PHYSIOLOGY

Monocrotaline-induced pulmonary arterial hypertension: the benefic effects of magnesium sulfate, Rosuvastatin and Sildenafil

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Abstract

Background. Pulmonary arterial hypertension (PAH) is characterized by several maladaptive mechanisms: endothelial dysfunction, oxidative stress, inflammation, pathological remodeling of the pulmonary arterioles, and cellular hypoxia. These mechanisms all favor progressive pulmonary vasculopathy and progressive right ventricle (RV) dysfunction.

Aim. This study aims to characterize the experimental model of monocrotaline-induced PAH in rats. Subsequently, by administering Sildenafil, Rosuvastatin, and Magnesium sulfate, we assessed the animals via ultrasonography and assayed biochemical parameters to evaluate the efficacy of the treatment.

Methods. 42 male Wistar rats were randomly allocated into six equal groups (n=7) and received a single subcutaneous MCT injection (60 mg/kg dose). Drug therapy with Sildenafil, Rosuvastatin, and Magnesium sulfate in different combinations was initiated 14 days after MCT injection. Fulton Index, RV anterior wall thickness, RV internal diameter, and pulmonary arterial acceleration time/ejection time (PAAT/PAET) were measured. The following biochemical parameters were also measured: endothelin 1(ET1), brain natriuretic peptide (BNP), nitric oxide (NO) metabolites, vascular endothelial growth factor (VEGF), and inducible nitric oxide synthase (iNOS).

Results. MCT-PAH was a successful experimental model that has fulfilled anatomical, pressure, and biochemical characteristics supporting this fact. Sildenafil monotherapy does not provide any substantial benefit in reducing MCT-PAH. The additive effects of Rosuvastatin + Sildenafil or Sildenafil + Magnesium sulfate significantly reduced the degree of RV hypertrophy and improved RV systolic pressures. However, there were also modest decreases in biochemical parameters compared to Sildenafil alone. The triple drug combination Sildenafil + Rosuvastatin + Magnesium sulfate shows significant results ($p<0,001$) compared to the previously described drug combinations. The lowest biochemical parameters were recorded: RV anterior wall thickness, RV internal diameter values, and a significant PAAT/PAET ratio improvement. Thanks to their benefits on vascular pathological remodeling, triple drug combinations implicitly reduce ET1, VEGF, NO metabolites, and iNOS values with statistical significance.

Conclusions. The beneficial pleiotropic effects of Rosuvastatin combined with Magnesium sulfate (thanks to its potent vasodilator and antioxidant effects) demonstrated its efficacy in this study by improving RV systolic pressures, RV hypertrophy, oxidative stress, and myocardial dysfunction biomarkers.

Keywords: monocrotaline, pulmonary arterial hypertension, magnesium sulfate, Rosuvastatin, Sildenafil

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Background and aims

Pulmonary hypertension (PH) belongs to the category of severe and rare vascular diseases and is characterized by increased morbidity and mortality with unfavorable prognosis in advanced stages. The diagnosis is based on a mean pulmonary artery pressure (PA) ≥ 20 mmHg [1]. According to etiology, PH is classified into five major groups. Group I includes pulmonary arterial hypertension (PAH), which comprises several subcategories: heritable, induced by drugs and toxins, idiopathic, or associated with connective tissue disease or congenital heart diseases. PAH diagnosis is also based on a pulmonary vascular resistance (PVR) >3 Wood units (WU) and pulmonary arterial wedge pressure (PAWP) ≤15 mmHg [1]. Pathophysiologically, PAH is characterized by several maladaptive mechanisms: endothelial dysfunction, oxidative stress, inflammation, pathological remodeling of the pulmonary arterioles, cellular hypoxia, and *in situ* thrombosis - all of which favor progressive pulmonary vasculopathy, progressive PAH, and progressive right ventricle (RV) dysfunction as a consequence [2].

Early diagnosis of this pathology, correct classification, and prompt treatment are essential for patients at risk of major cardiovascular events. However, despite all the research and medical advances, it remains a disease with a guarded prognosis.

In vivo, experimental models are essential for evaluating all PAH underlying pathophysiologic mechanisms and testing various innovative treatments. They remain a necessary cornerstone in this pathology's diagnosis, prognosis, and treatment.

Over time, many experimental animal models of induced PH have been developed that have attempted to replicate the human form. Still, none have met all the required characteristics: pathological remodeling and hemodynamic changes. A wide variety of PH-induced experimental models have been grouped into several categories: invasive *in vivo* models, non-invasive *in vivo* models, or genetically modified models. Surgically invasive models are not widely used because they are practically difficult to perform and involve pneumectomy, vascular shunt, and pulmonary artery (PA) ligation, which, however, are associated with a high mortality rate. Pneumectomy and vascular shunt favor increased blood flow in the remaining PA, which is vital in developing PH. Pneumectomy is often combined with MCT administration, intending to replicate the human PH phenotype of PAH as closely as possible [3]. The PA ligation technique is an ideal model for the evaluation of RV volume overload and right heart failure, thus avoiding the adverse effects of hypoxia or MCT. Genetically modified models have developed significantly over the last two decades. Numerous genes have been identified that are involved in the development of familial or idiopathic forms of PAH. The most common gene is BMPR2 (bone morphogenetic protein receptor type 2), which is found at a frequency of 80% in familial PAH and 20% in idiopathic PAH [3].

The non-invasive models involved *in vivo* are represented by the animal model favoring the development of PH - induced by exposure to chronic hypoxia and/or administration of a VEGF receptor antagonist - SUGEN 5416 (Semaxanib) for 3 weeks. SUGEN induces pulmonary vascular proliferation and neointimal plexiform lesion formation. Those two models are implicated in PH development, whose etiopathogenetic characteristics approximate the human group III form of PH. The administration of MCT, Bleomycin, and Mitomycin C are responsible for developing PH-induced form, but etiopathogenetic characteristics correspond to group I of PH.

Monocrotaline (MCT) is an alkaloid derived from the seeds of the *Crotalaria spectabilis* plant. Lalich et al. first discovered pulmonary arteritis in rats fed this plant [4]. Therefore, this model of MCT-induced PAH has been validated for over 60 years since it is a simple, predictable, easily reproducible, and inexpensive model that can lead to severe PAH [3]. A single subcutaneous dose of MCT (50-80mg/kg) is injected to develop the MCT-induced PAH model. The injected MCT is metabolized into toxic degradation products (MCT pyrrole) via cytochrome P450 3A4 in the liver. One of the limitations of this model is that significant hepatic and myocardial toxicity, pulmonary fibrosis, necrotizing pulmonary arteritis, and renal failure develop [3]. Although MCT is also used to create the PAH model, its pathophysiological characteristics are not superimposable with those of group I human PAH. At a pulmonary level, significant endothelial dysfunction and interstitial edema occur, which favor arterial muscularization, vasocontraction, and growth of the pulmonary vascular bed. This robust pulmonary vascular remodeling favors elevated PVR, RV systolic pressure, maladaptive RV hypertrophy [5,6], and the death of the animals 4-6 weeks after developing the experimental model [5]. Despite all these aspects, the induced PAH (MCT-PAH) rat model remains a valid way to evaluate the therapeutic response of various innovative therapies.

Regarding specific therapy with endothelin receptor antagonists, phosphodiesterase 5 inhibitors (PDE5is) such as Sildenafil have been known for over 10 years as a specific drug for group I of PAH, recommended by specialized guidelines [1]. However, it has also proven to be effective in MCT-induced PAH [7]. Sildenafil has an essential role in pulmonary arterioles, decreasing PVR due to its effective vasodilator effect [1,7].

Magnesium is an essential intracellular element and a natural calcium antagonist involved in various cellular processes. It plays an essential role in the normal vasoreactivity of the pulmonary vascular endothelium and is a potent vasodilator with antioxidant and anti-inflammatory properties [8–10]. Magnesium sulfate has demonstrated its

benefit in PH newborns and chronic hypoxia PH [8,10–12].

The efficacy and safety of statins, such as 3-hydroxy-3-methyl-glutaryl-coenzyme A (HMG-CoA) reductase inhibitors, have been evaluated over time in PAH therapy. Rosuvastatin, a hydrophilic statin, has demonstrated its *in vivo* benefits in numerous MCT-PAH clinical trials [13-15]. Through its pleiotropic effects - antioxidant, antiproliferative, hypolipidemic, and anti-inflammatory - it positively affects pulmonary arteriolar remodeling, decreases PVR, and implicitly maintains RV function [16-18].

This study aims to characterize the experimental model of MCT-PAH in rats. Subsequently, we administered Sildenafil, Rosuvastatin, and Magnesium sulfate to the animals and assessed them via ultrasonography. We also assayed some biomarkers to evaluate the treatment's efficacy.

Methods

Animals

The experiment was conducted on 42 male Wistar rats weighing 250 g. They were fed granulated Murigran (from Agropol, Poland) and had unrestricted access to water. The animals were housed in special cages, 7 in each cage. The experiment took place in the Physiology Department of the Iuliu Haţieganu Faculty of Medicine and Pharmacy in Cluj-Napoca. Animal housing, medication administration, and the entire experiment protocol were performed according to the International Animal Research Guidelines and were approved by the Veterinary Health Authority of Cluj-Napoca, Romania (no. 229/12.08.2020; 260/22.07.2020) and respected also Directive 86/609/EEC.

MCT treatment

Monocrotaline (MCT, Sigma-Aldrich, Ro) was dissolved in 1 molar (M) HCl and neutralized to 7.2 pH with 3 M NaOH [3,13]. A single subcutaneous (s.c.) injection (60 mg/kg dissolved in 3 ml/kg NaCl 0.9%) was administered according to the previous study [13] for the MCT-induced PAH rat model. The 60 mg/kg dose is known to induce robust vascular remodeling [5].

Experimental design

An overview of this MCT-induced PAH experimental design can be seen in figure 1.

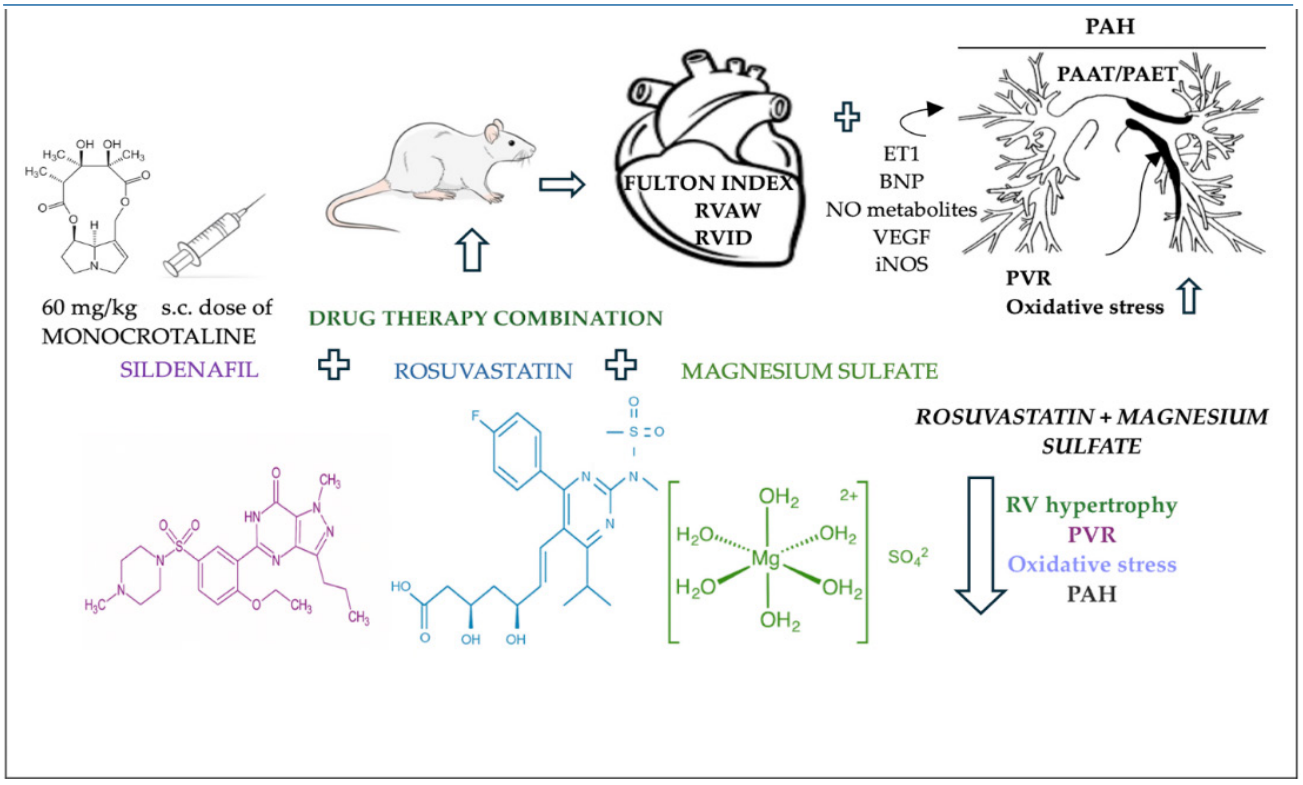

Figure 1. An overview of MCT-induced PAH experimental design. After rats were injected with 60 mg/kg s.c. dose of MCT, several drugs were administered: Sildenafil, Rosuvastatin, and Magnesium Sulfate in different combinations. The Fulton index was calculated as an index for RV hypertrophy. An echocardiographic assessment of cardiac anatomy was made. RV anterior wall thickness (RVAW) and RV internal diameter in diastole (RVID) were measured in diastole; pulmonary artery acceleration time (PAAT), pulmonary artery ejection time (PAET), and PAAT/PAET ratio were calculated. Biochemical parameters (ET1, BNP, NO metabolites, VEGF, and iNOS) were also calculated.

The animals were divided into 6 equal groups. The control group (C), consisting of 7 healthy rats, did not receive MCT. This group was housed in a special cage for 4 weeks and fed accordingly. Groups I - V were injected with MCT s.c., and various drug combinations were administered. Sildenafil (Sigma-Aldrich, Ro), Rosuvastatin (Sigma-Aldrich, Ro), and Magnesium sulfate (Sigma-Aldrich, Ro) were suspended in 1.0% methylcellulose and administered via intragastric (i.g.) gavage.

Group I received only s.c. injection with MCT on day 1 after inclusion in the study. Group II received MCT s.c. on day 1, and after 14 days, drug therapy with Sildenafil 25 mg/kg i.g. daily for another 2 weeks, was started. Group III received one injection of MCT s.c. and then, 2 weeks after, treatment with Sildenafil 25 mg/kg i.g. + Rosuvastatin 10 mg/kg i.g. Group IV was injected s.c. with MCT and, after 14 days, started drug therapy with Sildenafil 25mg/kg i.g.+ Magnesium sulfate 10% solution, 10 ml/kg i.g. daily. Finally, group V was injected s.c. with MCT on day 1 and, from day 14 onward, started drug therapy with Sildenafil 25 mg/kg i.g. + Rosuvastatin 10 mg/kg i.g. + Magnesium sulfate 10% solution, 10 ml/kg i.g. daily.

The Magnesium sulfate dose was chosen according to another MCT-induced PAH study [8], as was the Rosuvastatin + Sildenafil dose [13]. When Sildenafil is administered intragastrically, there is an intestinal and a hepatic passage. 70% of the absorption is realized in the intestine. Only a small dose of 0.626% is not absorbed. The recommended dose of Sildenafil is 25-30 mg/kg in pharmacokinetic studies to minimize adverse hepatic effects, as hepatotoxicity is dose-dependent [19]. According to pharmacokinetic studies, the 10 mg/kg dose of Rosuvastatin provides endothelial benefits by increasing eNOS expression and stimulating NO release at the vascular endothelial level. Doses exceeding 20 mg/kg have a severe toxic effect on the liver and have been linked to early death in lab rats [20]. The 10% Magnesium sulfate dose at 10 ml/kg (1000 mg/kg) is considered a high dose according to the literature in experimental models of PAH induced by monocrotaline (MCT) administration. Both the study by Wang et al. [8] and Chang et al. [10] revealed that Magnesium sulfate at this dose inhibits the progression of MCT-induced PAH in rats. Magnesium sulfate is an essential cation for the vascular endothelium due to its anti-inflammatory and vasodilator properties. It also attenuates endothelin1 (ET1)-dependent vasoconstriction and increased magnesium concentrations inhibit calcium (Ca^{2+}) entry into the cell, thereby improving vasodilation [21].

Four weeks after their inclusion in the study, only 28 rats survived. At 24 hours after the last drug administration, group C included 7 animals, group I included 4 animals, group II only 4 animals, group III only 4 animals, group IV included 5 animals, and group V only 4 animals.

Measurement of body mass

The animals were weighed upon inclusion in the study, 2 weeks after MCT injection, as well as at the end of the experiment.

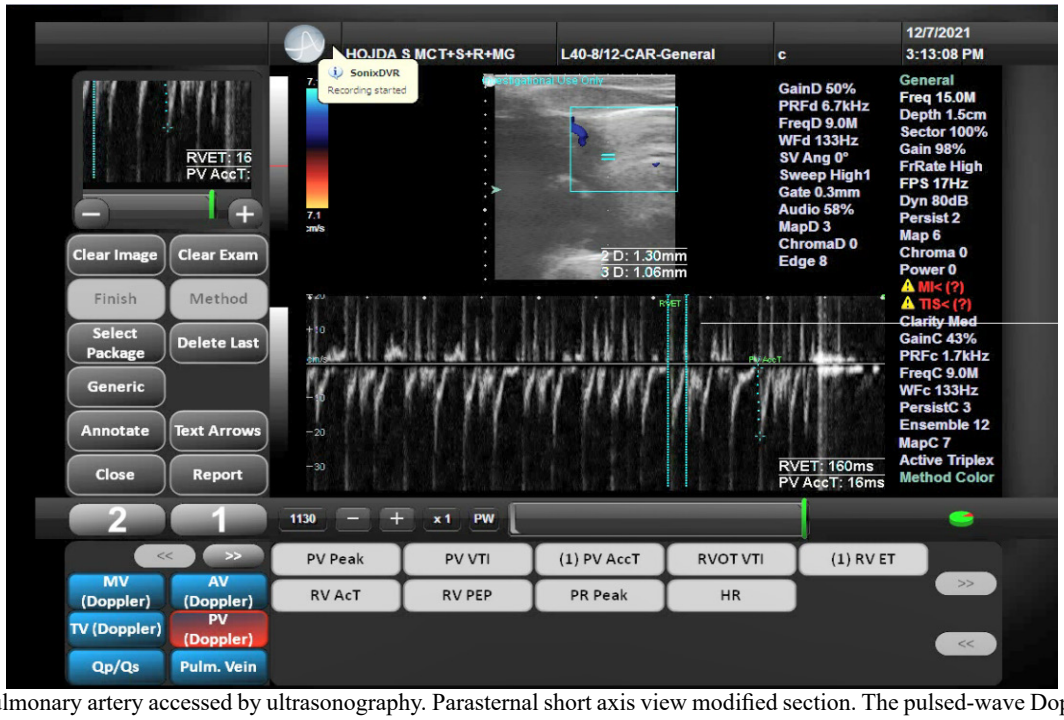

Figure 2. Pulmonary artery accessed by ultrasonography. Parasternal short axis view modified section. The pulsed-wave Doppler sample volume was placed in the center of PA for measurements: PAAT = PVAccT and PAET=RVET.

Echocardiographic measurements

The animals were sedated with pentobarbital sodium (30 mg/kg) via intraperitoneal injection and then placed in the supine position. For the echocardiographic assessment of rat cardiac anatomy, ultrasound measurements were taken with an Ultrasonix version 6.0.7 ultrasound scanner and a special 20 Mhz MicroScan probe. Additional boluses of pentobarbital sodium were administered to maintain sedation until the procedure was completed.

Parameters of RV hypertrophy and indirect parameters of RV systolic pressure were observed using sonography: from parasternal long-axis view using B-mode echocardiography, RV anterior wall thickness at end-diastole (RVAW), RV internal diameter in diastole (RVID) were measured in diastole; the pulmonary artery (PA) diameter was measured from the parasternal short-axis view modified section; the pulsed-wave Doppler sample volume placed in the center of PA for the pulmonary artery acceleration time (PAAT) and pulmonary artery ejection time (PAET) (Figure 2).

Measurements of organ mass

At the end of the study, the animals were euthanized with an overdose of pentobarbital sodium (100 mg/kg) administered via intraperitoneal injection. The animals were then weighed using a veterinary scale. The heart and lungs were removed through a medial thoracic incision. Immediately after removal, the hearts were blotted dry and weighed. The RV was then separated from the left ventricle and septum (LV+S) and weighed immediately. The Fulton index (the RV mass index - RVMI=RV/LV+S) was calculated as an index for RV hypertrophy.

Biochemical parameters

Endothelin 1 (ET1), inducible nitric oxide synthase (iNOS), brain natriuretic peptides (BNP), vascular endothelial growth factor (VEGF), and nitric oxide (NO) metabolites levels:

The levels of these biochemical parameters were measured with commercially available ELISA kits for rats (MCT, Sigma-Aldrich, Ro), and the NO (nitrite/nitrate metabolites) levels were calculated using the colorimetric method (Sigma-Aldrich, RO). BNP values were evaluated in the RV tissue sample, VEGF values were also evaluated in the PA and RV tissue samples, and the others were evaluated only in the PA tissue sample.

Statistical analysis

The normal distribution was tested with the Shapiro-Wilk test, and variance was measured with the F-test. The mean ± sample standard deviation (SD) was used to summarize the distribution of quantitative variables. Two groups were compared with Student's t-test or Mann-Whitney (U) tests. The statistical significance thresholds were $\alpha = 0.05$ (5%), $\alpha = 0.01$ (1%), and $\alpha = 0.001$ (0.1%).

We used StatsDirect software v.2.7.2 (StatsDirect Ltd., Wirral, UK) for statistical data analysis.

Results

Survival and MCT-PAH development

Four weeks after inclusion in the study, only 28 rats survived. Some of these rats died within the first few days after the s.c. MCT injection, and the majority within 10-14 days of administration. After initiating drug therapy, no rats died. Therefore, the side effects of MCT administration played a vital role for some of the rats in the experiment.

Several measurements of organ mass and echocardiographic measurements were taken to assess whether the experimental MCT-PAH model was successfully developed. In the following paragraphs, statistically significant data ($p<0.001$) will be presented.

Regarding the Fulton index, the ratio of RV /(S+LV) weight, considered an indirect marker of RV hypertrophy, there were statistically significant differences ($p<0.001$) between group C and group I (exposed only to MCT) and the rest of the groups, as shown in figure 3. This increased index is a consequence of RV volume and pressure overload. The MCT-PAH experimental model developed statistically significant RV hypertrophy ($p<0.001$).

Compared to group C, various echocardiographic parameters supporting MCT-PAH development in group I (exposed to MCT) were also assessed. In the statistical analysis of RVAW, statistically significant differences ($p<0.001$) were recorded between group C and groups I (exposed to MCT) and II (treated only with Sildenafil), as shown in figure 4. This echocardiographic parameter also demonstrated RV hypertrophy in MCT-PAH. In addition, in the statistical analysis of the RVID diameter, statistically significant differences ($p<0.001$) were observed between group C and groups I, II, and V, as seen in figure 5. There is an important dilation of the RV, the most significant occurring in group I (MCT).

In the statistical analysis of the PAAT/PAET ratio, statistically significant differences ($p<0.001$) between group C and groups I, II, and III were observed echocardiographically, as seen in figure 6. A PAAT/PAET ratio ≤ 0.25 predicts PH with 77% sensitivity and 80% specificity and correlates with RV systolic pressure >35 mmHg in hemodynamic measurements. It can, therefore, be noted that this experimental model registers increased values of RV systolic pressures. Based on the data presented above, it can be said that our MCT-induced PAH model is efficient, with all the necessary parameters for this statement having been achieved [22].

Measurement of body and organ mass after treatments

In the statistical analysis of the body mass values of the experiment animals compared to the control group, significant differences ($p<0.001$) were observed between group C and the groups under drug therapy (group II, group III, group IV, and V). There was a significantly lower body mass in the groups under treatment compared to group I exposed to MCT alone. Under medication, regardless of

the combination, there is a cachexia trend, secondary to the cardiovascular pathology induced by MCT but also secondary to the adverse effects of the medication.

In statistical analysis of the Fulton Index values, significant differences were observed between groups I-III, I-IV, and I-V ($p<0.001$). Without medication, the Fulton Index registers a ratio above 0.34. However, there is a progressive reduction below 0.34 under treatment, as shown in figure 3. Whether in group III (Sildenafil + Rosuvastatin) group IV (Sildenafil + Magnesium sulfate) or group V (Sildenafil + Rosuvastatin + Magnesium sulfate) there is a statistically significant ($p<0.001$) trend of reduction of this parameter. However, there are no statistically significant differences between the 3 drug combinations (groups III, IV, or V). Therefore, all drug combinations, unlike Sildenafil monotherapy, reduce the Fulton Index (considered as an indirect indicator of RV hypertrophy in the literature).

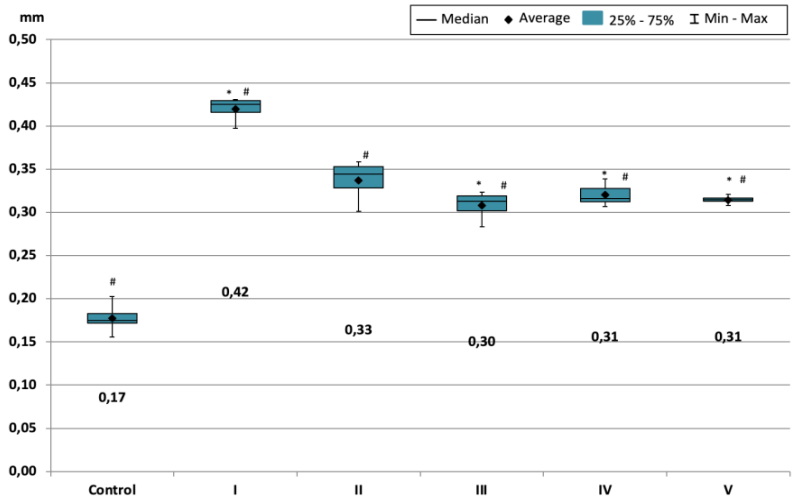

Figure 3. The image represents the Fulton Index, a marker of RV hypertrophy as the ratio of RV mass to LV mass plus septal mass (RV/S+LV) in rat group C and group I-V; group I – MCT without treatment; group II – Sildenafil; group III – Sildenafil + Rosuvastatin; group IV – Sildenafil + Magnesium sulfate; Group V – Sildenafil + Rosuvastatin + Magnesium sulfate; # ($p<0,001$) group C vs. groups I, II, III, IV and group V. * ($p<0,001$) group I vs. groups III, IV and V.

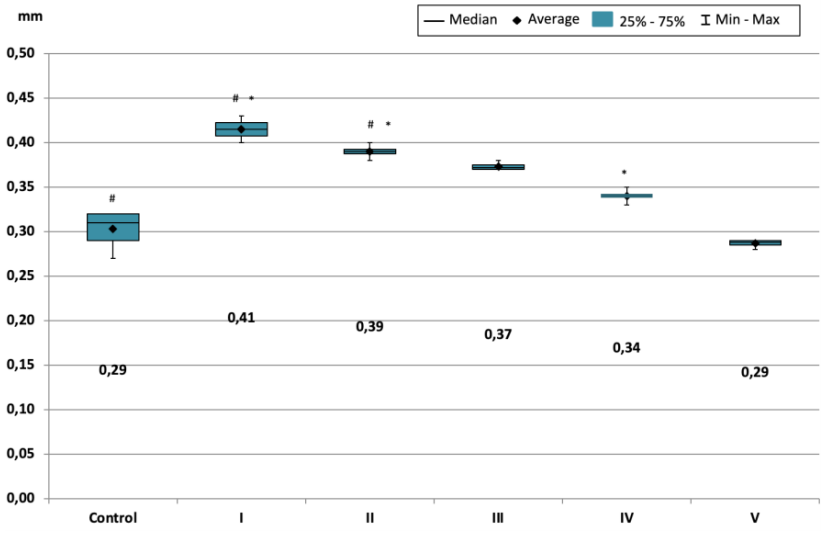

Figure 4. The image represents RVAW thickness (mm) and the progressive reduction of this parameter after initiating drug therapy; group I – MCT without treatment; group II – Sildenafil; group III – Sildenafil + Rosuvastatin; group IV – Sildenafil + Magnesium sulfate; Group V – Sildenafil + Rosuvastatin + Magnesium sulfate; # ($p<0,001$) group C vs. groups I, II. * ($p<0,001$) group I vs. group IV and group II vs. group IV.

Echocardiographic measurements after treatments

In the statistical analysis of RVAW, significant differences were observed between groups I-IV and II-IV ($p<0.001$). We also recorded a degree of RVAW hypertrophy, the highest appearing in group I, which was subjected only to MCT. Once drug therapy is started, there is a significant trend of progressive reduction in the thickness of this wall. Sildenafil + Magnesium sulfate (group IV) shows a statistically significant ($p<0.001$) reduction compared to Sildenafil alone (group II). Still, the lowest value is recorded in group V (as shown in figure 4).

In statistical analysis of the RVID, significant differences were observed between groups I-V ($p<0.001$). A degree of RV dilatation can be seen in the MCT-induced experimental group. The remaining drug combinations provide a modest benefit. However, triple therapy causes a statistically significant RVID improvement ($p<0.001$). This can be explained by the fact that a partial improvement of the pressure gradient under treatment positively influences the degree of RV dilatation (as shown in figure 5).

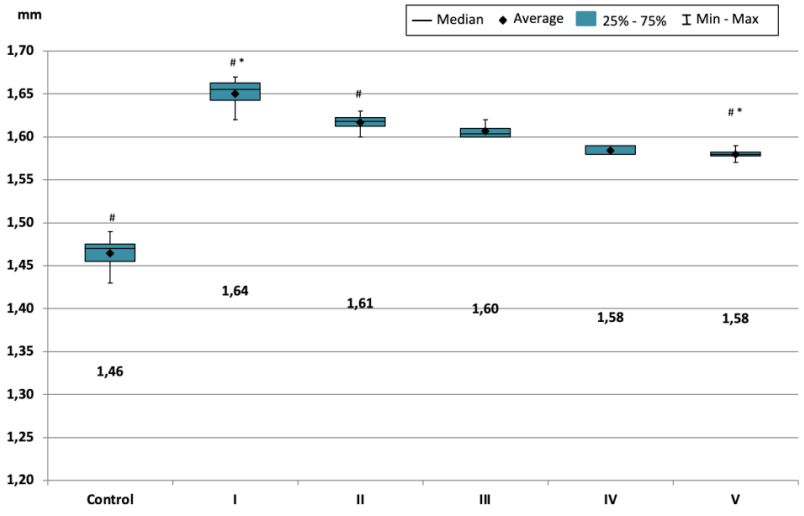

Figure 5. The image highlights how RVID (mm) shows a significant decrease when drug therapy started; group I – MCT without treatment; group II – Sildenafil; group III – Sildenafil + Rosuvastatin; group IV – Sildenafil + Magnesium sulfate; Group V — Sildenafil + Rosuvastatin + Magnesium sulfate; # ($p<0,001$) group C vs. groups I, II and V. * ($p<0,001$) group I vs. group V.

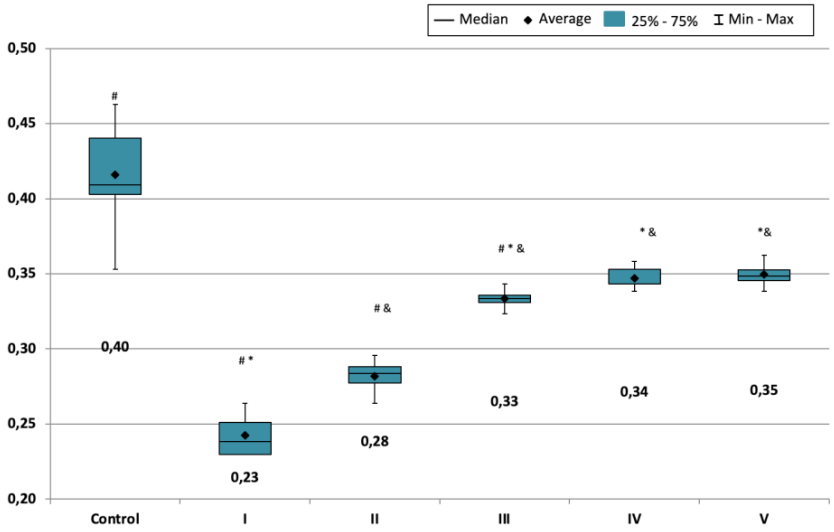

Figure 6. The image shows the graphical representation of the PAAT/PAET ratio in group C and groups I-V under treatment; group I – MCT without treatment; group II – Sildenafil; group III – Sildenafil + Rosuvastatin; group IV – Sildenafil + Magnesium sulfate; Group V – Sildenafil + Rosuvastatin + Magnesium sulfate; # ($p<0,001$) group C vs. groups I, II and III. * ($p<0,001$) group I vs. groups III, IV, and V. & ($p<0,001$) group II vs. groups III, IV, and V.

In the statistical analysis of the PAAT/PAET ratio, significant differences ($p<0.001$) were observed between groups I-III, I-IV, I-V, II-III, II-IV, and II-V. From the statistical data, we can observe significant differences between group I, exposed only to MCT, and the rest of the groups under dual or triple treatment combinations. Compared with group II (receiving only Sildenafil) it is evident that the combination Sildenafil + Magnesium sulfate (Group IV) or Sildenafil + Rosuvastatin + Magnesium sulfate (group V) improves this parameter. No significant differences exist between groups IV and V (as shown in figure 6). It can be concluded that Magnesium sulfate and Rosuvastatin improve PAH by reducing PVR, but the values obtained do not reach the parameters of the control group.

Biochemical parameters

In the statistical analysis of the ET1 values in the PA tissue sample, significant differences ($p<0.001$) were observed between groups C and III, III and IV. As can be seen in table I, the highest ET1 level appears in group III under treatment with Sildenafil + Rosuvastatin in comparison to group C. The lowest value was recorded in group IV (Sildenafil + Magnesium sulfate). Significant differences were recorded between these two groups ($p<0.001$), suggesting that Magnesium sulfate is essential in inhibiting ET1 production.

In the statistical analysis of iNOS values in the PA tissue sample, significant differences ($p<0.001$) were observed between groups C and I, I-V and II-V. Compared to group C, group I, exposed to MCT alone, recorded significant iNOS increases. However, the highest iNOS value appears in group II (under Sildenafil treatment), as shown in table I. The lowest iNOS value appears in group V (under triple therapy). In this case, the triple combination in group V (Sildenafil + Rosuvastatin + Magnesium sulfate) through the combination of Rosuvastatin with Magnesium sulfate results in a significant ($p<0.001$) reduction of this parameter.

In the statistical analysis of the BNP values in RV tissue samples, significant differences were observed between group C and group I, as well as between groups I-III, I-IV, and I-V, as can be seen in table I. The highest BNP value appeared in group I, exposed only to MCT, with a subsequent reduction trend of this value under medication. A significant decrease ($p<0.001$) could be observed in group IV (Sildenafil + Magnesium sulfate) and in group III (Sildenafil + Rosuvastatin). Rats under triple therapy (Sildenafil + Rosuvastatin + Magnesium sulfate) also showed a significant BNP value reduction. Therefore, Magnesium sulfate and Rosuvastatin in MCT-exposed rats significantly reduce BNP compared to Sildenafil alone.

In the statistical analysis of VEGF values in both PA and RV tissue samples, significant differences ($p<0.001$) were recorded between groups C and I, II, and III, as well as between groups I-III and III-V. As shown in table I, group I exposed only to MCT has a high VEGF level, which, once drug therapy is initiated, records a significant decrease ($p<0.001$) under Sildenafil + Rosuvastatin (group III) or under triple therapy (Sildenafil + Rosuvastatin + Magnesium sulfate). The triple drug combination leads to an important reduction of VEGF values, approaching group C. There were no differences between the PA and RV tissue samples evaluated.

In the statistical analysis of NO metabolites (nitrate and nitrite) values in the PA tissue sample, significant differences ($p<0.001$) between group C and groups I, II and V were recorded, as well as between groups I-III, I-V, II-III, and III-V. These statistical results show that the highest values of NO metabolites are recorded in group I exposed only to MCT, and in group II, treated only with Sildenafil, as shown in table I. Nevertheless, after administering Sildenafil + Rosuvastatin (group III) or (Sildenafil + Magnesium sulfate), group IV showed a significant decrease in these values. However, the lowest values of NO metabolites were recorded in the triple therapy group ($p<0.001$).

Table I. Values of the five tested biochemical parameters in the Control group and in groups I-V.

	ET1-PA (ng/ml)	iNOS-PA (ng/ml)	BNP-RV (pg/ml)	VEGF-PA (pg/ml)	NO metabolites-PA (µmol/l)
C	23.9±8.2 #	2.1±1.6 #	134.8±34.3 #	95.4±18.8 #	1.1±0.1 #
I	194.3±81.7	7.6±0.9 #*	522.4±22 #*	420.8±51 #*	5.7±0.3 #*
II	137.3±083.7	13.4±5.7 *	322.4±22	262.7±46.4 #	6.3±0.5 #&
III	275.7±23.3#*	2.1±0.09	226±6.6 *	229.3±15 #*&	3.2±0.1 *
IV	101.9±29.7 *	3.6±2.1	215.8±23.6 *	202.3±51.8	3.4±0.4
V	153.6±37	1.062±0.037 *	223.6±16.8 *	123±8.7 &	1.8±0.08 #*&

[1] Values are expressed as mean ± SD. Group I – MCT without treatment; group II – Sildenafil; group III – Sildenafil + Rosuvastatin; group IV – Sildenafil + Magnesium sulfate; Group V - Sildenafil + Rosuvastatin + Magnesium sulfate; # ($p<0.001$) group C vs. group I, II and III; * ($p<0.001$) group I vs. group III, IV and V. For ET-1 - # ($p<0,001$) group C vs. group III; * ($p<0.001$) group III vs. group IV. For iNOS - # ($p<0.001$) group C vs. group I; * ($p<0.001$) group II vs. group V. For BNP — # ($p<0.001$) group C vs. group I; * ($p<0.001$) group I vs. groups III, IV, V. For VEGF - # ($p<0.001$) group C vs. groups I, II and III; * ($p<0.001$) group I vs. group III and & ($p<0.001$) group III vs. group V. For NO metabolites — # ($p<0.001$) group C vs. groups I, II, and group V; * ($p<0.001$) group I vs. groups III and V, &($p<0.001$) group III vs. group V.

Discussion

Regarding the MCT-induced PAH experimental model in the present study, we can say it demonstrates both through measurement of organ mass and through echocardiographic measurements that it fulfilled many required characteristics: the Fulton index, an indirect marker of RV hypertrophy, recorded a mean value above 0.34 in group I that was exposed only to MCT. A Fulton index >0.34 predicts 94% sensitivity and 97% specificity PAH development and RV hypertrophy [22]. Ultrasonography remains an essential technique in evaluating experimental models since it is easy to use and noninvasive. It is subject to numerous technical errors and is meant for experienced medical personnel, and sedation is required. Clinical trials have demonstrated the validity of multiple echocardiographic parameters through invasive hemodynamic measurements [22,23]. Thus, statistically very highly significant increased values of both RVAW and RVID were observed in group I. For adult rats, a normal RVID range is 1.42 ±0.19 mm [23]. This RV dilatation results from PAH leading to increased PVR and negatively influencing all compensatory mechanisms that maintain normal RV function. The PAAT/PAET ratio of about 0.22 in group I is an essential echocardiographic criterion. In contrast, a PAAT/PAET ratio ≤ 0.25 predicts the development of PAH with 77% sensitivity and 80% specificity and correlates with RV systolic pressure >35 mmHg on invasive hemodynamic measurements [22,24]. Regarding biochemical parameters, in group I exposed only to MCT, the highest BNP values were found in RV tissues (BNP being a marker of myocardial ischemia and high ventricular wall stress). VEGF (endothelial dysfunction marker) recorded the highest values in both tissue types we evaluated (PA and RV) due to the pressure and volume overload to which RV myocytes are exposed and to the increased pressure of the PA.

Therefore, based on all the data described above, we can state that the MCT-PAH is a successful experimental model that has fulfilled anatomical, pressure, and biochemical characteristics that support this fact.

Regarding Sildenafil monotherapy benefits in the current study, they can be stated to be modest regarding all parameters evaluated. There was an inhibition of RV hypertrophy progression and a modest reduction in RV systolic pressure, but no significant results.

The Sildenafil + Rosuvastatin combination has been studied in MCT-PAH, where it has been shown to play a positive role in RV function and in inhibiting angiogenesis in the pulmonary vascular bed [13]. In the present study, the Sildenafil + Rosuvastatin combination in group IV resulted in the lowest body mass of the animals. It showed significant results on the Fulton Index compared to group I (no medication). It also demonstrated benefits on RVAW, RVID, and PAAT/PAET thickness compared to group II (on Sildenafil monotherapy). There were also modest decreases in BNP, VEGF, and NO metabolites (nitrite/nitrate) compared to Sildenafil alone. Therefore, the additive effects of Rosuvastatin + Sildenafil in our study are important in reducing the RV hypertrophy degree and improving RV systolic pressures. Hepatotoxicity due to statins can be prevented using low doses [13].

In this study, the dual combination of Sildenafil + Magnesium sulfate used in group IV showed a significant improvement in the echocardiographic parameters of RV hypertrophy. Still, it also significantly reduced systolic pressures in the PA. The PAAT/PAET ratio >0.25 also improved under the dual combination compared to Sildenafil monotherapy. Modest differences in the parameters described above were recorded between the dual combinations used: Sildenafil + Rosuvastatin or Sildenafil + Magnesium sulfate. The ET1 value is much lower in this group than in group III (Sildenafil + Rosuvastatin) described above. However, the decrease in other parameters (BNP, iNOS, VEGF, NO metabolites is modest compared to Sildenafil monotherapy or Sildenafil + Rosuvastatin combined).

The triple drug combination Sildenafil + Rosuvastatin + Magnesium sulfate shows highly significant results compared to the previously described drug combinations. The lowest RVAWd and RVIDd values (measured via echocardiography) were recorded in group V. Moreover, a PAAT/PAET ratio >0.25 was observed in groups IV and V. It can be concluded that Magnesium sulfate has important effects in reducing RV systolic pressure (thanks to its potent vasodilator and antioxidant effects). The lowest biomarker values - VEGF, NO metabolites, and iNOS - were also recorded in group V. However, a slight increase in BNP values can be observed in group V compared to group IV (but not statistically significant).

ET1 is a highly potent vasoconstrictor factor released by the vascular endothelium in response to pressure and volume overload in the right side of the heart. It is involved in this level's vascular remodeling, pulmonary arteriolar contraction, and fibrosis. Clinical trials have shown that increased levels of this biomarker correlate with hemodynamically measured mean pressure in PA [25]. At the same time, ET1 is an important prognostic marker correlating with PAH progression and treatment response [9,26]. The benefit of the endothelin receptor antagonist in PAH treatment has been proven in many clinical trials, and it is a therapeutic class recommended in medical guidelines [1]. In the present study, the highest ET1 value was recorded in group III, while the lowest appeared in group IV. The paradox is that Rosuvastatin has an unfavorable effect on the ET1 value. However, other studies support the benefit of Rosuvastatin in reducing oxidative stress and, thus, ET1 values [27]. It should be highlighted that this study is based on a small number of animals, and larger-scale studies are necessary.

Regarding BNP, we chose to evaluate the BNP

values in the RV tissue sample because BNP is a molecule released by cardiac myocytes in response to increased cardiac pressure, volume overload, and RV distension. The BNP assay of RV tissue provides targeted information on this PAH-induced myocardial stress. Magnesium sulfate and Rosuvastatin, or the triple combination, provide superior benefits in reducing this parameter [9,28].

VEGF is an angiogenic modulator involved in pathological vascular remodeling, with elevated values in patients with PAH [29]. VEGF values were also evaluated in the PA and RV tissue samples. VEGF is released into the bloodstream due to endothelial dysfunction and smooth muscle cell proliferation in the pulmonary circulation. It is involved in vascular remodeling; therefore, this study evaluated it in the PA tissue sample. Recent data have emphasized that cardiomyocytes from the heart are a source of VEFG-A, which express VEGFR1 and VEGFR2 receptors on the cell surface. VEGF-A activates cardiomyocytes by inducing contractility and inflammation and stimulates cytokine release. Increased amounts of VEGF have also been found in various cardiovascular pathologies; therefore, it is associated with unfavorable prognosis. We harvested RV tissue samples to objectify this fact. In this study, triple therapy significantly reduced this parameter.

Oxidative stress leads to pulmonary vascular endothelial diffusion and may alter vascular tone. PA endothelial cells regulate vascular tone and lung remodeling by producing vasodilators (NO) or vasoconstrictors (ET1) factors. As is well known, NO is produced through the oxidation of L-arginine by a family of enzymes: endothelial NOS (eNOS) and iNOS in various cells. Plasma NO levels are related to eNOS values or, in some pathologies, to iNOS values, which is hyperactivated by proinflammatory cytokines and oxidative stress [30]. iNOS is a key mediator of immune activation and inflammation, implicated in the progression of multiple pathologies [31]. The activation of iNOS will generate NO in a 1000-fold more significant measure than eNOS [32]. However, this hyperactivation has the opposite effect, as NO will interact with superoxide anion to produce peroxynitrites involved in tissue injury. Since these biochemical parameters are directly related to vascular endothelial dysfunction, we preferred to measure them from the PA tissue sample.

For this reason, NOS inhibitors have been researched in various clinical trials [33]. NO biotransformation occurs in the body via multiple pathways. The primary oxidative metabolites are nitrite and nitrate. The microenvironment redox conditions determine their level [34]. Circulating nitrite levels estimate endothelial NO formation, and nitrates may be useful in estimating nitrogen/NO turnover [35]. NO metabolites may be beneficial in certain clinical conditions because they can be reduced to NO once again [30,36]. All NO produced may contribute to the modulation of blood flow and vascular tone. The increased amount of NO metabolites in various diseases can be explained through the hyperactivation of iNOS due to proinflammatory status.

In this study, both iNOS and NO metabolites recorded the highest values in group II (treated only with Sildenafil). Still, a significant decrease can be noticed after the association of Rosuvastatin and/or Magnesium sulfate ($p<0,001$). This can be explained by the fact that Sildenafil monotherapy is ineffective in reducing oxidative stress at the cellular level in MCT-PAH rats. However, drug combinations with Rosuvastatin and Magnesium sulfate implicitly reduce these markers, thanks to their benefits on endothelial function, oxidative stress, and vascular pathological remodeling.

According to the latest diagnostic and treatment guideline for PH, published in 2022 [1], Sildenafil is a class I indication treatment in PAH. In this study, Sildenafil offers modest results in reducing RV hypertrophy parameters and RV systolic pressure measurement parameters. By the results obtained, this study does not dispute the vasodilator benefits offered by Sildenafil in clinical practice. However, this study may open new horizons toward other therapeutic approaches. Statins, through their pleiotropic effects associated with Sildenafil therapy, could enhance the benefits of Sildenafil in PAH patients. Magnesium is an essential intracellular cation with proven vasodilator, antioxidant, and anti-inflammatory effects. Magnesium supplementation, in addition to standard Sildenafil therapy, may be beneficial in reducing RV systolic pressures in PAH patients. In this study, the combination of these drugs showed the most spectacular results. This triple-drug combination could be a valid option in clinical practice, but rigorous research to balance the risk/benefit is needed. Particular attention should be paid to the side effects of statins; for this reason, a low-dose therapy could be used.

This study has numerous limitations: the low number of animals included and the high number of animals that died during the experiment. Only one dose of medication was used in this study. However, the minimum effective dose of Rosuvastatin or Magnesium sulfate is still being determined, and further studies are required. The liver and kidney toxicity of the drugs and arterial blood gas analysis have also not been assessed. Magnesium sulfate is a systemic vasodilator, and we did not measure blood pressure in this study. Therefore, this side effect remained unclear.

This article is unique because it is the first preclinical study to investigate this triple drug combination in an MCT-induced PAH rat model, with significant results in all studied parameters.

Conclusions

Therefore, Sildenafil monotherapy does not provide any substantial benefit in reducing MCT-PAH. The beneficial pleiotropic effects of Rosuvastatin with long-term administration demonstrated its efficacy

in this study by reducing PVR, RV hypertrophy, and biomarkers of oxidative stress and myocardial dysfunction. However, these benefits are enhanced in combination with Magnesium sulfate. Long-term administration of Magnesium sulfate has demonstrated efficacy in this study on rodents. Nevertheless, further research is needed to establish the therapeutic potential of Magnesium sulfate and Rosuvastatin in patients with PAH.

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