ORIGINAL CONTRIBUTIONS

IN VIVO AND *IN VITRO* ANTITUMOR EFFECTS OF NUTRIENT MIXTURE IN MURINE LEUKEMIA CELL LINE P-388

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Aim: Leukemia is characterized by uncontrolled marrow cell proliferation and metastatic foci. We investigated the antitumor potential of a nutrient mixture on malignant leukemia P-388 cells. *Methods:* The nutrient mixture containing lysine, proline, ascorbic acid, green tea extract and other nutrients is formulated to target key pathways in cancer progression. The cells were treated with the mixture, and tested at doses 0, 10, 50, 100, 500 and 1000 µg/ml in triplicates. The effects were evaluated by cell proliferation, Matrigel invasion, cell morphology and apoptosis. The *in vivo* effect was measured in male nude mice ($n = 12$) inoculated with P-388 cells. After randomly dividing in two groups, each group was fed regular and the nutrient mixture supplemented diet and the mice were sacrificed after four weeks. Results: The nutrient mixture decreased P-388 cell proliferation at 500 and 1000 µg/ml. Only 10% cells were viable at 1000 µg/ml. Matrigel invasion was significantly inhibited in a dose dependent manner with virtually total inhibition at 1000 µg/ml. Cell morphological features notably changed with dose increase to 1000 µg/ml. Analysis of apoptotic cells on live green caspase kit exhibited gradual increase with the increasing dose of the nutrient mixture, and at 1000 µg/ml 92% of P-388 cells were in late apoptosis. Tumors in the group of mice supplemented with the nutrient mixture had 50% lower weight compared to the tumors in control group ($p = 0.0105$). Histopathologically, both the groups of tumors were similar, yet size of tumors in the group treated with the nutrient mixture was considerably smaller. *Conclusion:* These results indicate that the nutrient mixture exhibited significant action against multiple targets in P-388 leukemia and may have potential in human leukemia.
Key Words: leukemia, P-388, nutrient mixture, green tea extract, apoptosis, metastasis.

Leukemia, acute or chronic, is a malignancy of bone marrow characterized by uncontrolled proliferation of while blood cells, resulting in formation of metastatic foci. The process of penetration through extra cellular matrix (ECM) barrier and blood vessel walls is often mediated through matrix metalloproteinases (MMPs) enzymes. Acute lymphoblastic/lymphocytic leukemia (ALL) is a fast growing tumor of immature lymphocytes in the bone marrow, while chronic lymphocytic leukemia (CLL) is much slower developing malignancy of partially matured cells. Leukemic lymphocytes replace the normal marrow cells thereby diminishing red, white blood cells and platelets. Once diagnosed, lymphocytic leukemia rapidly invades and metastasizes to lymph nodes, spleen, liver, central nervous system and testis in males. Children in the first decade of life have the highest incidence of ALL and the incidence of all types of leukemia increases with age. Some risk factors include genetic diseases like Down syndrome, blood disorders as myelodysplastic syndrome, therapeutic and environmental exposure to chemicals and radiation. Viral infections with human T-cell lymphoma/leukemia virus-1 (HTLV-1) and Epstein-Barr virus (EBV) also increase the risk of this particular type of leukemia. Without treatment, most patients with ALL would live only few months. Although there are several treatment options available, ALL turns rapidly fatal in adults [1]. In 2010, the American Cancer Society estimated there would be 5,330 new cases of ALL diagnosed in the USA and 1,420 deaths would occur. While, only 25% of those diagnosed would be adults, 75% of those who die would be adult ALL patients [2]. Intense chemotherapy may help initial remission in 80–90% patients; yet, frequent relapses reduce the disease free survival rates to mere 30–40%. Although newer therapies are being developed for improving this statistics in children and adults, many of the chemotherapy options have damaging side effects. This is of critical importance in childhood leukemia, due to long-term reported side effects such as secondary malignancies in later life, cardiomyopathies, and neurological, endocrine as well as musculoskeletal dysfunctions. Therefore, we thought it would be helpful to evaluate natural compounds for their efficacy in leukemia treatment.

Destruction of extracellular matrix (ECM) has been known as a precondition for cancer cell growth, invasion and metastasis. Rath and Pauling [3] proposed intervention through natural inhibitors of plasmin-induced proteolysis, such as lysine and analogues. Our previous studies have indicated that synergistic anticancer effect of ascorbic acid, proline, lysine, and epigallocatechin gallate (EGCG) was greater than that of the individual nutrients [4]. Further studies have proven significant anticancer activity of the combination of nutrients against a number of solid cancer cell lines by blocking cancer growth, tissue invasion, and MMPs expression both *in vitro* [4–6] and *in vivo* [7–9].

In the current study we investigated the antitumor potential of nutrient mixture (NM) mainly containing lysine, proline, ascorbic acid, and green tea extract and other nutrients, on lymphoblastic murine leukemia cell lines P-388 by measuring *in vitro* cell proliferation,

Abbreviations used: ALL – acute lymphoblastic/lymphocytic leukemia; EBV – Epstein-Barr virus; ECM – extracellular matrix; EGCG – epigallocatechin gallate; HTLV-1 – human T-cell lymphoma/leukemia virus-1; MMPs – matrix metalloproteinases; NAC – N-acetyl cysteine; NM – nutrient mixture; PMA-phorbol 12-myristate 13-acetate; SD – standard deviation.

Matrigel invasion, modulation of MMPs expression, apoptosis and in *in vivo* studies. We used the NM combination in P-388 leukemia cells, which is the mouse model used for highly malignant leukemia similar to acute leukemia in humans. The NM is a combination of several nutrients formulated to target the key physiological pathways in cancer progression and metastasis. For example, the ECM integrity is dependent upon adequate collagen formation and its stability. In this aspect ascorbic acid and the amino acids lysine and proline are necessary for the formation and optimum structure of collagen fibers. Manganese and copper are also essential cofactors in collagen formation process. Collagen stability can be controlled by lysine [3] and by N-acetyl cysteine (NAC) through its inhibitory effect on MMP-9 activity [10] and invasiveness of tumor cells [11]. In addition, selenium has been shown to interfere with MMP expression and tumor invasion [12] and to induce selective apoptosis of cancer cells. Ascorbic acid is proven to inhibit cell division and growth through production of hydrogen peroxide [13]. Green tea extract is known to be a promising agent in controlling angiogenesis, metastasis, and other aspects of cancer progression [14, 15]. Because arginine is a precursor of nitric oxide (NO), any deficiency of arginine can limit the production of NO, which predominantly acts as an inducer of apoptosis [16]. In addition we also found that a combination of ascorbic acid, lysine, and proline used with EGCG enhanced the anti-invasive activity of 20 µg/ml EGCG to that of 50 µg/ml [17]. 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. Also, we have reported that the combined effects of these individual nutrients on decreasing proliferation of neoplastic cells were superior to the effects of each individual component or when they were randomly combined [18].

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 magnesium, calcium, 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 mg; copper 2 mg; manganese 1 mg.

In vitro study

Cancer cell line and cell culture. Leukemia P-388 cell line obtained from ATCC (American Type Culture Collection, Rockville, MD) was cultured in modified DEM media supplemented with 10% horse serum, and antibiotics (100 mg/ml penicillin and 100 mg/ml streptomycin from Gibco, BRL (Long Island, NY)). The cells were treated with the NM, dissolved in media and tested at 0, 10, 50, 100, 500 and 1000 µg/ml NM in triplicate 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. Phorbol 12-myristate 13-acetate (PMA), 100 µg/ml, was added to the cells to induce MMP secretion. The plates were then returned to the incubator.

Gelatinase Zymography. Gelatinase zymography was used to determine the MMPs activity in conditioned media. Zymography was performed in 10% Novex precast SDS-polyacrylamide gel (Invitrogen Corporation, Carlsbad, CA) 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 $CaCl_2$ 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.

Invasion analysis using Matrigel. Invasion studies were conducted using Matrigel™ (Becton-Dickinson, Franklin Lakes, NJ) inserts in 24-well plates. Suspended in medium, P-388 cells were supplemented with NM, 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_2 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 the insert was washed, and the media and washing were collected gently in the well. The media was spun and the cells were counted.

Morphology. Morphological studies were conducted after 24 h treatment of the cells with NM at the concentrations ranging from 50–1000 µg/ml. The cells were stained by hematoxylin & eosin and examined by light microscopy.

Apoptosis. Apoptosis in NM treated cells was determined using Poly Caspase Detection Kit. Cells were challenged with the NM at 0, 50, 100, 500 and 1000 µg/ml concentrations 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 examined by fluorescence microscopy and counted. Green colored cells represent viable cells, while yellow orange colored

cells represent early apoptosis stage and red color represents late apoptosis stage.

In vivo study

Experimental design: Male athymic nude mice (NCr-nu/nu), approximately 6 weeks of age, were purchased from Simonsen Laboratories (Gilroy, CA) and maintained in micro-insulated cages under pathogen-free conditions on a 12 h light/12 h dark schedule for a week. All animals were cared for in accordance with institutional guidelines for the care and use of experimental animals.

After housing for a week, the mice (*n* = 12) were inoculated with 0.5 x 10^6 P-388 cells in 0.2 ml of PBS in the right flank. After injection, the mice were randomly divided in two groups; group A was fed regular diet while group B was fed the regular diet supplemented with 0.5% of NM. During the study, the mice consumed, on an average, 4 g of their respective diets per day. Thus, the experimental animal received approximately 20 mg of NM per day. After 4 weeks, the mice were sacrificed and their tumor were excised and processed for histology.

Histology. Tissue samples were fixed in 10% buffered formalin. All tissues were fixed in paraffin and cut at 4–5 μm slides. Sections were deparaffinized through xylene, dehydrated by alcohol and stained with hematoxylin and eosin (H&E) for evaluation using a standard light microscopy.

Statistical analysis. Results were expressed as means ± SD for the groups. Data was analyzed by independent sample "t" test.

RESULTS

In vitro studies

Cell proliferation study. NM was not toxic to P-388 leukemia cells at concentrations up to 100 μg/ml and exhibited a dose response decline in cell proliferation and viability at 500 μg/ml and 1000 μg/ml. As seen from Fig. 1, only 70% and 10% cells were viable at 500 and 1000 μg/ml NM respectively.

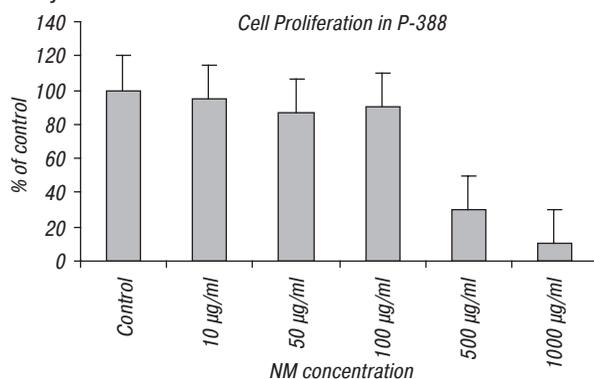**Fig. 1.** Effect of NM on cell proliferation of murine leukemia cell line P-388; NM exhibited no toxicity at concentrations up to 100 μg/ml; while the cell viability considerably decreased at concentrations 500 μg/ml and 1000 μg/ml

Gelatinase zymography. Zymography did not demonstrate expression of MMP-2 or MMP-9 enzymes. No activity was detected even after treatment with PMA (100 ng/ml) (data not shown).

Invasion through Matrigel. The NM significantly inhibited invasion of P-388 cells through Matrigel compared with control. With increasing concentrations, the inhibition of invasion progressed from 80% at 500 μg/ml to total inhibition at 1000 μg/ml (Fig. 2).
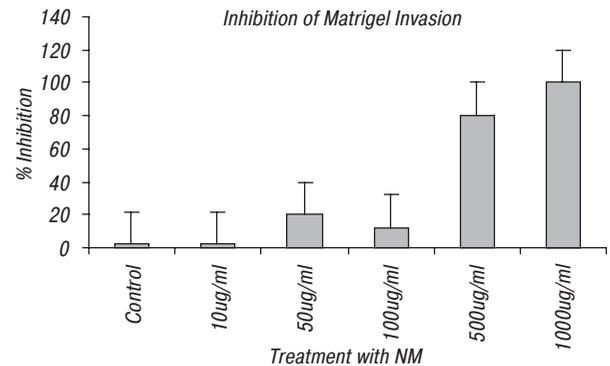

Fig. 2. NM significantly inhibited Matrigel invasion of P-388 cells compared to the control. Virtually 100% inhibition was observed at 1000 μg/ml of NM

Morphology. Mild changes in P-388 cell morphology were noticed at 500 μg/ml NM and significant changes were noted at 1000 μg/ml NM (Fig. 3).

Apoptosis analysis. Dose dependent NM effect on apoptosis of P-388 cells was observed (Fig. 4, a–e). Moderate amount of cell apoptosis was observed in cells exposed to 50 μg/ml NM and 100 μg/ml NM (Fig. 4, b, c). However, the extent of apoptotic cells increased significantly with increasing doses of NM up to 1000 μg/ml. (Fig. 4, c–f) Quantitative analysis of live, early and late apoptotic cells is shown (Fig. 4, g). Exposure to 50 μg/ml NM resulted in 61% live cells and 19% late apoptotic cells, and along with the increasing concentrations of NM, the percentage of late apoptotic cells gradually increased up to 92% at 1000 μg/ml NM while the percentage of live cells decreased accordingly from 6% at 1000 μg/ml.

In vivo studies

Tumor growth. Nude mice supplemented for 4 weeks with 0.5% NM in their diets, developed significantly smaller tumors than the control group as shown in Fig. 5. Tumors in NM supplemented mice demonstrated 50% lower weight compared to those developed in the control group of mice. The average weight of tumors in control group was 1.02 g *versus* 0.54 g in the NM supplemented group (*p* = 0.0105). Nude mice from both groups showed no loss in body weight over the study period.

Histopathology. Histopathological reports stated that the tumors from control animals were large and irregularly round and present in the dermis and subcutis of the skin. The tumor is composed of solid sheets of irregularly round cells with large irregularly round hyperchromatic nuclei and scant bluish cytoplasm. These findings are consistent with malignant lymphoma. Mitotic figures are common as are irregular foci of tumor necrosis. The tumors from NM treated group of mice were reported to have similar histology, but their size was considerably smaller (Fig. 6).
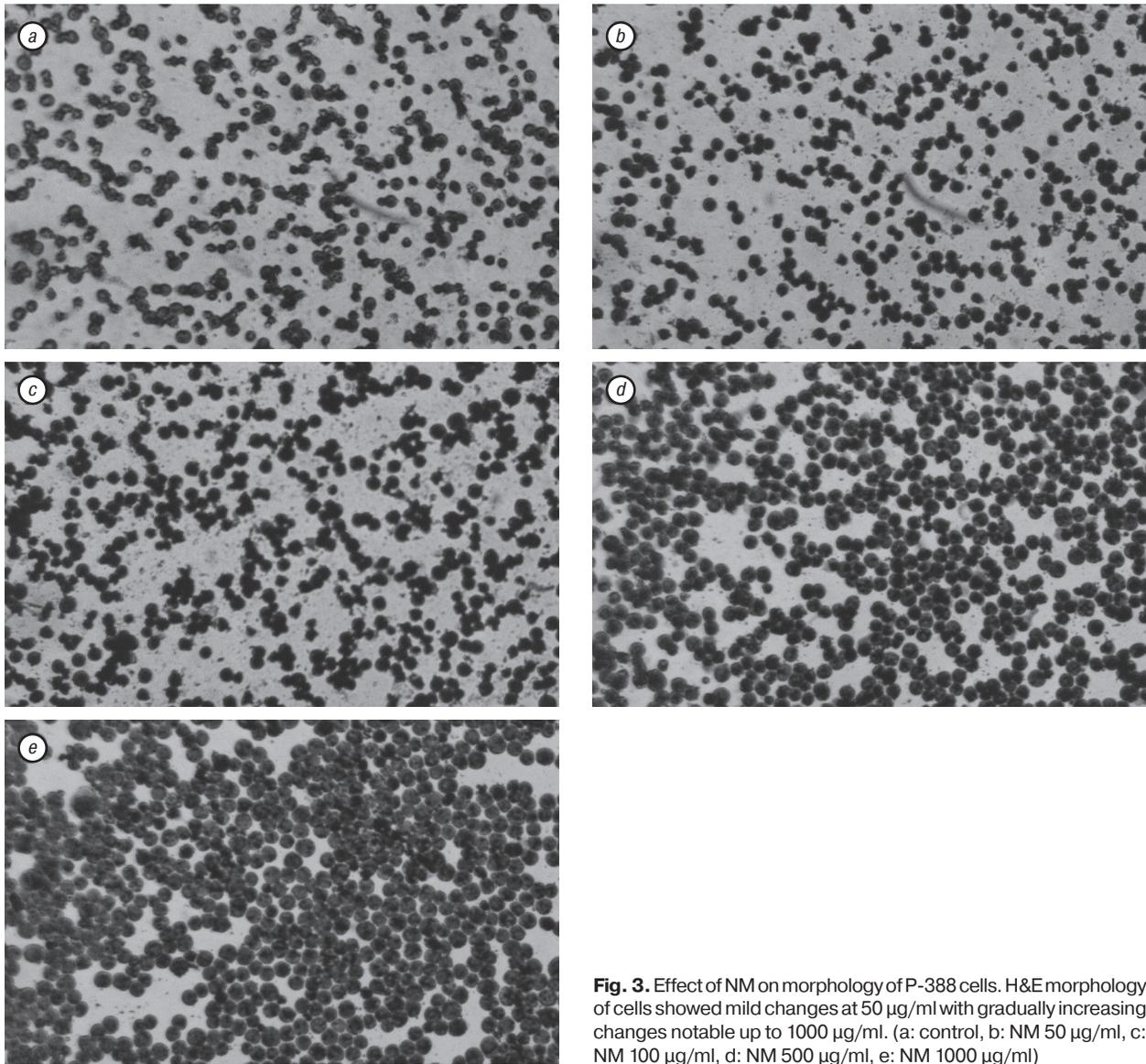
Fig. 3. Effect of NM on morphology of P-388 cells. H&E morphology of cells showed mild changes at 50 μg/ml with gradually increasing changes notable up to 1000 μg/ml. (a: control, b: NM 50 μg/ml, c: NM 100 μg/ml, d: NM 500 μg/ml, e: NM 1000 μg/ml)

DISCUSSION

In this study, the use of NM demonstrated significant inhibition of P-388 leukemia cells as measured by various *in vitro* and *in vivo* parameters. NM exhibited slight toxicity at 50 μg/ml and significant (70–90% over the control) antiproliferative effect at 500 and 1000 μg/ml respectively. Matrigel invasion was inhibited at 500 μg/ml NM by 80% and virtually complete inhibition was achieved at 1000 μg/ml NM. *In vivo* studies verified the efficacy of NM, and the tumors developed in NM treated mice were by 50% smaller than these in control group.

The studies have demonstrated that the invasive and metastatic abilities of cancer cell correlate with MMPs expression, and thus treatment methods aimed at the control of proteolytic activity of ECM allows the opportunity to focus on common mechanisms of metastasis, angiogenesis and tumor growth. Rath and Pauling [3] postulated that nutrients such as lysine and ascorbic acid (Vitamin C) could act as natural inhibitors of ECM proteolysis and, by stabilizing the connective tissue, have the potential to modulate tumor growth and me-

tastasis. Ascorbic acid, lysine, proline, manganese, and copper are correlated with the support of collagen formation and ascorbic acid and green tea extract have been linked to the MMPs expression of cancer cells. Although significant MMPs activity was not noted in the current P-388 cell study, the importance of MMPs in acute leukemia is important. Kuittinen [19] studied the role of MMPs in various types of leukemias. Cancer cells secrete proteases that can degrade the ECM. MMPs, especially MMP-2 and MMP-9 lead to the ECM degradation, resulting in cancer progression [20].

The nutrients in NM can utilize their antitumor potential through several mechanisms, including the inhibition of MMPs as well as strengthening of connective tissue surrounding cancer cells, which can act as "Tumor encapsulating effect". The inhibitory effects of individual nutrients tested have been reported in clinical and experimental studies. Ascorbic acid has been reported to exert cytotoxic and antimetastatic actions on malignant cell lines [21–23], as well as its low levels are reported in cancer patients [24, 25]. The dose dependent inhibitory effect of NM on cell proliferation was consistent with its dose dependent
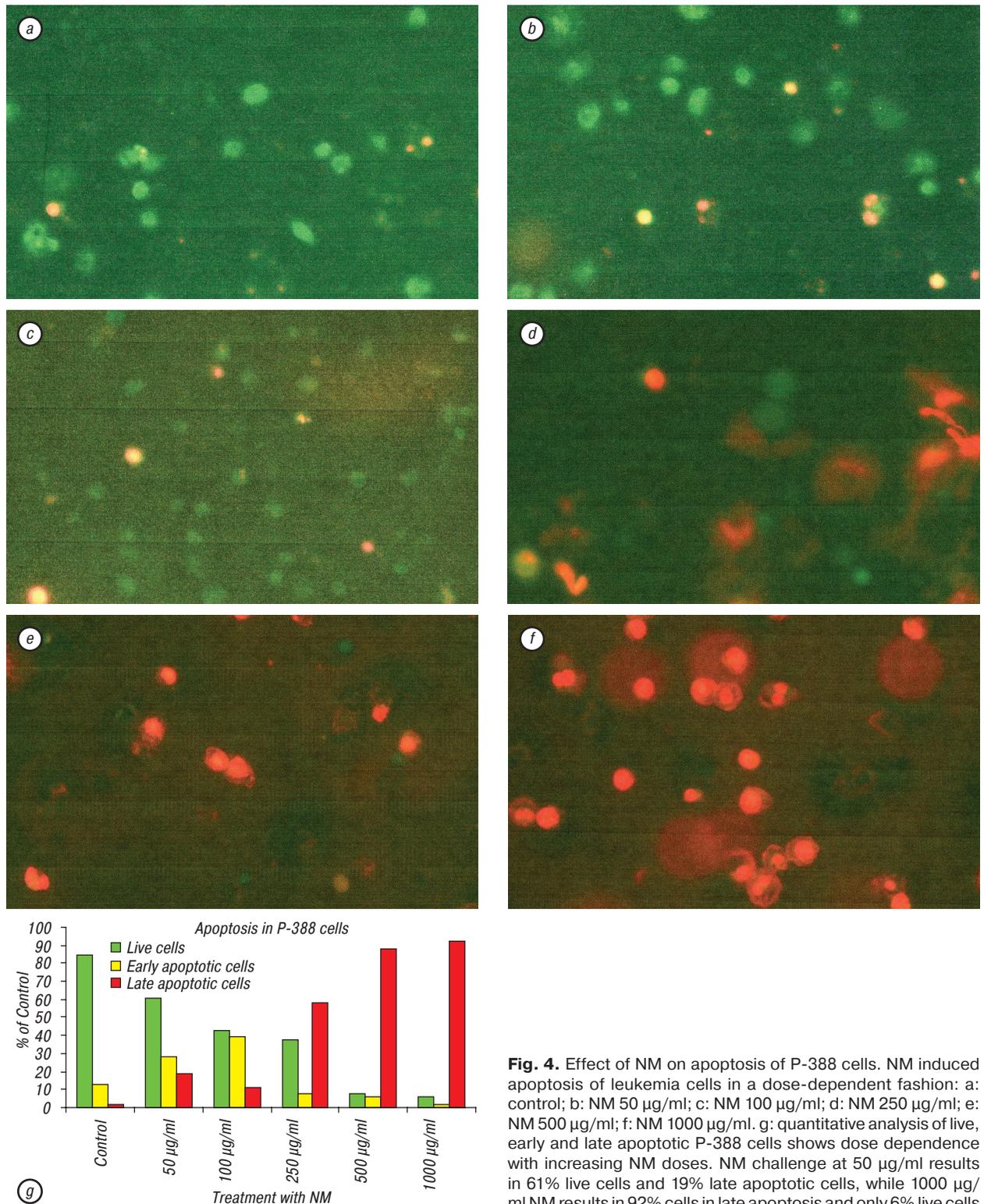
Fig. 4. Effect of NM on apoptosis of P-388 cells. NM induced apoptosis of leukemia cells in a dose-dependent fashion: a: control; b: NM 50 µg/ml; c: NM 100 µg/ml; d: NM 250 µg/ml; e: NM 500 µg/ml; f: NM 1000 µg/ml. g: quantitative analysis of live, early and late apoptotic P-388 cells shows dose dependence with increasing NM doses. NM challenge at 50 µg/ml results in 61% live cells and 19% late apoptotic cells, while 1000 µg/ml NM results in 92% cells in late apoptosis and only 6% live cells

inhibition through Matrigel as well as increased apoptosis. Higher intake of green tea is shown to be associated with reduced risk of leukemia [26]. Individual case studies have also reported objective and positive clinical response in CLL patients [27].

Ascorbic acid, EGCG and lysine have been shown to induce apoptosis in HTLV-1 positive and negative leukemia cells, in three separate studies [28, 29, 30]. However, we thought that individual nutrients are not as potent as their synergy. Our previous studies have shown that synergistic anticancer effect of ascorbic acid, proline, lysine, and EGCG on several cancer cell lines in tissue culture were greater than that of individual nutrients. In another study, the combination of the nutrients used in NM has also shown similar response in HTLV-1 positive leukemia cells [31]. The current study initiated to test this phenomenon, has proven that increasing concentrations of NM induced 92% cell apoptosis. Moreover, in contrast to the chemotherapy, which causes indiscriminate cellular
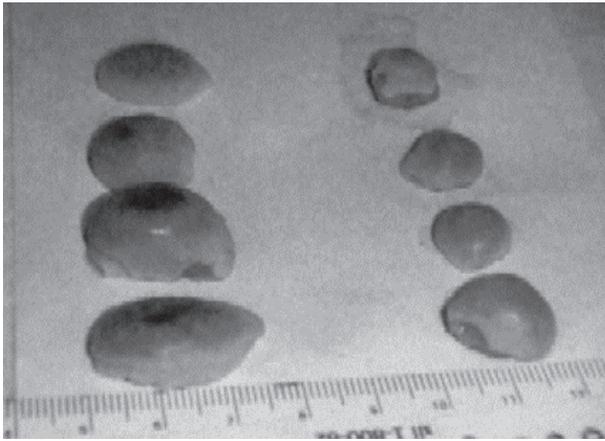
Fig. 5. Effect of 0.5% NM supplementation on tumor volumes in athymic mice inoculated with P-388 leukemia cells. Tumors in NM treated group were significantly smaller and weighed 50% less than that of control group (*p* value = 0.0105)
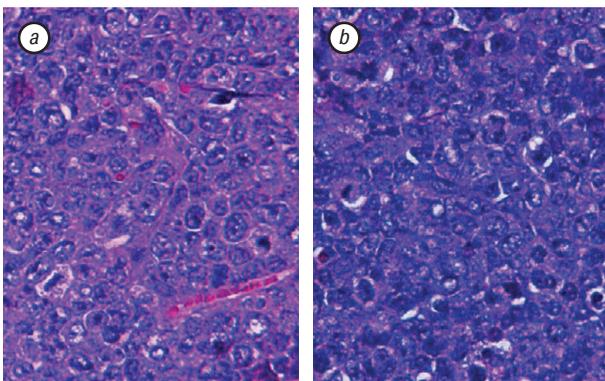

Fig. 6. Tumor histology: a — control group, b — 0.5% NM treated group (magnification 40X). Fewer foci of angiogenesis and increased areas of necrosis seen within the NM supplemented group

destruction and ECM damage, morphological studies have shown that even at the highest concentrations of NM the P-388 cancer cells were not affected showing that this formulation is non-toxic to cells. The *in vivo* results further confirmed the findings when NM fed mice developed 50% smaller tumors.

According to 2010 estimates, 43,050 patients are expected to be diagnosed with leukemia in the USA and 21,840 of them would die because of it. While there are medications that may modify the prognosis, all of the drugs cause damaging side effects. On this background, our study suggests that NM is a potential candidate for therapeutic evaluation in lymphocytic leukemia and could be a studied for other types of cancer as well.

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