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Research Article

Induction of Apoptosis in Human Lung Cancer Cells (A-549) by a Novel Nutrient Mixture *via* Upregulation of Caspase Enzymes

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Abstract

Lung cancer is the leading cause of death in western world, and the incidence continues to increase. Lung cancer is frequently diagnosed when it is extensively metastasized and has a dismal prognosis. Chemotherapy being the mainstay of treatment has not been able to increase quality of life or survival of patients. A novel nutrient mixture containing green tea extract, ascorbic acid, lysine, and proline exhibited anti-cancer effects in various cancers. In our earlier studies, the nutrient mixture considerably reduced the tumor weight and tumor burden in athymic mice using A-549 lung cancer cells. Furthermore, our studies also showed that prior supplementation of nutrient mixture was able to reduce urathane induced lung cancer in male A/J mice. Based on the observation, we questioned whether cell apoptosis was partially responsible for this phenomenon. The lung cancer cells A-549 were cultured in complete Ham F-12K medium 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. The nutrient mixture did not show significant inhibition of cell proliferation. However, H&E staining at the dose of 100 µg/ml showed a few cellular changes, 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. Our results suggest that NM may be a new supplemental strategy and deserves further investigation as a potential therapeutic agent in lung cancer.

Keywords: Lung cancer; A-549 cells; Apoptosis; Cytotoxicity; MTT; Live green caspase

Introduction

Worldwide, lung cancer is the most common cause of death from cancer and the incidence of lung cancer continues to increase. Although cigarette and tobacco smoking substantially increase the risk of developing lung cancer, the lifetime chance of developing lung cancer for a male is 1 in 15 and for females, the risk is slightly lower, 1 in 17. According to 2018 estimates of the American Cancer Society the 234,030 people may get a diagnosis of lung cancer in the United States and it may account for approximately 154,050 deaths [1]. Small Cell Lung Cancer (SCLC) and Non Small Cell Lung Cancer (NSCLC) are the two major categories of lung cancer.

Only 16% of the lung cancer cases are diagnosed at early stage. Most of the diagnoses occur when the cancer has already metastasized. More than 50% of patients die within one year of being diagnosed. Despite scientific advances surgery, chemotherapy, and radiation are still main modalities of treatment for lung cancer. Yet, both SCLS and NSCLC have poor prognosis with a 5-year survival rate of metastasized lung cancer has not improved more than 4% over several decades [2]. There is therefore an urgent need to change the direction in cancer research and develop new, original and safer approaches.

A number of plant-based phytochemicals are increasingly being used as important treatment methods of cancers, due to their antitumor actions including induction of apoptosis. We have developed strategies to inhibit cancer development and its spread using a unique combination of naturally occurring nutrients. The antitumor Nutrient Mixture (NM) combination containing lysine, proline, ascorbic acid, and green tea extract has demonstrated anti-carcinogenic activity against a number of cancer cell lines.

In our earlier studies, we found that a Nutrient Mixture (NM) significantly affect tumor weight and tumor burden in athymic mice using A-549 lung cancer cells [3]. Additionally, our studies also showed that prior supplementation of nutrient mixture was able to reduce urathane, chemically induced lung cancer in male A/J mice [4].

In the current study, we examine, whether the antitumor effects of the NM are due to induction of apoptosis via caspases. Activation of caspase enzymes is a distinctive feature of the early stage 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

Composition of the Nutrient Mixture (NM)

The stock solution of the NM (total weight 4.4g) consists of: vitamin

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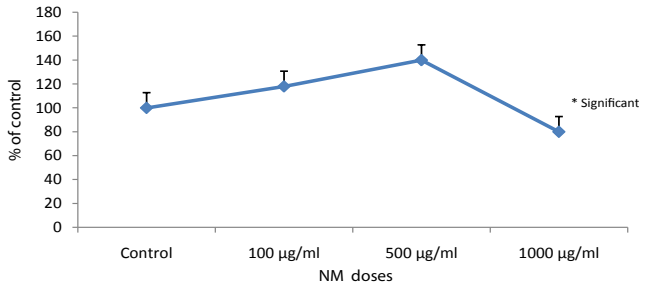


Figure 1: Effect of NM on cell viability: MTT assay using lung cancer (A-549) cells treated with 0, 100, 500, and 1000 µg/ml concentrations of NM respectively (p=0.008).

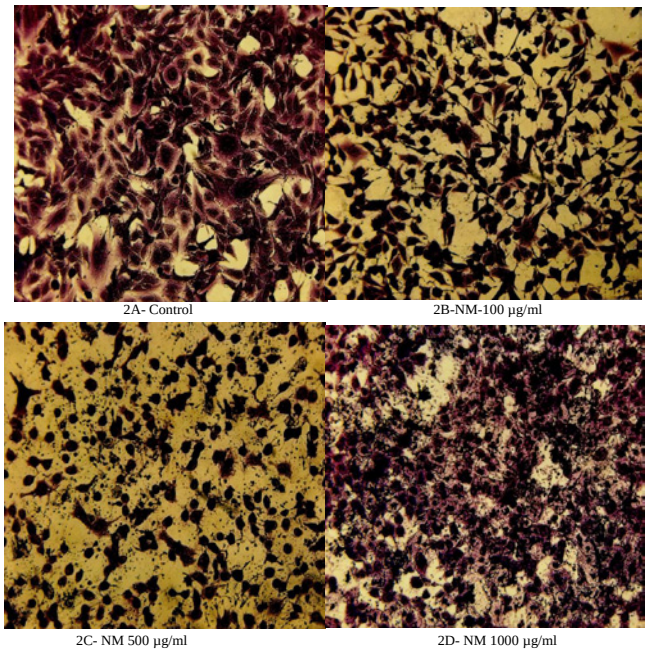


Figure 2:2A through 2D- Effect of NM on morphological changes: H&E staining of lung cancer (A-549) cells treated with 0, 100, 500, and 1000 g/ml concentrations of NM respectively.

C (as ascorbic acid and as 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, Manganese 1 mg.

Cell line and culture: Human lung cancer cells A-549 were obtained from ATCC (American Type Culture Collection, Rockville, MD) and were maintained in Ham F-12K medium, supplemented with 10% Fetal Bovine Serum (FBS), 100 U/mL penicillin and 100 µg/ml streptomycin. FBS and the antibiotics were obtained from Gibco (Long Island, NY). At near confluence the cells were treated with NM in triplicate at concentrations of 0, 100, 500, and 1000 µg/ml.

MTT assay

MTT assay was carried out as follows. The cell suspensions were plated in 24-well tissue culture plates (Nunc, Denmark) at a

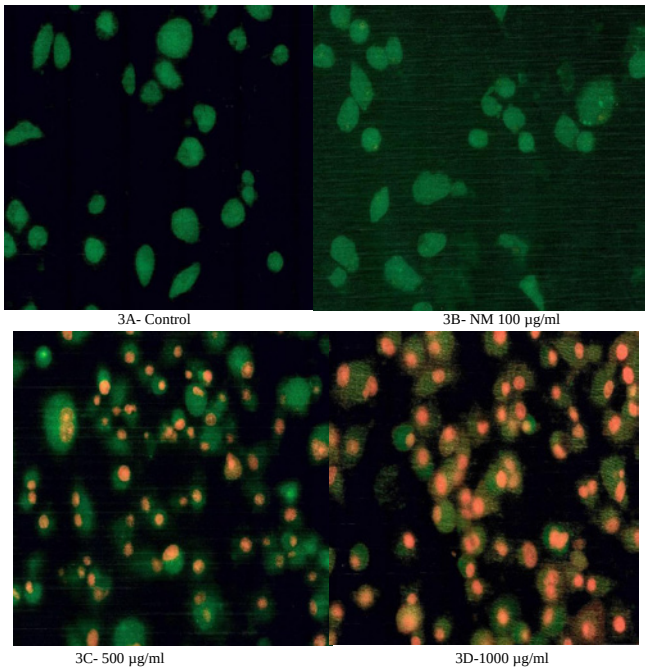


Figure 3: Effect of NM on apoptosis of lung cancer (A-549) cells. 3A-3D - Photomicrographs of cells treated with 0, 100, 500, and 1000 µg/ml concentrations of NM respectively.

concentration of 3×10^4 cells/well. After incubating the plates for 24 hours at 37°C in a humidified incubator, the cells were treated with the NM at concentrations of 0, 100, 500 and 1000 µg/ml for 24 hours. 500 µl of MTT assay reagent (Sigma No. M-2128-0.5 mg/ml in media) was added to each well followed by 2-hour incubation at 37°C. Following incubation, the solution was carefully aspirated from the wells, the formazan product was dissolved in 1 ml DMSO, and the absorbance (OD) was measured on a microplate reader at a wavelength of 570 nm in a BioSpec 1601 Shimadzu spectrometer. The OD 570 of the DMSO solution in each well was considered to be proportional to the number of cells.

H&E stainin

The cells were cultured in 24-well plates and were treated with NM in test concentrations at 0, 100, 500 and 1000 µg/ml respectively. After 24-hour incubation, the cells were washed with PBS, fixed with methanol, and then stained with Haematoxylin and Eosin (H&E), and images were captured by microscope.

Apoptosis and live green caspase assay

The cells were grown to near confluence and treated with the NM dissolved in media at 0, 100, 500 and 1000 µg/ml for 24 hours. The media was removed and was washed with PBS and treated with the caspase reagent as specified in the manufacturer's instructions (Molecular Probes Image-IT Live Green Poly Caspases Detection Kit 135104, Invitrogen). The cells were photographed under the fluorescence microscope and counted. Green colored cells represent viable cells, 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

Morphology and viability study

Figure 1shows that NM did not have significant effect on the growth of A-549 cells. There was a minimal stimulation of A-549 cells upto 500 µg/ml. However reduction in cell growth to 80% of the control was seen at 1000 µg/ml (p=0.008).

H&E staining

H&E staining revealed an apoptotic pattern with a dose dependent incresing fashion in A-549 cells treated with NM at 100, 500, and 1000 µg/ml as seen in (Figure 2A-D). The apoptotic changes seen on H&E staining included characteristic morphological changes such as shrinkage of the cytoplasm, and darkly stained nuclei with intensely acidophilic cytoplasm. These changes were seen to be increasing incrementally, that is, slight changes noticed at 100 µg/ml, moderate to significant changes as the NM dose increased to 500 and 1000 µg/ml.

Apoptosis

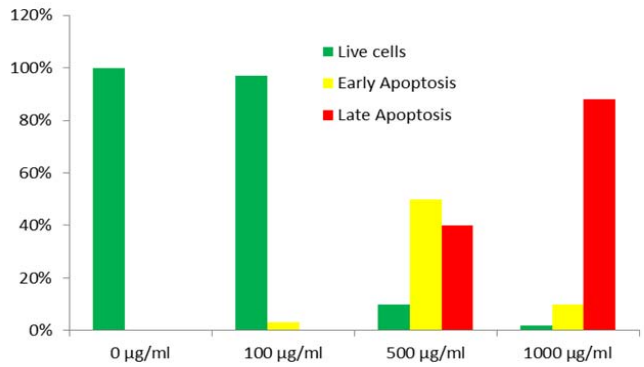
Analysis with the Live Green Caspase revealed a dose dependent increase in apoptosis of A-549 lung cancer cells. Although initially at 100 µg/ml, very few A-549 cells were seen were seen in early apoptotic stage, this percentage progressively increased. Significant apoptosis was observed at 500 µg/ml, at 1000 µg/ml. (Figure 3A)through (Figure 3D)

Quantitative analysis of the data revealed the percentage of apoptotic cells increasing with increased dose of NM (Table 1). There were minimal, 3% of apoptotic cells at 100 µg/ml. However with increasing doses of the NM, the percentage of A-549 cells at 500 µg/ml of NM showed only 10% lung cancer cells were alive, the cells in early apoptotic phase increased up to 50% and 40% cells were in late apoptosis. At NM concentration of 1000µg/ml, fewer than 2% of A-549 cells were alive, 10% of the cells were seen in early apoptosis and 88% lung cancer cells were seen in late apoptosis.

Discussion

The signs and symptoms of lung cancer are very widely from person to person. In more than 85% of all diagnosed lung cancer patients, local or distant metastasis is already present at the time of diagnosis. When diagnosed in an early stages, the 5-year survival of lung cancer not more than 55%, and in advanced stages this rate drastically drops to 4% [2]. Only some patients may have the choice of surgery if the cancer is not extensively metastasized. In majority of the cases, chemotherapy is the mainstay of treatment for lung cancer, and it has not shown significant benefit with regards to the survival.

We have developed strategies to inhibit cancer growth and its progression by inhibiting invasion, metastasis, and inducing apoptosis by using an innovative combination of synergistic nutrients that include natural substances like ascorbic acid, lysine, proline, and green tea extract. It was postulated that this nutrient combination exerts potential synergistic anticancer activity rather than using a single nutrient. The Nutrient Mixture (NM) used in the study was specifically developed to combine the individual anti-tumorigenic and pro-apoptotic properties of the component



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

Table 1: Quantitative analysis of the data showing percentage of apoptotic cells compared to the dose of NM.

NM dose	Live cells	Early Apoptosis	Late Apoptosis
0 µg/ml	100%	0%	0%
100 µg/ml	97%	3%	0%
500 µg/ml	10%	50%	40%
1000 µg/ml	2%	10%	88%

micronutrients. Recently, more attention has been paid to anticancer activity by inducing apoptosis and a number of plant based materials and phytochemicals have shown anticancer activity by inhibiting proliferation, invasion, metastasis, and by inducing apoptosis. Ascorbic acid is well known for its antioxidant and free radical scavenging functions and detoxification of xenobiotics [5]. Previous studies have proven that the action of ascorbic acid in cancer prevention [6-9] includes its role in collagen synthesis, basement membrane integrity and hyaluronidase inhibition [10,11] which may be important in inhibiting tumor spread and micrometastases. Green tea catechins, including Epigallocatechin Gallate (EGCG), have been proven to be chemo preventive agents *in vitro* and in many *in vivo* animal models of induced carcinogenesis [12]. EGCG alone is also a potent anti-cancer agent and has been reported to have a growth inhibitory effect against human cancer cell lines [13-15]. However, it has been observed in earlier studies that a specific combination of substances such as EGCG, ascorbic acid, and proline and lysine, show anticancer effect which is much more pronounced in combination than any of the individual nutrients alone [16].

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 p^{53} genes [17]. One of them is based on the distinctive features of early stage of apoptosis, which is the activation of caspase enzymes. The study of apoptosis by activation of caspase enzymes is an emerging area of research. The family of caspase aspartate – specifically, cysteine proteases is emerging as playing a central role in apoptosis. Some examples of these important caspases are caspase -3, -7, -8 ,-9, -10 and so on [18,19]. Studies have shown that natural substances such as quercetin, curcumin induces apoptosis in lung cancer by

inducing cell cycle arrest *via* DNA damage and stress on endoplasmic reticulum through caspase induction [20,21]. Moreover, different combinations of drugs and synthetic compounds are also being studied to induce apoptosis in lung cancer and increase the efficacy of the chemotherapy drugs like paclitaxel [22].

Activation of caspase enzymes is a characteristic feature of the early stages of apoptosis, which participates in a series of reactions in response to pro-apoptotic signals and result in the cleavage of protein substrates and in the subsequent disassembly of the cell. Increased caspase activity was detected upon treatment with 100, 500 and 1000 µg/ml of NM in a dose dependent fashion, which progressed from mild to moderate changes of apoptosis at 100 µg/ml, to significant (88-90%) apoptosis at 1000 µg/ml concentration of the NM.

Although lung cancer could be treated in the early stages, patient outcome is extremely poor once the cancer has metastasized. Our previous *in vitro*studies have indicated that NM was able to inhibit growth of A-549 by down regulating MMP-2 and -9 expression, and upregulating the MMP inhibitors [23]. Additionally, our *in vivo*studies have proven that the NM is significantly able to reduce development of urathane induced lung tumor in male mice. It showed that after long term consumption of NM supplemented diet, the mice injected by carcinogen urathane, developed almost 50% fewer tumors than the control group [24].

Furthermore, NM has also proven to be a safe therapeutic agent *in vivo* as well. Our other studies have shown that MN was ineffective in inducing enzymes in vital organs such as heart, kidney and liver, even at high concentrations. This shows that NM is non-toxic [25].

Conclusion

These results suggest that the NM could have supplemental benefit in the treatment of lung cancer by inducing apoptosis.

Acknowledgement

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