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LOW-DOSE VALPROIC ACID ENHANCES ENDOTHELIAL CELL PROLIFERATION AND ACCELERATES RE-ENDOTHLIALIZATION IN BALLOON-INJURED RAT CAROTID ARTERY

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ABSTRACT: Re-endothelialization after stent implantation and vascular injury is a critical step in the process of vascular healing. Proliferation of human umbilical vein endothelial cells (HUVECs) is a major factor influencing the induction of re-endothelialization. Valproic acid (VPA) is a widely used anti-epileptic drug. VPA was recently shown to modulate expression of various genes involved in angiogenic activity. However, accumulating evidence suggests that significant dose-dependent antiproliferative effects of VPA can occur and partial cytotoxic effects may restrict the use of VPA to local high-dose (≥ 1mM) devices such as a drug eluting stent. Therefore, we evaluated the effects of low-dose VPA on proliferation of HUVECs and vascular smooth muscle cells (VSMCs) *in-vitro* and neointima formation in balloon-injured rat carotid arteries *in-vivo*. Treatment of HUVECs with low-dose VPA (≤ 1mM) promoted proliferation, whereas VSMCs were not affected. Particularly, VPA 100 nM and 100 μM promoted endothelial proliferation by 151.41 ± 15.40% and 163.28 ± 4.82%, respectively, compared with the control group. These changes were preceded by increased expression of extracellular signal-regulated kinase 1/2 (ERK1/2), phosphatidylinositol 3-kinase (PI3K)/Akt (Akt), and endothelial nitric-oxide synthase (eNOS). VPA was also found to accelerate re-endothelialization, thereby inhibiting neointima formation in balloon-injured rat carotid arteries. VPA reduces neointima formation by promoting re-endothelialization. The results of this study have significant implications for treating restenosis following revascularization.

INTRODUCTION: Drug-eluting stents (DESs) provide a significant reduction in restenosis rate compared to bare metal stents (BMSs) [1]. However, DES implantation has been correlated with delayed vascular healing with impaired restoration of endothelial coverage and chronic inflammatory response, which may contribute to late and very late stent thrombosis [2].

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Although the mechanism of late stent thrombosis is poorly understood, re-endothelialization is a principal factor in maintaining luminal patency because endothelial cells provide critical vascular structure. Therefore, accelerating re-endothelialization is not only important for remodeling injured vessels, but also for reducing neointima formation and preventing restenosis.

Valproic acid (VPA; 2-propyl-pentanoic acid) has been used as an antiepileptic drug and has been employed clinically for treating schizophrenia, bipolar disorders, and various forms of headache [3]. VPA is a histone deacetylase inhibitor that exerts its effects by modifying chromatin structure and

thus gene expression [4]. VPA has also been shown to affect various signaling pathways such as Extracellular signal-regulated kinase 1/2 (ERK1/2), phosphatidylinositol 3-kinase (PI3K)/Akt (Akt), and endothelial nitric-oxide synthase (eNOS) [5]. In different cells, VPA was shown to stimulate activation of the ERK1/2 which plays an integral role in cellular survival signaling. In endothelial cells (ECs), VPA was shown to promote cell survival and angiogenesis by enhancing expression of phospho-ERK1/2. Indeed, VPA affects angiogenesis both *in-vitro* and *in-vivo* by changing the expression of the PI3K/Akt and eNOS pathways in ECs [6]. Phosphorylation of ERK1/2, PI3K/Akt, and eNOS occurs upstream of angiogenesis [7].

However, significant dose-dependent inhibitory effects of VPA have been detected and strong antiproliferative potential of VPA can be seen after a local high-dose administration due to partial cytotoxic effects. Whether low-dose VPA affects HUVEC proliferation and re-endothelialization *in-vitro* and *in-vivo* is unknown. Therefore, the present study was designed to investigate the effect of low-dose VPA on HUVEC and VSMC proliferation *in-vitro* and to examine the mechanisms of VPA action at the molecular level using immunoblotting. Additionally, we examined the ability of VPA to accelerate re-endothelialization and reduce restenosis using a model of vascular neointima hyperplasia induced by rubbing the endothelia of the common carotid artery with a balloon catheter in rats.

MATERIALS AND METHODS:

Cell Culture and Reagents: HUVECs were obtained from Scien Cell Research Laboratory (San Diego, CA, USA) and grown on Matrigel (BD Biosciences, Heidelberg, Germany) coated culture plates. HUVECs were cultured at 37℃ with 5% CO_2 in Endothelial Cell Growth Medium (EGM-2, Lonza Inc., Walkersville, MD, USA) supplemented with human recombinant epidermal growth factor (hEGF), human fibroblast growth factor-basic with heparin (hFGF-B), ascorbic acid, vascular endothelial cell growth factor (VEGF), hydrocortisone, human recombinant insulin-like growth factor (long R3-IGF-1), heparin, gentamicin, amphotecin, and 2% fetal bovine serum (FBS) or, when indicated, in Endothelial Cell Basal Medium (EBM-2 Lonza Inc., Walkersville, MD, USA) supplemented with 1% FBS (Sigma, St. Louis, MO, USA). For experiments, cells that had been passaged no more than 10 times were used. VSMCs were isolated from rat aortic vascular smooth muscle from primary culture. Isolation was carried out as follows: an enzyme mixture containing 1 mg/mL type II collagenase, 0.25 mg/mL elastase, 1 mg/mL soybean trypsin inhibitor, and 2 mg/mL bovine serum albumin (BSA) was prepared in DMEM/F12 (Lonza Inc., Walkersville, MD, USA) pre-warmed to 37℃ and incubated with an aorta sample, which had been obtained from 6-week-old Sprague Dawley (SD) rats (Charles River, Japan) and cut into small pieces.

All materials, including the enzyme mixture, were obtained from Sigma (St. Louis, MO, USA). Digestion was carried out for 30–45 min with stirring at 37℃. The cell suspension was centrifuged at 240 ×*g* (1000 rpm in Jouan) for 5 min at 4℃, and the pellet was washed with DMEM/F12 medium containing 10% FBS and 1% antibiotic–antimycotic agent (Sigma). The pellet was then resuspended in 5ml of fresh medium. Cells were then grown in DMEM/F12 medium containing 10% FBS and 1% antibiotic–antimycotic agent. The cells in passage numbers between 3 and 10 were used for all experiments. VPA was purchased from Sigma-Aldrich Chemical Co.

Proliferation Assay: HUVECs (1×10^4 cells/well) were seeded onto a 24-well plate coated with 2% gelatin (Sigma-Aldrich) and incubated with endothelial growth medium (EGM) containing all necessary growth factors and serum supplement until 60% confluence. The cells were washed three times using phosphate buffered saline (PBS) and were starved for 24 h in EBM-2 basal media supplemented only with 1% FBS. Cells were continuously incubated at 37±1℃/ 5% CO_2 for 24h. The cells were treated with serial dilutions of VPA (1 nM, 10 nM, 100 nM, 1 µM, 10 µM, 100 µM, and 1 mM) and VEGF (20 ng/mL). After 2 days of incubation, Cell Counting Kit-8 (CCK-8, Dojindo, Kumamoto, Japan) was added. Next, 50 µL of the CCK-8 solution was rapidly added to each well and further incubated for 2 h. Subsequently, the plate was shaken slightly and

absorbance was measured at 460 nm using a microplate reader (Molecular Devices Co., Menlo Park, CA). Cell viability was calculated as a percentage relative to non-treated control cells. VSMCs were seeded in 24-well plates at a density of 1×10^4 cells/well and incubated with DMEM/F12 (Lonza) containing 10% FBS. Following 24h of incubation, the cells were starved for 24 h in DMEM/F12 (Lonza) supplemented only with 0.5% FBS. After day 1, the cells were treated with VPA under the same conditions as those for HUVECs.

Immunoblotting: HUVECs were plated at a density of 6×10^5 cells in a gelatin-coated 10-cm diameter dish. When confluence was reached, the cells were starved overnight in EBM-2 medium containing 1% FBS followed by treatment with the indicated dosages of VPA. Lysates of cells were prepared as follows. The cells were washed twice with PBS and added to 200 µL of New England Biolabs (Ipswich, MA, USA) buffer containing 62.5 mM Tris-HCl, 2% sodium dodecyl sulfate (SDS), 10% glycerol, 50 mM dithiothreitol, 0.01% bromophenol blue (BPB), 1 mM phenylmethylsulfonyl fluoride, 2 mM sodium orthovanadate, and deionized water. The cells were scraped and transferred into Eppendorf tubes, and the cell suspension were sonicated for 10 s. Protein concentration was determined using the Bradford assay, and equal amounts (15 µg) of samples were loaded into each lane. Samples were separated using 10-12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a nitrocellulose membrane (Amersham, Buckinghamshire, UK). After blocking in TBST containing 5% non-fat dry skimmed milk for 1 h, the blots were incubated with the following primary antibodies overnight at 4°C: ERK (Cell Signaling Technologies, Beverly, MA, USA), p-ERK-1/2 (Cell signaling, Beverly, MA), Akt (Cell Signaling), p-Akt (Cell signaling, Beverly, MA), eNOS (BD), p-eNOS (Cell Signaling) and anti-tubulin (Calbiochem/Merck, Darmstadt, Germany). After washing in TBST, blots were incubated for 1 h in horseradish peroxidase (HRP)-conjugated secondary antibodies and visualized using the enhanced chemiluminescence western blotting detection system (Amersham).

Rat Carotid Artery Injury Model: This study was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of the Samsung Biomedical Research Institute (SBRI). SBRI is an Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC International) accredited facility and abides by the Institute of Laboratory Animal Resources (ILAR) guide.

To produce a common carotid artery intima denudation model, the male Sprague Dawley rats (n = 8, 350 – 400 g) were anesthetized using sodium ketamine and xylene, the left common carotid artery (CCA) and external carotid artery (ECA) were exposed through a midline neck incision, and the ECA was dissected. A 2F Fogarty embolectomy balloon catheter (Baxter Health Care, Palo Alto, CA) was introduced into the right common carotid artery by way of the external carotid artery. The balloon was inflated with 0.2 mL normal saline to expand the common carotid artery and was withdrawn to the external carotid artery in the inflated state 3 times. Upon catheter removal, the external carotid artery was ligated and the neck incision was closed. After operation, the animals were treated using antibiotics to prevent infection (0.12 million units of penicillin G benzathine every animal, i.m). VPA was delivered intra-arterially using the dip-spin technique immediately following balloon injury. This was followed by 2 weeks of continuous subcutaneously delivery using an osmotic pump (Alzet, model 2002, Alza, Vaacaville, CA, USA) containing VPA (2 mg or 20 mg) or vehicle in 200 µL of PBS. Two weeks after balloon injury, 3 mL of blood were collected from the blood, the rats were sacrificed, and right and left carotid arteries were prepared.

Immunostaining: For immunohistochemical analysis, cross-sections of injured carotid artery were fixed using 4% (w/v) paraformaldehyde (Sigma) at room temperature for 1 day. The segments were paraffin-embedded, and the paraffin-sectioned slides were treated with xylene to remove the paraffin, rehydrated with 70%, 50%, and 30% ethanol and PBS for 2 min each at room temperature, and washed twice with PBS. To quench endogenous peroxidase activity, slides were immersed in PBS containing 0.2% H_2O_2 for 15 min at room temperature and then washed three times in PBS. The samples were then incubated in PBS containing 10% (v/v) normal goat serum in 0.5%

Triton X-100 for 30 min at room temperature. To detect endothelial cells, sections were stained with von Willebrand factor (1:100 Dakopatts, Hamburg, Germany) in PBS containing 1% normal goat serum and 0.5% Triton X-100 overnight at 4°C. Subsequently, sections were incubated with biotinylated anti-rabbit immunoglobulin G (IgG) (1:200 R&D Systems, Minneapolis, MN, USA) for 30 min at room temperature before washing. Diaminobenzidine was used as the chromogen, and slides were counterstained using hematoxylin.

Statistics: Data are expressed as the mean ± SD. Results were analyzed using GarphPad Prism statistics software (GraphPad Software, Inc., San Diego, CA, USA). A *P*-value of less than 0.05 was considered statistically significant.

RESULTS:

Valproic Acid Enhanced Proliferation of HUVEC but not SMC: Endothelial cell proliferation is a crucial step in re-endothelialization. To determine whether endothelial cells are affected by VPA, proliferation was evaluated using CCK-8. VPA enhanced HUVEC proliferation in a dose-dependent manner, whereas it did not affect the proliferation of VSMCs **Fig. 1**. Particularly, the growth of HUVECs treated with VPA (100 nM and 100 μM) increased by 153 ± 13.98% and 163 ± 4.82%, respectively, compared with the control group **Fig. 1A**. Although growth was not constant, all groups treated with VPA demonstrated higher viability and proliferation than the control group in HUVECs.

Unexpectedly, VPA had no effect on VSMCs at low concentrations except at 1 mM **Fig. 1B**. In HUVECs and SMCs, an antiproliferative effect was observed after incubation with VPA at a high concentration (≥ 1 mM) (data not shown). Significant dose-dependent effects were detected in HUVECs proliferation following treatment with VPA.

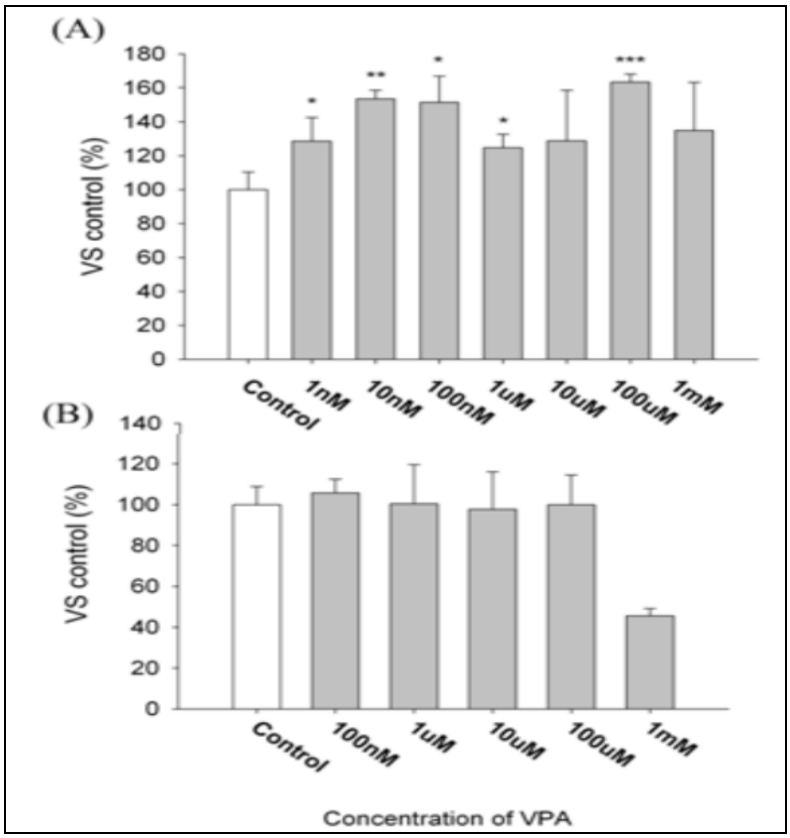

FIG. 1: EFFECTS OF LOW-DOSE VALPROIC ACID (VPA) ON THE PROLIFERATION OF ENDOTHELIAL CELLS AND SMOOTH MUSCLE CELLS UNDER DIFFERENT MEDIUM CONDITION. HUVECS AND SMCS WERE INCUBATED WITH VPA (1 NM TO 1 MM) FOR 2 DAYS. (A) INFLUENCE OF VPA ON HUVEC PROLIFERATION UNDER STARVATION (1% FBS) CONDITIONS. (B) INFLUENCE OF VPA ON SMC PROLIFERATION UNDER STARVATION (0.5% FBS) CONDITIONS. RESULTS ARE PRESENTED AS THE MEAN ± S.E.M OF DATA OBTAINED IN THREE INDEPENDENT EXPERIMENTS. (C, CONTROL, ****P* < 0.05; *****P* < 0.01;****P* < 0.001; PAIRED STUDENT'S *T* TEST).**

Valproic Acid Increases Phosphorylation of ERK, Akt, and eNOS in HUVECs: Mitogen-activated protein kinase (MAPK) pathways are commonly involved in various cellular processes such as proliferation, differentiation, and apoptosis in endothelial cells (22-26). To determine the optimum concentration of VPA and compare this value with the results of the proliferation test, cells were treated with serial dilutions of VPA (1 nM, 10 nM, 100 nM, 1 µM, 10 µM, 100 µM, and 1 mM) and VEGF (20 ng/mL) for 30 min. The phosphorylating activity of ERK-1/2 and Akt were similar to those observed in the proliferation test. Compared with VEGF (positive control), phosphor-ERK, Akt, and eNOS expression was less enhanced. However, compared with control, signal enhancement was observed following VPA treatment **Fig. 2A**. To investigate kinase signaling related to EC proliferation after incubation with VPA, we determined the level of phosphorylated ERK-1/2, Akt, and eNOS. HUVECs were incubated under starvation conditions (1% FBS) overnight and then incubated with 100 nM VPA for varying periods. Increased phosphorylation of ERK-1/2 and Akt were detected within 5m in after addition of VPA and reached a maximum after 30min **Fig. 2B**. p-eNOS increased after 1 h. We also determined the amounts of pERK, pAkt, and p-eNOS in HUVECs treated with vehicle at different times (data not shown); however, gene expression was not detected.

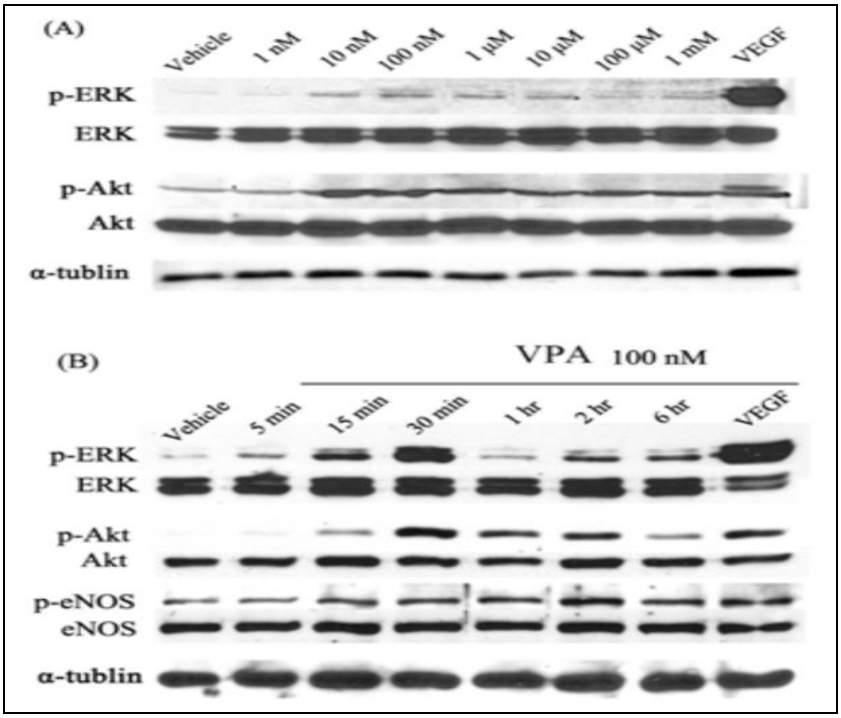

FIG. 2: EFFECTS OF LOW-DOSE VPA ON ERK/AKT/ENOS PATHWAYS IN ENDOTHELIAL CELLS. (A) REPRESENTATIVE WESTERN BLOT SHOWING ERK/AKT/ENOS PATHWAY IN HUVEC AFTER OVERNIGHT CULTIVATION IN STARVATION (1% FBS) MEDIUM WITH SEVERAL CONCENTRATION (1 NM TO 1 MM) FOR 30 MIN. (B) REPRESENTATIVE WESTERN BLOT SHOWING ERK/AKT/ENOS PATHWAY IN HUVEC AFTER OVERNIGHT CULTIVATION IN STARVATION (1% FBS) MEDIUM WITH VPA (100 NM) FOR DIFFERENT TIME. WESTERN BLOTS ARE REPRESENTATIVE OF THREE INDEPENDENT EXPERIMENTS (VEGF; POSITIVE CONTROL)

VPA Inhibits Neointima Formation in Balloon-Injured Carotid Artery Model: Visual examination of the representative neointima sections of balloon-injured carotid arteries with immunohistochemistry staining revealed that VPA affect neointima formation **Fig. 3A**. Two weeks after balloon injury, neointima areas were 0.20 ± 0.04 mm^2, 0.14 ± 0.07 mm^2, and 0.12 ± 0.09 mm^2 in case of vehicle, 2 mg VPA, and 20 mg VPA, respectively **Fig. 3B**, which accounts for the significantly lower stenosis area in rats treated with VPA. Hematoxylin and eosin (H&E) staining was used to verify the absence of stenosis in the entire artery. Particularly, when the amount of VPA was higher (20 mg), a more positive effect was observed on preventing stenosis.

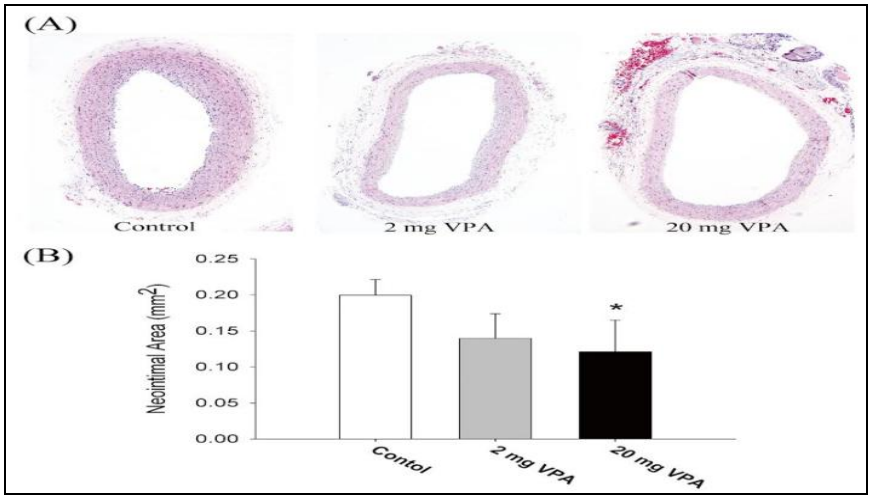

FIG. 3: INHIBITORY EFFECT OF VPA ON NEOINTIMA HYPERPLASIA OF RAT CAROTID ARTERIES INDUCED BY BALLOON INJURY. THE REPRESENTATIVE NEOINTIMA HYPERPLASIA SECTIONS OF RAT COMMON CAROTID ARTERIES (CCA) STAINED WITH HEMATOXYLIN–EOSIN (ORIGINAL MAGNIFICATION 100×) DEMONSTRATED THE NEOINTIMA HYPERPLASIA OF CCA ON DAY 15 AFTER BALLOON-INJURY IN MODEL GROUP, THE ALLEVIATED NEOINTIMA HYPERPLASIA IN VPA (2 MG OR 20 MG)-TREATMENT (FOR 14 DAYS) GROUP (A); DATA TAKEN FROM THE IMAGE MANIPULATION SYSTEM SHOWED THE CHANGES OF THE AVERAGE VALUES IN NEOINTIMA AREA AFTER VPA-ADMINISTRATION AT DIFFERENT DOSES FOR 14 DAYS (B). DATA ARE MEAN ± S.E.M OF 6 OR 8 RATS. (*P < 0.05; PAIRED STUDENT'S T TEST).

VPA Accelerates re-endothelialization in Balloon- Injured Carotid Artery Model: To investigate the effect of VPA treatment on re-endothelialization of the denuded region after injury, immunohistochemistry staining was used to detect the von Willebrand factor (vWF) **Fig. 4A**, which is a constitutively expressed glycoprotein produced in the endothelium. The control group showed incomplete endothelial formation, whereas a more complete and continuous monolayer of vWF-positive cells was observed in the carotids of rats treated with 20 mg VPA; this shows that VPA promotes re-endothelialization **Fig. 4B**. The degrees of re-endothelialization were 21.81 ± 8.99% in the control group and 60.63 ± 7.36% in the group treated with 20 mg VPA.

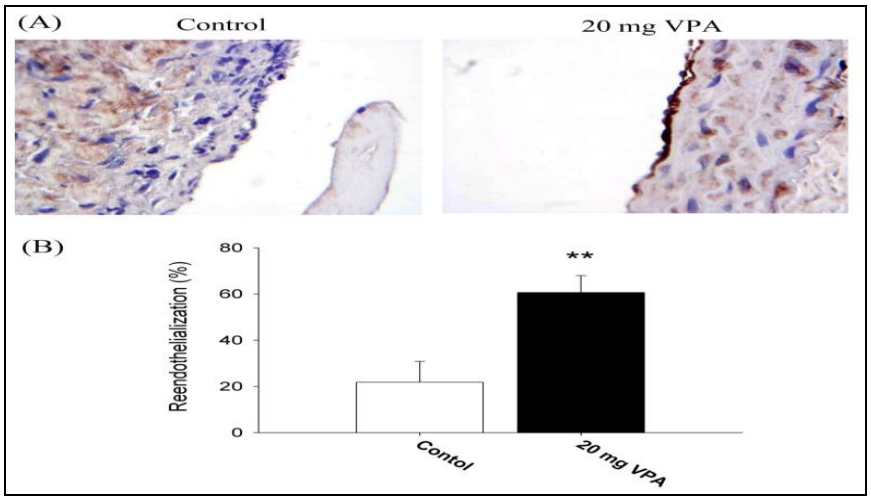

FIG. 4: EFFECT OF VPA ON RE-ENDOTHELIALIZATION IN NEOINTIMA OF BALLOON-INJURED ARTERIES OF RATS. REPRESENTATIVE SECTIONS (ORIGINAL MAGNIFICATION 400×) OF CAROTID ARTERIES WITH IMMUNOHISTOCHEMISTRY STAINING FOR VWF SHOWED ALMOST NO RE-ENDOTHELIALIZATION IN CONTROL VASCULAR WALL AND IN THE VPA (20 MG)-TREATMENT GROUP (A). DATA TAKEN FROM THE IMAGE MANIPULATION SYSTEM DEMONSTRATED THE AREA OF REENDOTHELIALIZATION RATIO AFTER VPA (20 MG)-ADMINISTRATION FOR 14 DAYS (B). RE-ENDOTHELIALIZATION RATIO WAS THE NUMBER OF VWF-POSITIVE CELL AREA /TOTAL AREA × 100%. DATA WERE MEAN ± S.E.M OF 6 OR 8 RATS. (*P < 0.05; PAIRED STUDENT'S T TEST).

DISCUSSION: Evidence from recent studies suggests that the anti-proliferative effects of DESs delay re-endothelialization within the stent, increasing the risk of stent thrombosis. Late or very late thrombosis following SES stenting is related to a lack of stent strut endothelial coverage [8]. Moreover, increased proliferation of smooth muscle cells plays a key role in pathologies involving intima thickening, such as atherosclerosis and restenosis after vascular intervention [9]. Therefore, drugs for restenosis that promote HUVEC proliferation but have no effect on VSMCs should be developed. Re-endothelialization after stent implantation and vascular injury is a critical step for vascular healing. Although the predominant mechanism of stent endothelialization remains unclear, the biological process of vascular healing after bare-metal stent implantation is a dynamic interaction between the prolonged inhibitory effect caused by the drug on smooth muscle and EC proliferation [10]. VPA is an antiepileptic drug that is frequently prescribed due to its safety and effectiveness. VPA have also shown to inhibit angiogenesis and apoptosis of endothelial cells at high VPA concentrations [11]. Moreover, partial cytotoxic effects restrict the use of VPA to local high-dose devices such as drug eluting stents [12]. Because of the cytotoxic effects, VPA was used in concentrations ≤ 1 mM, which is in the range of therapeutically achievable plasma concentration.

In this study, HUVEC proliferation was enhanced at a low VPA concentration (≤1 mM), specifically 100 nM and 100 μM. A VPA concentration of 100 nM activated expression of phosphor-ERK 1/2, Akt, and eNOS in HUVECs. The ERK-1/2 and Akt pathways are well known as key players in endothelial cell angiogenesis and survival [13].

Nitric Oxide (NO) modulates endothelial cell migration, proliferation, survival, lumen/network formation, and vasodilatation [14]. *In-vitro* and *in-vivo* experiments have found that eNOS triggers endothelial cell migration, proliferation, and differentiation [6]. Our observations are in accordance with previous reports showing that VPA activates the ERK-1/2, and Akt pathways in several cell types. Additionally, immuno-precipitation experiments have demonstrated that Akt and eNOS are highly associated and that Akt directly activates eNOS via phosphorylation at Ser1177 [15]. Therefore, low-dose VPA induces HUVEC proliferation by activating the ERK/Akt/eNOS pathways. In VSMCs, no proliferative effect was found after incubation with VPA at a low-concentration (≤ 1 mM). A significant antiproliferative effect in VSMCs was detected after incubation with VPA at concentration of 1 mM due to cytotoxicity. These data indicate that VPA has significant dose-dependent effects on HUVEC proliferation but not on VSMC proliferation.

To evaluate the effect of VPA on re-endothelialization and neointima hyperplasia, we used a common and well-established rat carotid artery model caused by injury induced by balloon catheter dilatation. Neointima formation occurs as a response to injury to vascular tissue and is mediated by thrombotic and inflammatory mediators, growth factors, cytokines, and, importantly, oxidative stress. As demonstrated in previous studies, the endothelium is a critical factor protecting vessels from thrombosis and atherosclerosis [16]. In the present study, we demonstrated that VPA promotes re-endothelialization, thereby inhibiting neointima formation in injured arteries.

Our *in-vitro* data of phosphor-ERK1/2, Akt, and eNOS up-regulation following VPA treatment in HUVECs can be explained by enhanced re-endothelialization that inhibits neointima formation to injured vascular walls. Consistent with *in-vitro* findings, previous studies demonstrated that NO has a variety of well-documented biological activities that may interfere with the pathomechanisms of restenosis, e.g., promote adaptive vascular dilatation, inhibit of VSMC proliferation and migration, as well as inhibit adhesion molecule expression, platelet aggregation, and pro-inflammatory cytokine synthesis [16, 17]. Several protein kinases, such as Akt, AMP-activated protein kinase (AMPK), and protein kinase A (PKA), are known to activate eNOS. Indeed, there is evidence that cell survival (PI3K/Akt) and mitogenic (Ras/Ref/MEK/ERK) cascades act as independent pathways, but that cross talk occurs between these cascades [18]. These results suggest that the ERK1/2, PI3K/Akt and eNOS pathways are functionally involved in VPA-

induced signaling cascades leading to proliferation of human endothelial cells, thereby inhibiting neointima formation. However, additional concentrations must also be tested to determine the most efficient concentration *in-vivo*.

CONCLUSION: We showed that low-dose VPA increases expression of ERK/Akt/Enos phosphorylation in endothelial cells. Because the ERK/Akt/eNOS pathway plays important roles in HUVECs proliferation, VPA can accelerate re-endothelialization and inhibit neointima formation in balloon-injured arteries of rats. We propose that the proliferative potential of VPA can be adjusted to the device being used to treat vascular disease, such as DES and grafts.

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CONFLICT OF INTEREST: The authors declare no conflict of interest.

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