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Research Article

A Nutrient Mixture Induces Caspase Dependent Apoptosis in Human Synovial Sarcoma Cells

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Abbreviations NM: Nutrient Mixture; ATCC: American Type Culture Collection; FBS: Fetal Bovine Serum; EGCG: Epigallocatechin gallate; EGC: Epicatechin-3-gallate; EC: Epicatechin

Abstract

Synovial sarcoma is a rare soft tissue cancer occurring more frequently in adolescent and young adults than older people. The primary sites being the joints of the arms and the legs.

A novel Nutrient Mixture (NM) containing green tea extract, ascorbic acid, lysine, and proline exhibited anti-cancer effects in various cancers. In our earlier studies, the NM considerably reduced the tumor weight and tumor burden in synovial sarcoma. Based on the observation, we investigated whether this phenomenon and the anti-cancer effects were due to the induction of apoptosis. Synovial sarcoma cell line SW982 was cultured in complete DME media and the cells were treated with NM at 0-1000 µg/ml concentration. Cell cytotoxicity was measured by MTT assay, morphology by H&E staining, and the apoptosis by Green Caspases. NM showed no significant cytotoxicity at 100 µg/ml, slight toxicity at 500 µg/ml and maximum at 1000 µg/ml. H&E staining at the NM dose of 100 µg/ml showed a few cellular changes characteristic to apoptosis, while significant changes pertaining to apoptosis morphology were observed at 500 and 1000 µg/ml. Live Green Caspases analysis showed cells in early and late apoptosis with increasing doses of NM. Since there are no satisfactory treatments and cures for synovial sarcoma patients and the 5-year survival rate is low (55-75%), we think that addition of NM could add a new option for the patients of synovial sarcoma and deserves further clinical investigation.

Introduction

Synovial sarcoma is a rare soft tissue cancer occurring more frequently in adolescent and young adults than older people. The primary sites of origin of synovial sarcoma are the joints of the arms and the legs (50%), but it can also occur in the trunk (40%) and head/neck areas (10%). It is slightly more common in men than women. Due to the elasticity of this soft tissue cancer, tumors can grow rather large before they are noticed. Initially, synovial sarcoma presents as a painless lump, which becomes more painful as the tumor grows and presses against nearby nerves and muscles. The only reliable method of determining whether the tumor is malignant is by biopsy. Treatment depends upon location and stage of the disease. The most common treatment is surgery and involves removing the entire tumor and nearby muscle and tissue. Sometimes surgery is accompanied by radiation and chemotherapy. Despite various treatment modalities, synovial sarcoma has a high recurrence rate and local and distant metastasis occurs in about 50% of the cases [1]. The conventional treatment options have failed to show significant improvement in the long-term survival rate or the quality of life for the patient. The prognosis is poor with 5- year survival rate ranging from 55-75%.

In an earlier study we found that the nutrient mixture significantly affected the tumor growth and tumor burden in human synovial sarcoma cell line SW982 [2].

In the current study, we investigated whether the antitumor effects of the nutrient mixture are due to induction of caspase dependent apoptosis in human synovial sarcoma. Activated caspase is a distinctive feature of early stages of apoptosis. These enzymes participate in a series of reactions that are triggered in response to pro apoptotic signals and result in the cleavage of protein substances and in the subsequent assembly of the cells.

Materials and Methods

Cell culture

Human synovial sarcoma cells SW982 were obtained from ATCC and grown in modified Eagle medium supplemented with 10% fetal bovine serum, penicillin G sodium (100 U/ml), streptomycin (100 µg/ml), and amphotericin (0.25 µg/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% C02. At near confluence, the cells were treated with the nutrient mixture dissolved in media and tested in triplicate at 0 µg/ml, 100 µg/ml, 500 µg/ml, and 1000 µg/ml. The plates were then returned to the incubator. Culture media components were purchased from Gibco (Grand Island, NY). All other chemicals used were purchased from Sigma.

Components of nutrient mixture

The stock solution of the NM (total weight 4.4g) used for testing was composed of the following in the quantities indicated: Vitamin C (as ascorbic acid, magnesium ascorbate, calcium ascorbate 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 (from green tea leaves obtained from US Pharma Lab with total polyphenol 80%, Catechins 60%, Epigallocatechin Gallate [EGCG] 35%, and Caffeine 1%); Selenium 30 µg; Copper 2 mg; and Manganese 1 mg.

MTT assay

Cell proliferation was evaluated by MTT assay [3], a colorimetric assay based on the ability of viable cells to reduce a soluble yellow tetrazolium salt [3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide] (MTT) to a blue formazan crystal by mitochondrial succinate dehydrogenase activity of viable cells. This test is a good index of mitochondrial activity and cell viability. After MTT addition (0.5mg/ml), the plates were covered and returned to the 37°C incubator for 2 hours, the optimal time for formazan product formation. Following incubation, the supernatant was carefully removed from the wells, and the formazan product was dissolved in 1 ml DMSO and absorbance was measured at 570 nm in a Bio Spec 1601 Shimadzu spectrometer. The OD570 of the DMSO solution in each well was considered to be proportional to the number of cells. The OD570 of the control (treatment without supplement) was considered to be 100%.

H&E staining

The cells were cultured in 24-well plates and were kept either untreated (control group) or treated with NM at concentrations of 100, 500 and 1000 µg/ml (treatment group). After 24-hourincubation, the cells were washed with PBS, fixed with cold methanol, and then stained with haematoxylin and eosin for 5 minutes each. The stained cells were then observed and imaged by microscopy.

Apoptosis and live green caspase assay

The SW982 synovial sarcoma cells were grown to near confluence and either left in media alone, or challenged with the NM dissolved in media at 100, 500 and 1000 µg/ml, and incubated for 24 hours. 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 Caspases Detection Kit 135104, Invitrogen). Camptothecin 10 µM was used as positive control to demonstrate apoptosis. The cells were photographed under the fluorescence microscope and counted. Green colored cells represent viable cells, while yellow-orange and red colors represent early and late apoptotic cells, respectively.

Statistical Analysis

The results were expressed as mean ± Standard Deviation (SD) for the groups. Data was analyzed by the independent t-test.

Results

Cell proliferation

NM has no significant effect on the proliferation of SW982 synovial sarcoma cells, up to 100 µg/ml concentration. However, it exhibited 20% anti proliferative effects starting at 500 µg/ml ($p < 0.003$), and 26% at 1000 µg/ml concentration of NM ($p < 0.006$) (Figure 1).

Apoptotic morphology by H&E staining

H&E staining revealed a similar apoptotic pattern in dose dependent fashion in synovial sarcoma SW982 cells treated with NM doses at 100, 500 and 1000 µg/ml. This included characteristic morphological changes such as the shrinkage of the cytoplasm, and darkly stained nuclei with intensely acidophilic cytoplasm. These changes were dose dependent and slight changes were noticed at 100 µg/ml as compared to the control and moderate to significant changes were seen as the NM dose increased to 500 and 1000 µg/ml as shown in (Figures 2A through 2D).

Apoptosis

Analysis with the Live Green Caspase kit revealed a dose dependent increase in apoptosis of synovial sarcoma SW982 cells with 14% of cells in late stages of apoptosis at 100 µg/ml, and this concentration increased to up to 82% of cells in the late stages of apoptosis as represented in table 1.

Photomicrographs shown in Figures 3A through 3D demonstrate the increasing percentage of apoptotic cells represented by red. Figure 3E represents the Camptothecin used for comparison. Quantitative analysis of the data revealed the percentage of apoptotic SW982 cells increasing with an increased dose of NM (Figure3F).

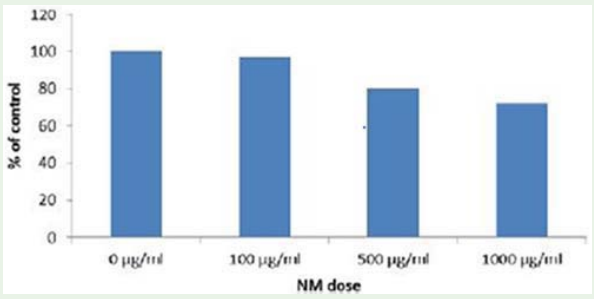

Figure 1: MTT Assay of SW982 by NM.

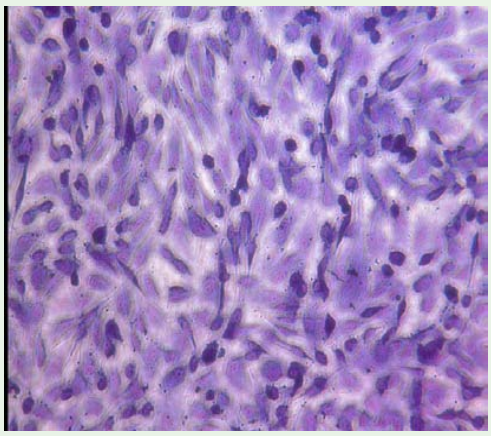

Figure 2A: Control.

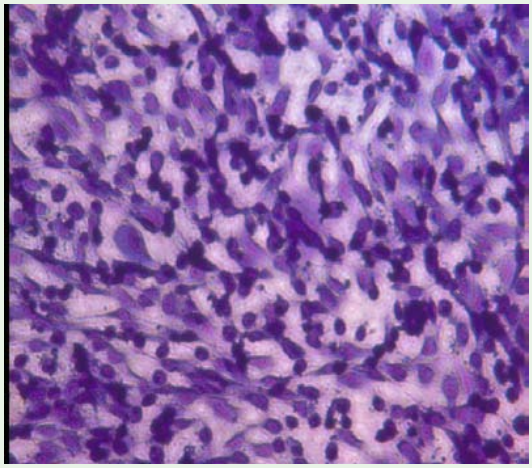

Figure 2B: NM-100 µg/ml.

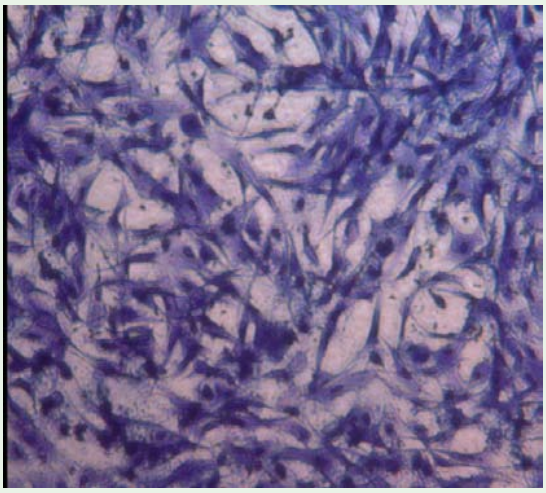

Figure 2C: NM 500 µg/ml.

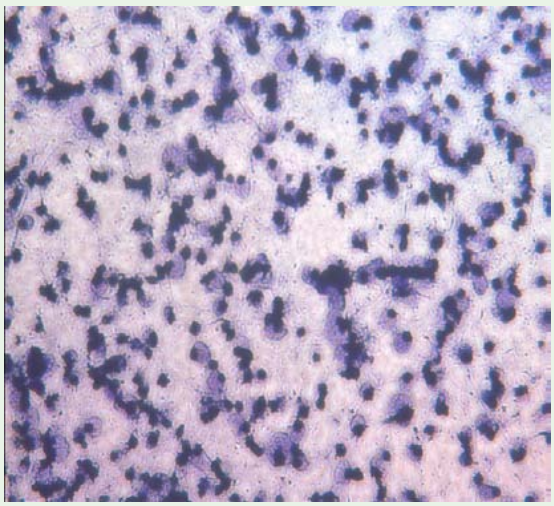

Figure 2D: NM 1000 µg/ml.

Table 1: Stages of Apoptosis.

NM dose	Live cells	Early Apoptosis	Late Apoptosis
0 µg/ml	98%	2%	0%
100 µg/ml	45%	39%	16%
500 µg/ml	32%	12%	56%
1000 µg/ml	10%	8%	82%
Camptothecin	10%	85%	5%

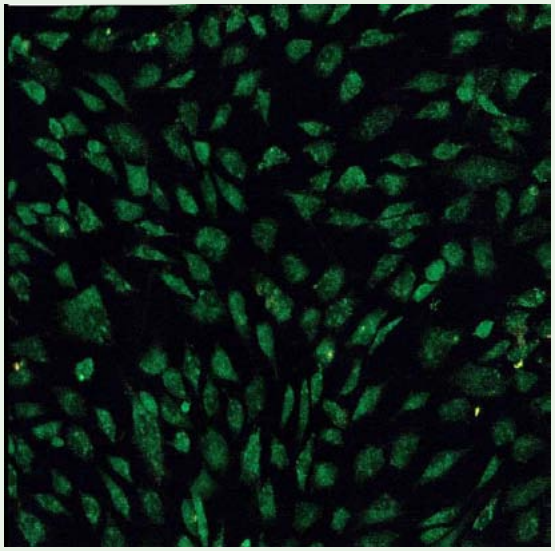

Figure 3A: Control.

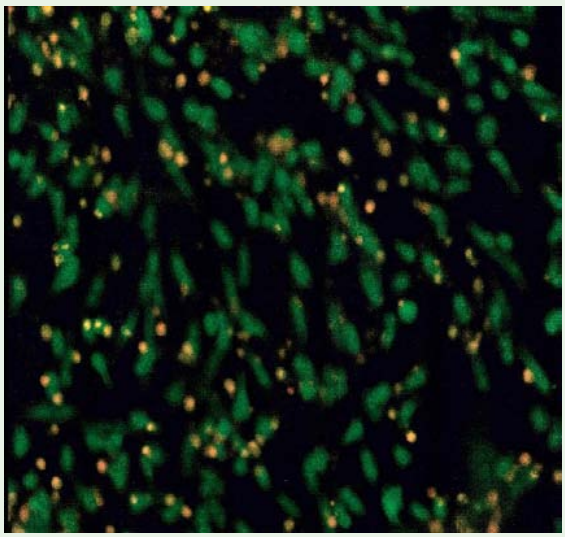

Figure 3B: NM 100 µg/ml.

3F- Analytical representation of the differential distribution of cells in early or late phases of apoptosis upon treatment with 0, 100, 500 and 1000 g/ml NM concentrations.

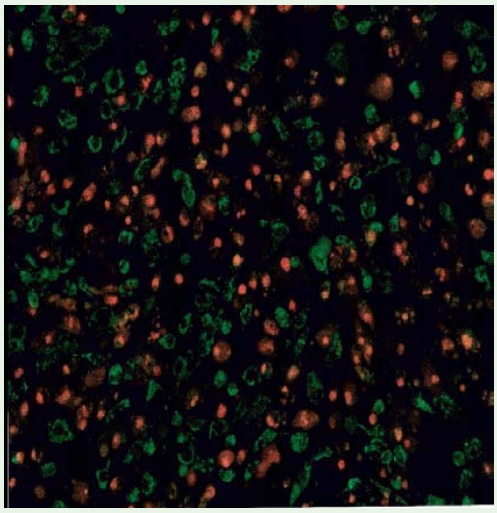

Figure 3C: 500 µg/ml.

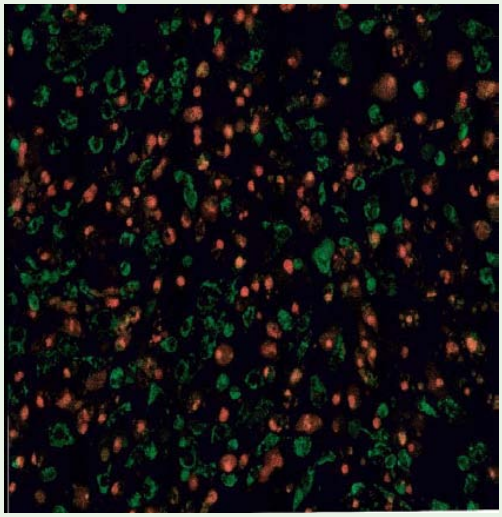

Figure 3D: NM 1000 µg/ml.

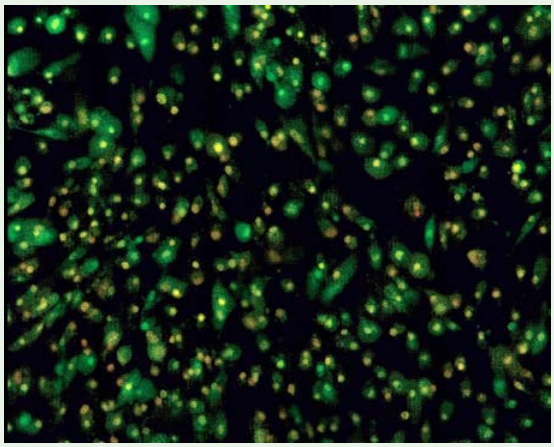

Figure 3E: Camptothecin (10µM).

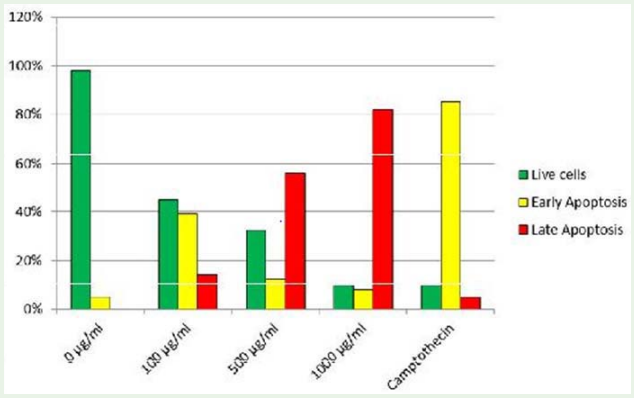

Figure 3F: Analytical representation of the differential distribution of cells in early or late phases of apoptosis upon treatment with 0, 100, 500 and 1000 g/ml NM concentrations.

Discussion

The stimulation of suppressed apoptotic pathways in cancer cells and the induction of apoptosis is a predominant mechanistic approach to target cancer. It has previously been reported in both in vivo and in vitro studies that NM inhibited the tumor growth and tumor burden of synovial sarcoma cells in nude mice [2]. In the current study, we investigated whether this underlying antitumor effect of NM was due, in part, to its action of inducing apoptosis via activation of caspase enzymes. The stimulation of suppressed apoptotic pathways in cancer cells and the induction of apoptosis is a predominant mechanism to target cancer. Although NM did not have significant toxic effect on cell proliferation of SW982 cells, NM induced dose dependent and significant apoptotic changes with H&E staining. The cellular characteristics representing apoptosis such as cell shrinkage, nuclear condensation, cell membrane asymmetry, and condensation of cytoplasm were observed with H&E staining. We also studied the effect of NM on inducing apoptosis using the in vitro Live Green Caspases detection method and by using camptothecin as a positive control. Camptothecin is a potent inhibitor of topoisomerase I, required for DNA synthesis. It is generally used as an anti cancer treatment agent to induce apoptosis in solid tumors. As seen from the photomicrographs in figure 3, the green colored cells are viable cells, yellow colored cells are in early apoptosis and the red colored cells are in late stages of apoptosis. Quantitative analysis indicated that the proportion of apoptotic cells increased with the increasing concentrations of NM. From these results, it is clear that NM at 100, 500 and 1000 µg/ml concentrations can induce dose dependent changes in cytotoxicity, morphology and apoptosis of SW982 synovial sarcoma cells. Our study demonstrated that the specific mixture of tested nutrients induced significantly more apoptosis in SW982 cells than the control and also more than camptothecin.

Apoptosis, also known as programmed cell death, is a complex process that occurs in several pathological situations. Various methods have been developed to study apoptosis using multiple up regulation and down regulation of specific genes such as Bax and p53 genes [4]. One of them is based on the distinctive features of the early stage of apoptosis, which is the activation of caspase enzymes. The family of caspase aspartate, specifically cysteine proteases, is emerging which plays a central role in apoptosis. Some examples of these

important caspases are caspase - 3,-7,-8,-9 and -10 [5,6]. Paradoxin, an antimicrobial peptide, inhibited cell proliferation and induced apoptosis by decreasing the activity of caspase -3 and -7. Although multiple nutrients have anti-cancer properties, a study combining the micronutrients in the appropriate quantities for synergistic and enhanced action is lacking.

A number of plant-based phytochemicals are increasingly being used in the treatment of cancer due to their antitumor properties including induction of apoptosis. The NM used in this study was specifically developed to combine the individual anti-tumorigenic and pro-apoptoticproperties of the component micronutrients. The inhibitory effects of the individual nutrients comprising the novel nutrient formulation have been reported in both experimental and clinical studies. Ascorbic acid is increasingly recognized as an agent with broad biological function. Among its well-known functions are its antioxidant and free radical scavenging functions and detoxification of exogenous compounds [7]. Previous studies have described the mechanisms of action of ascorbic acid in cancer prevention, including a role in collagen synthesis and basement membrane integrity and hyaluronidase inhibition, which may be important in inhibiting tumor spread and micrometastases [8,9]. The green tea catechins such as ( )-epigallocatechin-3-gallate (EGCG), ( )-epicatechin-3-gallate (ECG), and ( )-Epicatechin (EC) have been proven to be chemopreventive agents in vitro and in many in vivo animal models of induced carcinogenesis [10]. EGCG on its own is also a potent anti-cancer agent and has been reported to have a growth inhibitory effect against certain human cancer cell lines including synovial sarcoma [11-13].

However, it has been observed in previous studies that a specific combination of nutrients such as ascorbic acid, EGCG, lysine and proline show a synergistic anti-cancer effect which is much more effective than any of the individual nutrients alone [14].

The toxicity and limited efficacy associated with the current cancer treatments such as chemotherapy and radiation and the efficacy of natural compounds have been extensively documented [15]. Furthermore, in contrast to the toxic effects of current cancer treatments such as chemotherapy and radiation, NM has been shown to be a safe therapeutic agent in vivo as well. Our studies have shown that vital organs such as the heart, kidneys and liver, are not affected even at high concentrations of NM demonstrating that this formulation is non-toxic [16]. Thus, treatment with NM can serve as a multipronged approach to target synovial sarcoma and is a potential candidate for further clinical investigation.

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