Cilostazol as a noninferiority pharmacologic option to paclitaxel in early intimal hyperplasia inhibition after venous balloon angioplasty in a rabbit model: a preliminary study

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ABSTRACT

The development of venous intimal hyperplasia (VIH) has not been fully studied. At present, there are no drugs approved for VIH inhibition; to investigate such alternatives, we aimed to compare paclitaxel with cilostazol in VIH early inhibition in a preliminary experimental model of balloon angioplasty. Twenty-eight male New Zealand rabbits were randomly divided into two groups: cilostazol (A) and paclitaxel (B), which underwent femoral vein barotrauma by a 4 mm balloon angioplasty. The VIH model was previously tested in controls obtaining an 80% increase of subintimal area (SIA) compared with veins without injury (from 0.12 mm^2 [standard deviation (SD), 0.05] to 0.86 mm^2 [SD, 0.08]). Group A received 20 mg/kg twice daily; group B angioplasty was performed with a single-dose paclitaxel-coated balloon. Seven days later rabbits were euthanized, and vein tissue samples were taken for histological analysis. The primary end point was SIA measure expressed in mm^2, and the anticipated difference between treatments was 0.21 mm^2. Other measurements were immunohistochemistry expression of hypoxia inducible factor-1 alpha, platelet derived growth factor, and smooth muscle actin, as surrogates of cell migration and oxidative stress. SIA of group A was 0.33 mm^2 (SD, 0.15; 95% CI, 0.24-0.42 mm^2), and that of group B was 0.31 mm^2 (SD, 0.14; 95% CI, 0.22-0.40 mm^2). Both drugs showed a reduction of 61% and 63%, respectively, in SIA, compared with controls. The difference between both drugs was 0.0193 mm^2 (95% CI, −0.1175 to 0.156 mm^2); the statistical difference was found in hypoxia inducible factor-1 alpha expression between both groups. (JVS—Vascular Science 2020;1:200-6.)

Clinical Relevance: Although veins have a thinner middle layer compared with arteries, smooth muscle cells appear to play an important role in venous stenosis after angioplasty. The study of smooth muscle cell response after barotrauma may have clinical applications in the endovascular treatment of venous stenosis, because at the moment, there is no medication indicated to prolong patency after venous endovascular procedures, for example in May Thurner syndrome. Paclitaxel and cilostazol seem to have a promising role. Finally, the present study could inspire a research line to reduce stent placement and increase patency after venous angioplasty.

Keywords: Intimal hyperplasia; Paclitaxel; Cilostazol; Veins; Balloon angioplasty

Intimal hyperplasia (IH) is a physiological response of arteries and veins characterized by an intra- and extraluminal thickening of the vessel wall due to smooth muscle cell (SMC) migration and proliferation.[1] Venous IH (VIH) has not been thoroughly studied even though it has different stimuli compared with arterial IH, and it is responsible for 30%-50% of autologous peripheral bypass failure and over a half of arteriovenous fistulae hemodialysis access failure.[2,3] VIH has also been related with long-term occlusion of stents deployed to treat benign obstructive venous disease and thrombus organization.[4,5]

Although until now there are no drugs approved for VIH inhibition, different strategies have been used to attempt the reduction of IH after arterial revascularization procedures: cryo-angioplasty, cutting balloons, and the use of balloons and stents coated with cell-proliferation inhibitor drugs such as paclitaxel.[6] In this case, paclitaxel

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has been used to treat venous stenosis in arteriovenous fistulas.[7] Even though arterial data cannot be completely applied to venous pathology, if we consider a strategy to inhibit VIH, it is possible that paclitaxel-coated devices might represent the first choice, because there is an unspoken trend to extrapolate knowledge of the arterial field into the venous one.[8] For its part, cilostazol is a phosphodiesterase III inhibitor and promotes the increase of adenosine 3′-5′ cyclic monophosphate in platelets and SMCs, avoiding the activation of these cells. Also, cilostazol improves endothelial regeneration, and these mechanisms could inhibit VIH proliferation.[9]

In view of these findings, there is a need to identify therapeutics alternatives to inhibit VIH. This study was carried out to investigate the role of cilostazol and paclitaxel as inhibitors of VIH. We aim to compare cilostazol and paclitaxel VIH inhibition in an experimental model of balloon angioplasty barotrauma in rabbit femoral veins.

ARTICLE HIGHLIGHTS

- **Type of Research:** Prospective preliminary experimental study
- **Key Findings:** In our experimental model, induced barotrauma by femoral vein balloon angioplasty in 14 New Zealand rabbits resulted in vein wall subintimal area (SIA) growth greater than 80% (from 0.12 mm^2 [standard deviation (SD), 0.05] to 0.86 mm^2 [SD, 0.08] at seventh day). Same characteristics rabbits were randomized to study SIA inhibition; in the group treated with cilostazol, SIA decreased by 61% compared with controls (mean of 0.33 mm^2; SD, 0.15). In the group treated with a single-dose paclitaxel-coated balloon, SIA was reduced by 63% (mean of 0.31 mm^2; SD, 0.14). Noninferiority was shown between both drugs in SIA inhibition (SIA difference between both drugs was 0.0193 mm^2 [95% confidence interval, −0.1175 to 0.156 mm^2]; delta was 0.21 mm^2).
- **Take Home Message:** Cilostazol and paclitaxel may be feasible alternatives for early venous intimal hyperplasia inhibition.

METHODS

Twenty-eight male New Zealand white rabbits (*Oryctolagus cuniculus*) were assigned randomly to a noninferiority experimental preliminary study between cilostazol and paclitaxel evaluating subintimal area (SIA) inhibition. The study was carried out in the experimental surgery laboratory of the medicine faculty of the Universidad Nacional Autónoma de México, approved by the ethics and investigation commission of the same university (Project No. FM/DI/106/2017), and was conducted according to Mexican official standard of animal protection and animal care complied with the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council, Washington, DC, National Academy Press, 1996).[10-14] Morphometric and densitometric analysis was conducted in the Pathology Service of the American British Cowdray Hospital and the biochemistry laboratory at the National Institute of Medical Sciences and Nutrition "Salvador Zubirán" in Mexico City.

VIH model. The development of our model has been previously described by our team.[13] For this protocol, a pilot study was conducted in 14 rabbits to obtain controls. Under general anesthesia with isoflurane and induction with ketamine, the rabbit groin was opened by 3 cm oblique incision and femoral vein was isolated, after distal clamping it was punctured to put a 4 Fr sheath, then a 0.014 inches guidewire was inserted through the vessel. Plain old balloon angioplasty (POBA) was performed with a 4 mm diameter × 2 cm length balloon (Joker; Eurocor, Bonn, Germany) insufflated to 14 Atm (atmospheres) for 3 minutes, distending femoral vein approximately 300% more than its normal diameter. A venography was performed with a direct injection of 2.5 mL of iodinated contrast (Ultravist; Bayer, Germany) diluted 2:1 with saline; images were taken to confirm the pulmonary and peripheral venous system patency. Endovascular devices were removed, and the veins were closured with a single point of the 8-0 polypropylene vascular suture. Heparin was not used during the procedures. Seven days later the rabbits were killed, and tissue samples were obtained from the injured vein for microscopic analysis. SIA was defined as the space between the endothelial cell surface and the internal elastic lamina, which forms the adventitial border of the middle layer. The contralateral femoral vein was harvested to verify the normal femoral vein wall thickness, referred to as veins without injury. All femoral veins were patent; in the angioplasty samples, the mean of SIA was 0.86 mm2, and in contralateral veins, the mean of the medial layer area was 0.1 mm2 ($P < .001$).

Cilostazol and paclitaxel groups. Twenty-eight rabbits with same characteristics of controls (same interventionalist and same equipment) were randomized in clusters before the procedure using a sequence generated in an online portal, performing the same VIH induction procedure as previously described. A total of 14 rabbits were assigned to the paclitaxel group, and the rest to the cilostazol group. In the first group, drug-coated balloon angioplasty was performed with a 4 mm diameter and 20 mm length paclitaxel-coated balloon (Freeway; Eurocor), and controlled insufflation was performed in the same way at 14 Atm for 3 minutes.

In the cilostazol group, POBA was performed with the same size balloon under equal surgical conditions (Joker; Eurocor) and oral administration of cilostazol 20 mg/kg

divided into two doses in a day for 7 days after POBA (a veterinarian gave the diluted cilostazol to the rabbit with a pipette and confirmed that it has been swallowed by the animal).

Morphometric analysis. Seven days after angioplasty rabbits were killed and 20-mm-long tissue samples were obtained from the injured vein. Fixation, tissue processing, and embedding were performed following the protocol established by Choy et al.[15] The explanted vein was cut perpendicular to the long axis of the vessel into three or four paraffin blocks of 2-3 mm, and embedded segments were cut with a thickness of 4-6 μm each and mounted on glass slides. Samples were evaluated with hematoxylin-eosin, Masson and Verhoeff stains. Histological analysis was performed by two pathologists, blinded to the assignment group. The primary outcome was SIA; to measure it, pictures were taken at a ×4 magnification of all circumferential cuts and were digitally processed using the ImageJ program (National Institutes of Health) previously calibrated at millimeters to pixels to obtain means of every tissue. The software recognizes the SIA by color and automatically returns the result of the area (Fig 1). These measures were performed in all cross-sections of each femoral vein.

Immunohistochemical analysis. Rabbit polyclonal antibodies (Ab) of alpha-smooth muscle actin (α-SMA; ACTA2, 1:500 dilution; Sino Biological Inc, Beijing, China) were used for SMC characterization, and Ab of platelet-derived growth factor (PDGF-A [E-10]: sc-9974, 1:500 dilution; Santa Cruz Biotechnology, Santa Cruz, Calif), as well as Ab of hypoxia-induced factor (HIF-1 alpha [28b]: sc-13515, 1:500 dilution; Santa Cruz Biotechnology), as surrogates of oxidative stress and SMC proliferation signals.[16] The HiDef Detection HPR Polymer System (954D-10, 7.0 mL kit; Cell Marque, Rockling, Calif) was used on all samples to visualize the immunohistochemical reaction. Appropriate positive and negative controls were used for each antibody, from cell lines and tumor tissues. The expression reading of each antibody was performed by two different pathologists blinded to assignment who completed a qualitative analysis and reported in concordance the following findings: negative result, characterized by complete negativity or less than 50% of the "target" cells with less intensity than the control, weakly positive result, more than 50% of the "target" cells with less intensity than the control; and lastly, a positive result with more than 50% of the "target" cells with greater intensity than the control.[17]

Sample size and statistical analysis. Sample size was calculated by values obtained in the pilot study and the reports of Kanamasa et al.[18] SIA was expected in the paclitaxel group of 0.5 mm2, with a standard deviation (SD) of 0.18 mm^2. It was anticipated that the SD for cilostazol would be 0.18 mm^2. The noninferiority margin between cilostazol and paclitaxel was a difference of 0.21 mm^2, it corresponds to 30% of the distance between 0.8 mm^2 and 0.01 mm^2 and because endovascular revascularization is considered successful when the residual stenosis is equal or less than 30%. Type I errors of 5% and 80% power were considered.

Baseline data were evaluated according to means, SD, medians, and proportions; the normality of the values was determined by bias and kurtosis and values were compared with Student's *t*-tests, Mann-Whitney, and the χ^2 test between both groups. Results of the SIA were expressed as mean and standard deviations, and the 95% confidence interval (95% CI) was calculated. To consider noninferiority, the 95% CI of the difference between both drugs was calculated and compared with the established limit of 0.21 mm^2. The χ^2 test was used to stablish differences in immunohistochemistry results, and the Kruskall-Wallis test was used to compare both drug groups with controls. In all scenarios, a P value of $<.05$ was considered statistically significant.

RESULTS

In controls, the VIH induced by angioplasty was a 60% (SD, 10%) increase in thickness of the subintimal space compared with contralateral veins without barotrauma. Regarding groups, Table I shows the baseline characteristics of each. All veins were patent 7 days after balloon angioplasty; morphometric analysis could be performed in all samples. There was superficial wound dehiscence (skin only) in one rabbit of each group at the fifth and sixth postoperative days; they were not excluded from the study. Morphometric measurement results of both groups and controls are summarized in Table II.

Paclitaxel and cilostazol had a reduction in the expression of IH of 61% and 63%, respectively, compared with controls. The SIA difference between both drugs was 0.0193 mm^2 (95% CI, −0.1175 to 0.156 mm^2); the CIs did not exceed the clinically significant difference raised of 0.21 mm^2 (Fig 2). Interindividual variation was 0.06 with a SD range of 0.04-0.08 mm^2 per rabbit. Interobserver kappa was 0.95.

Immunohistochemistry results are shown in Table III (Fig 3). Interobserver kappa was 0.80. The χ^2 test was not significant between paclitaxel and cilostazol regarding the expression of SMA and PDGF. It was significant in the difference of HIF-1 alpha ($P = .001$), for the positive values. The differences between the results of all antibodies between both drugs and controls were significant ($P = .001$), except in PDGF for cilostazol.

DISCUSSION

Few experimental studies in the venous circulation have been conducted to understand early intimal response after extensive venous injury, and until now, there are no approved drugs exclusively for IH inhibition in artery or vein pathology, but in view of these findings, cilostazol and paclitaxel could be potential options to

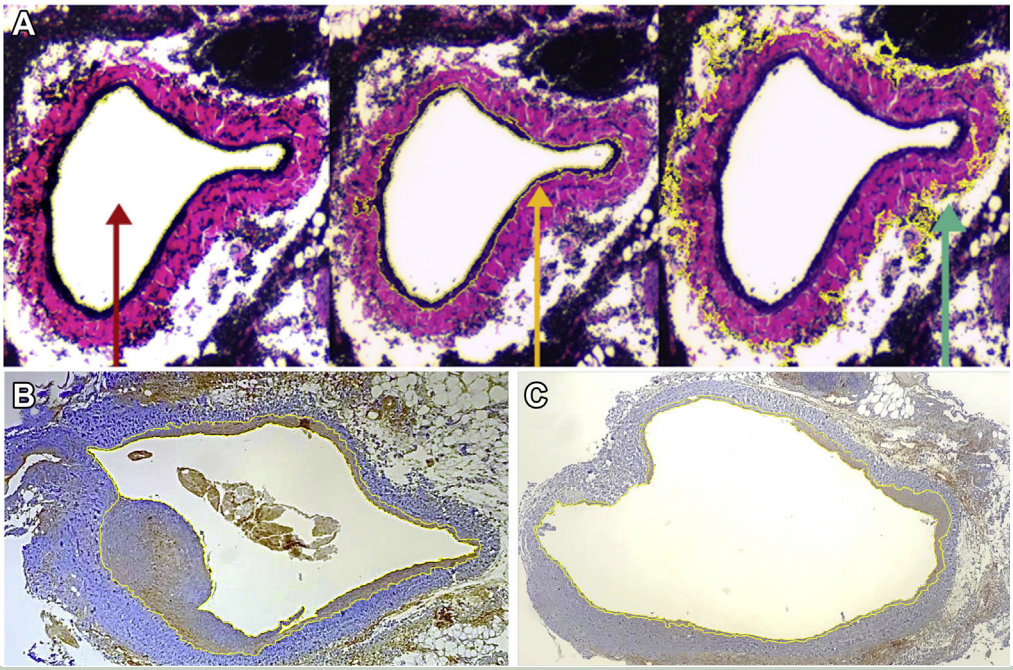

Fig 1. A-B, Measurement of morphometric data. **A,** Cross-sectional segment of femoral vein (increased 40 times) with elastic fiber staining showing contrast between vessel lumen (white color), subintimal space (darker color), and the rest of the vessel (pink color). The *red arrow* represents the lumen area, the *orange arrow* the subintimal area, and the *green arrow* the complete vessel area. **B,** Cross sectional segment of femoral vein with intimal hyperplasia. **C,** Cross sectional segment of femoral vein without intimal hyperplasia.

Table I. Basal variables of rabbits in each group

Variables	Control group (n = 14)	Cilostazol group (n = 14)	Paclitaxel group (n = 14)	*P* value
Age (months)	10.77 (2.01)	10.75 (2.21)	10.9 (3.13)	N/S
Weight (kg)	3790 (333)	3677 (334)	3820 (320)	N/S
Extern vein diameter before angioplasty	1.3 mm (0.1)	1.3 mm (0.1)	1.29 mm (0.1)	N/S

N/S, Not significant.

Table II. Morphometric analysis between groups

Groups	Subintimal areal, mm^2 (SD)
Cilostazol (N = 14)	0.33 (0.15)
Paclitaxel (N = 14)	0.31 (0.14)
Controls (N = 14)	0.86 (0.08)
Veins W/I (N = 14)	0.16 (0.05)

SD, Standard deviation; *W/I*, without injury.

develop further studies.[19] Although these drugs differ in pharmacokinetics and pharmacodynamics as well as the prescription indications, in real practice, they have been used concomitantly after endovascular revascularization. A retrospective study showed trends to poor patency when cilostazol was not prescribed after the drug-coated balloon angioplasty peripheral procedure, and other studies reported improved patency of paclitaxel-eluting stenting after cilostazol concomitant treatment.[20,21] However, more studies are needed to increase the evidence about its efficacy inhibiting VIH.

On the other hand, the results of immunohistochemistry allow us to make some inferences about the mechanism of action of both drugs in the venous response to barotrauma, which has not been studied in a comparative way. In the case of SMA, this was used as a surrogate marker of SMC characterization, in this study, there was no difference in their expression between the two drug groups.[19]

The reason for evaluating PDGF at the tissue level was to study its role in each vascular layer after injury, mainly expressed in the endothelium. In this study, control veins showed great discontinuity of this marker, which was interpreted as an endothelial and media layer lesion. Cilostazol has been shown to have a beneficial effect on vascular re-endothelization and angiogenesis, upregulating some growth factors like PDGF.[20,21]

HIF-1 alpha is a marker that has been more recently related in the genesis of IH, its expression promotes angiogenesis as well as apoptosis and cell migration.[22]

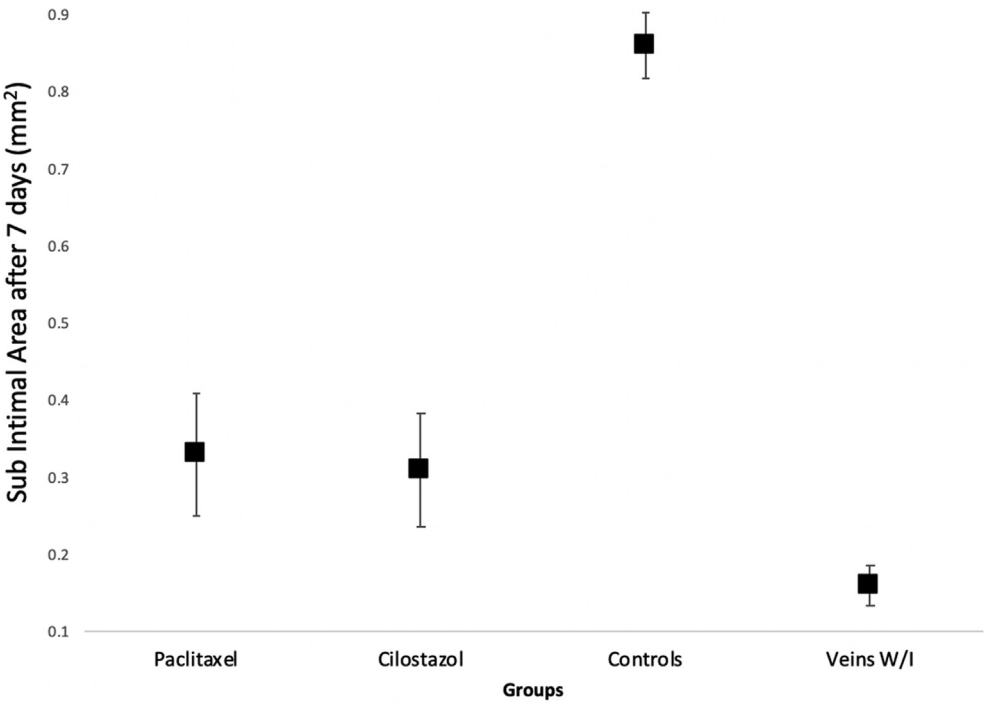

Fig 2. Interval plot of the subintimal area between both groups and controls, confidence interval of 95%.

Table III. Qualitative results of immunohistochemistry

Group	Qualitative result	ACTA2	PDGF	HIF-1 α
Cilostazol (N = 12)	Negative	1	2	0
	Weakly positive	8	7	3
	Positive	3	3	9
Paclitaxel (N = 12)	Negative	1	2	2
	Weakly positive	4	9	9
	Positive	7	1	1
Controls (N = 14)	Negative	2	2	3
	Weakly positive	7	9	4
	Positive	5	3	7
Veins W/I (N = 14)	Negative	11	6	10
	Weakly positive	1	5	2
	Positive	0	1	0

ACTA2, Smooth muscle actin; *HIF-1α*, hypoxia inducible factor 1 alpha; *PDGF*, platelet-derived growth factor; *W/I*, without injury.

Ortega et al[23] describe that venous SMCs are sensitive to hypoxia. Distension of the venous wall and loss of normal fluid shear stress can also lead to cellular hypoxia.[23] Studies mention a greater increase at the serum level of this marker in the context of IH; in turn, proliferation and migration of SMC decrease.[16] In this experiment, cilostazol had more positive samples compared with paclitaxel. This is because HIF-1 alpha and paclitaxel may have a different interaction; for example, solid tumors (mostly with hypoxic or necrotic centers) express higher levels of HIF-1 alpha, and they respond less to treatment with taxanes.[24]

This experimental study has several limitations: the IH phenomenon is very controlled in experimental models; in the clinical scenario, other factors could be related to the development of hyperplastic events. Tissue distortion caused by histological preparation can lead to measurement biases. The transverse measurement at 7 days after injury does not include the entire phenomenon of IH. A longitudinal study with several measurements would have provided a better understanding of how IH behaves over time. The cilostazol doses used (20 mg/kg of weight) do not correlate with the recommended doses in humans, which could limit to the transfer of the results from the experimental model to the clinical field.

Immunohistochemical reports are qualitative; and these studies do not determine protein expression with quantitative certainty. The staining of the different antibodies in the same way is multifactorial, so that causality cannot be inferred. There are more specific stains to test the effect of drugs on cell proliferation, such as proliferating cell nuclear antigen and KI-67. Future studies with these markers are required.

CONCLUSIONS

Cilostazol does not seem to be inferior to paclitaxel for IH inhibition in femoral veins of New Zealand rabbits at 7 days after angioplasty barotrauma. The mechanism of action of these drugs might have implications in oxidative stress; in addition, this research field might improve

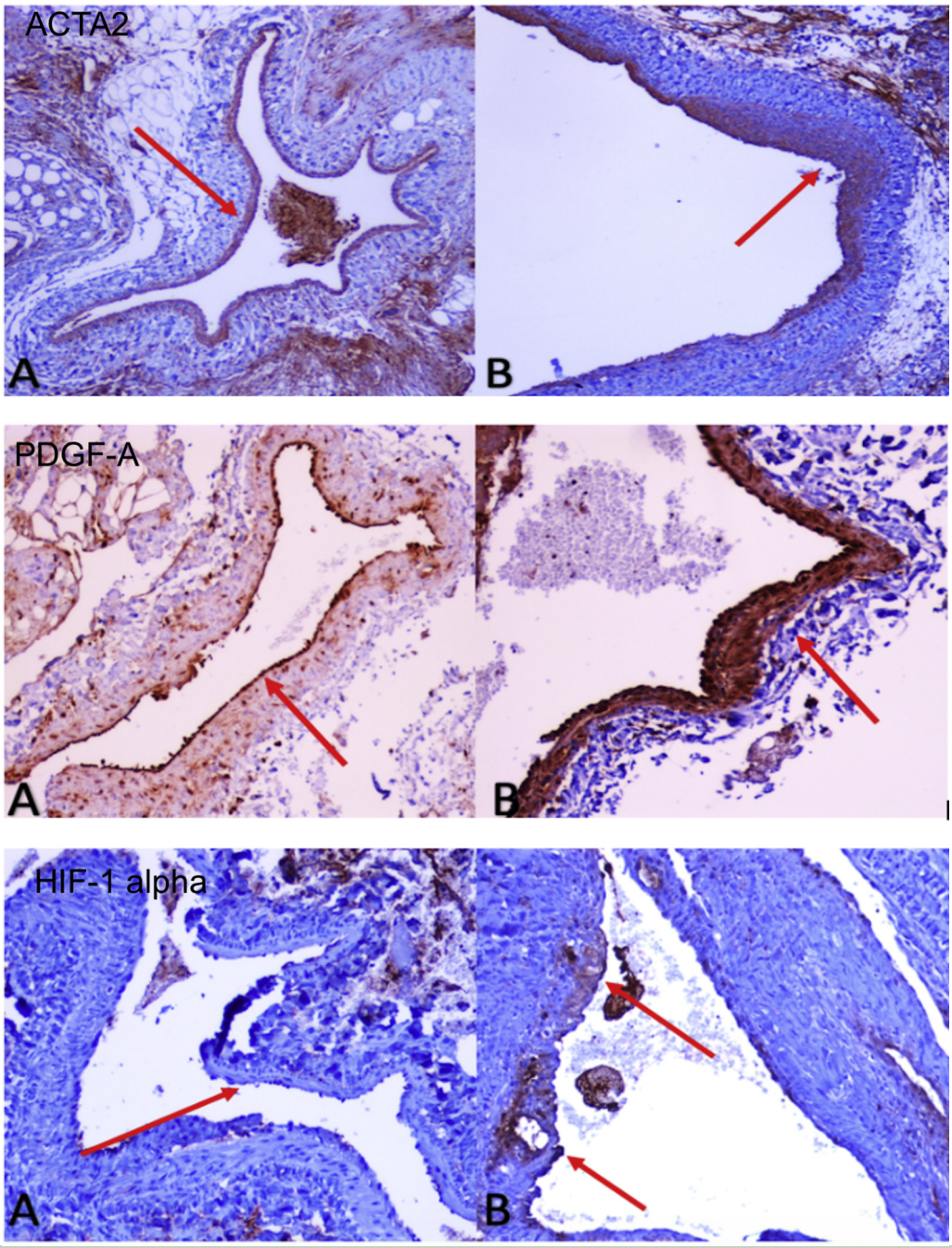

Fig 3. Immunohistochemical results. First row: rabbit polyclonal antibodies (Ab) of alpha-smooth muscle actin (*ACTA2*). **A,** Negative result. **B,** Positive result. Second line: Ab of platelet-derived growth factor (*PDGF-A*). **A,** Negative result. **B,** Positive result. Third line: Ab of hypoxia induced factor (*HIF-1 alpha*). **A,** Negative result. **B**, positive result.

our understanding and clinical experience with the aim to increase the patency and durability of endovascular venous procedures.

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