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Apoptosis of Human Fibrosarcoma Cells HT-1080 Triggered by a Novel Nutrient Mixture via Induction of Caspases

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Abstract

Fibrosarcoma is an aggressive and highly malignant cancer of connective tissue with the lung being the most common site of metastasis. Surgery, chemotherapy, and radiation are the mainstay of treatments, yet the prognosis is very poor. 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 HT-1080 fibrosarcoma tumors induced in athymic mice. Based on the observation, we investigated whether this phenomenon and the anti-cancer effects were due to the induction of apoptosis. Human fibrosarcoma cells HT-1080 were 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 cytotoxicity at 100µg/ml, slight toxicity at 500µg/ml and maximum at 1000µg/ml. H&E staining at the NM dose of 100µg/ml showed a few cellular changes characteristic to apoptosis, while significant changes pertaining to apoptosis morphology were observed at 500 and 1000µg/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 chemotherapeutic strategy for fibrosarcoma patients and deserves further investigation as a potential agent in the treatment of this malignancy.

Keywords: Fibrosarcoma; HT-1080; Apoptosis; Cytotoxicity; MTT; Live Green Caspase

Abbreviations

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

Introduction

Fibrosarcoma is a tumor of the soft tissue of mesenchymal origin and begins in fibrous tissues which hold to the bones, muscles, and other organs. Fibrosarcoma is a very aggressive and highly metastatic cancer primarily developing in metaphysis of the long bones and the most common site of metastasis is the lungs. It predominantly affects children, adolescents, and young adults [1]. According to the American Cancer Society, approximately 12,310 new cases of soft tissue sarcomas, including fibrosarcoma, are estimated to be diagnosed in 2016, and close to 5000 people are expected to die due to this disease [2]. While the exact cause of fibrosarcoma is unknown, the genetic mutations may play a role. The most common mutation includes allele loss, point mutations, and chromosomal translocations [3,4].

Surgery, chemotherapy, and radiation are the main modalities of treatment for fibrosarcoma, however, overall this cancer has poor prognosis [5]. Prognosis in fibrosarcoma patients depends on pathologic grading and it worsens progressively with increasing grade fibrosarcoma. However, even when diagnosed and operated on early, the probability of cancer recurrence of fibrosarcoma at metastatic sites is greater than 70% after surgery [1]. The average 5-year survival rates range from 50-80% for moderate to low-grade fibrosarcoma, and drops to 30% for high-grade fibrosarcoma. Due to resistance to chemotherapy drugs and limitation of other current treatment modalities, there is an urgent need for safe and effective treatment approach.

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.

In an earlier study, we found that the Nutrient Mixture (NM) significantly affects the tumor weight and tumor burden in the human fibrosarcoma in athymic mice [6]. 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 NM

The stock solution of the NM (total weight 4.4g) used for testing

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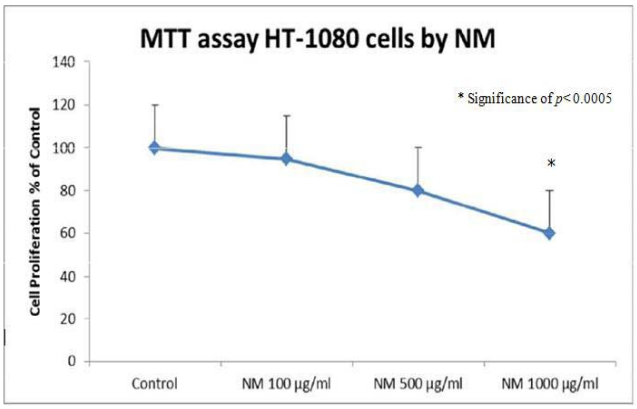


Figure 1: Effect of NM on cell viability: MTT assay using fibrosarcoma (HT-1080) cells treated with 0, 100, 500, and 1000g/ml concentrations of NM respectively ($p < 0.0005$).

was composed of the following in the quantities indicated: Vitamin C (as ascorbic acid and as Magnesium ascorbate, Calcium ascorbate and Palmitate ascorbate) 700mg, L-lysine 1000mg, L-proline 750mg, L-arginine 500mg, N-acetyl cysteine 200mg, Standardized green tea extract 1000mg (from green tea leaves obtained from US Pharma Lab with total polyphenol 80%, Catechins 60%, Epigallocatechin gallate [EGCG] 35%, and Caffeine 1%), Selenium 30µg, Copper 2mg, and Manganese 1mg.

Cell culture

The Fibrosarcoma cell line, HT 1080 was obtained from ATCC (American Type Culture Collection, Rockville, MD, USA). The cells were cultured in DME medium, and supplemented with 10% FBS, and antibiotics. At near confluence the cells were treated with NM in triplicate, at concentrations of 0, 100, 500, and 1000µg/ml.

MTT assay

MTT assay was carried out as described earlier [7]. Briefly, cell suspensions were plated in 24-well tissue culture plates (Nunc, Denmark) at a concentration of 3 × 104 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 100, 500 and 1000µg/ml for 24 hours and the control group was left untreated. 500µl of MTT assay reagent (Sigma No. M-2128-0.5mg/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 570nm 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 Staining

The cells were cultured in 24-well plates and were kept either untreated (control group) or treated with NM at a concentrations of 100, 500 and 1000µg/ml (treatment group). After 24-hour incubation, 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.

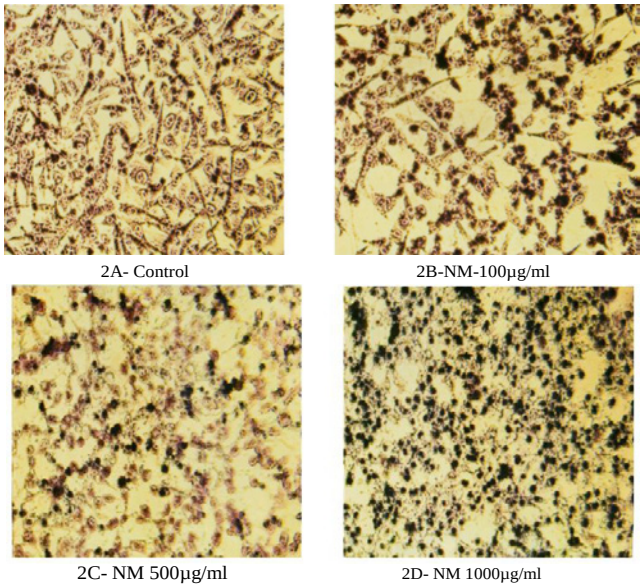


Figure 2: 2A through 2D- Effect of NM on morphological changes: H&E staining of fibrosarcoma (HT-1080) cells treated with 0, 100, 500, and 1000g/ml concentrations of NM respectively.

Apoptosis and live green caspase assay

The cells were grown to near confluence and either left in media alone, or challenged with the NM dissolved in media at 100, 500 and 1000µg/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). 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.

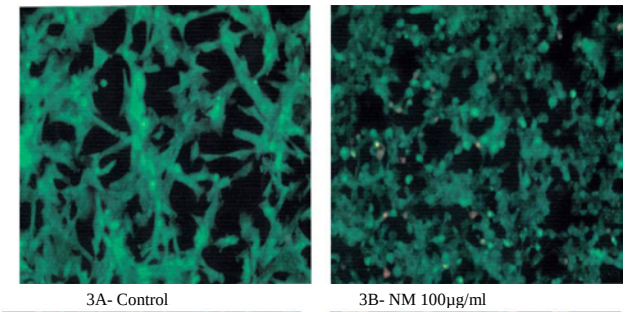
Results

Cell viability study

NM was slightly toxic to HT-1080 cells at 100µg/ml and moderately toxic at 500 and 1000µg/ml. As can be seen in Figure 1, the cell viability showed a dose dependent response with 95% viable cells at 100µg/ml, 80% at 500µg/ml NM, and 60% at 1000µg/ml, respectively, as compared to the untreated control group ($p<0.0005$) (Figure 1).

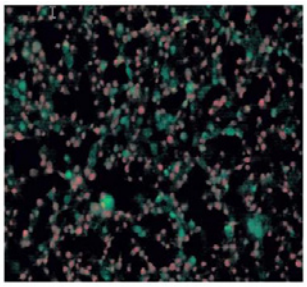
Apoptotic morphology by H&E staining

H&E staining revealed a similar apoptotic pattern in dose dependent fashion in HT-1080 fibrosarcoma cells treated with NM at 100, 500, and 1000µg/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 that is slight changes noticed at 100µg/ml, moderate to significant changes as the NM dose increased to 500 and 1000µg/ml as shown in (Figure 2A through 2D).

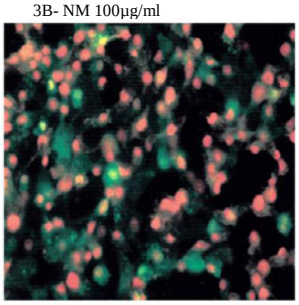


3A- Control

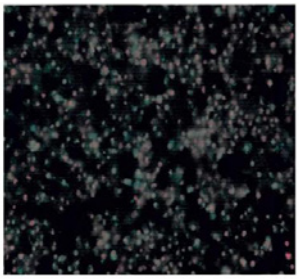
3B- NM 100µg/ml



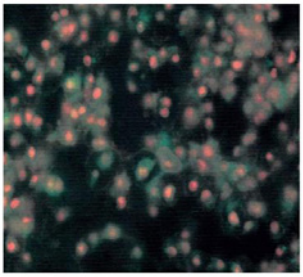
3C- 500µg/ml



3C1- NM 500µg/ml (high mag)



3D-1000µg/ml



3D1- NM 1000µg/ml (high mag)

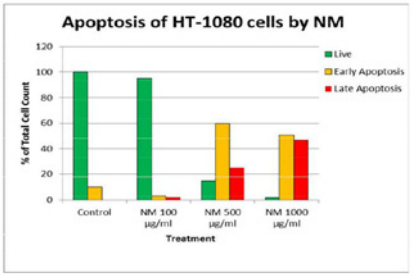


Figure 3: Effect of NM on apoptosis of fibrosarcoma (HT-1080) cells. 3A-3D - Photomicrographs of cells treated with 0, 100, 500, and 1000g/ml concentrations of NM respectively. 3C1, 3D1- High magnification photomicrographs of apoptosis in HT-1080 at 500 µg/ml and 1000µg/ml. 3E- Analytical representation of the differential distribution of cells in early or late phases of apoptosis upon treatment with 0, 100, 500 and 1000g/ml NM concentrations.

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

Apoptosis

Analysis with the Live Green Caspase revealed a dose dependent increase in apoptosis of fibrosarcoma HT-1080 cells, with a slight apoptosis observed at 100µg/ml, progressively increasing apoptosis was observed at 500µg/ml, at 1000µg/ml. (Figure 3A through 3D Figure 3C1 and 3D1 show higher magnification of NM 500 and 1000µg/ml resp).

Quantitative analysis of the data revealed the percentage of apoptotic HT-1080 cells increasing with increased dose of NM. There were minimal percentage of apoptotic cells were observed at 100µg/ml, the NM dose of 500µg/ml showed only 15% fibrosarcoma cells were alive, the cells in early apoptotic phase increased up to 60% and 25% cells were in late apoptosis. At NM concentration of 1000µg/ml, 51% of HT-1080 cells were seen in early apoptosis and 47% fibrosarcoma cells were seen in late apoptosis. It was observed that there was a corresponding decrease in percentage of live cells and at 1000µg/ml NM, the live cells were reduced to 2% (Figure 3E).

Discussion

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 [8]. One of them is based on the distinctive features of 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, -10 and so on [9,10]. Paradoxin, an antimicrobial peptide, inhibited cell proliferation and induced apoptosis by decreasing the activity of caspase 3 and 7. Huang TC et al., has suggested that paradoxin may be a paternal agent for inducing apoptosis in fibrosarcoma HT-1080 cancers due to caspase inducing action [11]. Similarly, Jin et al., showed that a benzylurea derivative markedly inhibited growth and induced apoptosis through cleavage of caspase-3 and PARP in fibrosarcoma HT-1080 cells [12]. In a comparative study using 2-hydroxybenzoate zinc (2HBZ) and acetylsalicylic acid, Mahdi et al., demonstrated that 2HBZ is a potent agent to induce apoptosis via activation of caspase-3 enzymes [13].

In our earlier *in vivo* studies, the NM significantly inhibited the tumor growth and tumor burden of human fibrosarcoma HT-1080 cells in xenograft in athymic mice [6]. 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. At 100µg/ml dose, NM exhibited no toxicity. However, at 500µg/ml and at 1000µg/ml the NM showed a moderate and significant toxicity. Moreover, NM induced dose dependent apoptotic morphological characteristics such as cell shrinkage, nuclear condensation, cell membrane asymmetry, and condensation of cytoplasm. We also studied the effect of NM on inducing apoptosis using the *in vitro* Live Green Caspase detection method. As seen from the photomicrographs, 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 1000µg/ml concentrations can induce dose dependent changes in cytotoxicity, morphology and apoptosis of HT-1080 fibrosarcoma cells.

The NM used in the study was specifically developed to combine the individual anti-tumorigenic and pro-apoptotic properties 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 [14]. 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 [15,16]. 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 [17]. 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 [18]. 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 [19].

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 other 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 [20]. Thus, treatment with NM can serve as a multipronged approach to target fibrosarcoma and should be further investigated with human clinical trials.

References

1. Papagelopoulos PJ, Galanis EC, Trantafyllidis P, Boscainos PJ, Sim FH, Unni KK. Clinicopathologic features, diagnosis, and treatment of fibrosarcoma of bone. *Am J Orthop.* 2002; 31: 253–257.
2. American Cancer Society. Information and Resources for Cancer.
3. Ferrari A, Casanova M, Bisogno G, Cecchetto G, Meazza C, Gandola L, et al. Malignant vascular tumors in children and adolescents: a report from the Italian and German Soft Tissue Sarcoma Cooperative Group. *Med Pediatr Oncol*. 2002; 39: 109–114.
4. Collin CF, Friedrich C, Godbold J, Hajdu S, Brennan MF. Prognostic factors for local recurrence and survival in patients with localized extremity soft-tissue sarcoma. Semin Surg Oncol. 1988; 4: 30–37.
5. Weitz J, Antonescu CR, Brennan MF. Localized extremity soft tissue sarcoma: improved knowledge with unchanged survival over time. J Clin Oncol. 2003; 15: 2719–2725.
6. Roomi MW, Ivanov V, Kalinovsky T, Niedzwiecki A, Rath M. *In Vivo* and *In Vitro* Antitumor Effect of Ascorbic Acid, Lysine, Proline, Arginine and Green Tea Extract on Human Fibrosarcoma Cells HT-1080, Medical Oncology. 2006; 23: 105-112.
7. Roomi MW, Shanker N, Niedzwiecki A, Rath M. Induction of Apoptosis in the Human Prostate Cancer Cell Line DU-145 by a Novel Micronutrient Formulation, Open Journal of Apoptosis. 2015.
8. Basu A, Haldar S. The relationship between BcI2, Bax and p53: consequences for cell cycle progression and cell death. Molecular human reproduction, Oxford Univ Press. 1998; 4: 1099-1109.
9. Li J, Yuan J. Caspases in apoptosis and beyond, Oncogene. 2008; 27: 6194–6206.
10. McIlwain DR, Berger T, Mak TW. Caspase Functions in Cell Death and Disease, Perspectives in Biology, November 21, Published by Cold Spring Harbor Laboratory Press. 2016.
11. Huang TC, Lee JF, Chen JY. Pardaxin, an antimicrobial peptide, triggers caspase-dependent and ROS-mediated apoptosis in HT-1080 cells. Mar Drugs. 2011; 9: 1995-2009.
12. Jin H, Ren K, He HW, Liu X, Song DQ, Shao RG. The benzoylurea derivative F13 inhibits cell growth, migration and invasion through inducing expression of ERK1/2-mediated RECK in fibrosarcoma HT-1080 cells. Anticancer Drugs. 2010; 21: 372-380.
13. Mahdi JG, Pepper CJ, Alkarrawi MA, Mahdi AJ, Bowen ID. Sub-millimolar concentration of the novel phenol-based compound, 2-hydroxy benzoate zinc, induces apoptosis in human HT-1080 fibrosarcoma cells. Cell Prolif. 2010; 43: 95-102.
14. Hanck AB. Vitamin C and cancer. Progress in Clinical and Biological Research. 1988; 259: 307-320.
15. Cameron E, Pauling L. The orthomolecular treatment of cancer I. The role of ascorbic acid in host resistance. Chemico-Biological Interactions. 1974; 9: 273-283.
16. Liao S, Kao YH, Hiipakka RA. Green tea: biochemical and biological basis for health benefits. Vitamins and Hormones. 2001; 62: 61–94.
17. Valcic S, Timmerman BN, Alberts DS, Wachter GA, Krutzch M, Wymer J, et al. Inhibitory effects of six green tea catechins and caffeine on the growth of four selected human tumor cell lines. Anticancer drugs. 1996; 7: 461-468.
18. Hara Y. Green tea: Health Benefits and applications book, Mercel Dekker Publications, New York. 2001.
19. Netke SP, Roomi MW, Ivanov V, Niedzwiecki A, Rath M. A specific combination of ascorbic acid, lysine, proline and epigallocatechin gallate inhibits proliferation of extracellular matrix invasion of various human cancer cell lines. Research communications in Pharmacology and Toxicology: Emerging Drugs. 2003; 2: 37-50.
20. Roomi MW, Ivanov V, Netke SP, Niedzwiecki A, Rath M. Serum markers of the liver, heart, and kidney and lipid profile and histopathology in ODS rats treated with nutrient synergy. J. Am. Coll. Nutr. 2003; 22: 477.

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