Chapter 1

Programmed Cell Death in Renal Cancer Cell O-786 by a Novel Nutrient Mixture by Down Regulating u-PA, MMPs and Up Regulating TIMPs

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Abstract

Renal cell carcinoma (RCC) is highly erratic and unpredictable even when diagnosed at an early stage. The incidence of RCC is higher among African Americans than among Caucasians. The male to female ratio is 2:1. Except for blood in the urine, other symptoms such as abdominal, back or flank pain, weight loss, and abnormal blood counts, are vague signs and symptoms that delay the diagnosis. Therefore, at the time of the diagnosis, the RCC is already metastasized reducing the five-year-survival to 60% in RCC patients.

A novel nutrient mixture (NM) containing green tea extract, ascorbic acid, lysine, and proline exhibited anticancer effects in various cancers. In this study, we investigated whether the anti-cancer properties of NM were due to the induction of apoptosis. The RCC cells (786-0) 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, u-PA, MMP and TIMPs were measured by gelatinase, fibrin, and reverse zymography, cell morphology by H&E staining, apoptosis by Live Green Caspases. NM showed no significant effect on RCC cell growth, exhibiting slight toxicity at 100 µg and significant at 500 and 1000 µg/ml. RCC 786-0 cells treated at 500 and 1000 µg/ml NM and stained by H&E demonstrated obvious apoptotic cells with shrinkage and darkly stained and condensed nuclei and strong

acidophilic cytoplasm. Using Live Green Caspases kit a significant number of early and late apoptotic RCC cells were seen at 500 and 1000 µg/ml NM. Our results suggest that NM induces apoptosis, which in turn down regulated u-PA and MMP and up regulated TIMPs. Therefore, NM may be a new therapeutic strategy for kidney cancer patients and deserves further investigation as a potential agent in the treatment of this malignancy.

Keywords

Kidney Cancer; 786-0; Apoptosis; Cytotoxicity; MTT; Live Green Caspase

Introduction

Kidney cancer is one of the most lethal and most common urological cancers in both men and women worldwide. The American Cancer Society estimates 62,700 new cases of kidney cancer will be diagnosed in the US in 2016, and approximately 14,240 deaths are estimated to be because of kidney cancer [1]. Males tend to get kidney cancer twice as often as females (2:1 ratio). Other than gender, some common risk factors for kidney cancer include smoking, obesity, and exposure to chemicals like benzene, asbestos, and certain pesticides. Being African American, or having a family history of hypertension, lymphoma or previous kidney cancer, and some genetic conditions further increases the risk of developing kidney cancer. Since the kidney tumors tend to grow quickly without any specific symptoms, they are usually removed before any specific diagnostic biopsy is done [2].

There are different cellular types of kidney cancer; however, renal cell carcinoma (RCC) also known as renal cell adenocarcinoma is the most common cell type and is an aggressive form of cancer. Nine out of ten kidney cancers are renal cell adenocarcinomas. Except for blood in the urine, other symptoms such as abdominal pain, back or flank pain, weight loss, and abnormal blood counts, are vague signs and symptoms delaying the diagnosis. RCC is erratic and unpredictable even when diagnosed early. Generally, kidney cancer is associated with good prognosis if treated when still localized to the kidney; however, once metastasized, prognosis is poor. Due to the lack of specific symptoms, 20-30% of the patients already have metastatic cancer at the time of diagnosis. The most common sites of RCC metastasis are the lungs, liver, and bones. The conventional treatment options are limited to combinations of surgery, chemotherapy, and radiotherapy, which do not provide complete cure and the five-year survival of metastatic RCC is reduced to 60%. There is an urgent need for an effective approach to kidney cancer [3-6].

Two families of proteases, the matrix metalloproteinase (MMPs) and the urokinase plasminogen activators (u-PA), are actively involved to facilitate the process of metastasis in many cancers including kidney cancer. MMPs, particularly MMP-2 and MMP- 9 play a key role

in tumor invasion and metastasis due to their ability to degrade the extra cellular matrix (ECM) and the basement membrane components [7,8]. Several studies have proven that elevated levels of MMPs and u-PAs are associated with tumor growth, cancer progression, and metastasis, thereby eventually shortening the patient survival [9-11]. Tumor cell invasion requires the critical steps of cell attachment, degradation of the extra cellular matrix (ECM) and migration through the disrupted matrix. The serine protease u-PA, a 55- kDa serine protease consisting of two di-sulphide bridges linked to polypeptides, is cleaved to the active chain (33 kDa) by various stimuli. The protease u-PA converts plasminogen to plasmin, which is capable of promoting tumor growth and angiogenesis by degrading the ECM and the basement membrane and activating pro-MMPs. Thus, the u-PA and MMP systems represent a potential target for anticancer strategies [11-13]. In addition, the MMP activity is regulated by specific tissue inhibitors of metalloproteinases (TIMPs). There is a critical natural balance maintained between MMP and the TIMP levels in the body [14,15].

Unlike many other cancers, RCC is resistant to conventional chemotherapy and radiation treatments and sometimes to immunotherapy. We attempted to determine whether the NM could be used as an additional approach in combating RCC. 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 this study, we investigated whether the anti-cancer effects of NM on 786-0 are due to induction of apoptosis and if the apoptosis is caused by down-regulation of u-PA and MMPs and up-regulation TIMPs.

Materials and Methods

Nutrient Mixture

The nutrient mixture (NM), prepared by VitaTech (Hayward, CA, USA) was composed of the following ingredients in the relative amounts 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 (80% polyphenol) 1000 mg; selenium 30 µg; copper 2 mg; manganese 1 mg.

Cancer Cell Line and Culture

Human Renal Cell adenocarcinoma cell line 786-0 was obtained from American Type Culture Collection (ATCC, Rockville, MD). The cells were grown cultured in RPMI media supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin (Gibco BRL, Long Island, NY), in twenty-four well Costar tissue culture plates obtained from Costar (Cambrdige, MA). At

near confluence, the cells were then challenged with NM at 0, 50, 100, 250, 500 and 1000 µg/ml concentration in triplicate at each concentration and the results were compared. All other reagents used were of high quality and were obtained from Sigma, unless otherwise indicated.

Gelatinase Zymography

Gelatinase zymography was performed as described earlier in 10% Novex pre-cast SDS polyacrylamide gel (Invitrogen Corp.) in the presence of 0.1% gelatin under non-reducing conditions [16].

Fibrin Zymography

Fibrin zymography was used to analyze u-PA activity on 10% SDS- PAGE gels containing fibrinogen (5.5 mg/ml) and plasminogen (50 µg/ml). After electrophoresis, the gels were washed twice with 2.5% Triton X-100 for 30 min. The gels were then incubated overnight at 37° C with 0.1% glycine buffer pH 7.5 and then stained with 0.5% Coomassie Brilliant Blue R250 and destained. Electrophoresis of u-PA and tissue plasminogen activator(t-PA) were conducted for comparison. Fibrinzymograms were scanned using CanoScan 9950F Canon Scanner.

Reverse Zymography

TIMPs were analyzed by reverse zymography on 15% SDS gels containing serum-free conditioned medium

from cells, as described earlier [17]. Briefly, after electrophoresis the gels were washed twice with 2.5% Triton-X for 30 min at room temperature to remove SDS. The gels were then incubated at 37˚C overnight in 50 mMTris-HCl and 10 mM $CaCl_2$ at pH 7.6 and stained with 0.5% Coomassie blue R25, destained and scanned.

Scanning of Gelatinase, Reverse and Fibrin-Zymograms

Gelatinase, reverse and fibrin zymograms were scanned using CanoScan 9950F Canon scanner at 300 dpi. The intensity of the bands was evaluated using the pixel-based densitometer program Un-Scan-It, Version 5.1, 32-bit, by Silk Scientific Corp. (P.O. Box 533, Orem, UT, USA), at a resolution of 1 Scanner Unit (1/100 of an inch for an image that was scanned at 100 dpi). The pixel densitometer calculates the optical density of each pixel (values 0-255) using the darkly stained background of the gel as a pixel value of 0. A logarithmic optical density scale was used since the optical density of the film and gels is logarithmically proportional to the concentration. The pixel densitometer sums the optical density of each pixel to give the band density.

MTT Assay

MTT assay was carried out as described earlier [18]. Briefly, cell suspensions were plated in 24-well tissue cul-

ture plates at a concentration of 3×10^4cells/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, 50, 100, 250, 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.

Morphology (H & E Staining)

The cells were cultured in 24-well plates and were treated with NM at a concentrations of 0, 50, 100, 250, 500 and 1000 µg/ml. After 24-hour incubation, the cells were washed with PBS, fixed with cold methanol, and then stained with haematoxylin and eosin (H&E) for 5 minutes each. The stained cells were then observed and imaged by microscopy.

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 50, 100, 250, 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). 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

Nutrient Mixture Decreased the Viability of Kidney Cancer Cells

Our results demonstrated that the nutrient formulation induced slight toxicity to RCC 786-0 cells in a dose dependent manner. As can be seen in Figure 1, the NM showed insignificant toxicity to RCC 786-0 cells at 100 µg/ml, 10% at 500 µg/ml, 22% at 1000 µg/ml ($p<0.003$).

Apoptotic Morphology by H & E Staining

H&E staining revealed an apoptotic pattern in dose dependent fashion in RCC 786-0 cells treated with NM at 100, 500, and 1000 µg/ml. Though the NM was not toxic to RCC 786-0 at lower doses, the cells exposed to NM at 500

µg/ml and 1000 µg/ml showed obvious apoptosis by H&E staining as seen in figure 2 (A-D). 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 100 µg/ml, moderate to significant changes as the NM dose increased to 500 and 1000 µg/ml.

Effect of NM on MMP Secretion by RCC 786-0 Cells

RCC cells exhibited two bands corresponding to MMP-2 and MMP-9, with enhanced MMP-9 secretion when induced by PMA (100 ng/ml). NM decreased the secretion of both MMP-2 and MMP-9 in dose dependent fashion (Figure not shown).

Effect of NM on u-PA Activity on RCC 786-0 cells

The cells showed one band corresponding to subunit 1 of u-PA at 55 kDa. NM inhibited the u-PA secretion even at 100 µg/ml (linear trend R2 = 0.4286) as shown in Figure 3A, 3B.

Effect of NM on TIMP Activity

Reverse zymography revealed up-regulation of TIMP-2 expression with NM treatment in kidney cancer RCC 786-0 cell line in a dose-dependent manner. While no

activity was expressed at 50 or 100 µg/ml, the expression gradually started increasing from 250 µg/ml and reached maximum at 1000 µg/ml NM, as seen in Figure 4A, 4B for reverse zymogram and densitometry analyses.

Apoptosis by Live Green Caspase Kit

Analysis with the Live Green Caspase revealed a dose dependent increase in apoptosis of RCC 786-0 cells, with a slight apoptosis observed at 100 µg/ml, progressively increasing apoptosis was observed at 500 µg/ml, at 1000 µg/ml. (Figure 5A through 5F. Figure 5G shows prominent apoptotic changes in a single nucleus at a higher magnification). A significant number of early and late apoptotic cells were evident when RCC 786-0 cells were exposed to higher doses of NM. Quantitative analysis with live green caspase kit revealed a clear dose dependent increase in apoptosis of RCC 786-0 cells. 81.6% apoptotic cells were seen at 500 µg/ml (14.8% in early and 68.8% in late stage apoptosis) followed by 95.7% at 1000 µg/ml (5.7% in early and 90% late apoptosis stage), simultaneously significant decrease in live cells (Figure 5H).

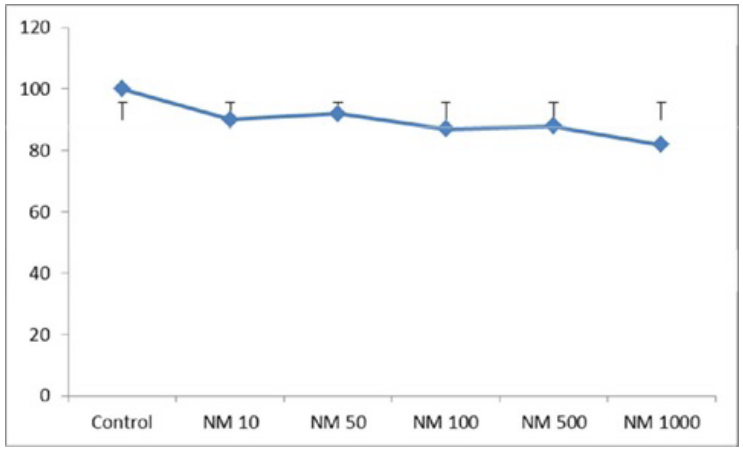
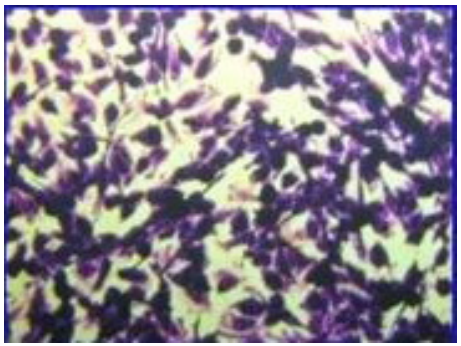


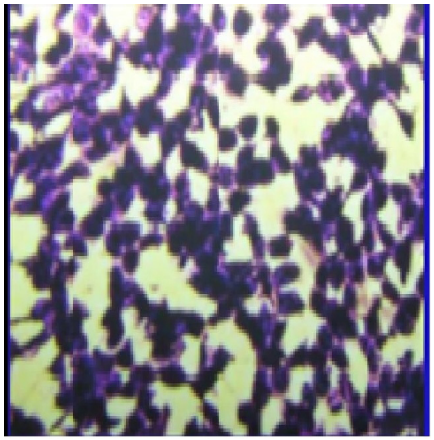
Figure 1: Effect of NM on cell viability: MTT assay using RCC (786-0) cells treated with 0, 10, 50, 100, 500, and 1000 µg/ml concentrations of NM respectively. ($p < 0.003$) The NM shows no significant effect on renal cancer cell proliferation.



2(A) Control.

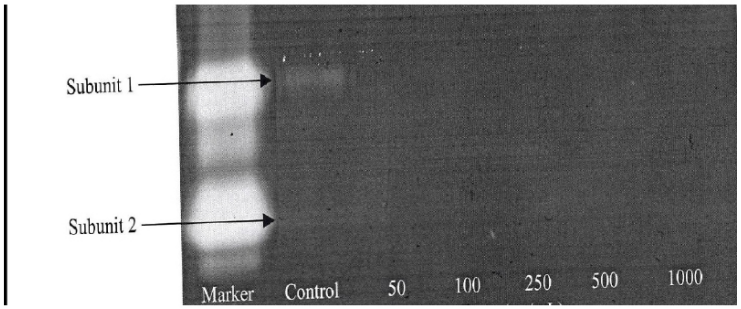
2(B) NM 100 µg/ml.

2(C) NM 500 µg/ml.

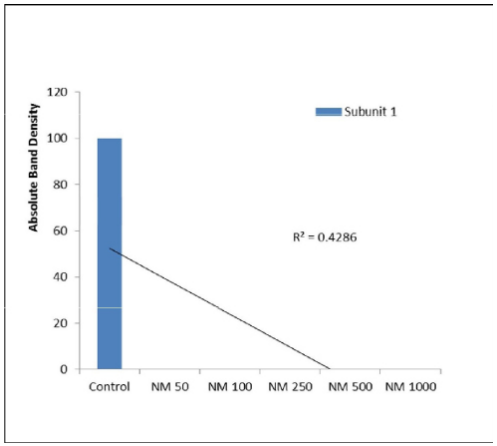


2(D) NM 1000 µg/ml.

Figure 2: A-2D: Effect of NM on morphological changes: H&E staining of RCC (786-0) cells treated with 0, 100, 500, and 1000 µg/ml concentrations of NM respectively.

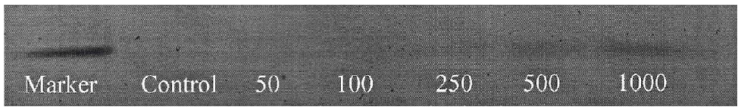


3(A) The RCC Cells Showed One band corresponding to subunit 1 of u-PA at 55 kDa.

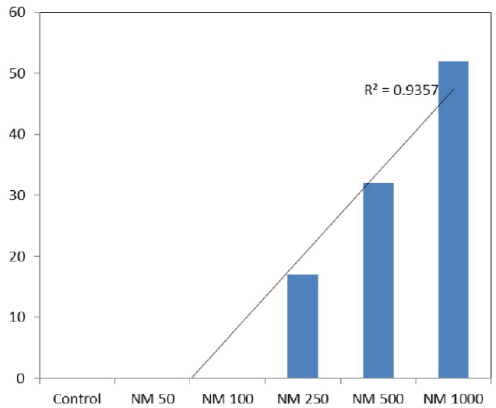


3(B) Correlation between the effects of NM on RCC 786-0.

Figure 3: Effect of NM on u-PA expression in RCC 786-0 cells. NM exhibited complete inhibition of u-PA (subunit 1) expression in RCC786-0 cells starting at 50 µg/ml.

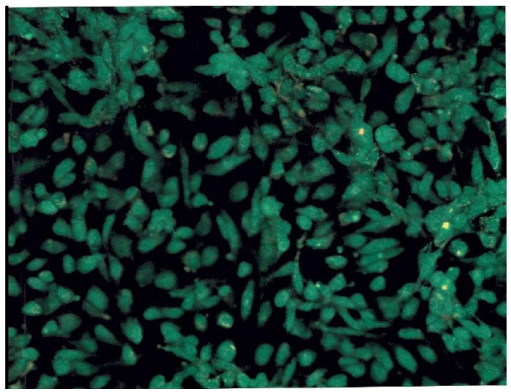


4(A) Zymography Showing increased TIMP expression with increasing doses of NM.

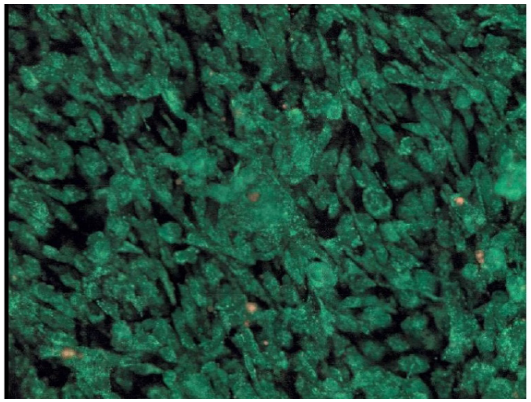


4(B) Densitometric analysis of RCC 786-0 cells and TIMP expression.

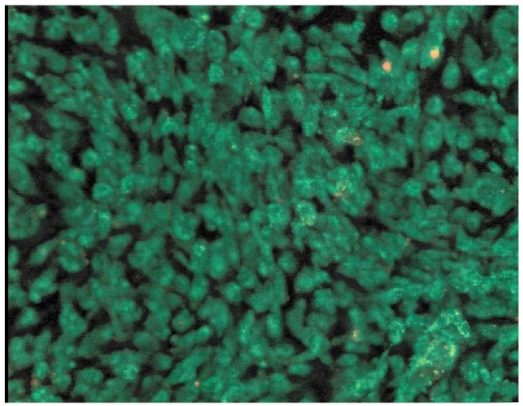
Figure 4: Effect of NM on RCC 786-0 cells TIMP-2 expression. NM shows increase in TIMP-2 with increasing doses. No activity was noted at 50 or 100 µg/ml of NM, the TIMP expression increased from 250 µg/ml reaching maximum at 1000 µg/ml.



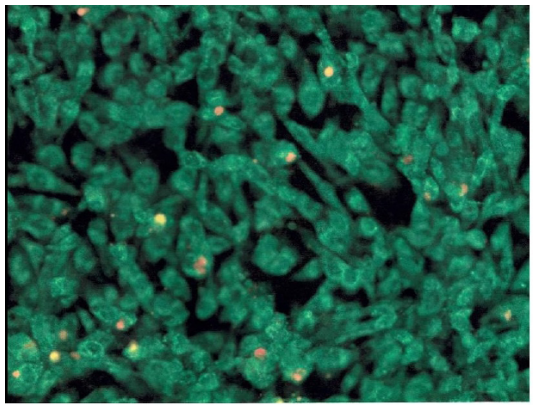
5(A) Control.



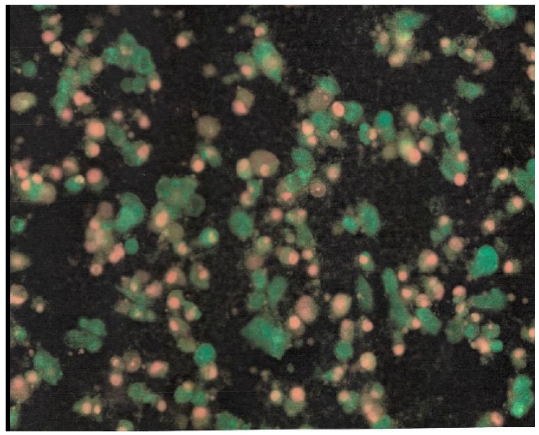
5(B) NM 50µg/ml.



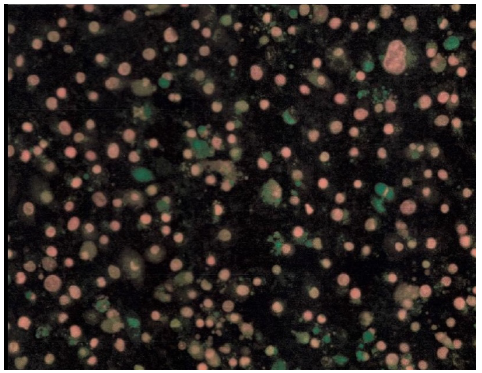
5(C) NM 100µg/ml.



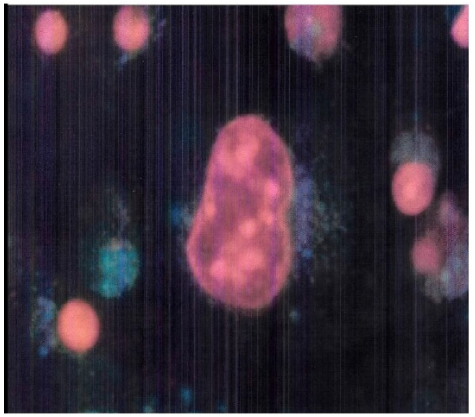
5(D) NM 250 µg/ml.



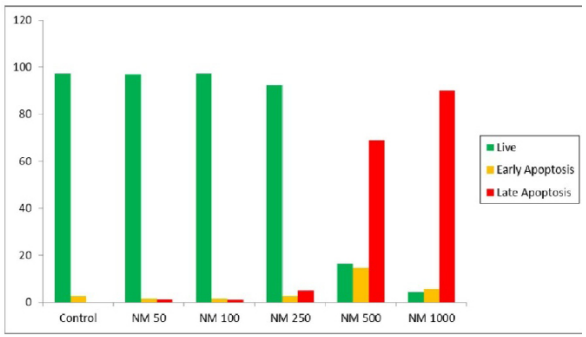
5(E) NM 500 µg/ml.



5(F) NM 1000 µg/ml.



5(G) A single nuclerus showing apoptotis.



5(H) Quantitative analysis of the apoptosis.

Figure 5: Apoptosis of renal cancer cells 786-0 with increasing doses of NM. NM exhibited dose dependent increase in apoptotic and dead cells with increasing doses, reaching maximum 90% dead cells at 1000µg/ml of NM.

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 [19]. 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 [20,21]. Studies have shown that natural substances such as resveratrol, cantharidin, and synthetic agents like Docetaxel induces apoptosis in renal cell carcinoma by cell cycle arrest and caspase induction [22,23,24,25].

In this study, we noticed, that although NM was non-toxic to 786-0 cells at 100 µg/ml, it consistently exhibited toxicity at 250, 500 and 1000 µg/ml. These results were consistent with the morphological and apoptotic changes as seen under microscopy. H&E staining showed progressive changes in cell shrinkage, rounding of the nucleus, condensation of cytoplasm and acidophilic nucleus. Similar changes were further reported using the Live Green Caspase assays.

Tumor cell invasion requires the critical steps of cell attachment, degradation of the ECM and migration through the disrupted matrix. The two families of proteases, MMP and u-PA, play key roles in tumor cell invasion [26,27,28]. Experimental studies have demonstrated the role of urokinase plasminogen, especially cell surface u-PA, as an initiator of ECM proteolysis and associated tumor cell invasion. The protease u-PA converts plasminogen to plasmin, which is capable of promoting tumor growth and angiogenesis, degrading the ECM and basement membrane and activating pro-MMPs [14]. Our study demonstrated that the specific mixture of tested nutrients significantly inhibited u-PA secretion in kidney

cancer cell lines RCC 786-0. In our previous studies, we have demonstrated dose-dependent decrease in MMP secretion [16].

In contrast to the associated toxicity and limited efficacy of standard cancer chemotherapy and radiation therapy, the efficacy and safety of natural dietary and botanical compounds in cancer prevention has been extensively documented [29]. Epidemiological studies have suggested that consumption of a plant based diet has been associated with the prevention of cancer. Diet and nutritional supplementation have often been analyzed by several studies for beneficial effect in reduction of all types of cancers [30,31]. Studies have also indicated that individual the phytochemicals and micronutrients such as epigallocatechin gallate (EGCG—present in green tea), ascorbic acid, N-acetyl cysteine and selenium individually have anticancer properties.

Rath and Pauling proposed using nutrients such as lysine and ascorbic acid to target plasmin-mediated connective tissue degradation as a universal approach to inhibit tumor growth and expansion [23]. Subsequent studies confirmed this approach and led to identifying a novel formulation composed of lysine, ascorbic acid, proline, green tea extract, and other micronutrients (NM), which has shown significant anticancer activity against a large number of cancer cell lines, blocking cancer growth, tissue invasion and MMP expression both in vitro and in vivo [24]. 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 NM have been reported in both experimental and clinical studies. Ascorbicacidis 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 [34]. Previous studies have described the mechanisms of action of ascorbic acid in cancer prevention, which includes a role in collagen synthesis and basement membrane integrity and hyaluronidase inhibition, and which may be important in inhibiting tumor spread and micrometastases [35,36].Thegreenteacatechinssuchas(-)-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 [37]. 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 [38]. Although all these nutrients have anti-cancer properties, a study combining the micronutrients in the appropriate quantities for synergistic and enhanced action is lacking. 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 [39]. The NM

was formulated by selecting nutrients that acton critical physiological targets in cancer progression and metastasis, as documented in both clinical and experimental studies. Combining these micronutrients expands metabolic targets, and maximizes biological impact with lower doses of components.

Due to the limitations of the current treatment modalities in tackling kidney cancer, there is an urgent need for safe and effective therapeutic approaches. The NM containing lysine, proline, ascorbic acid, and green tea extract has shown a wide range of pharmacological properties, including a broad spectrum of antitumor activity against a number of human cancer cell lines. In this study, we investigated whether the underlying antitumor effect of NM was due to apoptosis in human renal cancer, by studying its effect on caspases, which in turn is caused by down-regulation of u-PA, MMP, and up-regulation of TIMPs.

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 [40]. Thus, treatment with NM can serve as a multipronged approach to target renal cell carcinoma and should be further investigated with

human clinical trials.

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