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Subchronic toxicity of the pulmonary hypertension model due to low-dose monocrotaline in rats

[Toxicidad subcrónica del modelo de hipertensión pulmonar debido a dosis bajas de monocrotalina en ratas]

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Abstract

Context: The study of pulmonary hypertension is mainly based on an experimental model that induces this condition using monocrotaline. Even though this model has been in use for decades, the toxic effect of low dose monocrotaline in other systems is not well described.

Aims: To evaluate the renal and hepatic effects of monocrotaline in order to be able to better predict the pharmacodynamic impact that it could have.

Methods: Two groups of rats were used, the first one received monocrotaline following pulmonary hypertension protocol (30 mg/kg) and the second one received saline 0.9%. At day 60 blood from the vena cava was obtained and liver and kidney were extracted for histologic exam. Fulton index (right ventricle hypertrophy measurement) was used to confirm pulmonary hypertension.

Results: The monocrotaline group presents focal interstitial lymphoid infiltration and regeneration foci in the kidney as well as venous congestion of the liver in some of the animals, these changes were not found in the control group. Kidney and liver function tests showed no significant differences. These results show that low-dose monocrotaline model for pulmonary hypertension generates changes on liver and kidney; however, these alterations were not consistent, making it a viable model for evaluating new drugs in this condition.

Conclusions: The present study demonstrates that the low dose of monocrotaline (30 mg/kg) in animals exposed for 60 days does not cause consistent changes in liver and kidney; there were findings in some animals that could be caused by cardiovascular changes generated by pulmonary hypertension.

Keywords: animal models; monocrotaline; pulmonary hypertension; right ventricular hypertrophy, subacute toxicity.

Resumen

Contexto: El estudio de la hipertensión pulmonar se basa principalmente en un modelo experimental que induce esta condición usando monocrotalina. Aunque este modelo ha estado en uso durante décadas, el efecto tóxico de las dosis bajas de monocrotalina en otros sistemas no está bien descrito.

Objetivos: Evaluar los efectos renales y hepáticos de la monocrotalina con el fin de mejorar la predicción del impacto farmacodinámico que podría tener.

Métodos: Se utilizaron dos grupos de ratas, el primero recibió monocrotalina siguiendo el protocolo de hipertensión pulmonar (30 mg/kg) y el segundo recibió solución salina al 0.9%. En el día 60 se obtuvo sangre de la vena cava y se extrajeron hígado y riñón para examen histológico. Se utilizó el índice de Fulton (medición de hipertrofia del ventrículo derecho) para confirmar la hipertensión pulmonar.

Resultados: El grupo de monocrotalina tuvo focos de infiltración linfoide intersticial focal y focos de regeneración en el riñón, así como congestión venosa del hígado en algunos de los animales, estos cambios no se encontraron en el grupo de control. Las pruebas de función renal y hepática no mostraron diferencias significativas. Estos resultados muestran que el modelo de dosis bajas de monocrotalina para la hipertensión pulmonar genera cambios en el hígado y los riñones; sin embargo, estas alteraciones no fueron consistentes, por lo que es un modelo viable para evaluar nuevos medicamentos en esta condición.

Conclusiones: El presente estudio demuestra que la dosis baja de monocrotalina (30 mg/kg) en animales expuestos durante 60 días no ocasiona cambios consistentes en el hígado y los riñones; Hubo hallazgos en algunos animales que podrían ser causados por cambios cardiovasculares generados por la hipertensión pulmonar.

Palabras Clave: hipertensión pulmonar; hipertrofia ventricular derecha; modelos animales; monocrotalina; toxicidad subaguda.

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INTRODUCTION

Pulmonary hypertension (PH) is a chronic disease characterized by increased pulmonary arterial pressure and pulmonary vascular resistance, with subsequent right heart failure and death. PH is a high-cost disease that requires further research in its pathophysiology, in order to innovate in pharmacological therapy aiming to increase life expectancy and to decrease the costs associated with healthcare (Alves et al., 2017). Currently, it is considered to be a systemic disease with multiple organ involvement, and basic research should consider this approach in experimental models (Chen et al., 2018).

Since monocrotaline (MCT) generates acute pulmonary vascular damage, which progresses to myocardial hypertrophy and right heart failure (Rafikova et al., 2016), the MCT-induced PH model is the most used animal model in preclinical research, and although there are other models, literature endorses its use permanently (Absi et al., 2019; Jin et al., 2019), because it reproduces the progressive pathophysiology of the disease and the metabolic changes prior to hemodynamic symptoms (Nakai et al., 2019).

Acute toxicity by MCT was investigated due to reports of accidental consumption of herbal preparations and infusions (Copple et al., 2002). The dose used in these studies (up to 300 mg/kg) is several times higher than the one used in PH induction protocols; and mortality in animals was very high, even a few hours after injection (Lachant et al., 2018). At these doses, MCT is metabolized by CYP450, producing dehydroMCT (DHM), which is an alkylating compound that causes alterations at the level of proteins and nucleic acids (Wilson et al., 1992). DHM causes extrahepatic oxidative cell damage (Gomez-Arroyo et al., 2012).

With lower doses, the data of sub-acute and chronic systemic effects may be less significant. Therefore, it is necessary to systematically evaluate the effects of MCT with a lower dose, but still able to generate PH (30 mg/kg); and which allows the PH model to be evaluated for a longer time (Maarman et al., 2013). Based on the aforementioned, this study aims to evaluate the sub-chronic systemic effects of MCT, in order to contribute to the knowledge of the PH model for future research with pharmacological candidates for this disease. The description of the systemic effects of MCT is necessary to maintain the validity of the PH model.

MATERIAL AND METHODS

Animals

There were used sixteen adults male Wistar rats, 6 weeks of age and weighing between 250 and 300 g. Animals were housed in the University animal care facility for at least 1 week before being used in the experiments. Animals were kept in a 12-hour light/dark cycle, at a room temperature of 22°C; they received standard rodent food and water *ad libitum*. The research protocol was approved and supervised by the Animal Ethics Committee of institutional biomedical experimentation (Reference number 018-015). The study was conducted in accordance with the policy for experimental and clinical studies and the guidelines for animal use, care and experimentation established by the Universidad del Valle (Singh, 2012; Tveden-Nyborg et al., 2018).

According to the recommended sample calculation for this type of experiments, each group had 8 animals. The sample was calculated with the formula $\log\beta/\log\rho$ where β was the probability of committing a type II error (0.05) and ρ was the proportion of animals with pulmonary hypertension; Additionally, a 10% probability of loss was added (Jones-Bolin, 2012).

Experimental design

The animals were randomly assigned to groups. The group of hypertensive rats MCT (n: 8) received a single subcutaneous injection of monocrotaline (30 mg/kg) (Sigma-Aldrich, Poole, United Kingdom), dose that has been used to induce pulmonary hypertension with subsequent right

ventricular failure (Fujita et al., 2018). The negative control group of non-hypertensive rats (n=8) received a single subcutaneous injection of 0.9% saline solution.

During 60 days, the weight was checked twice a week, as well as the intake of food and water. Reproduction of the pulmonary hypertension model was confirmed by the Fulton index (Ma et al., 2016). At the end of the experiment, the animals were anesthetized with xylazine/ketamine (0.3/10 mg/kg), and right ventricle catheterization was performed to check pressure, consistent with the PH model.

Laboratory evaluation

The levels of blood urea nitrogen (BUN) and serum creatinine (Cr) were evaluated with an automatic chemistry analyzer, the blood was mixed with trichloroacetic acid, 3% (w/v), in a 1:3 ratio for 10 minutes, and centrifuged at 2000 g for 10 minutes; creatinine was determined in the supernatant, and BUN levels were determined directly from blood samples, as presented in previous studies (Banday et al., 2008). Hepatoxicity was assessed using blood levels quantification of ALT, AST and ALP-AMP by means of an AutoAnalyzer; these results are expressed in U/L, bilirubin levels were obtained with the Van den Bergh method (Ahlfors, 2000).

Morphological evaluation

After 60 days, the animals were euthanized with overdose of chemical anesthetics (3 times the anesthetic dose) and then exsanguination was performed with perfusion of 0.9% saline solution, followed by 4% paraformaldehyde buffer. For the evaluation of cardiovascular changes produced by MCT, right ventricular hypertrophy was estimated with the Fulton index; The right ventricle (RV) was dissected and separated from the left ventricle (LV) and the septum (S). The samples were weighed with an analytical balance (OHAUS, Switzerland) to determine the extent of ventricular hypertrophy expressed with the RV / (LV + S) radius (Ma et al., 2016). Livers and kidneys were removed and weighed in order to obtain their ratios of weight (liver/body weight - kidney/body weight).

Histological evaluation

After the procedures described above, the liver, kidneys and lungs of each animal were embedded in paraffin for further processing. Fixed tissues were washed twice for 30 minutes in PBS and were dehydrated in a Leica TP1020 tissue processor in 12 steps using 70%, 80%, 95% (2 times), 100% ethanol (4 times for 1 hour each), Xilol (2 times) and two steps in paraffin (Thermo Scientific Histoplast PE 8330) at 58°C for 40 minutes.

Finally, once prepared and assembled (Thermo Shandon Histocentre 3), the paraffin blocks were stored at room temperature. All samples included in paraffin were sectioned with a thickness of 4 µm with a Thermo Scientific MX35 Premier blade, in a Leica RM2245 Microtome. Subsequently, the tissues to be dyed with colorations (hematoxylin eosin, Masson's Trichromic) were mounted on Microscope Slides Frosted® plates (CAT.NO.7105)

Signs of nephrotoxicity were evaluated, including tubular atrophy, interstitial fibrosis, interstitial inflammation and glomerular morphology. The following signs of hepatoxicity were evaluated: inflammatory infiltrate, hepatocyte atrophy, necrosis, apoptosis, disruption of the lattice and regenerative changes.

These findings were evaluated by a blinded pathologist, and they were assessed with four different fields of slide under magnification (4×, 10× and 40×); a control case was indicated to compare with normal results (Copple et al., 2003).

Statistical analysis

It was made a description of the data, expressed as mean values (standard error of mean). Group comparisons were made with the unpaired Student's T test. A value of $p<0.05$ was considered statistically significant. The calculations were performed with Graph Pad Prism 6.0 software (GraphPad software, San Diego California USA).

RESULTS

None of the animals died before two months. From the non-hypertensive rats' group, one animal was discarded for reasons unrelated to the experiment. There were no differences in the monitoring of the weight of the animals in both groups.

Morphological changes

To determine cardiovascular changes, ventricular hypertrophy was measured, and the MCT group was found to have an increase in the Fulton index, demonstrating the impact of pulmonary hypertension on cardiac muscle cells. In the present study there was no difference in renal or hepatic ratios, thus dismissing the presence of nephro- and hepato-megaly in rats with exposure to a dose of 30 mg/kg MCT (Table 1).

Blood biochemistry

Due to the insufficient volume obtained for analysis or coagulation of the blood sample, 5 exams were not taken into account. A total of five blood samples of the MCT group and five of non-hypertensive rats' group were analyzed. The results were expressed as mean value (standard error). There were no differences between the MCT and the control group in none of the parameters evaluated (Table 2).

Histological findings

No findings consistent with renal alterations were found in the control group. In the MCT group, there were observed focal interstitial lymphoid infiltrate and renal regeneration foci that are associated with renal tubular alteration. No interstitial fibrosis or tubular or glomerular atrophy was found.

In the negative control group, no liver abnormalities were found. In the livers of the MCT group, poor hepatocyte apoptosis was observed, as well as vascular congestion and erythrocytes in sinusoids; these findings are consistent with the established PH and the consequent cardiac failure (Fig. 1).

Table 1. Liver, kidney and heart morphological evaluation.

Variable	Negative control	MCT
Liver ratio	3.53 (0.23)	3.20 (0.09)
Kidney ratio	3.25 (0.25)	2.78 (0.16)
Fulton index	0.23 (0.005)	0.43 (0.03)*

Each value represents the mean ± SEM of eight animals per group. *p<0.05 significant difference respect to the negative control. Student´s t-test was performed to compare the groups.

Table 2. Blood biochemistry results.

Parameter	Negative control	MCT
Total protein (g/dL)	5.53 (0.30)	6.28 (0.20)
Creatinine (mg/dL)	0.74 (0.03)	0.61 (0.06)
Alanine aminotransferase (U/L)	45.80 (10.80)	57.00 (3.70)
Aspartate aminotransferase (U/L)	139.00 (20.20)	160.10 (19.40)
Alkaline phosphatase (U/L)	170.00 (10.20)	174.70 (18.40)
Total bilirubin (mg/dL)	0.20 (0.03)	0.35 (0.11)
Direct bilirubin (mg/dL)	0.25 (0.11)	0.20 (0.09)

Each value represents the mean ± SEM. MCT (n= 5) Negative control (n=5). Student´s t-test was performed to compare the groups and no significant differences were found. g: grams; dL: deciliter; U: units; L: liter; mg: milligrams.

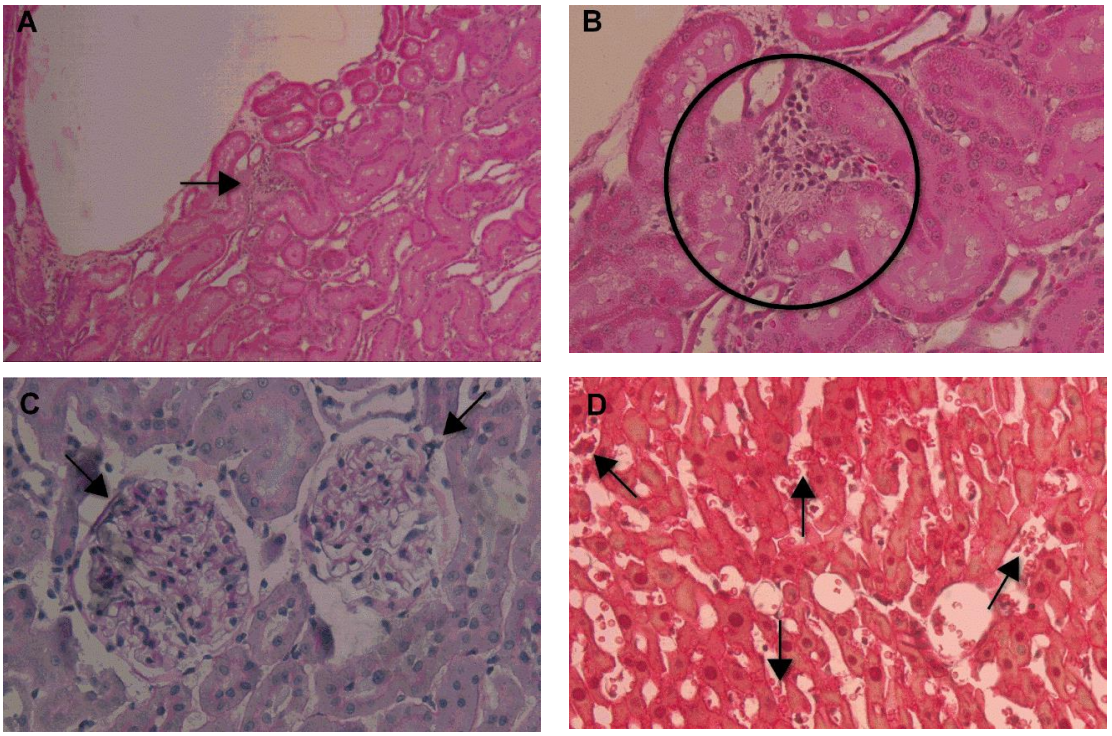

Figure 1. Representative photomicrographs of liver and kidney of rats with monocrotaline (30 mg/kg) per 60 days.

(A) Represents cells lymphoproliferative process at 10× (arrow); **(B)** represents lymphoid infiltrate in renal tissue at 40×; (**C)** represents focal renal regeneration at 40×, the arrows indicates flattened epithelium accompanied by nuclear crowding; **(D)** Represents hepatic vascular congestion at 40×, the arrows shows red blood cells in sinusoids. Photomicrographs are representative of the experimental group. Hematoxylin and eosin and periodic-acid-Schiff stain in kidney and Masson trichrome stain for liver.

DISCUSSION

The MCT-induced PH model has turned over 50 years, and it has been the most commonly used model in experimental studies of pathophysiology and pharmacology. With it, progress has been made in the knowledge of the disease; and a large part of the medications currently used have been tested in preclinical studies with this model (Hill et al., 2017).

Over time, the concept of PH has changed, from being a vascular disease to a systemic disease. Therefore, the PH study models must be validated in that direction. Systemic aspects of this toxic model have not been taken into account in large part of the experimental studies carried out so far (Lachant et al., 2018). In this study, it was used a low dose to induce PH, in order to avoid 60-day mortality and reduces the impact of cardio-pulmonary changes in other organs. This is also intended to consider a baseline from a systemic point of view when promising molecules be tested. There has been reported the potential for acute toxicity of MCT due to ingestion of large quantities of herbal medicine preparations of *Crotalaria spectabilis* (from which MCT is extracted). For this reason, MCT was implemented with high doses, to assess acute toxicity (Ahlfors, 2000).

MCT is used in different models as an inductor. In the pulmonary fibrosis model, it is applied a subcutaneous dose of 60 mg/kg at 30 days (Hill et al., 2017). In liver, different models of liver damage have been used to simulate venous occlusive damage; it has been done in dogs with 60 mg/kg i.p (Lachant et al., 2018); different doses have been used in rats for oxidative hepatotoxicity, one of

300 mg/kg i.p., and another of 10 mg/kg i.p. (Honório Junior et al., 2012). For cardiac damage and generation of right ventricular hypertrophy, the rats have been subjected to doses of 30 mg/kg s.c. (Baybutt et al., 2007). These models can be contrasted with this study, in which with a dose of 30 mg/kg s.c. with a follow-up of 60 days, there were evidenced vascular congestion and erythrocytes in hepatic sinusoid, without generating liver enzyme alterations, evidenced with the similarity in the values obtained from ALT, AST and ALP-AMP in the study groups. However, the histological findings could be secondary to established heart failure, rather than primary liver toxicity.

The protocols for PH have different doses and follow-up times; and toxic potential in other systems should be considered. Rats exposed to the traditional PH model (dose of 60 mg/kg) have a high mortality rate (Amin et al., 2014), and the cause of death is not completely clear. This "cause" has been questioned by Gómez-Arroyo et al. (2012) who ask the following question: "Are (they) rats that die from PH, or rats with PH that die from toxicity?". The present study allows to answer partially this question; it determined that at a low dose, the alterations generated in the kidney and liver are probably the result of heart failure. Additionally, it is plausible that the mechanism of action by which PH is produced in the MCT model is not purely vascular, as it is not in the current disease, especially considering that one of the hallmarks of PH is right heart hypertrophy, which is produced by MCT regardless of PH (Baybutt et al., 2007).

The high mortality of the traditional model limits the observation time, which is typically between two and three weeks. It should be noted that the model of the current research demonstrated successful induction of PH despite being a lower dose than the usual one (60 mg/kg) (Cho et al., 2010; Malikova et al., 2016); and that the follow-up period was 60 days, which improves the period for the assessment of sub-chronic toxicity. With the 30 mg/kg dose, right ventricular hypertrophy is generated (Baybutt et al., 2007), as well as an increase in the right ventricular pressure. It is necessary that the survival of the model overcome the acute stage of the disease, in order to carry out trials with medications, considering that in humans, PH is a chronic disease.

A dose-effect relationship of acute hepatic and renal systemic toxicity of MCT has been described (Weiss et al., 2019). The relevance of the PH model should be evaluated; especially in the pharmacology area, where renal and hepatic metabolism must be intact, to avoid errors in the interpretation of results. The low dose of MCT that was used in the present investigation, would be a valid alternative to simulate the progression of PH, and to increase the follow-up time, as evidenced in this research; where there was no mortality in the period of study, and the only loss was a consequence of reasons other than the experiment.

From the liver damage, elevated transaminases in liver congestion secondary to heart failure have been reported in a 60 mg/kg dose model (Xiao et al., 2017), and sinusoidal hepatic obstruction at a dose of 90 mg/kg (Ruiter et al., 2013).

PH is considered as a systemic disease (Copple et al., 2003; Hill et al., 2017) and the pharmacological targets are not limited to the cardiopulmonary system. These new target hypotheses are tested in preclinical models (Amin et al., 2014), which in the case of PH has induction with MCT as a major exponent; and to interpret the experimental results properly, it is necessary to know the baseline of changes in rats at systemic level.

Since the studies regarding toxicity by MCT have been carried out in the framework of toxicity by plants, acute models have been implemented at high or chronic doses, with oral delivery route; therefore, comparing the results obtained with these are less significant. However, it should be noted that some histological findings are similar on the present study, including vascular congestion, regeneration and infiltrates on a smaller scale (Nakai et al., 2019); these similarities also occurred in that the findings are not consistent in all animals evaluated; and although the route of delivery may explain some differences, it is postulated that other changes could be due to the time of observation and dose, demonstrating an initial damage in

this model, which would have advanced during the time or (have) developed at higher doses.

As previously mentioned, high doses can cause severe liver damage, including hepatocyte necrosis, endothelial damage of the central vein, sinusoidal hemorrhage and lymphocyte infiltration (Copple et al., 2002). These data contrast with the findings of this research, which may show the beginning of damage due to hepatic vascular congestion, but without consistency in the animals of the experimental group; this situation improves the hypothesis that with a low dose of MCT, it is possible to study pharmacological targets of PH, without causing multi-organ alterations that interfere with the obtained results.

As a limitation of this study, it is worth mentioning the difficulty of obtaining sufficient blood samples for biochemical tests in all the studied animals; this reduced the analysis of these variables. In addition, quantifications of the results obtained in the histological analysis were not performed; this because in the qualitative pathology analysis, it was concluded that there was no need for this assessment, due to the similarity of images obtained that did not show differences between the groups at study.

CONCLUSIONS

The present study demonstrates that the low dose of MCT (30 mg/kg) in animals exposed for 60 days does not cause consistent changes in liver and kidney; there were findings in some animals that could be caused by cardiovascular changes generated by PH. These findings will allow a low dose of MCT to be used for the study of PH during times longer than those regularly used (3 - 4 weeks), making it possible to simulate the chronicity of the disease in the study of new pharmacological targets.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Contribution	Benavides-Cordoba V	Silva-Medina M	Varela MX	Palacios Gómez M
Concepts or ideas	x		x	x
Design	x	x	x	x
Definition of intellectual content	x	x	x	x
Literature search	x	x	x	x
Experimental studies	x	x	x	x
Data acquisition	x	x	x	x
Data analysis	x			x
Statistical analysis	x			x
Manuscript preparation	x			x
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Manuscript review	x	x	x	x

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