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Original Article

Arginine-conjugated albumin microspheres inhibits proliferation and migration in lung cancer cells

Hung-Yen Lee[1,3], Kamal A Mohammed[1,2], Eugene P Goldberg[3], Najmunnisa Nasreen[1,2]

[1]Division of Pulmonary, Critical Care & Sleep Medicine, Department of Medicine, [2]NF/SGVHS, Malcom Randall VA Medical Center, University of Florida, P. O. Box 100225, [3]Biomaterials Center, Department of Materials Sciences and Engineering, University of Florida, P. O. Box 116400, Gainesville, FL 32611-6400, USA

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Abstract: Arginine is one of the essential amino acid involved in numerous biosynthetic pathways that significantly influence tumor growth. It has been demonstrated that arginine is effective to inhibit proliferation of cancer cells when an appropriate dose is applied. Generally, induction of cell death requires high concentration of arginine while low concentration of arginine facilitates cell proliferation. In addition to the apoptosis induced by metabolism of arginine, it has also been reported that in an ideal solution environment, arginine may assemble into arginine clusters to kill cancer cells. Therefore, to make the arginine an effective anticancer agent, arginine/albumin microspheres were designed and synthesized to provide a localized high concentration of arginine on tumor sites. In addition, the arginine/albumin mesospheres (AAMS) are also expected to provide an arginine-rich surface on microspheres, which is similar to the arginine cluster, to effectively inhibit tumor growth. In this study, the AAMS were synthesized through a water/organic solvent emulsion system and the surface properties were characterized. The *in vitro* effects of AAMS on A549, CRL-2081, MAK9 lung cancer cells (LCC) proliferation, migration, and tumor growth were determined. The expression of oncogenic protein EphA2 and transcription factor slug was also determined. AAMS significantly inhibited the cell proliferation, cell migration and tumor growth in all the three LCC, while same concentration of free arginine promoted the LCC tumor growth and migration. Our studies indicate that the synthesized AAMS has a more effective inhibiting effect on proliferation, migration and tumor growth of LCC than freely released arginine. The expression of EphA2 receptor mRNA was significantly decreased when compared to control cells. In addition the mRNA expression of transcription factor slug was also inhibited by AAMS suggesting that AAMS affects the expression of EphA2 and slug and may regulate LCC proliferation and migration. These data suggests that the AAMS can be an ideal delivery vehicle for therapeutic interventions against LCCs.

Keywords: Arginine-conjugated albumin, microspheres, inhibition, proliferation, migration, lung cancer

Introduction

Arginine and its metabolic products are involved in biosynthetic pathways that influence carcinogenesis and tumor generation [3, 20]. Arginine was shown to inhibit cell proliferation in various cancer cells including lung, breast, and gastric cancer cell lines [9, 27, 36]. The mechanisms of cell proliferation inhibition by arginine are still not clear and the reactions induced by arginine in tumor biology are complex. The nitric oxide (NO) generated through arginine metabolism has been thought as an important molecule to influence the cancer cell proliferation and tumor growth. NO stimulates or inhibits cell proliferation, through apoptosis, depending upon NO level and cell types [23, 27, 31, 36, 37]. Besides, high concentration of arginase, that converts arginine to ornithine and urea, has been found in the microenvironment of various malignant tumor tissues and it enables the tumor cells to escape the immune surveillance [32]. However, it has been reported recently that the concentration and delivery environment is crucial to make arginine, especially L-arginine, an efficient anticancer molecule [33]. Depending upon the environment in solution, arginine may assemble into molecular clusters displaying a hydrophobic surface by the alignment of its methylene groups [11, 35]. The hydrophobic surface of arginine clusters may interact with malignant cells disrupting

their membrane integrity and leads to necrosis. Unlike the metabolism of arginine, this non-metabolic process avoids the development of tumor resistance and can be more efficient to kill different types of malignant cells if high concentration of arginine is present at the local milieu. Therefore, to make the arginine an effective anticancer agent, the delivery method is extremely important to provide an ideal local environment of arginine.

Lung cancer is one of the leading causes of cancer-related mortality and the five-year survival rate of lung cancer patients is only 16% [1]. In previous studies, the plasma arginine concentration has been demonstrated to be decreased in patients with lung cancer; however, the cause of the depletion has not been determined yet [26]. It has also been reported in vitro that arginine inhibits or stimulates the proliferation of lung cancer cells [30, 33]. Whether inhibition or stimulation of lung cancer cell proliferation is dependent on the delivery media of arginine is not clear [33].

To improve the treatment of lung cancer, endobronchial intratumoral chemotherapy (EITC), a direct intratumoral injection using a bronchoscopic needle-catheter, has been explored clinically in recent years [4-7, 14]. This is achieved without the complications caused by systemic drug toxicity normally associated with conventional chemotherapy. In recent studies, in order to improve the effectiveness of intratumoral chemotherapy, various nano-meso-microsphere (MS) compositions loaded with drugs or biomolecules have been designed to prolong high intratumoral drug concentrations and to further minimize any risk of systemic toxicity [8, 18]. Microspheres made by different synthetic and biopolymers have been widely studied for their application of drug delivery in cancer treatment [16, 29]. Among the various biodegradable particulate carriers available for consideration, considering the similar nature of amino acid and peptide, we regard the most abundant natural plasma protein, serum albumin, as a most appealing biocompatible carrier for the effective localized delivery of arginine [21, 22]. Furthermore, the abundant functional groups on surface of human serum albumin (HAS) or bovine serum albumin (BSA) facilitate physiological absorption and covalent coupling to arginine and other biomolecules [15, 21]. In previous studies, we have demonstrated the low cytotoxicity of AMS and its effectiveness of drug-loaded AMS for the treatment of a Lewis lung carcinoma and a mammary adenocarcinoma [2]. In view of this, drug- or biomolecule-loaded albumin microspheres (AMS) are now being considered for bronchoscopic intratumoral treatment of lung cancer to provide localized, continuous and prolonged high drug concentration at target tumor sites. The goal is to improve the effectiveness of therapy, to minimize the undesired diffusion through systemic circulation, and to reduce the tumor burden. Here, to locally inhibit the cancer tumors, the arginine molecules incorporated into microspheres may be active by forming a hydrophobic surface and free arginine can be released from the microspheres by diffusion and by degradation of BSA matrix.

Here we report the L-arginine/BSA incorporated microspheres (AAMS) synthesized through a water/organic solvent emulsion system. This particle size in the range of 5 to 10 μm was selected based on our previous research indicating mesospheres were especially effective for IT injection and tumor perfusion. The anticancer effects of AAMS on A549 (a human lung carcinoma cell line), CRL-2081 (a malignant pleural mesothelioma cell line), and MAK9 (mouse lung carcinoma cell line), were investigated and compared with free released L-arginine. The effect of synthesized AAMS was investigated on cell proliferation, migration, and tumor formation. In addition the expression of oncogenic protein EphA2 and Slug have been determined.

Materials and methods

Cell lines and reagents

A549 cells, human lung adenocarcinoma epithelial cell line, CRL-2081, malignant mesothelioma cell lines were obtained from American Type Cell Collection (Manassas, VA). MAK9, mouse lung carcinoma cell lines was generated from primary lung tumor harvested from mice. The cells were cultured in RPMI-1640 medium (Sigma, St Louis, MO) containing 10% fetal bovine serum (FBS), penicillin (100 U/ml), streptomycin (100 μg/ml), fungizone (100 μg/ml), 0.25% D-glucose, 0.2% sodium bicarbonate and 1% sodium pyruvate as reported earlier [19, 25]. The L-arginine were purchased from Sigma, St Louis.

Synthesis of L-arginine/BSA mircospheres (AAMS)

A 5% w/v solution of cellulose acetate butyrate (CAB) (butyryl content 16.5-19.0%, Sigma, St Louis, MO) in 1,2-dichloroethane (DCE) (certified ACS grade, Fisher scientific) was used as the continuous organic phase. 16.0 ml of the CAB/DCE solution was added to a 50 ml polystyrene centrifuge tube. 1 ml mixture solution containing 50 μg of bovine serum albumin (BSA) (A2153, Sigma) and 50 μg of L-arginine were added into the continuous phase. The mixture solution in the tube was vortexed to create a dispersion so solution at 3000 rpm for 2 min. 32% w/w EM grade glutaraldehyde to BSA (Electron Microscopy Science, Hatfield, PA), was added through DCE into the emulsion solution to solidify the microspheres. The tube was placed on rotator for 8 hours to complete crosslinking. The centrifuge tube was then filled with about 50 ml of acetone (ACS certified, Fisher) and then centrifuged at 2000 rpm for 10 min to yield a clear supernatant fluid over a microspheres pellet. The acetone wash was repeated 3 times by dispersing MS in 50 ml of acetone and centrifuging each time. The appearance of MS was investigated by using optical microscope (Olympus, Center Valley, PA). The AMS control samples were prepared by the same procedure.

Particle size distribution, surface morphology and zeta potential of AAMS

The particle size distribution of AAMS in PBS was determined by Coulter LS 13320 Laser diffraction particle size analyzer (Beckman Coulter, Inc., Brea, CA). PBS was used as suspension fluid and AAMS were dispersed in PBS and sonicated before measurement. The laser obscuration range was between 8 to 12% during measurement. The AAMS were coated with a thin layer of gold/platinum and mounted on a metal stub. The surface morphology of AMS was investigated by scanning electron microscopy (SEM) with a JEOL JSM 6400 (Tokyo, Japan). The zeta potential of AAMS and AMS dispersed in molecular grade water were measured by using PALS Zeta Potential Analyzer (Brookhaven Instruments Corp., Holtsville, NY).

Quantitative RT-PCR

Total RNA was reverse transcribed into cDNA and PCR was performed using SYBR Green JumpStart Taq ReadyMix (Sigma, St. Louis) as reported earlier [24]. The primers used were sense 5'-TTCAGCCACCACAACATCAT-3, anti-sense 5'-TCAGACACCTTGCAGACCAG-3' (EphA2); sense 5'-AGGCACTGGCTTGGCTGCAC-3', anti-sense 5'-GCGTGGAATGGAGCAGCGGT-3' (slug); sense 5'-AGAGCTACGAGCTGCCTGAC-3', anti-sense 5'-AAAGCCATGCCAATCTCATC-3' (β-actin). Total cellular RNA was isolated from LCC transfected with EphA2 siRNA or control siRNA or untransfected LCC using RNeasy kit (QIAGEN, Maryland) according to manufacturer's recommendations as reported earlier [28]. 100 ng/μL, of RNA was reverse transcribed into cDNA. After reverse transcription, 80 μL RNase-free water were added to each sample. 10 μL of diluted cDNA product was mixed with 25 μL of SYBR Green JumpStart Taq ReadyMix, 0.5 μL of Internal Reference Dye, and 14.5 μL of specific oligonucleotide primers (80 nM final concentration) to total 50 μL volume for quantification real-time PCR. The quantification of real-time PCR were performed by SYBR Green method using the Applied Biosystems 7500 Real Time PCR System with the following profile: 1 cycle at 94°C for 2 min, 40 cycles at 94°C 15 sec, 60°C 1 min, 72°C 1 min, and the acquire the fluorescent signal from the elongation step. The real time PCR products were confirmed by electrophoresis on a 2% agarose gel. Data analysis was carried out by the ABI sequence detection software using the relative quantification. The threshold cycle (Ct), which is defined as the cycle at which PCR amplification reaches a significant value, is given as the mean value. The relative expression of each mRNA was calculated by the ΔCt method. The amount of the target genes relative to the β-actin mRNA was expressed as $2^{-(\Delta Ct)}$.

Proliferation rate of A549, CRL-2081 and MAK9 treated with AAMS

The effect of AAMS on cell proliferation was determined by using the WST-1 reagent (Roche, Indianapolis, IN) as reported earlier [17, 19]. 2,000 to 4000 cells were seeded in each well of the 96-well plate and incubated in media containing various concentrations of AAMS, free L-arginine and AMS. The media used in proliferation assay was phenol red-free RPMI-1640 containing 10% FBS. The proliferation rates were measured after 24, and 48 hours. Every experiment was done in triplicates.

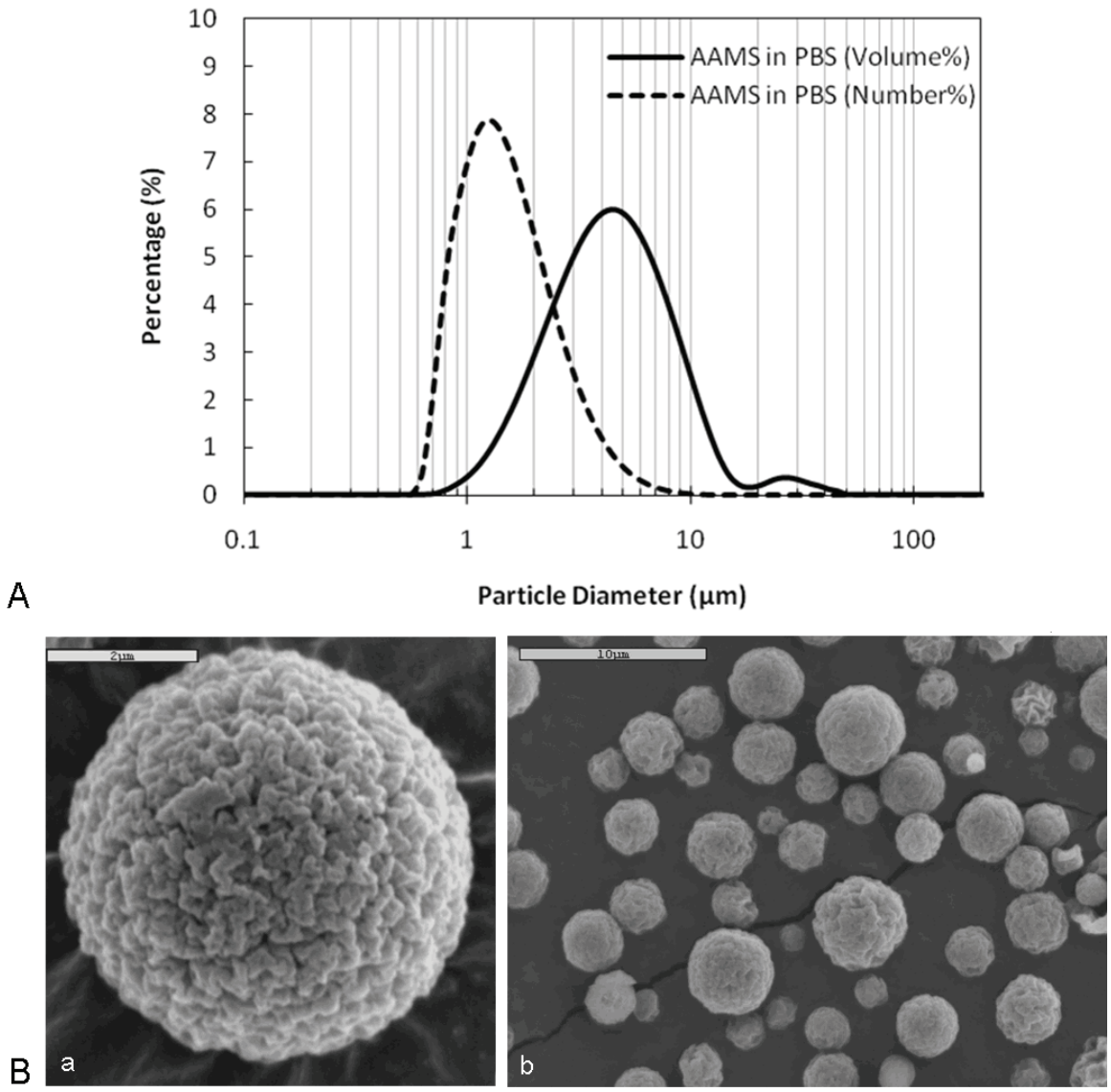

Figure 1. Panel A. Particle size distribution of arginine/albumin microspheres (AAMS) in PBS. Solid line represents the size distribution in volume percentage and dash line represents number percentage. The particle diameter (x-axis) is shown using a logarithmic scale based on 10. Panel B. SEM images of arginine/albumin microspheres (AAMS). a. Surface morphology of air-dried AAMS after acetone wash. b. Dry AAMS at lower magnitude.

Cell migration assay

About $1x10^5$ cells were seeded into each well of 6-well plate. After 48 hours of incubation, cells reached 100% confluency and then were incubated with serum free media containing free arginine, AMS and AAMS. After 24 hours of incubation, the cells were scratched by 200 µl pipette tips to create a wound on cell monolayer and then washed gently by PBS to remove cell debris. The wounded cells were then incubated with fresh media containing 5% FBS. The wound areas were photographed every 24 hours and every experiment was done in triplicates as reported earlier [19].

Three-dimensional tumor growth assay

Matrigel (BD, Franklin Lakes, NJ) was diluted with serum free media in the ratio 1:1. 200 µl of diluted Matrigel solution containing AAMS and free arginine were added into each well of 48-well plate and then allowed to gel at 37°C for at least 30 min. After gellization, A549, CRL-2081 and MAK9 cells at a density of 2,000 cells per well were plated in 200 µl of serum

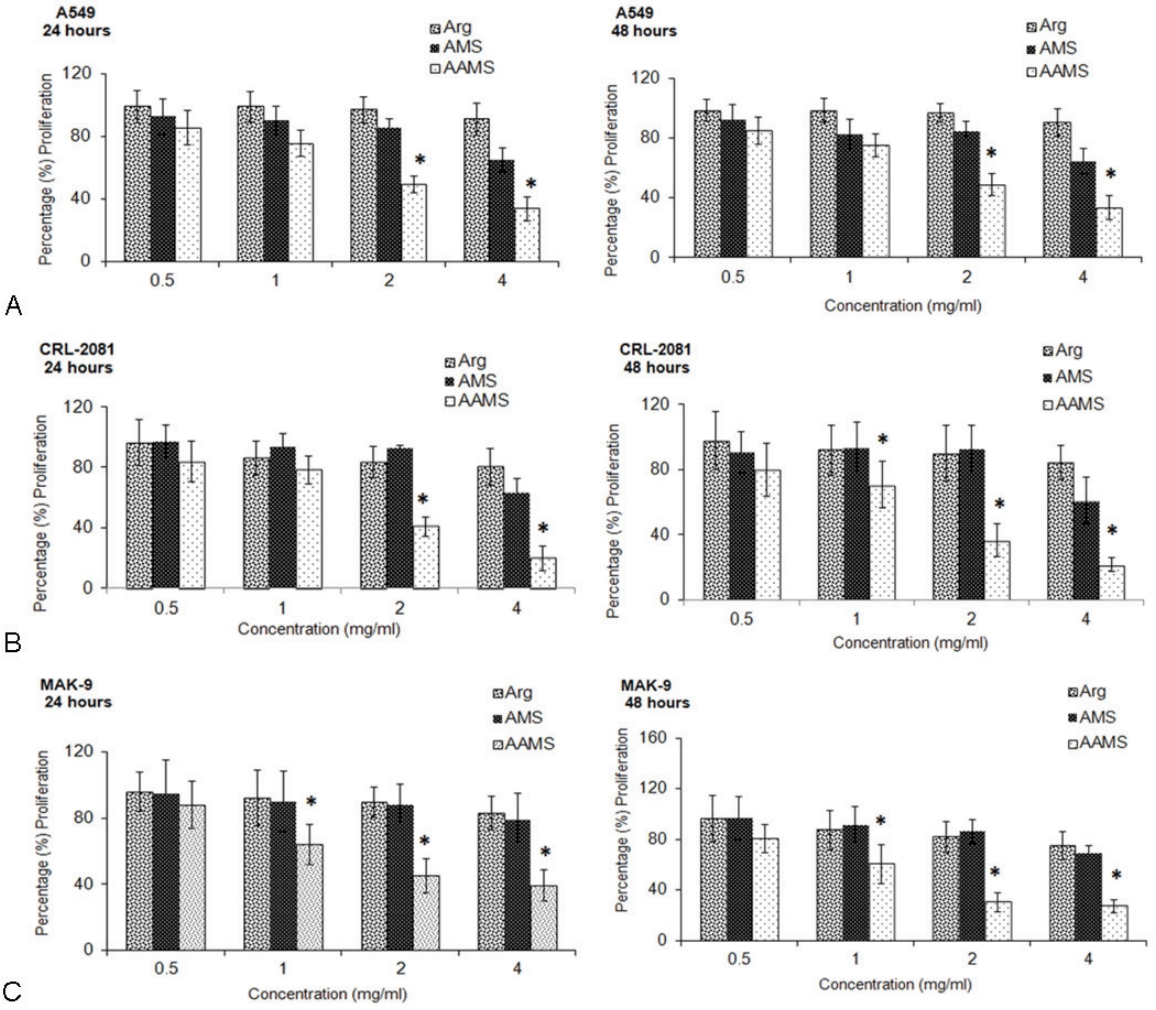

Figure 2. AAMS inhibits proliferation rate of Lung cancer cells: A549 (A), CRL-2081 (B), and MAK9 (C) treated with arginine (Arg), AMS and AAMS for 48 hours. AAMS effectively inhibited the cell proliferation in all the cell lines. Below the concentration of 4 mg/ml, arginine showed no significant inhibiting effect on cell proliferation within 24 hours. Compared to arginine, AAMS provided a fast and long effectiveness on cell proliferation inhibition after 48 hours of incubation. The inhibiting effects of the same concentrations of free arginine and AAMS are compared in these charts. However, the AAMS contains no more than 50% (w/w) of arginine in the microsphere matrix, which implies that the arginine from AAMS is more effective in inhibiting cell proliferation than two times more arginine freely released in environment. (The value of proliferation percentage = the cell number after treatment with reagents / the cell number without treatment. $*P < 0.05$ is considered significant when compared with LCC without treatment).

free media. Media were changed every 3 days with 2% serum media or serum free media as reported earlier [19]. Randomly chosen fields in each well were photographed every two days until the tumor colonies were formed.

Statistical analysis

The statistical analyses were performed by using SigmaStat 3.5 (SYSTAT Software, Inc. San Jose, CA). Results are expressed as mean ± SEM. Comparisons were made using one way ANOVA followed by Student-Newman-Keuls test for multiple comparisons. Differences were considered significant when P values were < 0.05.

Results

AAMS with narrowed size distribution in meso-scale and increased zeta-potential

We determined the volume percentage, number percentage, and particle size distribution of AAMS (**Figure 1A**). In the volume percentage size distribution, AAMS in PBS showed a mean

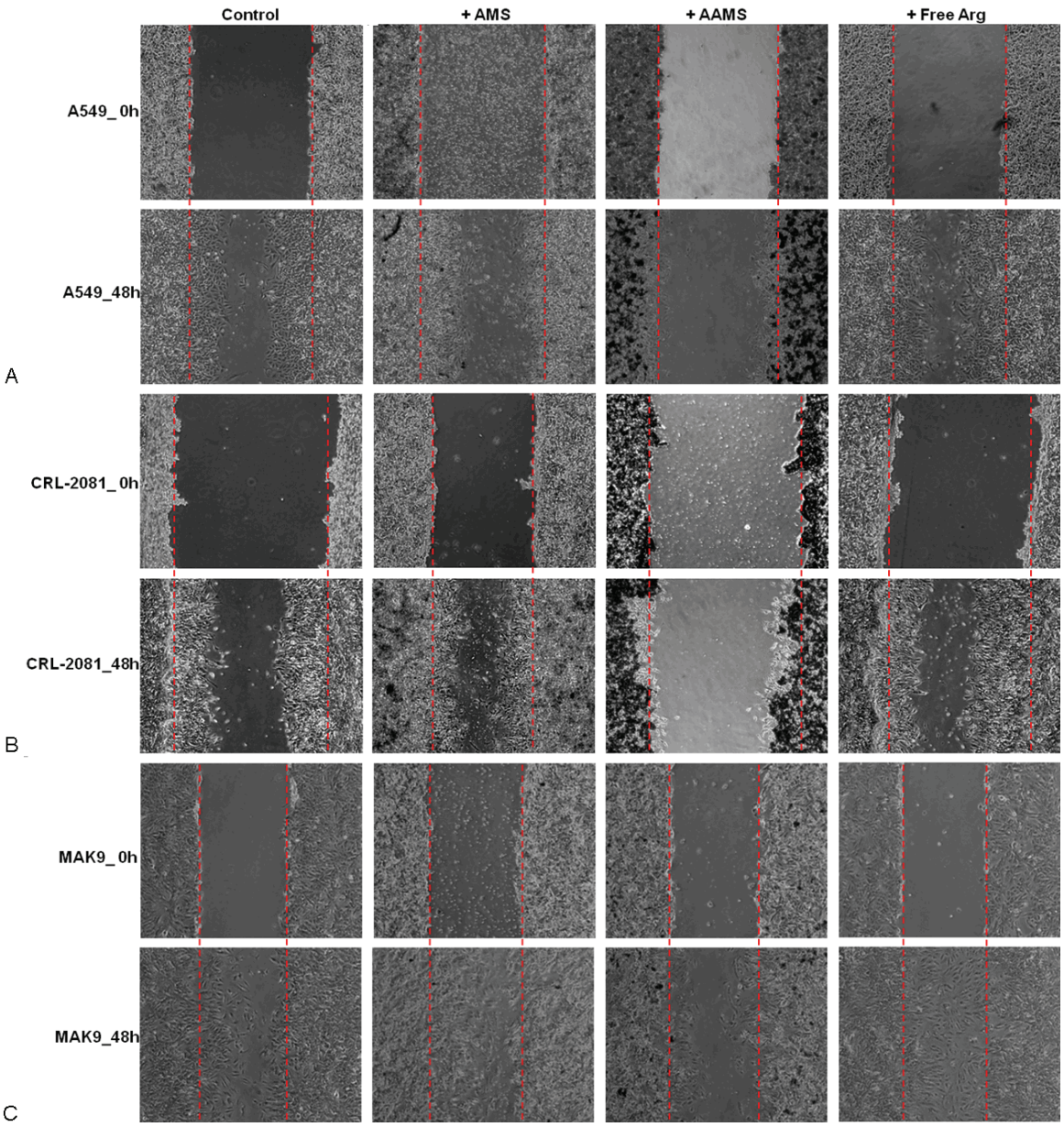

Figure 3. AAMS inhibits Cell migration of Lung cancer cells. Panel A. A549 cells, Panel B - CRL-2081, and Panel C - MAK9 cells, after 48 hours of incubation in wound healing assay. The inhibiting effect of AAMS and prompting effect of free arginine on cell migration were observed in all the cell lines. The cells were incubated with 4 mg AMS, AAMS, and free arginine in 2 ml culture media containing 5% serum. (0 h = pictures taken right after the wounds were created; 48 h = pictures taken after 48 hours of incubation).

particle size of about 5 μm with a standard deviation of 6.6. The mode of the distribution lied around 4 and about 95% of AAMS were synthesized in the size range between 1 to 10 μm. The SEM images of AAMS were shown in **Figure 1B**. The AAMS were acetone washed and then air-dried. AAMS showed a rough surface under SEM. From the SEM images, it was observed that most of the AAMS appeared as spheres during the preparation. The particle size of AAMS was confirmed to be distributed within the range of 1 to 10 μm under SEM. The zeta potential of AAMS and AMS were measured while dispersed in water. AMS had a negatively charged surface and the mean zeta potential was -37.58 mV. For the AAMS which had been incorporated with arginine, the particle surfaces were less negatively charged with

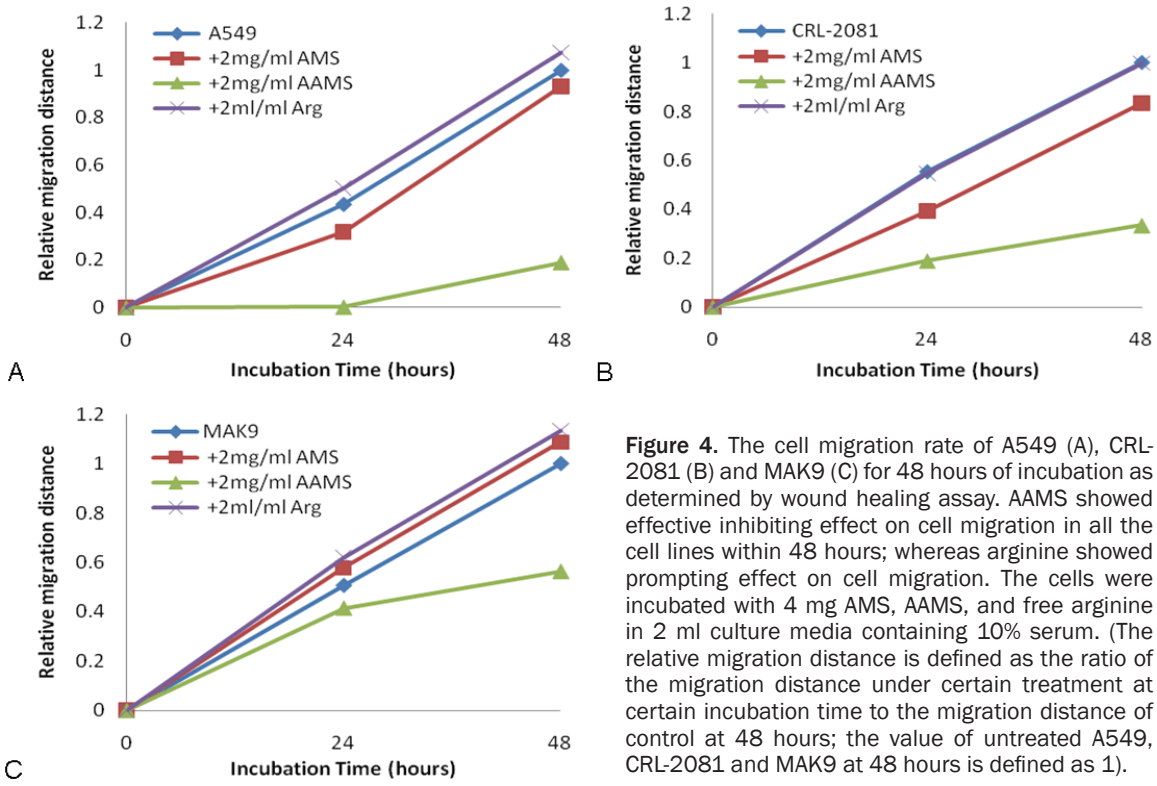

Figure 4. The cell migration rate of A549 (A), CRL-2081 (B) and MAK9 (C) for 48 hours of incubation as determined by wound healing assay. AAMS showed effective inhibiting effect on cell migration in all the cell lines within 48 hours; whereas arginine showed prompting effect on cell migration. The cells were incubated with 4 mg AMS, AAMS, and free arginine in 2 ml culture media containing 10% serum. (The relative migration distance is defined as the ratio of the migration distance under certain treatment at certain incubation time to the migration distance of control at 48 hours; the value of untreated A549, CRL-2081 and MAK9 at 48 hours is defined as 1).

a higher mean zeta potential value of -27.51 mV was noted. The standard errors of the mean zeta potential values were around 1.5.

AAMS inhibited cell proliferation of lung cancer cells

The cell proliferation of A549, CRL-2081 and MAK9 cell were determined by WST-1 assay. Free arginine (Arg), did not induce significant cell death in all the cells at a concentration of 4 mg/ml (about 23 mM) or lower for 48 hours. AAMS reduced cell proliferation with increasing concentration in media. **Figure 2**, demonstrated that AAMS showed the most effective inhibiting effect on the cell proliferation in all the cell lines. While treated with 2 mg/ml of AAMS, 50% cell proliferation in all cell lines had been inhibited at 24 and 48 hours. It can also be found that AAMS was more effective on inhibiting the cell proliferation of CRL-2081 which exhibit fibroblast-like morphology. Generally, when the concentration of arginine was lower than 4 mg/ml in media, arginine had no significant inhibiting effect on cell proliferation. However, when the concentration reached 4 mg/ml or higher, arginine dramatically reduce the living cell number to a low level (below 50%). At a concentration of 2 mg/ml or higher, the inhibiting effect of AAMS on the cell proliferation was two times more effective than free arginine at 24, and 48 hours. These data suggests that AAMS were more effective in suppressing the cell proliferation when compared to free arginine.

AAMS inhibited cell migration of lung cancer cells

In the wound healing assay, a cell-free wound area was created in the cell monolayer by scraping with a pipette tip as shown in **Figure 3**. The results of cell migration rate showed that at the concentration of 2 mg/ml, AAMS effectively inhibited the migration of A549, CRL-2081 and MAK9 cells invading into the cell-free area within 48 hours. However, the same concentration of free arginine had no inhibiting effect on cell migration of all the LCC; it even prompts cell migration and cause faster wound healing rate. The relative migration distance of LCC when treated with AAMS and Free Arginine over time was compared to control as demonstrated in **Figure 4**. These results suggest that AAMS had a significant effect on inhibiting the migration

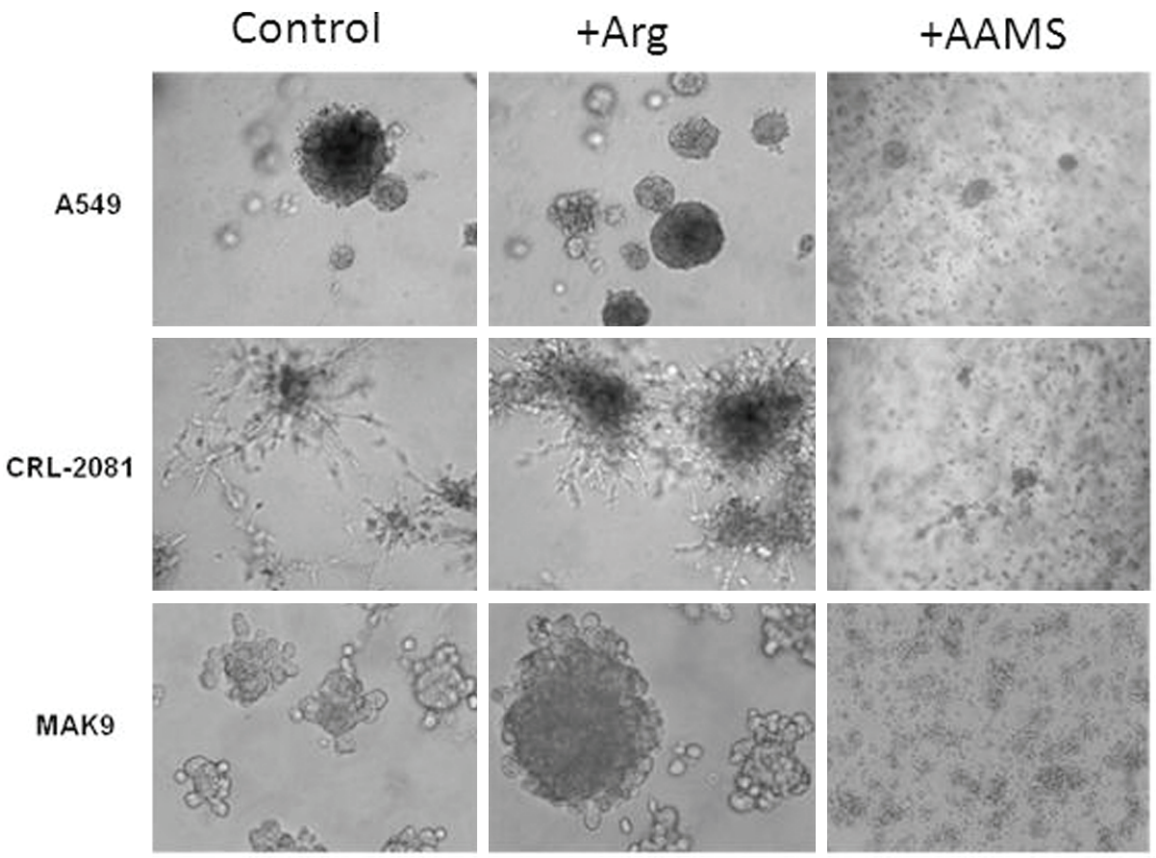

Figure 5. AAMS inhibits tumor colonies growth in Matrigel. Lung cancer cells were treated with AMS, AAMS and Arg and seeded on Matrigel. AAMS inhibited the formation of tumor of A549, CRL-2081 and MAK9 cells in the gel network. However, the free arginine added in Matrigel stimulated the growth in size and number of tumor colonies. The cells were incubated in Matrigel for 7-10 days, and the concentration of AAMS or arginine used in Matrigel was 2 mg/ml.

of A549, CRL-2081 and MAK9 cells when compared to free arginine.

AAMS inhibited tumor growth in matrigel

The cells were seeded into Matrigel containing 2 mg/ml free arginine, AMS and AAMS. The pictures of A549, CRL-2081 and MAK9 were taken after 10 days of incubation. The results of tumor growth in **Figure 5** showed that the free arginine prompted the tumor growth of all these cell lines. Free arginine showed a significant effect on the number, size and morphology of tumor colonies. In addition, AAMS reduced the number and size of tumor colonies in Matrigel. From the morphology of CRL-2081 cells treated with AAMS, it can be found that AAMS inhibited the tumor spreading from colonies and constrain the tumors into small round colonies. Taken together these data suggests that AAMS remarkably inhibited the tumor colonies formation in matrigel when compared to free arginine and control.

AAMS inhibits the mRNA expression of receptor EphA2 and slug in lung cancer cells

LCC were treated with AMS, AAMS and free arginine for the time period of 24 hours for the analysis of EphA2 and for 3 hours for the analysis of slug. The total RNA was isolated and subjected to quantitative PCR. The mRNA expression of EphA2 was inhibited in cells treated with AAMS when compared to control. Free arginine showed a significant increase in EphA2 expression in all the LCC when compared to AAMS **Figure 6A**. These data together suggests that the AAMS effectively inhibits the oncogenic protein EphA2 receptor expression and mediates anti-oncogenic effect on lung cancer cells.

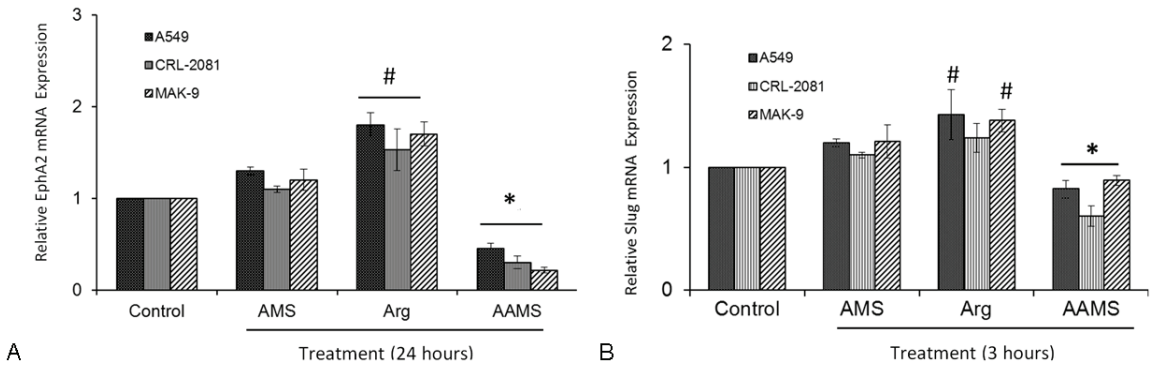

Figure 6. AAMS inhibits the mRNA expression of receptor EphA2. Lung cancer cells were treated with AMS, AAMS and Arg, total RNA was isolated. The relative expression of receptor EphA2 Panel A and Slug (Panel B) was determined by quantitive PCR. The data presented is the Mean ± SEM of three separate experiments. $^{*}P < 0.05$ is considered significant when compared with control; $^{\#}P < 0.05$ when compared with AAMS.

AAMS inhibits the mRNA expression of slug in lung cancer cells

Lung cancer cells were treated with various concentrations of AAMS and free arginine for 3hours and the total RNA was isolated and subjected to quantitative PCR. The expression of slug was inhibited in cells treated with AAMS when compared to control. Free arginine showed a moderate effect when compared to control **Figure 6B**.

Discussion

Arginine has been reported to inhibit or facilitate the proliferation of cancer cells based on the metabolism of arginine [9, 27, 30]. The mechanism of the cell death through the metabolic process of arginine has been shown to induce apoptosis [27, 36]. In a previous study, arginine has been shown to inhibit the proliferation of gastric cancer cells and induced apoptosis [27]. In addition, the induction of apoptosis was depends on the level of NO generated through the metabolism of arginine in various kinds of tumor cells. Generally, the induction of apoptosis requires high concentration of NO and low NO concentration can lead to resistance to the NO-induced apoptosis [37]. However, a recent study by *Shukla, et. al.,* pointed out another possible mechanism of arginine inhibiting tumor cell proliferation [33]. It has been indicated that the mechanism of cell death by arginine is via membrane damage leading to necrosis. In the *in vitro* study on various malignant cell types, damage to the cell membrane, deficiency of NO in supernatant, and the absence of apoptotic gene expression had been observed with arginine induced cell death. This indicates that necrosis, instead of the metabolically driven cell death apoptosis, is the most likely mechanism by which arginine inhibits the proliferation of cancer cells.

Disruption of cell membrane is the most likely mechanism that arginine effectively kills cancer cells. Arginine molecules in a concentrated arginine solution have a high tendency to aggregate and assemble into a molecular cluster due to the positively charged amphiphilic molecular structure [11, 35]. With the highly positively charged and hydrophobic surface, the arginine cluster can disrupt the negatively charged cell membrane and become an effective antitumor reagent. However, the local concentration and environment is a crucial factor to influence the cytotoxicity of arginine to cancer cells. The clinical use of arginine in cancer treatment is still questionable because the inhibiting effect of arginine on cancer cell proliferation is paradoxical. High concentration of arginine inhibits cancer cell proliferation, while on the contrary, inefficient dosage stimulates the proliferation. In previous study, it is observed that arginine tends to aggregate and induce tumor death at least at a concentration of 10 mM [35]. To achieve this, the delivery method of arginine to tumor sites is crucial to create a local environment with high concentration of arginine and make arginine an effective antitumor agent.

In this report, we synthesized the microspheres with 50% L-arginine and 50% BSA (w/w). The arginine was incorporated with BSA in the ma-

trix of microspheres and on the surface as well. Compared to the AMS, the surface of AAMS is more positively charged and stronger interaction between AAMS and cancer cells was observed during the experiments. The synthesized AAMS were expected to act as an antitumor agent by providing an arginine-rich surface on microspheres, similar to the arginine cluster surface, and also locally releasing arginine to the tumor sites. In this study, the AAMS showed a more effective inhibition on cell proliferation than freely released arginine. AAMS inhibited the proliferation of lung cancer cells including A-549, CRL-2081 and MAK9 with a relatively low concentration; moreover, the efficacy has been prolonged since the arginine incorporated within AAMS are less likely to be involved into metabolism. This indicates that the arginine incorporated on microspheres can be more effective to inhibit the proliferation of cancer cells than the free arginine in the environment.

The inhibition of tumor growth by AAMS was demonstrated by using the 3-dimensional tumor growth assay in Matrigel. The results showed that the AAMS effectively reduced the size and number of the tumor colonies in an incubation of 7 to 10 days, while the lung cancer cells treated with free arginine showed a prompted tumor growth. In CRL-2081, it is noteworthy that the AAMS in the environment not only reduced the number and size in tumor colonies but also had a significant influence on tumor morphology. In untreated cells and the cells treated with free arginine and AMS, the cells spread out from the tumor colonies and connected to other cells. However, it was observed that the cell spreading was inhibited in the cells treated with AAMS. It has been demonstrated that arginine inhibited proliferation of gastric cancer cells with increased apoptosis but no effect on tumor cell invasion [27]. However, our study showed that the AAMS we synthesized has a promising inhibiting effect on the cell migration of lung cancer cells, whereas the free arginine prompted the cell migration.

Receptor tyrosine kinase EphA2 promotes tumor growth. Earlier studies from our laboratory have demonstrated that lung cancer cells express receptor EphA2 [28, 34]. To understand the mechanisms involved on how arginine affects the lung cancer cells and inhibits proliferation we probed for the expression of oncogeneic protein EphA2 expression. The expression of EphA2 was significantly decreased when the LLCs were treated with AAMS when compared to free arginine. In addition we also looked if transcription factor slug is involved in arginine induced cell proliferation. The expression of slug has been decreased suggesting AAMS induced inhibition of cell proliferation is via slug. Slug is a zing-finger transcription factor of slug/snail superfamily. Elevated expression of slug promotes malignant cells to become aggressive by increased invasiveness and motility. In epithelial tumors such as breast cancer, lung cancer, pancreatic cancer, ovarian cancer and hepatocellular carcinoma slug promotes lympnode metastasis [10, 12, 13].

In conclusion, the arginine/albumin microspheres we synthesized here showed significant effectiveness on inhibiting proliferation and migration of the lung cancer cells including A549, CRL-2081 and MAK9 cells. AAMS acted as a more effective antitumor agent on the lung cancer cells than arginine. The AAMS showed constant inhibiting effects on the proliferation and migration of lung cancer cells; whereas the freely released arginine showed stimulation on cell proliferation and migration at lower concentrations. This implies that creating a microenvironment with high concentration of arginine is a more effective approach to kill cancer cells. In addition, AAMS has a high potential to be applied into an intratumoral therapy for different types of cancers due to its antitumor ability, strong interaction with cells, and most importantly, the high safety. Although the mechanism of AAMS inhibiting lung cancer cell proliferation are not clear and needs to be further investigated. The present data provides a promising foundation for future *in vivo* studies and offers a different point of view to explain how arginine inhibits lung cancer cells growth and migration.

List of abbreviations

AMS, Albumin mesospheres; Arg, Arginine; AAMS, Arginine Albumin Mesospheres; SEM, Scanning Electron Microscopy; RTKs, Receptor tyrosine kinase; RT-PCR, Reverse transcriptase polymerase chain reaction.

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Disclosure of conflict of interest

The authors declare that they have no competing interests.

Authors' contributions: NN and KAM designed the experiments and HL carried out all the experiments. HL compiled the results and drafted the manuscript along with NN. EPG and KAM participated in analysis and interpretation of the data and provided critical revision of the manuscript for important intellectual content. All authors read and approved the final version.

Address correspondence to: Dr. Najmunnisa Nasreen, Division of Pulmonary, Critical Care & Sleep Medicine, Department of Medicine, University of Florida, Gainesville, FL 32601, USA. Phone: 352-376-1611 ext. 6491; Fax: 352-392-7088; E-mail: nnasreen@medicine.ufl.edu

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