The 2-Adrenoceptor Agonist Terbutaline Stimulates Angiogenesis via Akt and ERK Signaling

DOI: 10.1002/jcp.25483
Handle: http://hdl.handle.net/1942/22810

Rapid Communication

The β2-adrenoceptor agonist terbutaline stimulates angiogenesis via Akt and ERK signaling[†]

Running head: Terbutaline stimulates angiogenesis

Stefanie Lemmens, Lauren Kusters, Annelies Bronckaers, Nathalie Geurtsand, Sven Hendrix[*]

Department of Morphology & Biomedical Research Institute, Hasselt University, Diepenbeek, Belgium

[*]Corresponding author:

Sven Hendrix, MD, PhD

Department of Morphology & Biomedical Research Institute

Martelarenlaan 42, 3500 Hasselt, Diepenbeek, Belgium

Tel: +32 11 26 9246; Fax: +32 11 26 9299

Email: sven.hendrix@uhasselt.be

Grant information:

Deutsche Forschungsgemeinschaft (SPP1394)
Fonds voor Wetenschappelijk Onderzoek FWO (G.0389.12, G0A5813, G.0834.11N, G0A58113FWO, 1.2.917.14N)
Agentschap voor Innovatie door Wetenschap en Technologie IWT (131230)

Received 7 April 2016; Revised 4 July 2016; Accepted 11 July 2016
Journal of Cellular Physiology

DOI 10.1002/jcp.25483

Abstract

Angiogenesis is associated with changes in endothelial cell (EC) proliferation and tube formation, controlled by extracellular receptor-activated kinase (ERK)/mitogen activated protein kinase (MAPK) and Akt signaling. Important regulators of these systems include hormones acting on G-protein-coupled receptors, such as beta 2-adrenoceptors (β2-ARs).

In central nervous system (CNS) trauma, the importance of β2-AR modulation has been highlighted, although the effects on revascularization remain unclear. Vascular protection and revascularization are, however, key to support regeneration.

We have investigatedthe angiogenic capacity of the specific β2-AR agonist terbutaline on ECs derived from the CNS, namely bEnd.3-cells.As angiogenesis is a multistep process involving increased proliferation and tube formation of ECs, we investigated the effects of terbutaline on these processes.We show that terbutaline significantly induced bEnd.3 tube formation in a matrigel*in vitro* assay. Moreover, administration of specific inhibitors of ERK and Akt signaling both inhibited terbutaline-induced tube formation. The proliferation rate of the ECs was not affected. In order to investigate the general effects of terbutaline in an organotypic system, we have used the chick chorioallantoic membrane (CAM)-assay. Most importantly, terbutaline increased the number of blood vessels in this *in ovo* setting. Although we observed a positive trend, the systemic administration of terbutaline did not significantly improve the functional outcome, nor did it affect revascularization in our spinal cord injury model.

In conclusion, these dataindicate that terbutaline is promising to stimulate blood vessel formation, underscoring the importance of further research into the angiotherapeutic relevance of terbutaline and β2-AR signalingafter CNS-trauma.

Key words: Angiogenesis, neovascularisation, terbutaline, tube formation

1. Introduction

The vascular system supports normal tissue function through the delivery of oxygen and nutrients, metabolic waste disposal and immune surveillance.Within the human body, three mechanisms of blood vessel formation can be distinguished: vasculogenesis, angiogenesis and arteriogenesis. Angiogenesis is not only a fundamental event during embryogenesis, but also during adult life, asit is a key process during wound healing (Bhadada et al., 2011). Under physiological conditions, angiogenesis does not take place because anti-angiogenic factors (e.g. angiostatin, endostatin) are in excess topro-angiogenicfactors (e.g. vascular endothelial growth factor, VEGF; basic fibroblast growth factor, bFGF). However, during wound healing and cancer development, pro-angiogenic factors are producedand the balance is skewedtowards blood vessel growth(Distler et al., 2003).Adult angiogenesis is associated with endothelial cell (EC) proliferation and migration, controlled by extracellular receptor-activated kinase (ERK)/mitogen activated protein kinase (MAPK) and Aktsignaling pathways (Bussolino et al., 1997; Iaccarino et al., 2002). In addition, EC apoptosis is strictly regulated during vascular growth by Akt signaling which protects ECs against apoptosis.

Damage to the vasculature and breakdown of the blood-brain barrier (BBB) are universal consequences after traumatic brain injury and spinal cord injury (SCI). Most importantly, the unfavorable regulation of pro-angiogenic and counterregulatory anti-angiogenic factors after CNS trauma is suspected to participate in the failure of revascularization and vessel stabilization (Graumann et al., 2011). Vascular protection and revascularization are key to support survival of sprouting and regenerating axons (Dray et al., 2009; Loy et al., 2002). Numerous angiogenic factors (e.g. vascular endothelial growth factor, VEGF) were already investigated for their effects after traumatic SCI, although with variable success (reviewed in (Graumann et al., 2011)). On the other hand, for beta 2-adrenoceptor (β2-AR) agonists such as isoproterenol (ISO), salmeterol and clenbuterol, it has already been shown that they have neuroprotective effects and they improve the neurological and functional outcome (Graumann et al., 2011; Junker et al., 2002; Loy et al., 2002; Qian et al., 2011; Zlotnik et al., 2012). These data indicate the importance of β2-AR modulation after CNS trauma, although previous studies did not focus on functional revascularization.

Beta-adrenoceptors are important G-protein-coupled receptors that belong to the adrenergic systemand are involved in cardiac and vascular function. They are implicated in important signaling pathways during angiogenesis, such as the Akt- and ERK pathway mentioned above. The β2-ARs are most abundantly expressed on the vasculature and modulate the release of nitric oxide (NO), which in turn causes vasodilation (Iaccarino et al., 2002). Their relevance to stimulate angiogenesis was recently confirmed as the expression of the receptors on endothelial progenitor cells (EPCs) and mature ECs coincides with increased proliferation and migration after stimulationwith the non-specific β-AR agonist isoproterenol (Galasso et al., 2013).

In this study, we have investigated the role of the short-acting and highlyspecific β2-AR agonist terbutaline ($C_{12}H_{19}NO_3$)in the control of angiogenesis within the context of CNS trauma. Specifically β2-ARs were stimulated as this adrenoceptor subtype is abundantly expressed on the vasculature. It is expressed on ECs where it plays a role in vasodilation, EC proliferation, and EC migration. In contrast to clenbuterol and zilpaterol (specific β2-AR agonists), terbutaline is not reported to be anabolic, thereby avoiding adverse effects such as cardiac hypertrophy (Delmore et al., 2010; Dutt et al., 2015; Lara-Pezzi et al., 2009). Salbutamol is another specific β2-AR agonist correlated with angiogenesis.However, this agonist was reported to decrease the number of blood vessels in the chorioallantoic membrane (CAM)-assay (Pullar, 2015). Therefore, we have selected terbutaline to investigate its angiogenic potential both *in vitro* and *in vivo*. Adult mouse brain ECs (bEnd.3) were chosen because they are derived from the brain and hence, they are more suitable to investigate angiogenesis in the context of CNS trauma and to relate our *in vitro* results with our *in vivo* results in a mouse model of SCI.

In a first set of *in vitro* experiments, we show that terbutaline stimulated tube formation of the bEnd.3 cells. Next, we evaluated whether terbutaline could enhance angiogenesis in the chorioallantoic membrane (CAM)-assay, which is an *in ovo* model for blood vessel development with a complexity that approximates the *in vivo* situation(Ribatti, 2008). Consistently, terbutaline treatment increased the number of blood vessels in the CAM assay. In conclusion, this *ex vivo* study suggests that terbutaline is a promising drug to improve angiogenesis via β2-AR stimulation. Most importantly, enhanced tube formation at the cellular level as well as increased neo-vascularization *in ovo* suggest the importance of β2-AR stimulation to induce angiogenesis in order to improve functional regeneration, although in depth research is necessary to reinforce the effects.

2. Experimental procedures

2.1. Cell culture mouse brain endothelial cells

The bEnd.3 microvascular endothelial cell line was purchased from the American Type Culture Collection (Ampule passage No: 22; ATCC, USA). They were cultured at 37°C at 5% CO2 in ATCC-formulated Dulbecco's Modified Eagle's Medium (DMEM; ATCC, USA) supplemented with 10% fetal calf serum (FCS; Life Technologies, Belgium), 100 IU.ml-1 penicillin and 100 µg.ml-1 streptomycin (P/S; Sigma-Aldrich, Belgium)in T-25 flasks (Greiner, Belgium). Medium change was performed every 3 days. When confluence was reached, the cells were sub-cultured after treatment with 0.05% trypsin/EDTA solution (Sigma-Aldrich, Belgium). All experiments were performed between passage No 22-30.

2.2. Tube formation assay

The effects of different concentrations of terbutaline (0.1; 1; 10 µM; Sigma-Aldrich, Belgium) on tube formation by the bEnd.3 cells were assessedusingIbidiangiogenesis µ-slides (Ibidi GmbH, Planegg/Martinsried, Germany). The inner wells of the µ-slide were coated with growth factor-reduced BD Matrigel™ Basement Membrane Matrix (BD Biosciences, Franklin Lakes, NJ). On top of the Matrigel 15,000 bEnd.3 cells were seeded, resolved in 50 µl of the desired experimental conditions.The cells were incubated with different concentrations of terbutaline (0.01; 1; 10 µM), terbutaline (1 µM) combined with the PI3K-inhibitor LY294002 (10 µM; Sigma-Aldrich, Belgium) or terbutaline (1 µM) combined with MEK-inhibitor U0126 (10 µM; Sigma-Aldrich). The inhibitors were added at the same time as terbutaline. DMEM without FCS was used as vehicle control. In addition, the pathway blockers have been applied to the controls to confirm their inhibitory capacity. The cultures were maintained for 6h at 37°C in a humidified atmosphere containing 5% CO_2. Next, 3 representative pictures per well were taken with an inverted phase-contrast microscope (Nikon Eclipse TS100, Japan) equipped with a ProgRes® C3 digital microscope camera (Jenoptik AG, Germany). Image analysis was performed using the Angiogenesis Analyzer tool from ImageJ (National Institutes of Health, USA). Three parameters were quantified: the total branching length, the number of nodes and the number branches and segments. The tubes form a network. A segment is a tube delimited by two junctions (formed by one or multiple nodes), whereas a branch has an open ending. The total branching length is the sum of the lengths of the branches and segments in one picture. The number of nodes is the number of crossings between the segments.

The number of branches and segments is the sum of the branches and segments in one picture. Per well, 3 representative pictures were analyzed and the mean values are presented.

2.3. MTT assay

The bEnd.3 cells were seeded in a 96-well plate (Greiner, Belgium) at a density of 7,500 cells per well in their normal culture medium. After attachment to the culture plate, cells were rinsed twice with phosphate buffered saline (PBS) and incubated with DMEM without FCS supplemented with different concentrations of terbutaline (0.01; 1; 10 µM), terbutaline (1 µM) combined with the PI3K-inhibitor LY294002 (10 µM; Sigma-Aldrich, Belgium) or terbutaline (1 µM) combined with MEK-inhibitor U0126 (10 µM; Sigma-Aldrich). DMEM without FCS was used as vehicle control. In addition, the pathway blockers have been applied to the controls to confirm their inhibitory capacity. After 6h or 72h of incubation, the different conditions were replaced by the conditions supplemented with 500 µg/ml MTT (MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma-Aldrich, Belgium). After 4h of incubation, the MTT solution was removed and a mixture of 0.01 M glycine in DMSO (Dimethyl Sulfoxide; Sigma-Aldrich, Belgium) was added to dissolve the formed formazan crystals. The absorbance was measured at a wavelength of 570 nm with a Benchmark microplate reader (Bio-Rad Laboratories, USA).

2.4. Chorioallantoic membrane assay

Fertilized chicken eggs (Gallus gallus; Wijverkens chicken farm, Halle, Belgium) were incubated for 3 days at 37°C in a humidified environment. On embryonic day 3 (E3), the developing CAM was detached from the egg shell by removing 3 ml of albumin. To evaluate whether the eggs were fertilized, a small window was created in the shell and immediately closed with cellophane tape. Six days later (E9), terbutaline (10; 50 and 250 nmol) was applied on plastic discs (8 mm diameter), which were allowed to dry under sterile conditions. Cortisone acetate (100 µg/disc; Dr. Ehrenstorfer GmbH, Germany) was applied to all discs in order to avoid an inflammatory response.The selected doses were based on earlier studies (Liekens et al., 2006). After an incubation period of two days (E11), the CAM was carefully dissected to evaluate angiogenesis. Pictures of each CAM were taken with a stereomicroscope (Wild M3Z Stereomicroscope, Switzerland) equipped with a ProgRes® C3 digital microscope camera (Jenoptik AG). All vessels intersecting a concentric circle (radius 3.5 mm) positioned in the treated area were counted by 3 independent investigators who were blinded to the experimental conditions.

2.5. BrdU cell proliferation assay

The bEnd.3 cells were seeded in a 96-well plate (Greiner, Belgium) at a density of 20,000 cells per well in their normal culture medium. After attachment to the culture plate, cells were rinsed with PBS and incubated with DMEM without FCS supplemented with different concentrations of terbutaline (0.01; 1; 10 µM). BrdU incorporation was measured after 6 and 48, following the manufacturer's instructions (BrdU Cell Proliferation Kit, Merck Millipore, Belgium).

2.6. Experimental spinal cord injury and terbutaline treatment

The *in vivo* experiment was performed using female C57BL/6j mice (9-10 weeks old; Janvier, France). They were housed in a conventional animal facility at Hasselt University under regular conditions, i.e. in a temperature-controlled room (20 ± 3 °C) on a 12 h light-dark schedule and with food and water ad libitum. All experiments were approved by the local ethical committee of Hasselt University and were performed according to the guidelines described in Directive 2010/63/EU on the protection of animals used for scientific purposes.

A hemisection injury was performed as previously described (Nelissen et al., 2014). Briefly, mice were anesthetized with 3% isoflurane (IsofFlo, Abbot Animal Health, Belgium) and received a subcutaneous injection of the analgesic buprenorphine Temgesic (0.1 mg/kg bodyweight; Val d'HonyVerdifarm, Belgium) before surgery. A partial laminectomy was performed at thoracic level 8 to expose the spinal cord. A bilateral hemisection injury was induced to the spinal cord by using iridectomy scissors to transect left and right dorsal funiculus, the dorsal horns and additionally the ventral funiculus. This "T-cut" hemisection results in a complete transection of the dorsomedial and ventral corticospinal tract and impairs several other descending and ascending tracts. Afterwards, muscles were sutured and the back skin was closed with wound clips (Autoclip®, Clay-Adams Co., Inc.). Glucose solution (20%) was given after the operation to compensate for any blood loss during surgery. All mice were placed in a temperature-controlled chamber (33 °C) until thermoregulation was established. Bladders were emptied manually until a spontaneous return of the micturition reflex.

After surgery, mice were distributed equally among the groups according to their Basso Mouse Scale (BMS) score. Mice were intraperitoneously injected twice a day, for 9 days with either terbutaline (5 mg/kg) or the vehicle control saline (0.9% NaCl).

Locomotor recovery of the animals was determined by an investigator blinded to the experimental groups using BMS (Basso et al., 2006). During the first week after injury, mice were scored daily and from the start of the second week until the end of the observation period (21 days post injury [dpi]), mice were examined every second day.

2.7. Immunohistochemistry and quantitative image analysis

At 21 dpi, mice were overdosed with Nembutal and transcardially perfused with Ringer solutioncontaining heparin, followed by 4% paraformaldehyde in phosphate-buffered saline (PFA, pH 7.4).Spinal cords were dissected and dehydrated by incubation in 5% sucrose in 4% PFA, followed by a30% sucrose solution in PBS. Next, the samples were embedded inTissue-Tek O.C.T. Compound (Sakura, Belgium) and frozen in liquid-nitrogen cooled isopentane.Immunohistochemicalstainings were performed on 10 µm thick saggitalcryosections of these spinalcords. Spinal cord sections were blocked with 10% normal goat serum and permeabilized with 0.05%Triton X-100 in PBS for 30 min at RT. Then, the spinal cord sections were incubated with the rat anti-CD31 primary antibody (1/1000; BD Pharmingen, BD Biosciences) overnight at 4°C in a humidifiedchamber. Following repeated washing steps with PBS, spinal cord sections were incubated with goatanti-rat Alexa 488 secondary antibody (1/250; Invitrogen) for 1 h at room temperature. Specificity ofthe secondary antibody was verified by including a control staining in which the primary antibodywas omitted (data not shown).Autofluorescence was controlled for by omitting the primary and the secondary antibody. Images were taken with a Nikon Eclipse 80i microscope and aNikon digital sight camera DS-2MBWc.Quantitative image analysis were performed on original unmodified photos using the ImageJ opensource software (NIH). For standardization, analyses were performed on 6-9 spinal cord sections (permouse) representing the lesion area, i.e. the lesion epicenter as well as consecutive sagittal sections,as previously described (Nelissen et al., 2014). Spinal cord lesion areas are subdivided in differentregions, i.e. the lesion epicenter, the rostral area and the caudal area. The CD31-positive area wasdetermined at the three locations by selecting the stained area with ImageJ.

2.8. Statistics

Statistical analyses were performed using JMP Pro 11 and GraphPad Prism 5.01 software (GraphPad Software, Inc.). For the tube formation assays, MTT assays and the BrdU-assays, Mixed model ANOVA was performed using JMP to exclude random effects caused by variability inside one group.

Only when this requirement was met, additional statistical analyses were performed on the data set by using GraphPad. Data sets were analyzed for normal distribution using the D'Agostino-Pearson normality test. This test indicated that all data sets were not-normally distributed. Therefore, statistical differences between two groups were analyzed via the nonparametric Mann-Whitney U test and to compare multiple groups, a one-way ANOVA with a Dunn's Multiple comparison test was used. Functional recovery *in vivo* and histological analyses of blood vessel density (CD31) were statistically analyzed using two-way ANOVA for repeated measurements with a Bonferroni post hoc test for multiple comparisons. Data were presented as mean ± standard error of the mean (SEM). Differences were considered statistically significant when $p<0.05$.

3. Results

3.1. Terbutaline stimulates tube formation by mouse brain endothelial cells

First, the angiogenic capacity of terbutaline was investigated by the tube formation assay. Tube formation is an important step in the angiogenic process. It takes place after vessel destabilization, EC proliferation and migration. Studying this step in cell culture *in vitro* provides insight into which specific part of the blood vessel development is affected by the treatment The tube formation assay was performed on bEnd.3 cells, which express the beta-2 adrenoceptor (β2-AR) (data not shown). Terbutaline stimulation of these ECs resulted in increased tube formation as compared to the vehicle control (arrows; figure 1A). Three parameters were quantified: the total tube length (total branching length), the number of nodes (# nodes) and the number of tubes (# branches + # segments). In every condition with terbutaline, all these parameters were significantly increased (figure 1B). For instance, the total branching length increased with 68.3% when 1 µM terbutaline was applied (mean total branching length: 168.3% ± 11.65%; figure 1B), compared to the control condition (mean total branching length: 100% ± 5.12%; figure 1B). Summarized, terbutaline stimulates tube formation by adult mouse brain ECs.

3.2. Terbutaline stimulates tube formation via Akt and ERK signaling

As terbutaline is a potent stimulator of tube formation *in vitro*, we aimed to unravel the pathways through which terbutaline exerts these effects. Therefore, we inhibited Akt or ERK,which are closely involved in the angiogenic process. LY294002 (LY) has been used because it is a potent inhibitor of phosphoinositide 3-kinases (PI3Ks), which are upstream of

Akt in the Akt pathway. U0126 (U) is a highly selective inhibitor of MAPK/ERK kinase (MEK) 1 and MEK2, upstream of ERK1/2 in the respective pathway. Inhibition of both the Aktsignaling or the ERKsignaling significantlyprevented tube formation induced by terbutaline (figure 2Aand 2B, respectively). Quantification revealed that when LY was applied, the total branching length, the number of nodes and the number of tubes where reduced significantly by 80.99%; 80.1% and 68.73% respectively, compared to the condition where terbutaline alone was applied (terb 1 µM + LY 10 µM: 65.21% ± 10.31% vs. terb 1 µM: 146.2% ± 9.366% for the total branching length; terb 1 µM + LY 10 µM: 64.22% ± 13.4% vs. terb 1 µM: 144.4% ± 11.2% for the number of nodes; terb 1 µM + LY 10 µM: 64.47% ± 11.61% vs. terb 1 µM: 133.2% ± 9.481%; figure 2C).

Application of U reduced the tube formation by 114.64%; 115.25% and 95.16% for the total branching length, the number of nodes and the number of tubes respectively, compared to the condition where terbutaline alone was applied (terb 1 µM + U 100 µM: 31.56% ± 7.602% vs. terb 1 µM: 146.2% ± 9.366% for the total branching length; terb 1 µM + U 100 µM: 29.15% ± 7.172% vs. terb 1 µM: 144.4% ± 11.2% for the number of nodes; terb 1 µM + LY 10 µM: 38.04% ± 10.07% vs. terb 1 µM: 133.2% ± 9.481%; figure 2C). These results imply that the tube formation induced by terbutaline is dependent on Akt signaling and ERK signaling.

The inhibitors were also applied in the absence of terbutaline to evaluate their influence on tube formation as such. The Akt blocker alone significantly reduced the total branching length, the number of nodes and the number of tubes compared to the vehicle control, whereas the ERK blocker only decreased the number of tubes (figure 2D). However, the Akt blocker caused only a small inhibition of the standard tube formation, whereas it caused a large reduction of the terbutaline induced tube formation (e.g. 32,72% in the standard tube formation assay vs. 114,64% in the terbutaline induced tube formation for the total branching length).When applied to a condition (NGM + 10% FCS) known to induce tube formation, the blockers reduced the tube length, number of nodes and number of tubes, although not significantly, indicating their proper functioning (figure 2D).

3.3. Terbutaline slightly decreases the metabolic activity of the ECs via the ERK pathway

Measuring the metabolic activity gives a first impression of the proliferation capacity and cell viability. The ECs were incubated for 6h and 72h with terbutaline. The metabolic activity of the ECs after 6h incubation with terbutaline was only decreased when 10 µM had been applied, compared to the vehicle control. The other concentrations did not affect the metabolic

activity of the cells (Figure 3A). After 72h incubation, the metabolic activity was decreased in all conditions (Figure 3B). Again, the involvement of the Akt and ERK pathway were assessed by blocking them with the respective inhibitors LY and U. Addition of the Akt blocker LY did not change the effects of terbutaline on the metabolic activity of the cells, although it decreased the metabolic activity of the cells on its own (figure 3C and E). In contrast, when the ERK blocker U was applied, the metabolic activity returned to levels similar to the vehicle control (figure 3D), whereas the blocker alone did not affect the metabolic activity of the cells (figure 3F). To control for the proper function of the blockers, they were added to the normal growth conditions of the cells (NGM; with 10% FCS).

The metabolic activity was reduced after application of the blockers, compared to the NGM without the blockers, indicating that the blockers did function properly (Figure 3 E-F). Taken together, these results indicate that, on the short term (6h), terbutaline only slightly affects the metabolic activity when applied in higher concentrations (10 µM). The long term effects (72h) include reduction in the metabolic activity of the ECs, involving the ERK pathway.

3.4. Terbutaline does not affect the proliferation rate of the ECs

To determine whether terbutaline affects proliferation, a BrdU cell proliferation assay was performed. Therefore, the ECs were incubated with terbutaline for 6h and 48h. The data show that terbutaline does not affect the proliferation of the bEnd.3 cells after 6h and 48h (figure 4A-B).The time points were chosen because they correspond with the timing of tube formation and the timing of reaching a significant difference in proliferation between the vehicle control and the normal growth conditions with 10% FCS (figure 4C-D). In conclusion, terbutaline does not affect proliferation of bEnd.3 cells.

3.5. Terbutaline stimulates vessel formation in ovo

Next, we examined the more generaleffects of terbutaline on neo-vessel formation in a microcirculatory preparation, namely the chick chorioallantoic membrane (CAM).Briefly,on day 9 after fertilization, terbutaline has been applied on plastic discs (8-mm diameter), which wereplaced on the CAM. A solution of cortisone acetate (100 µg/disc) has been added to all discs to prevent an inflammatory response. A dried control disc loaded with dilution buffer (MQ) was used as vehicle control. After 48h incubation, the CAM was removed, and angiogenesis wasevaluated. All vessels intersecting a concentric circlepositioned in the treated area were counted (figure 5A). The results indicate that terbutaline increases the number of

blood vessels in all the investigated concentrations, although only for 250 nmolthe increase reached statistical significance compared to the vehicle control MQ (figure 5B-C). These data highlight that terbutaline not only stimulates tube formation in an isolated system of ECs*in vitro*, but it also significantly increases vessel formation in an organotypic and more general context.

3.6.Terbutaline alone does not affect functional or vascularregeneration after SCI

To unravel its potential as a therapeutic treatment strategy for CNS trauma, terbutaline has been applied in our SCI mouse model. Functionalrecovery has been assessed using the BMS scoring system. Systemic administration of terbutaline seems to increase the BMS score from day 3 post injury onwards, compared to the BMS of the control mice which only received NaCl, although the observed differences were not statistically significant (figure 6A).

To assess the effects of terbutaline on angiogenesis *in vivo*, we examined spinal cord sections for their CD31 expression (figure 6B). CD31, also known as platelet endothelial cell adhesion molecule-1 (PECAM-1),is a commonly used marker to detect ECs. Quantification of the CD31-positive area showed that both terbutaline- and NaCl-treated mice express CD31 at the same level perilesional and at the lesion site (figure 6C).

4. Discussion

In the present study, we show that terbutaline promotes angiogenesis bystimulating the β2-AR on bEnd.3 cells *in vitro* and stimulates blood vessel formation in the CAM assay, an *in ovo* model compromising of a complex vasculature (figure 7).

We have specifically stimulated endothelial β2-ARs with terbutalinein order to stimulate tube formation. We have used the bEnd.3 cells as these are the most relevant cells to investigate *in vitro* the pro-angiogenic effects of terbutaline in the context of CNS trauma and to relate the data to our SCI mouse model. Although HUVEC cells are commonly used in *in vitro* angiogenesis assays, they are not ideal as a model for the vasculature of the adult mouse CNS.

Previous studies using the general non-specific β-AR agonist isoproterenol (ISO) either increased tube formation by mature ECs and EPCs or reduced tube formation in a different experimental set-up using different EC types and different outcome parameters(Galasso et al., 2013; Iaccarino et al., 2005; Iaccarino et al., 2002; O'Leary et al., 2014). Another complicating factor is the non-specific stimulation of both β1- and β2-ARs by ISO. Both

signaling pathways exert distinct and often contradictory effects in multiple biological systems (Sorriento et al., 2011). In addition, the β2-AR is the most abundantly expressed AR subtype on blood vessels and ECs (Sorriento et al., 2011). It is therefore crucial to study selective β2-AR signaling in angiogenesis.

Three β2-AR agonists have been reported so far to play a role in angiogenesis. Clenbuterol and zilpaterol are known to stimulate the release of pro-angiogenic factors from macrophages (Verhoeckx et al., 2006). However, these are controversial agents because of their anabolic capacity (Delmore et al., 2010; Dutt et al., 2015). In contrast to clenbuterol and zilpaterol, terbutaline is not reported to be anabolic, avoiding adverse effects such as cardiac hypertrophy (Delmore et al., 2010; Dutt et al., 2015; Lara-Pezzi et al., 2009). Salbutamol is another specific β2-AR agonist correlated with angiogenesis. However, it was reported to decrease the number of blood vessels in the CAM-assay (Pullar, 2015). In contrast, specific β2-AR stimulation with terbutaline induced tube formation by bEnd.3 cells and blood vessel formation in the CAM.

Further analyses of the pathways involved in terbutaline-induced tube formation showed that the inhibition of both AKT and ERK significantly reduced the tube formation by bEnd.3 cells, implying that both pathways are important during EC tubulogenesis. This is in accordance with previous findings showing that AKT and ERK signaling are required for the tube formation of choroid-retinal ECs (Jin et al., 2013).

To explore the effects of terbutaline on other key steps of angiogenesis, we performed MTT-assays and BrdU-assays. The MTT-assay revealed that short term application (6h) of low doses of terbutaline (<10 µM) does not affect the metabolic activity. Long term application (48-72h; 0.01 µM-10 µM terbutaline) reduced the metabolic activity without effects on proliferation. These data suggest that terbutaline reduces the viability of the ECs. Indeed, it has been shown that stimulation of β2-ARs induces apoptosis of human aortic ECs via p38/MAPK signaling (Iaccarino et al., 2005). Accordingly, we show that inhibition of MAPK/ERK signaling reversed the effects of terbutaline on metabolic activity, indicating the involvement of this pathway in terbutaline-mediated proliferation and cell viability. In addition, also other angiogenic factors, such as angiopoietin 2 (Ang2), transforming growth factor β (TGFβ) and angiotropin, play dualistic roles in angiogenesis. For Ang2 it depends on the co-stimulatory molecules. For instance, in the presence of VEGF, application of

angiopoietin 2 mediates an increase in the capillary diameter, induces migration and proliferation of ECs. In the absence of VEGF, however, it causes apoptosis of ECs and regression of blood vessels (Lobov et al., 2002). Like angiopoietin 2, TGF-β can act as an angiostatic or angiogenic molecule.Comparable to terbutaline, angiotropin is not mitogenic for ECs, but it promotes tube formation and stimulates angiogenesis in chorioallantoic membrane and cornea pocket assays (Hockel et al., 1988).

To confirm the angiogenic potency of terbutaline in an organotypicmodel with fully functional blood capillaries, we applied the compound on the CAM. Here, the number of blood vessels was increased by 15% after terbutaline treatment compared to vehicle. In contrast, the non-specific β-AR agonist ISO and the specific β2-AR agonist salbutamolreduced angiogenesis by 29-45% in the CAM-assay (O'Leary et al., 2014; Pullar, 2015). Moreover, Pullar*et al* showed an increase in angiogenesis after application of the β2-AR antagonist ICI 118,551 on the CAM (Pullar et al., 2012). These studies, together with our data, highlight that terbutaline is the most promising specific β2-AR agonist to stimulate blood vessel formation.

The discrepancies could be explained either by pharmacokinetic differences between the different compounds or by important methodological differences in the study of these factors. As mentioned above, ISO non-specifically activatesboth β-adrenoceptor subtypes. For this reason, we have chosen to specifically stimulate the β2-ARs with terbutaline. In addition, the time points of application of the compounds, the incubation times as well as the method of analyses differ between the different studies. In previous studies the compounds were already applied on day 5 after fertilization (O'Leary et al., 2014; Pullar et al., 2012; Pullar, 2015). We applied terbutaline at day 9 after fertilization because only then is the capillary plexus of the CAM formed and associated with the overlying epithelial cells to mediate gas exchange. Before this time point, the blood vessels are immature and lack a complete basal lamina and smooth muscle cells. It is interesting to note that terbutaline stimulates angiogenesis in two models involving different species, namely tube formation by mouse ECs and blood vessel formation in the chick CAM. Since many angiogenic processes are evolutionary well-conserved, the results of this study, namely the pro-angiogenic capacity of terbutaline in two different species, give a first indication about the potential clinical relevance of the compound.

Finally, we have evaluated the therapeutic potency of terbutaline *in vivo*. Therefore, we applied terbutaline intraperitoneally in an SCI mouse model and monitored recovery of locomotor function in addition to revascularization. Although we observed a suggestive positive trend, the systemic administration of terbutaline did not significantly improve the functional outcome, nor did it affect revascularization. Interestingly, previous reports showed that β2-AR agonists like clenbuterol enhance functional recovery after SCI (Bai et al., 2010). However, the effects on angiogenesis were not investigated. Although terbutaline and clenbuterol have similar chemical structures, they have different pharmacokinetic properties (Smith, 1998). These pharmacokinetic differences most likely have played a role in the observed discrepancies. In addition, the use of clenbuterol has been associated with various adverse effects like tachycardia and muscle tremors (Brett et al., 2014). Therefore, it is currently not considered as a promising treatment strategy for SCI.

The discrepancy between very promising *in vitro* findings and only a suggestive, non-significant improvement of the functional outcome *in vivo* may result from differences between the *in vitro* and *in vivo* situation as well as from the non-traumatic nature of the *in vitro* models (ECs and CAM) compared to the highly inflammatory post-traumatic nature of the *in vivo* injury model (SCI). After SCI, an overall non-permissive environment for the growth of axons and blood vessels is created due to the inflammation in combination with the lack of oxygen and nutrients, etc. We conclude that terbutaline as a monotherapy may not be potent enough to overcome these challenges and to induce proper revascularization of the spinal cord. A more complex approach is necessary, as adequate recovery of function can only be achieved when revascularization of the spinal cord is complemented with axonal regeneration and plasticity, elimination of glial scar tissue and remyelination.

An *in vivo* study by Thaker*et al*. suggested that terbutaline may indirectly influence angiogenesis by modulating VEGF secretion of non-endothelial cells. In this study mice bearing ovarian cancer cells were treated daily with 5 mg/kg terbutaline. The authors show that terbutaline treatment leads to higher VEGF production by the carcinoma cells, which resulted in enhanced tumor vascularization as revealed by CD31-staining (Thaker et al., 2006). In addition, Iaccarino*et al.* have shown in two studies that β2-AR activation may either directly stimulate EC proliferation *in vitro* or indirectly by increasing their VEGF release (Iaccarino et al., 2005). In our *in vivo* model it cannot be excluded that VEGF secretion was stimulated, however, we do not see any increase in vessel density (CD31) in the injured spinal cord after terbutaline treatment *in vivo*.

In conclusion, we provide novel evidence that the specific β2-AR agonist terbutaline can stimulate angiogenesis *in vitro*. The stimulation of tube formationis mediated via theERK- and Akt-pathway.Moreover, terbutaline is able to stimulate neo-vascularization*in ovo*. Compared to other β-AR modulators, terbutaline appears to be the most promising compound as it acts specifically on the β2-AR, whereas ISO stimulates both β1- and β2-ARs. In addition, adverse effects like muscle hypertrophy can be avoided as unlike clenbuterol and zilpaterol, it is not reported to be an anabolic agent. Salbutamol should be avoided because it acts as an anti-angiogenic agent. Taken together, our findings make terbutaline the most promising β2-AR agonist to stimulate angiogenesis, whilst underlining the importance of further research into the angiotherapeutic relevance of terbutaline and β2-AR signalingin CNS trauma models.

Abbreviations

AR (adrenoceptor)
ANG (angiopoietin)
Akt (protein kinase b)
BBB (blood brain barrier)
bFGF (basic fibroblast growth factor)
BMS (Basso mouse scale)
BrdU (5-bromo-2'-deoxyuridine)
CAM (chorioallantoic membrane)
CD31 (Cluster of differentiation 31)
CNS (central nervous system)
DMEM (Dulbecco's Modified Eagle's Medium)
DMSO (dimethyl sulfoxide)
DPI (days post injury)
E (embryonic day)
EC (endothelial cell)
ECM (extracellular matrix)
ELISA (Enzyme-linked immunosorbent assay)
EPC (endothelial precursor cell)
ERK (extracellular-signal-regulated kinases)
FCS (fetal calf serum)
FGF-2 (fibroblast growth factor 2)

GPCR (G-protein coupled receptor)

GFR (growth factor reduced)

ISO (isoproterenol)

LY (LY294002Akt blocker)

NaCl (Sodium chloride)

NGM (normal growth medium)

MQ (MilliQ)

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)

PBS (phosphate buffered saline)

PECAM-1 (platelet endothelial cell adhesion molecule)

PFA (paraformaldehyde)

P/S (penicillin/streptomycin)

RT (room temperature)

SEM (standard error of the mean)

TBS-T (tris-buffered saline with tween)

Terb (terbutaline)

TGFβ (transforminggrowth factor beta)

U (U0126, ERK blocker)

VEGF (vascular endothelial growth factor)

Acknowledgements

This study was supported in part by grants from Deutsche Forschungsgemeinschaft (SPP1394) and from 'FondsvoorWetenschappelijkOnderzoek – Vlaanderen' (FWO) to SH (G.0389.12, G0A5813, G.0834.11N, G0A58113FWO), from FWO to NG (1.2.917.14N), and from 'AgentschapvoorInnovatie door WetenschapenTechnologie' (IWT) to SL.

Conflicts of interest statement

The authors declare no conflicts of interest.

References

Basso DM, Fisher LC, Anderson AJ, Jakeman LB, McTigue DM, Popovich PG. 2006. Basso Mouse Scale for locomotion detects differences in recovery after spinal cord injury in five common mouse strains. Journal of neurotrauma 23(5):635-659.

Bhadada SV, Goyal BR, Patel MM. 2011. Angiogenic targets for potential disorders. Fundamental & clinical pharmacology 25(1):29-47.

Bussolino F, Mantovani A, Persico G. 1997. Molecular mechanisms of blood vessel formation. Trends in biochemical sciences 22(7):251-256.

Delmore RJ, Hodgen JM, Johnson BJ. 2010. Perspectives on the application of zilpaterol hydrochloride in the United States beef industry. Journal of animal science 88(8):2825-2828.

Distler JH, Hirth A, Kurowska-Stolarska M, Gay RE, Gay S, Distler O. 2003. Angiogenic and angiostatic factors in the molecular control of angiogenesis. Q J Nucl Med 47(3):149-161.

Dray C, Rougon G, Debarbieux F. 2009. Quantitative analysis by in vivo imaging of the dynamics of vascular and axonal networks in injured mouse spinal cord. Proceedings of the National Academy of Sciences of the United States of America 106(23):9459-9464.

Dutt V, Gupta S, Dabur R, Injeti E, Mittal A. 2015. Skeletal muscle atrophy: Potential therapeutic agents and their mechanisms of action. Pharmacological research 99:86-100.

Galasso G, De Rosa R, Ciccarelli M, Sorriento D, Del Giudice C, Strisciuglio T, De Biase C, Luciano R, Piccolo R, Pierri A, Di Gioia G, Prevete N, Trimarco B, Piscione F, Iaccarino G. 2013. beta2-Adrenergic receptor stimulation improves endothelial progenitor cell-mediated ischemic neoangiogenesis. Circulation research 112(7):1026-1034.

Graumann U, Ritz MF, Hausmann O. 2011. Necessity for re-vascularization after spinal cord injury and the search for potential therapeutic options. Current neurovascular research 8(4):334-341.

Hockel M, Jung W, Vaupel P, Rabes H, Khaledpour C, Wissler JH. 1988. Purified monocyte-derived angiogenic substance (angiotropin) induces controlled angiogenesis associated with regulated tissue proliferation in rabbit skin. The Journal of clinical investigation 82(3):1075-1090.

Iaccarino G, Ciccarelli M, Sorriento D, Galasso G, Campanile A, Santulli G, Cipolletta E, Cerullo V, Cimini V, Altobelli GG, Piscione F, Priante O, Pastore L, Chiariello M, Salvatore F, Koch WJ, Trimarco B. 2005. Ischemic neoangiogenesis enhanced by beta2-adrenergic receptor overexpression: a novel role for the endothelial adrenergic system. Circulation research 97(11):1182-1189.

Iaccarino G, Cipolletta E, Fiorillo A, Annecchiarico M, Ciccarelli M, Cimini V, Koch WJ, Trimarco B. 2002. Beta(2)-adrenergic receptor gene delivery to the endothelium corrects impaired adrenergic vasorelaxation in hypertension. Circulation 106(3):349-355.

Jin J, Yuan F, Shen MQ, Feng YF, He QL. 2013. Vascular endothelial growth factor regulates primate choroid-retinal endothelial cell proliferation and tube formation through PI3K/Akt and MEK/ERK dependent signaling. Molecular and cellular biochemistry 381(1-2):267-272.

Junker V, Becker A, Huhne R, Zembatov M, Ravati A, Culmsee C, Krieglstein J. 2002. Stimulation of beta-adrenoceptors activates astrocytes and provides neuroprotection. European journal of pharmacology 446(1-3):25-36.

Lara-Pezzi E, Terracciano CM, Soppa GK, Smolenski RT, Felkin LE, Yacoub MH, Barton PJ. 2009. A gene expression profile of the myocardial response to clenbuterol. Journal of cardiovascular translational research 2(2):191-197.

Liekens S, Bronckaers A, Hernandez AI, Priego EM, Casanova E, Camarasa MJ, Perez-Perez MJ, Balzarini J. 2006. 5'-O-tritylated nucleoside derivatives: inhibition of thymidine phosphorylase and angiogenesis. Molecular pharmacology 70(2):501-509.

Lobov IB, Brooks PC, Lang RA. 2002. Angiopoietin-2 displays VEGF-dependent modulation of capillary structure and endothelial cell survival in vivo. Proceedings of the National Academy of Sciences of the United States of America 99(17):11205-11210.

Loy DN, Crawford CH, Darnall JB, Burke DA, Onifer SM, Whittemore SR. 2002. Temporal progression of angiogenesis and basal lamina deposition after contusive spinal cord injury in the adult rat. The Journal of comparative neurology 445(4):308-324.

Nelissen S, Vangansewinkel T, Geurts N, Geboes L, Lemmens E, Vidal PM, Lemmens S, Willems L, Boato F, Dooley D, Pehl D, Pejler G, Maurer M, Metz M, Hendrix S. 2014. Mast cells protect from post-traumatic spinal cord damage in mice by degrading inflammation-associated cytokines via mouse mast cell protease 4. Neurobiology of disease 62:260-272.

O'Leary AP, Fox JM, Pullar CE. 2014. Beta-Adrenoceptor Activation Reduces Both Dermal Microvascular Endothelial Cell Migration Via a cAMP-Dependent Mechanism and Wound Angiogenesis. Journal of cellular physiology.

Pullar CE, Le Provost GS, O'Leary AP, Evans SE, Baier BS, Isseroff RR. 2012. beta2AR antagonists and beta2AR gene deletion both promote skin wound repair processes. The Journal of investigative dermatology 132(8):2076-2084.

Pullar GSLPaCE. 2015. b2-Adrenoceptor Activation Modulates Skin Wound

Healing Processes to Reduce Scarring. Journal of Investigative Dermatology 135:279-288.

Qian L, Wu HM, Chen SH, Zhang D, Ali SF, Peterson L, Wilson B, Lu RB, Hong JS, Flood PM. 2011. beta2-adrenergic receptor activation prevents rodent dopaminergic neurotoxicity by inhibiting microglia via a novel signaling pathway. J Immunol 186(7):4443-4454.

Ribatti D. 2008. Chick embryo chorioallantoic membrane as a useful tool to study angiogenesis. International review of cell and molecular biology 270:181-224.

Sorriento D, Trimarco B, Iaccarino G. 2011. Adrenergic mechanism in the control of endothelial function. Translational medicine @ UniSa 1:213-228.

Verhoeckx KC, Doornbos RP, Witkamp RF, van der Greef J, Rodenburg RJ. 2006. Beta-adrenergic receptor agonists induce the release of granulocyte chemotactic protein-2, oncostatin M, and vascular endothelial growth factor from macrophages. International immunopharmacology 6(1):1-7.

Zlotnik A, Klin Y, Gruenbaum BF, Gruenbaum SE, Ohayon S, Leibowitz A, Kotz R, Dubilet M, Boyko M, Shapira Y, Teichberg VI. 2012. beta2 adrenergic-mediated reduction of blood glutamate levels and improved neurological outcome after traumatic brain injury in rats. Journal of neurosurgical anesthesiology 24(1):30-38.

Figure legends

Figure 1: Terbutaline stimulates tube formation by bEnd.3 cells. A) The bEnd.3 cells, seeded on growth factor reduced (GFR)-matrigel in Ibidi-slides, were treated for 6h with different concentrations of terbutaline (0.01; 1; and 10 µM). Normal growth medium without FCS was used as vehicle to dilute terbutaline. Representative pictures are shown of the vehicle control-treated and the terbutaline 1 µM-treated ECs. Black arrows mark the formed tubes. **B)** Images were evaluatedwith the Angio-analyzer of ImageJ to quantify the total branching length, the number of nodes and the sum of the number of segments and branches. Scale bar= 500 µm. Data were normalized to control and represented as mean ± SEM; n= 8 wells/condition; *p<0.05 and **p<0.01.

Figure 2: Terbutaline stimulates tube formation via Akt and ERK pathway signaling. A-B) The bEnd.3 cells were seeded onto a GFR-matrigel and treated for 6h with terbutaline (1 µM), either or not in combination with the Akt blocker LY (10 µM) (A) or the ERK blocker U (100 µM) (B). The vehicle control contains NGM without FCS. Representative pictures are shown of the vehicle control, terb 1 µM, terb 1 µM combined with LY 10 µM and terb 1 µM combined with U 100 µM. Black arrows mark the formed tubes. **C)** Images were evaluated with the Angio-analyzer of ImageJ to quantify the total branching length, the number of nodes and the sum of the number of segments and branches. **D)** For the control conditions, the bEnd.3 cells were treated for 6h with the Akt blocker (10 µM) or the ERK blocker (100 µM) alone. The vehicle control contains NGM without FCS. The blockers were also combined with the normal growth medium of the cells (NGM) which contains 10% FCS. FCS is known to induce tube formation and can be considered as a positive control. Images were evaluated as described previously. The controls shown in these graphs are identical to the controls in figuresA-C. For reasons of readability, they are shown in separate graphs. Scale bar= 500 µm. Data were normalized to control and represented as mean ± SEM; n= 24 wells/condition for the controls and terb 1 µM, n= 8-10 wells/condition for the combinations with LY and U; *p<0.05; **p<0.01 and ***p<0.001.

Figure 3: Terbutaline slightly decreases the metabolic activity of the ECs via the ERK pathway. A-B) The bEnd.3 cells were treated for 6h or 72h with terbutaline (0.01; 1; 10 µM). The vehicle control contains NGM without FCS. **C-D)** To investigate the involvement of the Akt and the ERK pathway, terbutaline (1 µM) has been incubated in combination with the Akt blocker (10 µM; C) or the ERK blocker (10 µM; D) for 72h. **E-F)**The bEnd.3 cells were

treated for 72h with the Akt blocker LY (10 µM; E) or the ERK blocker U (10 µM; F) alone. The vehicle control contains NGM without FCS. The blockers were also applied to the NGM containing 10% FCS. The controls shown in these graphs are identical to the controls in figure 3. For reasons of readability, they are shown in separate graphs. Data were normalized to control and represented as mean ± SEM; n= 22-30 wells/condition; *p<0.05; **p<0.01 and ***p<0.001.

Figure 4: Terbutaline does not affect the proliferation of the bEnd.3 cells. A-B) The bEnd.3 cells were treated for 6h or 48h with terbutaline (0.01; 1; 10 µM). The vehicle control contains NGM without FCS. **C-D**) In addition, the bEnd.3 cells were grown in NGM with 10% FCS for 6h and 48h. The controls shown in these graphs are identical to the controls in figure A and B. To increasereadability, they are shown in separate graphs. Data were normalized to control and represented as mean ± SEM; n= 10-15 wells/condition: ***p<0.001.

Figure 5: Terbutaline stimulates angiogenesis in the CAM-assay. A) On day 9 after fertilization, terbutaline has been applied onto the CAM on sterile plastic discs.A control disc loaded with dilution buffer (MQ) is placed on the CAM as vehicle control. After 48h incubation, the CAM is dissected and photographed. All vessels intersecting a concentric circle (radius 3.5 mm) positioned in the treated area are counted double blinded and independently by two investigators (arrows). **B)** Terbutaline stimulatesblood vessel formation, compared to the vehicle control. **C)** Quantification reveals that terbutaline, when applied in an amount of 250 nmol, significantly increases the number of blood vessels, compared to the control. A trend towards increased angiogenesis is also visible for the other conditions, although statistical significance has not been reached. Scale bar: 500 µm. Data are represented as mean ± SEM; n= 12-23 CAMs/condition. *p<0.05.

Figure 6: Terbutaline alone does not affect functional or vascular regeneration after SCI.A) The first 9 days after SCI, mice were injected twice a day with either terbutaline (5 mg/kg) or with the vehicle NaCl. Functional recovery was analyzed according to the BMS. The data are presented as mean values ± SEM; n= 5-7. **B)**Spinal cord cryosections were stained for CD31.Representative pictures at the lesion siteare shown. Scale bar= 300 µm. **C)** A color threshold was set in order that the image analysis software could automatically detect the intensively stained CD31+ blood vessels. Quantification of the CD31+ area (µm²) is shown. The data are presented as mean values ± SEM; n = 6-9.

Figure 7: Terbutaline stimulates angiogenesis both *in vitro* and *in ovo*. Summarized, the short-acting, highly specific β2-AR agonist terbutaline significantly induced bEnd.3 tube formation in thematrigel*in vitro* assay. Administration of specific inhibitors of ERK and Akt signaling both inhibited terbutaline-induced tube formation.Most importantly, terbutaline increased the number of blood vessels in the*in ovo* setting, underscoring the general effects of terbutaline in an organotypic system. In conclusion, these data indicate that terbutaline is a promising β2-AR agonist to stimulate blood vessel formation.

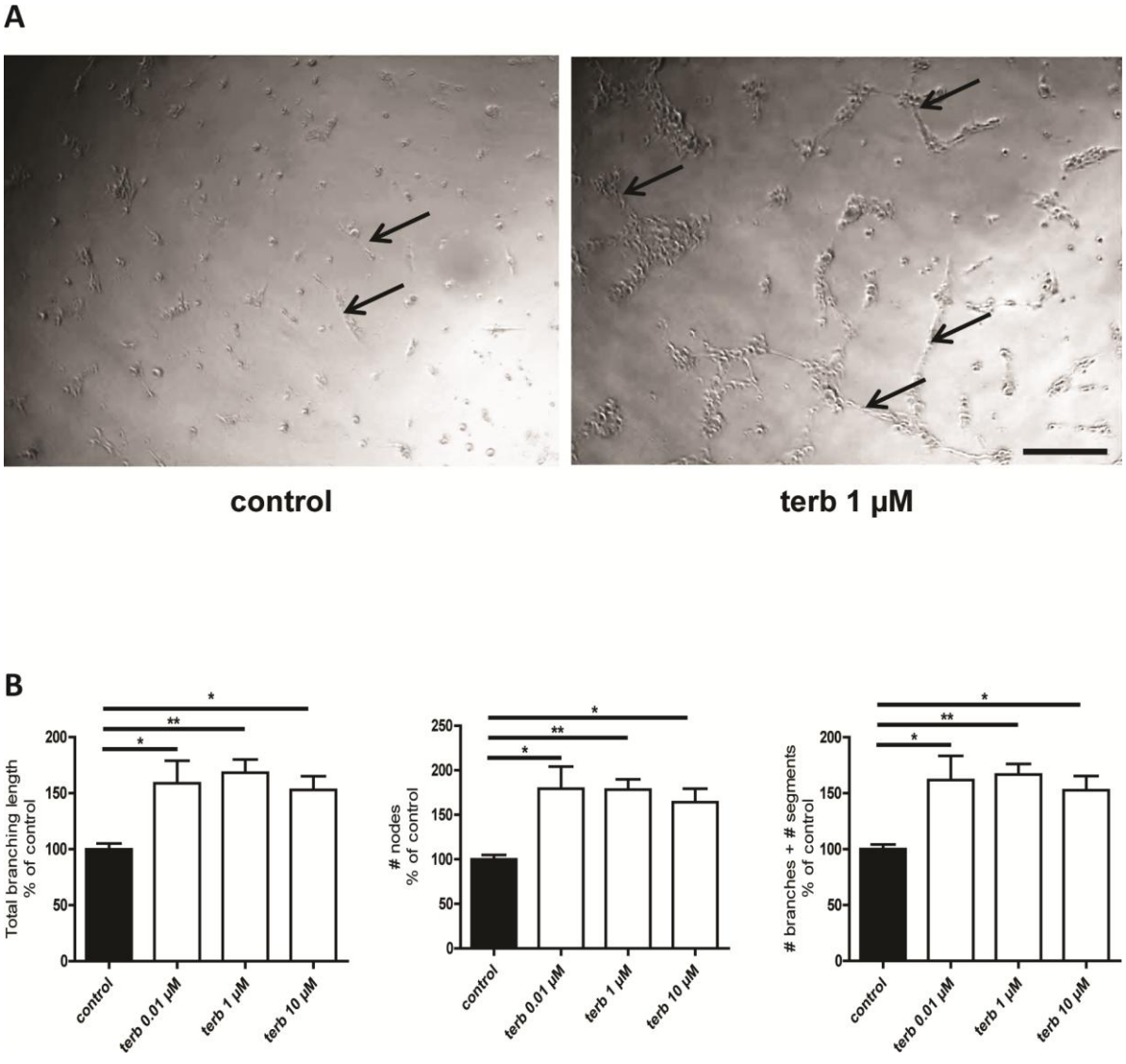

Figure 1

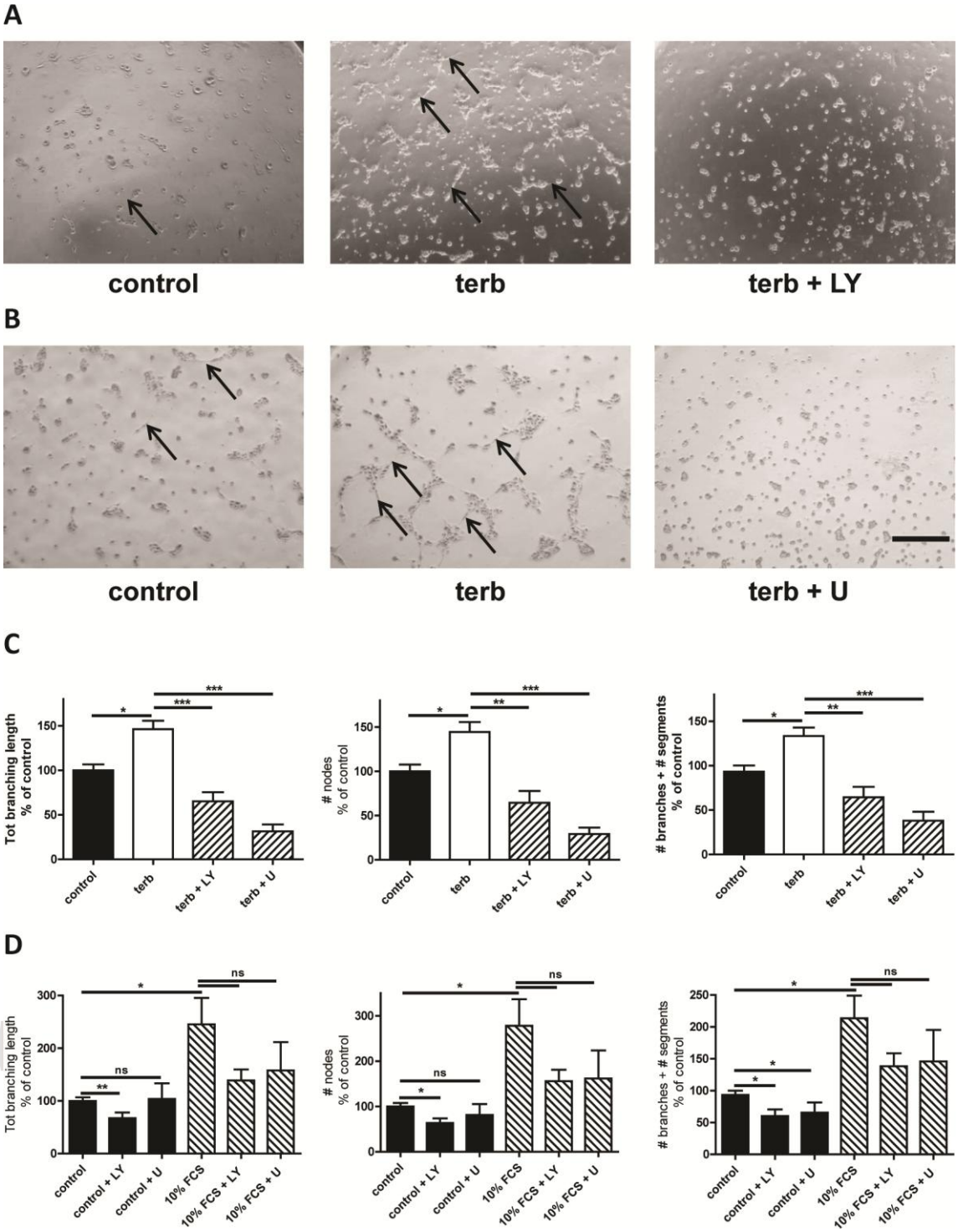

Figure 2

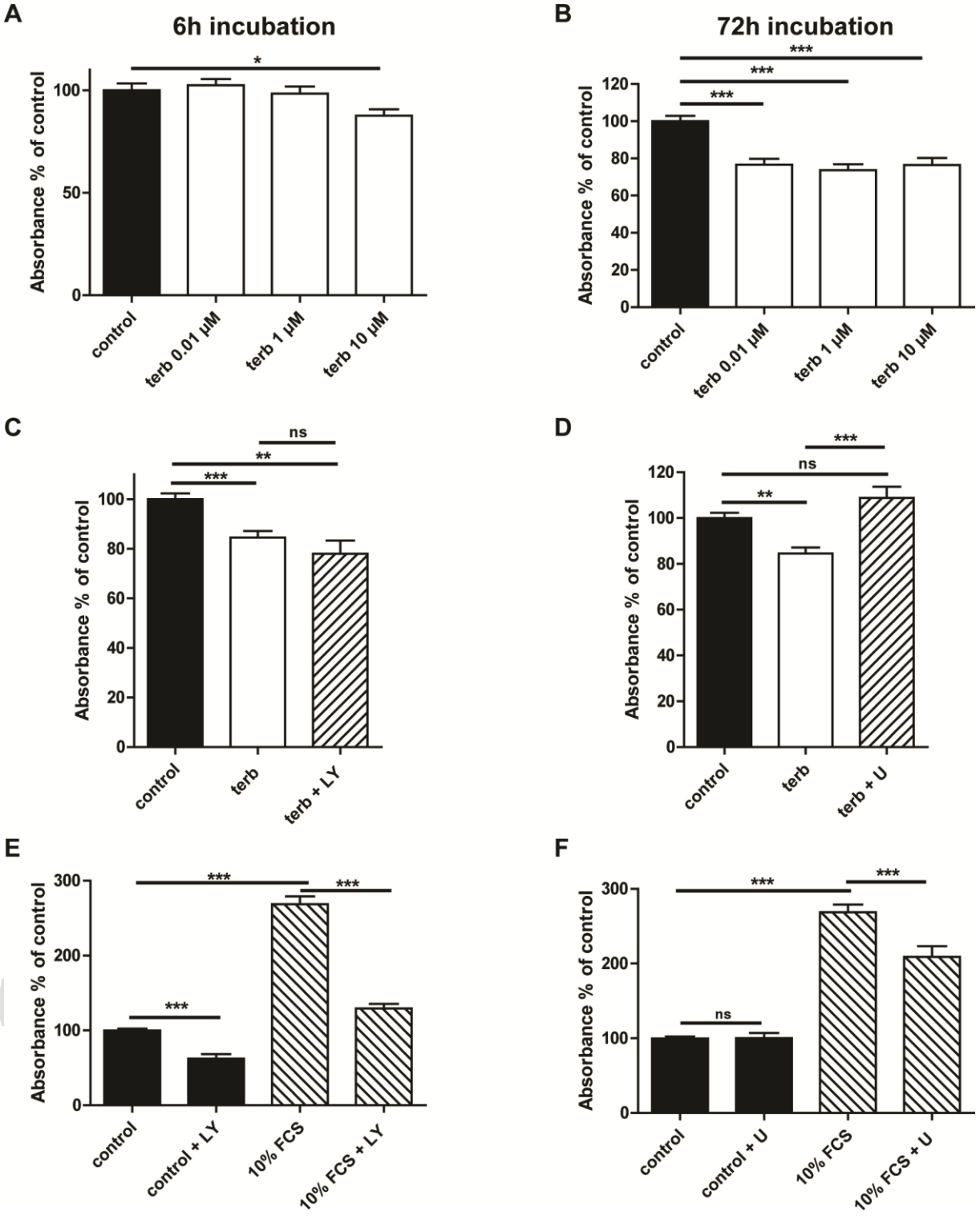

Figure 3

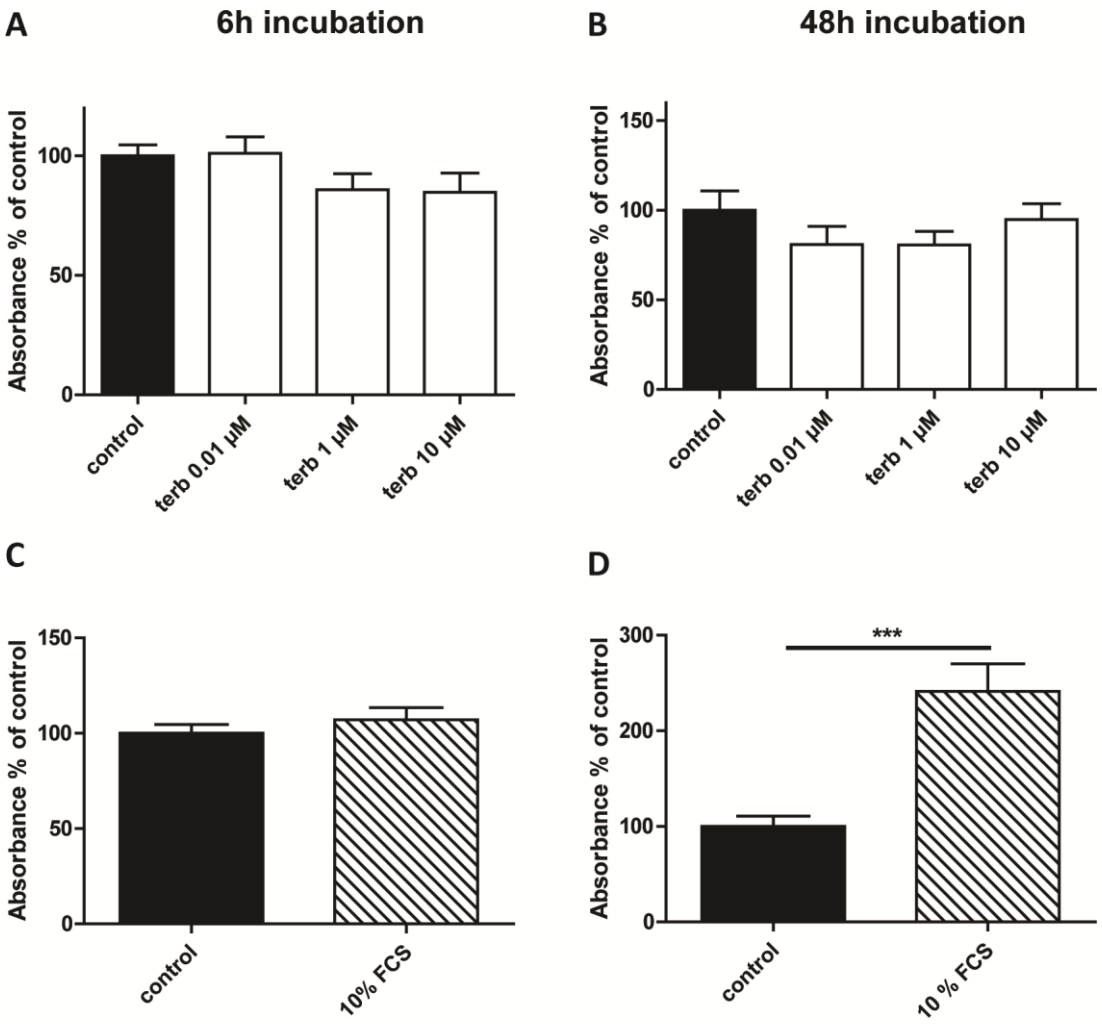

Figure 4

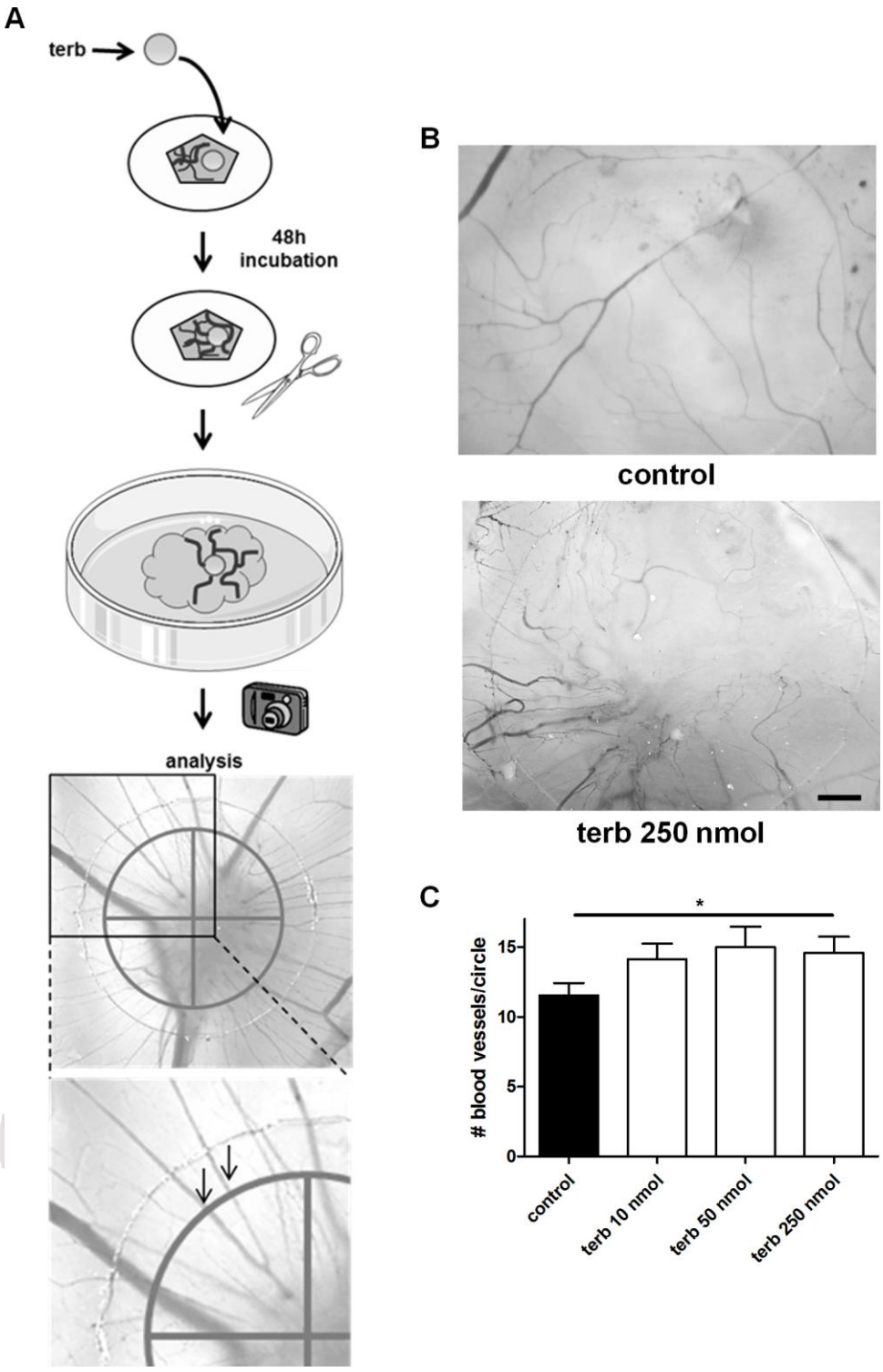

Figure 5

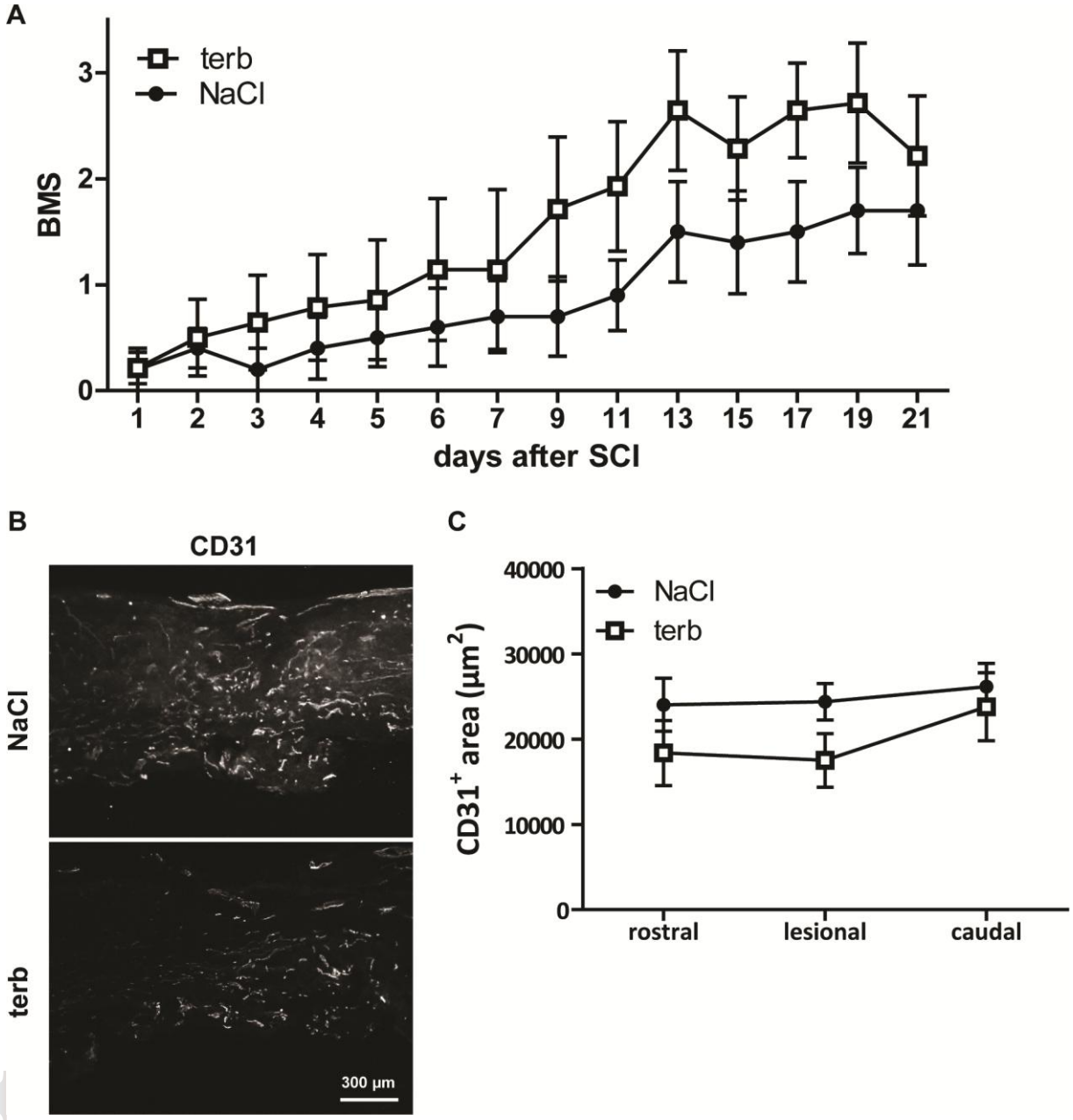

Figure 6

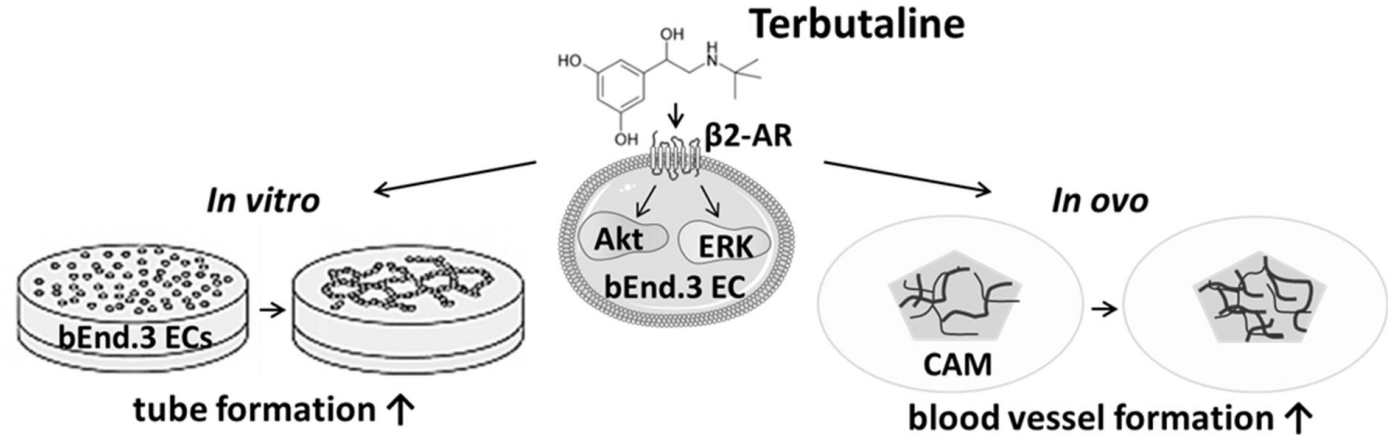

Figure 7