

DOWN REGULATION OF u-PA BY A NUTRIENT MIXTURE IN HEMANGIOMA (EOMA) CELLS BY INDUCING CASPASE-DEPENDENT APOPTOSIS

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Hemangiomas are the most common congenital vascular and benign tumor in infants and children. Most hemangiomas do not cause major symptoms to require intervention, however, the larger hemangiomas have tendency to bleed and may require surgical removal. Experimental studies have demonstrated the role of urokinase plasminogen activator (u-PA), especially cell surface u-PA, as an initiator of extra-cellular matrix proteolysis and associated tumor cell invasion. *Aim:* To examine, whether the antitumor effects of a specific nutrient mixture are due to induction of apoptosis by inhibition of u-PA. *Materials and Methods:* A nutrient mixture containing lysine, proline, ascorbic acid, and green tea extract which has showed anticancer activity against a number of cancer cell lines was used as an experimental composition. EOMA cells were grown in appropriate media with antibiotics in 24-well tissue culture plates. At near confluence, the cells were treated with nutrition mixture at 10, 100, 1000 µg/ml in triplicate. Analysis of u-PA activity was carried out by fibrin zymography. Morphological changes and caspase activation associated with apoptosis induction was checked by H&E staining and Live Green caspase assay, respectively. Apoptosis inducing anticancer drug camptothecin (10 µM) was used as positive control. *Results:* The nutrition mixture exhibited dose response toxicity with maximum toxicity 55% ($p < 0.001$) at 1000 µg/ml. EOMA cells expressed u-PA, which was inhibited by nutrition mixture in a dose-dependent manner. The caspase analysis revealed a dose dependent increase in apoptosis of EOMA hemangioma cells, with an increasing apoptosis observed at 100 µg/ml, and maximum at 1000 µg/ml. Cells treated with nutrition mixture showed significantly more apoptotic changes than the control or camptothecin-treated cells. *Conclusion:* These results suggest NM as a potential therapeutic agent as it specifically targets and induces apoptosis in hemangioma cells.

Key Words: nutrient mixture, urokinase plasminogen activators, hemangioma, EOMA, caspase, apoptosis.

A hemangioma is a vascular malformation, an abnormal buildup of blood vessels in the skin or internal organs. Hemangiomas are the most common congenital vascular and benign tumor in infants and children. They are three to five times more common in female children than in males and occur more frequently in Caucasian infants than in Asian-American or African-American infants. Between 4–10% of infants have at least one hemangioma. Although a hemangioma can appear anywhere on the skin, 60% of them occur on scalp and face, and about 25% occur on chest, and trunk areas, which are the most common sites of superficial hemangiomas. The most common site of internal organ hemangiomas is liver, kidneys, lungs, colon, and brain. Generally, most hemangiomas do not cause any symptoms to require intervention. Some of the larger hemangiomas have tendency to bleed and may require surgical removal. The exact cause of hemangioma is unknown. Sometimes, hemangiomas can have genetic origin and can be inherited in a family as an autosomal dominant characteristic with various genetic syndromes [1, 2].

While there are different theories regarding the causes of hemangioma, researchers think that hemangiomas are an abnormal proliferation of blood vessels and a manifestation of aberrant angiogenesis [3]. Abnormal cell proliferation and angiogenesis require

the following steps for tumor formation: attachment of cells to the extra-cellular matrix (ECM), the eventual degradation of ECM for cell migration through the disrupted matrix. The two families of proteases, matrix metalloproteinases (MMP), and urokinase plasminogen activator (u-PA), play key roles in tumor cell invasion. 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 [4]. Consumption of plant based diet has been associated with prevention of the development and progression of various tumors.

In other studies, we found that a nutrient mixture (NM) containing lysine, proline, arginine, ascorbic acid, and green tea extract demonstrated significant antiangiogenic activity [5]. Our previous study also reported significant antiangiogenic properties of NM in hemangioma cells *in vivo* and *in vitro*. In that study, we found that the NM significantly affects the tumor growth in the hemangioma (EOMA) cells in athymic mice [6]. In the current study, we examine, whether the antitumor effects of the NM are due to inhibition of u-PA and induction of caspase-dependent apoptosis.

MATERIALS AND METHODS

Composition of the NM. The stock solution of the NM (total weight 4.4 g) used for testing was composed of the following in the quantities indicated:

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Abbreviations used: ECM – extra-cellular matrix; MMP – matrix metalloproteinases; NM – nutrient mixture; u-PA – urokinase plasminogen activator.

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 1000 mg (derived from green tea leaves, was obtained from US Pharma Lab; the certificate of analysis indicated the following characteristics: total polyphenol, 80%, catechins, 60%; epigallocatechin gallate (EGCG), 35% and caffeine, 1%), selenium 30 mg, copper 2 mg, and manganese 1 mg.

Cells and cell culture. Hemangioma cell line (EOMA) was obtained from ATCC (American Type Culture Collection, Rockville, MD, USA) and maintained in Dulbecco's Modified Eagle's Medium (DMEM) and supplemented with 10% FBS, 100 U/ml penicillin and 100 mg/ml streptomycin. The media and sera were obtained from ATCC, and antibiotics from Gibco BRL (Long Island, NY, USA). The cells were grown at 37 °C in 5% CO₂.

MTT assay. MTT assay was carried out as described earlier [6]. Briefly, cell suspensions were plated in 24-well tissue culture plates (Nunc, Denmark) at a concentration of 3×10^4 cells/well. After incubating the plates for 24 h at 37 °C in a humidified incubator, the cells were treated with the NM at concentrations of 10, 100 and 1000 µg/ml for 24 h and the control group was left untreated. MTT assay reagent, 0.5 mg/ml (Sigma, USA) was added (500 µl per 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 (Scintec Instruments, Manassas, VA, USA). The OD₅₇₀ of the DMSO solution in each well was considered to be proportional to the number of cells.

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). The sample used was the cell culture supernatant treated with NM. 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 were conducted for comparison. Fibrin zymograms were scanned using CanoScan 9950F Canon Scanner (Orem, Utah, USA).

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 50, 500 and 1000 µg/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 min each. The stained cells were then observed and imaged by microscopy.

Apoptosis and Live Green Caspase Assay. The EOMA hemangioma cells were grown to near confluence and either left in media alone, or challenged with

the NM dissolved in media at 0, 100, and 1000 µg/ml, and incubated for 24 h. 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). Camptothecin 10 µM was used as positive control to demonstrate apoptosis. 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 for the groups. Data was analyzed by the independent *t*-test.

RESULTS

Cell viability study. The MTT assay demonstrated dose-dependent toxicity with increased NM concentration — 10%, 30%, and 55% at 10, 100, and 1000 µg/ml, respectively. NM exhibited dose response toxicity on EOMA cells, with maximum toxicity of 55% ($p < 0.001$) over the control at 1000 µg/ml. There was significant negative correlation between NM concentration and cell viability, with $p < 0.0001$ (Fig. 1).

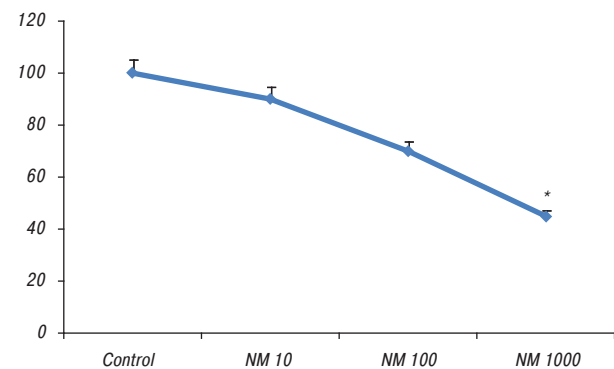


Fig. 1. Effect of NM on viability of EOMA cells. * $p < 0.001$ compared with control cells

Effect of NM on u-PA activity on hemangioma EOMA cells. The cells showed two bands corresponding to subunit 1 and 2 of u-PA at 55 kDa for subunit 1. NM inhibited the u-PA secretion in a dose dependent manner starting at 250 µg/ml and maximum inhibition at 1000 µg/ml. The u-PA subunit 1 showed linear trend $R^2 = 0.8571$ and for the u-PA subunit 2 the linear trend $R^2 = 0.7714$ as shown in Fig. 2, a, b.

Apoptotic morphology by H&E staining. H&E staining revealed a similar apoptotic pattern in dose dependent fashion in hemangioma EOMA cells treated with NM at 0, 50, 500 and 1000 µg/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 50 µg/ml, moderate to significant changes as the NM dose increased to 500 and 1000 µg/ml as shown in (Fig. 3).

Apoptosis. Analysis with the Live Green Caspase revealed a dose dependent increase in apoptosis of EOMA hemangioma cells, with a slight apoptosis

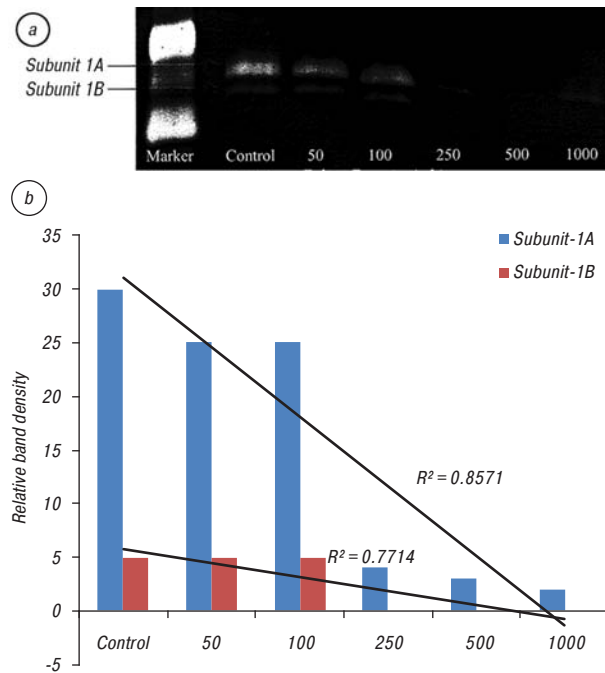


Fig. 2. Effect of NM on u-PA activity on hemangioma EOMA cells: a — inhibition of u-PA secretion; b — linear regression lines

observed at 100 µg/ml, and maximum at 1000 µg/ml (Fig. 4).

DISCUSSION

In our earlier *in vivo* studies, the NM significantly inhibited the growth of hemangioma (EOMA) cells 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. NM induced significant apoptosis in the EOMA cells. Moreover, the apoptotic changes such as cell shrinkage, nuclear condensation, cell membrane asymmetry, and condensation of cytoplasm were also evident and increased in a dose-dependent fashion. We also studied the effect of NM on inducing apoptosis using the *in vitro* Live Green Caspase detection method and by using camptothecin as a positive control. Camptothecin is a potent inhibitor of topoisomerase I, a molecule required for DNA synthesis. It is generally used as an anticancer treatment agent to induce apoptosis in solid tumors.

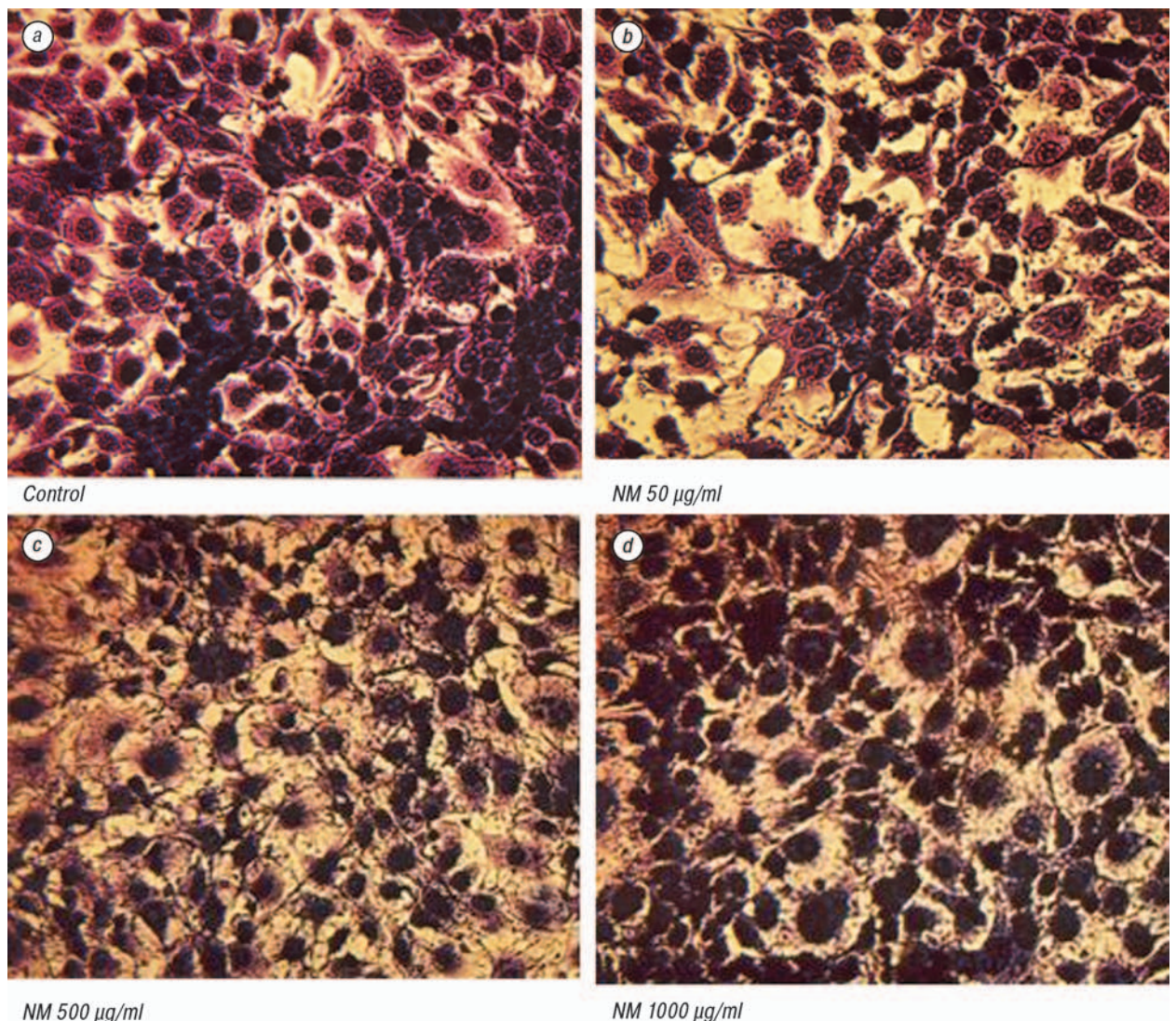


Fig. 3. Effect of NM on morphology of EOMA cells: a — control; b — NM, 50 µg/ml; c — NM, 500 µg/ml; d — NM, 1000 µg/ml. H&E staining, magnification × 200

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. Our study demonstrated that the specific mixture of tested nutrients induced significantly more apoptosis in EOMA cells than the control and also more than camptothecin. NM also inhibited u-PA secretion in hemangioma (EOMA) cells.

Congenital hemangiomas are abnormal development of blood vessels during embryogenesis. Although hemangiomas may resolve spontaneously, some of the larger or internal hemangiomas are treated either surgically or by drugs. The goal of drug therapy with corticosteroids or vincristine is to stop endothelial cell proliferation and angiogenesis, and induce apoptosis to initiate regression. Degradation of ECM is essential process in angiogenesis and vascular remodeling during fetal maturation. Studies have shown that elevated levels of protease enzymes, such as u-PA are associated with tumor growth, progression, angiogenesis, and metastasis. Endothelial cell proliferation requires the critical steps of cell attachment, degradation of ECM and migration through the disrupted matrix. The serine protease u-PA (a 55-kDa serine protease consisting of two disulphide bridges linked to polypep-

tides), is cleaved to the active chain (33 kDa) by various stimuli. Furthermore, u-PA also activates and mobilizes the angiogenesis inducing factors to the site of hemangioma. This enzyme also modulated endothelial cell function including migration and growth through a mechanism independent of its proteolytic activity [7]. Plasmin is critical for hemangioma formation, but may not be critical for capillary formation by normal endothelial cells. u-PA converts plasminogen to plasmin and the plasmin generated in this acts as a chemotactic agent for endothelial cells [7]. Therefore, we examined the actions of NM on inhibition of u-PA and observed the dose dependent reduction of u-PA. Development of antiangiogenic remedies is one of the potential targets to treat hemangiomas.

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 ex-

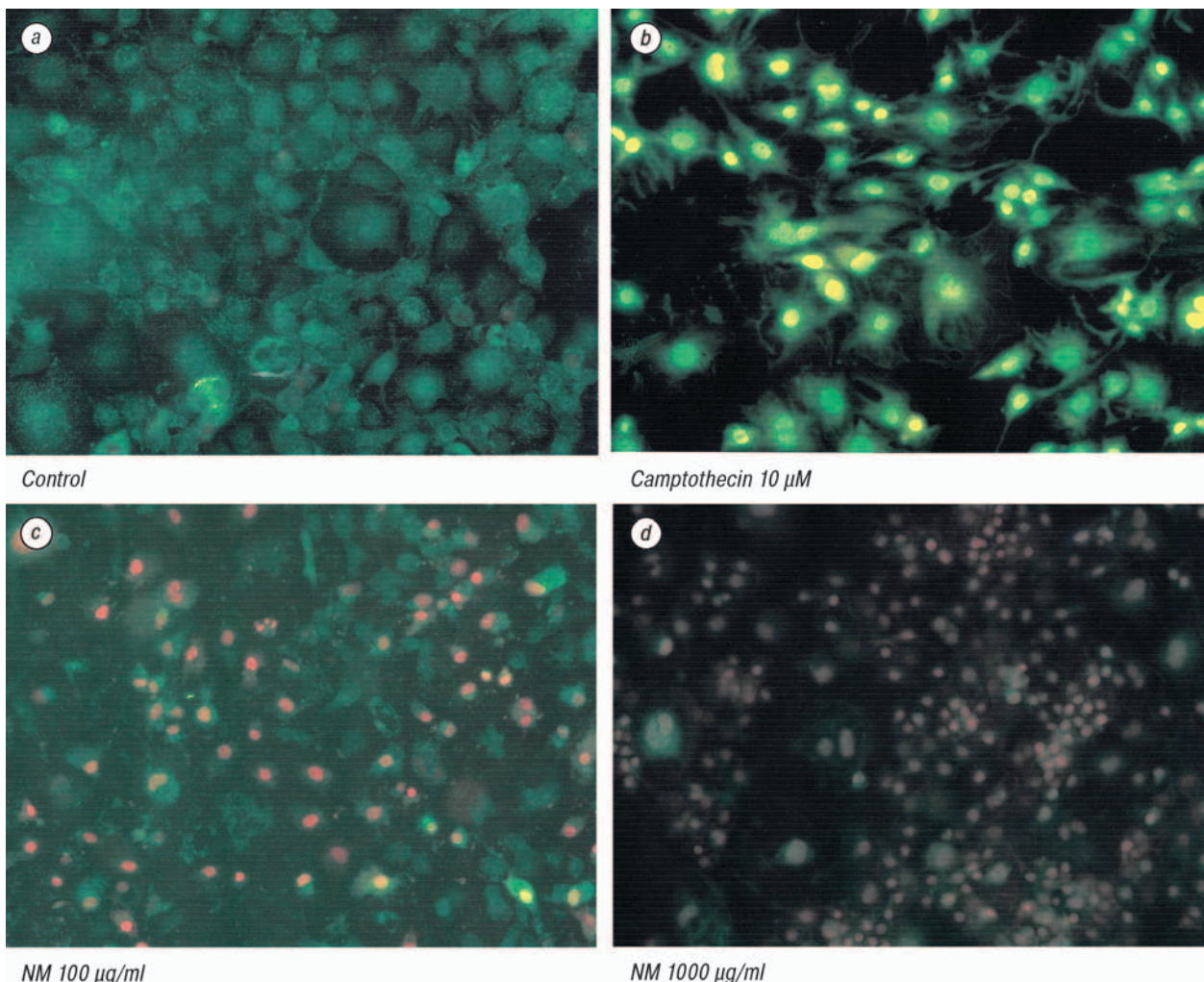


Fig. 4. Effect of NM on apoptosis by caspase induction on EOMA cells: *a* — control; *b* — camptothecin, 10 μM; *c* — NM, 100 μg/ml; *d* — NM, 1000 μg/ml (magnification × 200)

amples of these important caspases are caspase-3, -7, -8, -9, -10 and so on [9, 10]. Although multiple nutrients have anticancer 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 anticancer effect which is much more effective than any of the individual nutrients alone [11]. The NM was formulated by selecting nutrients that act on critical physiological targets in cancer progression and metastasis, as documented in several studies. Combining these micronutrients expands metabolic targets, and maximizes biological impact with lower doses of components [12, 13].

Surgery and other modalities used in the current treatment of hemangiomas are associated with risks and side effects. Therefore, they are used only in severe cases. We have demonstrated that NM is safe according to our studies in female nude mice and where the administration of NM did not have adverse effects on vital organs such as heart, liver, kidneys or the functional serum markers [14]. With the proven safety of NM and the results of our current study we think that NM may have potential in treating hemangioma without side effects of current treatments.

REFERENCES

1. **Drolet BA, Swanson EA, Frieden IJ; Hemangioma Investigator Group.** Infantile hemangiomas: an emerging health issue linked to an increased rate of low birth weight infants. *J Pediatr* 2008; **153**: 712–5, 715.e1.
2. **Bowers RE, Graham EA, Tomison KM.** The natural history of the strawberry nevus. *Arch Dermatol* 1960; **82**: 667–70.
3. **Mulliken JB, Glowacki J.** Hemangiomas and vascular malformations in infants and children: a classification based on endothelial characteristics. *Plast Reconstr Surg* 1982; **69**: 412–22.
4. Hemangiomas and vascular malformations: an atlas of diagnosis and treatment. R Mattassi, DA Loose, M Vaghi, eds. Springer-Verlag Mailand, 2015. 485 p.
5. **Roomi MW, Roomi N, Ivanov V, et al.** Anti-angiogenic effects of a nutrient mixture on human umbilical vein endothelial cells. *Oncol Rep* 2005; **14**: 1399–404.
6. **Roomi MW, Kalinovsky T, Niedzwiecki A, Rath M.** Antiangiogenic properties of a nutrient mixture in a model of hemangioma. *Exp Oncol* 2009; **31**: 214–9.
7. **Günthert U, Birchmeier W, Schlag PM.** Attempts to understand metastasis formation I: Metastasis-related molecules. Introduction. *Curr Top Microbiol Immunol* 1996; **213** (Pt 1): V–VII.
8. **Basu A, Haldar S.** The relationship between Bcl2, Bax and p53: consequences for cell cycle progression and cell death. *Mol Hum Reprod* 1998; **4**: 1099–109.
9. **Li J, Yuan J.** Caspases in apoptosis and beyond. *Oncogene* 2008; **27**: 6194–206.
10. **McIlwain DR, Berger T, Mak TW.** Caspase functions in cell death and disease. *Cold Spring Harb Perspect Biol* 2013; **5**: a008656.
11. **Netke SP, Roomi MW, Ivanov V, et al.** A specific combination of ascorbic acid, lysine, proline and epigallocatechin gallate inhibits proliferation of extracellular matrix invasion of various human cancer cell lines. *Res Commun Pharmacol Toxicol* 2003; **8**: 37–50.
12. **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 J Apoptosis* 2015; **4**: 11–21.
13. **Roomi MW, Ivanov V, Kalinovsky T, et al.** *In vivo* and *in vitro* antitumor effect of ascorbic acid, lysine, proline, arginine and green tea extract on human fibrosarcoma cells HT-1080. *Med Oncol* 2006; **23**: 105–11.
14. **Roomi MW, Ivanov V, Netke SP, et al.** 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 (Abstr #86).