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High concentration of C-type natriuretic peptide promotes VEGF-dependent vasculogenesis in the remodeled region of infarcted swine heart with preserved left ventricular ejection fraction☆

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ABSTRACT

Background: Vasculogenesis is a hallmark of myocardial restoration. Post-ischemic late remodeling is associated with pathology and function worsening. At the same time, neo-vasculogenesis helps function improving and requires the release of vascular endothelial growth factor type A (VEGF-A). The vasculogenic role of C-type natriuretic peptide (CNP), a cardiac paracrine hormone, is unknown in infarcted hearts with preserved left ventricular (LV) ejection fraction (EF). We explored whether myocardial VEGF-dependent vasculogenesis is affected by CNP.

Methods and results: To this end, infarcted swine hearts were investigated by magnetic resonance imaging (MRI), histological and molecular assays. At the fourth week, MRI showed that transmural myocardial infarction (MI) affected approximately 13% of the LV wall mass without impairing global function (LVEF > 50%, n = 9). Increased fibrosis, metalloproteases and capillary density were localized to the infarct border zone (BZ), and were associated with increased expression of CNP ($p = 0.03$ vs. remote zone (RZ)), VEGF-A ($p < 0.001$ vs. RZ), BNP, a marker of myocardial dysfunction ($p < 0.01$ vs. RZ) and the endothelial marker, factor VIII-related antigen ($p < 0.01$ vs. RZ). In vitro, CNP 1000 nM promoted VEGF-dependent vasculogenesis without affecting the cell growth and survival, although CNP 100 nM or a high concentration of VEGF-A halted vascular growth.

Conclusions: CNP expression is locally increased in infarct remodeled myocardium in the presence of dense capillary network. The vasculogenic response requires the co-exposure to high concentration of CNP and VEGF-A. Our data will be helpful to develop combined myocardial delivery of CNP and VEGF-A genes in order to reverse the remodeling process.

1. Introduction

The presence of structural remodeling in hearts with preserved left ventricular (LV) ejection fraction (EF) following myocardial infarction (MI) is associated with a high rate of hospitalization [1]. Left ventricular (LV) remodeling after myocardial infarction (MI) occurs in response to cell loss and increased mechanical and neuro-humoral stress, and involves alterations in both ventricular architecture and paracrinicity [2,3]. In particular, post-infarction LV remodeling involves myocyte survival and hypertrophy, increased release of humoral factors (i.e.: growth factors, cytokines, hormones), increased myocardial recruitment and activation of stem/progenitor cells [4], mast cells [5], macrophages and fibroblasts [6], altered collagen deposition and degradation by metalloproteinases and impaired cellular cross-talk. This scenario may affect triggers of myocardial repair and regeneration. Neo-vasculogenesis is a hallmark of myocardial restoration in remodeled heart and its extent may affect contractile function [7]. However, the events that regulate capillary formation and organization during late remodeling in infarcted hearts with preserved LV function are still poorly understood [8,9].

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A key trigger of the pathways leading to post-ischemic vasculogenesis is the release of vascular endothelial growth factor type A (VEGF-A) [10]. During prolonged VEGF-A release, the enlargement of pre-existent collateral network and the formation of new vessels is insufficient to rescue chronically ischemic myocardium [11]. It is therefore conceivable that other endogenous paracrine signals might control the extent of vasculogenic response locally [3].

Cardiac natriuretic peptides (NPs) are autacoids with auto-/paracrine effects that protect cardiomyocytes from ischemia–reperfusion injury [12], yet their role in post-ischemic myocardial vasculogenesis is poorly understood.

Despite a great deal of interest in the relationship between cardiac remodeling and local release of brain natriuretic peptide (BNP) [13], less interest has been focused on C-type natriuretic peptide (CNP), which is expressed in cardiomyocytes [14], coronary endothelial [15] and smooth muscle cells [16] and might play a homeostatic role in the heart through activation of natriuretic peptide receptor-B (NPR-B) [17,18].

At present, evidence for cardioprotective effects of CNP is controversial. For example, recent studies have reported that activation of the CNP/NPR-B pathway following MI promotes apoptosis of cardiomyocytes [19], as well as anti-proliferative and anti-hypertrophic effects in adult cardiac cells [17,20]. Conversely, prolonged administration of exogenous CNP limits late cardiac remodeling after MI in vivo [21]. Furthermore, the expression of CNP is higher in the LV region with a better myocardial function in a pre-clinical model of non-ischemic heart failure (HF) [22]. However, to our knowledge, the relationship between CNP and VEGF-dependent vasculogenesis in post-ischemic remodeled myocardium has not been investigated. The aim of this study was to explore whether CNP expression is related to post-ischemic vasculogenic response, and, more specifically, whether VEGF-dependent vasculogenesis is affected by CNP. To this end, a swine model of MI, multimodal evaluation of regional LV late remodeling by magnetic resonance imaging (MRI), histology and molecular biology, and in vitro assays on HUVECs culture were employed.

2. Methods

2.1. Study design

Fourteen healthy male adult farm pigs (35–40 kg) were randomly assigned to one of two experimental groups: 1) MI (n = 9), or 2) sham-operated (control, n = 5). Three animals in the MI group died from ventricular fibrillation after coronary ligation. Four weeks after surgery, we investigated the cardiac performance of sedated animals by MRI (1.5 T, Signa Excite HD; GE Medical Systems, Waukesha, WI). Animals were then sacrificed by the infusion of a cold cardioplegia solution in order to collect cardiac tissue for molecular and histological analysis. In addition, we performed in vitro experiments to investigate the vasculogenic role of CNP during cardiac remodeling.

The authors of this manuscript have certified that they comply with the Principles of Ethical Publishing in the International Journal of Cardiology.

2.2. Myocardial infarction model and tissue collection

General anesthesia was induced with propofol (2–4 mg/kg, intravenously [iv]) and maintained with 1–2% isoflurane in 60% air and 40% oxygen. After thoracotomy, we induced MI by permanent surgical ligation of the left anterior descending (LAD) coronary artery below the second diagonal branch [23]. Sham operated animals were not subjected to LAD occlusion. LV tissue samples were collected from the infarct BZ and non-infarcted RZ from infarcted hearts, and from the LV of healthy pigs (N), and were immediately placed in ice-cold RNA stabilization agent (RNAlater) and stored at −80 °C.

This protocol was approved by the Italian Ministry of Health and was in accordance with Italian law (DL-116, Jan. 27, 1992), which conforms to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

2.3. Cardiac MRI measurements

Cine-MRI images were acquired using a non-breath-hold electrocardiogram (ECG)-gated, steady-state free precession pulse sequence (Fast Imaging Employing Steady State Acquisition) [23]. Global (end-diastolic and end-systolic volumes, ejection fraction) and regional LVWT and LV functional parameters were measured as described previously [23–25]. To detect and quantify post-infarct myocardial fibrosis, Gd-DE images were acquired in two-dimensional segmented inversion recovery-prepared gradient echo-sequence, 10 min after administration of contrast agent Gd-DTPA (0.2 mmol/kg iv), in short-axis views. The infarcted myocardium was quantified as the percentage of total myocardium. Data were analyzed in blinded fashion, under the supervision of cardiologists with expertise in the interpretation of MRI data.

2.4. Molecular analysis

RNA extraction: Total RNA was extracted by the acid guanidinium thiocyanate-phenol-chloroform method from LV tissue samples obtained from pig hearts with RNeasy Midi kit (Qiagen S.p.A, Milano, Italy) as previously described [22,26–28]. The RNA samples were stored at −80 °C for subsequent use in gene expression studies.

RT-PCR: Following DNAse treatment, first strand cDNA was synthesized using the iScript cDNA Synthesis kit (Bio-Rad, Hercules, CA, USA), using about 1 μg of total RNA as a template.

Real-Time (RT)-PCR was performed on a 96-well iQ Cycler RT-PCR Detection System (Bio-Rad). Annealing temperature varied, depending on the primer pair used (Table 1). A series of PCRs were performed using primer pairs for CNP and NPR-B. In the same samples, we also evaluated expression of BNP and TNF-α as an internal and inflammatory marker, respectively. Primer pairs were designed with Primer Express Version 2.0 (Applied Biosystems) and details are shown in Table 1. All reactions were performed in duplicate.

2.5. Histological analysis

Five micrometer-thick sections of LV tissue samples from the infarct BZ, RZ and healthy myocardium were used for immuno-/histological analysis. Serial slices were stained with hematoxylin/eosin and Van Gieson's stain to assess myocardial architecture and fibrosis, respectively, and to detect CNP expression. For each sample, two independent observers evaluated a minimum of three serial sections. Myocardial expression of CNP was detected by immunohistochemistry, using a rabbit anti-CNP antibody (CNP-53, Phoenix Pharmaceuticals, Inc., CA, USA) diluted 1:800 in 1% bovine serum

Table 1
Primers pairs.

Gene	Primers	Annealing T°	GeneBank
C-type natriuretic peptide (CNP)	5′-AAGACCGTTTATCATTCCATTTC-3′ 5′-GCTTCTGACCTTGCCAAA-3′	60°	M64758
B-type natriuretic peptide (BNP)	5′-TTCTTGTTGCCAGTCCTC-3′ 5′-GCTAGTGAATGCTGTCCAA-3′	64°	M25547
Natriuretic peptide receptor-B (NPR-B)	5′-TAAGAATGAGCATTACCGTACC-3′ 5′-GCGAGCATCCAGATACAG-3′	60°	DQ487044
Tumor necrosis factor - α (TNF-α)	5′-TGACCACCACCAAGAATT-3′ 5′-TGTTCTGAAGTATTCCGATTG -3′	60°	NM_214022
Glyceraldehyde 3-phosphate dehydrogenase(GAPDH)	5′-TCGGAGTGAACGGATTTG-3′ 5′-CCTGGAAGATGGTGATGG-3′	59°	AF017079
Cyclophilin A (CypA)	5′-GGGTGGTGACTTCACACGCCA-3′ 5′-TTGGGAACCGTTTGTGTTGGGGC -3′	60°	AY266299
TATA-binding protein (TBP)	5′-GATGGACGTTCGGTTTAGG-3′ 5′-AGCAGCACAGTACGAGCAA-3′	59°	DQ178129
Beta-actin (ACTB)	5′-TCTGGCACCACACCTTCT-3′ 5′-TGATCTGGGTCATCTTCTCAC-3′	60°	DQ178122

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albumin/phosphate buffered saline (BSA/PBS). Specimens were examined using a semi-quantitative scale of immunoreactivity, consisting of no (−), low (+) and high (++) staining. Photomicrographs were taken using a DFC480 digital camera (Leica Microsystem, Cambridge, UK). Myocardial expression of MMP-9 was detected by immunohistochemistry, using a rabbit anti-MMP-9 antibody (MMP-9, Santa Cruz Biotechnology, Inc., CA, USA). Expression of VEGF-A was measured as marker of angiogenesis, using a rabbit anti-VEGF antibody (VEGF, Santa Cruz Biotechnology, Inc.) diluted 1:100 in 3% BSA/PBS.

The density of the vascular network was evaluated by immunohistochemical visualization of factor VIII-related antigen. Myocardial factor VIII-related antigen expression was detected using a mouse anti-factor-VIIIa antibody (Ventana Medical Systems, Inc., AZ, USA), as recommended by the Ventana staining procedure. Counts were performed by two independent researchers using a light microscope at 400× magnification on 10 representative fields. Negative controls were obtained by incubating specimens in the absence of the primary antibody.

2.6. Myocardial collagen expression

Myocardial collagen mRNA expression was measured by PCR using collagen (coll)-3α primer (273 bp) (sense: 5′-TGGCCCTGTTTGCTTTTTAT-3′, antisense: 5′-CCATTGAGACAT TTGAAATTGG-3′, 24 cycles, annealing temperature 61 °C) in BZ, RZ and N samples.

2.7. Cell isolation and culture

Neonatal cardiac myocytes were isolated from hearts of three-day-old Wistar rats by enzymatic digestion, as described previously [14]. Briefly, the beating heart was explanted from anesthetized rats and placed in ice, rinsed with 10 ml HBSS (Lonza Group Ltd., Switzerland). The heart was minced into 1-mm^3 pieces and digested with trypsin (50 μg/ml, Sigma) and collagenase IV (1 mg/ml). Tissue digestion was filtered through a Sigma Cell Strainer. Isolated cells were centrifuged at low speed (65 ×g) to minimize non-cardiomyocyte contamination. The supernatant, containing the cardiomyocyte-enriched fraction was plated in cell culture medium at a density of 1.5×10^5 cells/ml. Cardiomyocytes were identified through immunofluorescent staining with an anti-alpha sarcomeric actin antibody (Sigma, A7811, clone EA-53) and they were used when the yield reached 100%.

2.8. Cell viability assay

HUVECs from Cambrex Bio Science Inc (Walkersville, MD, USA), and cardiomyocytes were seeded in 24-well plates and treated with or without CNP 10 nM, 100 nM or 1000 nM. After 24, 48, 72 and 96 h of treatment with or without CNP, soluble tetrazolium salt [3–(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT) (Sigma-Aldrich Corp., St. Louis, MO, USA) was added to each well for 3 h. The supernatant fluid was then removed and dimethylsulfoxide was added. After shaking the samples to dissolve formazan, absorbance was measured at 570 nm using a microplate reader. All measurements were performed in triplicate.

2.9. VEGF assay

The release of $VEGF_{165}$ from HUVECS exposed to conditioned medium was assayed using a commercially available enzyme-linked immunosorbent assay (ELISA; R&D Systems, Minneapolis, MN, USA), according to the manufacturer's protocol. All samples were assayed at least in triplicate.

2.10. In vitro vasculogenesis assay

The in vitro vasculogenesis assay was performed as described previously [29]. To evaluate the effect of CNP on capillarogenesis, CNP 10 nM, 100 nM, 1000 nM was added to the culture medium. In control conditions, HUVECs were cultured in the presence of endothelial growth medium-2 (EGM-2). To evaluate the effect of conditioned medium on HUVEC-derived vasculogenesis, conditioned medium was mixed with EGM-2 (1:1) without fetal bovine serum. Capillary-like structures were observed over the following 24-hour period and were photographed using a light microscope equipped with a digital sight camera (Nikon). To monitor the same field at different time points, we randomly selected four or five spots on the bottom of the culture wells with a fine-point marker and used these marks as a guide for further analysis. The length of all the capillaries within a selected area was drawn by hand on the computer screen, and the length was calculated by converting the pixels to micrometers, according to calibrated scales that were digitized in the same way as the vessels. We also measured the number of branching points that subdivide a vascular zone into smaller regions and play an important role in revascularization of ischemic tissue [30].

2.11. Caspase-3 activity

Caspase-3 activity was measured using a commercial kit (Fluorimetric Caspase 3 Assay Kit, Sigma-Aldrich Corp., St. Louis, MO, USA), according to the manufacturer's instructions. Measurements were performed in triplicate.

2.12. Data analysis and statistics

Several reference genes were tested and GeNorm software was used to establish the most stably expressed genes in each chamber, as described previously [31,32].

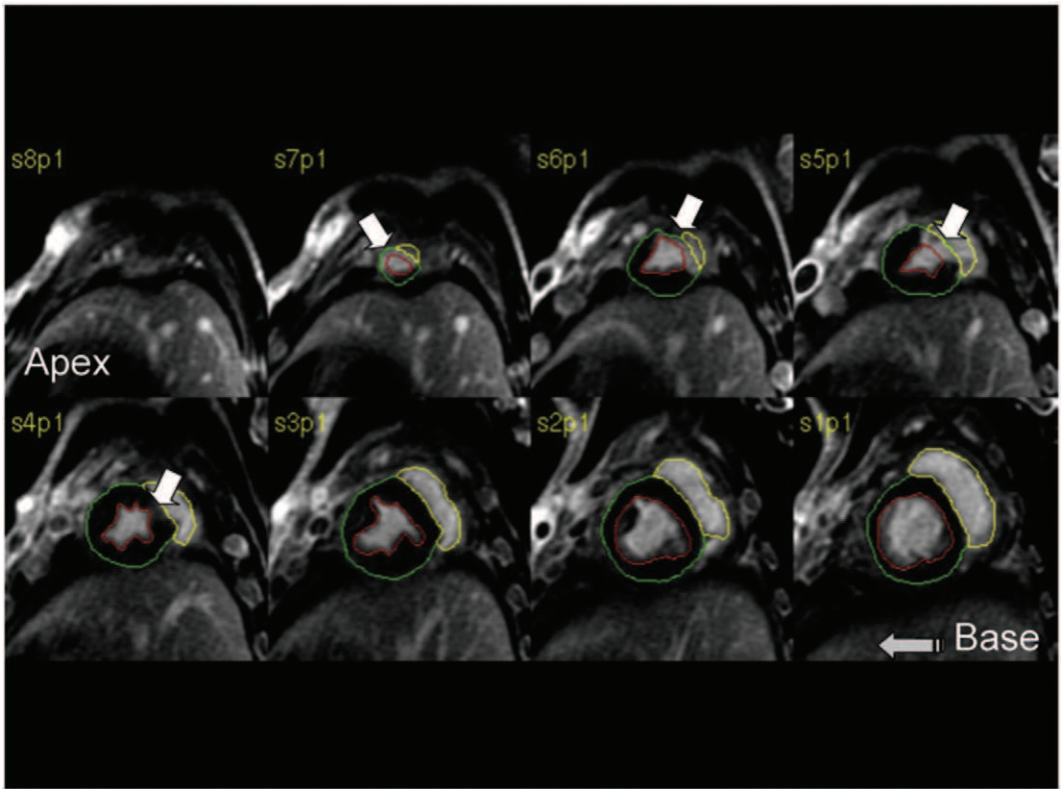

Fig. 1. Representative examples of LV Gd-DE MRI short-axis images used to measure the dimension of the infarct scar. White arrows indicate the LV infarct scar from left ventricular base to apex.

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The geometric mean of the four most stably expressed genes (cyclophilin A [CypA], TATA-binding protein [TBP], beta actin [ACTB], and glyceraldehyde-3-phosphate dehydrogenase [GAPDH]) was used for normalization of each gene mRNA expression in the samples. The relative quantification was performed by the ΔΔCt method using the iQ5 Software (BioRad). Differences between more than two independent groups were analyzed by Fisher's test after ANOVA. The results are expressed as mean ± s.e.m. and $p < 0.05$ was considered significant.

3. Results

3.1. In vivo cardiac MRI measurements

We measured the infarct size and extent of ventricular remodeling in infarcted pig hearts and compared cardiac performance parameters in infarcted and control hearts using in vivo gadolinium-delayed contrast-enhanced (Gd-DE) magnetic resonance imaging (MRI), four weeks after coronary ligation. In infarcted hearts, transmural infarction affected 13 ± 1% of the LV wall mass, as shown in Fig. 1. Four weeks after coronary ligation, LV ejection fraction and volume were not significantly different in infarcted and sham-operated hearts (Table 2). In contrast, end-systolic left ventricular wall thickening (LVWT) in the infarct border zone (BZ) was reduced compared with the non-infarcted remote zone (RZ) that was located opposite the infarcted region, and healthy myocardium (N) in sham-operated animals (Table 2).

3.2. Regional expression of CNP and NPR-B in infarcted myocardium

Four weeks after MI, expression of CNP mRNA, revealed by Real-Time PCR, was significantly increased in the infarct BZ compared with the RZ, and was similar between the RZ of infarcted hearts and healthy myocardium in sham-operated animals (N: 2.44 ± 2.07; BZ: 15.46 ± 7.62; RZ: 2.19 ± 0.79; $p = 0.03$ BZ vs. RZ; Fig. 2a). These findings were confirmed by immunohistochemical analysis (Fig. 3a).

Conversely, NPR-B mRNA expression was similar in infarct BZ, RZ and controls (N:1.33 ± 0.11; BZ: 1.25 ± 0.22; RZ: 1.34 ± 0.39; Fig. 2b).

Expression of BNP mRNA, a marker of ventricular dysfunction, was significantly higher in the infarct BZ than in controls ($p = 0.0016$) and RZ ($p = 0.0016$) (N: 0.04 ± 0.02; BZ: 16.23 ± 4.60; RZ: 2.25 ± 1.32; Fig. 2c). We also measured the expression of mRNA for the inflammatory marker, tumor necrosis factor (TNF)-α. TNF-α mRNA expression was similar in the infarct BZ, non-infarcted RZ, and normal myocardium (N: 0.41 ± 0.25; BZ: 1.60 ± 0.19; RZ: 0.17 ± 0.04).

3.3. Regional myocardial structure and fibrosis

Activation of the remodeling process following MI was demonstrated by increased expression of matrix metalloproteinase-9 (MMP-9) in the infarct BZ compared with normal myocardial tissue from sham-operated animals (Fig. 4; N: −/+ cardiomyocytes, − vessels; BZ: ++ cardiomyocytes, ++ vessels; RZ: + cardiomyocytes, − vessels). In addition, expressions of the angiogenic cytokine vascular endothelial growth factor A (VEGF-A) and the endothelial cell marker factor VIII-related antigen were significantly higher in the infarct BZ compared with healthy myocardium (Figs. 5, 6). We also evaluated the density of the vascular network, defined as the overall length of capillaries and the number of branch points, which was significantly higher in the infarct BZ compared with healthy myocardium (N: 11.4 ± 085; BZ: 20.4 ± 1.47; RZ: 17.4 ± 2.8; $p < 0.001$ N vs. BZ; $p = 0.04$ N vs. RZ).

Deposition of interstitial collagen was increased in the infarct BZ compared with the non-infarcted RZ (Fig. 3b), which was consistent with the findings from the in vivo Gd-DE images shown in Fig. 1. In addition, collagen (coll)-3α gene expression was slightly increased in the BZ of infarcted LV compared with healthy myocardium.

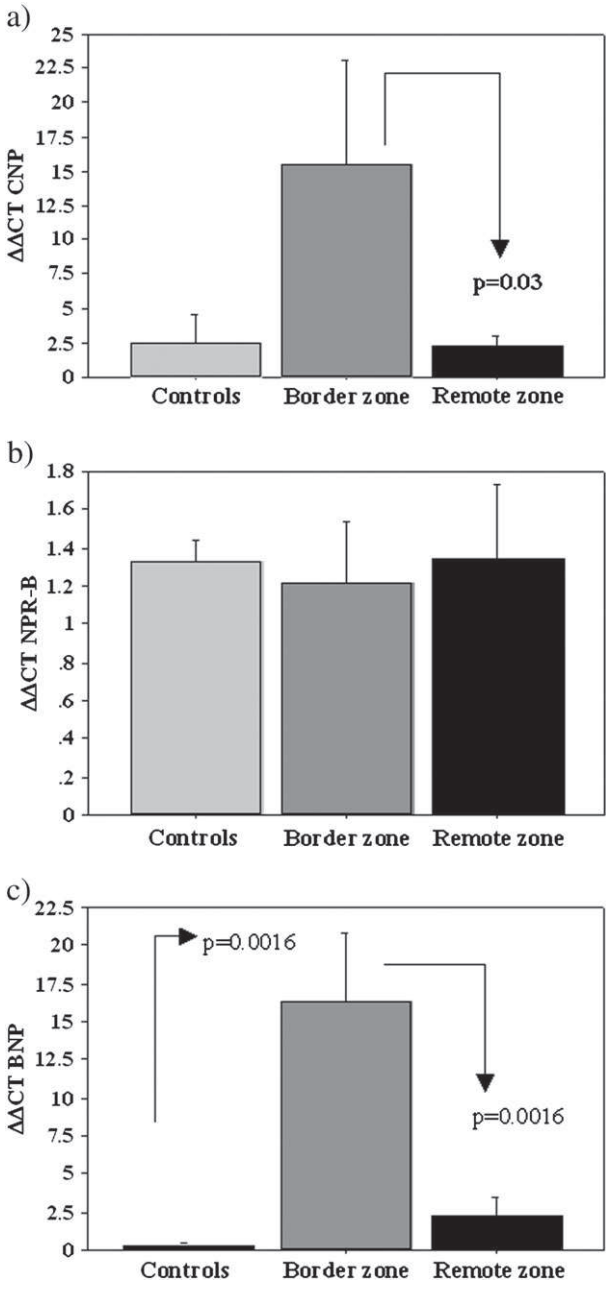

Fig. 2. Expression of natriuretic peptides and their receptor in cardiac tissue of normal animals and in animals with MI. a), b) and c) show the levels of CNP, NPR-B, and BNP mRNA, respectively measured by RT-PCR in healthy myocardium and in infarct BZ and RZ.

Table 2
Cardiac MRI measurements.

	Normal	Myocardial infarction
Heart rate (beats/min)	90 ± 5.77	89.60 ± 8.2
Mean aortic pressure (mm Hg)	118.33 ± 10.7	124.43 ± 11.5
ES volume (ml)	21.71 ± 5.5	24 ± 1.7
ED volume (ml)	60.86 ± 4.2	65.5 ± 4.5
Cardiac output (l/min)	4.62 ± 0.3	4.72 ± 0.4
Stroke volume (ml)	39.15 ± 1.34	41.5 ± 3.2
Ejection fraction (%)	64.32 ± 6.85	63 ± 1.5
End-systolic wall thickening Border zone (%)	64.16 ± 9.69	*8.4 ± 16.6
End-systolic wall thickening Remote zone (%)	68.45 ± 12.5	62 ± 9.3

* $p<0.05$.

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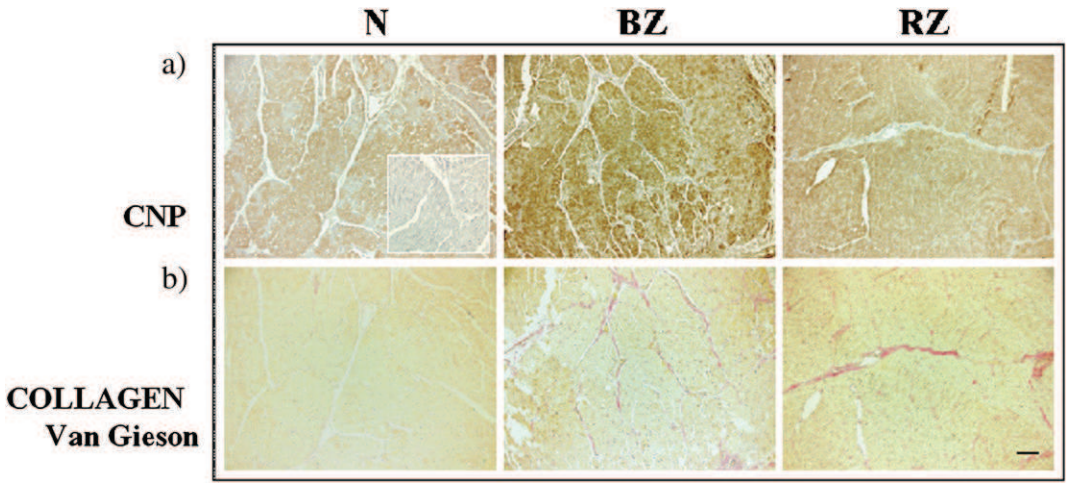

Fig. 3. Immunohistochemical staining of CNP and collagen in healthy myocardium and in BZ and RZ from infarcted hearts. a) The CNP immunoreaction (*brown*) shown by BZ of MI was higher (++) compared with N and RZ (+). Negative control (square) did not show any immunoreaction (−). (Scale bar 100 μm) b) Van Gieson's stain: collagen staining (*red*) was increased in BZ with respect to the N and RZ (Scale bar 100 μm).

Together, these findings indicate that changes in myocardial structure following MI were confined to the infarct BZ.

3.4. In vitro effects of CNP on vasculogenesis

To evaluate the effects of CNP on vasculogenesis, we incubated human umbilical vein endothelial cells (HUVECs) with different doses of CNP. 100 nM CNP, but not 10 nM or 1000 nm, significantly decreased total capillary length and the number of branching points per field compared with untreated cells ($p < 0.05$; Figs. 7, 8).

HUVECs cultured in conditioned medium, obtained from cardiomyocytes exposed to CNP 100 nM or 1000 nM for 24 h, formed fewer capillary-like structures than untreated cells and had a significantly lower total capillary length and number of branching points per field compared with untreated cells (Figs. 7, 8).

3.5. Effects of CNP on VEGF release in vitro

We also measured the level of the angiogenic cytokine $VEGF_{165}$ in cultured HUVECs exposed to conditioned medium, obtained from cardiomyocytes exposed to different concentrations of CNP (10 nM, 100 nM, and 1000 nM) for 24 h. Following exposure to conditioned medium from cardiomyocytes exposed to 1000 nM CNP, the concentration of $VEGF_{165}$ was significantly increased ($p < 0.05$), whereas the level was reduced by CNP 100 nM, as shown in Fig. 8.

3.6. Effects of CNP on caspase-3 activity

To determine if CNP induced apoptosis, we measured the activity of the apoptosis marker caspase-3 in HUVECs exposed to CNP 10, 100, or 1000 nM and conditioned medium obtained from cardiomyocytes exposed to CNP 10, 100, or 1000 nM. CNP, either directly or in conditioned medium did not induce caspase-3 activity under any experimental condition (Fig. 8). Interestingly, low doses of CNP significantly reduced the growth of endothelial cells compared with untreated cells ($p < 0.05$), whereas the proliferation of endothelial cells was not affected by exposure to conditioned medium.

4. Discussion

In addition to cell loss, insufficient angiogenesis is locally responsible for severe myocardial remodeling following MI. In vivo experiments have shown impaired myocardial perfusion in LV regions of infarcted heart in the presence of capillary density [23,33]. Despite this evidence of functionally inadequate vasculogenesis, several pre-clinical trials of gene therapy have shown that over-expression of myocardial VEGF might prevent [34] or limit [35] late myocardial remodeling through induction of angiogenesis. It is conceivable that the magnitude of VEGF-dependent vascular response is influenced by local biological factors. Even though endogenous CNP influences

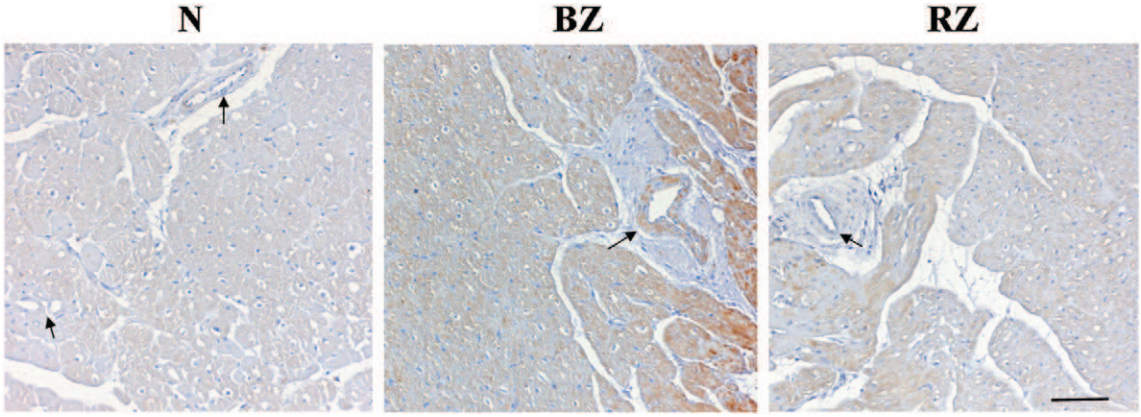

Fig. 4. Section of cardiac tissue from normal myocardium, infarct BZ and RZ stained for MMP-9. The arrows show the vessels. Scale bar: 100 μm. (N: −/+ cardiomyocytes, − vessels; BZ: ++ cardiomyocytes, ++ vessels; RZ: + cardiomyocytes, - vessels).

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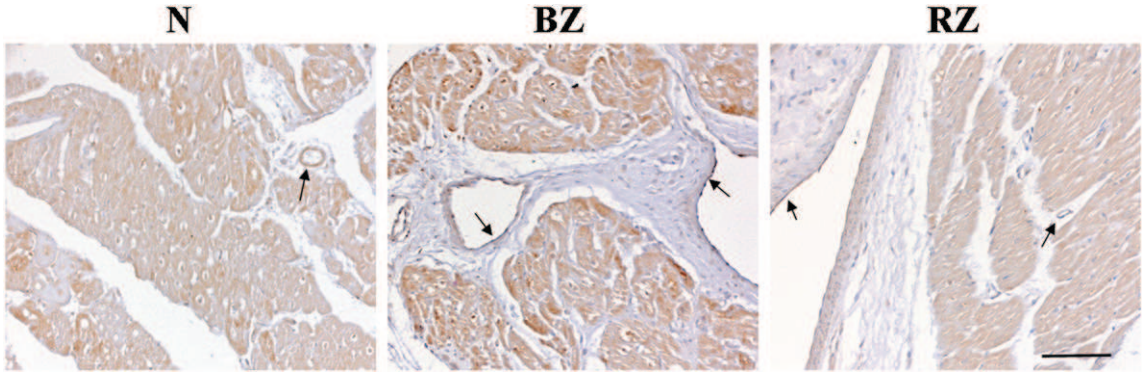

Fig. 5. Immunohistochemical expression of VEGF in normal myocardium, infarct BZ and RZ. The arrows show endothelial cells. VEGF expression was significantly higher in the infarct BZ compared with healthy myocardium. Scale bar: 100 μm.

the viability and proliferation of cardiac cells, its vasculogenic role in myocardial remodeling is still undefined.

Our findings showed that CNP and VEGF were over-expressed in the LVBZ. At the fourth week after MI, the expression of CNP and VEGF was associated with increased LV capillary density, higher BNP concentration, and extracellular matrix remodeling. The expression of CNP in the non-infarcted RZ was not increased and was similar to that of normal healthy myocardium from sham-operated animals. These findings suggest that in addition to the vasorelaxant effect of BNP [36], CNP acts as an endogenous modulator of VEGF-dependent vasculogenesis during the development of post-ischemic remodeling, even in absence of global cardiac dysfunction.

We have previously shown constitutive expression of CNP in cardiomyocytes and endothelial cells [14], and CNP administration has been reported to significantly limit myocardial collagen deposition in infarcted LV regions in vivo [21]. However, to date, the regional impact of post-ischemic remodeling on endogenous transcription, and the action of CNP in the LV infarct area had not been investigated.

Our data show that increased CNP transcription occurred mainly in cardiomyocytes bathed in a rich pattern of collagen type III and I fibers. Thus, it is conceivable that ischemic environment elicits the adaptive expression of CNP in cardiomyocytes. Although CNP protects cardiomyocytes from ischemic insult through the NP receptor C-type (NPR-C) [37], the predominant cardiac CNP receptor is NPR-B [18,27,38]. In LV remodeled regions of our animal model, the NPR-B mRNA expression was normal, whereas NPR-C mRNA was increased three-fold compared to RZ (data not shown).

Several autocrine and paracrine signals are modulated during post-ischemic myocardial remodeling [3], and these may tune the CNP/NPR-C signal pathway activity. Concerning pro-inflammatory mediators, it has been established that cytokines play an important role during early remodeling, up to the second week after acute MI. The level of cytokine expression is correlated with infarct size, and is significantly lower in small versus large infarcts [39]. In our model, 4 weeks after MI, the TNF-α level in the LVBZ was similar to non-infarcted myocardium, regardless of the presence of regional ischemic remodeling shown by the increased expression of MMP-9 in the same LV region. These data are consistent with previous reports [40–42].

Regional myocardial remodeling depends on the magnitude of the angiogenic response in the healing area, which is mediated mainly by VEGF-A [2,43]. After MI, VEGF-A is rapidly expressed in the LVBZ and persists for up to three months [44]. Our results show that increased VEGF expression is localized to the coronary endothelial cells in the remodeled LV myocardium, where CNP is also expressed. Thus, CNP-VEGF cross-talk between endothelial cells and cardiomyocytes is possible.

Our in vivo findings are clinically relevant for two reasons: first, our model resembles the clinical condition of MI with preserved LVEF [9]. A recent study did not find any significant correlation between infarct size and LV ejection fraction, when the infarct size was less than 15%; in our animals, the infarct scar size is 12–13% of the LV mass. Second, the absence of myocardial reperfusion in our model is consistent with clinical persistence of severe microvascular obstructions in patients undergoing thrombolytic therapy and angioplasty after acute transmural MI, worsening the clinical outcome [39,45,46].

However, our in vivo findings in part contest those of Doi et al. who reported that VEGF suppressed endothelial CNP expression and release in a dose-dependent manner [47]. Therefore, it was helpful to investigate the CNP-VEGF relationship in vitro. We found that CNP 100 nM significantly inhibited of the growth, sprouting and vascular formation of HUVECs without affecting their viability, whereas CNP 1000 nM maintained vasculogenesis of viable cells and was associated with higher expression of VEGF-A. Conversely, the exposure of HUVECs to medium from cardiomyocytes exposed to CNP for 24 h halted capillarogenesis without affecting cell growth or inducing activation of caspase-3. Hence, maintenance of the growth and viability of endothelial cells does not ensure an adequate vasculogenesis. In

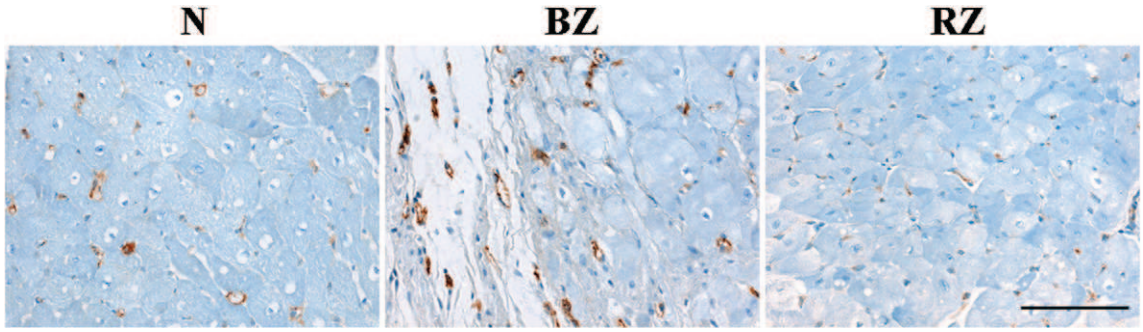

Fig. 6. Immunohistochemical expression of the endothelial marker factor VIII-related antigen in normal myocardium, infarct BZ and RZ. Expression of Factor VIII-related antigen was significantly higher in the infarct BZ compared with healthy myocardium. Scale bar: 100 μm.

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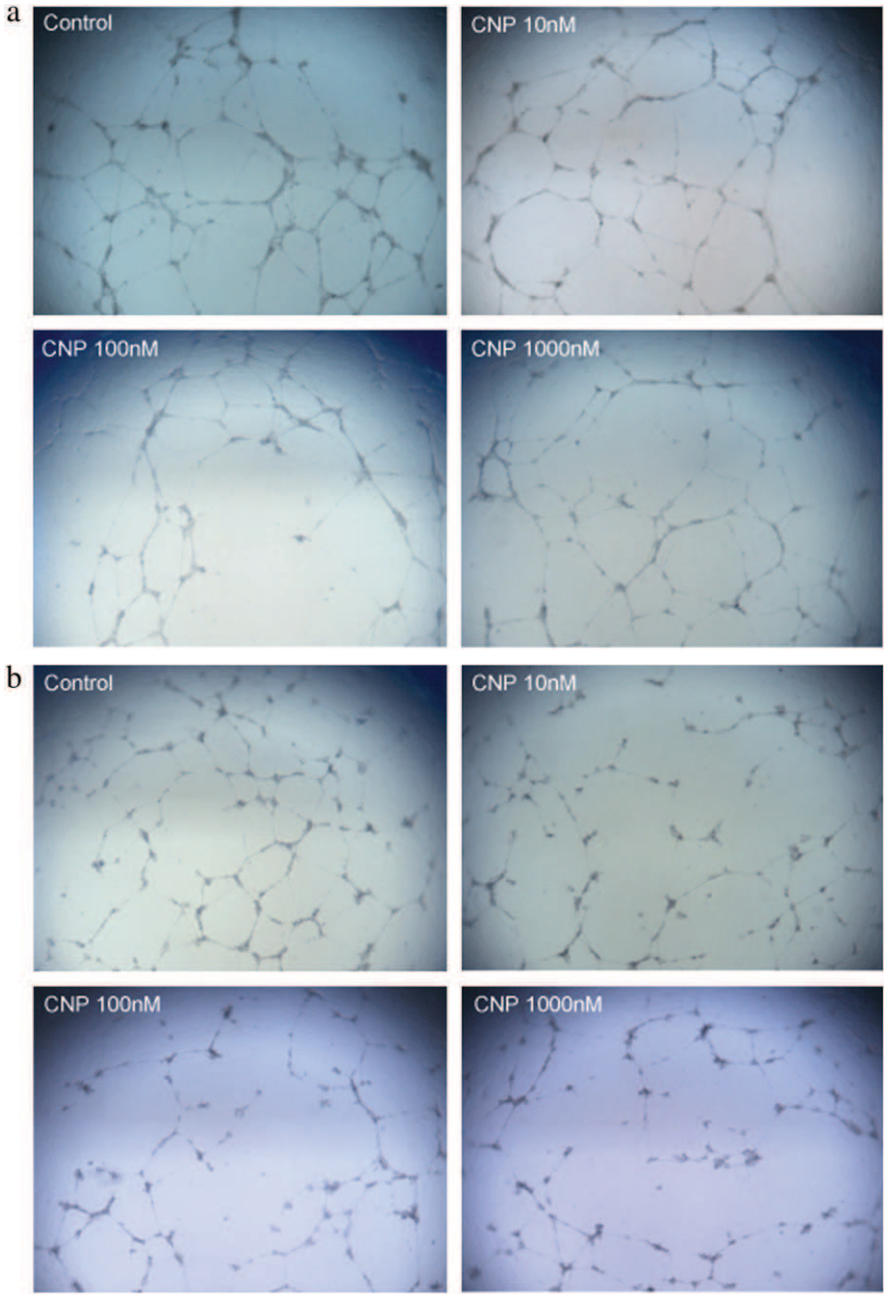

Fig. 7. In vitro capillarogenesis assessed in a) HUVECs exposed to vehicle (control) or CNP at increasing doses (10, 100, 1000 nM), and b) in HUVECs exposed to conditioned medium obtained from cardiomyocytes cultured for 24 h in the absence (control) or presence of different concentrations of CNP.

regard to downstream factors of CNP-VEGF cross talk, it is conceivable that a high level of VEGF released by cardiac cells exposed to CNP enhances the vasculogenic response. Surprisingly, we found that the in vitro vasculogenic response of HUVECs was significantly reduced in presence of culture medium containing a high level of VEGF-A. Our data argue that the capillarogenesis requires the co-exposure of endothelial cells to VEGF-A and a high concentration of CNP.

It is possible that CNP-dependent anti-apoptotic effects are also enhanced by VEGF. In fact, VEGF inhibits caspase-3-dependent apoptosis of cardiomyocytes in the absence of mechanical stress [34,48].

4.1. Limitations

Our investigation provides new information generated by a multimodal in vivo and in vitro analysis. However, an important limitation of the study is the use of wild type experimental models, with no changes in genetic profile, to study in vivo and in vitro the local CNP-VEGF relationship. This was very convenient to obtain a consistent experimental preparation in order to investigate the vasculogenic role of CNP in the absence of adaptive responses associated with genetic manipulations. Further studies will be necessary to test whether knockdown or upregulation of myocardial CNP expression locally affects the vasculogenic response in remodeled myocardium.

4.2. Conclusions

Our findings suggest that combined effects of CNP and VEGF-A play a role in vasculogenesis and cell survival in the local vasculogenic response in remodeled myocardium without mechanical stress due to global LV dysfunction. High levels of CNP are adequate to maintain

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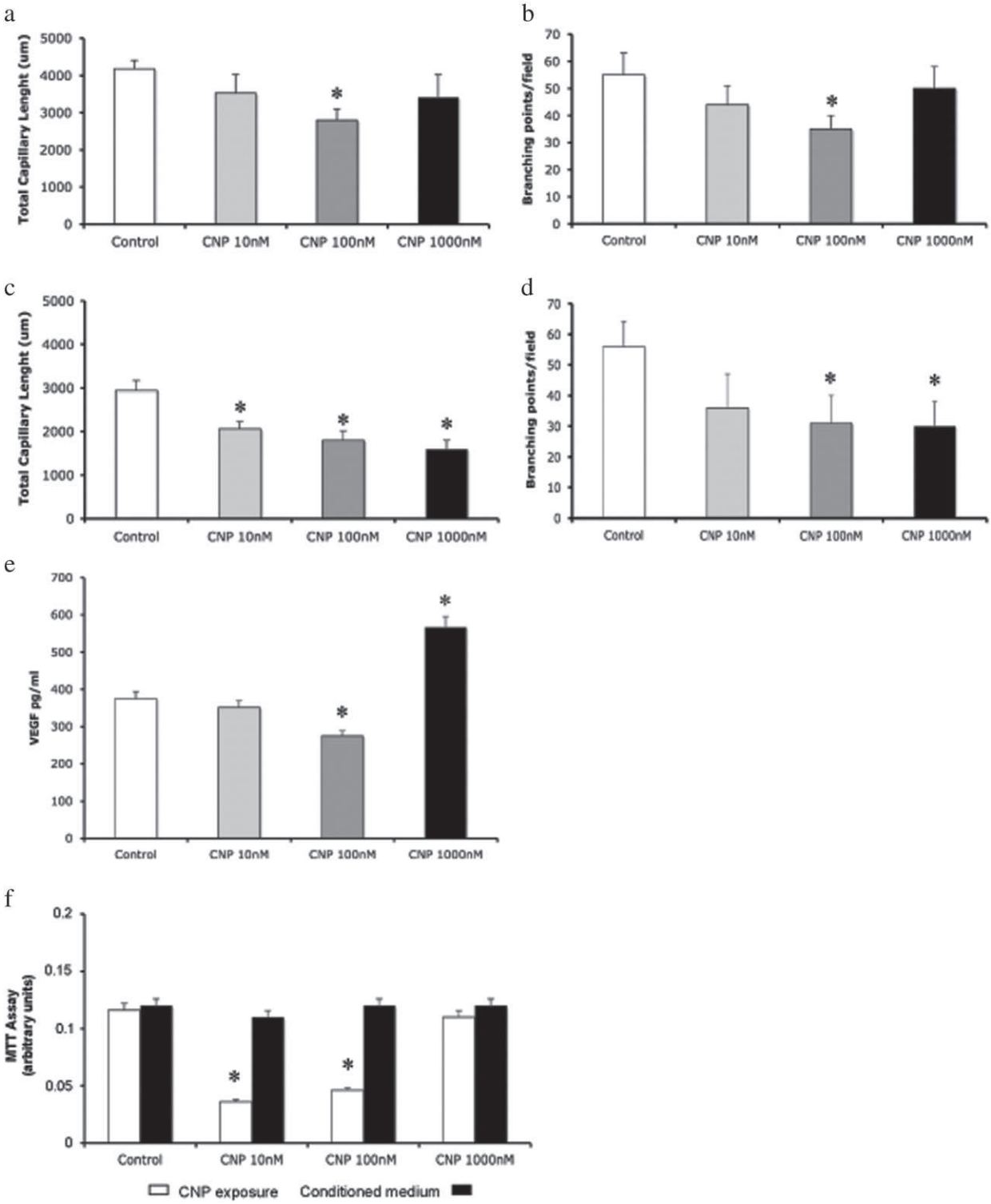

Fig. 8. Morphological characteristics of capillary-like networks in HUVECs exposed to CNP (a, b) or to conditioned medium (c, d), evaluated using NIS-Elements D Nikon software (version 3.06). VEGF release (e) was assessed by ELISA in conditioned medium obtained from cardiomyocytes cultured for 24 h in the absence (control) or presence of CNP. (f) HUVECs growth and proliferation were assessed by MTT assay in each experimental condition. Data are expressed as mean ± standard error; n = 3, *significantly different from control ($p < 0.05$).

VEGF-dependent vasculogenesis without affecting the survival and growth of endothelial cells. Conversely, a high level of VEGF-A alone is insufficient to maintain vasculogenesis in HUVECs. The next challenge will be to determine how CNP leads to modulation of the VEGF pathway, and whether combined myocardial delivery of CNP and VEGF genes might reverse the remodeling process.

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