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BASIC SCIENCE

New anti-angiogenic compound based on chemically modified heparin

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Abstract

Purpose The purpose of this study was to measure the anti-angiogenic effect of N-desulfated Re–N-acetylated, a chemically modified heparin (mHep).

Methods In vitro assays (cell tube formation, viability, proliferation, and migration) with endothelial cells were performed after 24 h of treatment with mHep at 10, 100, and 1000 ng/mL or saline. In vivo tests were performed after laser-induced choroidal neovascularization (CNV) in rats, followed by an intravitreal injection (5 µL) of mHep (10, 100, 1000 ng/mL) or balanced salt solution. Immunofluorescence analysis of the CNV was performed after 14 days.

Results mHep produced a statistically significant reduction in cell proliferation, tube formation, and migration, without cell viability changes when compared to saline. Mean measures of CNV area were 54.84×10^6 pixels/mm ($\pm 12.41 \times 10^6$), 58.77×10^6 pixels/mm ($\pm 17.52 \times 10^6$), and 59.42×10^6 pixels/mm ($\pm 17.33 \times 10^6$) in groups 100, 1000, and 10,000 ng/mL, respectively, while in the control group, mean area was 72.23×10^6 ($\pm 16.51 \times 10^6$). The P value was 0.0065. Perimeter analysis also demonstrated statistical significance ($P = 0.0235$) with the mean measure of 93.55×10^4, 94.23×10^4, and 102×10^4 in the 100 ng/mL, 1000 ng/mL, and control groups, respectively.

Conclusions These results suggest that mHep N-DRN is a potent anti-angiogenic, anti-proliferative, and anti-migratory compound with negligible anticoagulant or hemorrhagic action and no cytotoxicity for retina cells. This compound may serve as a candidate for treating choroidal neovascularization.

Keywords Angiogenesis inhibitors · Choroidal neovascularization · Vascular endothelial growth factor · Heparin

Key messages

- Heparin is a glycosaminoglycan that modulate the angiogenesis and regulate vessel growth via ECM interactions.
- Chemically-modified heparin demonstrated anti-angiogenic, anti-proliferative and anti-migratory effects in vitro study.
- Choroidal neovascularization area was significantly reduced when treated with chemically-modified heparin compared to control group, in an animal model.

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Introduction

Choroidal neovascularization (CNV) is an important cause of vision loss, especially in age-related macular degeneration (AMD), which is the leading cause of vision loss in older adults in industrialized countries [1]. In neovascular AMD, new vessels sprout from the choroid through Bruch's membrane and enter the retina. This process leads to photoreceptor loss and damage to sensory retina followed by a submacular fibrotic scar [2]. The pathogenesis of CNV is controlled by angiogenic agents such as growth factors, cytokines, and extracellular matrix (ECM) components, including glycosaminoglycans (GAGs) [3, 4].

GAGs interact with several proteins controlling and modulating biological activities, including angiogenesis. Heparin and heparan sulfate (HS) are GAGs that modulate the angiogenesis and regulate vessel growth via ECM interactions, providing signaling for endothelial cell proliferation, survival, and migration after binding to integrin [4, 5]. The dynamic remodeling of ECM by metalloproteinase is related to vascular tube formation [6, 7]. Angiogenesis modulation also occurs through the interaction of GAGs with angiogenic growth factors and cytokines such as VEGFs, FGFs, TGF-β, IFN-γ, and TNF-α [8]. VEGF-A is one of the most significant growth factors involved in angiogenesis, and it contains a heparin-binding domain [9, 10]. VEGF-A's stability increases when bound to heparin, with bioavailability and protein half-life control, suggesting that the heparin-binding domain targets diseases related to VEGF [11, 12]. These findings combined with the properties of GAGs to bind and modulate angiogenic factors suggest a motive for studying and designing new synthetic GAG analogs to inhibit angiogenesis.

The development of new drugs that target the angiogenic cascade of CNV could significantly impact patients' health and quality of life with AMD [13]. CNV treatment has been based on anti-vascular endothelial grown factor (anti-VEGF) inhibitors; however, all available medications carry treatment burdens, including tachyphylaxis and nonresponse with vision loss over time [14]. Therefore, the search for new therapies to treat CNV is of fundamental importance. Among various compounds, heparin is known for its anticoagulant activity; however, it also has anti-inflammatory activity, including inactivation of chemokines, inhibition of the activation and recruitment of inflammatory cells, and modulation of the synthesis of matrix metalloproteinases (MMPs) [15]. Heparins display anti-angiogenic activity, inhibiting capillary tube formation by endothelial cells (ECs); this inhibitory feature depends on the heparins' molecular weight or structure [16, 17].

Despite its great potential as an anti-inflammatory and anti-angiogenic agent, heparin's clinical use is limited by its vigorous anticoagulant activity and risk of hemorrhagic complications. Therefore, chemically modified heparin derivatives, devoid of anticoagulant and hemorrhagic activity, may be preferable to heparin for controlling inflammation and neovascularization. Chemically modified heparins are created after depolymerization, desulfation, and acetylation to maintain anti-angiogenic potential and to remove interference on hemostasis. N-sulfate groups and O-sulfate are removed separately, and the resultant free amino groups are acetylated [18].

Previously, we showed that a heparinoid with low content of 2-O-sulfate groups isolated from marine shrimp presented negligible anticoagulant and hemorrhagic activities, with reduced acute inflammatory and angiogenesis processes [19]. Based on these findings, the present study was designed to determine the effects of intravitreal chemically modified heparin (N-desulfated Re–N-acetylated heparin) on modulation angiogenesis.

Materials and methods

Modified heparin preparation and characterization

Unfractionated heparin (UFH) from porcine intestinal mucosa (Bioiberica, Spain) was used to generate the N-desulfated Re–N-acetylated heparin (N-acetyl heparin). Heparin desulfation and re–N-acetylation was performed as previously described [20]. Essentially, UFH was N-desulfated by solvolytic desulfation of the heparin pyridium salt in DMSO:Methanol at 60 °C. The end product was precipitated with cold ethanol saturated with sodium carbonate. The precipitate was then dissolved in an aqueous solution of saturated sodium carbonate and re–N-acetylation was carried out by the addition of acetic anhydride at 0 °C. The final product was precipitated and desalted using gel permeation chromatography connected to a FPLC system (GE) (Figs. 1–6).

The heparin structure was determined by 2D (heteronuclear single quantum coherence, 13C-1H HSQC) nuclear magnetic resonance (NMR). Briefly, samples of 10 mg/mL in deuterium oxide (99,9%, Cambridge Isotope Laboratories Inc., Andover, MA, USA) were used for spectra acquisition at 22 °C in a Bruker Avance NEO 500 MHz spectrometer [21, 22]. The average molecular weight of both compounds is equivalent (around 16 kDa) as the chemical modifications are optimized to cause minimal depolymerization (Fig. 1). The UFH degree of sulfation is 2.3, and no free amino groups were detected on the N-acetyl heparin, suggesting a level of N-acetylation > 95% (based on NMR sensitivity).

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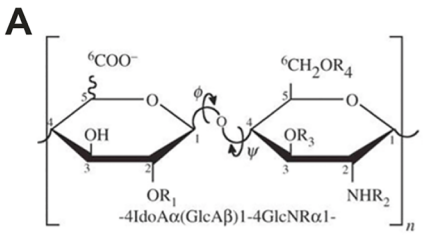

	R1	R2	R3	R4
Heparin	Sulfate	Sulfate	OH	Sulfate
N-Acetyl Heparin	Sulfate	Acetyl	OH	Sulfate

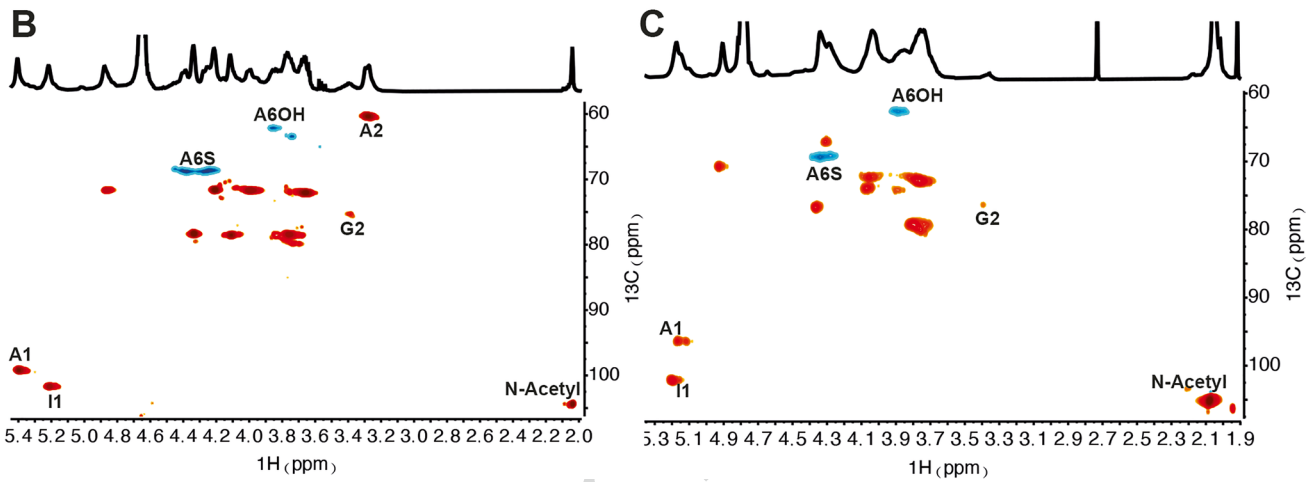

Fig. 1 Characterization of N-desulfated Re–N-acetylated heparin (mHep). **A** Predominant disaccharide repeats in UFH and N-acetyl heparin. **B** 13C-1H heteronuclear single quantum coherence spectroscopy (HSQC) NMR spectrum of UFH. I denotes iduronic acid; G, glucuronic acid; and A, glucosamine; and **C** 13C-1H HSQC spectrum of N-acetyl heparin. I denotes iduronic acid; G, glucuronic acid; and A, glucosamine

Cell culture and animals

Adult human retinal pigment epithelial cells (ARPE-19) were cultured in DMEM/F12 medium (Invitrogen, San Diego, CA, USA) supplemented with 10% fetal bovine serum (FBS) (Cultilab, Campinas, SP, Brazil), 15 mM HEPES, 2.0 mM L-glutamine, 0.5 mM sodium pyruvate, and 20 mM sodium bicarbonate in 5.0% CO_2 atmosphere. RAECs (rabbit aortic endothelial cells) were cultured in F12 medium (Invitrogen) supplemented with 10% FBS (Cultilab) and 20 mM sodium bicarbonate in 2.5% CO_2 atmosphere [23]. All cultures were plated on Falcon culture dishes (BD Falcon, San Jose, CA, USA).

For in vivo experiments, male heterozygote pigmented Zucker rats weighing 200 g were used. Animals were maintained on a 12:12-h dark–light cycle at room temperature and had free access to standard chow and water. All experiments were performed following the association for Research in Vision and Ophthalmology (ARVO) and the Animal Care Ethics Committee of the Federal University of Sao Paulo (number: 5726120717).

Endothelial cell proliferation assay

Cell proliferation was assessed by direct cell counting. ECs were plated at 1×10^4 cells per well in 24-well plates and cultured for 16 h in F12 medium containing 10% FBS; the cells were then starved for 48 h in F12 medium containing 0.2% FBS. Afterwards, ECs were stimulated with 10% FBS and different concentrations of mHep (10, 100, or 1000 ng/mL) or saline (control), harvested after 24 h, and counted in a cell counter chamber. Experiments were performed in triplicate, and values were expressed as means ± SEM.

Cytotoxicity assay

Cytotoxicity was assessed by using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) assay. 10^5 ARPE-19 or 2×10^4 ECs were plated in 96-well plates and cultured for 5 days using the respective cell culture media as described at the 2.1 item. The media was replaced by

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fresh medium, containing 10% FBS and different amounts of mHep (10, 100, and 1000 ng/mL in 200 µL/well) or saline (control) and maintained for 24 h in the CO_2 atmosphere. Afterwards, the medium was aspirated and the cells washed 2× with PBS, and serum-free medium containing MTT (0.5 mg/mL) was added. ARPE or ECs were incubated with the MTT for 2 h followed by isopropanol extraction and the absorbance measured at 570 nm using an ELISA plate reader (EL·800; BioTek Instruments, Winooski, VT, USA).

Cell migration assay

Conventional transwell plates contain 24-well inserts (Corning Life Sciences, Tewksbury, USA) with each well consisting of an upper and a lower chamber separated by a microporous membrane containing randomly distributed 8-µm pores. The upper chamber of the inserts were seeded with ECs (5×10^4 cells/well) in F12 supplemented with 0.2% FBS and various concentrations of mHep (10, 100, or 1000 ng/mL) or saline (control); the lower chamber was filled with F12 medium supplemented with 10% FBS, and the plate were maintained for 16 h at 37 °C, 2.5% CO_2 [24]. Then, inserts were washed with PBS, the upper part of the insert was cleaned with a cotton swab and fixed with paraformaldehyde, permeabilized with methanol, and stained with 4′,6-diamidino-2-phenylindole (DAPI). The nuclei present at the bottom of the insert were counted by the analysis using ImageJ (NIH, Bethesda, MD, USA).

Angiogenesis assay: capillary-like tube formation

Matrigel (Corning® Matrigel Matrix) was thawed at 4 °C on ice and plated on 24-well plates and incubated at 37 °C for 16 h to gel. ECs (10^5 cells) were seeded on top of the jellified Matrigel in F12 medium containing 10% FBS and different amounts of mHep (10, 100, 1000 ng/mL) or saline (control). The cultures were maintained at 37 °C in a 2.5% CO_2 humidified atmosphere for 16 h. The experiment was performed in triplicate. Capillary-like tube formation was analyzed under an inverted light microscope at 100× magnification. Six images were randomly taken in different areas of the well and quantified by two different observers. The total length of connected cells forming capillary structures was quantified using ImageJ software and expressed as mm tube length [25].

Induction of choroidal neovascularization

Zucker rats were anesthetized with an intraperitoneal injection of a mixture of 80 mg/kg of ketamine and 8 mg/kg of xylazine. The pupils were then dilated by topical application of 1% tropicamide plus 2.5% phenylephrine (Allergan, Guarulhos, SP, Brazil). The animals were sited at the slit lamp, and a handheld coverslip associated with 50 µL of 2% methylcellulose was used as a contact lens. Photocoagulation was executed in the right eye, using an argon laser (532 nm; Quantel Medical, Cournon-d'Auvergne, France), power 120 mW, spot size 100 µm, and duration 100 ms. Four lesions around the optic disc were performed. The aim of the laser shot was to rupture Bruch's membrane, indicated by an air bubble at the moment of the laser application [25].

Intravitreous chemically modified heparin injection

Immediately following the laser procedure, the animals received an intravitreous injection of mHep using a micro-syringe (Hamilton Co, Reno, NV, USA). They were assigned to groups (1:1) according to the dose: 100, 1000, and 1000 ng/mL of mHep in 5 µL of balanced salt solution (BSS) (Alcon, Sao Paulo, Brazil) or only intravitreous BSS. The injection was performed under a stereomicroscope (Stemi 508, Carl Zeiss, Oberkochen, Germany) to visualize and confirm proper placement. After 24 h of CNV induction and intravitreous injections, the animals were anesthetized to perform fundus examination after dilation of the pupil. Animals with vitreous or retinal hemorrhages and traumatic lens injury were excluded from the study. The animals were maintained for 14 days at the animal facility and then euthanized and submitted to immunofluorescence analysis of the posterior part of the eye.

Flatmount immunofluorescence analysis

Zucker rats were subjected to CNV induction and mHep treatment as described above. After 14 days, the rats were euthanized with anesthesia overdose (10×), and eyes were enucleated. The eyecups were fixed with 2% paraformaldehyde for 30 min and washed in 0.1 mM glycine in PBS, the retina was removed, and the eyecups were incubated with anti-von Willebrand factor (1:50, sc-8068; Santa Cruz Biotechnology, Santa Cruz, CA, USA) in PBS containing 0.1% saponin at room temperature for 2 h. The eyecups were washed 6 times with PBS and incubated for 30 min with anti-rabbit IgG conjugated with Alexa Fluor 488 (Molecular Probes) in PBS and flat-mounted in Fluoromount-G (Electron Microscopy Sciences, Hatfield, PA, USA). Confocal microscopy (Leica SP8, Wetzlar, Germany) was used to analyze the flatmount slides.

The neovascularization area and perimeter were measured by two individual analyzers, using ImageJ software (National Institutes of Health) on a pixels/mm^2 scale [25].

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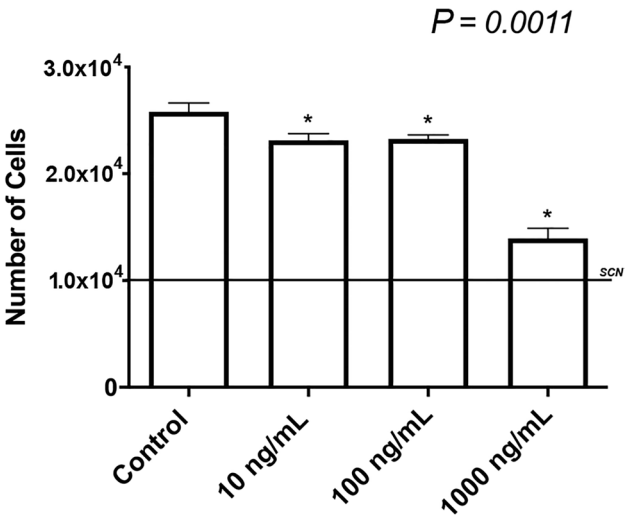

Fig. 2 N-desulfated Re–N-acetylated heparin (mHep) inhibits endothelial cell proliferation. Comparison between control and mHep were significantly different (P values = 0.0011). Differences between lower concentrations of mHep (10 and 100 ng/mL) were also significantly different when compared to 1000 ng/mL ($^{*}P < 0.05$; Bonferroni's post-test.)

Statistics

The statistical analysis was based on one-way ANOVA with Bonferroni's post-test, using GraphPad Prism 9 for Mac (GraphPad Software, Inc., La Jolla, CA, USA), with data expressed as means ± standard error of the mean. A 95% confidence interval with a 5% level of significance was adopted; results with P values < 0.05 were considered significant.

Results

mHep inhibits EC proliferation

EC proliferation was assessed by direct cell counting after exposure to various mHep concentrations. We found that all concentrations of mHep significantly inhibited fetal bovine serum (FBS's) ability to stimulate EC proliferation compared with control group (BSS) after 24 h (Fig. 2, $P = 0.0011$). The higher dose of mHep (1000 ng/ml) significantly reduced EC proliferation when compared to 10 and 100 ng/mL ($P < 0.05$).

mHep does not affect cell viability

Cell toxicity with subsequent impairment on cell viability could justified the inhibition of EC proliferation; however, the chemically modified heparin did not demonstrate cell viability significant changes for both ECs (Fig. 3A; $P = 0.1251$) and ARPE-19 (Fig. 3B; $P = 0.1699$) compared to BSS.

mHep inhibits EC migration

mHep decreased the migration rate of ECs when compared to control. Treatment of ECs with 10 and 1000 ng/mL mHep decreased the migration rate by 1.5 × compared to control, while the 100 ng/mL group decreased migration by 2.0 ×. These results were statistically significant ($P = 0.0003$; Fig. 4). There was no difference in the migration rate of ECs when the mHep groups were compared to one another.

mHep inhibits capillary tube formation

All doses of the mHep inhibited capillary tube formation compared with the control (Fig. 5A–D). The decrease in total mm of tube length was statistically significant ($P = 0.002$) for all doses of the mHep (Fig. 5E). No significant effect was observed when various concentrations of mHep were compared; however, they were significantly different when compared BSS to 100 and 1000 ng/mL ($P = 0.0144$ and 0.0014, respectively).

In vivo *studies*

Forty rats were allocated into four groups, BSS or mHep (100 ng/mL, 1000 ng/mL, and 10000 ng/mL). In the BSS group, two rats presented with vitreous hemorrhage during the

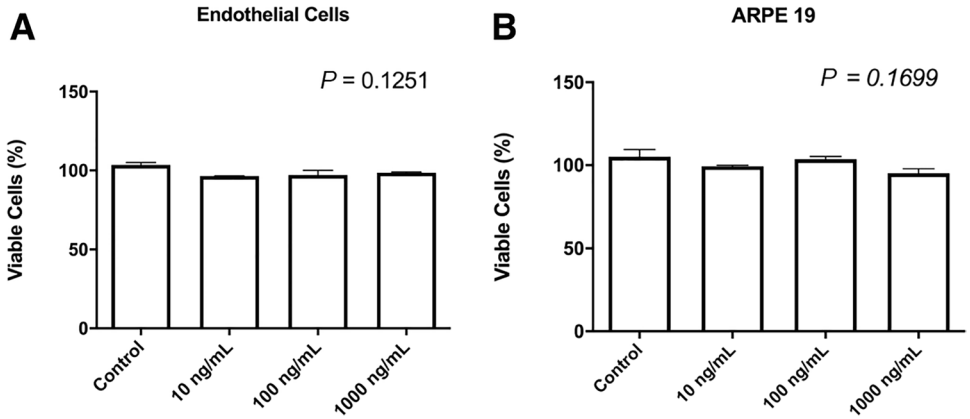

Fig. 3 Chemically modified heparin does not promote cell death. The MTT assay assessed cell viability assay that was performed in endothelial cells (**A**) and retinal pigmented cells (ARPE-19) (**B**). P values (0.1251 and 0.1699, respectively) represent the results of control group compared to mHep (10, 100 and 1000 ng/mL)

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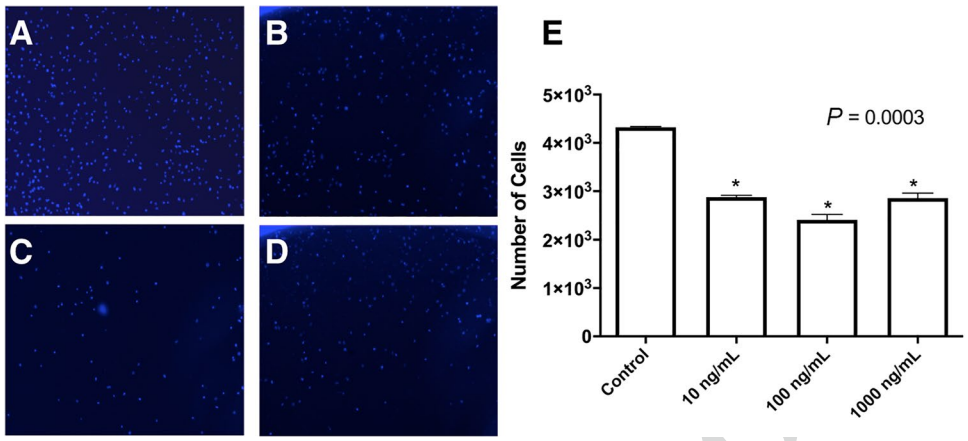

Fig. 4 N-desulfated Re–N-acetylated heparin inhibits endothelial cell migration. Endothelial cell migrations were analyzed in the presence of balanced salt solution (**A**) or various concentrations of the mHep: 10 ng/mL (**B**), 100 ng/mL (**C**), and 1000 ng/mL(**D**). *P* values = 0.0003 (ANOVA); $^{*}P < 0.05$ using Bonferroni's post-test

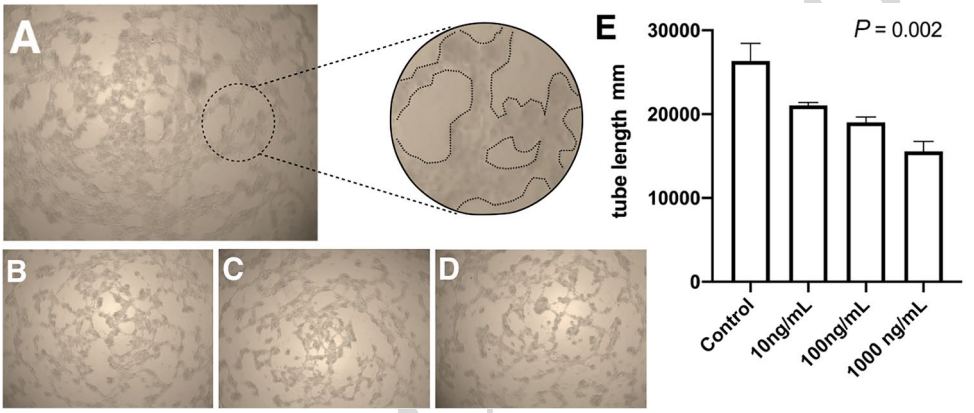

Fig. 5 N-desulfated Re–N-acetylated heparin inhibits tube formation (Matrigel-based capillary formation assay). Tube formation was examined under balanced salt solution (**A**) or various concentrations of the MHEP; 10 ng/mL (**B**), 100 ng/mL (**C**), and 1000 ng/mL(**D**). An example of the tubular structure was delimited by black lines in the magnification insert (**A**). The total length of tubular structures was measured and expressed as mm tube length (**E**) and were significantly different (*P* = 0.002)

laser procedure and were excluded; 38 rats completed the study (eight in control and ten each in the experimental groups).

No signs of hemorrhages, infection, or severe inflammation were noticed in clinical examination at 14 days after intravitreal injection. During the process of enucleation and eye cup fixation, we clinically analyzed the vitreous, and no signs of severe intraocular inflammation (vitritis) or vitreous hemorrhages were notice.

The neovascularization area comparison demonstrated significant differences between control and treatment groups (Fig. 6A–D). Mean measures of membrane area were 54.84×10^6 pixels/mm^2 ($\pm 12.41 \times 10^6$), 58.77×10^6 pixels/mm^2 ($\pm 17.52 \times 10^6$), and 59.42×10^6 pixels/mm^2 ($\pm 17.33 \times 10^6$) in groups 100, 1000, and 10,000 ng/mL, respectively, while in the control group, mean area was 72.23×10^6 ($\pm 16.51 \times 10^6$). The *P* value in the ANOVA analysis was 0.0024 (Fig. 6E). In the Bonferroni's multiple comparisons test, control × 100 ng/mL, control × 1000 ng/mL, and control × 1000 ng/mL demonstrated *P* values of 0.0028, 0.0404, and 0.0322, respectively. Perimeter analysis also demonstrated a significant difference ($P = 0.0235$).

Discussion

Heparin is a heterogeneous N- and O-sulfated glycosaminoglycan with anticoagulant activity widely used to treat and prevent thrombosis. In addition to anticoagulant activity, heparin can bind and modulate several proteins, including pro-angiogenic factors. Because of this property, heparin and its analogs have been studied as anti-angiogenic medications [5, 25].

The treatment of choroidal neovascularization with crude heparin could have consequences such as submacular hemorrhage, with irreversible vision loss due to the barrier effect, tractional changes (clot contraction), and toxicity (hemosiderin) [26]. To avoid anticoagulant activity and hemorrhagic effects of heparin, maintaining

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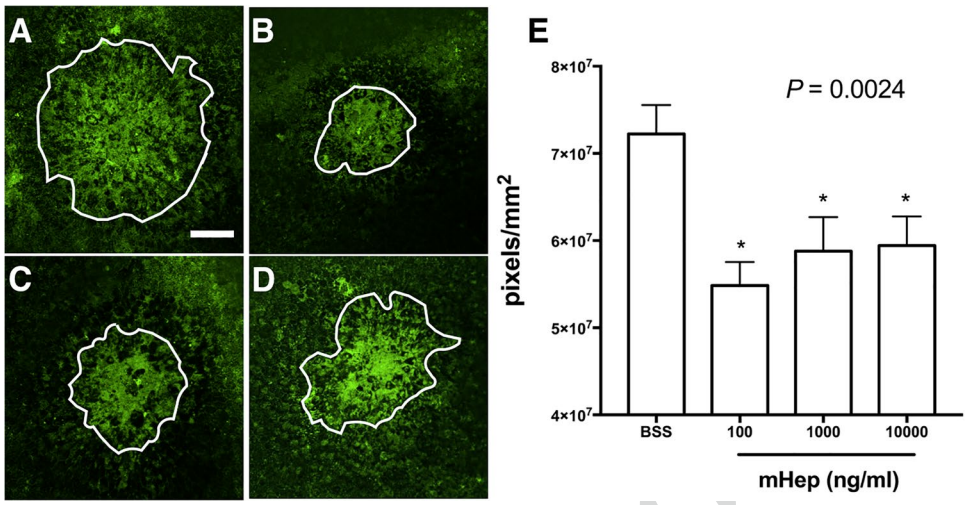

Fig. 6 Choroidal neovascularization analyses after intravitreous injection of chemically modified heparin. Choroidal neovascularization area was manually measured with ImageJ. Balanced salt solution (**A**) or various concentrations of the MHEP were compared; 100 ng/mL (**B**), 1000 ng/mL (**C**), and 10,000 ng/mL (**D**). Bar: 100 µm. *P* value ($P = 0.0024$) demonstrated a significant difference of CNV area (**E**); * represents $P < 0.05$ using Bonferroni's post-test

anti-angiogenic and anti-inflammatory effects, chemically modified heparins have been created after depolymerization, desulfation, and acetylation processes.

In the present study, we determined the anti-angiogenic potential of chemically modified heparin using in vitro and in vivo assays with N-desulfated Re–N-acetylated heparin (mHep) that presented no anticoagulant or hemorrhagic effects [27].

The in vitro studies were designed to evaluate EC proliferation, migration, and finally capillary-like tube formation, all of which are essential steps of angiogenesis.

The first steps of angiogenesis are related to EC proliferation and migration, followed by capillary morphogenesis [28, 29]. Targeting the proliferation and migration of the ECs or morphogenesis can be practical approaches to prevent CNV. Our results demonstrated that the treatment of ECs with all tested doses of mHep reduced endothelial cell migration and proliferation and capillary-like tube formation compared to the control group.

Heparin reduces angiogenesis and metastasis due to its ability to modulate growth factors and their receptors, including FGF, VEGF, and TGF-b, thereby controlling various aspects of vascular development and angiogenesis [3, 30]. Heparin impairs EC proliferation via modulating VEGF signaling, as shown by Dao et al. [31] where the authors treated ECs with heparin and observed a decreased VEGF-mediated activation of VEGFR2 and mitogenic effect in vitro. Cohen et al. [32] showed that heparin binds to VEGF and interferes with mitogenic activity. In contrast, chemically modified heparin lacking the 2-O-sulfate groups bound VEGF but inhibited its mitogenic activity [25]. EC proliferation decreased in the presence of a shrimp heparinoid with low content of 2-O-sulfate, possibly explaining its potent anti-angiogenic effect.

N-desulfated Re–N-acetylated heparin, like the 2-O-desulfated-heparin, might competitively inhibit the interaction between growth factors and cell surface heparin sulfate (HS) proteoglycans. Low-molecular-weight heparin reduced cancer cell migration in A549 cells, mediated by interference with two major PAR-1 downstream signaling pathways, MAPK/ERK and PI3K/Akt [33]. Our results agree with those in the literature, as we showed that heparin significantly reduced cell proliferation and migration.

Mousa and Mohamed [34] showed that tinzaparin, low-molecular-weight heparin, potently inhibited angiogenesis. This effect was dose-related and depended on relatively higher-molecular-weight tinzaparin fragments. These experiments suggested that heparin's effect is mediated via cellular release of tissue factor pathway inhibitor. Previous studies from our group showed strong anti-angiogenic effects of a heparinoid lacking 2-O-sulfate groups. This heparinoid interferes with the binding and modulation of FGF-2, EGF, and VEGF in endothelial cells, altering proliferation, modification of the 2-D network organization in capillary-like structures, and reduction of the CNV area in an animal model. [25]

No cytotoxic effects were detected in ARPE-19 cells or ECs when measuring mitochondrial reductase using the MTT test. The lack of cytotoxicity in ARPE-19 cells indicates a new intravitreous anti-angiogenic compound because the retinal pigment epithelium participates in the maintenance of photoreceptors metabolism with a critical role in retinal function [35].

Because mHep has anti-angiogenic activity in vitro and showed no cytotoxicity in retina cells, in vivo studies were performed using an intravitreous injection of mHep in laser-induced choroidal neovascularization in pigmented rats to evaluate the anti-angiogenic effect of this heparin.

After the laser shot in the retina, the injection of 5 µL of solution containing only BSS or combined with 100 ng, 1000 ng, or 10,000 ng mHep was applied intravitreously in rats. After 2 weeks, the eyes were enucleated, the posterior segments were dissected, and the choroidal neovascular area was analyzed using immunofluorescence with anti-vWF, an EC marker. We found significant CNV reduction in all

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animals treated with mHep compared to control, mainly at the low dose of 100 ng. This finding suggests the potential activity of mHep to treat CNV by reducing angiogenesis. HS/heparin binds and modulates heparin-binding growth factors and their receptors, controlling various aspects of vascular development and angiogenesis. This anti-angiogenic potential could be attributed to mHep and VEGF interaction because heparin-related molecules competitively bind to heparin-binding growth factors, limiting their interactions with cell surface heparan sulfate proteoglycans and cytokine receptors, consequently blocking angiogenesis [36].

Low-molecular-weight heparin is a potent inhibitor of FGF-2 and VEGF-mediated EC proliferation [37]. HS oligosaccharides have been demonstrated to suppress EC migration, tube formation, and signaling induced by VEGF165 and FGF-2 [9, 38]. Our group's previous studies showed that a heparinoid with low content of 2-O-sulfated groups binds VEGF, FGF-2, and EGF, reducing angiogenesis in EC culture and a laser-induced choroidal neovascularization animal model [25].

Taken together, these findings suggest that the mHep is a potent anti-angiogenic, anti-proliferative, and anti-migratory compound with negligible anticoagulant or hemorrhagic activity. It showed no cytotoxicity in retinal cells, suggesting that this compound is a candidate for treating neovascular AMD and other angioproliferative diseases.

Author contribution Vinicius Kniggendorf: conceptualization, methodology, formal analysis, investigation, and writing.

Maria Eduarda Perrud Souza: investigation.

Thatiane Russo: investigation.

Marcelo Andrade de Lima: investigation.

Alex Treiger Grupenmacher: investigation.

Caio V. Regatieri: conceptualization, methodology, formal analysis, investigation, writing, supervision, and project administration.

Juliana L. Dreyfuss: conceptualization, methodology, investigation, writing, supervision, and project administration.

Animal Care Ethics Committee of the Federal University of Sao Paulo number: 5726120717.

All experiments were performed following the association for Research in Vision and Ophthalmology (ARVO).

Declarations

Conflict of interest The authors declare no competing interests.

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