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Ac_{2-26} peptide and serine protease of *Bothrops atrox* similarly induces angiogenesis without triggering local and systemic inflammation in a murine model of dorsal skinfold chamber

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1. Introduction

Angiogenesis is the process of new blood vessels formation from the pre-existing vasculature (Staton et al., 2009). Also known as neovascularization, this dynamic process involves extracellular matrix degradation, dilation of existing blood vessels, migration and proliferation of endothelial cells, formation of vessel lumen, expression of adhesion molecules and, finally, stabilization of new vascular walls by other types of cells (Laschke et al., 2006) This is a key event in the maintenance of tissue homeostasis, remodelling and repair. The angiogenic process is fundamental to physiological events, such as embryogenesis and organogenesis, as well as physiopathological conditions, including tissue regeneration, cicatrization, and tumour progression (Bergers and Benjamin, 2003; Karamysheva, 2008).

Neovascularization in non-physiological contexts is mostly dependent on immune modulators, such as immune cells and the cytokines/chemokines (Szade et al., 2015). However, these mediators interact in complex pathways and their exact role in the angiogenic process is still controversial, as inflammatory cells and signals act either promoting or inhibiting the formation of new capillaries (Apte et al., 2006; Sanberg et al., 2010; Szade et al., 2015). Regardless the stimulatory or inhibitory effect, these mediators certainly play a role in mediating the pro- and anti-angiogenic balance, and investigating them is crucial to provide new alternatives for public health issues such as the application of bioengineering tools therapeutic strategies which success depends on angiogenesis, including tissue/organ transplantations and the treatment of diseases associated with impaired vascular irrigation (Liu et al., 2017; Said and Geoffrey, 2013; Mimura et al., 2016). Studies in this field have pointed the anti-inflammatory protein annexin A1 (AnxA1), as well as the serine protease isolated from *Bothrops atrox* venom (BaSp) as potent inductors of neovascularization (Bhat et al., 2016; M Yi and Schnitzer, 2009).

Endothelial cells produce and are modulated by both AnxA1 and serine proteases (Drewes et al., 2012; Kamal et al., 2005; Pin et al., 2012; Sharma and Sharma, 2007). So far, it is known that the endogenous AnxA1 induces endothelial proliferation and migration (Ming Yi and Schnitzer, 2009), while serine proteases from snake venom (SPSVs) are well-characterized as a class of toxins that affect the blood coagulation system (Serrano and Maroun, 2005). Although it is clear that AnxA1 and serine proteases display an important interaction with the vasculature, few investigations have assessed the effects of administrating these mediators over angiogenesis in an in vivo system.

We hypothesize that the mimetic peptide from AnxA1, Ac_{2-26}, in association with BaSp increases angiogenesis. Herein, a dorsal skin

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chamber model was performed in mice to investigate the effects of Ac_{2-26} and BaSp over the formation of new blood vessels, focusing on the modulations of immune mediators related to the angiogenic process with the possibility of revealing potential applications of these mediators in the induction of these highly dynamic and complex processes.

2. Material and methods

2.1. Animals

Female wild-type (WT) Balb/c mice, weighting 25–30 g, 6–8 weeks old, were kept on a 12 h light-dark cycle and allowed food and water *ad libitum*. The experiments were performed in strict accordance with the Brazilian laws of Ethical of protection and this study was approved by the Ethics Committee in Animal Experimentation of São Paulo State University (IBILCE/UNESP) (Nº. nº112/2015).

2.2. Ac_{2-26} peptide and serine protease of Bothrops atrox

The Ac_{2-26} peptide (Ac-AMVSEFLKQAWFIENEEQEYVQTVK; Raynal and Pollard, 1994) was obtained by Thermo Fisher Scientifics, Waltham, MA. The serine protease, given by the specialist Professor Dr. Raghuvir Krishnaswamy Arni (Laboratory of Molecular Biology, Physics Department, IBILCE/UNESP), was purified from the venom of *Bothrops atrox* using a combination of size exclusion chromatography on a Sephacryl S-200 column and affinity chromatography on bezamidine sepharose 4 fast flow following published procedure (Bhat et al., 2016).

2.3. Standardization of treatment doses

The concentration used for the peptide Ac_{2-26} was 1 mg/kg, according to preliminary studies realized in our laboratory (Girol et al., 2013). It was realized tests (1 mg/kg and 0.4 mg/kg) to establish the appropriate concentration of serine protease (BaSp). The concentrations used was 0.4 mg/kg, considered appropriated to the continuity of the experiments.

2.4. Study design: dorsal skinfold chamber

The dorsal skinfold chamber was implanted in mice under anaesthesia, as previously described by Harder et al. (2004). Saline (10 μL) (control), Ac_{2-26} peptide (1 mg/kg, 10 μL) and/or BaSp (0.4 mg/kg, 10 μL) were locally applied as previously described by Drewes et al. (2012). Treatments (n = 5 animals/group) were carried out on the 4th, 5th and 6th days after chamber implantation, to avoid potential inflammatory cells recruitment due to the surgical manipulation. Animals were immobilized in a polycarbonate tube and the microcirculatory network in the window was photographed using a digital camera (NIKON D3100, 12 Mega Pixels). The images obtained before (day 4) and after treatment (day 9) were quantified following published procedure (Dellian et al., 1996; Girol et al., 2013). Results were expressed as percentage of vessels in comparison with the control group of mice (Control-treated animals).

After treatments, animals were again anesthetized with ketamine (100 mg/kg, Ketalar/Parke-Daves) and xylazine (10 mg/kg; Rompum/Bayer), sacrificed and skin removed for histological processing and enzymatic and cytokine analyzes.

2.5. Inflammatory cells quantification in blood

After euthanasia, the blood of each animal was collected by puncture of the vena cava using a syringe containing EDTA (10%). From the collected blood, 10 μL was added to 190 μL of Türk's liquid and the solution homogenized. From this solution, an aliquot was obtained for quantification of polymorphonuclear cells (PMNs) and mononuclear cells (PBMCs) in the Neubauer chamber (Bittencourt et al., 2006).

2.6. Histopathological analysis

Skin samples were fixed in 4% buffered formalin for 24 h, dehydrated in graded ethanol and embedded in paraffin for histopathological and immunohistochemical analysis. Skin fragments sections of 3 μm were stained with hematoxylin–eosin and analyzed using a highpower objective (20×) on an Axioskop 2-Mot Plus Zeiss microscope (Carl Zeiss, Jena, Germany).

2.7. Mast cells analysis in skin fragments

To evaluate the presence of metacromatic cytoplasmic granules in the mast cells, tissue samples were fixed in 0.5% glutaraldehyde and 4% paraformaldehyde, 0.1 M sodium cacodylate buffer pH 7.4 for 24 h at 4 °C. They were then washed in the same buffer, dehydrated in an increasing series of methanol (Merck, Germany) at 20 °C and pre-included in a 100% methanol mixture with LRGold resin (London Resin CO, Reading, Berkshire, UK), (24 h) at 20 °C. Afterwards, they were included in pure LRGold resin for 24 h at 20 °C and exposed to ultraviolet light (Oliani et al., 2001). 0.5 μm sections were obtained on the Leica RM2265 microtome and then stained with 1% toluidine blue in 1% borax solution (TAAB Laboratories, IL) for mast cell analysis. Quantification was performed throughout the tissue extension at 40× magnification, and the total number of cells divided by the area. The photomicrographs were obtained from the microscope ZEISS AXIOSKOP 2, from the Laboratory of Immunomorphology, IBILCE - UNESP, São José do Rio Preto.

2.8. Immunohistochemistry assay

For analysis and quantification of macrophages, skin sections (3 μm) were subjected to immunohistochemical reactions using Anti-ED-1 antibody (1:500, monoclonal, AbD Serotec, MCA1957, Bio-Rad Laboratories, Inc). For endogenous peroxidase blockade, 12% hydrogen peroxide solution and 10% normal goat serum (Goat Normal Serum, Sigma-Aldrich) were used. Fragments, preheated in an oven at 60 °C for 1 h, were dewaxed and subjected to antigen exposure by incubation in citrate buffer (0.01 M, pH 6.0). The material was incubated with primary antibody (diluted 1:200 in 5% TBS-BSA) for 1 h at room temperature and then with the HRP-conjugated secondary antibody (Goat anti-mouse Abcam, United Kingdom) in a humid chamber and room temperature for an hour. The expression of the labelled substrings was revealed by DAB (3,3-diaminobenzidine, Liquid DAB Substrate Kit, Invitrogen, Paisley, UK) and hematoxylin counter-treated. Quantification of macrophages was based on the number of positive cells in 10 fields per animal analyzed, photographed with a 63× objective. Analyzes were carried out in the image analyzer, using AXIOVISION software, and the results expressed as mean ± S.E.M.

2.9. Myeloperoxidase measurement

Neutrophil infiltration was indirectly assessed by myeloperoxidase (MPO) measurement. Briefly, skin fragments were stored at −80 °C for dosing of myeloperoxidase tissue levels (MPO). Samples were homogenized in 5% EDTA/NaCl buffer (pH 4.7) and

centrifuged at 10.000 rpm, 4 °C for 15 min. The pellet was resuspended in a 0.2% NaCl, 1.6% NaCl-5% glucose and 0.5% hexadecyl trimethyl ammonium bromide (pH 5.4) solution. Three repetitions of thermal shock (immersion in N_2 and, after, in a water bath at 37 °C) were performed before the last centrifugation. The samples were then distributed into 96-well plates, developed with 3,3′,5,5'-tetramethylbenzidine (TMB, Millipore, USA) and read in the spectrophotometric apparatus at 450 nm. The concentration of MPO in pg/mL/mg was compared to the HRP curve diluted in $NaPO_4$.

2.10. Multiplex assays for cytokine analysis

The quantification of IL-1β, IL-4, IL-6, IL-10, TNF-α inflammatory mediators and vascular endothelial growth factor (VEGF) in the skin and plasma fragments was assessed by the Luminex MAGPIX xMAP multiplex instrument (Millipore Corporation, Billerica, MA, USA). Tissue fragments were macerated in liquid nitrogen and placed in tubes of 1.5, in which 500 μl of a protease inhibitor solution (GE Healthcare, Amersham, UK) and Tween 20 (1 μL) (Sigma-Aldrich, Poole, Dorset, UK) was added.

The samples were incubated for 1 h at 4 °C under constant stirring and then centrifuged at 12.000 g for 15 min at 4 °C. The supernatant was removed, centrifuged and maintained at −20 °C until use. The protein concentration in the supernatant was measured using a Bradford assay (Biorad, Hemel Hempsted, UK). Standards, control solutions and samples were prepared following the manufacturer's instructions (MILLIPLEX HCYTOMAG-60K kit) and added on the test plate with magnetic beads coated with specific antibodies.

After overnight incubation at 4 °C, the biotin-bound detection antibody was added followed 1 h later by streptavidin conjugated to phycoerythrin. The reading was performed by LUMINEX xMAP MAGPIX. The concentration of the analytes was determined by MAGPIX xPONENT software (Millipore Corporation, Billerica, MA, USA).

2.11. Statistical analyses

Data were analyzed using Prisma® GraphPad software version 5.00. The results are presented as mean ± standard error of the mean (S.E.M.) and statistical analysis was performed by analysis of variance for multiple comparisons (ANOVA), followed by the Bonferroni adjustment or Student's t-test. *P* values less than 0.05 were considered to indicate statistically significant differences.

3. Results

3.1. Post treatment with the Ac_{2-26} peptide and/or serine proteases from B. atrox increase angiogenesis process

Initially, the angiogenic potential of the Ac_{2-26} peptide and/or BaSp was evaluated by quantification of blood vessels before (day 4) and after 9 days (Fig. 1A) of treatments using the dorsal chamber model (Fig. 1B). The dorsal skin chamber was well tolerated by the animals. No signals of infection or rejection were noticed during the 9 days of experiments. The captured images (days 4 and 9) showed a significant increase in the number of blood vessels in the Ac_{2-26}, BaSp and BaSp + Ac_{2-26} groups relative to the control (Fig. 1C). Fig. 1D illustrates histological blood vessels results following nine days of Ac_{2-26} peptide and/or BaSp administration.

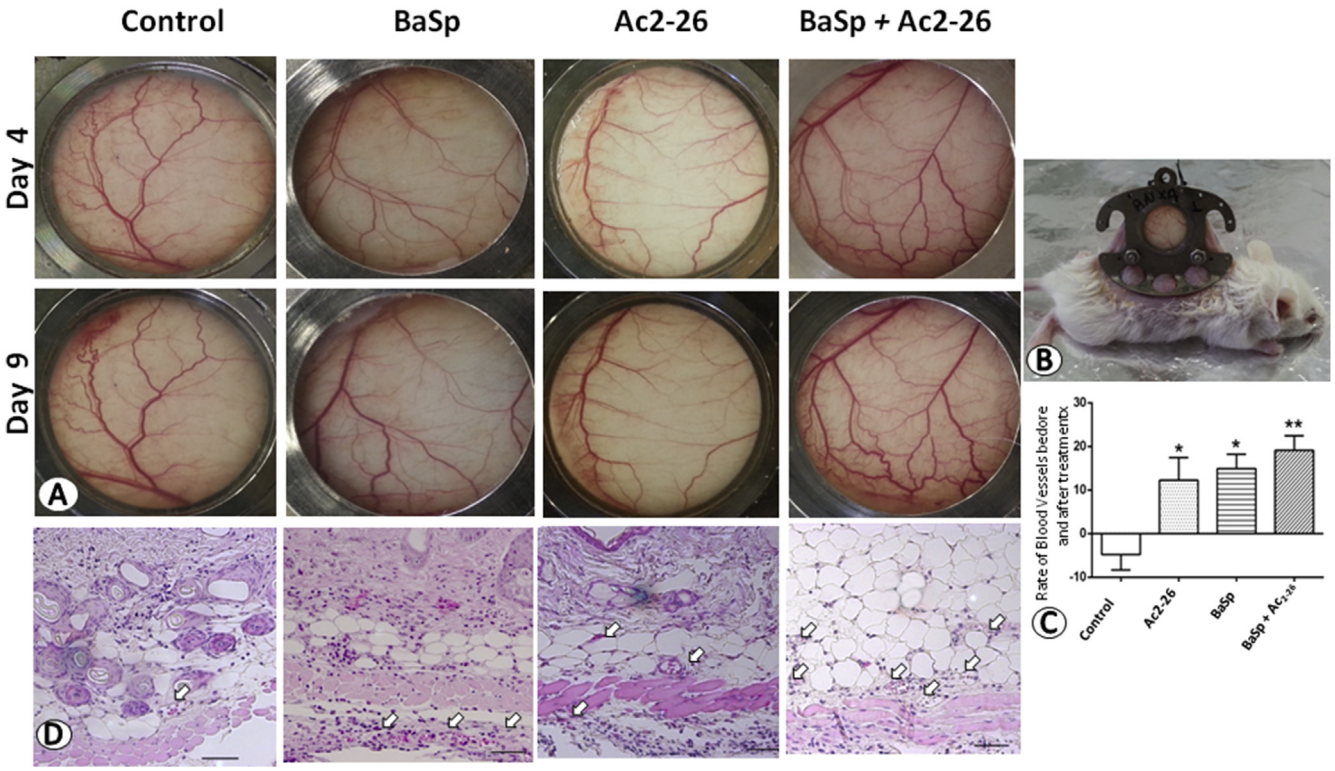

Fig. 1. Effects of treatments on the microcirculatory network of the skin. Control: saline solution; BaSp (0.4 mg/kg); Ac_{2-26} (1 mg/kg); BaSp + Ac_{2-26}. Blood vessels before (day 4) and after treatment (day 9) (1A). Dorsal skin chamber experimental model (B). Growth rate of vessels: increase after treatments in relation to control (C). Histopathological analysis of skin fragments (D). Blood vessels (white arrows). Staining Haematoxylin and Eosin. Bars: 50 μm. Values are mean ± S.E.M (n = 5 animals/group). *P < 0.05, **p < 0.01 vs Control.

Table 1
Analysis of plasma cytokines and inflammatory cells after different treatments.

Plasma				
	Control	Ac_{2-26}	BaSp	BaSp + Ac_{2-26}
VEGF (pg/ml)	2.99 ± 0.23	3.46 ± 0.15	3.57 ± 0.39	3.88 ± 0.44
TNF (pg/ml)	14.44 ± 0.57	15.02 ± 1.73	19.50 ± 5.37	14.82 ± 0.50
IL-1β (pg/ml)	82.08 ± 13.29	90.86 ± 9.53	70.54 ± 7.39	97.77 ± 9.51
IL-6 (pg/ml)	82.16 ± 31.82	54.84 ± 17.75	72.07 ± 30.73	56.68 ±13.91
IL-10 (pg/ml)	11.41 ± 2.24	10.48 ± 1.26	14.88 ± 3.67	12.53 ± 2.16
PMN (× 10^5/ml)	5.30 ± 1.56	12.50 ± 4.67	7.50 ± 2.04	9.25 ± 1.09
PBMC (× 10^5/ml)	8.00 ± 2.01	12.50 ± 4.67	18.50 ± 5.50	17.25 ± 4.08

The inflammatory potential and recruitment of inflammatory cells in plasma after topical treatment with Ac2-26 (1 mg/kg) and/or BaSp (0.4 mg/kg) diluted in 10 μl of saline solution was evaluated after implantation of the dorsal chamber. The animals were treated on days 4, 5, and 6, and after day 9 sacrificed for blood and plasma collection, as described in item 3.4 of the material and methods. The results were not significant. Values of IL-1β, IL-6, IL-10, TNF and VEGF expressed as mean ± S.E.M of pg/ml values of PMN and PBMC expressed as mean ± S.E.M of cell numbers x 10^5/ml (n = 5 mice/group).

3.2. Systemic plasma cytokine levels and quantification of inflammatory cells

We investigated the presence of the cytokines IL1-β, IL-6, IL-10 and TNF-α to evaluate the systemic (plasma) effect in the production of these proinflammatory mediators in the different experimental groups. Furthermore, quantification of circulating leukocytes was performed to evaluate the systemic effect on recruitment of inflammatory cells by quantification of PMN and PBMC using the Neubauer chamber. The treatments used did not induce significant differences in the production of these cytokines or in the recruitment of inflammatory cells at systemic levels (Table 1).

3.3. Local cytokine levels and recruitment of inflammatory cells in skin fragments

In skin fragments, we also investigated the presence of the pro-angiogenic cytokines, VEGF and TNF-α. We also investigated the presence of the cytokines IL-1β, IL-6, IL-10 to evaluate the local effect in the production of these proinflammatory and growth mediators. Furthermore, the potential inflammatory response was assessed by colorimetric analysis of myeloperoxidase activity (MPO). The treatments used did not induce significant differences in the production of these cytokines or in the recruitment of neutrophils at local level (Table 2).

3.4. Analysis of mast cells after treatments with Ac2-26 and/or BaSp

Mast cells are strategically located at the host's interface with the environment, such as the skin and mucosa. Skin fragments, included in the LRGold resin and stained with Toluidine Blue, revealed the presence of mast cells with intact and degranulated metachromatic cytoplasmic granules located in the dermis in all experimental conditions (Fig. 2A–D). Treatment protocols (Ac_{2-26}, BaSp and BaSp + Ac_{2-26}) did not induce significant differences in the total number of mast cells (Fig. 2E) or degranulation of these cells (Fig. 2F).

Table 2
Analysis of cytokines and myeloperoxidase activity (MPO) after different treatments in skin fragments.

Skin				
	Control	Ac_{2-26}	BaSp	BaSp + Ac_{2-26}
VEGF (pg/ml/g)	0.36 ± 0.10	0.50 ± 0.10	0.29 ± 0.05	0.23 ± 0.08
TNF (pg/ml/g)	0.65 ± 0.21	1.86 ± 0.77	0.69 ± 0.18	0.94 ± 0.49
IL-1β (pg/ml/g)	5.50 ± 2.09	9.12 ± 3.08	10.10 ± 4.37	3.76 ± 2.22
IL-6 (pg/ml/g)	7.10 ± 2.00	7.70 ± 2.02	10.43 ± 4.02	9.73 ± 2.15
IL-10 (pg/ml/g)	0.25 ± 0.02	0.37 ± 0.05	0.31 ± 0.03	0.44 ± 0.17
MPO (ng/ml/mg)	0.11 ± 0.02	0.09 ± 0.00	0.09 ± 0.00	0.10 ± 0.03

The inflammatory potential of the topical treatment with Ac2-26 (1 mg/kg) and/or BaSp (0.4 mg/kg) diluted in 10 μl of saline solution was evaluated after implantation of the dorsal chamber. The animals were treated on days 4, 5, and 6, and after day 9 sacrificed for blood and plasma collection, as described in item 3.4 of the material and methods. The results were not significant. Values of IL-1β, IL-6, IL-10, TNF and VEGF expressed as mean ± SEM pg/ml/g and MPO expressed as mean SEM of ng/ml/mg. (n = 5 mice/group).

3.5. Ac_{2-26} and/or BaSp decrease macrophages recruitment in Balb/c mice skin

To analyze and quantify the total number of macrophages, immunohistochemical reactions were performed for ED-1 positive macrophages after treatments with peptide Ac_{2-26} and/or BaSp. Our results showed a significant decrease in the number of total macrophages/mm^2 in the Ac_{2-26}, BaSp and BaSp + Ac_{2-26} groups in relation to control (Fig. 3). Furthermore, there was a significant decrease in the number of macrophages in the BaSp + Ac_{2-26} group in relation to the administration of the Ac_{2-26} peptide alone.

4. Discussion

Angiogenesis is a key element for successful transplantation, regeneration and repair of damaged tissues, post-ischemia recovery, wound healing, embryogenesis and tissue engineering (Laschke et al., 2006; M Yi and Schnitzer, 2009). Controlling the non-physiological neovascularization is an important challenge to overcome, as this process is intimately associated with inflammatory mediators, which are also pivotal inductors of damage and transplants rejection (Reinders et al., 2006; Szade et al., 2015). The present investigation shows that the topical administration of the mimetic peptide Ac_{2-26} of AnxA1 protein and the BaSp isolated from *B. atrox* venom induces the formation of new capillaries without triggering local and systemic inflammation. This is a relevant report, as Ac_{2-26} and BaSp might be both applicable to the development of new therapies in order to promote successful tissue vascularization.

AnxA1 is a glucocorticoid-induced protein initially reported as an inhibitor of inflammation (Flower, 1988). More recently, this protein has been studied as a pro-angiogenic factor. Indeed, the endogenous AnxA1 mediates endothelial cell proliferation and migration, interacting with cell cycle pathways and motile cytoskeletal components (Alldridge and Bryant, 2003; Hayes et al., 2004; Pin et al., 2012; Ming Yi and Schnitzer, 2009). Apparently, the AnxA1-induced angiogenesis is rather associated with effects over the cytoskeleton and proliferating pathways than with inflammatory mediation, as the formation of new blood vessels regulated by AnxA1 is independent of inflammatory conditions

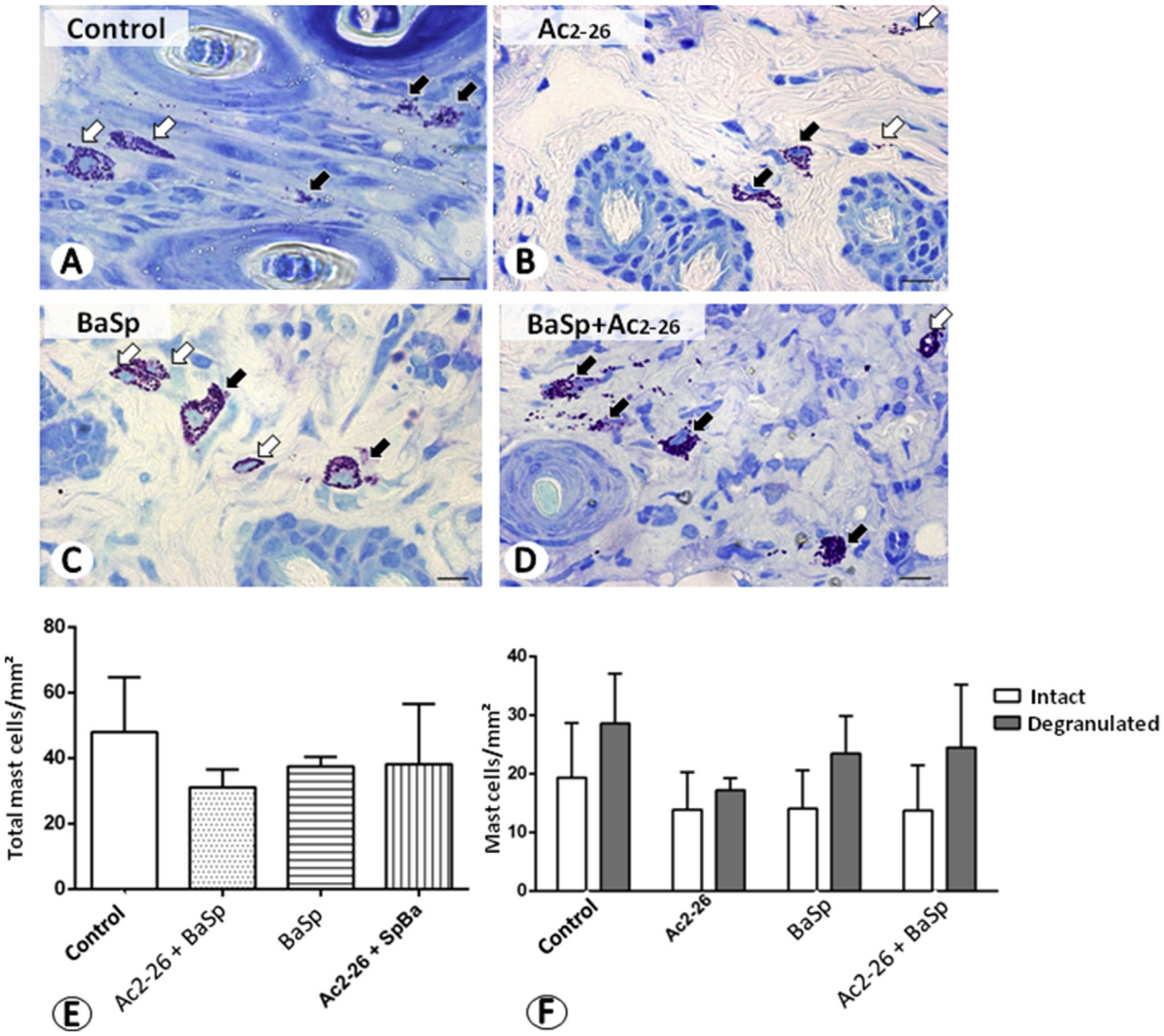

Fig. 2. Analysis of mast cells on skin after topical treatments with (A) Control. (B) Ac_{2-26}. (C) BaSp. (D) BaSp + Ac_{2-26}. Intact mast cells (white arrows). Degranulated mast cells (black arrows). Stain: Toluidine blue. Bars: 20 μm. Quantification: Total mast cells (E). Intact and degranulated mast cells/mm^2 (F). Values expressed in Mean ± S.E.M. (n = 5 animals/group). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(Ming Yi and Schnitzer, 2009). In our investigation, the mimetic peptide of AnxA1, Ac_{2-26}, displayed the same roles attributed to the endogenous AnxA1 in mediating downregulation of pro-inflammatory cytokines, recruitment and transmigration of leukocytes and mast cell activation (Bing et al., 2016; Gastardelo et al., 2009; Stuqui et al., 2015). After Ac_{2-26} treatment, we observed increased number of capillaries accompanied by unaltered levels of IL-1β, IL-4, IL-6, IL-10 and TNF-α, circulating leukocytes (PMNs/PBMCs), tissue neutrophils and total/degranulated mast cells. Ac_{2-26} is already known to reproduce the anti-inflammatory effects of the endogenous AnxA1, however, there is no literature about how this peptide affects neovascularization and, to our knowledge, this is the first report about the Ac_{2-26} pro-angiogenic property.

The results obtained from Ac_{2-26} treatment were similar to the observed after BaSp topical administration. Serine proteases are well known by their pro-angiogenic role, as Kunitz-type serine protease inhibitors, such as Amblyomin-X and PIVL, suppress endothelial cell migration, adhesion and tube formation in vitro (Drewes et al., 2012; Morjen et al., 2014). The snake venom serine proteases (SVSPs) extracted from *B. brazilis* and *B. atrox* (BaSp) were also shown to increase in vitro spreading and tubulogenesis of human umbilical vein endothelial cells (HUVEC) in a Matrigel® system (Bhat et al., 2016).

Despite their pro-angiogenic role, serine proteases are not pro-inflammatory enzymes. In mice, SVSPs from *B. jararaca* do not regulate paw edema, leukocyte-endothelium interactions in the cremaster muscle or the transmigration of leukocytes into the peritoneal cavity (Zychar et al., 2010). The inhibition of endogenous serine proteases by Amblyomin-X does not alter the levels of prostaglandin E_2 (PGE_2) and nitric oxide (NO) as well (Drewes et al., 2012). Indeed, our findings also show that the BaSp does not alter the recruitment of leukocytes into blood (PMN and PBMC) and tissue (neutrophils and mast cells), the degranulation of mast cells or the expression of the cytokines IL-1β, IL-4, IL-6, IL-10 and TNF-α. These results were interestingly reproduced in both presence and absence of the mimetic peptide Ac_{2-26} and suggest a direct role of

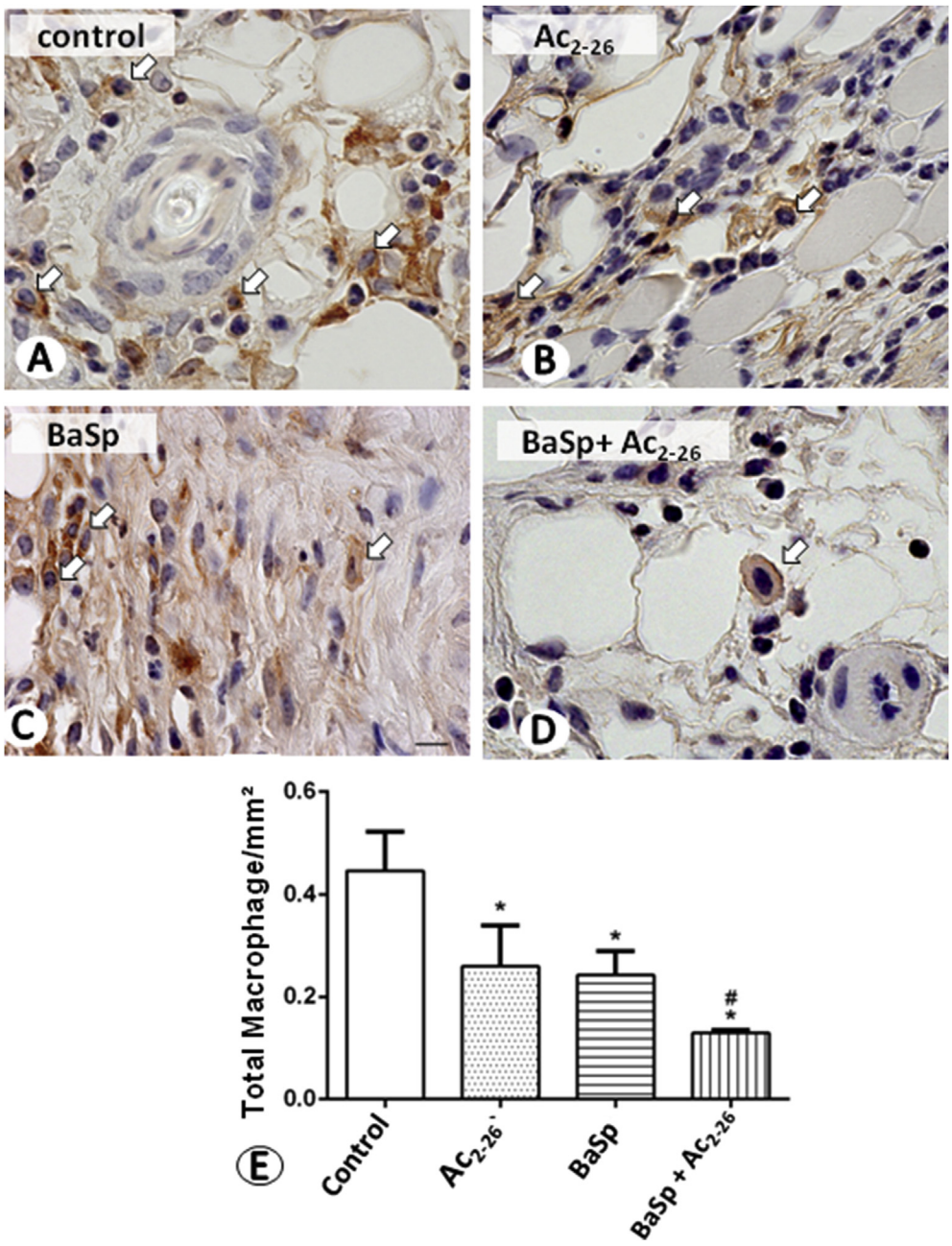

Fig. 3. Macrophages analyzes after treatments with Ac_{2-26} and/or BaSp. Immunohistochemistry and quantitative analysis of macrophages/mm^2 (day 9). (A) Control. (B) Ac_{2-26}. (C) BaSp. (D) BaSp + Ac_{2-26}. Macrophages (white arrows). Counterstaining: Hematoxylin. Bars: 10 μm. (E) Total macrophages/mm^2 in skin fragments. Values are mean ± S.E.M (n = 5 animals/group). *P < 0.005 vs Control, #p < 0.05 vs Ac_{2-26}.

both mediators over endothelial cells, once other angiogenic messengers were not affected by the treatment.

Synergistic effects between Ac_{2-26} and BaSp were not reported until the present study, however, an increasing body of evidence about their effects over cell proliferation and immune mediation seems to show that they act though similar manners to promote angiogenesis. Recently, it was demonstrated that BaSp mediates the neovascularization via activating Phosphatidylinositol 3-Kinase/Protein Kinase Akt (PI3K/Akt) (Bhat et al., 2016). This pathway stimulates the activation of endothelial nitric oxide synthase (eNOS), which in turn promotes endothelial cell migration and neovascularization (Kawasaki et al., 2003). The PI3K pathway is also activated by the binding of the protein AnxA1 with its formyl-peptide receptors (FPRs) 1 and 2, which can also be targeted by the peptide Ac_{2-26} (Gavins et al., 2007; Richard et al., 2009). By that binding and/or other interactions with phospholipids, AnxA1 might directly upregulate the PI3K/Akt pathway (Gavins et al., 2007; Yi and Schnitzer, 2009). These data suggest that BaSp and AnxA1/Ac_{2-26} may act similarly to stimulate cell proliferation and migration, as we show similar results from the topical administration of both compounds, combined or not. Further mechanistic investigations must be carried out to confirm this suggestion.

Considering the classical description of VEGF and macrophages in angiogenesis induction, it is interesting to observe that BaSp and/or Ac_{2-26} increased the number of new blood vessels simultaneously with unaltered levels of VEGF and decreased number of

macrophages. VEGF is a well characterized pro-angiogenic cytokine that induces endothelial cell growth, proliferation, differentiation and survival (Carmeliet and Jain, 2011; Olsson et al., 2006). Macrophages are also known as potent mediators of neovascularization by degrading the extracellular matrix (ECM), secreting signals of vascular guidance, inflammatory cytokines related to angiogenesis and the VEGF itself. Additionally, macrophages are a relevant source of VEGF (Kalucka et al., 2017), and by that we may suppose that we did not detect an increase in the levels of this cytokine as a result of the diminished number of these VEGF-producing cells in the same experimental groups in which we observed significant angiogenesis. Concerning the role of macrophages in angiogenesis, this cell has been surprisingly associated with inhibition of angiogenesis in some specific conditions. In a model of age-related macular degeneration (AMD), macrophages were correlated with inhibition of neovascularization (Apte et al., 2006). Other authors suggested the relevance of macrophages in the process of abnormal vasculature regression in the anterior segment of the eye (Lobov et al., 2006). Indeed, the lack of either macrophage chemoattractant protein-1 (MCP-1) or its cognate C-C chemokine receptor-2 (CCR-2) in mice culminated in spontaneous choroidal neovascularization (CNV) (Ambati et al., 2003). Although there are no reports about the role of macrophages in the angiogenic process promoted by BaSp and/or Ac_{2-26}, our results show a simultaneous increase in the number of new blood vessels and a decrease in tissue macrophages, therefore, there could be other specific conditions in which macrophages exhibit anti-angiogenic properties.

In summary, our data indicate that the BaSp and/or Ac_{2-26} peptide administered topically in the skin promote new blood vessel growth in similar manners, as this process does not induce systemic or local inflammatory responses. Our study provides important contribution for the possible therapeutic use of these mediators in processes that require tissue angiogenesis and regeneration. This hypothesis should be confirmed with other experimental models in order to completely validate its relevance in physiological angiogenesis.

Ethical statement

The experiments were performed in strict accordance with the Brazilian laws of Ethical of protection and this study was approved by the Ethics Committee in Animal Experimentation of São Paulo State University (IBILCE/UNESP) (Nº. nº112/2015).

Competing financial interests

The authors declare no competing financial interests.

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