New genetic risk factors and therapeutic possibilities in case of recurrent stenosis after carotid interventions

Ph.D. Doctoral Thesis

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1. INTRODUCTION

Longterm success of surgical and endovascular treatment of atherosclerotic stenosis is limited by restenosis, that still represents a complication not yet been completely solved. The incidence of restenosis varies according to the method (stenting, endarterectomy), the treated vascular region, gender, concomittant diseases and to several other factors, but the pathomechanics and risk factors are similar. Restenosis occurs in about 30-50% after conventional balloon angioplasty, in 10-30% after treatment with bare metal stents, while the use of drug-eluting stents further suppressed the frequency of recurrent stenosis.

Endothelial injury and ischemic/reperfusion injury during vascular interventions trigger the pathological processes leading to restenosis, that consists of the following four overlapping stages: (1) platelet activation and aggregation, (2) inflammation, (3) vascular smooth muscle cell (VSMC) migration and proliferation and (4) extracellular matrix (ECM) deposition. Neointimal hyperplasia caused by VSMC migration/proliferation and ECM deposition is primarily responsible for lumen loss. After conventional angioplasty, additional elastic recoil contributes to lumen loss, that is a passive phenomenon immediately after dilation. According to multricentric trials, the use of drug-eluting stents covered by anti-inflammatory or antiproliferative drugs (like sirolimus and paclitaxel, respectively) significantly reduced coronary instent restenosis. The patomechanism described above, regards the early restenosis occurring within 1-2 years after the interventions, while late restenosis is rather due to the progression of primary atherosclerosis.

Risk factors for restenosis can be divided into systemic (e.g. diabetes, female gender) and local (e.g. type and length of the atherosclerotic plaques) ones. In addition, evidence is growing, that restenosis development can be genetically determined as well (e.g. glycoprotein receptor, angiotensin converting enzyme or mannan-bindig lectin gene polymorphisms).

Considerable proportion of ischemic strokes are caused by significant stenosis of the internal carotid artery. Carotid endarterectomy (CEA) had been confirmed to provide protection against stroke in patients with symptomatic as well as asymptomatic severe carotid stenosis. In recent decades carotid artery angioplasty and stent placement has emerged as an alternative to surgery. Equality of surgical and endovascular carotid interventions are controversial, they are rather complementary than

competitive methods in the treatment of carotid disease. Carotid artery stenting (CAS) is preferred in high risk patients or for stenoses surgically not accessible.

Restenosis following both carotid interventions is a common complication, that is the topic of this Ph.D. thesis. We investigated the nitric-oxide (NO) - cyclic guanosine monophosphate (cGMP) - protein kinase G pathway and the role of estrogen receptor alpha (ESR1), that are – among others – important signaling pathways involved in restenosis formation.

Several beneficial effects of estrogen in the response to vascular injury has been shown in *in vitro* and *in vivo* studies. The vasoprotective effects of estrogen fulfil across estrogen receptor alpha. Recent contributions described the possible role of the estrogen receptor alpha gene polymorphisms in restenosis development. Nitric oxide presents vascular protective effects as well, and benefical role of NO has been described in several experimental restenosis model as well.

2. OBJECTIVES

In general, the aim of our work was to reveal new risk factors which could affect recurrent stenosis after surgical and endovascular carotid interventions as well as to investigate new therapeutic possibilities to decrease neointimal hyperplasia. Thus, our work consist of a clinical and an experimental part.

Based upon the observations that vascular wall damage and ischemia/reperfusion injury may trigger neointima formation, and upon our previous findings, that immediate complement activation follows carotid endarterectomy (but not carotid artery stenting) and is related to the clamping time, we aimed to study the difference in early restenosis rates following carotid endarterectomy and carotid artery stenting. Thus, we performed a retrospective clinical study recruiting all patients treated during one year at our cardiovascular surgery department.

As mentioned in the introduction, genetic factors (such as gene polymorphisms) can exhibit additional risk for developing restenosis, besides other systemic and local risk factors. Based upon recent studies which describe the beneficial role of estrogens (through estrogenreceptor-α) in response to vascular injury, as well as another study describing the role of estrogenreceptor-α polymorphisms in coronary restenosis, we aimed to

investigate the affect of two single nucleotide polymorphisms of the estrogenreceptor-α gene in recurrent carotid stenosis. We performed a non-randomized prospective study including patients who underwent CEA or CAS with one year ultrasonographic follow-up.

Smooth muscle cell migration and proliferation are essential in the generation of neointimal hyperplasia. Recent studies suggest, that decreased cGMP signaling may be associated with neointimal proliferation, and upregulation of the NO-cGMP-PKG pathway prevent neointimal growth. In our experimental study we investigated the possible beneficial effect of an oral phosphodiesterase-5 inhibitor treatment (which is able to enhance cGMP signaling through its decreased breakdown) on a microsurgical carotid endarterectomy model in the rat.

3. METHODS

3.1. Clinical studies

Patients

In our retrospective study, 685 patients undergoing carotid interventions during 2004 at the Department of Cardiovascular Surgery, Semmelweis University, had been elected in order to compare early restenosis rates after CEA and CAS. In the prospective study, 172 patients had been involved between 2005 and 2007, in order to determine the ESR1 gene polymorphisms and their relation to recurrent carotid stenosis.

Preoperative evaluation

All patients underwent preoperative duplex ultrasound (US) before carotid revascularization. The common carotid (CCA), internal carotid (ICA) and external carotid arteries (ECA) on both sides were examined in the standard fashion. Velocities were measured in the distal CCA and the ICA. The diagnostic criteria for ICA stenosis was based on peak systolic velocities (PSV), end diastolic velocities (EDV) as well as internal carotid artery/common carotid artery (ICA/CCA) PSV ratios. After US examination**,** diagnostic angiography was performed in CAS patients if it was possible in the same sitting with stent placement. Before surgery, CTA

(computed tomography angiography), MRA (magnetic-resonance angiography) or DSA (digital subtraction angiography) was performed.

Carotid interventions

During the study period eversion, carotid endarterectomy was the treatment of choice for carotid surgery. The eversion carotid endarterectomy was carried out under general anaesthesia. The ICA was obliquely transected at its origin in the carotid bulb and was everted cranially, than the plaques were removed. This was followed by endarterectomy of the common and external carotid arteries. After this, the ICA flap was reinserted in its original place. Indication for CEA was in accordance with the American Heart Association guidelines. After evaluation of the risk factors patients were treated with adequate medical therapy before and after surgery, besides currently accepted anticoagulant and antiplatelet prophylaxis.

Carotid artery stenting was carried out under local anaesthesia, usually in the same sitting with diagnostic angiography. Whenewer it was possible, the femoral artery was punctured to get access to the carotid arteries. Heparin (10 000 U) was administered intraarterially at the beginning of the procedure; patients with primary lesions received routine prophylactic i.v. atropine (0.5 mg) before balloon dilation. A cerebral protection device (FilterWire, EX Boston Scientific) was used to prevent distal embolization during the stenting procedure. Wallstent was used in 90% of cases, Precise and Xact stent were used approx. in 5%-5% of cases. Indication for CAS was in accordance with the American Heart Association/American Stroke Association guidelines. Patients were discharged on either the first or second postprocedural day. Acetylsalicylate (100 mg) and clopidogrel (75 mg) were given for at least 3 days before the procedure and afterwards, clopidogrel was continued for 6 weeks, and aspirin was continued permanently.

Follow-up

In 512 cases (368 carotid endarterectomy performed on 347 patients and 144 carotid artery stenting performed on 140 patients) from the 685 cases involved in the retrospective study, ultrasonographic check-up had been performed (mean follow-up time was 18.4 months, range from 6 months to 38 months). In the prospective study, follow-up was performed in

all patients (90 CAS, 82 CEA) of both the CAS and CEA groups by carotid duplex scan, resulting in a 12 months median follow-up time.

After carotid endarterectomy, ICA restenosis between 50-69% was diagnosed when ICA PSV was 125-230cm/sec. Additional criteria included ICA/CCA PSV ratio of 2.00-4.00 and ICA EDV of 40-100cm/sec. ICA restenosis over 70% was diagnosed when the ICA PSV was greater than 230cm/sec. Additional criteria included ICA/CCA PSV ratio >4.00 and ICA EDV >100cm/sec. For CAS patients, the ulrasonographic criteria were slightly different, as follows: a 50%-69% ICA instent restenosis was diagnosed when ICA PSV was 225-350cm/sec. Additional criteria included ICA/CCA PSV ratio of 2.50-4.75 and ICA EDV of 75-125cm/sec. A ≥70% ICA instent restenosis was diagnosed when the ICA PSV was greater than 350cm/sec. Additional criteria included ICA/CCA PSV ratio >4.75 and ICA EDV >125cm/sec.

Genotyping

Total genomic DNA was extracted from EDTA-anticoagulated blood samples of 172 patients by the manual method of Miller. A fragment of 1300 base pairs (bp) of the ESR1 gene was amplified by polymerase chain reaction. The PCR products were digested overnight with PvuII and XbaI restriction enzymes (Fermentas International Inc., Burlington, Ontario, Canada) at 37°C, producing fragments of 1300 bp (C allele) or 850+450 bp (T allele) and of 1300 bp (G allele) or 900+400 bp (A allele), respectively. The cleavage products were electrophoresed on 2% agarose gel and stained with ethidium bromide. The restriction fragments were detected with visual inspection on an ultraviolet table.

Statistics

Data were collected in Microsoft Excel 2003 and were analyzed with Statistica 8.1 (StatSoft, Tulsa, USA) software. As many of our data exhibited a non-Gaussian distribution (as tested by Shapiro-Wilk`s test), we used the Mann-Whitney U-test to compare continuous variables. Categorical values were compared using the chi-square test and Fischer's exact test. After univariate analysis, multivariate logistic regression was carried out to determine risk factors for restenosis. All analyses were performed two-tailed, and $p<0.05$ was considered as significant.

3.2. Experimental studies

Rat carotid endarterectomy model

After adequate anesthesia with an intraperitoneal injection of ketamine hydrochloride (100 mg/kg) and xylazine hydrochloride (5 mg/kg), the right common carotid artery was exposed under a dissecting microscope via a midline cervical incision. A longitudinal arteriotomy was made with a corneal blade and extended to 6 mm with microscissors after clamping of the right common carotid artery. To denude the endothelium, a sterile cotton-tipped applicator immersed in saponin (0.1%) was rubbed on the inner vessel surface. The arteriotomy was closed with a running 9-0 Ethilon monofilament nylon suture (Ethicon, Inc, Somerville, NJ). The superficial cervical muscles and skin were closed with running 4-0 absorbable sutures. After 3 weeks, the carotid arteries were perfusion-fixed and harvested for histological evaluation.

Treatment

In the experimental study, we investigated the role of NO-cGMP signaling in neointima formation. Oral phosphodiesterase-5 inhibitor had been administered to nine rats, while nine rats (control group) received placebo. In addition, nine other animals underwent a sham operation without carotid intervention.

Histology, immunohistochemistry, TUNEL, EIA

Morphometry was performed in three hematoxylin-eosin stained cross-sections of each animal (proximal, mid, and distal region of the operated vessel segment). Luminal, intimal, and medial dimensions were computed using the internal and external elastic laminae as delimeters. The percentage of stenosis was calculated by the ratio between the neointimal area and the original lumen area. In addition, the neointima/media area ratio was calculated. Immunohistochemical analysis had been performed by using antibodies against TGFß$_1$, alpha smooth muscle actin and cGMP. Stainings had been evaluated by a semiquantitative scoring system. Terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) assay was used to detect DNA damage/apoptosis in the

neointimal cells. cGMP concentration had been measured in plasma probes conserved at -80 °C.

Statistics

Statistical analysis was performed by using the Origin 7 statistical software product. Two group comparisons were determined by Student's t-test, as they exhibited normal distribution. A value of $p<0.05$ was considered statistically significant.

4. RESULTS

4.1. Clinical studies

Results from the retrospective studies suggest, that incidence of severe (≥70%) restenosis is higher after CEA than after CAS (10.05 % in patients undergoing CEA and 3.47 % in patients undergoing CAS, $p<0.05$). On the other hand, postoperative neurologic complications, especially transient ischemic attack, had been recorded more often after CAS than following CEA (7.60 % in patients undergoing CAS and 2.20 % in patients undergoing CEA, $p<0.05$). Evaluation with the Kaplan-Meyer life table analysis showed a significant difference in freedom from severe (≥70%) restenosis between the CEA and CAS groups in favour of the stent; $p=0.006$ (Figure 1). After further adjustment of data according to lesion type treated with stents, results were as follows: there was a greater difference in the incidence of severe (≥70%) restenosis between CEA and those CAS patients who underwent treatment with primary stents; $p=0.002$ (Figure 2). It is also supported by the observation that the incidence of instent restenosis of greater than 50% was higher in patients stented for postendarterectomy restenosis than in subjects stented for primary stenosis (13.6% vs. 6%, respectively; $p>0.05$). Univariate analysis showed female gender to be associated with severe restenosis irrespectively of the interventional technique (OR, odds ratio: 2.02 [CI, confidence interval: 1.07-3.80], $p=0.028$). The odds ratio was found to be higher when only those patients were selected who underwent CEA (OR: 2.61 [CI: 1.28-5.32], $p=0.006$).

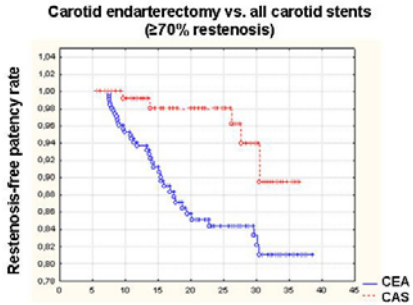

Figure 1.
Restenosis-free patency rate
CEA vs. all CAS

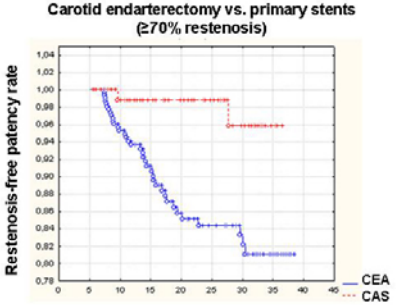

Figure 2.
Restenosis-free patency rate
CEA vs. primery CAS

According to the genetic association study, significantly higher restenosis rate has been found in patients carrying AA genotype of the XbaI polymorphisms of ESR1, as compared to patients carrying AG or GG genotypes (23.4% vs. 10.5%, p=0.02). Regarding the other polymorphism tested, we found significantly higher restenosis rate in patients carrying TT or TC genotypes as compared to patients with CC genotype (19.3% vs. 3.1%, p=0.02). These associations could be shown in the whole patient population, but especially in the subgroup of female patients undergoing carotid endarterectomy (Figure 3). Strong linkage disequilibrium was found between the PvuII and XbaI polymorphisms of the of ESR1 gene.

Genotype	All patients			Women			Men		
	All 172[a]	CEA 82[a]	CAS 90[a]	All 67[a]	CEA 42[a]	CAS 35[a]	All 105[a]	CEA 50[a]	CAS 55[a]
Restenosis rate % (lesions with restenosis/all lesions)									
AA	23.4% (18/77)	27.6% (8/29)	20.8% (10/48)	33.3% (11/33)	45.5% (5/11)	27.3% (6/22)	15.9% (7/44)	16.7% (3/18)	15.4% (4/26)
AG/GG	10.5% (10/95)	11.3% (6/53)	9.5% (4/42)	5.9% (2/34)	4.8% (1/21)	7.7% (1/13)	13.1% (8/61)	15.6% (5/32)	10.3% (3/29)
p-Value[*]	**0.02**	0.06	0.14	**0.005**	**0.01**	0.17	0.69	0.61	0.44
TT/TC	19.3% (27/140)	22.6% (14/62)	16.7% (13/78)	25.0% (13/52)	30.0% (6/20)	21.9% (7/32)	15.9% (14/88)	19.0% (8/42)	13.0% (6/46)
CC	3.1% (1/32)	0.0% (0/20)	8.3% (1/12)	0.0% (0/15)	0.0% (0/12)	0.0% (0/3)	5.9% (1/17)	0.0% (0/8)	11.1% (1/9)
p-Value[†]	**0.02**	**0.01**	0.40	**0.03**	**0.04**	0.50	0.25	0.22	0.68

[*] AA vs. non-AA (AG or GG).
[†] Non-CC (TT or TC) vs. CC.
[a] No. of lesions.

Figure 3.
ESR1 gene polymorphisms and restenosis after carotid interventions (genotypes of the XbaI polymorphism: AA, AG, GG; genotypes of the PvuII polymorphism: TT, TC, CC; CEA: carotid endarterectomy; CAS: carotid artery stenting)

We found gene dose dependency in the XbaI polymorphism of the ESR1 gene. Restenosis rates were 23.4% in patients carrying both A alleles, 12.2% in patients carrying only one A allele and 4.8% in patients without any A allele. In the smaller group of women only, these restenosis rates were 33.3%, 8% and 0%, respectively. Variant alleles (G and C alleles of XbaI and PvuII polymorphisms, respectively) were associated with significantly lower incidence of carotid restenosis. We demonstrated by multiple logistic regression analysis, that T-A haplotype carriers (homozygous or heterozygous) had an increased risk of restenosis after carotid interventions, which was independent from age, gender and and presence of recurrent stenosis. (adjusted odds ratio: 7.85, confidence interval: 1.01–60.98).

4.2. Experimental studies

We found intensive alpha-smooth muscle actin and transforming growth factor $ß_1$ immunoreactivity in the control neointima, which indirectly confirms the migration and proliferation of VSMC`s. Phosphodiesterase-5 inhibitor treatment significantly reduced the stenosis grade (24.64 ± 7.46% vs. 54.12 ± 10.30% in the control endarterectomy group; $p<0.05$) and the neointima/media area ratio (0.50 ± 0.15 vs. 1.03 ± 0.13 in the control endarterectomy group, $p<0.05$) as well as the expression of both neointimal markers (Figures 4, 5, 6).

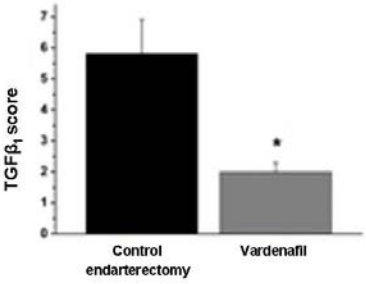

Figure 4.
$TGFβ_1$ immunohistochemistry
(*p<0.05 vs. control CEA)

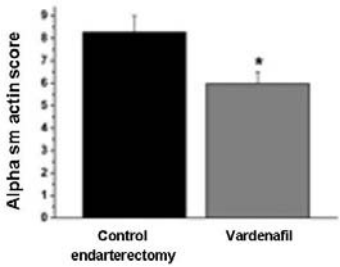

Figure 5.
Alpha-smooth muscle actin immunohistochemistry
(*p<0.05 vs. control CEA)

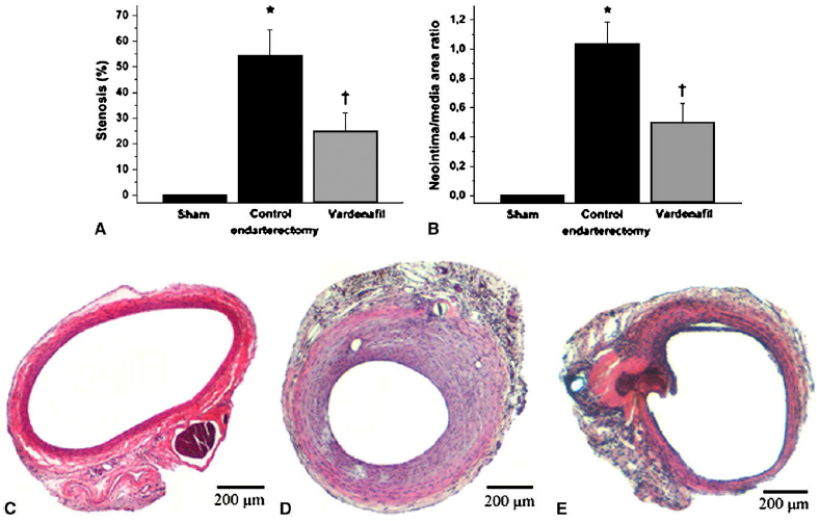

Figure 6.
Morphometric evaluation
A) Percent stenosis (neointima/original lumen area ratio) in the different groups.
* $p<0.05$ vs. sham; † $p<0.05$ vs. control endarterectomy
B) Neointima/media area ratio in the different groups.
* $p<0.05$ vs. sham; † $p<0.05$ vs. control endarterectomy
C) Representative histologic cross-section from the sham group
(HE, haematoxylin and eosin stain)
D) Representative histologic cross-section from the control endarterectomy group (HE)
E) Representative histologic cross-section from the treatment group (HE)

Phosphodiestersae-5 inhibitor treatment was correlated with local (neointimal) and systemic elevation of cGMP concentration, that was demonstrated by immunohistology and enzymeimmonoassay, respectively (Figures 7, 8). Plasma cGMP concetration was almost twice higher in the treatment group as compared to the control group (84.65 ± 12.77 pmol/ml vs. 43.50 ± 3.30 pmol/ml in the control endarterectomy group; $p<0.05$). On the other hand, we found no differenc in TUNEL-positive cell count between control and treated neointima.

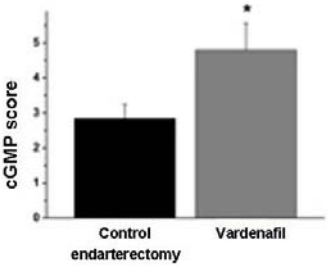

Figure 7.
cGMP immunohistochemistry
(*p<0,05 vs. control CEA)

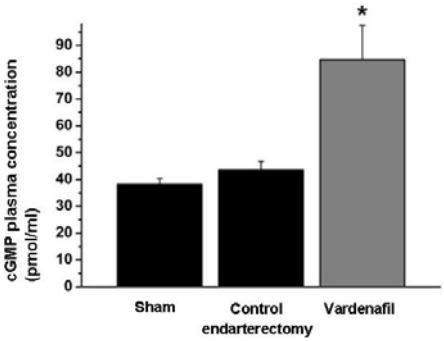

Figure 8.
cGMP plasma concentration
(*p<0,05 vs. sham and control CEA)

5. CONCLUSIONS

In the present work, conventional and new risk factors of restenosis following carotid artery revascularisation were investigated in human studies, and novel pharmacotherapeutic options were studied in an experimental model of carotid endarterectomy. We showed significant difference in the occurrence of clinically relevant restenosis between carotid endarterectomy and carotid artery stenting. Higher rate of recurrent stenosis after carotid endarterectomy were observed, where more pronounced endovascular damage occurs, accompanied by clamping of the carotid artery (ischemia/reperfusion injury). Our previous studies revealed more expressed complement activation after carotid endarterectomy than following carotid artery stenting, which can explain the higher restenosis rate observed after surgery. We also found a significant association between two common polymorphisms of the estrogen receptor alpha and restenosis; normal allele carriers were associated with higher incidence of recurrent carotid stenosis. Estrogen has several direct and indirect beneficial effects on vascular cells, that are mediated by estrogen receptor alpha. Altered estrogen effects due to the receptor expression can explain the genetic association showed in our present work. In a rat model of carotid endarterectomy we confirmed the therapeutic effects of the phosphodiesterase-5 inhibition in decreasing neointimal hyperplasia, that is mainly responsible for recurrent stenosis. We assume decreased estrogen effects and subsequently decreased NO-cGMP signalling in patients carrying TT/TC (PvuII) or AA (XbaI) genotypes, thus phosphodiesterase-5 inhibition may preferentially be beneficial in these patients. Our work presents important new data to local (endarterectomy or stenting) and systemic (gene polymorphisms) risk factors of restenosis that limits the longterm success of modern revascularisation procedures.

6. NEW FINDINGS

1. Incidence of both moderate and severe restenosis after carotid artery stenting was less common than after eversion carotid endarterectomy according to our single-center retrospective study. On the other hand, perioperative complications were recorded more often after CAS than following CEA.

2. Patients carrying AA genotype of the estrogen receptor alpha XbaI polymorphism had higher incidence of recurrent stenosis after carotid interventions as AG or GG genotype carriers. On the othe hand, TT and TC genotype carriers of the PvuII polymorphism exhibited higher risk for carotid restenosis than CC genotype carriers. These associations were more expressed in women undergoing carotid surgery.

3. Strong linkage disequilibrium was found between these polymorphisms. Homozygous carriers of the variant allels (GG and CC genotypes of the XbaI and PvuII polymorphisms, respectively) were associated with a lower incidence of early recurrent stenosis.

4. In a rat model of carotid endarterectomy**,** intensive alpha-smooth muscle actin and $TGF\beta_1$ immunoreactivity has been found in the control neointima. Phosphodiesterase-5 inhibitor treatment significantly reduced the expression of both neointimal markers, which indirectly confirms the reduced migration and proliferation of VSMC`s.

5. Vardenafil treatment significantly suppressed neointimal hyperplasia (neointima/media area ratio, percent stenosis) after surgical endarterectomy in the rat.

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