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Effect of Aminaphtone on *in vitro* vascular permeability and capillary–like maintenance

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Abstract

Aminaphtone (AMNA), a naphtohydrochinone used in the treatment of capillary disorders, may affect oedema in chronic venous insufficiency (CVI). Aim of study is to investigate the effect of AMNA on vascular endothelial permeability *in vitro* and its effects on three-dimensional capillary-like structures formed by Human Umbilical Vein Endothelial Cells (HUVECs).

HUVECs were treated with 50 ng/ml VEGF for 2h and AMNA for 6h. Permeability assay, VE-cadherin expression and Matrigel assay were performed.

VEGF-induced permeability was significantly decreased by AMNA in a range concentration of 1-20 µg/ml. AMNA restored VE-cadherin expression. Finally, 6h pre-treatment with AMNA significantly preserved capillary-like structures formed by HUVECs on Matrigel up to 48 h compared to untreated cells.

AMNA significantly protects endothelium permeability and stabilises endothelial cells organised in capillary-like structures, modulating VE-cadherin expression. These data might explain the clinical benefict of AMNA on CVI.

Keywords. Aminaphtone, Human Umbilical Vein Endothelial Cells, permeability, chronic venous insufficiency, capillary fragility, VE-cadherin

1. Introduction

Vascular permeability is an extremely complicated process that is affected by many different variables [1]. A rapid increase in vascular permeability occurs when the microvasculature is exposed to any of a number of vascular permeabilising factors, e.g. vascular endothelial growth factor A (VEGF-A), histamine, platelet activating factor [1]. This process has been implicated in many pathological conditions such as angiogenesis, ischaemic heart disease, acute and chronic inflammation, trauma, wound-healing [2].

The dynamic equilibrium between cell adhesion and cytoskeleton contraction plays an important role in maintaining the normal function of endothelial permeability [3]. The intercellular junctions of endothelial cells have an important barrier function regulating the permeability to small molecules and even to cells [4]. Several families of cytoadhesive proteins, such as the selectins, members of the immunoglobulin (Ig) superfamily, and cadherins, play major roles in mediating cell–cell interactions and therefore are candidates for receptors involved in capillary tube formation [5]. Endothelial cells express both VE-(vascular endothelial) and N-(neuronal) cadherins. Whereas VE-cadherin is localised at sites of cell-cell contact, N-cadherin is distributed diffusely over the cell surface and does not seem to contribute to cell-cell adhesion [6].

VE-cadherin and N-cadherin have been shown to participate in capillary tube formation. In particular, antibodies directed against VE-cadherin, but not against N-cadherin, PECAM-1 (Ig superfamily) or $\alpha_v\beta_3$ integrin, markedly inhibited the generation of capillary tubes [7, 8]. Moreover, it has been demonstrated that blocking VE-cadherin

function leads to a marked reorganisation of the actin cytoskeleton, increased monolayer permeability and enhanced neutrophil transmigration [7, 9].

Phlebotonics are known as venoactive drugs, whose mechanism of action is not well established despite the availability of numerous studies concerning their pharmacological and clinical properties. Phlebotonics **may have beneficial effects on oedema and on some signs and symptoms related to CVI such as trophic disorders, cramps, restless legs, swelling and paraesthesia when compared with placebo [10]**.

Aminaphtone ($C_{18}H_{15}NO_4$, AMNA), a naphtohydrochinone used in the treatment of capillary disorders, has been reported to be effective in reducing vessel permeability and the healing of leg ulcers in patients with CVI **and other pathological conditions [11, 12]**. **In particular, in patients with idiopathic cyclic oedema syndrome, a clinical conditions that exhibits a vascular capillary hyperpermeability accompanied by oedema caused by the interstitial retention of fluid, a significant reduction in limb size was obtained after treatment with AMNA (P<0.0001) [12]. AMNA acts on venous, capillary and lymphatic levels, reducing hyperpermeability, increasing venous resistance, and regulating lymphatic flux. This mechanisms of action result in reduced venous stasis, improved venous return, and, therefore, inducing tissues protection against toxins and free radicals, as demonstrated in a work of De Anna et al. in patients with chronic venous and lymphatic stasis [11]. Finally, the use of AMNA was associated with significant improvements in quality of life compared with placebo in CVI patients [13].**

Aim of the present study is to investigate the effect of AMNA on *in vitro* model of vascular endothelial permeability and to evaluate if the drug is able to stabilise capillary-like structures formed by HUVECs in an extracellular matrix model.

2. Materials and methods

2.1. Aminaphtone

AMNA was generously gifted by Baldacci Spa, Pisa, Italy. Lyophilised AMNA 0.1 g was diluted with dimethyl sulphoxide to obtain a 100 mg/ml concentration. The solution was then diluted with Medium 199 (Lonza, Basel, Switzerland) supplemented with 1 % foetal bovine serum (FBS; Hyclone, South Logan, UT, USA), penicillin/streptomycin, HEPES (Lonza, Basel, Switzerland), glutamine (Sigma-Aldrich 3050 Spruce St., St. Louis, Missouri 63103 United States), heparin (Epsoclar) and bovine retina-derived extract as a complete growth medium.

2.2. Cell isolation and culture

HUVECs were isolated from fresh human umbilical cords by incubation with collagenase, (Invitrogen, Auckland, New Zealand) and cultured on gelatine-coated flasks in Medium199 (Lonza) containing 20% heat-inactivated FBS (Hyclone), sodium heparin (Epsoclar), penicillin/streptomycin (Lonza), HEPES (Lonza), glutamine (Sigma-Aldrich 3050 Spruce St., St. Louis, Missouri 63103 United States) and bovine retina-derived extract in humidified atmosphere (37 °C, 5% CO2). The P4–P5 cultures

were cultivated until sub-confluence was reached. Thereafter, HUVECs were exposed to the different experimental conditions.

2.3. Cell viability

HUVECs, $3x10^4$ cells/well, were seeded in 96-well plates in complete growth medium. After 72 h, cells were washed with PBS and treated for 6 h with different concentrations of AMNA (0 – 1 – 5 – 10 – 30 – 50 – 70 – 100 µg/ml) in growth medium containing 1% FBS. After treatment, cells were washed twice with phosphate buffer saline (PBS) and viability was evaluated by WST-1 assay. WST-1 assay is a colorimetric assay based on the cleavage of tetrazolium salt (WST-1, 4-[3-(4-Iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolium]-1,3-benzene disulphonate, Roche Applied Science, Mannheim, Germany) by mitochondrial dehydrogenases in viable cells. Briefly, after treatment, HUVECs were incubated with tetrazolium salt (10 µl/well) for 4 h at 37 °C, 5% CO_2. Then, the formazan dye formed was quantified by measuring the optical density at 450 nm (reference wavelength: 650 nm), by a multiplate reader (Titertek). The absorbance directly correlated to the number of metabolically active cells. Viability was expressed as percent of viable cells, calculated from the ratio of absorbance after treatment to mean absorbance for the untreated control.

2.4. Vascular permeability assay

Vascular permeability was performed by Millipore assay kit (EMD Millipore Corporation Division Headquarters, Massachusetts, USA) according to the manufacture

protocol. Briefly, 80% confluent HUVECs, were detached and plated on inserts (3 x 10^4 cells/insert) and incubated for 72 h at 37 °C, 5% CO_2, up to form a monolayer. Cells were then washed and treated with different concentrations of AMNA (0.5-1-5-10-20 µg/ml) for 6 h with or without 50 ng/ml VEGF (Recombinant Human $VEGF_{165}$, Peprotech EC Ltd, London, UK) for 2 h, in growth medium with 1% FBS. At the end of incubation, all treatments were removed and an high molecular weight fluorescein isothiocyanate-dextran (FITC-dextran, MW 70KDa) was used to evaluate permeability. Plate was incubated in the dark for 20 min at room temperature. Permeation was arrested by removing the inserts from the wells. The levels of FITC-dextran was recorded by a sensitive fluorescence plate reader (Fluoroskan, Thermo Fisher ®), (Ex: 485 nm; Em: 538). The amount of diffused dextran has determined using calibration curves established just with the stock solution.

2.5. Western blot analysis of VE-cadherin

To determine VE-cadherin and VE-cadherin phosphorylation expression, HUVECs were seeded in appropriated density in 6 multiwells and cultured in complete growth medium until confluence. Cells were afterwards washed and pre-treated with 10 µg/ml AMNA for 6 h and then with 50 ng/ml VEGF for 2 h in medium with 1 % FBS. At the end of each treatment cells were lysed at 4°C for 1 h in lysis buffer (50 mM Tris–HCl pH 7.4, 150 mM NaCl, 1 mM PMSF, 1mM EDTA, 5 mg/ml Aprotinin, 5 mg/ml Leupeptin, 1% Triton X-100, 1% Sodium Deoxycholate, 0.1% SDS) and centrifuged at 10,000 X g for 15 min. The protein content of the supernatants was measured by

Bradford method. Aliquots containing 50 mg of cell lysate proteins were subjected to 7.5% SDS-PAGE under reducing conditions and blotted onto nitrocellulose membrane filters. The blots were blocked for 2 h at 37 8C in 2% BSA, 1mM Na2HPO4, 1.7mM NaH2PO4, 150mM NaCl, 0.05 Tween-20 (PBS-T). The nitrocellulose membranes were washed once with PBS-T for 15 min, three times for 5 min with 1mM PBS and immunoblotted for 2 h at room temperature with rabbit polyclonal anti-VE Cadherin (phospho Y685) antibody (Abcam Ltd) or with rabbit monoclonal anti-VE-cadherin antibody (Cell Signaling Technology, Boston, USA). After one washing with PBS-T and three washing with PBS, the blots were incubated for 1 h at room temperature with HRP labelled anti-rabbit IgG, washed with PBS, developed for 2 min with ECL reagent (Pierce) and exposed to Biomax film (Kodak Co.). The densitometry data of the protein bands were obtained using ImageJ software, and the objective protein expression was evaluated by the densitometry ratio of the objective proteins to β-actin.

2.6. Immunofluorescent detection of VE-cadherin

The distribution of VE-cadherin in HUVEC monolayers was assessed by immunofluorescent techniques using previously described procedure [14] with modifications. Cells were placed on multiwell slides. Confluent cultures of HUVECs were pre-treated with AMNA (10 μg/ml) for 6 h and with VEGF (50 ng/ml) for 2 h in medium with 1 % FBS. At the end of treatment, cells were washed three times with PBS containing 1 mM $CaCl_2$ and 1 mM $MgCl_2$ (DPBS) and then fixed with 4% formaldehyde at room temperature for 15 min. The formaldehyde was then aspirated

and the cells were washed three times with DPBS. The washed cells were then permeabilised with 0.5% Triton X-100 for 5 min at room temperature. Non-specific binding sites were blocked by incubation with 3% BSA in DPBS for 5 min at room temperature. The BSA was then aspirated and the cells were immediately incubated with primary mouse monoclonal antibodies against human VE-cadherin (10 mg/ml in DPBS) (Cell Signaling Technology, Boston, USA) for 1 to 12 h at 37°C in a humidified chamber. At the end of incubation the primary antibody were removed, cells were washed three times and incubated at 37°C for 30 min with FITC- conjugated anti-mouse secondary antibody (Cell Signaling Technology) diluted 1:200 and rhodamine-phalloidin for F-actin staining (Cytoskeleton, Inc.) diluted 1:400 in DPBS. The chamber slides were then washed and mounted with Vectashield mounting medium containing DAPI (Vector Laboratories, UK). Slides were viewed under a fluorescence microscope (Nikon) equipped with a digital CCD camera and 20X objective. Images were recorded using AxioVision (Carl Zeiss MicroImaging, GmbH) software.

2.7. *In vitro* capillary tube maintenance

To evaluate the capability of AMNA to maintain capillary tubes formation, HUVECs were treated with 10 µg/ml AMNA for 6h and then $7x10^4$ cells/well were plated on Matrigel (BD Biosciences) for 48 h. Matrigel was thawed on ice at 4 °C and used to coat 24-well plates for 30 min at 37 °C. Cells were monitored and optical microscopy images were captured at 2, 8, 24 and 48 h. Five independent images were taken for each well.

To quantify angiogenic structures formed by HUVECs, computational morphometric analysis was performed [10]. Briefly, representative fields from each experimental setting were taken. Number of meshes (number of closed meshes) and master segment length (total length of the network) of capillary tubes, in five random fields for each sample and time of acquisition, were calculated using Angiogenesis Analyzer for ImageJ software [13]. Results for different experimental groups were expressed as mean ± SD from three independent experiments. Representative pictures at higher magnification were also taken.

2.8. Statistical analysis

Data are presented as mean ± SD of independent experiments. Comparisons were made using Student's t-test or by ANOVA when appropriate. Values of $P<0.05$ were considered statistically significant. Statistical analysis was carried out using the Graph-Pad Prism 5 software (GraphPad Software, Inc., La Jolla, CA 92037 USA).

3. Results

3.1. Effect of Aminaphtone on cell viability

To determine non-toxic concentration of AMNA, HUVECs were incubated in the presence of different concentrations of drug (1–100 µg/ml). Dose-dependent HUVEC viability indicated that no significant cytotoxic effects were observed at AMNA

concentrations below 30 µg/ml. At concentration up to 30 µg/ml there was a rapid reduction in cell viability, **Figure 1.**

3.2. Effect of Aminaphtone on endothelial cell permeability

The effect of different concentrations of AMNA on VEGF-induced HUVEC permeability has been shown in **figure 2.** VEGF-induced cell permeability was significantly reduced at a range concentration of 1-20 µg/ml AMNA ($P<0.05$ vs VEGF), restoring normal condition. AMNA alone not affect cell permeability.

3.3. VE-cadherin fluorescent detection

To evaluate the effect of AMNA on VE-cadherin localisation, cells were fixed and processed for immunofluorescence (**Figure 3)** As showed in figure, VE-cadherin was localized as bright intense immunostaining at all cell–cell contacts (control and AMNA treatment). VEGF stimulation for 2 h reduced the staining of VE-cadherin at cell–cell contacts, that was now expressed in an irregular manner, and a loss of cell–cell adhesions. On the contrary, AMNA pre-treatment restored VE-cadherin staining at cell-cell contacts that was distributed in a striped fashion, like controls.

3.4. VE-cadherin expression

To correlate the immunolocalization changes with protein expression, Western blot analysis of total VE-cadherin and phospho-VE-cadherin were performed (**Figure 4**). Immunoblotting demonstrated that the levels of total VE-cadherin remained the same in

all treatment conditions. However, phosphoY685-VE-cadherin resulted reduced by AMNA pre-treatment (P<0.005 vs VEGF) and increased by VEGF treatment (P<0.001 vs control), as confirmed by densitometry analysis. These data suggest a potential protective role of AMNA on VE-cadherin degradation induced by VEGF.

3.5. In vitro capillary tube maintenance

HUVECs, when seeded on Matrigel, formed capillary-like structures. This process has a rapid onset, beginning within 1 h and is completed by 8-12 h; after this time the network starts to rearrange and completely disappears within 24-48 h.

Figure 5 shows the effect of 10 μg/ml AMNA 6h pre-treatment on capillary-like structure maintenance, formed by HUVECs. In **figure 5 A**, AMNA significantly reduced degradation of endothelial cells organised in endothelial cell cords on Matrigel at 24 h and 48 h compared with control. **Figure 5 B**, shows the number of meshes and master segment length of capillary tubes formed by HUVECs. After 48 h, there was a significant difference in number of meshes and master segment length between control and AMNA-treated cells (P<0.01).

4. Discussion

CVI is characterised by venous hypertension associated with decrease in microcirculatory blood flow and increase in post-capillary permeability of the veins, leading to oedema of the legs and feet [15]. The most used phlebotropic drugs, or oedema-protecting drugs, are the gamma-benzopyrone family (flavonoids) [16]. Results of clinical

studies show that flavonoids decrease capillary permeability and increase capillary resistance, which could partly be explained by inhibition of leukocyte activation, migration, and adhesion [16, 17]. As reported by Ramelet et al. (2000), this inhibition is linked to a significant decrease in plasma levels of endothelial adhesion molecules (VCAM-1 and ICAM-1) after flavonoids treatment [18]. Beside the clinical outcome in patients with CVI, AMNA has shown interesting endothelial-protective properties, inducing down-regulation of sELAM-1 and sVCAM-1 in patients with systemic sclerosis [19]. Moreover, AMNA is a molecule with a demonstrated anti-endothelin1 (ET-1) effect *in vitro*, interfering with the transcription of the pre-pro-ET-1 (PPET-1) gene [20]. Furthermore, ET-1 is known to acts as a permeability factor in the vascular endothelium [21-23]. It is known that HUVECs treated with VEGF increased their ET-1 production in a concentration and time-dependent manner [24]. In addition, Chen et al. (2000), demonstrated that VEGF increased both ET-1 mRNA and ET-1 protein expression along with endothelial cell permeability in HUVECs cultured in normal glucose condition. Interestingly, they reported that ET-1 induces a disruption of the endothelial cell junctions [25]. Moreover, in a recent work, we demonstrated that AMNA reduced significantly ET-1 secretion in HUVECs [26].

In this study, AMNA was able to restore vascular permeability induced by VEGF in a concentration range of 1-20 µg/ml. Inhibition of VE-cadherin function induces a reorganisation of the actin cytoskeleton leading to a loss of junctional VE-cadherin localization, reduces cell-cell adhesion and increases permeability [27]. It is know that VEGF stimulation reduces VE-cadherin staining at the adherent junction [7, 27-29].

Moreover, it is generally accepted that the tyrosine phosphorylation of VE-cadherin is associated with weak junctions and impaired barrier function. In particular, it has been proved that VEGF enhance the permeability of HUVEC monolayers and increase tyrosine phosphorylation of VE-cadherin [30]. In our study, AMNA pre-treatment restored VE-cadherin localization at the cell-cell contacts. In addition, protein expression of total VE-cadherin remained the same in all treatment conditions, whereas phospho-tyrosin 685-VE-cadherin resulted increased by VEGF treatment (compared to control) and reduced by AMNA pre-treatment.

Finally, HUVECs, when seeded on Matrigel, formed capillary-like structures. This process has a rapid onset, beginning within 1 h and is completed by 8-12 h; after this time the network starts to rearrange and completely disappears within 24-48 h. We used Matrigel assay to evaluate the effect of AMNA pre-treatment on degradation time of capillary-like structures formed by HUVECs. In accordance with the role of VE-cadherin in both formation and maintenance of capillary tubes in fibrin collagen gel-over-gel assays [7], our data showed that AMNA improved stability of endothelial cells organised in capillary-like structures, as observed after 48 h.

4.1. Limitations and future directions

One potential limitation of this study was the completely absences of *in vitro* studies on the mechanism of action of AMNA and its intracellular pathway involved. Here, for the first time, the influence of AMNA on VE-cadherin modulation was suggested.

Moreover, we lack of clinical experience of oedema reduction.

Future direction may be an *in vitro* study on endothelial cells cultured from veins of patients with CVI to set up a personalized study. Finally, a multicentric clinical studies would be essential to confirm the effect of AMNA on oedema reduction mainly in leg ulcer.

4.2. Conclusion

AMNA is used to reduce lower limbs oedema in chronic venous insufficiency.

On the basis of our experience, we can suppose that the effect of AMNA is due to protection of VE-cadherin degradation induced by VEGF and to its effect on ET-1 expression. These properties of AMNA may also explain the stability of endothelial cells organised in extracellular matrix.

Disclosures

The authors declare no conflicts of interest.

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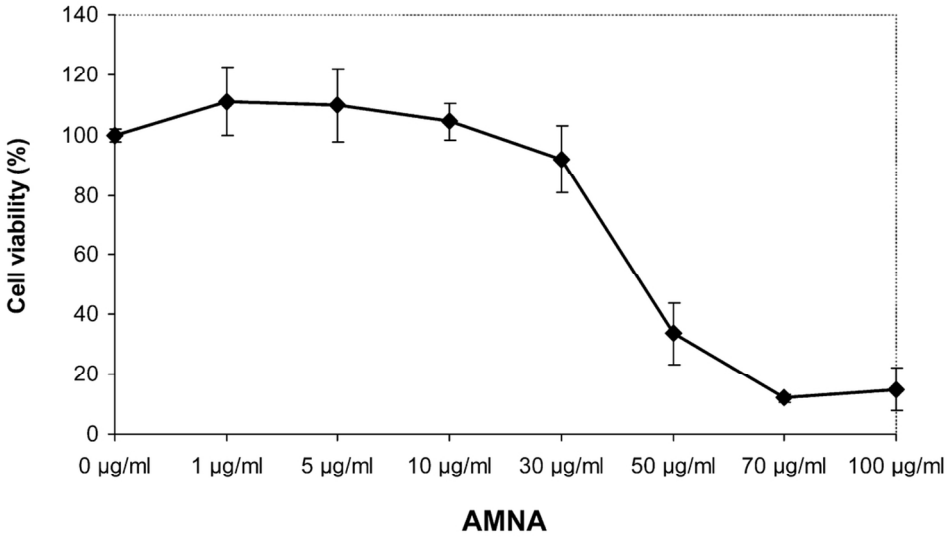

Figure 1. Dose-dependent effect of AMNA on cell viability. HUVECs were treated with different concentration of AMNA (1 – 5 – 10 – 30 – 50 – 70 – 100 µg/ml) for 6 h. Cell viability was evaluated by WST-1 colorimetric assay (Abs 450 nm). Data are expressed as percentage of control of 3 independent experiments ± SD.

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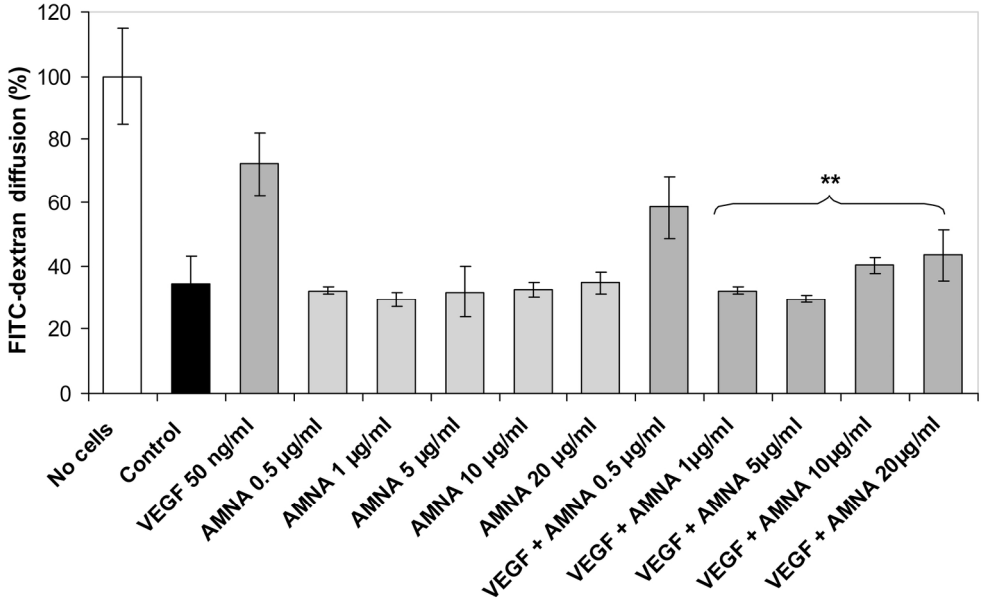

Figure 2. Permeability of HUVEC monolayers to FITC-dextran. Cells were grown to confluency on Transwell filters and pre-treated with different concentrations of AMNA (0.5 – 1 – 5 – 10 – 20 µg/ml) for 6h and than with or without VEGF (50 ng/ml) for 2h. Permeability was measured using FITC-dextran (MW 70 KDa) as described in Materials and Methods. Data are expressed as percentage of FITC-dextran diffusion of empty filter (no cells) and are representative of three independent experiments. Ex 485 nm; Em 538 nm. **P < 0.05 vs VEGF.

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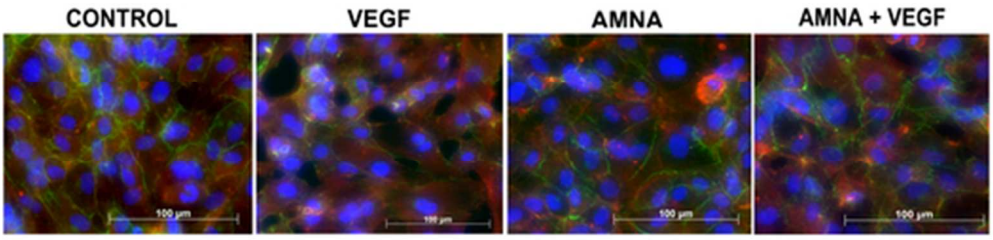

Figure 3. VE-cadherin localization in HUVECs. Confluent cell monolayers were pre-treated with AMNA (10 μg/ml) for 6 h and with VEGF (50 ng/ml) for 2 h. Cells were immunostained for VE-cadherin (green) and F-actin (red). Cell nuclei were counterstained with DAPI (blue). All fluorescence images were taken at five different fields/well, and images are representative of three independent experiments (magnification 20X).

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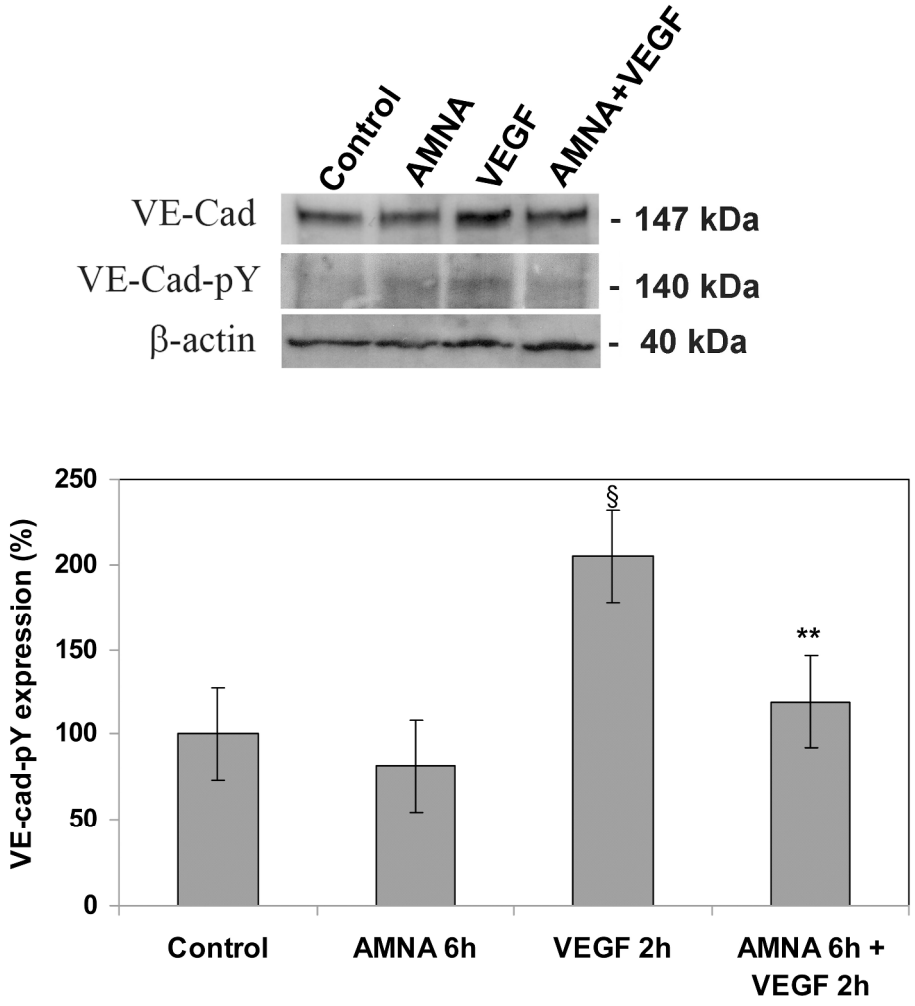

Figure 4. VE-cadherin expression. HUVECs were cultured until confluences and pre-treated with 10 µg/ml AMNA for 6 h and then with 50 ng/ml VEGF for 2 h in medium with 1 % FBS. Western blot analysis of cell lysate was performed. Anti-VE Cadherin (phospho-Y685) at 140 kDa and anti-VE-cadherin antibody at 147 kDa were detected. Quantitative analysis has been normalized to beta-actin (as control). Samples derive from the same experiment and the gels and blots were processed in parallel. **P<0.005 vs VEGF and § P<0.001 vs control.

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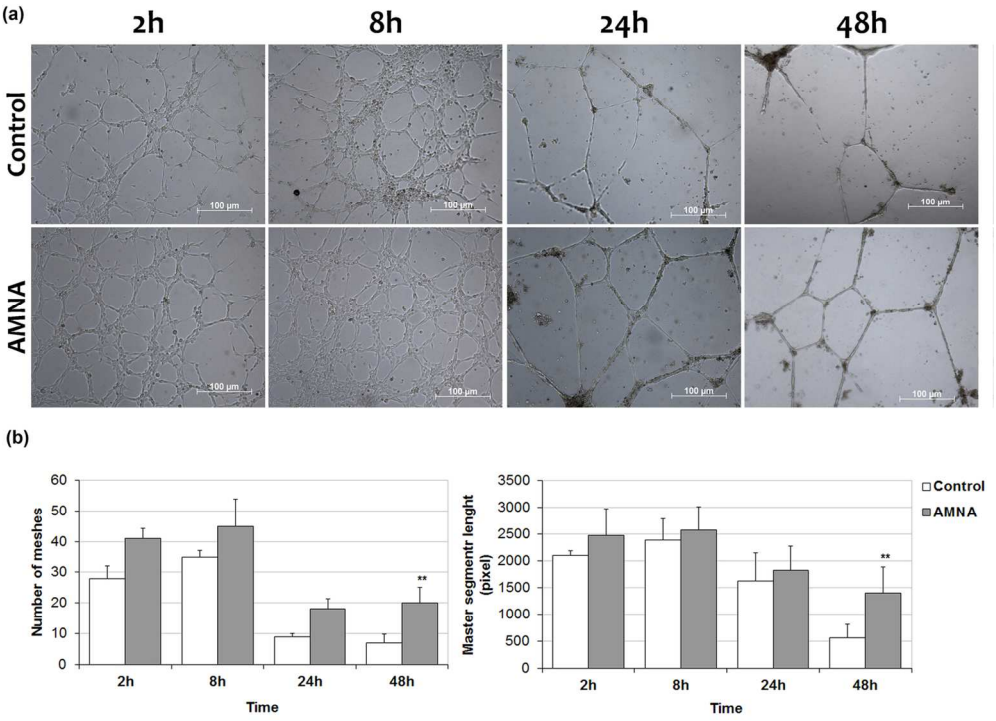

Figure 5. Capillary-like structures maintenance. Cells were treated for 6 h with 10 µg/ml AMNA and then 7x104 cells/well were plated on Matrigel for 48 h. Images were acquired after 2-8-24-48 h. Images are representative of 5 random-field (original magnification 5X) (A). Optical microscope images were analysed through Angiogenesis Analyzer for ImageJ software to measure number of meshes and master segment length of capillary tubes formed by HUVECs after 2-8-24-48 h (B). Five random fields for each sample and time of acquisition were analysed. Values in the graph are the mean from three independent experiments ± SD. **P<0.01 vs control.

137x99mm (300 x 300 DPI)

Declarations

Competing interests: The Authors declares that there is no conflict of interest

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Ethical approval: NA

Guarantor: RD

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