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Different effects of catechin on angiogenesis and inflammation depending on VEGF levels☆

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Abstract

Although physiological and pathological angiogenesis develop through similar processes, during pathological angiogenesis, proangiogenic factors are exacerbated. Polyphenols have been considered therapeutic tools for conditions exhibiting enhanced angiogenesis. However, the possibility that these compounds may also prevent vascularization in physiological situations is a major drawback for their use. The purpose of the current study was to investigate the effects of 0.1–100 μM catechin on endothelial cells (EC) and vascular smooth muscle cells (VSMC) regarding angiogenic and inflammatory processes. Catechin modulation of angiogenesis and inflammation was also evaluated *in vivo* using different models of angiogenesis: one physiological (skin wound-healing assay) and another one resembling pathological angiogenesis, exhibiting higher vascular endothelial growth factor (VEGF)-A stimulation (Matrigel plug assay). The *in vitro* results showed that 100 μM catechin increased viability (to 165.58% and to 165.34%) and decreased apoptosis (53.45% and 92.65%) and proliferation (33.19% and 23.36%) of EC and VSMC, respectively. Catechin affected migration and invasion, tending to increase both in EC and decreasing them in VSMC; however, it did not change sprouting angiogenesis. Nevertheless, catechin diminished *in vitro* inflammatory modulators such as tumor necrosis factor α (58.66% for human umbilical vein endothelial cells and 85.46% for human aortic smooth muscle cells) and nuclear factor kappa-B (38.43% for VSMC). The *in vivo* results demonstrated that catechin did not change angiogenesis and inflammation in skin wound-healing model and substantially decreased these processes in Matrigel plug assay. Altogether, the current study showed that catechin has different effects in angiogenesis and inflammation depending on VEGF-A levels. The absence of adverse effects in mature vasculature favors catechin potential use against pathological situations where angiogenesis is stimulated.

Keywords: Angiogenesis; Inflammation; Blood vessels; Endothelial cells; Smooth muscle cells; Catechin

1. Introduction

Angiogenesis refers to the growth and remodeling process of an existent vasculature to form a new branching network, characteristic of mature blood vessels. In the presence of a proangiogenic stimulus, local endothelial cells (EC) change their shape, degrade and invade the extracellular matrix. During this process, EC proliferate, forming tubular structures that coalesce with other newly forming vessels. These new structures are ultimately covered by pericytes or vascular smooth muscle cells (VSMC), giving rise to mature and stable blood vessels that enable adequate blood flow and prevent further sprouting. A decrease in pericyte coverage of blood vessels has been associated with increased vessel permeability and tumor metastasis [1,2]. The interplay between the two vascular wall cells, EC and VSMC, is now getting more attention. Under physiological conditions, angiogenesis is highly regulated by the balance between a huge number of pro- and antiangiogenic factors, acting synergistically to maintain functionality of blood vessels [3]. In the adult, angiogenesis maintains physiological homeostasis and tissue integrity during wound healing, inflammation, endometrial growth in menstrual cycle and following ischemia [3,4]. Despite angiogenesis being mandatory for the repair of damaged tissue, when deregulated, it can induce tissue damage in inflammatory disorders and other conditions like diabetes, cardiovascular disease and cancer [5,6].

Vascular endothelial growth factor (VEGF)-A is the best studied and the most potent proangiogenic factor known. VEGF-A belongs to a family of vascular endothelial growth factors together with VEGF-B, VEGF-C, VEGF-D and also PlGF. The cellular signalization stimulated by these molecules occurs through three transmembrane receptors: VEGFR1, VEGFR2 and VEGFR3 [7]. VEGF-A stimulates physiological and pathological angiogenesis, especially through VEGFR2 signalization. It is also a ligand for VEGFR1, through which receptor it can also regulate inflammation and angiogenesis [8]. During angiogenic stimulation, several cell types like inflammatory, tumor and mural cells produce VEGF-A, which increases vascular permeability and proliferation and sprouting of EC. VEGF-B and PlGF bind with high affinity to VEGFR1. VEGF-B seems important in embryonic angiogenesis and in energy metabolism [8,9]. PlGF is particularly implicated in pathological angiogenesis [8]. VEGF-C and VEGF-D are ligands for

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VEGFR2 and VEFGR3. The increase in vascular permeability induced by VEGF-C and VEGF-D has been associated to activation of VEGFR2, but their important function as regulators of lymphangiogenesis has been related to VEGFR3 activation [10,11]. However, Tammela et al. demonstrated recently that VEGFR3 is highly expressed on sprouting EC, sustaining proliferation, migration and viability of EC independently of VEGFR2 stimulation, suggesting that VEGFR3 may be also implicated in the angiogenic process [12]. Inflammatory cells produce diverse inflammatory and angiogenic factors such as tumor necrosis factor (TNF)α, nuclear factor kappa-B (NFκB) and VEGF-A. The inflammatory reaction stimulated by TNFα involves NFκB stimulation, which also regulates VEGF-A expression [13]. VEGF-A also mediates inflammation [14], reinforcing the close interrelation between inflammation and angiogenic processes and VEGF-A production. Skin wound repair requires the formation of new blood vessels, with VEGF-A being a key player for this physiological process. Although physiological and pathological angiogenesis develops through many similar processes, pathological angiogenesis as occurs in tumors, due to angiogenic stimulus like hypoxia and inflammation-related events, does not regress. In contrast, it persists stimulated by tumor-secreted factors [1]. In tumor angiogenesis, VEGF-A functions not only as a paracrine mediator as in physiological angiogenesis, but also as an autocrine factor [15], playing crucial roles in both angiogenesis and inflammation.

Inflammation has a central role in fighting pathogens and in wound healing. It is a complex biological process involving several cell types and inflammatory mediators. When not properly controlled, it can result in adverse effects on surrounding tissues and in chronic inflammation. Several evidences demonstrate that inflammation and angiogenesis depend on each other [16]. EC-immune cells cross-talk results in the regulation of blood vessels formation and functionality by immune cells and microenvironment [1,17].

It is increasingly recognized that several fruits, vegetables and some beverages like tea, wine and beer possess health-promoting effects that have been attributed to polyphenol compounds, ubiquitously found in the plant kingdom. Indeed, apart from their physiological roles in plants, these compounds are important components in the human diet. Several biological activities of polyphenols have been described. They seem to have beneficial effect on pathologies like cancer, cardiovascular diseases and diabetes and in the prevention of the establishment of neurodegenerative diseases, obesity and aging [18–22]. Experimental studies have been focused on the anti-inflammatory, antioxidant and anticancer activities of plant polyphenols, showing that they may also modulate cell signaling [23,24]. Epigallocatechin-3-gallate (EGCG), a polyphenol from green tea, inhibits interleukin (IL)-1β-dependent proinflammatory signal transduction via NFκB-dependent pathway in epithelial cells [24]. It also down-regulates cyclooxygenase-2 in stimulated human mammary epithelial cells [25] by decreasing the activation of extracellular signal-regulated protein kinase and p38 mitogen-activated protein kinases. EGCG and other catechins reduce nitric oxide (NO) production by inhibition of the inducible nitric oxide synthase (iNOS) expression and activity in rodent cell lines after stimulation with lipopolysaccharide [26], apparently by preventing binding of NFκB to the promoter of the iNOS gene.

Tea and wine are two of the most popular consumed polyphenol-rich beverages worldwide. Of all polyphenols present in nature, flavonoids comprise the most abundant group and, within this group, flavanols are the most frequent ones. Catechin (Cat) and epicatechin are the most common flavanols in fruits. Cat is especially abundant in fruits such as cocoa, grape, apple, apricot and cherry and in beverages such as tea and red wine [27].

The antiangiogenic effect of plant-derived polyphenols has been a matter of study in the last decade. Namely, inside the flavanol group, EGCG, the most abundant flavanol in green tea, has been extensively studied concerning anticancer properties and angiogenesis modulation.

However, studies regarding the effects of Cat in angiogenesis and particularly in both EC and VSMC are almost inexistent to date.

Taking these findings together, the purpose of the current study was to investigate the effects of this abundant polyphenol, Cat, on several steps of the angiogenic process in both EC and VSMC. The *in vivo* modulation of angiogenesis and inflammation by this polyphenol was also evaluated using two different models of angiogenesis: a physiological one (skin wound-healing assay) and another one resembling pathological angiogenesis, exhibiting higher VEGF-A stimulation (Matrigel plug assay), as VEGF-A is the most potent and well-known stimulator of both physiological and pathological angiogenesis.

2. Methods and materials

2.1. Cell cultures

Human umbilical vein endothelial cells (HUVEC) were obtained from ScienceCell Research Labs (San Diego, CA, USA). Cells were used between passages 3 and 8 and cultured in M199 medium (Sigma-Aldrich, Portugal) supplemented with 20% fetal bovine serum (FBS) (Invitrogen Life Technologies, Scotland, UK), 1% penicillin/streptomycin (Invitrogen Life Technologies, Scotland, UK), 0.01% heparin (Sigma-Aldrich, Portugal) and 30 μg/ml endothelial cell growth supplement (Sigma-Aldrich, Portugal) in plates coated with 0.2% gelatin (Sigma-Aldrich, Portugal). They were maintained at 37°C in a humidified 5% carbon dioxide atmosphere. Human aortic smooth muscle cells (HASMC) were obtained from ScienceCell Research Labs (San Diego, CA, USA), kept between passages 2 and 8 and cultured in Dulbecco's modified Eagle's medium, supplemented with 10% FBS and 1% penicillin/streptomycin and cultured at 37°C in a humidified 5% carbon dioxide atmosphere. Cat (Sigma-Aldrich, Portugal) was dissolved in ethanol and then added to cell culture medium at a concentration of 0.1–100 μM, established according to the viability assays performed. Cat and vehicle (ethanol) were added to cell cultures in medium supplemented with 2% FBS and 1% penicillin/streptomycin. Control cells were incubated with vehicle (ethanol). Ethanol concentrations were kept below 0.1% in every culture.

2.2. Cell viability

HUVEC and HASMC were allowed to grow until 70%–80% confluence and then incubated with 0.01–100.0 μM Cat or ethanol for 24 h. After the incubation period, cells were washed twice with phosphate-buffered saline solution and subjected to 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay as previously described [28]. Briefly, cells were incubated with MTT solution at a final concentration of 0.5 mg/ml for 3 h and then lysed in dimethylsulfoxide. Optical density was measured at 540 nm, and the background absorbance measured at 660 nm was subtracted. All samples were assayed at least in three independent experiments in duplicate, and the mean value for each experiment was calculated. The results are given as mean±S.E.M. and are expressed as percentage of control, which was considered to be 100%.

2.3. Cell apoptosis

HUVEC and HASMC (1×10^4 cells/ml) were grown on glass coverslips and incubated with different concentrations (1–100 μM) of Cat for 24 h. TUNEL assay was performed using the *In Situ* Cell Death Detection kit (Roche Diagnostics, Switzerland), as reported before [28,29]. The percentage of stained cells was evaluated by counting the cells stained with TUNEL (apoptotic cells) divided by the total number of nuclei stained with DAPI (Roche Diagnostics, Switzerland) at a ×200 magnification field.

2.4. Cell proliferation

HUVEC and HASMC cultures (1×10^4 cells/ml) were established on glass coverslips and were allowed to grow until 70%–80% confluence following treatment procedures with 1–100 μM Cat for 24 h. Cell proliferation analyses were carried out using cellular incorporation of 5'-bromodeoxyuridine (BrdU), a thymidine analogue. After incubation with BrdU solution at a final concentration of 0.01 mM for 24 h, the number of proliferating cells (positive for BrdU), after immunohistochemistry methods using anti-BrdU-specific antibodies (BrdU *In Situ* Detection Kit, BD Biosciences Pharmingen, USA), was evaluated at the microscope according to the manufacturer's instructions.

2.5. Migration assay

HUVEC and HASMC cultures (1×10^4 cells/ml) were established on glass coverslips and allowed to form confluent monolayers. A scratch was applied through the monolayer using a sterile 200-μl pipette tip. Cells were washed and treated with 100

μM Cat or 0.1% ethanol for 24 h. Cell migration was evaluated by counting the number of cells that migrated through the injured area.

2.6. Invasion capacity

The invasive cell behavior in the presence of 1–100 μM Cat for 24 h was quantified *in vitro* using a double-chamber assay by counting the number of cells that invaded a Transwell BD-Matrigel basement membrane matrix inserts (BD-Biosciences, Belgium), according to manufacturer's instructions. FBS was used as a chemoattractant. Results represent the ratio between invading cells in polyphenol-treated cultures compared to invasion in control cultures for the same initial amount of cultured cells.

2.7. Capillary-like structures formation

Cells were cultured on growth-factor-reduced Matrigel-coated plates (GFR-Matrigel, BD Biosciences, Belgium) for 24 h as previously described [29]. Briefly, HUVEC were cultured on GFR-Matrigel-coated plates for 24 h in medium containing 1–100 μM Cat or vehicle. When cultured on Matrigel, EC assemble into capillary-like structures. The number of cord-like structures was then counted on an inverted microscope. Each cord portion between the ramifications was considered one cord unit. Mean values were obtained by evaluating the whole cultures of each well under the same treatment. Treatments were performed as described above. A semiquantitative measurement of cord formation in GFR-Matrigel cultured HUVEC was developed as previously described [29].

2.8. Aortic ring assay

Aortic ring assay was performed as an *ex vivo* assay to evaluate microvessel outgrowth. In brief, thoracic aorta were removed from normal Wistar rats and washed in phosphate-buffered saline. After removing fibroadipose tissue, aortas were cross-sectioned, washed in cell media and placed on Matrigel-coated 24-well plates, embedded on Matrigel (BD Biosciences, USA) and cultured in adequate cell medium for 24 h. Cat or ethanol was then added to cell cultures in medium supplemented with 2% FBS. Aortic rings were visualized on an inverted microscope and photographed 4 days after incubations.

2.9. TNFα quantification

TNFα was quantified in 200-μl culture medium in contact with HUVEC and HASMC after 24 h of cell treatment with 50–100 μM Cat or 0.1% ethanol by enzyme-linked immunosorbent assay (ELISA) (TNF-α/TNFSF1A Immunoassay, R&D Systems, Abingdon, UK) according to the manufacturer's instructions. Quantification was performed at 450 nm and 550 nm using a plate reader (Thermo Electron Corporation, Multiskan Ascent, USA).

2.10. NFκB activity assay

NFκB activity was determined by ELISA assay. HUVEC and HASMC were treated with 50–100 μM Cat or 0.1% ethanol for 24 h. Nuclear extracts were prepared from the nuclear extraction kit (Active Motif, CA, USA). NFκB activity was measured using TransAM NFκB p65/p50 transcription factor assay kit (Active Motif, CA, USA). In brief, nuclear extract samples (5 μg) were added to biotinylated oligonucleotide containing the NFκB consensus site. This mixture was then added to a streptavidin-coated 96-well plate. Sample wells were incubated with NFκB p65 subunit primary antibody followed by incubation with HRP-conjugated secondary antibody. Quantification was performed at 450 nm and 650 nm using a plate reader (Thermo Electron Corporation, Multiskan Ascent, USA).

2.11. NO determination

NO level was determined as the concentration of nitrate plus nitrite in the extracellular medium in contact with HUVEC and HASMC after 24 h of cell treatment with 50–100 μM Cat or 0.1% ethanol and also in animal serum by colorimetric assay. Serum was incubated with equal volume of Griess reagent in a 96-well microtiter plate for 15 min at room temperature. Measurement was performed in a spectrophotometer plate reader at 550 nm. Data were expressed as NO concentration (μM).

2.12. In vivo studies

Animal experiments were conducted according to accepted standards of humane animal care [Declaration of Helsinki, European Community guidelines (86/609/EEC) and Portuguese Act (129/92) for the use of experimental animals]. All the authors involved in animal studies in the present study had received accreditation from the Portuguese Veterinarian Administration as a competent person for animal experimentation (investigator–coordinator) since 2009.

2.13. Skin wound-healing assay

Wistar rats (Charles River, Wilmington, MA, USA), 8–12 weeks old, were used and kept individually in their cages during the study. After general anesthesia, dorsal skin of the rat was shaved and cleaned. Full-skin-thickness longitudinal incisions (1.5 cm) were created on the dorsal surface of the rat, and the wound edges were closed with surgical sutures at 0.5-cm intervals. Cat or vehicle (ethanol+water, 1+5; C) was administered topically (50 μl of a 50-μM solution) daily. Rats were examined daily for wound-healing progression. After 7 days, wounded tissue was collected for histology studies, and blood was also used for evaluation of inflammatory factors. Skin wound tissue specimens were then collected, fixed in 10% neutral-buffered formalin and embedded in paraffin. Histological and immunohistochemistry analyses were performed in 5-μm tissue sections.

2.14. Immunohistochemistry analysis

Microvessel density was evaluated in each formalin-fixed paraffin-embedded wounded tissue section. Tissue slides were incubated with an anti-von-Willebrand factor antibody (Millipore, MA, USA). Capillaries were then counted in the three tissue sections for each animal and normalised to the total area of the tissue section. Negative controls were carried out by omission of the primary antibody in tissue sections expressing the marker.

2.15. Determination of N-acetylglucosaminidase (NAG) activity

The NAG enzyme is present at high levels in activated macrophages. Inflammation can be evaluated by measuring the levels of the lysosomal NAG enzyme in the serum. Serum was incubated for 10 min at 37°C with 100 μl of *p*-nitrophenyl-*N*-acetyl-beta-D-glucosaminide solution in a 96-well plate. The reaction was stopped by the addition of 0.2 M glycine buffer (pH 10.6), and the substrate hydrolysis was measured at 405 nm.

2.16. IL-1β measurement

IL-1β was quantified in mice and rats serum by ELISA (IL-1β-EASIA kit, BioSource, Nivelles, Belgium) according to the manufacturer's instructions.

2.17. Matrigel plug assay

A mixture of Matrigel and heparin without (negative control, C−) or with recombinant vascular endothelial growth factor (VEGF-A) (positive control, C+) and 100 μM Cat was subcutaneously inoculated into C57BL/6 mice (purchased at Charles River, Wilmington, MA, USA). The animals were euthanized after 7 days; the Matrigel plug was removed, weighed and photographed; and the amount of hemoglobin (Hb) in the homogenized plug was measured, as described below. Mice blood was also collected for evaluation of inflammatory factors.

2.18. Hb determination

The Hb content of the plug was evaluated after homogenization of the plug in a water–heparin solution, which was then centrifuged at 1500*g* for 15 min at 20°C. The supernatant (100 μl) was used to measure the Hb content according to Drabkin's method (Sigma-Aldrich, Portugal) at 540 nm.

2.19. Statistical analyses

Every cell experiment was performed at least in three independent experiments. Quantifications are expressed as mean±S.E.M. and as percentage of control, which was considered to be 100%. Statistical significance of difference between various groups was evaluated by analysis of variance followed by the Bonferroni test. For comparison between two groups, Student's *t* test was used. A difference between experimental groups was considered significant with a confidence interval of 95% whenever $P \leq .05$.

3. Results

3.1. Micromolar concentrations of Cat increased viability and diminished apoptosis and proliferation in HUVEC and HASMC

Viability was assessed by MTT assay in the two cell types upon treatment with 0.01–100 μM of Cat at 80% confluent cultures (Fig. 1A). Cat (10 and 100 μM) increased the viability of HUVEC and HASMC (increase to 165.58%±5.04% and to 165.34%±31.12% of control to 100 μM).

To understand whether these effects of Cat were related to apoptosis or cell growth, we next investigated the apoptotic and proliferative potential activity of this polyphenol. Incubation of either cell culture with 0.1–100.0 μM Cat for 24 h resulted in a significant

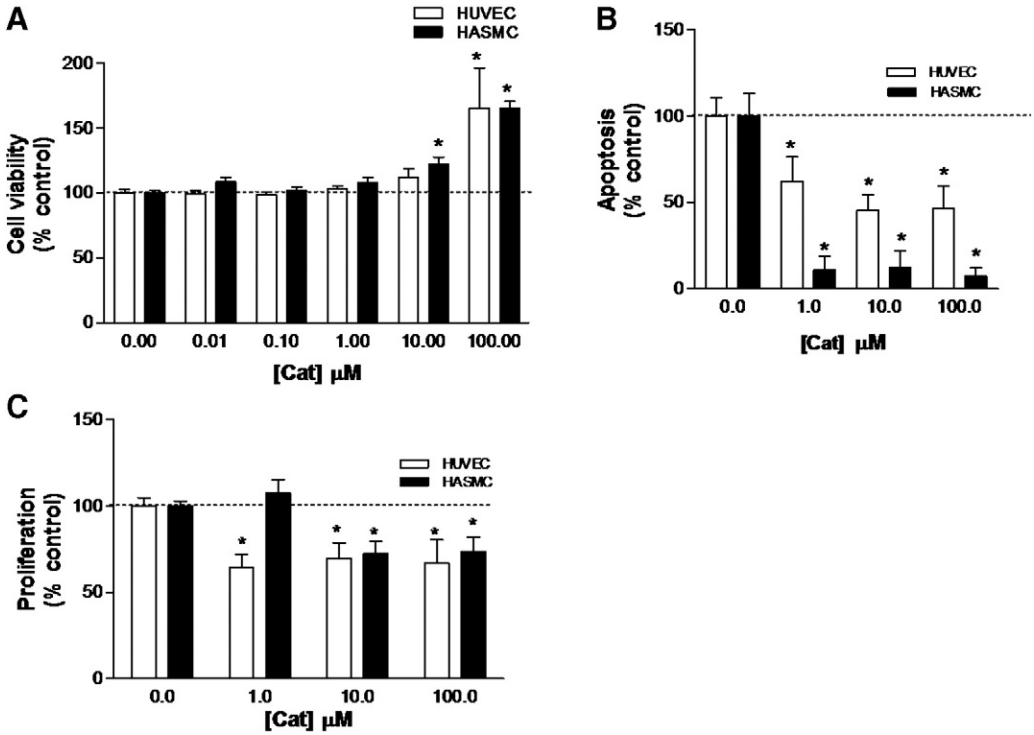


Fig. 1. Effect of Cat on (A) viability, (B) apoptosis and (C) proliferation of HUVEC and HASMC. Cells were incubated for 24 h with 0.01–100 µM Cat or vehicle (0.00). Cells' viability was evaluated by MTT assay, apoptosis was evaluated by TUNEL, and the percentage of proliferative cells was examined by the ratio between BrdU-stained cells and hematoxylin-stained nuclei in every culture. Results are means±S.E.M. of independent experiments ($4 \leq n \leq 8$) and are expressed as percentage of control. *$P \leq .05$ vs. control.

decrease in apoptosis, with statistical significance upon 1.0 µM Cat in HUVEC and 10 µM Cat in HASMC (53.45%±12.88% and 92.7%±4.85% for HUVEC and HASMC, respectively). This effect was consistently higher in HASMC (Fig. 1B). In addition, incubation with 1.0–100.0 µM Cat for 24 h also decreased cell proliferation in HUVEC (33.19%±13.56% decrease) and HASMC (23.36%±8.39% decrease) for 100 µM as assessed by BrdU assay and illustrated in Fig. 1C.

3.2. Cat increased migration of HUVEC but diminished HASMC migration and invasion capacity

Cell motility and extracellular matrix invasion are fundamental steps within the angiogenic process. Therefore, we next investigated the effects of Cat on migration and invasion capacity using the injury and double-chamber assays, respectively.

As illustrated in Fig. 2A, treatment with 100 µM Cat for 24 h increased the migration capacity of HUVEC cells in culture, while reducing HASMC migration in the same conditions. Interestingly, treatment with Cat significantly diminished invasion capacity in both cell cultures at 1.0 µM (39.66%±6.29% for HUVEC and 56.62%±14.41% for HASMC decrease) as analyzed by transwell assays (Fig. 2B). Nevertheless, as Cat concentration increased, there was a tendency to increase invasion capacity of HUVEC (to 135.43%±47.59% at 100 µM), although this did not reach statistical significance. On the contrary, invasiveness of HASMC treated with Cat tends to remain lower than invasiveness of control group but, once again, without statistical significance (reduction to 84.09%±23.94% relative to control, $P=.531$, for 100 µM) (Fig. 2B).

3.3. Formation of capillary-like structures was not affected by Cat treatment

Endothelial cells must differentiate and reorganize, assembling into vascular capillary structures, in order to form a new blood vessel. HUVEC are able to assemble into highly branched capillary-like structures when cultured on GFR-Matrigel. Therefore, we tested whether Cat treatments were able to alter the formation of *in vitro* vascular-like structures. Well-organized interconnected tubular structures were observed in every culture (Fig. 3A). Quantitative analysis revealed that no significant difference in the number of structures formed was found in any Cat-treated culture comparatively to control group (vehicle, ethanol 0.1% or 0.0 µM), except for 10 µM Cat that was able to increase the number of capillary-like structures formation to 159.70%±17.83% (Fig. 3B).

3.4. Cat did not change vessel outgrowth in aortic ring assay

Knowing that angiogenesis involves not only endothelial cells but also other cell types such as vascular smooth muscle cells present in the surrounding environment, we next confirmed the *ex vivo* effect of Cat on angiogenic sprouting by incubating rat aortic rings with 100 µM Cat or vehicle (ethanol 0.1%). Cat treatment resulted in a small network of angiogenic vessels surrounding the aortic rings very similar to that observed in ethanol-treated group (control) (Fig. 4). This finding suggests that Cat does not affect vessel formation as already observed in *in vitro* endothelial assembly into capillary-like structures (see Fig. 3A).

3.5. Cat modulated inflammatory markers in HUVEC and HASMC in a different manner

Given the strong cross-talk between angiogenesis and inflammation together with the fact that most natural polyphenols are often anti-inflammatory agents as well, we next analyzed the effects of Cat on inflammatory markers. The proinflammatory effects of TNFα are primarily due to its ability to activate NFκB that plays a central role in the inflammatory process, regulating the expression of several

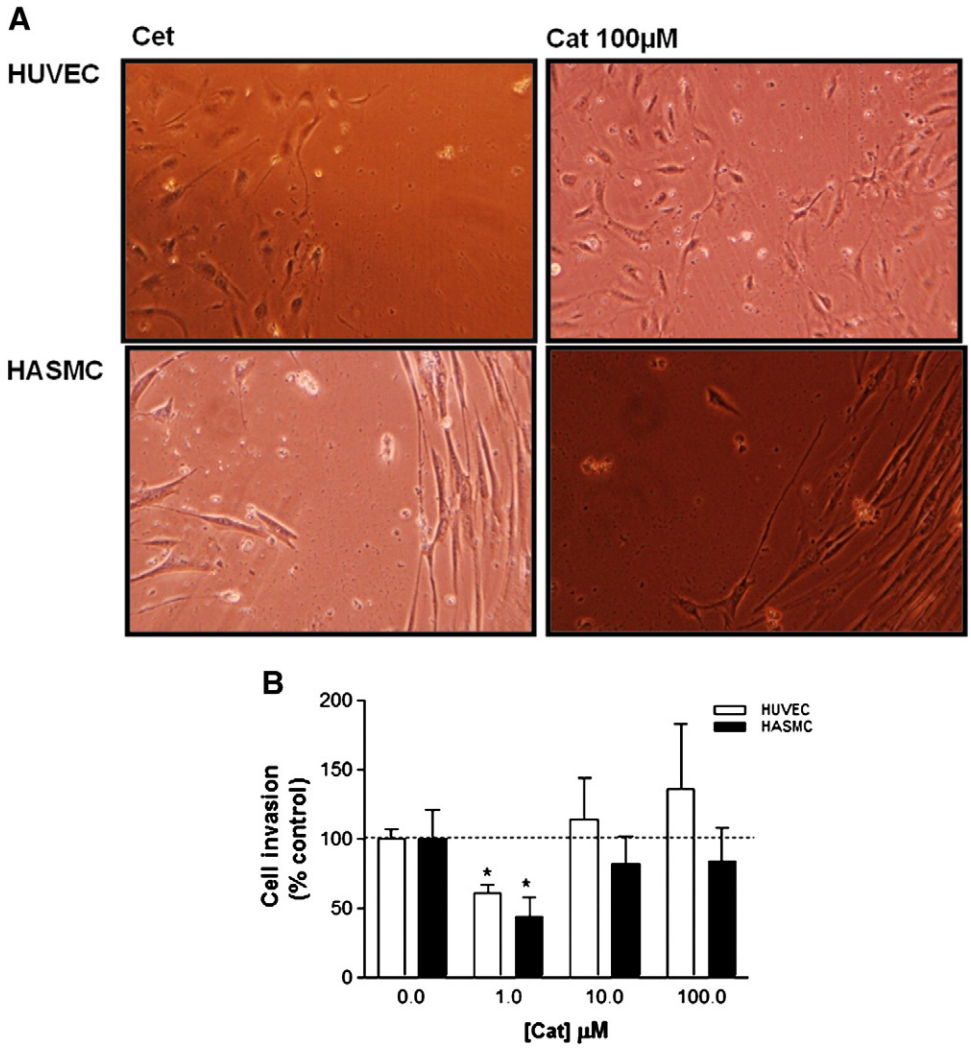


Fig. 2. Effect of Cat in HUVEC and HASMC (A) migration and (B) invasion. Cells were incubated for 24 h with 1.0–100 μM Cat or vehicle (Cet or 0.0). Cell migration was visualized by injury assay in confluent cell cultures. Pictures are representative of independent studies. Invasion was measured using a double-chamber assay. Results are means±S.E.M. of independent experiments ($4 \leq n \leq 8$) and are expressed as percentage of control. *$P \leq .05$ vs. control.

inflammatory and angiogenic-related genes. Thus, TNFα seems to be a good candidate to investigate Cat's effect on this cross-talk. The results revealed a reduction in the TNFα levels after 100-μM Cat treatment of HUVEC (58.66%±16.20% reduction) and an even more pronounced reduction in HASMC (85.46%±9.95% reduction) as can be observed in Fig. 5A.

NFκB activity was only decreased in HASMC, reaching maximal inhibition with 100 μM Cat (38.43%±6.01% inhibition) comparatively with control group (Fig. 5B). NFκB was slightly decreased in HUVEC, although it did not reach statistical difference relative to control (Fig. 5B). In addition, exposure of either HUVEC or HASMC to 50–100 μM Cat slightly decreased extracellular NO levels (decreases of 9.63%±0.84% and 5.43%±0.81% for 100 μM, for HUVEC and HASMC, respectively) as can be seen in Fig. 5C.

3.6. Cat did not affect angiogenesis or inflammation in skin wound-healing assay

Skin wound healing is a process involving the formation of new extracellular matrix, cell infiltration and tissue remodeling. Inflammation and angiogenesis are two physiological conditions crucial to this process.

We used this experimental model, with no VEGF-A stimulation, to investigate if the *in vivo* effect of Cat in neovascularization and inflammation changes according to VEGF-A stimulation. The healing process was completed on day 7 postinjury, and no differences were observed in the wound areas topically treated with Cat compared to vehicle. The histological sections used for quantification of blood vessels in the tissue revealed that treatment with Cat did not affect the formation of granulation tissue at the incision (Fig. 6A and B). Indeed, topical Cat did not change microvessel density in the vicinity of the incision area (6.85±0.52 vessels/mm^2) compared to the control group (6.02±0.49 vessels/mm^2, respectively) (Fig. 6C).

In agreement with these findings, the activity of NAG enzyme in rat's serum was also similar in the two treatments, revealing an identical systemic inflammatory status in rats treated with 100 μM Cat (87.71%±6.32%) or ethanol 0.1% (100.0%±11.24%) as illustrated in Fig. 6D. These findings were corroborated by an identical profile obtained for IL-1β levels in rat serum (Fig. 6E). On the other hand, rat serum NO decreased significantly after topical 100-μM Cat

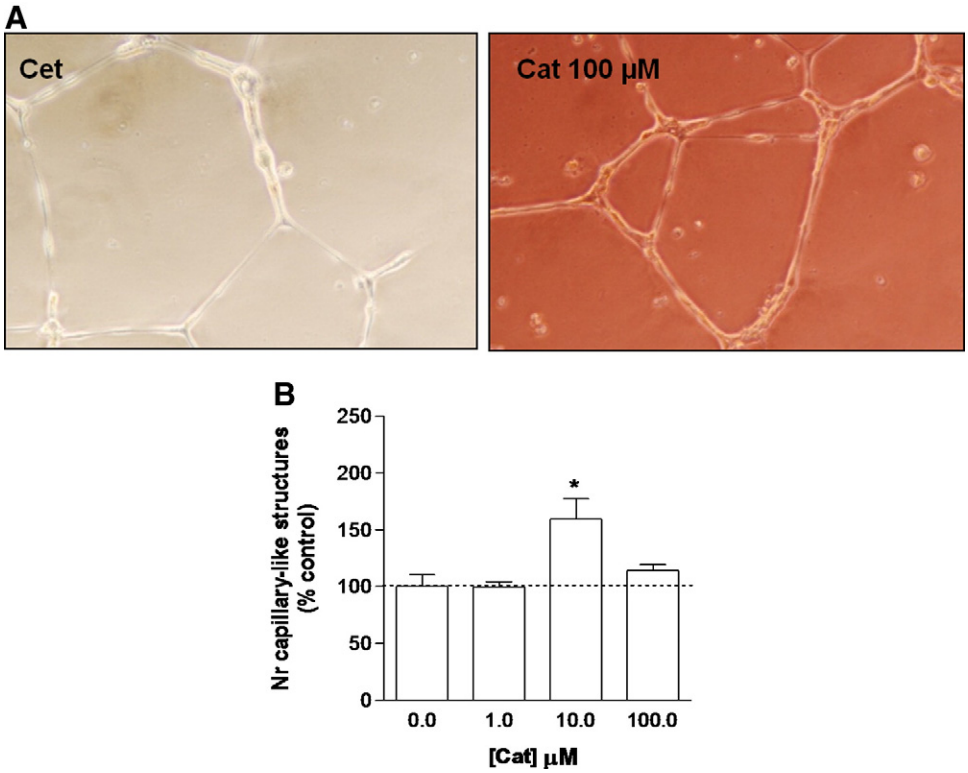


Fig. 3. Formation of capillary-like structures. HUVEC were grown on GFR-Matrigel and incubated with 1–100 µM Cat or vehicle (Cet or 0.0) for 24 h. (A) Capillary-like structures formation, after treatment with 100 µM Cat, visualized under a phase-contrast microscope. Figures are representative of the whole cultures. Magnification: ×200. (B) Semiquantification of capillary-like structures assembly. Results are means±S.E.M. of independent experiments ($3 \leq n \leq 5$) and are expressed as percentage of control. *$P \leq .05$ vs. control.

administration when compared with controls (63.47%±3.65%) as observed in Fig. 6F.

3.7. Cat diminished VEGF-A stimulated angiogenesis and inflammation in Matrigel plug assay

The other *in vivo* model used to evaluate the modulation of angiogenic process by Cat was the Matrigel plug neovascularization assay. As illustrated in Fig. 7A and B, VEGF-A-containing Matrigel (positive control, C+) presented extensive neovascularization. Matrigel implants containing recombinant VEGF-A and Cat resulted in a strong inhibition of vascular development, as quantified by Hb plug content (Fig. 7A and B). The plugs implanted with Cat showed an angiogenic response between both positive and negative controls (55.30%±10.03%), as highlighted by the red color distributed in the whole plug (Fig. 7B).

Accordingly, analysis of the inflammatory enzyme NAG activity in the serum of mice implanted with Matrigel plugs revealed that systemic inflammation decreased in the presence of Cat-containing plug (decrease of 83.48±1.04% of control) (see Fig. 7C).

4. Discussion

The present study investigated the effect of the flavanol Cat in several steps of the angiogenic process, like proliferation, migration, invasion and capillary differentiation capacity, simultaneously in HUVEC and HASMC cultures. *In vivo* models were also used to test Cat effect on physiological angiogenesis (skin wound-healing assay) and pathological angiogenesis, presenting higher VEGF-A stimulation (Matrigel plug assay).

We were able to demonstrate in subconfluent cell cultures that 100 µM Cat increased HUVEC and HASMC viability. This was probably due to a decrease in apoptosis. Proliferation of both cell cultures was also reduced upon 10-µM Cat incubation in both cell cultures, though to a less extent. These results suggest that Cat stabilizes HUVEC and HASMC in the absence of a prominent proangiogenic environment. A

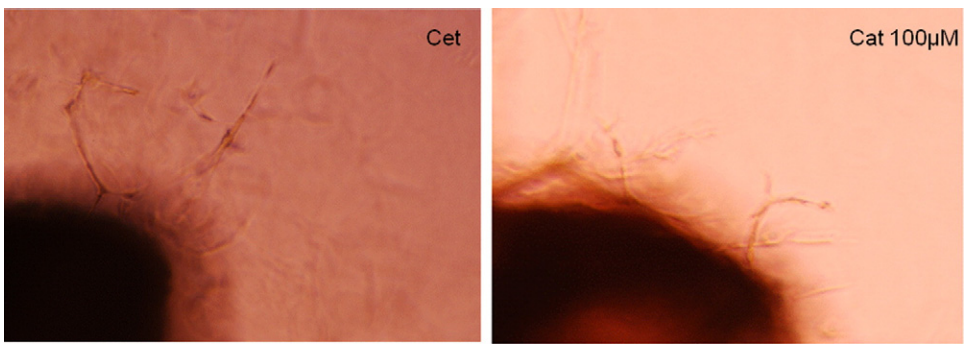


Fig. 4. Aortic ring assay showing vascular structures formed from the aorta after 100 µM Cat or ethanol treatment (Cet) (magnification ×200).

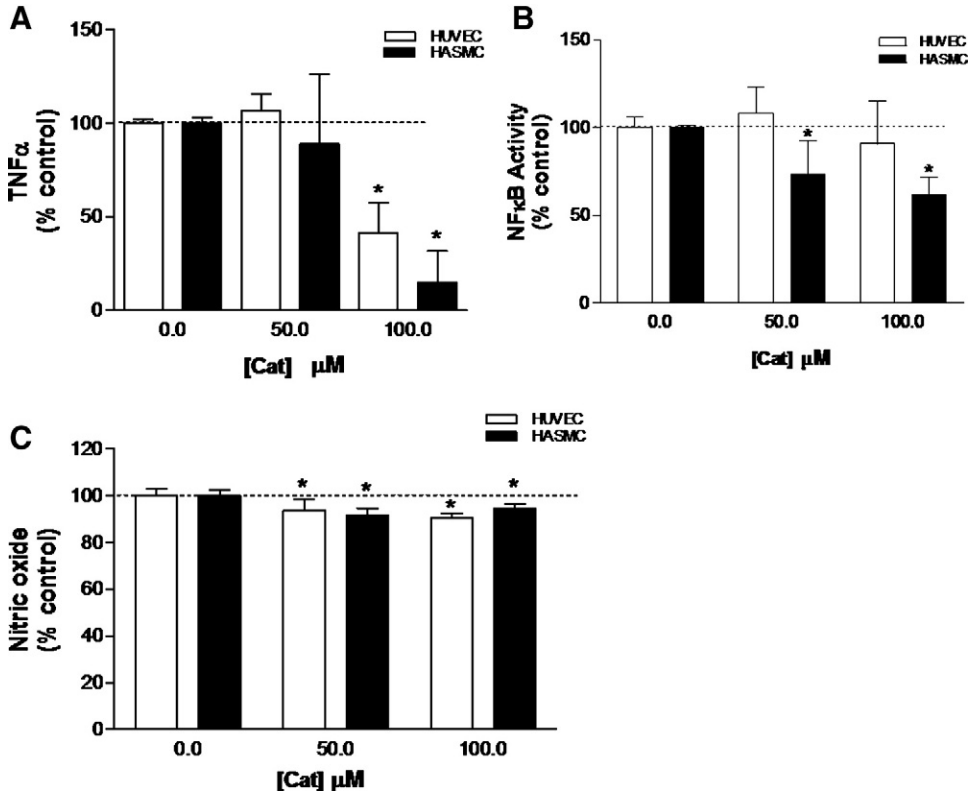


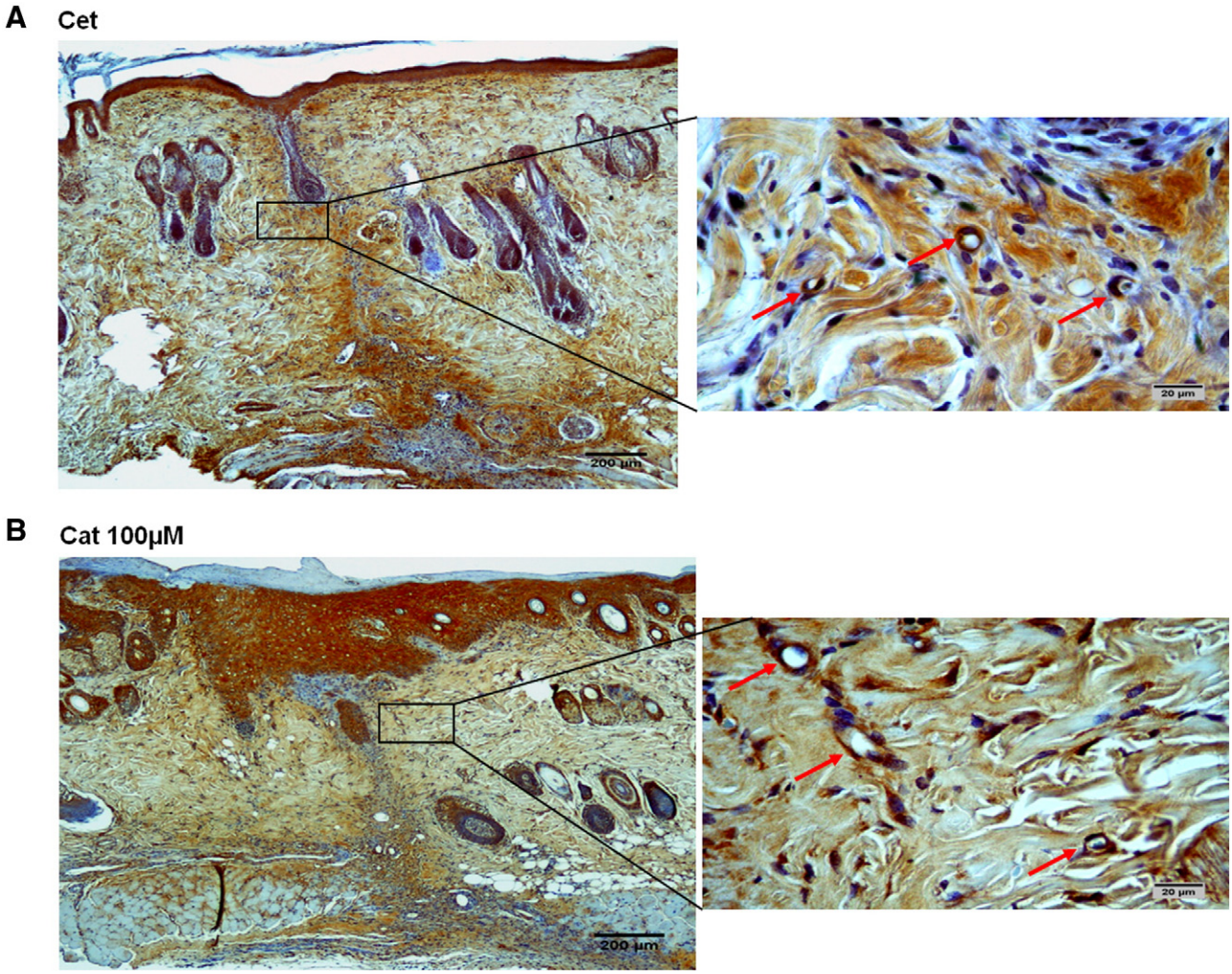
Fig. 5. Cat effect on inflammation. HUVEC and HASMC were treated with vehicle (0.0) or 50–100 μM Cat during 24 h. Inflammatory markers were then evaluated in extracellular medium. (A) TNFα was measured by ELISA, and quantification was performed at 450 nm and 550 nm. (B) NFκB activity was determined using TransAM NFκB p65/p50 transcription factor assay kit, and quantification was performed at 450 nm and 650 nm. (C) NO level was determined as the concentration of nitrate plus nitrite by Griess reaction, and measurements were performed at 550 nm. Results are means±S.E.M. of independent experiments ($3 \leq n \leq 6$) and are expressed as percentage of control. *$P \leq .05$ vs. control.

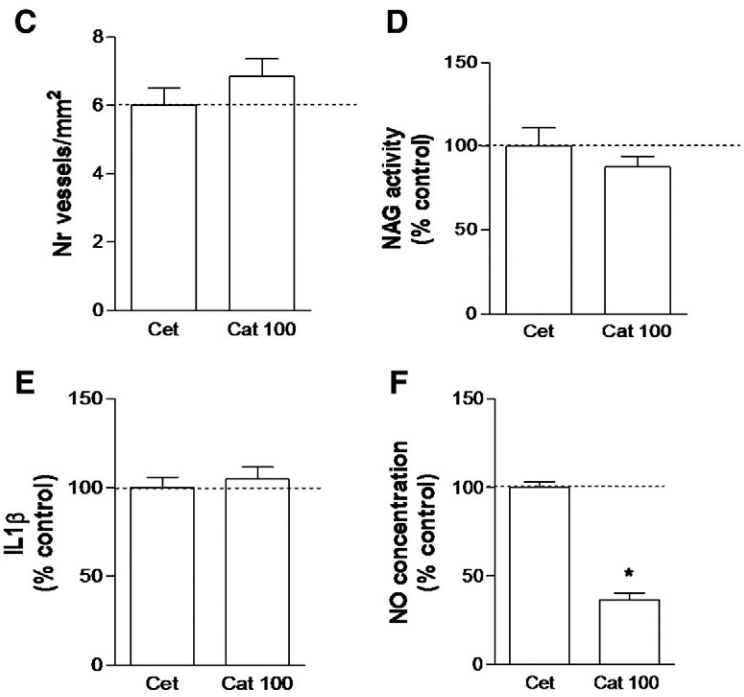
deeper look into the results reveals that most of the effects were observed at 1.0 μM Cat. The physiological relevance of these results depends on Cat bioavailability after consumption of nutritional sources. Cat plasma concentration after human ingestion of 200 ml of red wine is about 2 μM [30], and total polyphenol intake is about 1 g/day. So, as Cat is abundant in many fruits and vegetables, it can reach serum concentrations higher than 2 μM. Nevertheless, polyphenols may accumulate in cells, tissues and membranes at higher concentrations since catechins can bind to lipid bilayers through relatively strong interactions [31,32]. So, Cat concentrations used in this study include physiological concentrations and also higher Cat doses that may be relevant for therapeutic applications.

Our results further showed that Cat increased HUVEC migration and tended to augment invasion capacity at higher concentrations, although never reaching statistical significance. Strikingly, at 1.0 μM Cat, HUVEC invasion capacity was significantly decreased. Opposite effects within this range of Cat concentrations upon blood vessel function have been observed by other authors [33]. Strikingly, HASMC migration and invasion capacity tended to decrease with the same treatment. Given the role of VSMC proliferation and migration towards the blood vessel intima layer in the formation of atheroma plaque, the results obtained with HASMC indicate that Cat may protect from cardiovascular diseases. The reduced migration and invasion of VSMC in the presence of green tea polyphenols have already been described by several authors [34–36].

The assembly of capillary-like structures by HUVEC was not affected by Cat incubation at most concentrations. These findings were confirmed using aortic ring assay, a more accurate method to examine the complex interactions occurring between EC and VSMC within a blood vessel wall [37]. In agreement, 100 μM Cat resulted in no changes in the vascular network surrounding the aortic rings, comparing to control. These results may indicate that the relations between EC and VSMC, important in blood vessel stabilization, may not be the principal target of Cat effect on blood vessels. In contrast to our results, it was recently described that Cat inhibits new vessel sprouting from rat aortic rings, strongly supporting the efficacy of Cat in suppressing neovascularization. Nevertheless, these studies were performed in stimulated cultures [38], leading to the assumption that the effect of Cat in neovascularization might depend on whether angiogenesis stimuli are present or not, rather than on the presence of interplay between EC and VSMC.

A state of chronic inflammation is one of the major angiogenic stimulators [6,16]. TNFα is a key inflammatory cytokine involved in systemic inflammation and is stimulated in the acute phase reaction, being produced mainly by macrophages. Treatment of both HUVEC and HASMC with 100 μM Cat resulted in a very significant decrease in TNFα secretion to the extracellular medium. TNFα signaling is mediated in part by NFκB, which plays a central role in the regulation of genes associated with inflammation, cell survival and proliferation. Treating HUVEC with 50–100 μM Cat did not significantly change NFκB activity. In turn, Cat reduced NFκB activity in HASMC. The decrease in TNFα and NFκB activity, even in normal cell environment (in the absence of inflammatory or angiogenesis stimuli), may be interesting regarding resolution of inflammation and angiogenesis in physiological conditions. Other authors described similar effects for polyphenol-treated cells, but generally, this effect is only observed in stimulated cells [39]. Accordingly, Kurbitz et al. described that epicatechin-3-gallate inhibited TNFα-induced activation of NFκB and consequently inhibited secretion of proinflammatory factors and invasion of pancreatic ductal adenocarcinoma cells [40]. It is,


A
Cet
200 µm
20 µm
B
Cat 100µM
200 µm
20 µm


C
Nr vessels/mm2
0
2
4
6
8
Cet
Cat 100
D
NAG activity (% control)
0
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Cet
Cat 100
E
IL1β (% control)
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NO concentration (% control)
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Cat 100

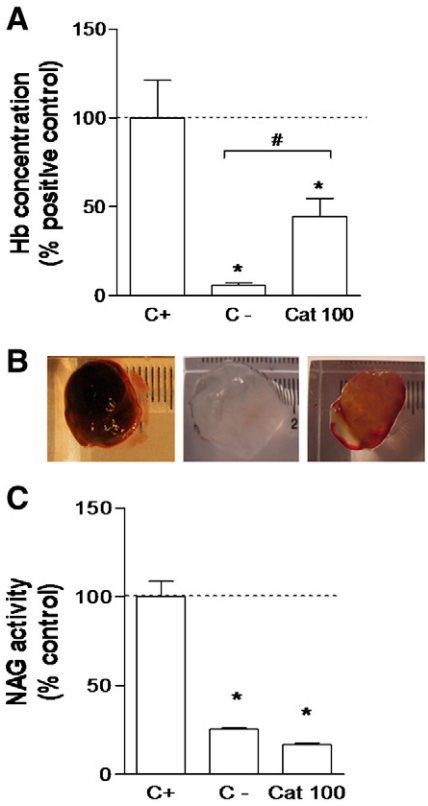

Fig. 7. *In vivo* evaluation of angiogenesis and inflammation by Matrigel plug assay. A mixture of Matrigel and heparin without VEGF (negative control, C−); or of Matrigel, heparin and VEGF (positive control, C+); or of Matrigel, heparin, VEGF and 100 μM Cat was injected subcutaneously into C57BL/6 mice. (A) Quantification of the Hb amount in the homogenized plugs by Drabkin's method. (B) Representative images of macroscopic visualization of Matrigel plugs. (C) Determination of NAG activity in mice serum 7 days after plugs implantation. Results are means±S.E.M. of independent experiments ($5 \leq n \leq 10$) and are expressed as percentage of control. *$P \leq .05$ vs. positive control; #$P \leq .05$ vs. negative control.

hence, possible that the results on invasive capacity already described upon HUVEC and HASMC treatment may involve NFκB activity.

NO is produced in large amounts by iNOS after immunological stimuli, which is up-regulated by TNFα and NFκB in inflammation. In agreement, chronic overexpression of NO is a feature of inflammatory conditions such as diabetes, cardiovascular diseases and various carcinomas. In this work, NO levels suffered a small but significant reduction after HUVEC and HASMC treatment with Cat, supporting TNFα and NFκB putative role previously observed. These findings are corroborated by other studies performed in different models that also reported inhibition of NO by this compound [38,39,41]. Nevertheless, we do not know whether this reduction of NO levels can reach physiological relevance.

Altogether, the *in vitro* results showed that Cat increased viability but decreased apoptosis of HUVEC and HASMC. It did also seem to alter migration and invasion in a distinct manner, increasing it in HUVEC and decreasing it in HASMC. Cat did not change *in vitro* sprouting and did not seem to interfere with EC-VSMC interplay. It diminished *in vitro* inflammatory modulators, affecting both EC and VSMC, but generally manifesting a more pronounced effect in HASMC.

Comparing our *in vitro* results to what have been recently described for Cat [38] led us to hypothesize that Cat effects in angiogenesis depended on the presence or absence of angiogenic stimuli. To test this, two different *in vivo* models were performed. Using the physiological skin wound-healing model, we could demonstrate that after topical administration of Cat for 7 days, the number of vessels formed in the injured area was similar to the control group, confirming that it did not affect angiogenic process, according to results obtained for capillary-like structures and aortic ring assay. Additionally, inflammatory marker levels such as NAG and IL-1β were also not changed in Cat-treated animals. This may be explained by the fact that the inflammatory phase of the wound-healing process that starts just upon injury induction should have already been resolved by day 7.

Only a decrease in NO serum levels could be observed after 7 days of treatment. These findings are in agreement with the ones described by Sanae et al. in which administration of Cat in inflammatory sites led to vessel contraction activity and inactivation of NO [33], indicating a benefic effect of this polyphenol.

Matrigel plug assay is a highly stimulated angiogenic model (comprising abundant VEGF-A stimulus). Cat was able to substantially prevent the recruitment of new blood vessels within the plug and also diminished macrophages activation, supporting the anti-angiogenic and anti-inflammatory effects described by others for Cat [38] and for other polyphenols [28,42,43].

Altogether, the current study was able to show that Cat presents distinct effects in blood vessel wall cells, depending on the presence or absence of angiogenesis and inflammation stimuli, such as VEGF-A. The absence of adverse effects in mature vasculature by Cat emphasizes its potential use against pathological situations where angiogenesis is stimulated. Molecular studies are now mandatory in order to elucidate the modulation of angiogenesis by Cat in physiological as well as in pathological conditions.

Acknowledgments

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Fig. 6. *In vivo* skin wound-healing assay. Longitudinal incisions were created on the dorsal surface of the rats, and vehicle (Cet) or 100 μM Cat was administered topically daily. After 7 days, wounded tissue was collected for angiogenesis evaluation. Micrographs of wound tissue sections highlighting thickness and appearance of granulation tissue, fulfilling the incision with different treatments (magnification: ×40): (A) control animals and (B) rats treated with Cat. Amplification of wound area (magnification: ×200): tissue section micrograph using von-Willebrand factor for evaluation of blood vessels. Red arrows indicate blood vessels. (C) Quantification of blood vessels present in three tissue sections, for each animal, and normalized to the total area of the tissue section. (D) NAG activity, (E) IL-1β levels and (F) NO determination in rat serum. Results are means±S.E.M. of independent experiments ($4 \leq n \leq 7$) and are expressed as percentage of control. *$P \leq .05$ vs. control.

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