

## ANTINEOPLASTIC EFFECTS OF NUTRIENT MIXTURE ON RAJI AND JURKAT T CELLS: THE TWO HIGHLY AGGRESSIVE NON HODGKIN'S LYMPHOMA CELL LINES

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Non-Hodgkin lymphomas incidence has increased more than 70% in last 25 years. Aggressiveness, higher relapse rate, and treatment complications pose significant barriers. Decreased food intake and side effects of treatments make cancer patients vulnerable to deficiency of essential nutrients such as vitamin C, lysine, and proline leading to the formation of weak extra cellular matrix susceptible to easy breakdown by matrix metalloproteinase enzymes. Inhibition of these enzymes has shown promise in stopping metastasis. *Aim:* In this study, we investigated the effects of a specific nutrient mixture, containing ascorbic acid, lysine, proline, green tea extract among others, in most aggressive forms of non-Hodgkin's lymphoma — Burkitt's lymphoma, and T-cell lymphoma — using Raji and Jurkat cells respectively. *Methods:* Nutrient mixture (NM) doses of 0, 10, 50, 100, 500, 1000 µg/ml, were used to study effects on cell proliferation, expression of matrix metalloproteinase, Matrigel™ invasion and apoptosis. *Results:* The results demonstrated that the dose response toxicity of the nutrient mixture on Raji cells gradually increased with increasing concentration. The nutrient mixture was non-toxic to Jurkat cells, however exhibited anti-proliferative properties at higher concentrations. Zymography demonstrated, NM had a significant inhibitory effect on matrix metalloproteinase-9 expression with total inhibition at 1000 µg/ml for Raji cells and at 500 µg/ml for Jurkat cells. The NM at 100 µg/ml completely inhibited Matrigel™ invasion for Raji cells, and at 1000 µg/ml for Jurkat cells. After the NM challenge virtually all Raji and Jurkat cells exposed to 1000 µg/ml were in late apoptosis. *Conclusion:* Considering the lack of treatment options and continually increasing incidence, NM could be further explored for its therapeutic potential in Burkitt's lymphoma and T-cell lymphoma.

**Key Words:** Burkitt's lymphoma, Raji cells, Jurkat T cells, MMP, apoptosis, nutrient mixture.

The American Cancer Society estimated in 2008, 66,120 Americans would be newly diagnosed with non-Hodgkin's lymphoma (NHL), contributing to approximately 19,160 deaths in 2008 [1]. Adult males and females are equally affected, while NHL contributes to about 7% of deaths in children and young adults below 20 years. Although a typical NHL patient is usually a white adult male, the incidence is more than doubled since 1970s and female patients contribute more for this rise.

The World Health Organization classifies NHL in 2 main categories — B cell and T cell origin. B cell lymphomas contribute to approximately 88% of the NHL cases, while the rest 12% originate from T cells [3]. Among several lymphoma subclasses, we have used Burkitt's lymphoma (Raji) and T-cell lymphoma (Jurkat) cell lines, both of which are fast growing, highly aggressive types of NHL, with poor prognosis and no satisfactory treatments [2]. Burkitt's lymphoma accounts for about 50% of childhood NHL and exhibits consistently aggressive clinical behavior. Despite advances in treatment options, the prognosis remains poor due to high relapse rate and the 5-year survival rates are below 30% [2].

Rath's work indicated that the most critical pathophysiology of cancer development is unre-

stricted cell proliferation and enzymatic degradation of the connective tissue, which can be controlled by nutrients essential for maintaining the connective tissue integrity. It has also shown that proteolytic degradation of extracellular matrix (ECM) is the key factor in stromal invasion of the tumors and eventual metastasis [4]. Matrix metalloproteinases (MMPs) enzymes are responsible for the tissue remodeling and degradation of the ECM [5, 6]. Studies have shown that the aggressiveness of the cancer indicated by invasiveness, grade and stage, is highly correlated with the expression of MMPs [7]. MMPs, especially MMP-2 and MMP-9, are identified as the most important types in this process. Rath and Pauling hypothesized that nutrients such as ascorbic acid and lysine could act as natural inhibitors of extracellular matrix proteolysis and, therefore, by stabilizing the connective tissue, have potential to modulate tumor growth and metastasis [4]. These nutrients can mediate their antitumor potential through several mechanisms, including the inhibition of MMPs and strengthening of connective tissue surrounding cancer cells, thereby encapsulating the tumor [8, 9]. In hematological malignancies, increased levels of MMP-2 and MMP-9 are linked to more aggressive and advanced stages of cancer [3]. Positive MMP-9 expression is seen to be associated with increased prevalence of B cell lymphoma symptoms and worse survival [3].

The nutrient mixture (NM) is formulated to target the key physiological pathways in cancer progression and metastasis. Ascorbic acid and the amino acids lysine and proline are necessary for the formation and optimum structure of collagen fibers and therefore ECM integrity. Manganese and copper are also

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*Abbreviations used:* ECM — extracellular matrix; EGCG — epigallocatechin gallate; MMP — matrix metalloproteinases; NAC — N-acetylcysteine; NHL — non-Hodgkin's lymphoma; NM — nutrient mixture; SD — standard deviation.

essential cofactors in collagen formation. Collagen stability can be controlled by lysine [4] and by N-acetyl cysteine (NAC) through its inhibitory effect on MMP-9 activity [11] and invasiveness of tumor cells [12, 13]. In addition, selenium has been shown to interfere with MMP expression and tumor invasion [14] and to induce selective apoptosis of cancer cells [36]. Ascorbic acid is proven to inhibit cell division and growth through production of hydrogen peroxide [15]. Green tea extract is known to be a promising agent in controlling angiogenesis, metastasis, and other aspects of cancer progression [16, 17]. Any deficiency of arginine can limit the production of nitric oxide, which predominantly acts as an inducer of apoptosis [18]. In addition we also found that a combination of ascorbic acid, lysine, and proline used with epigallocatechin gallate (EGCG) enhanced the anti-invasive activity of 20 µg/ml EGCG to that of 50 µg/ml [10]. Thus by including NAC, arginine, selenium, manganese, and copper along with the combination of ascorbic acid, lysine, proline and EGCG, we could obtain significant reduction in cell invasion at a much lower concentration of EGCG or other components. We have reported that the combined effects of these individual nutrients were superior to the effects of each individual component or when they were randomly combined [10].

We have used the NM to study various soft tissue cancer cell lines such as fibrosarcoma, cervical cancer among others [8, 9]. In the current study, for the first time, we used hematological cancer cells, human Jurkat T cells and human Burkitt's lymphoma Raji cell lines, because of the aggressiveness of these cancers. We investigated antitumor potential of NM *in vitro* on these two cell lines by measuring the cell proliferation, modulation of MMP secretion, cancer cell invasive potential, morphological changes and apoptosis.

## MATERIALS AND METHODS

**Composition of nutrient mixture (NM).** Stock solution of the NM prepared for testing was composed of the following: Vitamin C (as ascorbic acid, and as Mg, Ca, and ascorbyl palmitate) 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 was derived from green tea leaves 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 1 mg.

**Cell culture.** Human Burkitt's lymphoma Raji cells and human T Jurkat cells were obtained from the ATCC (American Type Culture Collection, Rockville, MD) and were grown in RPMI-1640 medium. The medium was supplemented with 10% fetal bovine serum, penicillin (100 U/ml) and streptomycin (100 mg/ml) and treated with the NM, dissolved in media and tested at 0, 10, 50, 100, 500 and 1000 µg/ml in triplicates at each dose. Cell viability and cytotoxicity was assessed by Trypan blue dye exclusion test after 24 h. Viable cell count was

expressed as a function of control. Data are shown as means of ± standard deviation.

**Gelatinase zymography.** Gelatinase zymography was used to determine the MMP activity in conditioned media. Zymography was performed in 10% Novex pre-cast SDS-polyacrylamide gel (Invitrogen) 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 min 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<sub>2</sub> at pH 8.0 and stained with 0.5% Coomassie Blue R250 in 50% methanol and 10% glacial acetic acid for 30 min 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 9950 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 (P. O. Box 533, Orem, UT-84059, USA), at a resolution of 1 Sanner Unit (1/100 of an inch for an image that was scanned at 100 dpi), and expressed as a percentage of control.

**Matrigel invasion studies.** Invasion studies were conducted using Matrigel™ (Becton-Dickinson) inserts in 24-well plates. Suspended in mediums, both Jurkat T cells and Raji leukemia cells were supplemented with nutrient, 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<sub>2</sub> for 24 h. After incubation, the media from the wells were withdrawn. The cells on the upper surface of the inserts were gently scrubbed. The outer surface of insert was washed and the media and washing was collected gently in the well. The media was spun and the cells were counted.

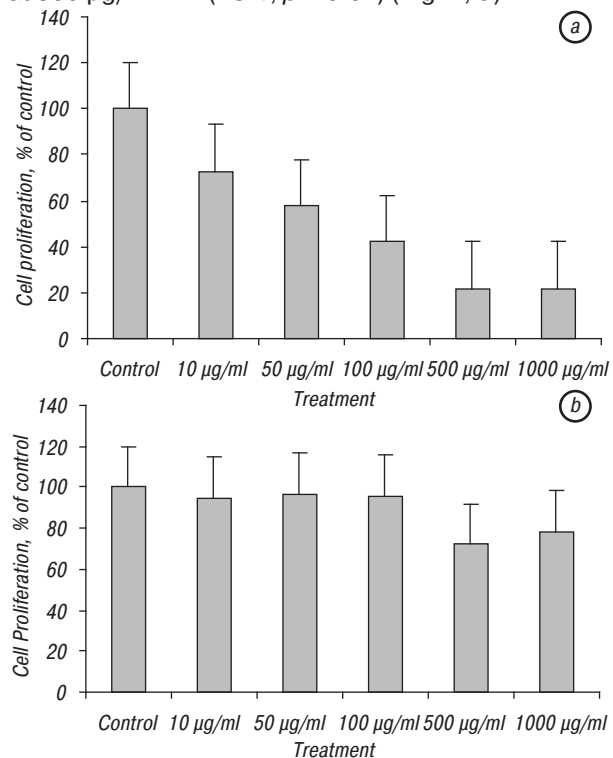
**Morphology and apoptosis.** Morphology of cultured cells for 24 h in control and test concentrations of NM was evaluated by H&E staining and photographed by microscopy. Apoptosis was determined by the method described in the live green Poly Caspase Detection Kit at different doses of NM. Cells were challenged with the NM at 0, 10, 50, 100, 500 and 1000 µg/ml concentration and incubated for 24 h. The culture was washed with PBS and treated with caspase reagent as specified in the manufacturer's protocol (Molecular Probes Image-IT™ Live green Poly Caspase Detection Kit 135104, Invitrogen). Cells were photographed under a fluorescence microscope and counted. Green colored cells represent viable cells, while yellow orange colored cells represent early apoptosis and red color represents late apoptosis.

**Statistical analysis.** Results were expressed as means  $\pm$  SD for the groups. Data was analyzed by independent Student's *t*-test.

## RESULTS

**Cell proliferation study.** The NM exhibited dose response toxicity on human Burkitt's lymphoma Raji cells when compared to the control: 27% at 10  $\mu$ g/ml ( $p=0.007$ ), 42% at 50  $\mu$ g/ml ( $p=0.002$ ), 58% at 100  $\mu$ g/ml ( $p=0.0003$ ), and 88% at 500  $\mu$ g/ml and 1000  $\mu$ g/ml ( $p < 0.0001$ ) of NM as shown on Fig. 1, a.

The NM was not toxic to Jurkat cells up to 100  $\mu$ g/ml but exhibited antiproliferative effect at 500  $\mu$ g/ml NM (28%,  $p=0.01$ ) (Fig. 1, b).

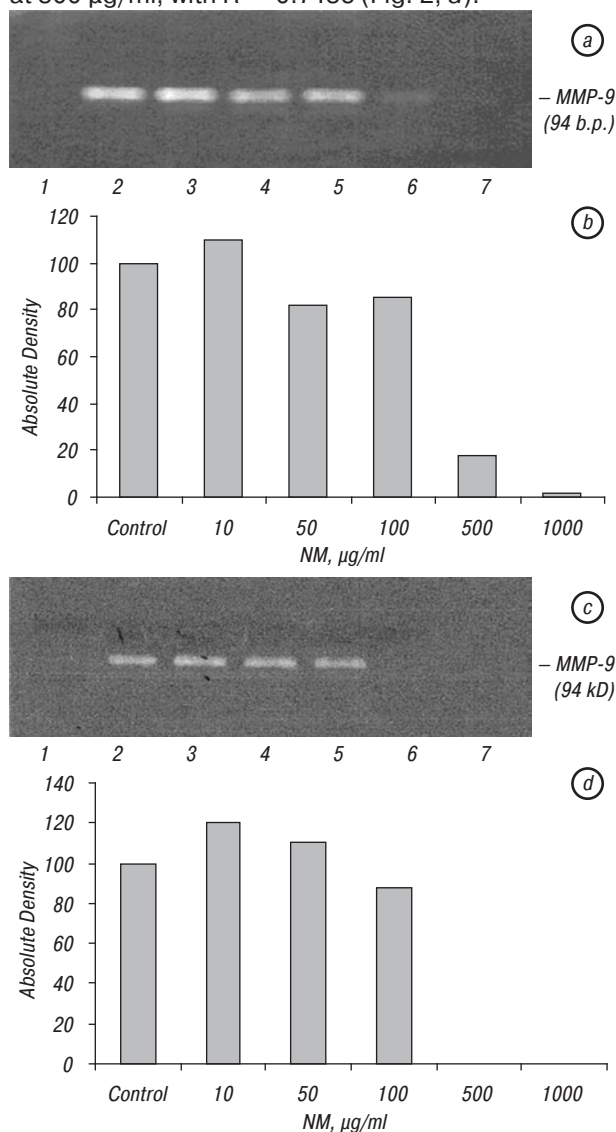


**Fig. 1.** Effect of NM on human Burkitt's lymphoma Raji cells (a) and Jurkat T cells (b) proliferation. a, The NM shows dose response toxicity on Raji cells when compared to the control. b, The NM was non-toxic to the cells up to 100  $\mu$ g/ml, but exhibited anti-proliferative effect at 500  $\mu$ g/ml

**Gelatinase zymography study.** Raji cells did not express any MMP by zymography. However, upon treatment with PMA (100 ng/ml), zymography exhibited only one band corresponding to MMP-9. The NM significantly decreased this expression of MMP-9 at 500  $\mu$ g/ml and completely at 1000  $\mu$ g/ml (Fig. 2, a) Densitometry analysis of relative activity of MMP-9 showed that the expression continued to be the same up to 100  $\mu$ g/ml and dramatically decreased at 500  $\mu$ g/ml and 1000  $\mu$ g/ml, with  $R^2 = 0.8094$  (Fig. 2, a).

The untreated Jurkat cells did not exhibit bands for either MMP-2 or MMP-9. However on treatment with PMA (100 ng/ml) the expression of MMP-9 was evident. Treatment with the NM showed dose dependent decrease in MMP-9 expression and virtual inhibition at 500  $\mu$ g/ml concentration (Fig. 2, c) Densitometry analysis on relative density of MMP-9 showed

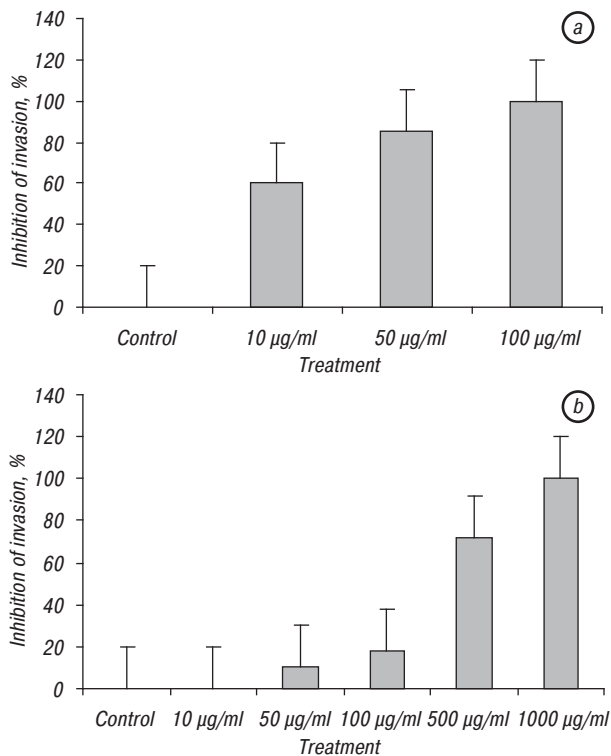
no change up to 250  $\mu$ g/ml and total inhibition at 500  $\mu$ g/ml, with  $R^2 = 0.7436$  (Fig. 2, d).



**Fig. 2.** Effect of NM on MMP-9 expression by human Burkitt's lymphoma Raji cells (a–b) and Jurkat T cells (c–d). a, MMP-9 expression in PMA (100 ng/ml) treated Burkitt's lymphoma Raji cells is significantly decreased at 500  $\mu$ g/ml and completely at 1000  $\mu$ g/ml. (Legend: 1 — markers, 2 — control, 3 — 10  $\mu$ g/ml, 4 to 7 — NM 50  $\mu$ g/ml, 100  $\mu$ g/ml, 500  $\mu$ g/ml, 1000  $\mu$ g/ml). b, Densitometry analysis: effect of NM on relative activity of MMP-9 in PMA treated Raji cells. c, Effect of NM on PMA (100 ng/ml) treated Jurkat T cells; NM exhibiting dose dependent decrease in MMP-9 expression with virtual inhibition at 500  $\mu$ g/ml (Legend: 1 — markers, 2 — control, 3 — 10  $\mu$ g/ml, 4 to 7 — NM 50  $\mu$ g/ml, 100  $\mu$ g/ml, 500  $\mu$ g/ml, 1000  $\mu$ g/ml). d, Densitometry analysis: effect of NM on relative activity of MMP-9

**Invasion study.** The NM significantly inhibited invasion of Raji cells through Matrigel. Compared with control, the NM showed 60% inhibition at 10  $\mu$ g/ml concentration ( $p=0.001$ ), 85% at 50  $\mu$ g/ml ( $p=0.0003$ ) and almost 100% at 100  $\mu$ g/ml ( $p=0.0001$ ) (Fig. 3, a).

Significant reduction of Jurkat T cells invasion through Matrigel was also seen in a dose-dependent fashion compared to control. Invasion was considerably inhibited at 500  $\mu$ g/ml NM (72%;  $p=0.0001$ ), and 100% at 1000  $\mu$ g/ml NM ( $p < 0.0001$ ) (Fig. 3, b).



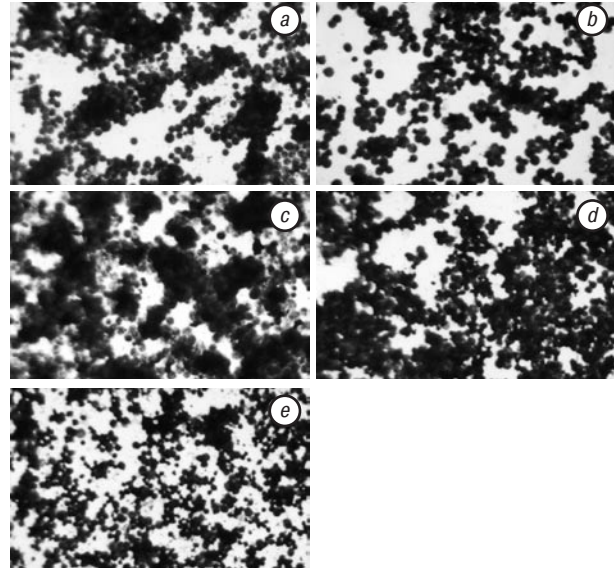
**Fig. 3.** Effect of NM on Matrigel Invasion by Burkitt's lymphoma Raji cells (a) and Jurkat T cells (b). Matrigel Invasion significantly decreased by NM in both cell types

#### Morphology study and detection of apoptosis.

Hematoxylin-eosin (H&E) staining showed no morphological changes in Raji cells, up to 100 µg/ml, but apoptotic cells were evident at 500 and 1000 µg/ml (Fig. 4, a–e). The apoptotic cells showed shrinkage, with condensed and darkly stained nuclei and strong acidophilic cytoplasm. The apoptosis was further confirmed by using the live scan green caspase kit. Dose dependent apoptosis of Raji cells was evident with the NM challenge (Fig. 5, a–e). Slight apoptosis of cells was observed in cells exposed to 50 µg/ml and 100 µg/ml (see Fig. 5, b, c). However, the extent of apoptosis was reflective of the increasing doses of NM from 500 to 1000 µg/ml (see Fig. 5, d, e). Quantitative analysis of live, early and late apoptotic cells is shown on Fig. 5, f. Moderate apoptosis with 51% live cells and 20% apoptotic cells are seen with exposure of 50 µg/ml NM. Percentage of dead cells increased up to 75% at 500 µg/ml and then to 81% at 1000 µg/ml while the percentage of live cells decreased with increase in dose of NM to 14% and 10%, respectively.

H&E staining showed slight morphological changes of Jurkat cells at 100 µg/ml (Fig. 6, c), and significant apoptosis was visible at 500 and 1000 µg/ml (Fig. 6, d, e). Results from Live green caspase analysis showed dose-dependent apoptosis in Jurkat T cells as well. There were a few apoptotic cells seen at 50 and 100 µg/ml (Fig. 7, b, c). However, the extent of apoptotic cells consistently increased from 500 µg/ml (Fig. 7, d) to 1000 µg/ml (Fig. 7, e). Quantitative analysis as shown in Fig. 7, f demonstrates

gradual increase in dead cells from 52% at 50 µg/ml up to 90% at 1000 µg/ml NM, significantly decreasing the live cells too.

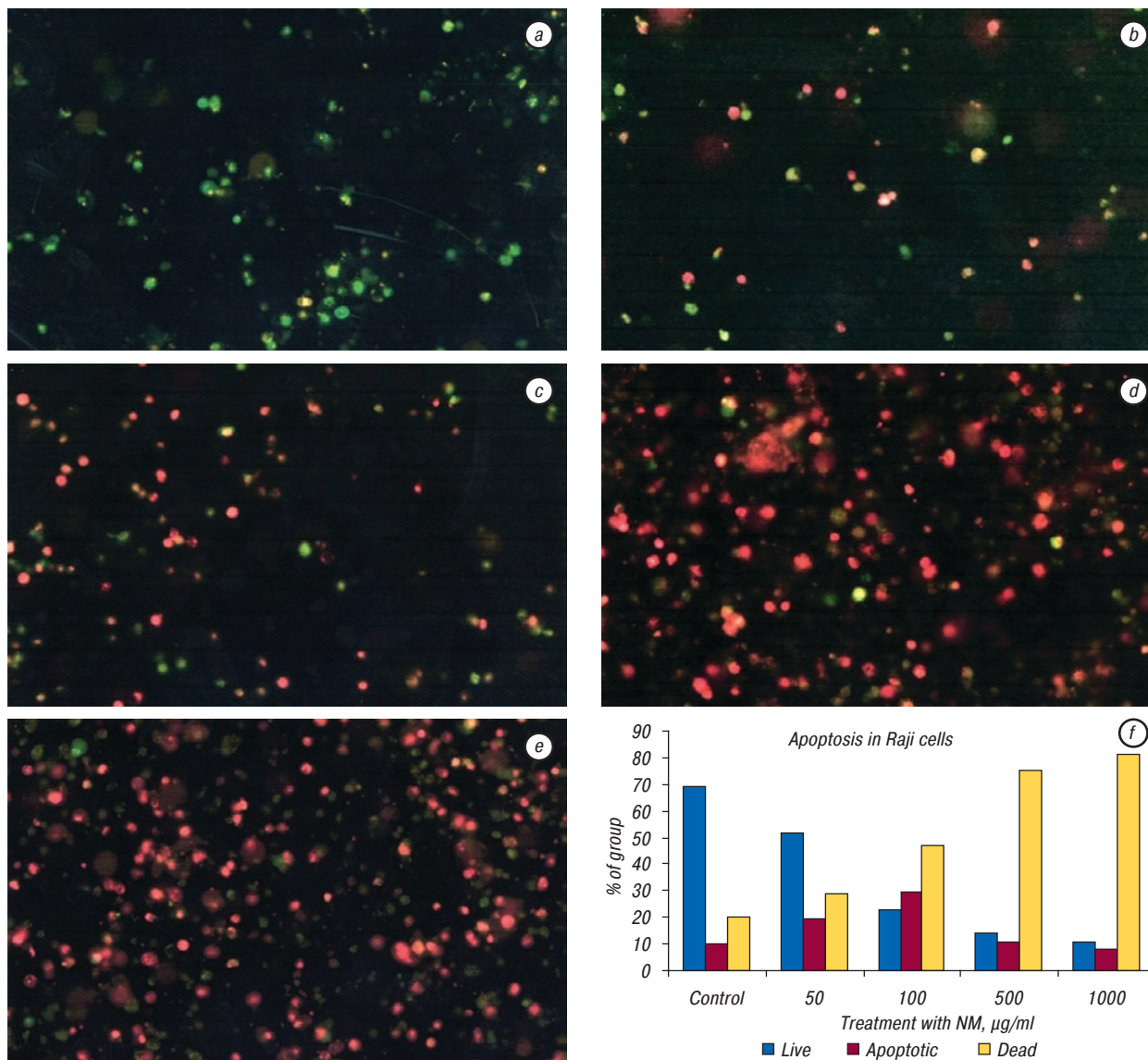


**Fig. 4.** Burkitt's lymphoma Raji cells morphology photomicrographs-H&E staining no morphological changes up to 100 µg/ml. Incremental changes were evident at 500 and 1000 µg/ml. a, Control; b, NM 50 µg/ml; c, NM 100 µg/ml; d, NM 5000 µg/ml; e, NM 1000 µg/ml.

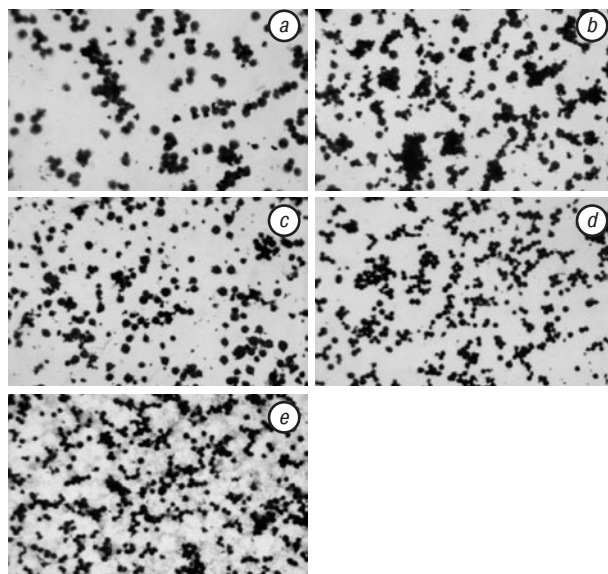
#### DISCUSSION

In this study, the use of NM demonstrated significant inhibition of both Raji and Jurkat T cells in various parameters of tumor progression such as cell proliferation, MMP expression, Matrigel invasion, morphological and apoptosis. The percentage inhibition gradually increased from 50 µg/ml to 1000 µg/ml NM in all cases and was observed to be dose dependant. MMP-9 expression was also inhibited with virtually complete inhibition at 500 µg/ml in both cell types. Cell apoptosis increased up to 90 to 100% in both Jurkat T cells and Raji cells, respectively.

Decreased food intake due to anorexia-cachexia syndrome complicated by side effects of chemotherapy treatments make cancer patients vulnerable to deficiency of essential nutrients such as vitamin C, lysine, and proline leading to the formation of weak ECM susceptible to easy breakdown by MMPs [4]. Research continues to explore synthetic compounds to target specific MMP enzymes as cancer therapies. However, we think that one of the most promising approaches to cancer would be targeting multiple steps involved in cancer growth and metastasis. Thus by encapsulating the tumor, strengthening of connective tissue, preventing angiogenesis, and blocking the MMP activity we believe that cancer growth could be arrested. Our previous research has proven that control of the proteolytic degradation of ECM using nutrient combination provides an opportunity to address these common mechanisms [19]. In this study we have used this approach to evaluate the effects of NM in hematological malignancies.

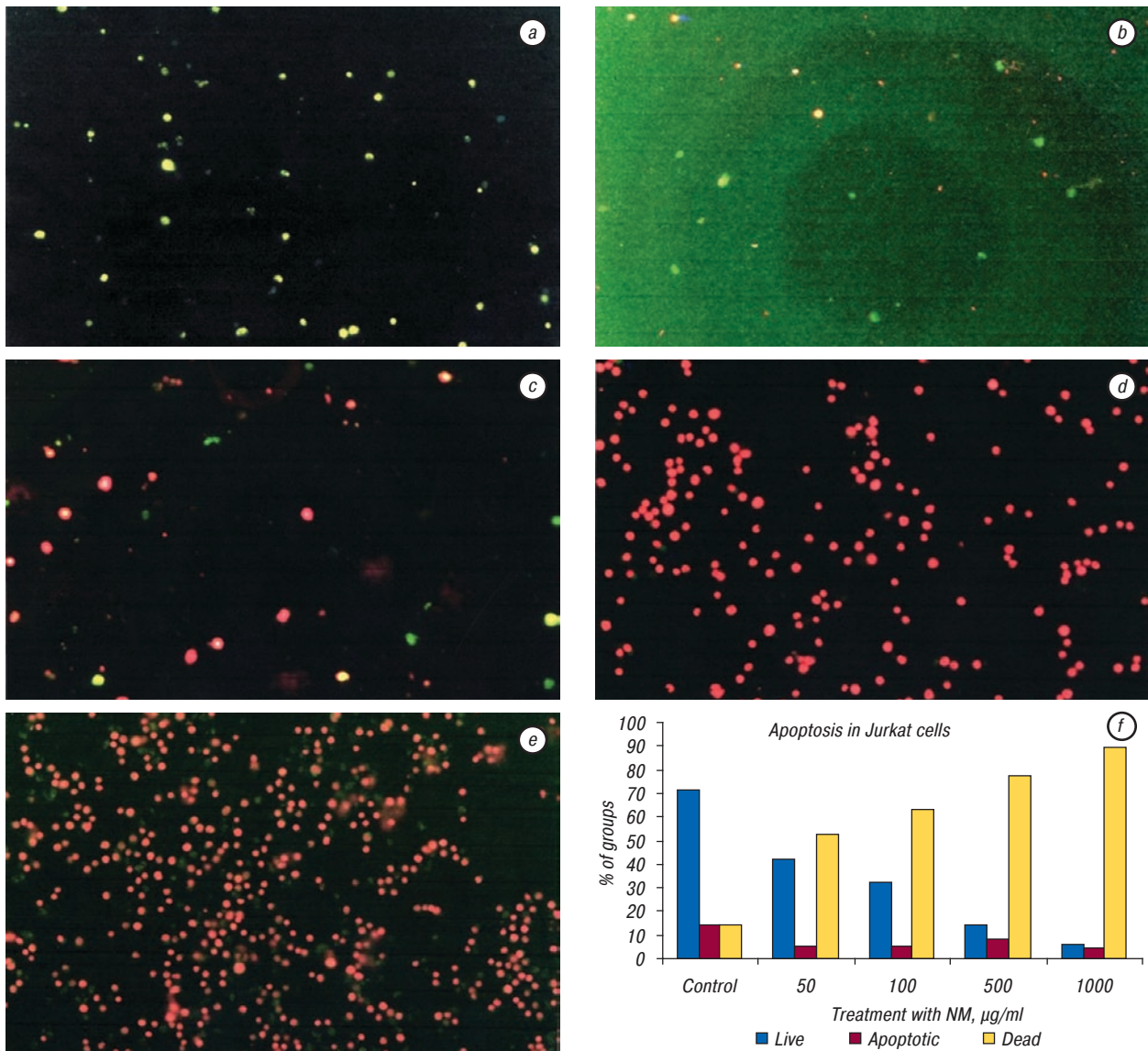


**Fig. 5.** Photomicrographs of apoptosis induction in Raji Cells by NM (Live green caspase detection kit): a, Control; b, NM 50  $\mu\text{g/ml}$ ; c, NM 100  $\mu\text{g/ml}$ ; d, NM 500  $\mu\text{g/ml}$ ; e, NM 1000  $\mu\text{g/ml}$ ; f, Quantitative analysis of live, early and late apoptotic cells shows minimal apoptosis at 50  $\mu\text{g/ml}$ , while profound apoptosis with increasing doses of NM



**Fig. 6.** Human Jurkat T cells morphology photomicrographs-H&E staining showed significant morphological changes as the concentration of NM increased. a, Control; b, NM 50  $\mu\text{g/ml}$ ; c, NM 100  $\mu\text{g/ml}$ ; d, NM 500  $\mu\text{g/ml}$ ; e, NM 1000  $\mu\text{g/ml}$ :

Collagen, being the main component of cellular basement membrane, plays a critical role in tumor invasion. Our studies have indicated that the ECM integrity is dependant upon adequate collagen formation and stability [10] supported by ascorbic acid, lysine and proline. Ascorbic acid is essential for hydroxylation and for regulation of collagen synthesis at a transcription level. Lysine, as an effective MMP blocker, helps to strengthen the connective tissue and prevent matrix invasion [4]. Lysine also prevents cancer cell migration by preventing MMPs binding to plasminogen-active sites, thereby blocking activation of plasmin by plasminogen [4]. However, suboptimal levels of ascorbic acid are possible and are already reported in cancer patients [20, 21] although it is cytotoxic to cancer cells and also possess anti-metastatic properties [22, 23]. Another important nutrient, proline, is a non-essential amino acid, yet, it is likely that the proline production cannot keep up with the increased demand during cancer. Researchers have also proven that EGCG and selenium induce selective apoptosis only of cancer



**Fig. 7.** Photomicrographs of apoptosis induction in Jurkat T cells by NM (Live green caspase detection kit): a, Control; b, NM 50 µg/ml; c, NM 100 µg/ml; d, NM 500 µg/ml; e, NM 1000 µg/ml; f, Quantitative analysis shows increased the apoptotic cells with increasing dose of NM. A few apoptotic cells seen at 50 and 100 µg/ml (b, c), and extent of apoptotic cells consistently increase to 1000 µg/ml

cells [24–26] Furthermore, based on the *in vivo* study, anti-angiogenic and apoptotic effects of green tea in NHL are also supported [27]. Utilization of such anti-angiogenic properties would be important considering that angiogenesis is postulated to have a role in clinical behavior and prognosis in childhood NHL [28]. Very recently, clinicians from Mayo Clinic (MN, USA), reported significant decrease in absolute lymphocyte count and reduced lymphadenopathy in patients with chronic lymphocytic leukemia after EGCG administration in their phase I clinical trials [29].

The role of MMPs in hematological malignancies was not completely understood until Kossakowska *et al.* [30] reported the correlation between increased expression of MMP-9 and higher prevalence of B-cell symptoms in NHL. Additionally, it is documented that increased MMP-9 is associated with poorer prognosis of NHL patients [28]. Therefore it is important that MMP-9 inhibitors should have a higher priority while exploring therapeutic approaches. Our current findings prove that NM can inhibit cell proliferation, expression of MMP-9

and enhance the apoptotic activity of malignant cells at much lower doses of nutrients than that used in most other studies due to the synergistic effect.

Current treatment strategies for various types of NHL differ according to the cell types, stage, and aggressiveness of the tumors. NHL has a greater tendency to metastasize to extra-nodal sites and about 40% of slow growing NHLs can quickly turn into highly. Five-year survival rates for Burkitt's lymphoma seem to have improved in the past few years, however long-term prognosis still remains poor and experimental treatments are sporadically reported [31]. Although stem cell transplants are a possible option, chemotherapy and radiation continue to be the mainstay of treatment. Yet higher relapse rate, involvement of central nervous system and treatment-associated complications dampen the prognosis in Burkitt's lymphoma [2, 3]. Fatalities attributed to are mainly due to aggressiveness of the disease. However inefficacy of current therapies to halt the progress and its side effects further contribute to the mortality rate. Although researchers are focusing

on developing selective medications most of them are in very initial phases. It could be years before patients have access to those, and even then the cost could be prohibitive. In the mean time, rising rates of NHL can have much higher economic impact on the society as children and young adults are involved.

On this background the results of our study strongly suggest that the NM is a potential candidate for therapeutic evaluation in highly aggressive. NM has shown the potential to inhibit MMP-9 expression, Matrigel invasion of Raji and Jurkat T cell lines, and increased selective apoptosis of malignant cells assuring safety for normal cells. Undeniably, additional animal and clinical trials are required; however development of such protocols could also serve as a model for other forms of lymphomas.

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