Universidade de Aveiro Departamento de Química
Ano 2012

Rita Marisa Nogueira Ferreira

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Terameprocol effect in the proliferation and apoptosis of SMCs in PAH

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Bioquímica Clínica, realizada sob a orientação científica do Doutor Tiago Alexandre Henriques Coelho, Professor Doutor da Faculdade de Medicina da Universidade do Porto, Departamento de Fisiologia e Cirurgia Cardiotorácica, e do Doutor Rui Miguel Pinheiro Vitorino, Investigador Auxiliar do Departamento de Química da Universidade de Aveiro.

Dedico este trabalho à minha família.

o júri

presidente

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palavras-chave hipertensão arterial pulmonar, células musculares lisas, proliferação celular, apoptose, survivina, terameprocol.

resumo A hipertensão arterial pulmonar (HAP) é uma doença rara e letal, caracterizada pela remodelação da vasculatura pulmonar, devido a uma proliferação celular excessiva e diminuição da apoptose. Estas alterações levam ao aumento da resistência e pressão arterial pulmonares, culminando em insuficiência cardíaca direita. O tratamento atual é limitado, não oferecendo uma cura e, portanto, é necessária mais investigação para encontrar novos alvos terapêuticos. Neste contexto, a via da survivina, uma molécula que inibe a apoptose e promove a proliferação e cujos níveis estão aumentados na HAP, parece promissora. O objetivo deste trabalho foi avaliar o efeito do terameprocol, um antagonista da survivina, na proliferação e apoptose de células musculares lisas na HAP. Foi utilizado o modelo experimental de HAP induzida por monocrotalina (MCT). Ratos Wistar foram injetados com MCT (60 mg/kg, sc) para a indução da doença e, após 21 dias foram sacrificados, o coração e os pulmões foram removidos e a artéria pulmonar do lobo superior esquerdo foi dissecada. A adventícia e íntima foram removidas da artéria e as células musculares lisas da média foram isoladas através de um método enzimático. Foi realizada uma cultura primária de células musculares lisas que foram tratadas com diferentes concentrações de terameprocol. Em seguida, foram realizados ensaios para avaliar a apoptose (TUNEL) e proliferação (BrdU e exclusão por *trypan blue*) das células musculares lisas. Os resultados mostraram que o terameprocol inibe a proliferação e induz a apoptose das células musculares lisas da artéria pulmonar de ratos com HAP induzida pela MCT, sugerindo que a via da survivina pode constituir um novo alvo terapêutico a ser investigado na HAP.

keywords

pulmonary arterial hypertension, smooth muscle cells, cellular proliferation, apoptosis, survivin, terameprocol.

abstract

Pulmonary arterial hypertension (PAH) is a rare and lethal disease, characterized by remodeling of the pulmonary vasculature due to excessive cellular proliferation and decreased apoptosis. These alterations lead to increased pulmonary arterial resistance and pressure, culminating in right heart failure. The current treatment is limited, not affording a cure and thus, more research is needed to find new therapeutic targets. In this context, targeting survivin, a molecule that inhibits apoptosis and promotes proliferation and reported to be increased in PAH, seems promising. The aim of this work was to evaluate the effect of terameprocol, an antagonist of survivin, in the proliferation and apoptosis of smooth muscle cells in PAH. We used the experimental model of PAH induced by monocrotaline (MCT). Wistar rats were injected with MCT (60 mg/kg, sc) to induce the disease and after 21 days, they were euthanized, their heart and lungs were removed and the pulmonary artery from the left upper lobe was dissected. The adventitia and intima were removed from the artery and the smooth muscle cells from the media were isolated through an enzymatic method. The resultant primary cultures of smooth muscle cells were treated with different concentrations of terameprocol. Then, assays were performed to evaluate smooth muscle cell apoptosis (TUNEL assay) and proliferation (BrdU and trypan blue exclusion assays). The results showed that terameprocol inhibits proliferation and promotes apoptosis of pulmonary artery smooth muscle cells from rats with MCT-induced PAH, suggesting that survivin pathway can be seen as a new therapeutic target to be explored in PAH.

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Abbreviations

5-HT	Serotonin
5-HT_{1B}	Serotonin type 1B receptor
5-HT_{2A}	Serotonin type 2A receptor
5-HT_{2B}	Serotonin type 2B receptor
6MWT	6-minute walk test
ActRII	Activin receptor type II
ADMA	Asymmetrical dimethylarginine
ALK-1	Activin receptor-like kinase 1
ANP	Atrial natriuretic peptide
AVD	Apoptotic volume decrease
BMP	Bone morphogenetic protein
BMPRII	Bone morphogenetic protein receptor type II
BNP	Brain natriuretic peptide
cAMP	Cyclic adenosine monophosphate
cav-1	Caveolin-1
CCBs	Calcium channel blockers
cGMP	Cyclic guanosine monophosphate
CREB	cAMP response element-binding
CTPH	Chronic thromboembolic pulmonary hypertension
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
ECs	Endothelial cells
eNOS	Endothelial nitric oxide synthase
ET-1	Endothelin-1
ET_A	Endothelin-1 type A receptor
ET_B	Endothelin-1 type B receptor
FBS	Fetal bovine serum
GTP	Guanosine triphosphate
HIF	Hypoxia-inducible transcription factor
HIV	Human immunodeficiency virus
IAP	Inhibitor of apoptosis

IPAH	Idiopathic pulmonary arterial hypertension
MCT	Monocrotaline
MCTP	Monocrotaline pyrrole
MMPs	Matrix metalloproteinases
mRNA	Messenger ribonucleic acid
NADPH	Nicotinamide adenine dinucleotide phosphate
NF-AT	Nuclear factor of activated T-cells
NF-kB	Nuclear factor kappa B
NO	Nitric oxide
NOS	Nitric oxide synthase
NSF	ATPase N-ethylmaleimide-sensitive factor
PAECs	Pulmonary artery endothelial cells
PAH	Pulmonary arterial hypertension
PAI-1	Plasminogen activator inhibitor-1
PAP	Pulmonary arterial pressure
PASMCs	Pulmonary artery smooth muscle cells
PCH	Pulmonary capillary haemangiomatosis
PDE	Phosphodiesterase
PDH	Pyruvate dehydrogenase
PDK	Pyruvate dehydrogenase kinase
PGI_2	Prostacyclin
PH	Pulmonary hypertension
PIP_2	Phosphatidylinositol 4, 5-biphosphate
PKA	Protein kinase A
PKC	Protein kinase C
PLC	Phospholipase C
PVOD	Pulmonary veno-occlusive disease
PWP	Pulmonary wedge pressure
RHC	Right heart catheterization
ROCK	Rho kinase
RV	Right ventricle
SDMA	Symmetrical dimethylarginine
sGC	Soluble guanylate cyclase
siRNA	Small interfering ribonucleic acid

Smac	Second mitochondrial activator of caspases
SMCs	Smooth muscle cells
SM-MHC	Smooth muscle-myosin heavy chain
SNAPs	Soluble NSF association proteins
SNAREs	SNAP receptors
TASK-1	Two pore-related acid-sensitive potassium channel-1
TF	Transcription factors
TGF-β	Transforming growth factor-β
TMP	Terameprocol
TNF-α	Tumor necrosis factor-α
t-PA	Tissue plasminogen activator
TXA_2	Thromboxane A_2
VDCC	Voltage-dependent calcium channels
WHO	World Health Organization
XIAP	X-chromosome-linked inhibitor of apoptosis

1. Introduction

Pulmonary arterial hypertension (PAH) is a disease that although rare, is fatal if untreated. It is characterized by vasoconstriction, thrombosis *in situ*, inflammation and a vascular remodeling that result from the excessive proliferation of endothelial cells, smooth muscle cells and fibroblasts of the pulmonary artery wall, and decreased cellular apoptosis [1, 2]. These alterations progressively lead to the pulmonary artery lumen obstruction, increasing pulmonary vascular resistance and imposing pressure overload to the right ventricle (RV). Despite the initial RV adaptation, heart failure and premature death will eventually occur [1, 3]. Several molecular pathways have been reported to be involved in these structural alterations of the pulmonary artery wall but only a few were found to be therapeutically relevant. For instance, treatments with prostacyclin analogs, endothelin-1 receptor blockers, or phosphodiesterase type 5 inhibitors were all shown to be of benefit [1]. Nevertheless, the great majority of patients remain symptomatic and mortality rates still elevated [3]. In order to develop effective therapeutic targets, more research is needed on the cellular and molecular mechanisms responsible for the pathobiology of this disease.

Survivin is an anti-apoptotic and proliferative molecule, reported to be increased in PAH. It was shown that inhibition of survivin function by inhaled adenoviral gene therapy with a survivin mutant (Thr34Ala) reduced the vascular remodeling observed in PAH induced by monocrotaline in an animal model [4]. Other strategies, such as terameprocol, were reported to inhibit the growth of tumors by targeting Sp1 regulated proteins, including cdc2 and survivin [5, 6]. As cancer, PAH is also a disease characterized by excessive proliferation and impaired apoptosis; however, the role of terameprocol in PAH is still unknown. Thus, the present work focus in the study of the effect of terameprocol in the proliferation and apoptosis of smooth muscle cells in an animal model of PAH induced by monocrotaline administration, through the use of primary smooth muscle cells cultures.

2. Pulmonary arterial hypertension

2.1. Definition, classification and epidemiology

Pulmonary arterial hypertension (PAH) was initially described by the German physician and pathologist Ernst von Romberg in 1891 and only in 1973 the World Health Organization (WHO) standardized clinical and pathological nomenclature of PAH. Successively WHO has continued with regular conventions on this issue (Evian 1998, Venice 2003 and Dana Point 2008) [7]. The disease is now defined as a mean pulmonary artery pressure greater than or equal to 25 mmHg at rest with a mean pulmonary-capillary wedge pressure less than or equal to 15 mmHg [8]. After the 2008 4th World Symposium on Pulmonary Hypertension classification, pulmonary hypertension (PH) was subdivided into six groups (Table 1) [9, 10].

Table 1 – Clinic classification of pulmonary hypertension according to the 4th World Symposium on Pulmonary Hypertension held in Dana Point in 2008.

Group 1. Pulmonary arterial hypertension (PAH)
1.1. Idiopathic PAH (IPAH)
1.2. Heritable
1.2.1. *BMPRII* gene
1.2.2. *ALK1,endoglin* genes (with or without hereditary hemorrhagic telangiectasia)
1.2.3. Unknown
1.3. Drugs and toxins induced
1.4. Associated with:
1.4.1. Connective tissue diseases
1.4.2. HIV infection
1.4.3. Portal hypertension
1.4.4. Congenital heart diseases
1.4.5. Schistosomiasis
1.4.6. Chronic haemolitic anaemia
1.5. Persistent pulmonary hypertension of the newborn
Group 1'. Pulmonay veno-occlusive disease (PVOD) and/or pulmonary capillary haemangiomatosis (PCH)
Group 2. Pulmonary hypertension due to left heart disease
2.1. Systolic dysfunction
2.2. Diastolic dysfunction
2.3. Valvular disease
Group 3. Pulmonary hypertension due to lung diseases and/or hypoxia
3.1. Chronic obstructive pulmonary disease
3.2. Interstitial lung disease
3.3. Sleep-disordered breathing
3.4. Chronic exposure to high altitude
3.5. Broncho pulmonary dysplasia
3.6. Developmental abnormalities
Group 4. Chronic thromboembolic pulmonary hypertension (CTPH)
Group 5. Pulmonary hypertension with unclear and/or multifactorial mechanisms
5.1. Haematologic disorders: myeloproliferative disorders, splenectomy
5.2. Systemic disorders: sarcoidosis, pulmonary Langerhans cell histiocytosis: lymphangioleiomyomatosis, neurofibromatosis, vasculitis
5.3. Metabolic disorders: Glycogen storage disease, Gaucher disease, thyroid disorders
5.4. Others: tumoral obstruction, fibrosing mediastinitis, chronic renal failure on dialysis

Legend: ALK1 = activin receptor-like kinase 1; BMPRII = bone morphogenetic protein receptor, type II; HIV = human immunodeficiency virus

PAH is a rare disease with an incidence of approximately 2.4 cases per million diagnosed per year and a prevalence of approximately 15 cases per million diagnosed per year, according to a French population study [11]. Two-thirds of affected persons are women and the majority of patients are diagnosed with 41-50 years of age [12]. In systemic sclerosis (a connective tissue disease), the prevalence of PAH has been estimated to be 7.8-23%. In addition, PAH can be diagnosed in 0.5% of patients with HIV infection and in 2-5% of patients suffering from portal hypertension. It is also estimated that 10% of patients with hemoglobinopathies (such as sickle cell disease and thalassaemia) develop moderate to severe pulmonary hypertension [7, 10].

2.2. Genetic association

A rare group of patients with hereditary hemorrhagic telangiectasia and idiopathic PAH present specific mutations in genes encoding activin receptor-like kinase type 1 (ALK1), a member of the transforming growth factor-β (TGF-β) superfamily, or endoglin, an endothelial cell-specific TGF-β superfamily coreceptor [13-15]. However, a more prevalent cohort of patients carries mutations in another member of the TGF-β superfamily, the bone morphogenetic protein receptor type II (BMPRII), that is encoded by *BMPRII* gene, located on the chromosome 2q33 [16].

In general, upon a ligand belonging to the TGF-β superfamily binds to a complex of type II and type I serine/threonine kinase transmembrane receptors, the receptor II phosphorylates the bound type I receptor, which, in turn, phosphorylates one of the Smad family proteins that translocate for the nucleus allowing their binding to DNA, and the regulation of the transcription of specific target genes (Figure 1) [17, 18].

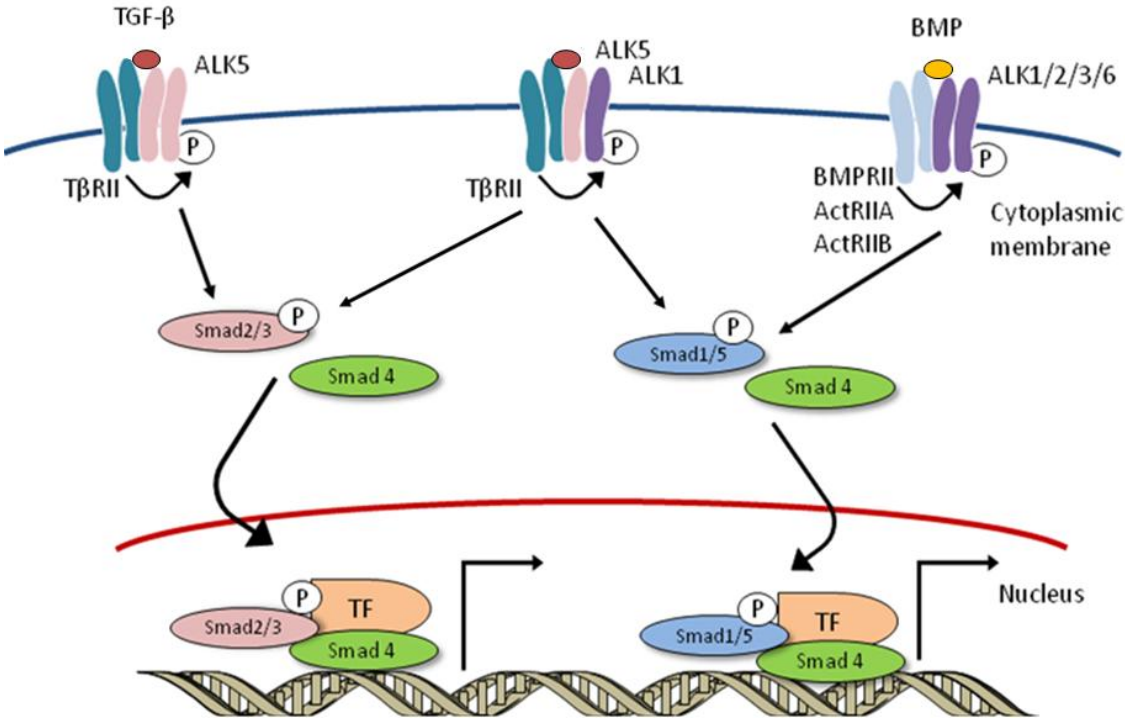

Figure 1 – Signal transduction by TGF-β family members. In most cells TGF-β interacts with TβRII (type II receptor) and ALK5 (type I receptor), but in endothelial cells it can also signal via ALK1 (type I receptor). BMPs signal via BMPRII, ActRIIA and ActRIIB (type II receptors), and type I receptors ALK1/2/3 and 6. ALK5 induces phosphorylation of Smad2 and Smad3, and ALK1/2/3 and 6 mediate phosphorylation of Smad1 and 5. Activated Smads form complexes with Smad4, which accumulate in the

nucleus, where they interact with transcription factors (TF) to regulate the expression of specific target genes (adapted from [18]).

Over 140 mutations in BMPRII have been reported in patients with heritable PAH [19]. The BMPRII protein comprises ligand-binding, kinase and cytoplasmic domains. Mutations have been identified in all of these regions, including partial gene deletions, missense, nonsense and frameshift mutations. The majority of mutations in BMPRII (approximately 70%) are nonsense or frameshift mutations, which are likely to result in the transcript being degraded [17]. Rudarakanchana *et al.* [20] showed that substitution of cysteine residues in the ligand binding or/and kinase domain of BMPRII prevented their trafficking to the cell surface, with retention in the endoplasmic reticulum. They also verified that in BMPRII mutants containing non-cysteine missense mutations in the kinase domain, the BMPRII attained the cell surface, but failed to activate a BMP/Smad response. In addition, missense mutations in the cytoplasmic region of BMPRII, not affect the location of BMPRII in the cell surface and BMPRII retained their ability to activate a BMP/Smad response.

In the adult, BMPRII is expressed predominantly in the pulmonary endothelial cells (ECs), medial smooth muscle cells (SMCs), and macrophages [21]. The mutations in BMPRII that lead to PAH exert their action predominantly by alterations in endothelium and/or smooth muscle [22]. Under normal conditions, BMP ligands bind BMPRII to suppress the growth of vascular SMCs [23, 24]. In addition, binding of BMP2 and BMP7 to BMPRII in pulmonary endothelium leads to protection from apoptosis [25]. It has been reported that inhibition of BMPRII using specific siRNA in human pulmonary artery ECs (PAECs) induced ECs apoptosis through activation of caspase-3 [25].

2.3. Physiological changes

The vascular wall is organized into three layers: an inner layer composed of endothelial cells (intima), a middle layer of smooth muscle cells (media), and an outer layer, mostly composed by fibroblasts (adventitia). In PAH it is verified structural changes in all these layers [26, 27], which include intimal hyperplasia, medial hypertrophy and hyperplasia, adventitia proliferation, formation of a layer of myofibroblasts and extracellular matrix between the endothelium and the internal elastic lamina (termed neointima) and the occurrence of plexiform lesions, which result from

the focal proliferation of endothelial cells and are lined by myofibroblasts, smooth muscle cells and connective tissue (Figure 2) [1, 27, 28]. With time, this vascular remodeling reduces the lumen diameter of pulmonary arteries leading to a rise in pulmonary vascular resistance and hence to an elevated pulmonary artery pressure (PAP). In addition, muscularisation of normally non-muscular arteries occurs [29]. Thrombosis *in situ* (Figure 2) and infiltration of inflammatory cells are also observed during PAH development [1].

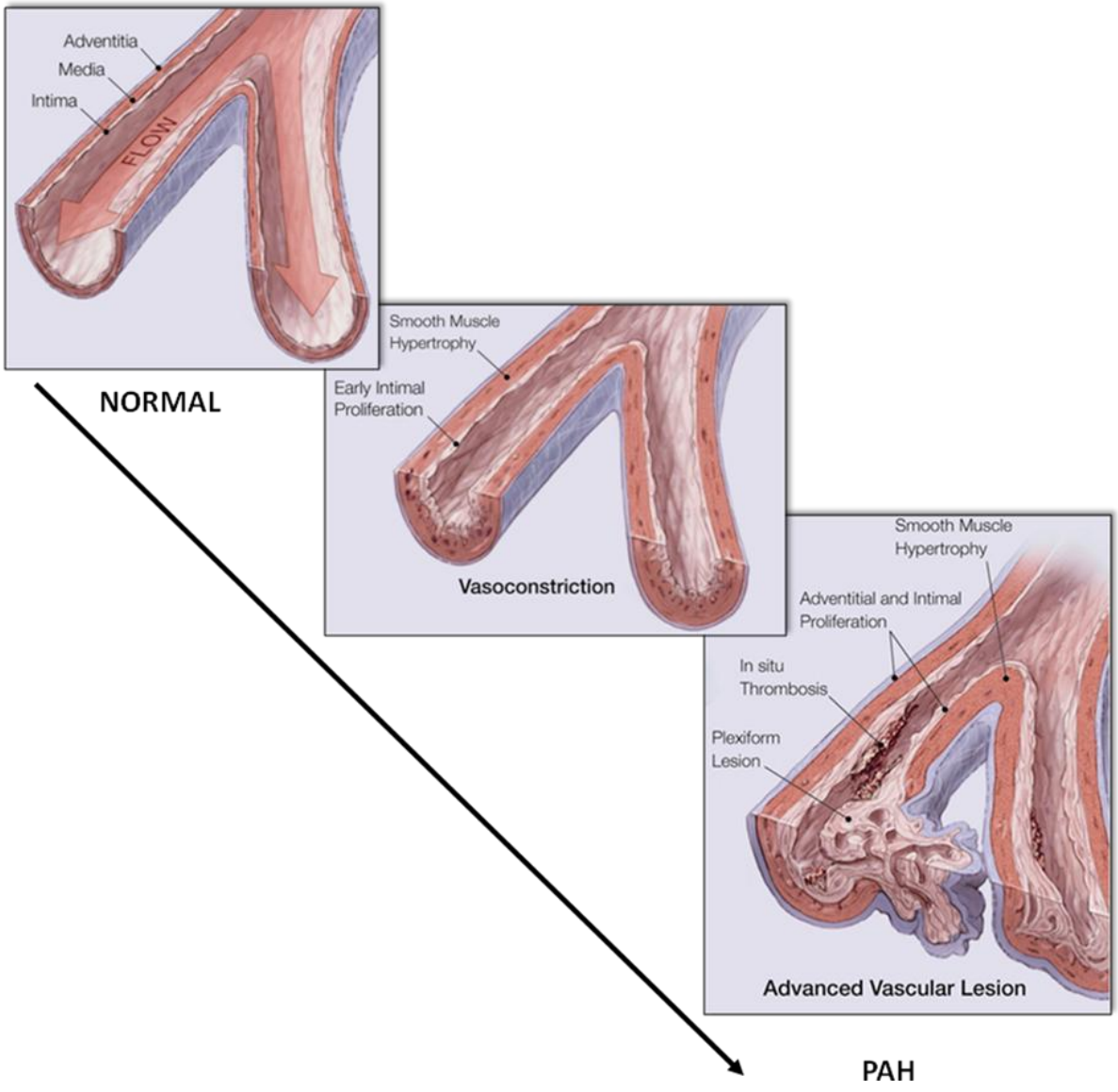

Figure 2 – Structural changes in the pulmonary arterial wall, in pulmonary arterial hypertension (adapted from [30]).

Right ventricular hypertrophy can also be verified during PAH. It develops when the right ventricle works against increased resistance in the pulmonary circulation. Cardiac hypertrophy develops in PAH, at first as a compensatory mechanism [27]. The right ventricle accumulates muscle mass (via elevated protein synthesis and an increase in myocyte size), and the right ventricle wall thickens [3]. In addition, it also assumes a more-rounded shape, compressing the left ventricle [31]. Cardiac output is initially maintained, but with persistent elevated resistance, progressive contractile dysfunction occurs and can lead to right heart failure [3].

2.4. Molecular mechanisms

In PAH it is verified an imbalance in the production of several mediators with important functions in controlling the vasoconstriction, cellular proliferation, apoptosis and platelet aggregation. Table 2 lists the changes reported in some of these mediators in PAH. Some of them will be focused in more detail in the section “Cellular changes”.

Table 2 – Cellular mediators and their changes in pulmonary arterial hypertension.

	Physiological effects				Levels in PAH		
Mediator	**Vascular constriction**	**Cell proliferation**	**Platelet aggregation**	**Apoptosis**	**Serum**	**Lung**	**Reference**
Prostacyclin (PGI_2)	-	-	-			↓	[26, 32]
Thromboxane A_2 (TXA_2)	+	+	+				[33]
Serotonin (5-HT)	+	+	+		↑		[34-37]
Nitric oxide (NO)	-	-	-	+			[38, 39]
Endothelin-1 (ET-1)	+	+			↑	↑	[26, 29, 40]
Vasoactive intestinal peptide	-	-	-	-	↓	↓	[26, 33, 41]
Adrenomedullin	-	-			↑		[33, 42]
Angiotensin-II		+					[43]
Urotensin-II	+	+					[44]
Atrial natriuretic peptide; Brain natriuretic peptide					↑		[45]
Angiopoietin-1				-		↑	[46, 47]
Vascular endothelial growth factor		+		-		↑↓	[48]
Platelet derived growth factor	+	+	-	-		↑	[38, 49, 50]

2.5. Cellular changes

In PAH progression it is verified structural alterations in the three layers of the vascular wall. In addition, functional alterations in the cell types that make part of these layers and also other cell types are verified during PAH development. Understanding the interplay between these cells is important to follow the development of the disease. Some changes that occur in endothelial cells, smooth muscle cells, fibroblasts, inflammatory cells and platelets in PAH are listed below.

2.5.1. Endothelial cells

Endothelial cells are known to be major regulators of vascular function [51]. They are equipped with mechanisms to sense differences in the oxygen (O_2) supply, which include O_2-sensitive NADPH oxidases, endothelial nitric oxide synthases, and heme oxygenases [52]. In addition, the hypoxia-inducible transcription factor (HIF) family is also involved in adaptations to changes in O_2 tension [53]. Although the mechanisms that mediate the damage to the endothelium are largely unknown, it seems that insults such as from chronic hypoxia, inflammation, viral infection, mechanical stretch or shear stress resulting from the blood flow, activates the endothelium [54] and results in accelerated endothelial cell apoptosis. The death of ECs leads to the appearance of apoptotic-resistant and hyper-proliferative ECs, which can lead to vascular remodeling and luminal obstruction [55, 56].

In PAH endothelial dysfunction can be reflected by a reduction in the production of vasodilators like NO and PGI_2, and increased production of vasoconstrictors like ET-1 and TXA_2 [51].

Nitric oxide is produced by a family of enzymes called nitric oxide synthases (NOS). There are three isoforms of NOS: endothelial (eNOS), inducible (iNOS) and neuronal (nNOS). In blood vessels, NO is produced and released by the endothelium. It was shown that eNOS is the primary mediator of the effects of NO in the pulmonary circulation [38]. The eNOS converts the amino acid L-arginine into two products; NO and L-citrulline. Upon its release by endothelium, NO diffuses into vascular SMCs where it stimulates soluble guanylate cyclase (sGC) to produce the second messenger cyclic guanosine monophosphate (cGMP) from guanosine triphosphate (GTP), leading to dilatation of blood vessels via dephosphorylation of myosin light chain. NO is a vasodilator with the ability to inhibit proliferation and platelet aggregation and promote apoptosis of vascular SMCs [38, 39].

Phosphodiesterases (PDE) are enzymes that regulate intracellular signals by degrading second messengers such as cGMP and cyclic adenosine monophosphate (cAMP). PDE type 5 is expressed predominantly in the lungs and pulmonary vasculature [38]. The NO pathway is impaired in several ways in PAH. NOS expression is depressed [57]. Moreover, PDE type 5 is increased in pulmonary artery SMCs (PASMCs), which leads to inactivation of cGMP [58]. The production of endogenous NOS inhibitors, asymmetrical and symmetrical dimethylarginines (ADMA and SDMA, respectively), is enhanced in PH [59]. Finally, sGC expression is upregulated in human PAH, as a compensatory mechanism [60].

Prostacyclin is a product of arachidonate metabolism [61, 62]. Following its synthesis and release, PGI_2 exerts its effects in a paracrine manner, is not stored, and is rapidly converted to an inactive metabolite, 6-ketoprostaglandin F1α [61]. In blood vessels, it is produced by endothelial cells and released to act upon neighboring vascular SMCs as well as circulating platelets, through plasma membrane prostacyclin receptors, which are G protein coupled receptors [61, 62]. PGI_2 binds to its receptor and leads to the activation of adenylyl cyclase, accumulation of the second messenger cAMP, and activation of its main intracellular effector, the cAMP-dependent protein kinase, PKA [62]. PGI_2 is a vasodilator that inhibits the proliferation of vascular SMCs and decrease platelet aggregation [26]. It was shown that PGI_2 synthesis is decreased in endothelial cells from PAH patients. Analysis of urinary metabolites of PGI_2 showed a decrease in the amount of excreted 6-ketoprostaglandin F1α in patients with idiopathic PAH [32]. Moreover, pulmonary ECs of PAH patients are characterized by reduced expression of prostacyclin synthase [63]. Prostacyclin receptor expression is also reduced in pulmonary artery smooth muscle in severe PH [64].

Endothelin-1 is mainly produced by endothelial cells and acts via two receptors: ET_A and ET_B. Both receptors are found in PASMCs and mediate vasoconstriction and proliferation, whereas only the ET_B receptor is present in endothelial cells and mediates NO and PGI_2 release, leading to vasodilatation. Signaling pathways involve a rapid increase in intracellular calcium [26, 29]. Lung and circulating ET-1 levels are increased in PAH patients [40].

Like prostacyclin, thromboxane results from arachidonate metabolism [62, 65]. TXA_2 is synthesized by platelets and endothelial cells and binds to its receptors, which are G protein coupled receptors, widely distributed among different cell types, such as platelets, endothelial cells, smooth muscle cells, cardiac myocytes

and epithelial cells [65, 66]. Thromboxane receptors activation is involved in a variety of processes, including thrombosis, modulation of the immune response, inflammatory lung disease, and hypertension. A multitude of signaling cascades are activated by TXA_2, and involve downstream effectors such as inositol triphosphate/diacylglycerol, cAMP, Ras, Rho, phosphoinositide-3 kinase, PKC and PKA. These different signaling pathways vary in a cell- and organ-specific manner [66]. TXA_2 is a vasoconstrictor that stimulates platelet aggregation and has mitogenic effects in SMCs [33]. It was verified an increase in the urinary excretion of 11-dehydro-thromboxane B_2 (a stable metabolite of thromboxane A_2) [32] and in total body synthesis of TXA_2 in patients with idiopathic PAH [67].

Importantly, an increase in ECs permeability seems to contribute to the pathogenesis of PAH [51]. The GTPases RhoA and Rac1 play opposing roles in the regulation of ECs barrier function. Stimuli such as thrombin activate RhoA/ROCK, which increases permeability. Mediators such as PGI_2 stimulate Rac1/p21-activated kinase, which promotes barrier integrity [51, 68]. It was shown that PAECs cultured from chronically hypoxic piglets demonstrate low Rac1 and high RhoA activities, which correlate with increased permeability [69]. Thus, increasing the permeability of ECs, mediators produced by these cells can be released and act on neighboring structures, including the smooth muscle cells in media layer. It has been shown that an altered production of the endothelial vasoactive mediators NO, PGI_2, ET-1, and TXA_2 can affect the growth of the SMCs, leading to the development of pulmonary vascular hypertrophy and structural remodeling [70]. In addition, ECs can be involved in the thrombosis process that contributes to PAH. They can regulate the thrombotic-antithrombotic balance and participate in the coagulation process by activating factor X, facilitating the formation of the thrombin-activating prothrombinase complex and activating the extrinsic pathway of coagulation via release of tissue factor [71]. On the other hand, ECs can also inhibit thrombosis and promote fibrinolysis. They produce and release NO and PGI_2, two important inhibitors of platelet aggregation, preventing thrombosis. Endothelial cells participate in the fibrinolytic process through the synthesis and release of the profibrinolytic tissue plasminogen activator (t-PA) that activates plasminogen in the fibrinolytic cascade. In addition, endothelial cells also produce the antifibrinolytic/prothrombotic plasminogen activator inhibitor (PAI)-1 [71].

PAH-related alterations in ECs also involve adaptations in mitochondrial metabolism. It was verified a shift in glucose metabolism from oxidative

phosphorylation to glycolysis, leading to the production of less energy [27]. Xu and colleagues verified, through positron tomography scan, a significantly higher uptake of glucose in PAECs from patients with idiopathic PAH compared with ECs from control individuals, confirming the increased glycolytic rate. In addition, they demonstrated that oxygen consumption, the number of mitochondria per cell and the mitochondrial DNA content of idiopathic PAH endothelial cells was lowered in comparison with ECs from control individuals [72]. The enzyme pyruvate dehydrogenase kinase (PDK) seems to be activated in PAH and is responsible for the phosphorylation and inhibition of pyruvate dehydrogenase (PDH), which is responsible for the oxidation of pyruvate, yielding acetyl-coenzyme A. PDK activation thus impairs the Krebs cycle and creates a glycolytic shift in glucose metabolism [1]. Dichloroacetate, which inactivates PDK, had been shown to regress experimental PAH, both in hypoxic and monocrotaline models [73, 74].

Alterations in the intracellular trafficking, which is mediated by a group of proteins, including membrane tethers, SNAREs (SNAP receptors) and SNAPs (soluble NSF association proteins), and the ATPase N-ethylmaleimide-sensitive factor (NSF) were also reported in PAH [75, 76]. It was verified that monocrotaline pyrrole (the bioactive form of monocrotaline)-treated PAECs in culture showed enlarged endoplasmic reticulum and Golgi stacks by electron microscopy within 48-96 h after the treatment [77]. In addition, it was shown the loss of the cell surface raft/caveolar protein caveolin-1 (cav-1) in PAECs in rat lungs within 48 h after the treatment with monocrotaline [78]. It has been showed that monocrotaline and hypoxia disrupt the molecular machinery of vesicular trafficking at the level of Golgi tethers, SNARES, and SNAPS (“Golgi blockade”) [79]. Consequences of this disruption include the trapping of cav-1 in the Golgi of PAECs, the loss of eNOS from plasma membrane caveolae and its sequestration in intracytoplasmic compartments, and loss of NO production [80]. Murata and colleagues [81] showed that eNOS was co-trapped in the Golgi with cav-1 in endothelial cells exposed to hypoxia.

2.5.2. Smooth muscle cells

Smooth muscle cells are stretch sensors, once cyclic stretch regulates the functions of vascular SMCs activating intracellular signaling networks, which regulate gene expression and thus cellular functions [82].

It was observed SMCs with different phenotypes. These phenotypes are characterized by changes in morphology, proliferation and migration rates, and in the expression of different marker proteins. The resulting phenotype diversity of SMCs, ranging from the contractile to the synthetic, appears to be related to genetic and environmental factors, such as extracellular matrix components and physical factors (stretch and shear stress) [83]. The different phenotypes observed between SMCs are seen in SMCs of different vessels as well as amongst SMCs within the same vessel [83]. Contractile SMCs are elongated, spindle-shaped cells, whereas synthetic SMCs are less elongated and present cobblestone morphology, also called epithelioid or rhomboid. Synthetic SMCs contain a high number of organelles involved in protein synthesis, whereas there are largely replaced by contractile filaments in contractile SMCs. In addition, synthetic SMCs exhibit higher growth rates and higher migratory activity than contractile SMCs [83].

There are some difficulties in identify SMCs phenotypes, because the markers used are also expressed in a variety of other cell types either under normal or pathologic conditions [84]. Currently, the two marker proteins that provide the best definition of a mature contractile SMC phenotype are smooth muscle-myosin heavy chain (SM-MHC) and smoothelin. SM-MHC expression has never been detected in non-SMCs *in vivo*. Smoothelin complements SM-MHC as a contractile SMC marker in that it appears to be more sensitive [83, 84]. Other markers expressed in a contractile SMC include α-smooth muscle actin (α-SMA), desmin, smooth muscle-calponin and h-caldesmon [83, 85]. In vascular repair during injury and/or vascular adaptation, it is verified that vascular SMCs "switch" to a non-contractile, proliferative and migratory phenotype. This phenotypic switch is under tight control and is often reversible, but under certain circumstances can contribute to vascular disease states, like hypertension [86].

Ions, including K^+, Ca^{2+}, Na^+ and Cl^- and their channels have been reported to have an important role in both apoptosis and cell proliferation of SMCs. An imbalance that favors proliferation instead apoptosis, leading to an increase in cell population is reported in several diseases, including PAH. Regarding the K^+ channels, the four main functional classes are: (i) voltage-gated (K_V) K^+ channels; (ii) Ca^{2+}-activated (K_{Ca}) K^+ channels; (iii) inwardly rectifying (K_{IR}) K^+ channels, of which ATP-sensitive (K_{ATP}) K^+ channels are a member and (iv) two-pore domain (K_{2P}) K^+ channels [87]. When K^+ channels are blocked (or K^+ channels gene expression is downregulated),

the membrane depolarizes and opens voltage-dependent Ca^{2+} channels (VDCC), promoting a Ca^{2+} influx, increasing cytosolic calcium concentration ($[Ca^{2+}]_{cyt}$) and causing PASMCs contraction. Conversely, when K^+ channels are activated (or K^+ channels gene expression is upregulated), the membrane hyperpolarizes and closes VDCC, inhibiting Ca^{2+} influx, decreasing $[Ca^{2+}]_{cyt}$ and causing vasodilatation [87]. Intracellular Ca^{2+} is also an important second messenger for cell migration and proliferation. Ca^{2+} influx through VDCC activates transcription factors, such as CREB, NF-AT and NF-kB that are involved in cell proliferation, protein synthesis and inflammation and Ca^{2+} is also required for cell-cycle progression. In apoptosis, K^+ channels mediate the K^+ efflux that is necessary for apoptotic volume decrease (AVD). K^+ efflux also leads to the decrease of $[K^+]_{cyt}$, releasing the inhibition of caspases [87]. It was reported a defect in the gene expression and attenuated function of K_V channels in PASMCs from patients with PH. Thus, a decrease in K^+ channels gene expression and/or function stimulates PASMCs proliferation by increasing $[Ca^{2+}]_{cyt}$ and inhibits PASMCs apoptosis by decelerating apoptotic volume decrease and attenuating cytoplasmic caspase activity. These alterations contribute to the vascular remodeling observed in PAH [88]. Tang *et al.* [89] reported the involvement of TASK-1 (two pore-related acid-sensitive potassium channel-1) in the ET-1-mediated depolarization in human PASMCs. They concluded that ET-1 depolarizes primary human PASMCs by phosphorylating (inhibiting) TASK-1. In addition, they presented a mechanism in which ET-1 binds to the G protein-coupled receptor ET_A, leading to the PKC-induced phosphorylation of TASK-1 channels through phospholipase C (PLC), phosphatidylinositol 4, 5-biphosphate (PIP_2) and diacylglycerol.

2.5.3. Fibroblasts

The adventitial compartment, mostly composed by fibroblasts may be considered a principal injury-sensing tissue of the vessel wall. In response to environmental stresses, such as hypoxia, vascular distention or vascular injury, resident fibroblasts are activated and undergo a variety of functional changes [84]. It has been reported that fibroblasts are able to (i) proliferate in greater propensity than SMCs in response to injury, (ii) differentiate in smooth muscle-like cells (i.e. myofibroblasts), which can accumulate in the adventitia and/or migrate to the medial and intimal layers of the vessel wall, (iii) increase and alter the profile of extracellular matrix (ECM)

deposition, (iv) synthesize and release molecules that act on neighbouring SMCs and ECs, and (v) express molecules/receptors that facilitate the recruitment of circulating leukocytes to the adventitial compartment [90]. The identification of fibroblasts is a difficult task, because markers frequently used, such as vimentin and α-smooth muscle actin, lack specificity and sensitivity. Vimentin does not distinguish fibroblasts from other cells of mesenchymal origin. α-smooth muscle actin has been used to identify myofibroblasts and is not generally observed in resting fibroblasts; in addition, it is observed in SMCs and in circulating smooth muscle precursors [90]. Activation of fibroblasts results in their differentiation into myofibroblasts. It was observed the appearance of myofibroblasts expressing α-smooth muscle actin in the adventitia in hypoxia-induced pulmonary hypertension [90]. Myofibroblasts are the principal producers of collagen and other extracellular matrix proteins, including fibronectin, tenascin-C and elastin. They appear to be capable of migration from the adventitia to the media or even to the intima and thus contribute to the thickening of these components [90]. Accumulation of myofibroblats in the intima of patients with PH has been observed [91]. In addition, it was shown an excessive deposition of ECM proteins in the adventitia in PAH. Marked increases in the production and accumulation of collagen and elastin were observed in the adventitia during the development of PH. Also, increases in the accumulation of fibronectin, tenascin-C and elastin in the adventitia of models of hypoxia-induced pulmonary hypertension were observed [90, 92]. Both tenascin-C and fibronectin are associated with fibroblast and SMC proliferation and have also been shown to contribute to differentiation in a myofibroblast. Fibronectin and tenascin-C deposition coincides with the expression and activity of matrix metalloproteinases (MMPs), a family of zinc enzymes produced by fibroblasts and macrophages responsible for degradation of the ECM components, including collagen, fibronectin, and various proteoglycans. These enzymes are also important to smooth muscle and endothelial cells migration and proliferation [90]. Excessive or inappropriate expression of MMPs may contribute to the pathogenesis of PAH, because MMPs may be necessary for the fibroblast to move through the adventitial matrix into the media and even intima. MMP activity is upregulated in adventitial fibroblasts from animals with hypoxia and monocrotaline-induced pulmonary hypertension [90, 93]. It has been reported that inhibition of MMP activity attenuates monocrotaline-induced pulmonary hypertension [94].

2.5.4. Inflammatory cells

There are several observations that argue for the role of inflammation in the pathogenesis of PAH. These include the association of PAH with several collagen vascular autoimmune disorders (e.g., scleroderma, systemic lupus erythematosus and connective disorders) and schistosomiasis; the presence of T cells, B cells and macrophages in plexiform lesions; the detection of auto-antibodies to endothelial cells and fibroblasts; raised blood cytokine and chemokine levels and the association of PAH with certain infections such as human herpes virus 8, human immunodeficiency virus and hepatitis C virus [1, 54, 95]. Studies by Austin *et al.* [96] showed a preponderance of CD3+ and CD8+ T-lymphocytes in lungs of PAH patients. Regarding B-cells, it was reported the detection of antibodies produced by B-cells against endothelial cells and fibroblasts in patients with PAH; in addition, it was also verified the activation of B-cells in peripheral blood in patients with idiopathic PAH [97-99].

Regarding the contribution of cytokines and chemokines to the development of PAH, patients with idiopathic or associated PAH exhibit higher circulating levels and pulmonary expression of interleukin (IL)-1β, IL-6 and TNF-α (tumor necrosis factor-α) than healthy individuals [54, 100, 101]. Transgenic mice overexpressing IL-6 have been shown to develop spontaneous pulmonary vascular remodeling and PH [102]. Also, transgenic mice overexpressing TNF-α develop severe PH and right ventricular hypertrophy [103].

The chemokine fractalkine (FKN/CX3CL1) and its receptor (CX3CR1), which is expressed by monocytes, mast cells, T cells, natural killer cells and other cell types, have been suggested to play important roles in monocyte/T-cell recruitment to the vessel wall [54, 104]. Balabanian *et al.* [105] have shown that CX3CR1 was upregulated in circulating T-cells from PAH patients compared to controls, elevated FKN plasma concentrations were found in PAH patients compared with controls, and an increase in FKN mRNA expression was detected in lung tissue of PAH patients compared to controls. They also showed that PAECs from PAH patients expressed FKN. In addition, Perros *et al.* [106] demonstrated that FKN is expressed by inflammatory cells surrounding pulmonary arterial lesions and that SMCs from these vessels have increased CX3CR1 expression. They also verified that cultured rat PASMCs express CX3CR1 and that FKN induces proliferation but not migration of these cells.

RANTES (Regulated upon Activation, Normal T-cell Expressed and Secreted), also known as CCL5 is another chemoattractant for monocytes and T-cells that has been showed to cause an induction of endothelin-converting enzyme-1 and ET-1 [104]. It has been showed an increased expression of RANTES mRNA in lung samples from PAH patients compared with control patients [107]. The authors of this study also verified that the major source of RANTES in PAH patients was endothelial cells from lung tissue.

Monocyte chemotactic protein (MCP-1), also known as CCL2 is produced by pulmonary vascular cells (SMCs and ECs) and can stimulate monocyte/macrophage migration and smooth muscle proliferation [54]. Sanchez *et al.* [108] showed that the levels of CCL2 was elevated in plasma and lung tissue of PAH patients, verifying an elevated CCL2 release from pulmonary ECs or SMCs. They also verified an enhanced monocyte migration in the presence of pulmonary endothelial cells from patients with idiopathic PAH. In addition, compared with controls, PASMCs from PAH patients demonstrated stronger proliferative responses to CCL2, in keeping with the finding that CCR2 (CCL2 receptor) was markedly increased in PASMCs from these patients. Antibodies directed against CCL2 have been shown to attenuate the development of PH, reducing infiltration of monocytes into the lung.

Stromal cell-derived factor-1α (SDF-1α), also known as CXCL12 is a small cytokine belonging to the chemokine family. SDF-1α activates leukocytes, and is often induced by proinflammatory stimuli such as lipopolysaccharides, TNF-α and IL-1 [54]. Young *et al.* [109] demonstrated that inhibition of the SDF-1α – CXCR4 (SDF-1α receptor) axis decreased pulmonary vascular cell proliferation and apoptosis and prevented the development of hypoxia-induced pulmonary vascular remodeling in neonatal mice.

It was reported that mast cells are a rich source of IL-4, as well as other factors that can stimulate B-cells to secrete auto-antibodies, including anti-endothelial cell antibodies; in addition, mast cells degranulation (activation) had been showed to be involved in the development of pulmonary vascular remodeling in chronic hypoxic rats [54, 110]. Dahal *et al.* [111] demonstrated that the accumulation and activation of mast cells in the lungs contribute to the development of PH in monocrotaline-rats and they observed that mast cells population was about 8 fold higher in idiopathic PAH patients compared with controls, with the majority of mast cells degranulated.

2.5.5. Platelets

It has been showed that multiple procoagulant aberrations are present in PAH patients, related to endothelial dysfunction, abnormalities of the coagulation cascade and disordered platelet function. These include increased levels of von Willebrand factor, plasma fibrinopeptide A, PAI-1, 5-HT, and TXA_2 and decreased levels of t-PA, thrombomodulin, NO, and PGI_2 [112]. Platelets not only participate in clot formation, but also are capable of releasing many vasoactive substances that promote vasoconstriction, as well as growth factors that stimulate proliferation of smooth muscle cells, endothelial cells, and fibroblasts [71]. One of the most important mediators released by platelets is serotonin.

Serotonin is produced by the gastrointestinal tract enterochromaffin cells and pulmonary neuroepithelial bodies and stored in platelets (the major repository of 5-HT) [26]. Circulating 5-HT levels are normally low, since free 5-HT is taken up for storage in platelets via the 5-HT transporter (5-HTT or SERT), or is metabolized in the liver [29]. 5-HT is also taken up by the 5-HTT in neurons, pulmonary artery smooth muscle and endothelial cells. Pulmonary artery endothelial and smooth muscle cells also express $5\text{-}HT_{1B}$, $5\text{-}HT_{2A}$ and $5\text{-}HT_{2B}$ receptors, which are G protein coupled receptors [34]. Serotonin is a vasoconstrictor that stimulates the proliferation of SMCs and fibroblasts [34, 35]. A significant rise of 5-HT in plasma is seen in pulmonary hypertensive patients and the level in platelets is low [36]. Activation of serotonin receptors $5\text{-}HT_{1B}$, $5\text{-}HT_{2A}$, and $5\text{-}HT_{2B}$ causes vasoconstriction, although $5\text{-}HT_{2B}$ is also implicated in the mitogenic action of 5-HT. The signaling pathways include mediators such as adenylate cyclase and PKA [34]. Mice deficient in $5\text{-}HT_{1B}$ receptors show reduced pulmonary vascular remodeling and absence of right ventricular hypertrophy on exposure to hypoxia [113]. Specific $5\text{-}HT_{2A}$ receptor blockade inhibits the development of PH in rats injected with monocrotaline and improves survival [114]. Human studies suggest that 5-HT mediates vasoconstrictive effects mainly by the $5\text{-}HT_{1B}$ receptor, which explains why a $5\text{-}HT_{2A}$ receptor antagonist has had only limited effect in the treatment of PAH [115, 116]. 5-HTT is encoded by a single gene on chromosome 17q11.2, and a variant in the upstream promoter region of this gene has been described. There are two polymorphic forms: the long (L) and the short (S). These polymorphisms affect 5-HTT expression and function with the L-allele inducing a greater rate of 5-HTT gene transcription than the S-allele. Isolated PASMCs expressing the LL genotype took up more 5-HT and had increased growth versus cells that were heterozygous (LS) or

homozygous for the short variant. The L-allelic variant was found to be present in homozygous forms in 65% of idiopathic PAH patients, but only in 27% of controls [117]. In experimental models, there is attenuated severity of pulmonary vascular disease in mice lacking the gene encoding the 5-HTT [118]. In contrast, overexpression of the serotonin transporter in a transgenic mouse worsens hypoxia-induced PAH [119].

2.6. Experimental models

Animal models have allowed the study of the pathogenesis of PAH, as well as the effects of drug intervention. An animal model should mimic the human disease and allows determination of relevant clinical, biochemical, hemodynamic and histopathological features. However, no model mimics exactly all the features of the human disease [120, 121] and thus, caution must be taken when extrapolating data obtained from animal models to human pathophysiology. As human PAH has many genetic and environmental causes, it may be appropriate to study different animal models to better understand the disease [120]. The most commonly used animal models of PAH are the monocrotaline model and the chronic hypoxic model [122]. Chronic hypoxia can be induced by exposing animals to normal air at hypobaric pressures or to oxygen-poor air at normal pressure [123]. This decrease in oxygen pressure elicits a strong pulmonary vasoconstrictor response [124]. Recently, Taraseviciene-Stewart and colleagues [125] described a rat animal model of PAH that involves a combination of vascular endothelial growth factor receptor blockade with SU5416 and chronic hypoxia exposure, demonstrating a severe pulmonary hypertension associated with precapillary arterial occlusion by proliferating endothelial cells. In addition, new animal models of PAH involve the use of genetically modified animals, since various mutations have been shown to be implicated in the development of PAH [124]. An example is the use of genetically engineered mouse overexpressing 5-HTT [119]. It follows a more detailed description of the features of the monocrotaline model, an animal model that has been used for over 40 years [126, 127].

2.6.1. Monocrotaline model

Monocrotaline (MCT) is a pyrrolizidine alkaloid present in the stems, leaves, and seeds of the *Crotalaria spectabilis* and in all the other plants of the *Crotalaria* genus, but in a lesser concentration. The toxicity of MCT is essentially

hepatic and cardiopulmonary, affecting both animals and humans. However, if applied topically or injected it does not cause localized toxicity. MCT will cause lesions in several organs after absorption and hepatic bioactivation [128]. Within the liver, MCT may undergo several chemical reactions, leading to toxic and non toxic products. Monocrotaline pyrrole (MCTP), also called dehydromonocrotaline, is a toxic metabolite of MCT formed by the cytochrome P450 3A in the liver [129].

Clinical signs of illness are usually not evident immediately after a single exposure of rats to doses of MCT that result in pulmonary hypertension. Within 3-7 days, rats show anorexia, listlessness, failure to gain weight and tachypnea. As lung injury and vascular remodeling progress, animals develop variable degrees of dyspnea, weakness, diarrhea, and peripheral cyanosis [127].

The pulmonary vascular endothelium is thought to be the early target of MCT intoxication based on circulatory proximity to the liver [130]. It was verified that hypertrophy and increased DNA synthesis in endothelial cells occur within 7-14 days after MCT (P) treatment [131, 132]. The term “megalocytosis”, which refers to enlarged cells with enlarged nuclei, reflects this phenotypic change caused by pyrrolizidine alkaloids [133]. In addition, intoxication with monocrotaline is associated with increased intimal expression of endothelin-1 [134], while the expressions of the ET_B and eNOS are decreased [135, 136]. Changes in the medial layer due to *C. spectabilis* or MCT (P) administration occur after intimal changes and are characterized by increased DNA synthesis, hypertrophy and hyperplasia of smooth muscle and extension of smooth muscle to normally non muscular pulmonary arteries [128, 137]. The medial expressions of the serotonin transporter and survivin, an inhibitor of apoptosis, are increased [4, 138], while there is a decreased expression of voltage-gated potassium channels, including Kv1.5 and Kv2.1 [73]. Also, the expression of BMPRII is decreased, but its restoration does not improve pulmonary hypertension [139]. Lesions of the adventitia caused by administration of MCT (P) or *C. spectabilis* to rats are initially characterized by the infiltration of inflammatory cells [128]. Lungs from monocrotaline-intoxicated rats present increased expression of several pro-inflammatory cytokines such as IL-1β, IL-6, MCP-1, ICAM-1 (intracellular adhesion molecule-1), E-selectin, and TGF-β. In addition, there is an adventitial increased production of extracellular matrix glycoproteins, like elastin, fibronectin, collagen, and tenascin-C [136, 140].

Rats chronically exposed to MCT in drinking water or food develop right cardioventricular hypertrophy [141]. Kay *et al.* [126] also demonstrated that the oral administration of *Crotalaria spectabilis* seeds to young rats induces pulmonary hypertension associated with right ventricular hypertrophy. In addition, administration of MCT 60 mg/ kg sc (subcutaneous) to rats results in right ventricle hypertrophy [142]. The macroscopic evidence of right heart enlargement is accompanied by an increased rate of ventricular protein synthesis [141]. It was observed marked increases in both cross-sectional area and cell length in myocytes from the right ventricle of rats treated with MCT. There is general agreement that the cardiac lesions occur as a physiologic response to an increased workload that results from a sustained elevation in pulmonary arterial pressure. Hypertrophy has not been reported in the left ventricle [128, 142]. In monocrotaline-induced pulmonary hypertension it was also shown a twofold increase in the Fulton index (ratio of right ventricular free wall weight divided by the sum of the septum plus left ventricular free wall weight). Regarding the hepatic lesions, animals intoxicated with monocrotaline present with hepatic necrotic lesions and cirrhosis [136].

Monocrotaline-induced pulmonary hypertension is similar to PAH in terms of hemodynamic and histopathological severity and high mortality. However, it differs from PAH by an initial permeability lung edema, with early loss of the endothelial barrier and striking inflammatory adventitial proliferation [136]. In addition, in PAH it is verified plexiform lesions, which are not observed in monocrotaline-induced pulmonary hypertension [122]. However, it has been reported the development of plexiform lesions in a rat model (rats with approximately 6 week old) that combined treatment with MCT and partial pneumonectomy [143]. A limitation of MCT model is that differences exist in monocrotaline sensitivity between rat strains as well as individual variances in the pharmacokinetics of monocrotaline involving degradation and hepatic formation of the pyrrole or conjugation and excretion [124]. However, the monocrotaline rat model continues to be a frequently used model of PAH as it offers technical simplicity, reproducibility and low cost compared to other models of PAH [144].

2.7. Diagnosis and prognosis

In the presence of a suspected PAH patient, multiple exams are required to confirm the diagnosis, clarify the specific etiology within the PAH group and also

evaluate the functional and hemodynamic impairment. Importantly, the diagnosis of PAH, and particularly of idiopathic PAH is a diagnosis of exclusion [145].

Regarding the clinical presentation, the symptoms of PAH are non-specific and include breathlessness, fatigue, weakness, angina, syncope, and abdominal distension. Symptoms at rest are reported only in advanced cases. The signs of PAH include RV third sound, tricuspid insufficiency murmur, prominent right ventricular impulse and accentuated pulmonic valve component. Jugular vein distention, hepatomegaly, peripheral edema, ascites and cool extremities can be present in a more advanced state of the disease [145, 146].

The electrocardiogram (ECG) may provide evidence of PH by demonstrating RV hypertrophy, and right atrial dilatation. RV hypertrophy on ECG is present in 87% and right axis dilatation in 79% of patients with idiopathic PAH. The absence of these findings does not exclude the presence of PH nor does it exclude severe haemodynamic abnormalities [145]. In 90% of patients with idiopathic PAH the chest radiography is abnormal at the time of diagnosis. Findings include central pulmonary arterial dilatation, which contrasts with loss of peripheral blood vessels. Right atrium and RV enlargement may be seen in more advanced cases [145]. Pulmonary function and arterial blood gases tests are also realized. Arterial blood gas analysis may help to exclude hypoxia as a contributor to pulmonary hypertension [146]. Pulmonary function tests are necessary to establish airflow obstruction or restrictive pulmonary pathology [146]. Transthoracic echocardiography provides several variables which correlate with right heart haemodynamics including PAP, and should always be performed in the case of suspected PH [145]. High-resolution computed tomography provides detailed views of the lung parenchyma and facilitates the diagnosis of interstitial lung disease and emphysema [145]. Cardiac magnetic resonance imaging provides a direct evaluation of RV size, morphology, and function and allows non-invasive assessment of blood flow including stroke volume, cardiac output, distensibility of pulmonary artery and RV mass [145]. Abdominal ultrasound should be used to exclude liver cirrhosis and/or portal hypertension [145].

In all the patients should be performed routine biochemistry, haematology and thyroid function tests. Serological testing is important to detect connective tissue diseases, HIV and hepatitis [145, 146]. Up to 2% of individuals with liver disease will manifest PAH and therefore liver function tests and hepatitis serology should be examined. Thyroid disease is commonly seen in PAH and should always be considered

[145]. A range of biomarkers have been described in PAH, which may be of diagnostic and prognostic significance. These include markers of heart failure, endothelial and/or platelet dysfunction and cardiac myocyte damage [147]. Examples are atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP). More recently, interest has turned to the N-terminal fragment of BNP (NT-proBNP) as an alternative biomarker to BNP, because it seems to provide the same information, but has advantages in terms of stability. Another biomarkers used are ET-1, acid uric, troponin T, NO, cGMP, D-dimer, von Willebrand factor and 5-HT [147].

Cardiac catheterization should be performed in patients with unexplained pulmonary hypertension, and remains the gold standard for PH diagnosis and quantification [146]. The following variables must be recorded during right heart catheterization (RHC): PAP (systolic, diastolic, and mean), right atrial pressure, PWP (pulmonary wedge pressure), and RV pressure. In PAH, vasoreactivity testing should be performed at the time of diagnostic RHC to identify patients who may benefit from long-term therapy with calcium channel blockers (CCBs). This test should only be performed with short-acting, safe, and easy to administer drugs with no or limited systemic effects. Currently the agent most used in acute vasodilator testing is NO; intravenous epoprostenol or intravenous adenosine may also be used as an alternative (but with a risk of systemic vasodilator effects). Only the patients that respond positively to these testing drugs can safely be treated with CCBs [145].

Assessment of functional exercise is needed to evaluate disease severity. Two commonly used tests are the 6 minute walk test (6MWT) and the cardiopulmonary exercise testing [148].

Thus, the knowledge of the clinical history of the patient and a careful physical examination are paramount to the diagnosis of pulmonary hypertension. Particular attention should be given to previous medical conditions, drug use and family history. In addition, all systems should be carefully reviewed [146]. Importantly, survival after diagnosis is estimated as low as 2.8 years, if left untreated and death occurs, in most cases, from right ventricular failure [7, 146].

2.8. Treatment

The knowledge of the etiology of the disease provides the basis for the pharmacological approach. However, since PAH appears to have a multi-factorial

nature, the treatment has remained primarily palliative till recently. With the advances in the knowledge of the pathogenesis of PAH over the last years, the treatment of this disease has improved significantly [148], emerging treatments that target the pathways underlying the development of PAH. However, these treatments may slow the progression of the disease but not afford a cure [149]. In table 3, it follows a description of some current therapies in PAH grouped according to the target pathway.

Table 3 – Current therapies for pulmonary arterial hypertension.

Drug	Route	Adverse effects and contraindications	Comments and recommendations	References
Supportive therapies				
Anticoagulants	O	Bleeding		[150, 151]
Diuretics	O	Hypotension	Used to reduce fluid retention	[152]
Oxygen	In		Useful in patients with hypoxia	[152, 153]
Digoxin	O	Arrhythmias, vomiting	Modest benefit on right ventricular function	[152, 154]
Inotropes	IV	Tachycardia	Mostly used in patients with end-stage right heart failure	[152]
Calcium-channel blockers				
Nifedipine	O	Hypotension, peripheral edema	Favorable as long-term treatment in patients with acute vasodilator responsiveness	[152, 155, 156]
Diltiazem	O			
Amlodipine	O			
Synthetic prostacyclin and prostacyclin analogues				
Epoprostenol	cIV	Jaw pain, headache, flushing, nausea, diarrhea, catheter-related sepsis	Beneficial effects can be sustained for years	[157-159]
Treprostinil	SC	Rash, infusion site pain, headache, diarrhea, nausea		[160, 161]
Beraprost	O	Headache, flushing	Only modest benefit in the short term	[162, 163]
Iloprost	In	Cough, flushing, headache, jaw pain,		[164]
Endothelin-1 receptor antagonists				
Bosentan	O	Hepatotoxicity, headache	Dual ET_A/ET_B antagonist, increasingly used as a first-line drug	[165-167]
Sitaxsentan	O	Hepatotoxicity, warfarin interaction	$ET_A >> ET_B$ antagonist	[168, 169]
Ambrisentan	O	Flushing, hepatotoxicity	$ET_A > ET_B$ antagonist	[170, 171]
Type 5 phosphodiesterase inhibitors				
Sildenafil	O	Headache, nasal congestion, visual disturbances	Increasingly used as a initial agent as well as in combination therapy	[172-175]
Tadalafil	O	Headache, nausea, dyspnea		[176]
Interventional procedures				
Atrial septostomy			Decompresses right heart and improves systemic output	[177]
Lung transplantation			Improves survival	

Legend: cIV, continuous intravenous; In, inhaled; IV, intravenous; O, oral; SC, subcutaneous.

Current treatments include supportive therapies, calcium channel blockers, synthetic prostacyclin and prostacyclin analogues, endothelin-1 receptor antagonists, type 5 phosphodiesterase inhibitors and interventional procedures. Supportive therapies

are adapted from other diseases with similar symptoms of PAH and have the aim of relief the symptoms. As discussed in the section 2.7., patients who may benefit from long-term therapy with CCBs are identified by their initial response to a vasodilator challenge. The patient could benefit from CCBs therapy if their response is positive. A response is considered positive if there is a decrease of at least 10 mmHg in the mean PAP, to a value of less than 40 mmHg with an increased or unchanged cardiac output. Importantly, only approximately 10-15% of patients with idiopathic PAH will meet the criteria for a positive response [145, 178]. Prostacyclin analogues, ET-1 receptor antagonists and type 5 phosphodiesterase inhibitors act primarily with the aim of improve vasodilatation.

Regarding surgical procedures, lung and heart/lung transplantation as well as atrial septostomy have been available for years to treat severe PAH. However, with the development of pharmacologic therapy, the need for these interventions has diminished, remaining the treatment of last resort, being used in patients who fail to respond favorably to pharmacologic therapy [179].

3. Survivin: a new target for PAH treatment

The therapies mentioned in the previous section, which focus in the promotion of vasodilatation, are not sufficient to offer a cure to PAH. Thus, more research is needed to find more therapeutic targets aiming effective treatments. There is an increasing interest in the last years to target the proliferative and apoptotic pathways, instead of vasoconstriction [180-182]. In this context, survivin, a molecule that performs functions both in cellular proliferation and apoptosis is a promising target [5, 183].

Survivin, a protein discovered in 1997 in human B-cell lymphoma, is the smallest element of the inhibitor of apoptosis (IAP) family, with 142 amino acids and 16.5 kDa [5, 183, 184]. The IAP proteins identified in humans include X-chromosome-linked IAP (XIAP), cellular inhibitor of apoptosis 1 and 2 (cIAP1, cIAP2), neuronal apoptosis inhibitor protein (NAIP), IAP-like protein 2, livin, BRUCE and survivin [183]. All IAP family members share two to three common structures of baculovirus IAP repeat (BIR) domains, containing 70-80 amino acids and localized at the N-terminus region, responsible for bind and inactivate caspases. However, survivin only contains a single BIR domain that stretches from amino acid residue 15 to 87. Most of IAPs, excluding survivin, possess other domains such as a carboxyl-terminal RING (really interesting new gene) domain, important for ubiquitination and proteossomal degradation of

caspases [183, 185, 186]. Alternative splicing of survivin pre-mRNA from chromosome 17q25 produces five different mRNAs, which encode five distinct proteins, survivin, survivin 2B, survivin ΔEx3, survivin 3B and survivin 2α. These survivin isoforms differ both in intracellular localization and in their ability to inhibit apoptosis [5, 183, 187].

Survivin plays a critical role in the regulation of cell division and viability. It has been reported that disruption of survivin locus in mice results in early embryonic lethality and loss of tissue viability [183, 188, 189]. Survivin shows a cell-cycle dependent expression at mitosis. There is an upregulation of survivin during the G2/M phase of the cell cycle. During mitosis, survivin exists as a multi-protein complex, known as the chromosomal passenger complex (CPC), and localizes to different components of the mitotic apparatus, including centromeres in prophase, kinetochores of the metaphase and anaphase spindle, and the midbody during cytokinesis [5, 183, 187, 188]. For these survivin functions on mitosis, it is crucial that a phosphorylation by cdc2, a mitotic kinase, on threonine 34 of survivin occurs. It has been reported that inhibition of survivin expression/function results in multiple mitotic defects, including cell cycle arrest at prometaphase, appearance of aberrant spindles, cytokinesis failure, and generation of multinucleated polyploidy cells. This reflects the important roles of survivin in multiple phases of mitosis [188].

Regarding the role of survivin in apoptosis, it was reported that in response to cell death stimulation, mitochondrial survivin is released to cytosol, where it prevents caspase activation and inhibits apoptosis [5, 183, 188, 190]. Transgenic animals expressing survivin are protected against apoptosis, *in vivo*, and molecular antagonists of survivin, such as, ribozymes, RNA interference, and dominant-negative mutants cause caspase-dependent cell death and enhance apoptosis [188]. The BIR2 domain of IAPs is capable of bind and inhibits caspases-3 and -7, whereas BIR3 is an inhibitory segment for caspase-9. BIR1 is not known to possess the inhibitory ability of caspase activation. The only BIR domain of survivin appears closely related in the structure to the BIR3 domain of XIAP, suggesting that survivin binds and inhibits caspase-9 [191]. Although some authors reported that survivin can directly bind and inhibit caspase -3, -7 and -9 activity, Song *et al.* [192] showed that survivin does not interact physically with caspases, but inhibits apoptosis through the interaction with Smac, in apoptosis induced by Taxol (a potent anti-tumor drug). Smac promotes caspase activation by binding and inhibiting IAPs activity. They proposed that during apoptosis survivin binds to Smac released from the mitochondria, thus reducing antagonism of Smac to

XIAP, and the free XIAP directly interacts with caspases, blocking cell death. This mechanism suggests that the anti-apoptotic effect of survivin may be mitochondria-dependent, because Smac is released from mitochondria. Survivin can also have other anti-apoptotic mechanisms. It has been reported that in response to cell death stimulation, survivin interacts with XIAP, and this complex increases XIAP stability against polyubiquitination and proteasomal degradation, enhancing its anti-caspase activity [193].

Regarding the use of survivin as a therapeutic target in PAH, there is not much information. McMurtry *et al.* [4], in addition to show that this pathway is up-regulated in patients with PAH and in rats injected with MCT, comparing with controls, they showed that inhaled adenoviral gene therapy with a survivin mutant (Thr34Ala) reverses MCT-PAH. They also reported that both *in vitro* and *in vivo* survivin targeting induces PASMCs mitochondria-dependent apoptosis and is associated with activation of potassium channels. An alternative to gene therapy is follows a pharmacological approach. In this context, terameprocol can be a promising option. Terameprocol (meso-tetra-O-methyl nordihydroguaiaretic acid, TMP) is a methylated derivate of nordihydroguaiaretic acid, found in the plant *Larrea tridentate*, reported to block cell cycle progression by inhibiting expression of the Sp1-dependent gene coding for cdc2 and promotes apoptosis by inhibiting expression of the survivin gene, whose expression is also highly dependent of the transcription factor Sp1 [6, 194]. This compound inhibits the growth of tumors by targeting cdc2 and survivin. Currently, trials are ongoing in patients with some cancer types [5, 183]. As cancer, PAH is characterized by an excessive proliferation and reduced apoptosis, which contributes to the vascular remodeling characteristic of this disease and thus, TMP can be a promising therapy and its effects in PAH should be evaluated.

4. Aims

Given the importance of the vascular remodeling in the progression of PAH, characterized by an excessive cellular proliferation and a decreased apoptosis, the aim of the present work was to evaluate the effects of terameprocol, a pro-apoptotic and anti-proliferative drug, in the proliferation and apoptosis of PASMCs in PAH. To achieve this goal, we evaluated the effect of the addition of different concentrations of terameprocol in the cellular proliferation and apoptosis in SMCs primary cultures isolated from animals injected with MCT.

5. Materials and methods

5.1. Experimental Design

The experimental design is summarized in figure 3.

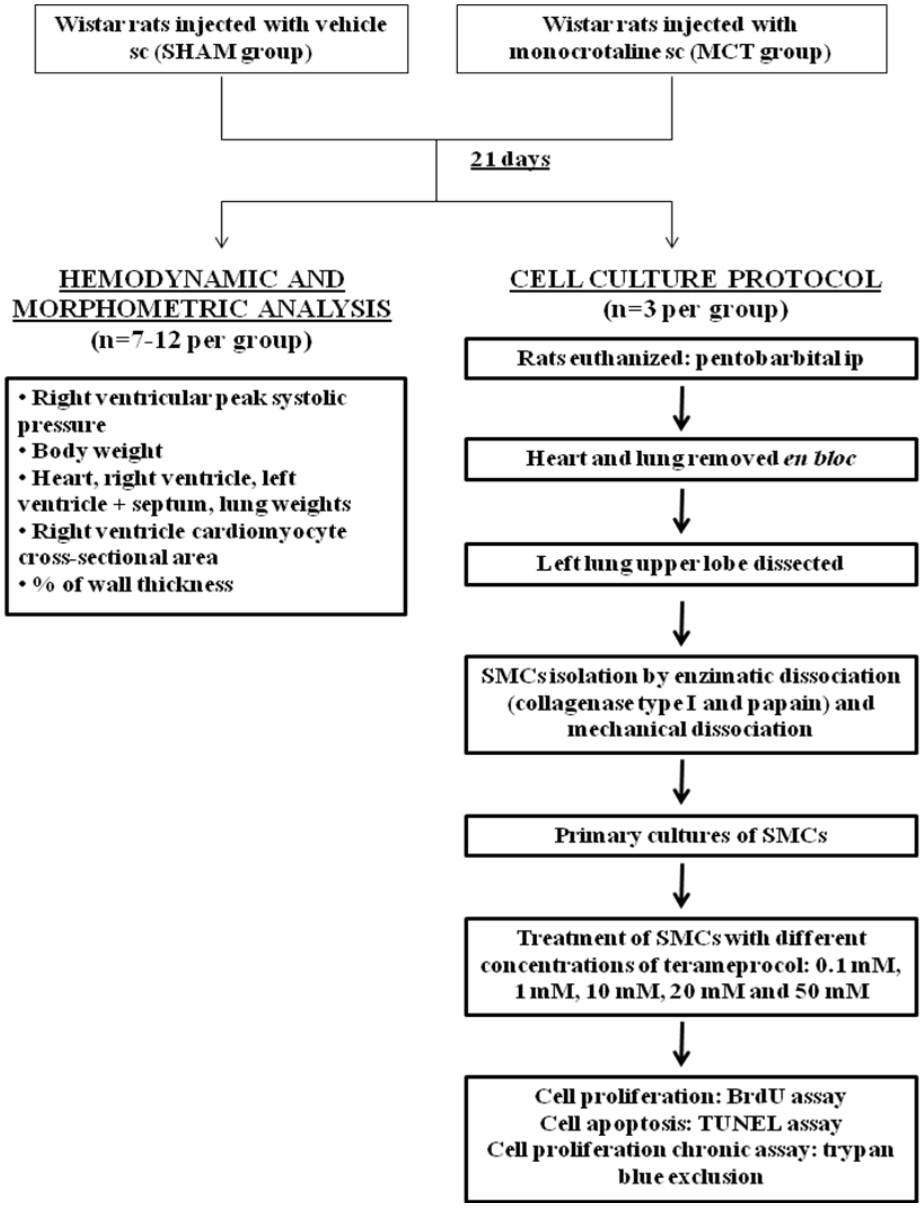

Figure 3 – Experimental protocol design (ip, intraperitoneal).

5.2. Animal and Experimental Protocol

Animal experiments were performed according to the Portuguese law for animal welfare and conform to the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* (NIH Pub. Nº 85-23, Revised 1996). Adult male Wistar rats (Charles River Laboratories; Barcelona, Spain) weighing 180–200 g were housed in a controlled environment under a 12:12 h light-dark cycle at a room temperature of 22ºC, with a free supply of food and water. Rats were randomly divided in two groups, one of which received a subcutaneous injection of MCT (60 mg/kg body weight; Sigma, Barcelona, Spain) (monocrotaline group) and the other an equal volume of vehicle (NaCl 0.9%; 2 ml/kg body weight) (sham group).

5.3. Hemodynamic evaluation

On day 21 after MCT/vehicle injection, 7-12 rats per group were anesthetized by inhalation of a mixture of sevoflurane (4 %) and oxygen, intubated for mechanical ventilation, and placed over a heating pad. Under binocular surgical microscopy (Wild M651.MS-D; Leica), the right jugular vein was cannulated for fluid administration (prewarmed 0.9 % NaCl solution) to compensate for perioperative losses. The heart was exposed through a median sternotomy, and the pericardium was widely opened. A pressure-volume (PV) catheter was implanted in the RV (PVR-1045, Millar Instruments, Houston, TX). After complete instrumentation, the animal preparation was allowed to stabilize for 15 minutes. Hemodynamic recordings were made under basal conditions with respiration suspended at end-expiration. Data was continuously acquired (MPVS 300, Millar Instruments), digitally recorded at 1000 Hz (ML880 PowerLab 16/30, Millar TM Instruments), and analyzed (PVAN 3.5, Millar Instruments). RV pressure was measured at peak systole (P_{max}).

5.4. Morphometric analysis

After hemodynamic assessment, animals were euthanized by exsanguination under anesthesia. The heart and lungs were excised and weighed. The RV free wall was dissected from the left ventricle (LV) + septum (S), under binocular magnification (x3.5), and weighed separately. Heart, lungs and RV weights were normalized to body weight (BW). RV weight was also normalized to that of LV + S. RV and right lung samples were immersion fixed in 4 % paraformaldehyde and embebbed in paraffin. Sections 4 μm thick were cut and stained with hematoxylin and eosin. RV free wall specimens were obtained from each heart at midway between the apex and base.

Studied samples were observed at microscope, photographed with a digital camera and measured with a digital image analyzer (cell^B life science basic imaging software, Olympus). All the measures were made directly at 400x magnification. RV samples were divided into five sections and the area of fifty cardiomyocyte (whose cross section included a nuclear profile) per sample was measured and averaged. On the pulmonary specimens, external diameter and medial wall thickness in muscular arteries (12-18 arteries/lung) were analyzed at 400x magnification. Orthogonal intercepts were used to generate eight random measurements of external diameter (distance between the external lamina) and sixteen random measurements of medial thickness (distance between the internal and external lamina). For each artery, medial hypertrophy was expressed as follows: % wall thickness = [(medial thickness x 2)/ (external diameter)] x 100.

5.5. Isolation and primary culture of rat pulmonary artery smooth muscle cells

Animals assigned to the cell culture protocol were euthanized with an intraperitoneal injection of pentobarbital (120 mg/kg) (n=3 per group), 21 days after MCT/vehicle injection. The heart and lungs were removed *en bloc* and the left upper lung lobe was removed and placed in a calcium enriched Hank´s Buffered Salt Solution (HBSS, Invitrogen, Madrid, Spain) (mM: KCl 5.4; NaCl 137; KH_2PO_4 0.44; $NaHCO_3$ 4.2; NaH_2PO_4 0.25; D-glucose 1; phenol red 0.2; $CaCl_2$ 2 and $MgCl_2$ 1). First order intrapulmonary artery was dissected under a stereomicroscope (Leica EZ4), in sterile conditions. After the artery was extracted from the lung, the adventitia was removed, the vessel was opened longitudinally and endothelium was removed by gently rubbing the inner surface with forceps tips. The artery, now mainly composed by medial layer, was submitted to an enzymatic dissociation process with papain (Sigma, Barcelona, Spain) and collagenase type 1 (Worthington Biochemical Corp., Lakewood, New Jersey), in a calcium-poor HBSS solution (25 μM $CaCl_2$ and 1 mg/mL BSA), with the aim to release smooth muscle cells. Then, a mechanical dissociation process was performed with a fire polished, silicone coated glass pipette, to release the cells. The cell-free tissue was removed and the solution was centrifuged (5 minutes, 250 g) to pellet the cells. Finally, cells were seeded in DMEM (PAN Biotech, Aidenbach, Germany) culture medium, supplemented with 1 % penicillin-streptomycin-amphotericin B (Invitrogen, Madrid, Spain), 1 % sodium pyruvate (Sigma, Barcelona, Spain) and 1 % nonessential amino acids (Sigma, Barcelona, Spain) and containing 10 % FBS (fetal bovine serum, Sigma,

Barcelona, Spain), in a 24-well culture plate (500 µL/well) and placed in an incubator (37 ºC, 5 % CO_2). The medium was changed after 24 h and then every 48 h. Cells were subcultured at 70-80 % confluence and they were then detached with trypsin (PAN Biotech, Aidenbach, Germany), passaged and cultured continuously. Cells between passages 2 and 4 were used for assays. Smooth muscle origin was confirmed by typical morphology (fusiform shape with hills and valleys) and detection of α-smooth muscle actin expression by immunocytochemistry (data not shown).

5.6. Assessment of pulmonary artery smooth muscle cell proliferation by BrdU assay

For BrdU assay, cells were seeded in 24-well plates at a concentration of $3x10^4$ cells/mL. After 72 h, the medium was removed and cells were incubated with different concentrations of terameprocol (Sigma, Barcelona, Spain) (µM: 0.1; 1; 10; 20 and 50) or DMSO (dimethylsulfoxide, Sigma, Barcelona, Spain) (vehicle) for 24 h in medium without FBS. The effect of terameprocol in cell proliferation was evaluated using the 5'-bromodeoxyuridine (BrdU) immunoassay (Roche Diagnostics). BrdU is a thymidine analogue and its incorporation in the DNA is a measure of cell proliferation. The BrdU assay was conducted following the 24 h incubation with terameprocol/vehicle. All protocol was followed according to manufacturer´s instructions. Each condition (concentration) was tested in triplicate and in cells from three animals per group. Results are expressed as percentage of control (medium with vehicle).

5.7. Assessment of pulmonary artery smooth muscle cell death by TUNEL assay

For TUNEL assay, cells were seeded in 24-well plates, with glass coverslips, at a concentration of $3x10^4$ cells/mL. After 72 h, the medium was removed and cells were incubated with different concentrations of terameprocol (µM: 0.1; 1; 10; 20 and 50) or DMSO (vehicle) for 24 h in medium without FBS. TUNEL (TdT-mediated dUTP Nick End Labelling) assay was performed using the In Situ Cell Death Detection kit (Roche Diagnostics), according to the manufacturer's instructions. After the 24 h incubation of cells with terameprocol/vehicle, cells were stained with TUNEL and DAPI. The percentage of apoptotic cells was calculated by dividing the number of cells stained with TUNEL (apoptotic cells) by the total number of nuclei stained with DAPI (Roche Diagnostics), in at least 10 different fields at 200x magnification. Each condition (concentration) was tested in triplicate and in cells from three animals per group.

5.8. Chronic assessment of pulmonary artery smooth muscle cell proliferation by trypan blue exclusion

Cells were seeded in 24-well plates at a concentration of $3x10^4$ cells/mL. The medium was replaced after 24 h and then every 48 h by a medium containing 10% FBS and terameprocol (μM: 20 and 50) or DMSO (vehicle). Cellular proliferation was evaluated by the performance of growth curves, through the diary counting of cells (during 6 days) in a hemocytometer, with exclusion by trypan blue. For each condition (concentration) cells from three wells were counted per day and it was used cells from three animals per group.

5.9. Statistical Analysis

Statistical analysis was performed using Sigma Stat 3.5 software. Values are presented as means ± standard error (SE) and were compared using two-way ANOVA. When the normality test failed, the two-way ANOVA was preceded by a logarithmic transform to obtain a normal distribution. When treatments were significantly different, the Holm-Sidak test was selected to perform pairwise multiple comparisons. A p value less than 0.05 was considered statistically significant.

6. Results

6.1. Characterization of monocrotaline – induced pulmonary arterial hypertension animal model

In order to characterize the animal model of MCT – induced pulmonary arterial hypertension from where smooth muscle cells were isolated, we performed a protocol to evaluate the hemodynamic and morphometric alterations. Results are showed in table 4 and figure 4.

Table 4 – Hemodynamic evaluation and morphometric analysis of monocrotaline-induced pulmonary arterial hypertension.

Hemodynamic evaluation		**Morphometric analysis**									
RVPSP (mmHg)		**BW (g)**		**HT/BW (g/Kg)**		**RV/(LV + S) (g/g)**		**RV/BW (g/Kg)**		**L/BW (g/Kg)**	
SHAM	MCT	SHAM	MCT	SHAM	MCT	SHAM	MCT	SHAM	MCT	SHAM	MCT
26.3 ± 1.2	38.9 ± 2.7*	290.6 ± 4.5	260.8 ± 4.9*	2.794 ± 0.069	3.282 ± 0.113*	0.302 ± 0.012	0.467 ± 0.049*	0.584 ± 0.013	0.911 ± 0.095*	4.782 ± 0.323	7.241 ± 0.464*

Data are given as means ± SE. SHAM, sham group; MCT, monocrotaline group; RVPSP, right ventricular peak systolic pressure; RV, right ventricle; LS + S, left ventricle plus septum; HT, heart; BW, body weight, L, lung. *p < 0.05 vs. SHAM.

As showed in table 4, 21 days after MCT/vehicle injection, MCT group presented a significantly higher right ventricular systolic pressure comparing with SHAM group (P<0.05), confirming that MCT was effective in inducing the increase in overload. In addition, table 4 summarizes morphometric analysis of MCT-induced PAH. MCT group presented a significantly lower body weight, on day 21 after injection, comparing with animals from the SHAM group (P<0.05). The ratio HT/BW was significantly higher in the MCT group (P<0.05). In addition, RV/ (LS + S) and RV/BW indexes were significantly higher in the MCT group, indicating the development of RV hypertrophy. The parameter L/BW was also significantly higher in the MCT group, comparing with SHAM group.

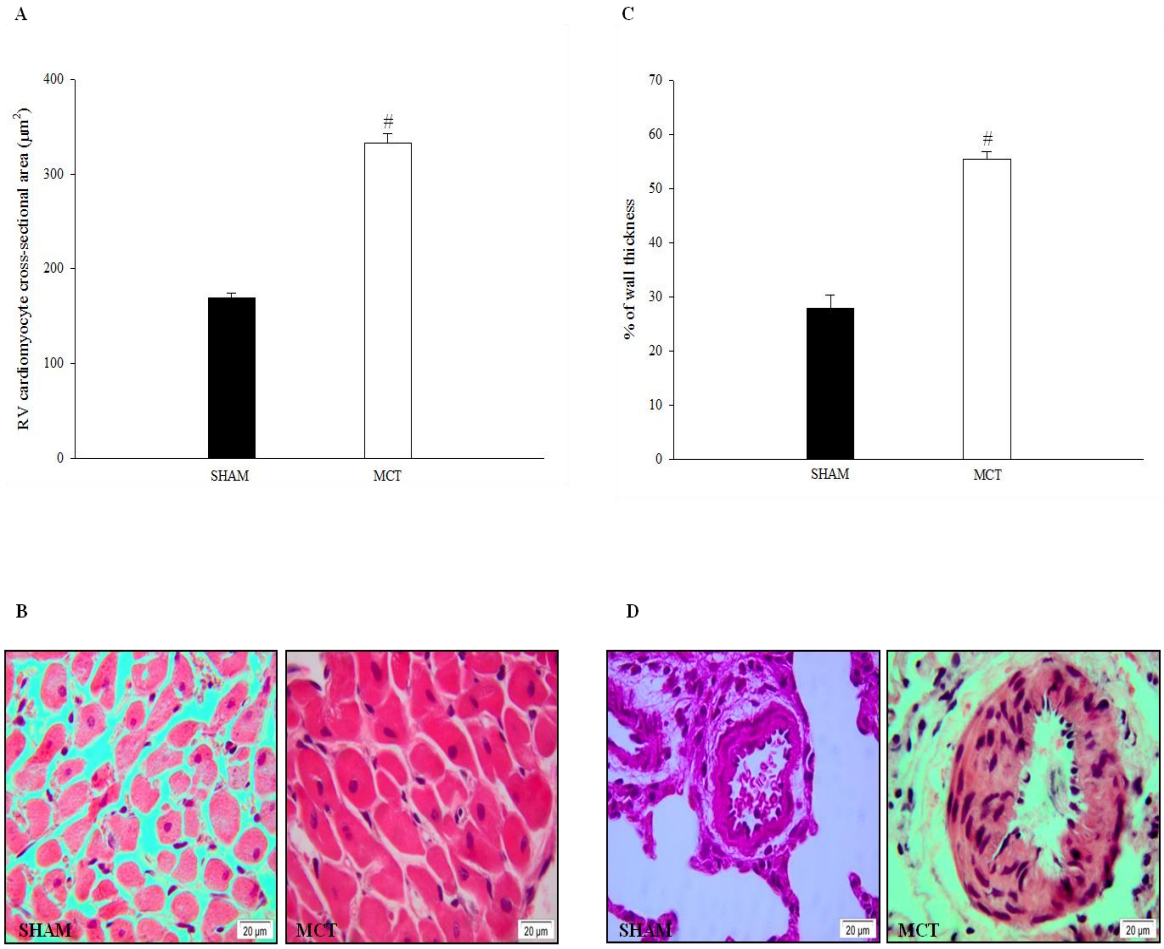

Figure 4 – Right ventricular cardiomyocyte cross-sectional area and pulmonary arterial wall thickness measurement (A and C, respectively) and right ventricular cardiomyocyte and pulmonary arterial wall hypertrophy assessed by microscopic observation (B and D, respectively). SHAM, sham group; MCT, monocrotaline group. Data are given as means ± SE. #p < 0.05 vs. SHAM.

Representative examples of right ventricle cardiomyocytes from rats 21 days after injection with vehicle and MCT are showed in figure 4B. As depicted in the graphic (Figure 4A), cardiomyocytes from MCT group had a significantly higher cross-sectional area comparing with SHAM group (P<0.05). Representative examples of morphology of pulmonary arteries from rats 21 days after injection with vehicle and MCT are showed in figure 4D. Measurement of pulmonary arterial wall thickness revealed increased pulmonary arterial wall thickness in MCT-treated groups in comparison to SHAM group (P<0.05) (Figure 4C).

6.2. Proliferation of pulmonary artery smooth muscle cells – acute studies

With the aim to evaluate the effect of terameprocol in the proliferation of smooth muscle cells primary cultures, isolated from rats with PAH induced by MCT, we performed the BrdU assay. The results are shown in figure 5.

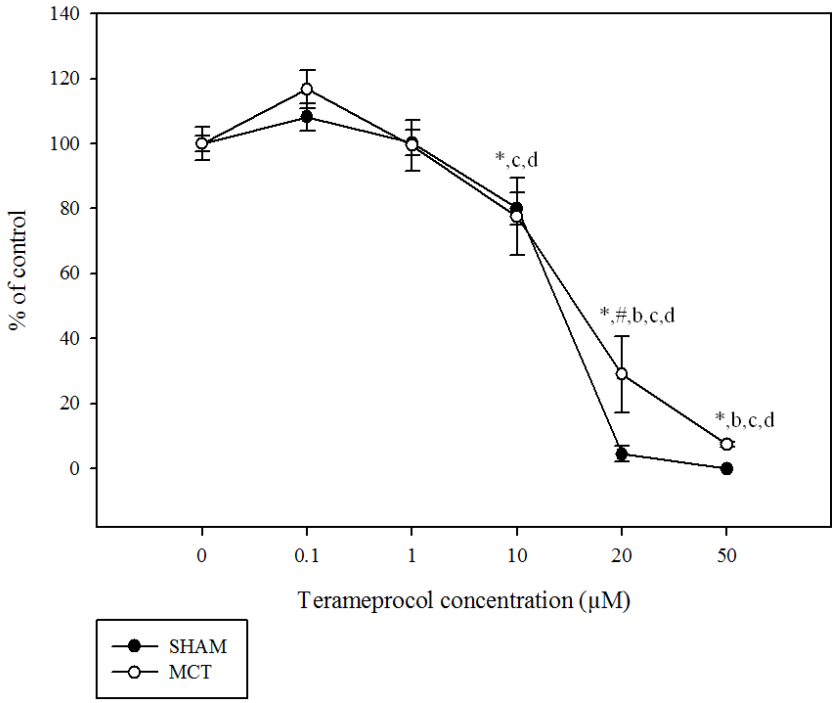

Figure 5 – Effect of terameprocol in pulmonary artery smooth muscle cell proliferation, evaluated by the BrdU assay. SHAM, sham group; MCT, monocrotaline group. Data are expressed as percentage of the absorbance of control (TMP 0 µM) from the same group, and given as means ± SE. *$p < 0.05$ vs. control of the same group; #$p < 0.05$ vs. SHAM of the same TMP concentration; $^{b}p < 0.05$ vs. 10 µM of the same group; $^{c}p < 0.05$ vs. 1 µM of the same group; $^{d}p < 0.05$ vs. 0.1 µM of the same group.

The results showed that the pattern of proliferation did not differ between the SHAM and the MCT groups. In addition, for the higher concentrations that were tested (10, 20 and 50 µM), terameprocol significantly inhibited cell proliferation, in a dose-dependent manner, in both SHAM and MCT groups. However, the inhibition of proliferation, using the lower terameprocol concentrations (0.1 and 1 µM) did not differ from the control (TMP 0 µM). For some terameprocol doses (0.1, 20 and 50 µM) it was verified a less marked inhibition of the proliferation of cells treated with MCT, comparing with

the cells treated with vehicle, being this result statistically significant for the TMP concentration of 20 μM.

6.3. Evaluation of pulmonary artery smooth muscle cell apoptosis

To evaluate the effect of terameprocol in the apoptosis of smooth muscle cells primary cultures, isolated from rats with PAH induced by monocrotaline, we performed the TUNEL assay. Results are plotted in figure 6.

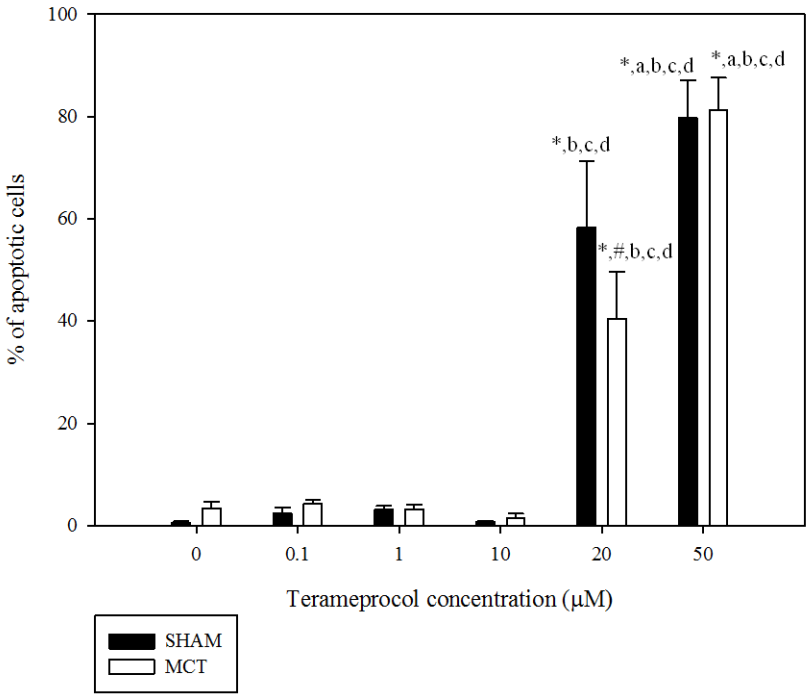

Figure 6 – Effect of terameprocol in pulmonary artery smooth muscle cell apoptosis, evaluated by the TUNEL assay. SHAM, sham group; MCT, monocrotaline group. Data are expressed as percentage of apoptotic cells and given as means ± SE. *$p < 0.05$ vs. control of the same group; #$p < 0.05$ vs. SHAM of the same TMP concentration; $^{a}p < 0.05$ vs. 20 μM of the same group; $^{b}p < 0.05$ vs. 10 μM of the same group; $^{c}p < 0.05$ vs. 1 μM of the same group; $^{d}p < 0.05$ vs. 0.1 μM of the same group.

Higher concentrations of terameprocol (20 and 50 μM) induced apoptosis of cells in both SHAM and MCT groups, in a dose-dependent manner. In contrast, the lower doses of terameprocol (0.1, 1 and 10 μM) had no effect on apoptosis from cells of both groups. In addition, at 20 μM of terameprocol, the percentage of apoptotic cells was significantly higher in the SHAM group, compared to the MCT.

6.4. Proliferation of pulmonary artery smooth muscle cells – chronic studies

Assays were performed during six days to evaluate the chronic cell response to terameprocol in the concentrations of 0, 20 and 50 μM. Results regarding SHAM and MCT groups are plotted in figure 7.

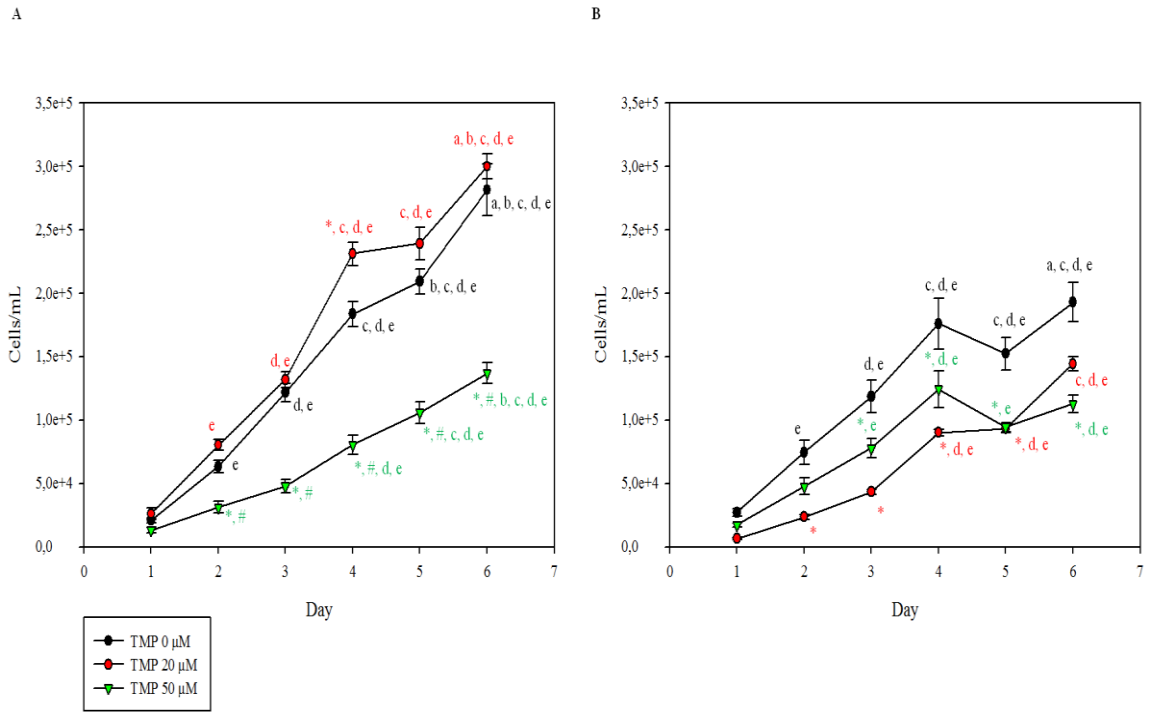

Figure 7 – Chronic effect of terameprocol in the proliferation of pulmonary artery smooth muscle cells from the sham (A) and monocrotaline (B) groups, evaluated by trypan blue exclusion method. Data are expressed as cells/mL and given as means ± SE. *p < 0.05 vs. control (TMP 0 μM) of the same day; #p < 0.05 vs. 20 μM of the same day; [a]p < 0.05 vs. day 5 of the same TMP concentration; [b]p < 0.05 vs. day 4 of the same TMP concentration; [c]p < 0.05 vs. day 3 of the same TMP concentration; [d]p < 0.05 vs. day 2 of the same TMP concentration; [e]p < 0.05 vs. day 1 of the same TMP concentration.

In sham group there was an increase in cell proliferation over time, for all the TMP concentrations tested (0, 20 and 50 μM). However, cells treated with TMP 50 μM proliferated significantly less than control cells (TMP 0 μM) and cells treated with TMP 20 μM. In contrast, cells treated with TMP 20 μM proliferated more than control cells. Regarding MCT group, it was observed that cell proliferation increased over time, in all the concentrations studied, but in day 5 it was observed a decrease in the case of the control and TMP 50 μM treated cells. For cells treated with TMP 20 and 50 μM, it was

observed that they proliferated significantly less than control cells. Cells treated with TMP 50 μM proliferated more than cells treated with TMP 20 μM until day 4.

7. Discussion

Despite the beneficial effects of current drugs in the relief of the symptoms and in the improvement of the survival [30, 149], a more emphasis has been given to therapies that focus in targeting vascular remodeling in PAH [181, 182]. In this context, Rho kinase inhibitors, dichloroacetate, simvastatin, tyrosine kinase inhibitors and survivin inhibitors are being explored as potential therapies to enhance apoptosis and decrease cellular proliferation [181]. Between these strategies, survivin is an attractive target, once it regulates both proliferation and apoptosis. The beneficial effect of targeting survivin pathway is well known in the area of cancer treatment [5, 183]. This is a disease that similarly to PAH is characterized by excessive cellular proliferation and impaired apoptosis. The fact that survivin expression is observed in the majority of tumors and rarely expressed in normal healthy adult tissues makes this molecule a target to cancer treatment [183]. In the last years the research for anti-survivin therapy in cancer increased and there are several drugs in clinical trials [5, 183, 195-198]. Strategies targeting survivin act by binding to survivin promoter, by inhibiting protein translation, or by interfering with survivin function and examples include: anti-sense oligonucleotides (LY2181308), ribozymes, small interfering RNAs, dominant-negative mutants (Cys84Ala and Thr34Ala), and small-molecule antagonists [183]. In the last group is included terameprocol, a compound that was found to inhibit Sp1-dependent gene coding for survivin and cdc2 [6, 194]. In addition, it was reported that TMP cause growth arrest in several cancer types, both *in vivo* and *in vitro*. Regarding the safety of this drug, previous data showed no relevant toxicity [199].

The beneficial effects of survivin pathway inhibition in cancer have encouraged researchers to explore this therapeutic approach in other diseases also marked by impaired proliferation and apoptosis, such as PAH. Regarding this issue, until now, there is only one study that evaluated the expression of survivin and the effects of its inhibition in PAH. McMurtry *et al.* [4] showed that this pathway is up-regulated in patients with PAH and in rats injected with MCT. In addition, they also reported that targeting survivin pathway, through gene therapy with a survivin mutant (Thr34Ala), inhibiting the phosphorylation necessary for survivin functions in proliferation and apoptosis, MCT-induced PAH was reversed. Thus, survivin inhibition can be considered a candidate therapeutic target for PAH. However, the use of a pharmacological approach instead of gene therapy targeting survivin was not yet explored. In this work we evaluated the effect of terameprocol in the proliferation and

apoptosis of PASMCs primary cultures in PAH. Regarding the results related with cellular proliferation, evaluated by the BrdU assay (Figure 5), it was verified that terameprocol inhibits PASMCs proliferation from SHAM and MCT animals in a dose-dependent manner. The results related with apoptosis, evaluated by TUNEL assay (Figure 6), showed that TMP induces PASMCs apoptosis, in both SHAM and MCT animals in a dose-dependent manner. In the case of cell proliferation, TMP inhibitory effect was observed from the concentration of 10 μM and continued until the maximum concentration tested. In the case of apoptosis, TMP induced markedly apoptosis only in the concentrations of 20 and 50 μM. However, in both studies, for the concentration of 20 μM, TMP showed to have an anti-proliferative and apoptotic effect more marked in cells from SHAM group, comparing with MCT group cells. Once the concentrations of 20 and 50 μM were those that showed to have more positive effects, in the acute studies, we then performed chronic assays to evaluate cell proliferation over time, during six days, through the trypan blue exclusion method. The results obtained were in agreement with the acute effect verified in the previous studies, showing an anti-proliferative effect of terameprocol. However, differences were observed between cells from SHAM and MCT groups. MCT cells showed a decrease in proliferation when treated with TMP 20 and 50 μM and this response was more marked in the case of TMP 20 μM treatment (Figure 7B). In contrast, with cells from SHAM group (Figure 7A), a decrease in proliferation was only observed in the case of cells treated with TMP 50 μM. Thus, once MCT cells responded to lower TMP concentrations than SHAM cells, this suggests that MCT cells can be more sensitive to TMP effects, which can be related to the survivin over-expression in these cells, fact that was previously observed by McMurtry and colleagues in tissue lung of PAH patients and rats injected with MCT, through immunohistochemistry analysis [4].

In the current study, we used the MCT animal model of PAH. This animal model has been used for years to study the pathogenesis of PAH, as well as the effects of drug intervention [121, 126, 127]. Monocrotaline is a pyrrolizidine alkaloid converted in the liver in a toxic metabolite (MCTP), which mainly affects the hepatic and cardiopulmonary systems [128, 129]. Several studies reported the effects of MCT administration by different routes, doses and animal species [126, 142, 200]. Although variations of these factors contribute to the severity of the lesions, the general spectrum of effects is maintained and includes right ventricular hypertrophy, vasoconstriction, ECs hypertrophy, SMCs hypertrophy and hyperplasia and inflammation [122, 128,

132]. Although there are some differences described between this animal model and human PAH (such as the absence of plexiform lesions in the experimental model) it has identical hemodynamic and some morphological features [122, 136]. In accordance, the results from hemodynamic evaluation revealed that MCT induced an increase in right ventricular peak systolic pressure (Table 4). This was associated with increased RV mass and cardiomyocyte hypertrophy, corroborating previous studies [126, 141, 142, 200]. The RV hypertrophy is the result of pressure overload secondary to the pulmonary artery wall changes that obstruct lumen arteries and lead to an increase in PAR and PAP [29]. This mechanism is firstly a compensatory response, reflecting RV adaptation to the sustained afterload elevation. However, depending on the duration of this afterload elevation, cardiac hypertrophy can progress from a compensatory state to a systolic or diastolic dysfunctional state, and can culminate in right heart failure [31]. Although LV weight was not determined in this study, previous studies reported that left ventricle hypertrophy does not occur with MCT administration, but alterations in the contractile phenotype are reported [142]. The increase in the index L/ BW (Table 4) in this study suggests cellular hypertrophy and/or hyperplasia in the lung due to MCT administration. In the present study, we observed pulmonary arterial wall alterations (Figure 4C and D), namely increased pulmonary arterial wall thickness in MCT group, results previously reported in other studies [200].

8. Conclusion

Aiming to evaluate the effect of terameprocol, an antagonist of survivin, in the proliferation and apoptosis of pulmonary artery SMCs in PAH, we used the monocrotaline animal model and studied the cellular response to this drug in primary cultures of this cell type. According to the results obtained it was possible to conclude that:

i) terameprocol inhibited the proliferation of PASMCs isolated from animals injected with MCT;

ii) terameprocol induced apoptosis of PASMCs isolated from animals injected with MCT;

iii) PASMCs isolated from animals injected with MCT seems to be more sensitive than PASMCs isolated from SHAM animals to the anti-proliferative terameprocol effect, suggesting a survivin over-expression in PASMCs primary cultures isolated from animals with MCT-induced PAH.

Taken together, data suggest that terameprocol might be seen as a new attractive therapeutic approach to PAH. In the future, it will be important to study the molecular pathways underlying these effects of TMP in PASMCs, and it will be interesting evaluate the effects of direct administration of TMP in rats with PAH induced by MCT.

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