2$ at pH 8.0 and stained with 0.5% Coomassie Blue R250 in 50% methanol and 10% glacial acetic acid for 30 minutes and destained. Protein standards were run concurrently and approximate molecular weights were determined by plotting the relative mobilities of known proteins.

Gelatinase zymograms were scanned using CanoScan 9950F Canon scanner at 1200 dpi. The intensity of the bands was evaluated using a pixel-based densitometer program Un-Scan-It, Version 5.1, 32-bit, by Silk Scientific Corporation (Orem, UT, USA), at a resolution of 1 Scanner Unit (1/100 of an inch for an image that was scanned at 100 dpi), and expressed as a percentage of control. The R^2 value (0 to 1), which represents how well the line of best fit falls on the dependent data, with R^2 = 1.0 being the best possible fit, was determined.

D. Matrigel invasion

Invasion studies were conducted using Matrigel (Becton Dickinson) inserts in 24-well plates. Suspended in medium, rhabdomyosarcoma 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% CO_2 for 24 hours. 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.

E. Morphology and apoptosis

Morphology of cells cultured for 24h in test concentrations of NM were evaluated by H&E staining and observed and photographed by microscopy. At near confluence, rhabdomyosarcoma cells were challenged with NM dissolved in media at 0, 100, 500, and 1000 μg/ml and incubated for 24 h. The cell culture was washed with PBS and treated with the caspase reagent as specified in the manufacturer's protocol (Molecular Probes Image-IT™ Live Green Poly Caspases Detection Kit 135104, Invitrogen). The cells were photographed under a fluorescence microscope and counted. Green-colored cells represent viable cells, while yellow orange represents early apoptosis and red late apoptosis.

F. Composition of NM

Stock solution of the nutrient mixture prepared for testing was composed of the following in the ratio indicated: 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 1000 mg (green tea extract derived from green tea leaves was obtained from US Pharma Lab. The certificate of analysis indicates the following characteristics: total polyphenol 80%, catechins 60%, EGCG 35%, and caffeine 1.0%); selenium 30 μg; copper 2 mg; manganese 1mg.

G. Statistical analysis

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

III. Results

A. Cell viability study

NM demonstrated insignificant effect on RMS cells at 10 μg/ml – 500 μg/ml. At 1000 μg/ml NM showed slight significant toxicity (20% over control, p=0.016) to RMS cells, as shown in **Figure 1**.

B. Gelatinase zymography study

Zymography demonstrated secretion of MMP-2 by unstimulated cells. Treatment of rhabdomyosarcoma cells with PMA (200 ng/ml) induced MMP-9 activity. NM inhibited secretion of both MMPs in a dose-dependent fashion with virtual total inhibition at 500 μg/ml, as shown in **Figures 2A and 2B.** Densitometry analysis on relative activity of MMP-2 showed 18% inhibition at 50 μg/ml, 45% at 100 μg/ml and 99% at 500 μg/ml NM, with R^2 = 0.8443 (**Figure 2C**). For MMP-9, relative activity per densitometry revealed 20% inhibition at 50 μg/ml, 46% at 100 μg/ml and 98% at 500 μg/ml NM, with R^2 = 0.8875 (**Figure 2D**).

C. Invasion study

NM significantly reduced the invasion of rhabdomyosarcoma cells through Matrigel in a dose-dependent fashion, with 75% inhibition at 10 μg/ml, 80% at 50 μg/ml, 92% at 100 μg/ml, and 100% at 500 μg/ml NM (p=0.02), as shown in **Figure 3**.

D. Morphology (H&E staining) and apoptosis (live green caspases detection kit)

H&E staining showed no morphological changes at 100 μg/ml NM, but apoptotic cells were evident at 500 and 1000 μg/ml, as shown in **Figures 4A-F**. The apoptotic cells showed shrinkage with condensed and darkly stained nuclei and strongly acidophilic cytoplasm. Using the live green caspase kit, dose-dependent apoptosis of pediatric rhabdomyosarcoma cells was evident with NM challenge, as shown in **Figures 5A-E**. Few apoptotic cells were observed in those cells exposed to 100 μg/ml NM; the number of apoptotic cells increased significantly in a dose-dependent manner at 250 μg/ml - 1000 μg/ml NM.

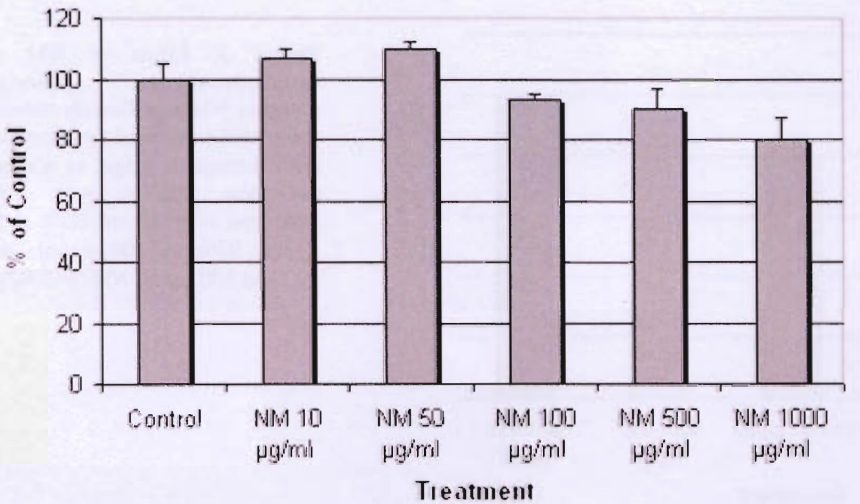
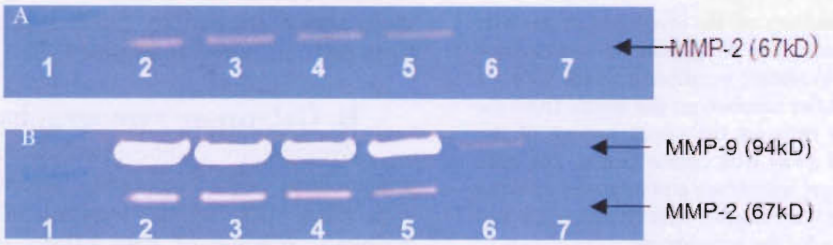
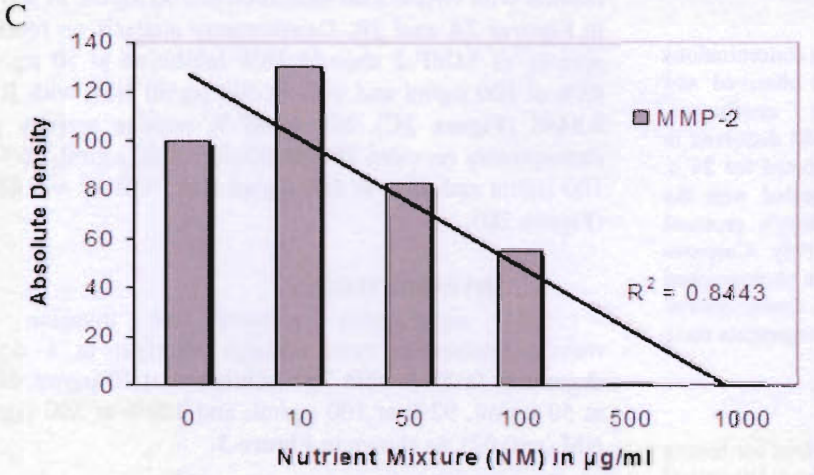


Figure 1. Effect of the NM on growth of rhabdomyosarcoma cells: 24h MTT assay. NM demonstrated insignificant effect on RMS cells at 10 μg/ml – 500 μg/ml. At 1000 μg/ml NM showed slight significant toxicity (20% over control, p=0.016) to RMS cells.





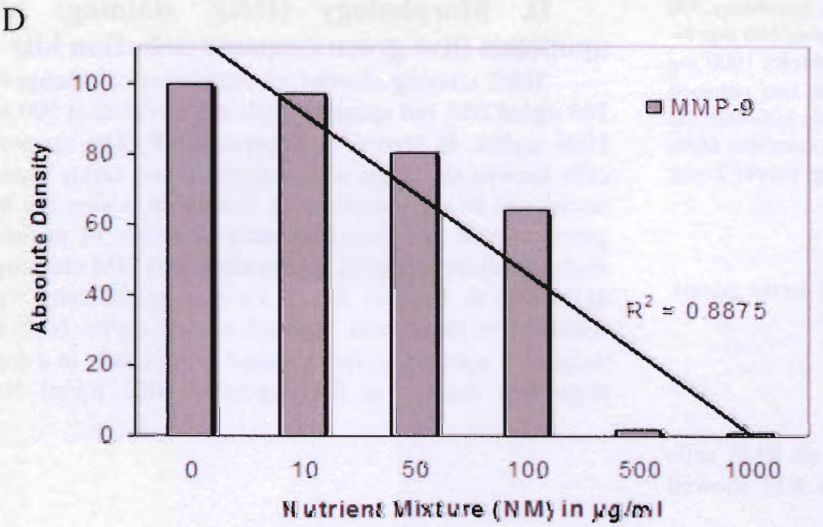


Figure 2. Effect of NM on MMP-2 and MMP-9 secretion by untreated rhabdomyosarcoma cells (**A**) and by PMA (200 ng/ml)-treated cells (**B**). Legend 1 – Markers, 2-Control, 3-7 NM 10, 50, 100, 500, 1000 µg/ml. Zymography demonstrated secretion of MMP-2 and PMA - induced MMP-9 activity. Densitometry Analysis. Densitometry analysis on relative activity of MMP-2 showed 18% inhibition at 50 µg/ml, 45% at 100 µg/ml and 99% at 500 µg/ml NM, with R^2 = 0.8443 (**C**). For MMP-9, relative activity per densitometry revealed 20% inhibition at 50 µg/ml, 46% at 100 µg/ml and 98% at 500 µg/ml NM, with R^2 = 0.8875 (**D**).

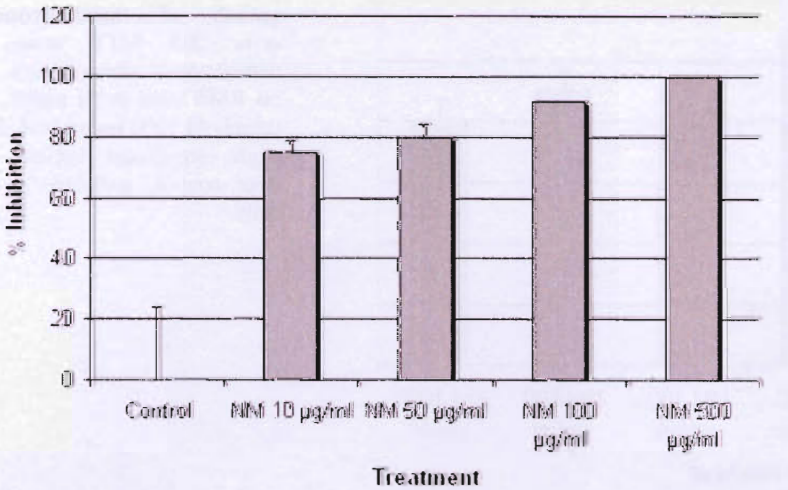


Figure 3. Effect of NM on rhabdomyosarcoma Matrigel invasion. NM significantly reduced the invasion of rhabdomyosarcoma cells through Matrigel in a dose-dependent fashion, with 75% inhibition at 10 µg/ml, 80% at 50 µg/ml, 92% at 100 µg/ml, and 100% at 500 µg/ml NM (p=0.02).

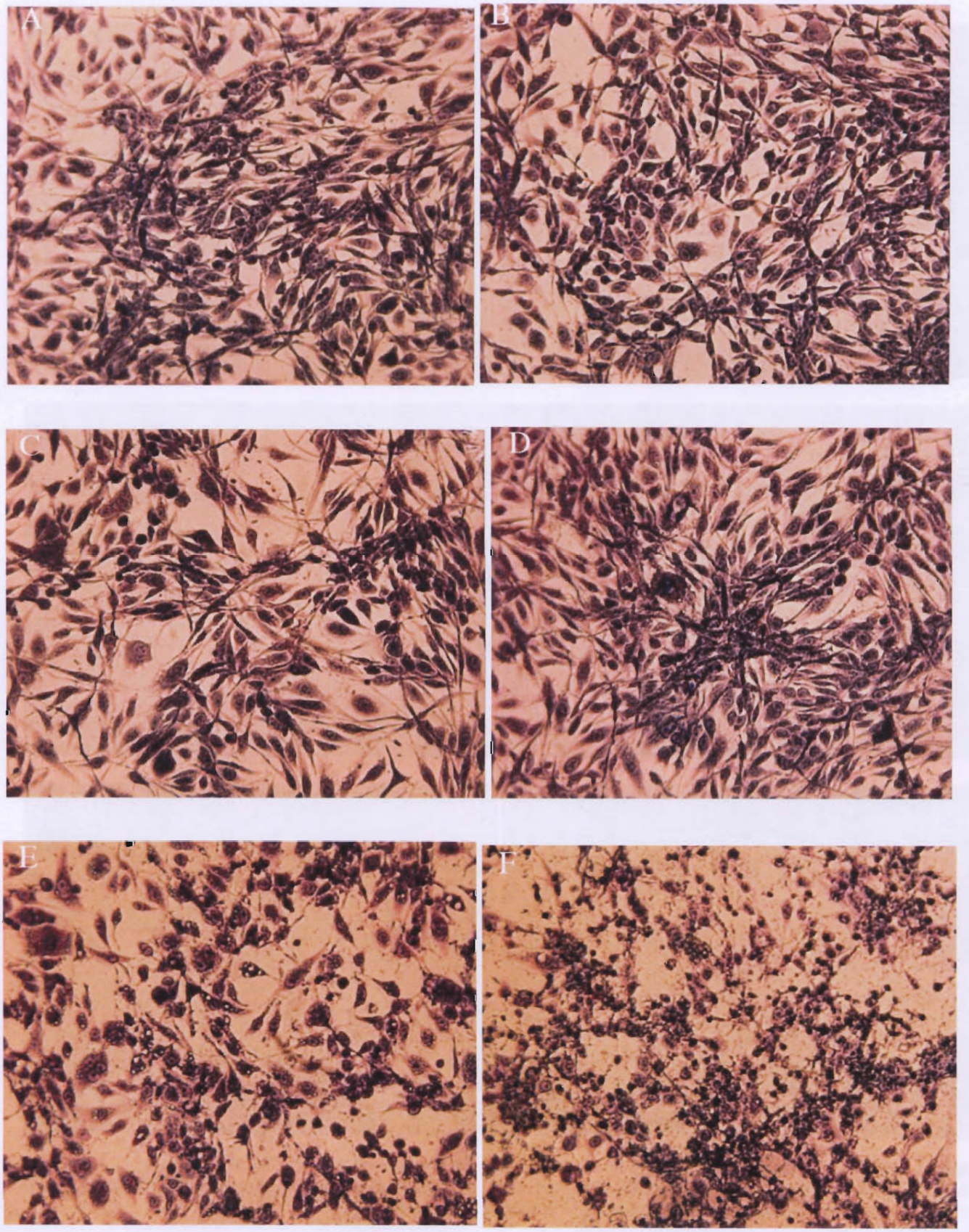


Figure 4. Effect of NM on rhabdomyosarcoma morphology (H&E staining): **A** – Control, **B** – NM 10 μg/ml, **C** – NM 50 μg/ml, **D** - 100 μg/ml, **E** - 500 μg/ml, **F** – 1000 μg/ml. H&E staining showed no morphological changes at 100 μg/ml NM, but apoptotic cells were evident at 500 and 1000 μg/ml. The apoptotic cells showed shrinkage with condensed and darkly stained nuclei and strongly acidophilic cytoplasm. Typical apoptotic cells indicated with arrows in E and F.

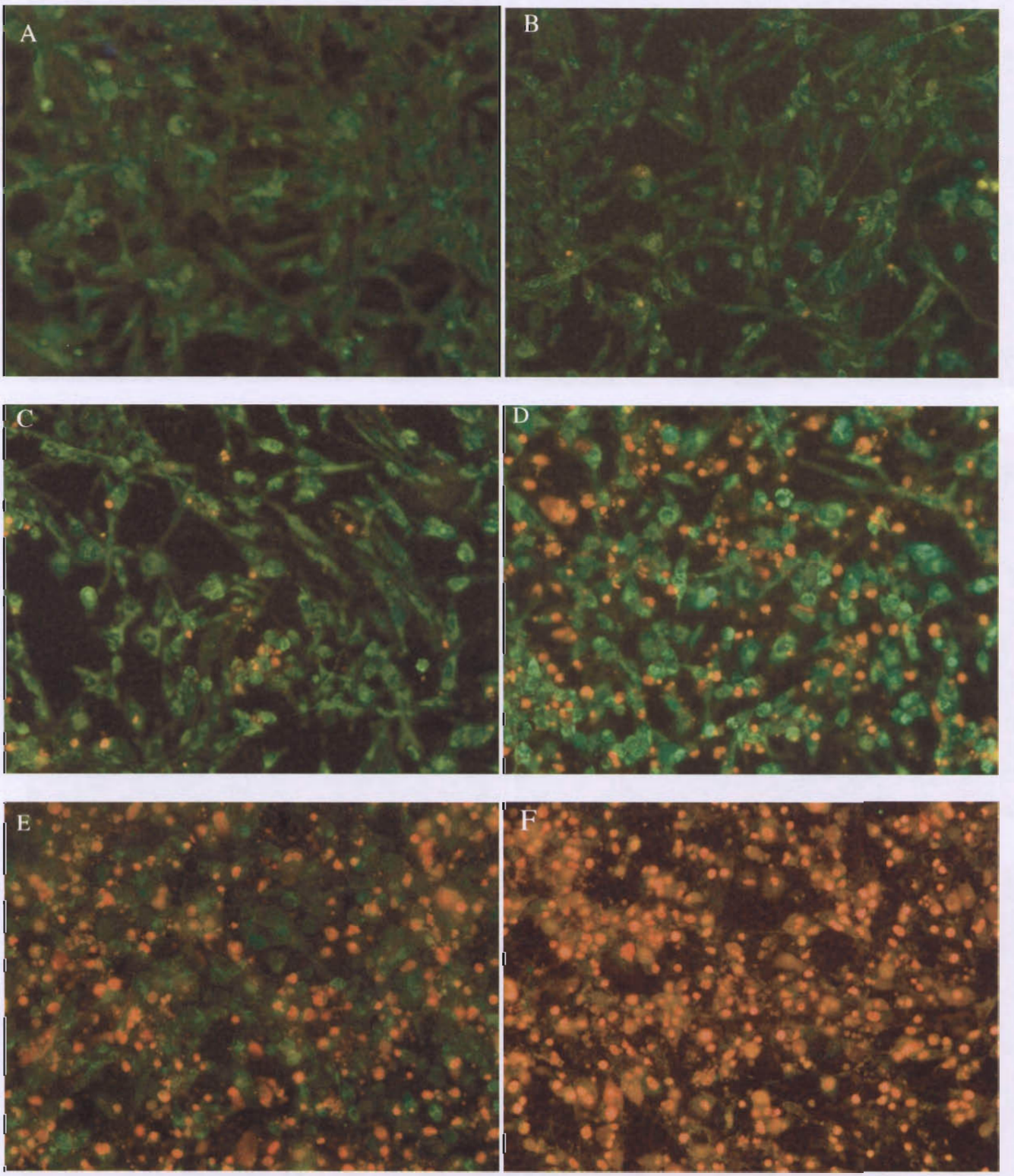


Figure 5. Effect of NM on rhabdomyosarcoma apoptosis (live green caspase detection kit): **A** – Control, **B** – NM 50 μg/ml, **C** – NM 100 μg/ml, **D** – NM 250 μg/ml, **E** – NM 500 μg/ml, 5**F** – NM 1000 μg/ml. Slight apoptosis of rhabdomyosarcoma cells was observed in cells exposed to 100 μg/ml, moderate at 250 μg/ml and profound at 500 and 1000 μg/ml NM.

Quantitative analysis of live, early and late apoptotic cells is shown in **Figure 6**. At 100 μg/ml NM, 95% of cells are viable and 5% apoptotic and at 250μg/ml NM 45% of cells are viable, 12% in early apoptosis, and 40% in late apoptosis. RMS cell apoptosis was further increased in cells exposed to 500 μg/ml NM: 28% viable, 16% early apoptosis, and 60% late apoptosis. Virtually all cells exposed to 1000 μg/ml NM were in late apoptosis: 10% early apoptosis and 90% late apoptosis.

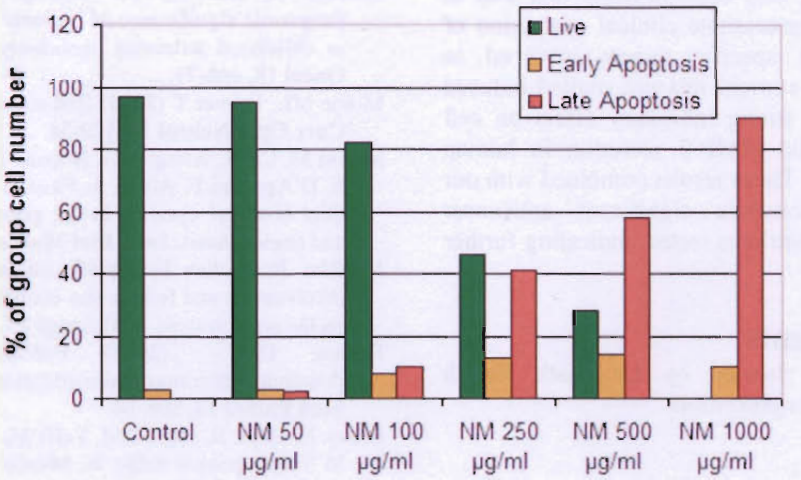


Figure 6. Effect of NM on rhabdomyosarcoma apoptosis (live green caspase detection kit): % of cells in various stages of apoptosis at NM 0, 50, 100, 250, 500, and 1000 µg/ml. At 100 µg/ml NM, 95% of cells are viable and 5% apoptotic and at 250µg/ml NM 46% of cells are viable, 13% in early apoptosis, and 41% in late apoptosis. RMS cell apoptosis was further increased in cells exposed to 500 µg/ml NM: 28% viable, 14% early apoptosis, and 58% late apoptosis. Virtually all cells exposed to 1000 µg/ml NM were in late apoptosis: 10% early apoptosis and 90% late apoptosis.

IV. Discussion

The nutrient mixture demonstrated significant inhibition of human rhabdomyosarcoma cell invasive parameters *in vitro*. Matrigel invasion and MMP-2 and MMP-9 secretion of rhabdomyosarcoma cells decreased in a dose-dependent fashion with complete inhibition of MMP-2 and -9 at 500 µg/ml, and of Matrigel invasion at 500 µg/ml, demonstrating strong antimetastatic potential. In addition NM demonstrated dose-dependent pro-apoptotic effects on rhabdomyosarcoma cells and profound induction of apoptosis and morphological changes at 500 µg/ml.

Matrix metalloproteinases (MMPs) have been implicated in the destruction of the extracellular matrix, neovascularization, tumor spread and metastases, and recent studies have shown overexpression of MMPs is associated with poor prognosis in cancer patients. Due to this, new treatment approaches have targeted universal pathomechanisms involved in cancer progression, such as control of proteolytic activity of the ECM as a potential strategy against cancer progression. A recent study reported that a high level of MMP-2 protein may contribute to the metastatic phenotype of aRMS showing potential for MMP-2 inhibition in the treatment of the aggressive alveolar subtype of rhabdomyosarcoma (Diomedi-Carnassei, 2004). While our study did not examine the effect of the nutrient mixture on inhibition of MMP-2 expression on the aRMS, MMP-2 secretion in the embryonal RMS cells was completely abolished at 500 µg/ml NM.

The nutrient mixture was formulated based on targeting different physiological processes involved in cancer progression and metastasis. For example, the ECM integrity is dependent upon adequate collagen formation and its stability. In this aspect, ascorbic acid and the amino acids lysine and proline are necessary for the formation and optimum structure of collagen fibers. Manganese and copper are also essential cofactors in collagen formation process. Collagen stability can be controlled by lysine (Rath and Pauling, 1992) and also by N-acetyl cysteine through its inhibitory effect on MMP-9 activity (Kawakami et al, 2001) and invasive activities of tumor cells (Morini et al, 1999). Also, selenium has been shown to interfere with MMP expression and tumor invasion (Yoon et al, 2001), as well as migration of endothelial cells through ECM (Morini et al, 1999). Ascorbic acid has been shown to inhibit cell division and growth through production of hydrogen peroxide (Chen et al, 2005). Green tea extract has shown to be a promising agent in controlling angiogenesis, metastasis, and other aspects of cancer progression (Hare, 2001; Landau, 1998; Yang, 1998). Since arginine is a precursor of nitric oxide (NO), any deficiency of arginine can limit the production of NO, which has been shown to predominantly act as an inducer of apoptosis, as in the case of breast cancer cells (Cooke and Dzau, 1997).

Based on the evidence available in literature and our own research, we have postulated that metabolic effects of a combination of ascorbic acid, lysine, proline, green tea extract, arginine, N-acetyl cysteine, selenium, copper and manganese would result from their synergy. For example, we found that a combination of ascorbic acid, lysine and proline used with EGCG enhanced the anti-invasive activity of 20 µg/ml EGCG to that of 50 µg/ml (Roomi et al, 2004). Thus by including nutrients like N-acetyl cysteine, arginine, selenium, manganese and copper in addition to ascorbic acid, proline, lysine and EGCG we could obtain significant reduction in cell invasion at a much lower concentration of EGCG.

Clearly, while five-year survival has improved with current multimodality pediatric rhabdomyosarcoma treatment, the associated toxicities are significant and can be fatal to the patient in later life. Likewise mutagenic

risks of radiation therapy and chemotherapy that lead to secondary malignancies necessitate clinical evaluation of novel agents aimed at specific targets involved in metastatic behavior. The nutrient mixture studied induced apoptosis and exerted a strong inhibitory effect on cell invasion and MMP-2 and MMP-9 secretion in human rhabdomyosarcoma cells. These results combined with our previous findings demonstrate significant anticancer potential of the specific nutrients tested, indicating further investigation *in vivo*.

Acknowledgments

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