



Inhibition of angiogenic initiation and disruption of newly established human vascular networks by juice from *Morinda citrifolia* (noni)

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Abstract

noni, the juice of the fruit from the *Morinda citrifolia* plant, has been used for centuries as a medicinal agent. We tested the effects of noni juice in a three-dimensional fibrin clot matrix model using human placental vein and human breast tumor explants as sources for angiogenic vessel development. Noni in concentrations of 5% (vol/vol) or greater was highly effective in inhibiting the initiation of new vessel sprouts from placental vein explants, compared with initiation in control explants in media supplemented with an equivalent amount of saline. These concentrations of noni were also effective in reducing the growth rate and proliferation of newly developing capillary sprouts. When used at a concentration of 10% in growth media, noni was able to induce vessel degeneration and apoptosis in wells with established capillary networks within a few days of its application. We also found that 10% noni juice in media was an effective inhibitor of capillary initiation in explants from human breast tumors. In tumor explants which did show capillary sprouting, the vessels rapidly degenerated (2–3 days) in those exposed to media supplemented with 10% noni.

Introduction

The term angiogenesis was first proposed by Folkman in 1971 to define the multistep process by which new vessels develop from pre-existing vascular networks [1]. In the normal adult, angiogenesis is most often associated with an existing pathology, the exception being the recurrent growth and sloughing off of the uterine lining during the ovulatory cycle. In widely diverse pathological conditions such as retinopathy, rheumatoid arthritis, psoriasis, wound healing and tumor development, the otherwise quiescent endothelial lining of blood vessels undergoes conversion to an angiogenic phenotype. It is thought that the molecular mechanism initiating this angiogenic ‘switch’ is an alteration in the balance of naturally occurring endothelial growth factors and inhibitors [2, 3]. Following the ‘switch’ to an angiogenic phenotype, endothelial cells must then proteolytically degrade the extracellular matrix that surrounds them, migrate and proliferate, form capillary

structures, and anastomose into a vascular network. Interruption or blockage of any of these successive steps could potentially prevent or ameliorate the pathologies in which angiogenesis plays a part. To this end, a number of compounds are currently being examined for their efficacy as anti-angiogenic agents [4–6]. An alternative, although related strategy, to blocking angiogenic initiation seeks to target the existing neovasculature of solid tumors [7, 8]. The purpose of this approach is to cause a cessation of nutrient delivery to tumor cells leading to extensive tumor cell death due to metabolic deprivation. A number of agents reported to elicit vascular interruption within tumors including vinca-alkaloids, colchicine, vinblastine, and flavone acetic acid (FAA), require high concentrations to be effective and often result in significant morbidity [9–11]. Combrestatin A4 phosphate can also damage tumor vasculature while sparing the surrounding vascular bed; although continued tumor growth in peripheral vascularized areas indicate that this agent may be most useful as an adjuvant to conventional therapy [12]. Ideally, an anticancer agent acting on the vasculature would inhibit the initiation of new vessel growth, thereby blocking metastasis and tumor expansion, while also attacking the vessels of the primary tumor itself.

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The *Morinda citrifolia* L. plant and its fruit have long been a medicinal staple of the islands of the South Pacific [13]. The juice of *Morinda* (noni) has been used by the Samoan, Tahitian and Hawaiian peoples in the treatment of diabetes, heart disease, high blood pressure, kidney and bladder disorders, while the fruit, leaves and bark are often applied as a poultice to sores, cuts and boils [14]. Recent studies have shown that noni juice given as daily intraperitoneal injections could significantly increase the lifespan of mice implanted with Lewis lung carcinoma cells. This finding was attributed to a stimulation of the rodents' immune system [15]. Other work has shown that an anthraquinone compound, damnacanthal, isolated from the chloroform extract of *Morinda* root can induce normal phenotypes in ras-transformed cells [16]. This report presents data on the anti-angiogenic effectiveness of the juice of the plant *Morinda citrifolia* L. or as it is more commonly known in the Hawaiian islands, 'noni'.

Materials and methods

Morinda citrifolia L.

The juice of *Morinda citrifolia* L. ('noni') was purchased commercially (Herbs' Herbs, Maui, Hawaii), pH adjusted to pH 7.4 with 50% NaOH, centrifuged at 130 k g_{av} for 18 h to remove residual cell debris, and sterilized using a 0.2 μ m Nalgene filter.

Angiogenesis model

Placental veins were dissected free from anonymously obtained discarded human placentas obtained under an LSUHSC IRB approved protocol. The trimmed vein segment was opened longitudinally to produce a flat film of full thickness venous tissue. Vein disks (2 mm diameter) were created with a sterile skin punch. The disks were then placed into standard 96-well plates (Corning Inc., Corning, New York) pre-loaded with a human thrombin solution (0.05 IU in 2 μ l/well) allowed to evaporate to dryness before use. Plating of the vein disks was completed within 3 h of delivery to optimize endothelial cell viability. Vein disks from individual veins were randomly distributed among treatment groups to minimize sampling error.

Immediately following placement in thrombin-loaded dry wells, vein disks were covered with 100 μ l of clot-forming medium. This medium contained fibrinogen (3 mg/ml) (Sigma Chemical Co., St. Louis, Missouri) and ϵ -caproic acid (0.5%) (Sigma Chemical Co., St. Louis, Missouri) in human placental vein angiogenesis medium (HPVAM). HPVAM consists of Medium199 (GibcoBRL, Gaithersburg, Maryland), an antibiotic/antimycotic solution (100 U penicillin, 100 U streptomycin sulfate and 0.25 μ g amphotericin β /ml) (GibcoBRL, Gaithersburg, Maryland), and endothelial growth media (25%) (GibcoBRL, Gaithersburg, Mary-

land). The mixture was allowed to clot by incubating in 6% CO₂, 94% air at 37 °C in a humidified incubator. After the media had jelled, the vein-containing clot was supplemented with 100 μ l HPVAM containing 20% fetal bovine serum (GibcoBRL, Gaithersburg, Maryland). Total well volume was 200 μ l.

Angiogenic evaluation

All wells were evaluated for angiogenic sprouting by examination at 20 \times or 40 \times magnification with a standardized reference grid by an unbiased observer using an inverted microscope every other day of the experiment. Angiogenesis was defined by two criteria. First, the number of wells, as a percentage of the total plated, which developed new vessel growth, was termed initiation. This was defined by the presence of at least three angiogenic sprouts of approximately 0.5 mm in length growing from the periphery of the vein disc. The angiogenic index (AI) based on a visual rating system was the second parameter used to quantify angiogenesis in this study. Each vein disc was divided into four quadrants and rated on the development of vessel growth in each quadrant from 0 to 4. Scores for all 4 quadrants were summed and the AI expressed as a numerical score ranging from 0 to 16. This method enabled us to objectively evaluate the large numbers of wells necessary for this study. Mean AI scores for a treatment group correlated well (90% or greater) among observers ($n=4$). There was also a high degree of correlation between the mean AI score and mean vessel length evaluated by image analysis.

Degradation of established networks

Following 7 days of growth in the HPVAM, 120 explants were randomly divided into test and control groups (60 wells each). Sixty wells were treated with medium containing 10% noni juice, 60 control wells received 10% 0.15 M NaCl. All wells were supplemented with new medium (containing either 10% noni juice, or 10% NaCl) and scored for extent of angiogenic growth every 2 days. Viability was also measured at the end of the experiments in the noni-treated and control groups using a tetrazolium salt-based assay (MTT Assay Viability kit Promega Corp., Madison, Wisconsin).

Apoptosis

Programmed cell death or apoptosis was assayed on 10 *Morinda*-treated and 10 control explants using the S7100 Apoptag Peroxidase *in situ* Apoptosis Detection Kit (Intergen Co., Purchase, New York). This kit is based on the TUNEL assay for the detection of DNA fragmentation, which employs enzymatic labeling of the free 3'-OH termini of single or double DNA strands using the enzyme terminal deoxynucleotidyl transferase (TdT). Increased numbers of 3'-OH ends can be found in nuclei undergoing fragmentation and the character-

istic morphological changes of apoptosis. The digoxigenin-labeled DNA ends can then be detected using anti-digoxigenin peroxidase. Briefly, tissue explants were fixed in 10% formalin and embedded in paraffin. Deparaffinized sections were treated for 15 min with proteinase K (20 $\mu\text{g}/\text{ml}$, Oncor). Endogenous peroxidase was quenched with 2% H_2O_2 in PBS for 15 min, followed by rinsing and blotting around the section. Equilibration buffer was applied for 15 s, and the excess fluid tapped off. Immediately working-strength terminal deoxynucleotidyl transferase was added and the slides were incubated in a humidity chamber at 37 °C for 1 h. Stop wash buffer was then added, and sections were incubated for an additional 10 min. Sections were washed 3 times in PBS and incubated for 30 min with two drops of anti-digoxigenin peroxidase. Slides were washed 3 times with PBS, and sections developed with DAB (4–6 min). Slides were then counterstained with hematoxylin, dehydrated in xylene, and mounted.

Statistics

Comparisons between noni-treated and control groups were performed using Student's *t*-test. These data met the assumptions for Student's *t*-test. All statistical tests were two-sided.

Results

Angiogenic vessel initiation in placental vein explants typically began within 2–4 days of plating. Although there was variation in the number of explants that became angiogenic under control conditions, most placentas yielded initiation rates of 70–95%. Experiments in which initiation rates fell below 70% were excluded. Figure 1 demonstrates angiogenic growth from placental explants under control conditions for a period of 2 weeks.

A dose–response effect of noni juice was established by testing the efficacy of various noni concentrations as inhibitors of angiogenic initiation and vessel growth. Figure 2A shows that when added to the growth medium at concentrations of 5% and 10%, noni was highly effective at suppressing angiogenic initiation, although at a concentration of 2.5% it appeared to lose its inhibitory effect. In contrast to its effect on vessel initiation, 2.5% noni in medium was effective ($P=0.005$) in reducing the growth and proliferation of capillaries (although less so than 5% and 10% noni) as shown in Figure 2B.

In placental explants that were allowed to grow for a week and then exposed to either 10% noni or 10% NaCl in HPVAM, it was observed that noni treatment resulted in both the inhibition of new growth and the breakdown of the newly developed vasculature (Figures 3A and 3B), while 10% NaCl-supplemented wells continued to grow in a normal fashion. Using the MTT viability assay, a minimal amount of stain was

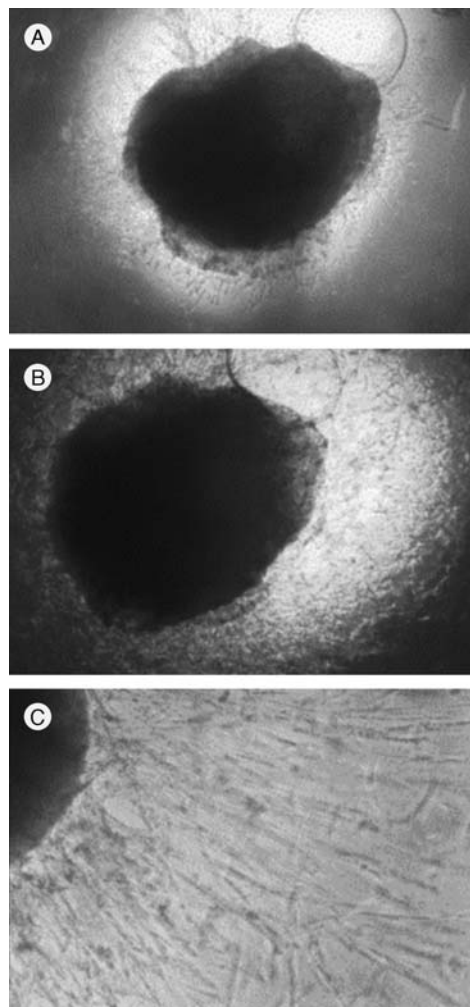


Figure 1. Development of angiogenic network over time. In (A) growth has proceeded under control conditions for 1 week (20 \times). In (B) the same explant can be seen after 2 weeks growth (20 \times). (C) Shows a close-up (40 \times) of the anastomosing network.

taken up by the disrupted vasculature of noni-treated explants, while controls invariably stained a dark blue, indicating that cell death was occurring in the noni treated wells (data not shown). In approximately 50% of the cases, the vascular degeneration was complete, so that no angiogenic vessels were observable following noni treatment (see Figure 3A). In the explants that remained angiogenic following 7 days of noni treatment, all showed a decrease in the size and complexity of the remaining vascular network (Figure 3B). The process of vascular degeneration resulted in what appeared to be small spherical blebs resembling those found during apoptosis (Figures 4 and 5). Using the TUNEL assay (see 'materials and methods'), we found that treatment with 10% noni in the media appeared to induce apoptosis in a large percentage of cells tested (Table 1).

Human breast cancer explants exposed to 10% noni in their medium demonstrated a highly significant reduction ($P=0.009$) in the percent of wells that developed an angiogenic response over 14 days in

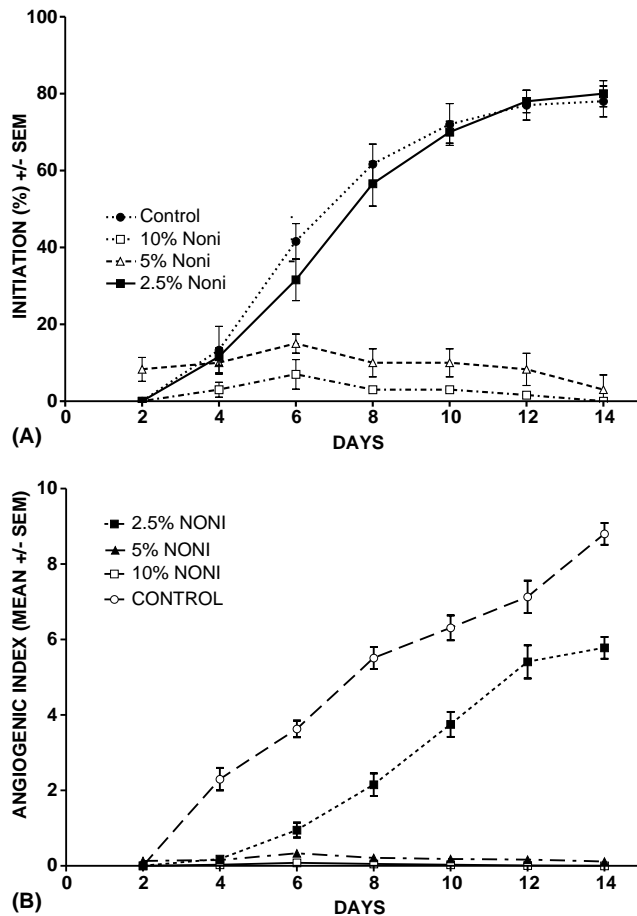


Figure 2. (A) Inhibition of initiation of angiogenesis in sprouts from human placental vein explants by various concentrations of noni juice. Noni juice was centrifuged at 130 k g_{av} , filtered through a 0.2 micron filter, and pH adjusted to 7.4. All noni tests represent data from 60 explants from 3 placentas for each noni concentration. Matched controls ($n = 60$) were given media with an equivalent volume (2.5%, 5%, or 10%) of 0.15 M NaCl added to ensure that observed effects were not due to variations in nutritional media. Control plotted in graph represents 5% supplementation with NaCl. Control 2.5% vs 2.5% noni $P = ns$; 5% control vs 5% noni $P < 0.001$; 10% control vs 10% noni $P < 0.001$. (B) Dose-response inhibition of angiogenic growth and proliferation by noni juice, prepared as described in Figure 2A. Media (HPVAM) was supplemented with varying concentrations of noni juice to yield three test dosages (10% noni $n = 120$, 5% noni $n = 120$, and 2.5% noni $n = 60$). Controls ($n = 120$), were cultured in HPVAM media supplemented with equivalent volumes (2.5%, 5%, or 10%) of 0.15 M NaCl to control for loss of nutritional media volume by noni supplementation in the test groups. Only the 5% control is shown in the graph. Control vs 2.5% noni $P = 0.005$; control vs 5% noni $P < 0.001$; control vs 10% noni $P < 0.001$.

culture when compared to controls (Figure 6A). In a similar fashion, wells that did initiate vessel growth showed markedly reduced development compared to controls ($P = 0.02$, Figure 6B).

Discussion

The results obtained in these studies demonstrate that juice from *Morinda citrifolia* is effective in: (1) inhibiting new angiogenic growth in human placental vein ex-

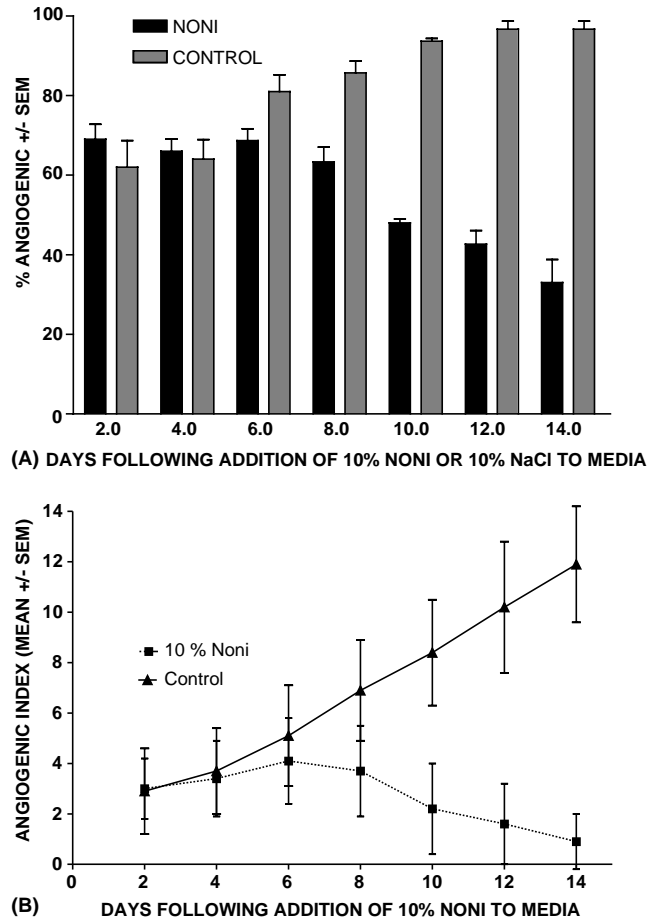


Figure 3. (A) Degradation of established angiogenic networks by noni juice. Following 7 days of culture in 100% growth media, 30 explants from three placentas were treated with 10% noni juice in growth media; 30 control explants from the same placentas received 10% NaCl in growth media. All media were pH balanced to 7.4. Media was changed every 2 days and plates were scored at the same time. Differences between two treatments are significant $P < 0.05$. (B) Degradation of established angiogenic capillary networks by noni juice. Following 7 days of culture in growth media, 30 explants from 2 placentas were treated with 10% noni juice in growth media; 30 control explants harvested from the same placentas, received 10% NaCl in growth media. All media were pH balanced to 7.4. Media was changed every 2 days and plates were scored at the same time. Differences between the treatments are significant $P < 0.05$.

plants; (2) reducing the rate of capillary proliferation and the development of vascular networks in vein discs in which angiogenesis does occur; (3) inducing apoptosis in newly formed angiogenic networks; and (4) suppressing both angiogenic incidence and vessel development in human breast cancer explants. In contrast with earlier investigations in which the growth of Lewis lung carcinoma cells implanted in mice were inhibited with intraperitoneal injections of noni, the effects of noni on angiogenesis do not appear to be mediated by the immune system, as no leukocytes are available in the human vein disk culture [15]. The effects also do not appear to be based on damnacanthal, as we found that chloroform extracts of noni were ineffective in angiogenic inhibition (data not shown) [16].

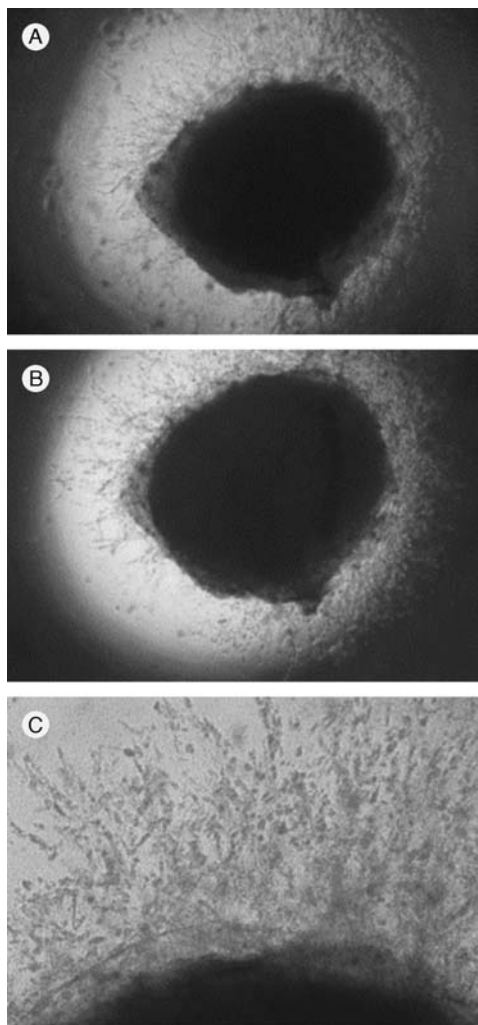


Figure 4. The effect of noni on an established capillary network is shown in these three illustrations. In (A) the growth of capillaries has proceeded under control conditions for one week (2×). (B) shows the effect of noni on the same explant after 7 additional days of 10% noni treatment (20×). (C) is a more highly magnified view of a portion of (B) in which the degradation of the vascular network is evident (40×).

Although it appears from the evidence presented that an induction of apoptosis is the means by which treatment with noni juice degrades newly developed capillary networks, it may well be that the block of angiogenic initiation relies on an entirely different mechanism. A block at any point in the cascade including: proteolytic matrix degradation; endothelial

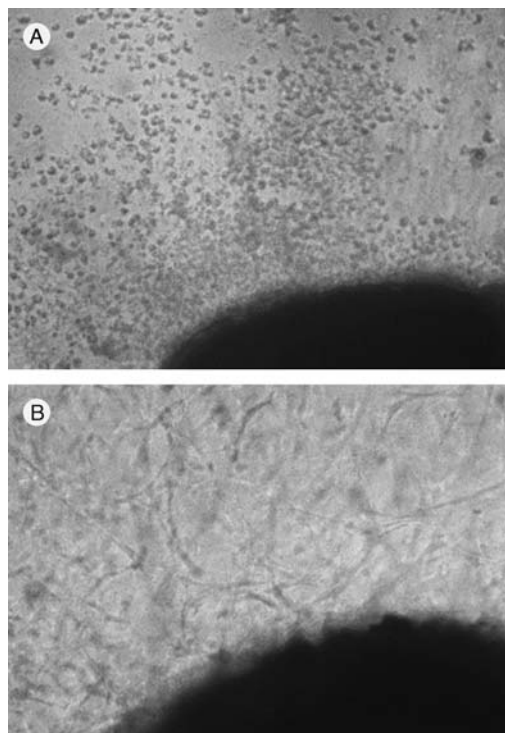


Figure 5. This pair of figures illustrates the induction of apoptosis as a result of 10% noni treatment for 7 days on the angiogenic explant in (A) while the explant in (B) continued to grow into a fully developed vascular network in a 10% NaCl-HPVAM environment. (40×).

cell proliferation; endothelial cell migration; capillary tube formation; and anastomosis into a vascular network could inhibit angiogenic initiation. Our finding that a concentration of 2.5% noni in the media in Figure 1, was ineffective in blocking initiation of angiogenesis (Figure 1A), but appeared to have a significant effect on the growth of vessels (Figure 1B) suggests that more than one process may be affected. In our early fractionation studies to ascertain the active anti-angiogenic ingredient(s) in noni, we found that the active factor did not reside in either the protein or lipid fractions, but when sodium meta-periodate was used to break up carbohydrate moieties, it also abrogated the effect of noni on angiogenesis (data not shown).

As noted earlier, endothelial cells can exist in both quiescent and activated states. Other investigators have shown that the state of confluence of endothelial cells can determine their sensitivity to the induction of

Table 1. Following 7 days of development in HPVAM, the growth media was removed and 10 explants were given a mixture of HPVAM and 10% NaCl, while 10 other explants were given 10% noni in HPVAM.

	Number explants	Number cells scored	% Cells apoptotic	% Nuclei intact
Noni	10	981	86%	14%
Control	10	643	6%	94%

After 7 days of this treatment the explants were fixed, sectioned, tested for apoptosis using the TUNEL assay and counterstained for viability using hematoxylin. Three random fields (40×) on each of the slides were scored for apoptosis (peroxidase staining) and intact nuclei (hematoxylin staining).

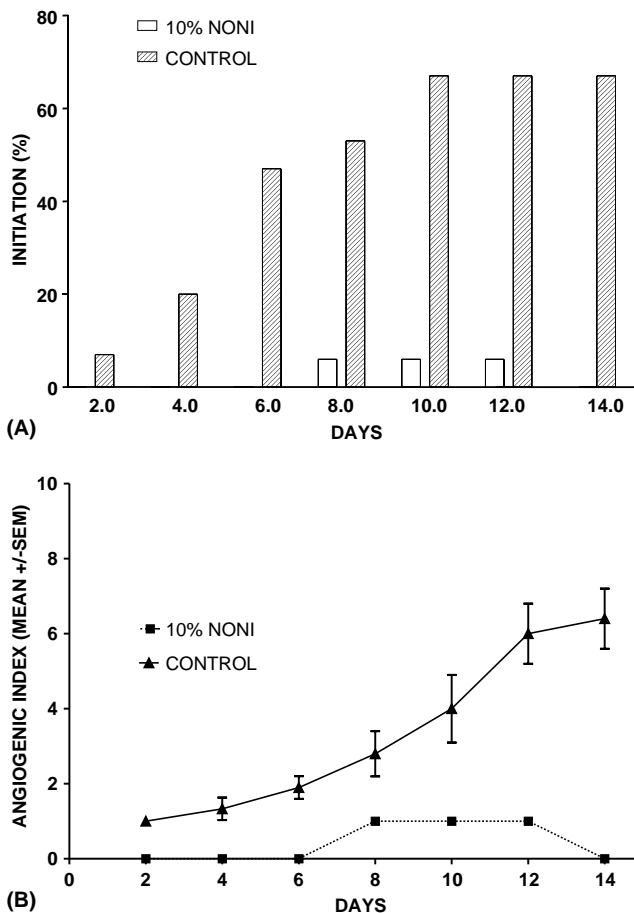


Figure 6. (A) From a human breast tumor, 34 1 mm explants were placed into 96-well culture plates and divided into a control group ($n = 17$) which received HPVAM+10% NaCl, and a test group ($n = 17$) which received HPVAM+10% Noni. Both sets of media were pH adjusted to 7.4 and sterile filtered. Differences between treated and control groups were significant at $P = 0.006$, using the Wilcoxon signed rank test. (B) From a human breast tumor; 34 1 mm explants were placed into 96-well culture plates and divided into a control group ($n = 17$) which received HPVAM+10% NaCl, and a test group ($n = 17$) which received HPVAM+10% noni. Both sets of media were pH adjusted to 7.4 and sterile filtered. Differences between noni treated and control were significant, $P = 0.02$.

apoptosis. One example of this is the induction of apoptosis by proteasome inhibition. Subconfluent proliferating primary endothelial cells required 340-fold lower concentrations of the proteasome inhibitor PSI to induce apoptosis than did confluent contact-inhibited cells [17]. If the mechanism of apoptotic induction resulting from noni treatment is similarly related to the state of confluency of the capillary endothelium, apoptosis of the established vasculature of solid tumors may be resistant to noni treatment. This would make it effective in blocking the growth of new sites of angiogenesis while leaving mature blood vessels intact. However, the susceptibility of recently formed capillaries seen in Figures 3 and 4 suggests that in recently developed vascular networks such as tumor vasculature, with a continuing high exposure to vascular growth factors, endothelial cells may exist largely

in a pro-angiogenic environment, thereby increasing the efficacy of apoptotic induction by agents such as noni.

One possible mechanism of action of noni, with respect to its anti-angiogenic properties is through interaction with cell surface structures such as the α_v integrins. Other investigators have shown that angiogenesis may be induced by numerous cytokines, which can interact with at least two distinct integrin mediated angiogenic pathways [18]. In this regards, noni may act as a competitive inhibitor with these growth factors if its active component is a carbohydrate as our preliminary data suggests. Recent findings have shown that numerous cytokines have carbohydrate recognition domains and their biological activity relies on carbohydrate binding [19]. It has also been demonstrated that integrin antagonists can promote tumor regression by inducing apoptosis of angiogenic blood vessels [20]. Whether this is indeed the functional mechanism by which noni acts is currently under active investigation.

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