

Tryptophan-5-hydroxylase, serotonin transporter protein and serotonergic receptors in dilated cardiomyopathy

Triptófano-5-hidroxilasa, transportador de serotonina y receptores serotoninérgicos en miocardiopatía dilatada

José A. Mondragón-Herrera¹, Yesenia Serrano-Hernández¹, Guillermo Valero-Elizondo², Armando Mansilla-Olivares¹, and Gabriel Manjarrez-Gutiérrez^{3*}

¹Laboratorio de Patología Molecular, Unidad de Investigación Biomolecular, Hospital de Cardiología; ²Servicio de Patología, Hospital de Cardiología;

³Unidad de Investigación en Enfermedades Neurológicas, Hospital de Especialidades. Centro Médico Nacional Siglo XXI, Instituto Mexicano del Seguro Social. Ciudad de México, México

Abstract

Background: Cardiomyocytes synthesize, utilize and reuptake serotonin, which is involved in the paracrine and autocrine modulation of heart activity and in the pathophysiology of some cardiovascular diseases. **Objective:** To determine the expression of tryptophan-5-hydroxylase (TPH) 1 and 2, serotonin transporter protein (SERT) and serotonergic receptors in hearts with dilated cardiomyopathy (DCM) compared to controls. **Method:** A comparative study was performed in six tissue blocks of the left ventricular free wall (LVWL) and inter-ventricular septum from patients who died of DCM and six who died of no cardiovascular diseases (controls). Five slices from each block were obtained to determine the expression of TPH1 and TPH2, SERT and serotonergic receptors with antibodies specific for immunofluorescence. Immunofluorescence was analyzed by Student's t-test, accepting a significance level of $p < 0.05$. **Results.** An increase in TPH1, TPH2, 5-HT_{2A} and 5-HT_{2B} receptors expression were observed in dilated structures compared to controls ($p < 0.05$). For dilated inter-ventricular septum, the 5-HT₄ receptor increased its expression ($p < 0.05$), and SERT in PLVI compared to controls ($p < 0.05$). **Conclusions.** These results suggest that the increases observed in the expression of TPH, SERT, and serotonergic receptors in hearts with DCM compared to controls could play an important role in the pathophysiology of MCD in humans.

Keywords: Dilated cardiomyopathy. Tryptophan-5-hydroxylase. Serotonin. SERT. Serotonergic receptors.

Resumen

Antecedentes: Los cardiomiocitos sintetizan, utilizan y recapturan serotonina, la cual participa en la modulación parácrina y autócrina de la actividad del corazón y en la fisiopatología de algunas enfermedades cardiovasculares. **Objetivo:** Determinar la expresión de triptófano-5-hidroxilasa (TPH) 1 y 2, transportador de serotonina (SERT) y receptores serotoninérgicos en corazones con miocardiopatía dilatada (MCD) en comparación con controles. **Método:** Estudio comparativo en seis bloques de la pared libre del ventrículo izquierdo (PLVI) y del septum interventricular de pacientes fallecidos por MCD y seis que murieron por enfermedades no cardiovasculares. Se obtuvieron cinco cortes de cada bloque para determinar la expresión de TPH1 y TPH2, SERT y receptores serotoninérgicos con anticuerpos específicos por inmunofluorescencia. La inmunofluorescencia fue analizada por la t de Student, aceptando un nivel de significancia de $p < 0.05$. **Resultados:** Se observó un aumento en la expresión de TPH1 y TPH2 y en los receptores 5-HT_{2A} y 5-HT_{2B} en las estructuras dilatadas en comparación con las controles ($p < 0.05$). El receptor 5-HT₄ aumentó su expresión en el septum interventricular dilatado ($p < 0.05$) y el SERT

*Correspondence:

Gabriel Manjarrez-Gutiérrez

E-mail: willisga@prodigy.net.mx; gmanjarrezg@gmail.com

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en la PLVI en comparación con los controles ($p < 0.05$). **Conclusiones:** Estos resultados sugieren que los aumentos observados en las expresiones de TPH, SERT y receptores serotoninérgicos en corazones con MCD en comparación con controles podrían desempeñar un papel importante en la fisiopatología de la MCD en los humanos.

Palabras clave: Miocardiopatía dilatada. Triptófano-5-hidroxilasa. Serotonina. SERT. Receptores serotoninérgicos.

Introduction

Several lines of evidence suggest that tryptophan-5-hydroxylase (5-HT) or serotonin regulates the cardiovascular function during embryogenesis and adult life¹⁻³. 5-HT is synthesized by intestinal enterochromaffin cells, it is secreted into the blood⁴ and stored in the platelets⁵. Plasmatic 5-HT can also be captured by sympathetic neuron⁶ and endothelial cells of the blood vessels^{7,8}. The effect of 5-HT on the cardiovascular system is complex; for instance, 5-HT has been related to bradycardia or tachycardia, hypotension or hypertension, vasodilation or vasoconstriction⁹⁻¹², and on the physiopathology of several cardiovascular diseases associated with the increase of plasmatic 5-HT levels, such as in carcinoid syndrome^{13,14}, valve disease^{15,16}, arrhythmia, atrial fibrillation, and atrioventricular block in patients that receive 5-HT reuptake inhibitors^{17,18}. It has been proven that the activation of the 5-HT receptor_{2B} in the heart, causes a proliferation of fibroblasts and subendocardial fibrosis^{16,17,19}. Additionally, 5-HT regulates cardiomyocyte activity in rats with heart failure by means of 5-HT receptors_{2A} and 5-HT₄; both induce a positive inotropic effect through the phosphorylation of the myosin lightweight chain^{20,21}. On the other hand, serotonin release by platelets significantly contributes to the regulation of cardiac fibroblasts, promoting their migration and differentiation through the activation of the 5-HT receptor_{2A}²². With real-time polymerase chain reaction and immunohistochemistry techniques, the expression of 5-HT receptors_{2B}, 5-HT_{1B}, and 5-HT_{2C} in the interstitial cells of heart valves in humans; these receptors can be activated by the levels of plasmatic serotonin^{16,23}. Furthermore, the presence of 5-HT producing mast cells and the expression of tryptophan-5-hydroxylase (TPH) during cardiogenesis²⁴ and in interstitial cell in the adult heart valves²⁵.

All these interesting data beg the question regarding the origin of 5-HT in the heart. In human beings, the myocardium contains approximately 0.4 µg of 5-HT/g of tissue²⁶, and their origin is not clear. It has been suggested that it comes from the local synthesis by

TPH2 in autonomic lymph nodes²⁷, or that it is captured by the serotonin transporter (SERT) of the plasmatic deposits. However, there is evidence for the presence of TPH1 and TPH2, SERT, and 5-HT serotoninergic receptors_{1B}, 5-HT_{2A} and 5-HT_{2B} in normal rats²⁸ and with diabetic cardiomyopathy²⁹. Recently, they have also been reported in hearts of deceased humans due to causes other than cardiovascular disease³⁰. Therefore, these findings, as a whole, suggest the presence of an intrinsic serotonergic system that participates in autocrine and paracrine modulation of heart function in humans²⁸⁻³⁰.

Based on these findings, the purpose of this work was to determine TPH1 and TPH2, SERT, and 5-HT serotoninergic receptors_{2A}, 5-HT_{2B} and 5-HT₄ in the cardiomyocytes of the free wall of the left ventricle and interventricular *septum* of deceased patients due to dilated cardiomyopathy (DMC) compared to a control group.

Method

A comparative study was performed in six tissue blocks of the left ventricular free wall (LVWL) and *inter-ventricular septum* from patients who died of DCM and six who died of no cardiovascular diseases (controls). Tissue samples were obtained from the pathology archives of the Cardiology Hospital del Centro Médico Nacional Siglo XXI del Instituto Mexicano del Seguro Social y del Hospital General de México, SSA. Since there is no history of a similar study in the literature, the sample size was adapted to the availability of the tissues. Five cuts of PLVI and SIV were obtained in each of the tissue blocks of each group, with the objective of determining the expressions of TPH1 and TPH2, SERT and serotoninergic receptors 5-HT_{2A}, 5-HT_{2B} and 5-HT₄ by immunofluorescence using specific antibodies for each of the molecules, which were observed and quantified on a Nikon Ti Eclipse inverted confocal microscope equipped with an AI imaging system, both controlled by the NIS Elements v.5.50 software.

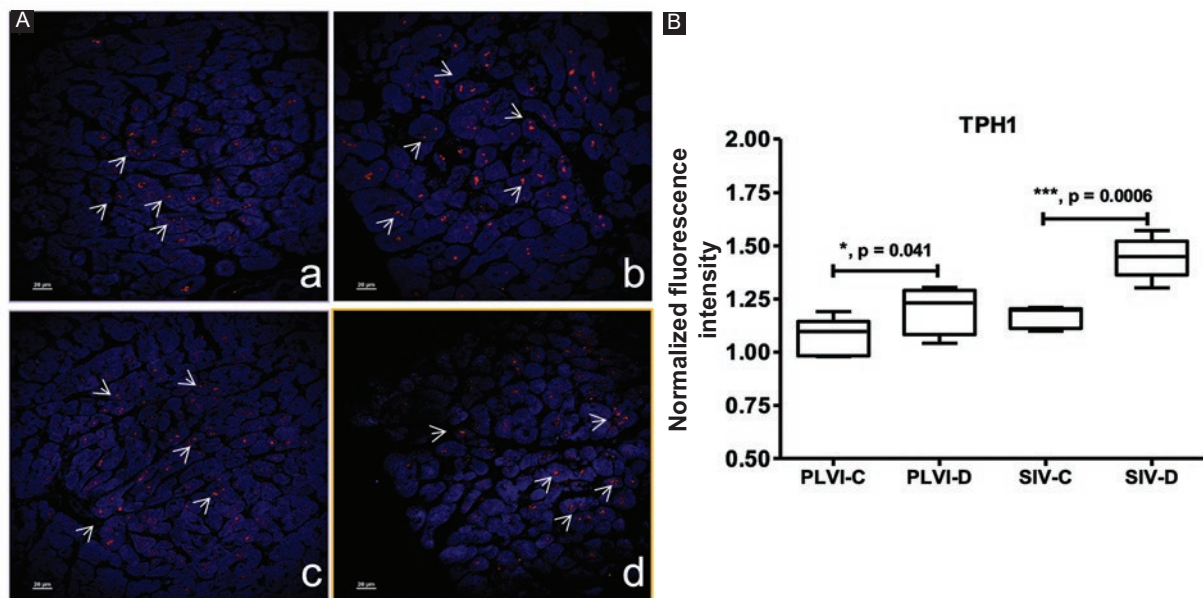


Figure 1. A: cross-transversal micrographs of PLVI (a, control; b, dilated) and SIV (c, control; d, dilated) showing TPH1 immunoreactive cardiomyocytes. The arrows point to cytoplasm immunoreactivity. Scale 40x, -20 μ M. **B:** IFN of PLVI and SIV. Each bar represents the medians $\pm$ standard deviation of five determinations in each group. The difference between groups was obtained by means of Student's *t* test. **p* < 0.05. ****p* < 0.001.

Immunofluorescence for TPH1 and TPH2, SERT, and 5-HT serotonergic receptors_{2A}, 5-HT_{2B} and 5-HT₄

Heart sections of 4 μ m of thickness were placed on electrically charged slides. Dewaxing of the sections was made in xylol and alcohol, and they were hydrated in PBS. Afterward, they were blocked with the Blocking Fluorescence Merk-Millipore, USA for 20 minutes in a wet chamber at 37°C. Afterward, the sections were incubated with monoclonal primary antibodies specific for TPH1 (Gene Tex International Corp) and TPH2 (Abcam, Cambridge, UK), and polyclonal for SERT (Chemicon, USA), 5-HT_{2A}, 5-HT_{2B} and 5-HT₄ (Santa Cruz, Biotechnology Inc, USA) at a dilution of 1:500 in a fluorescence block solution. The next day, the sections were washed with PBS-Tween at 0.1%, and then, they were incubated with Alexa Fluor 564 anti rabbit at a dilution of 1:500 for 2 hours. At the end, the sections were mounted with ProLong Diamond Antifade Mountant con DAPI (Life Technologies Corporation, USA), for immunofluorescence, and they were later analyzed with an inverted confocal microscope Nikon Ti Eclipse equipped with an AI image system, both controlled by the NIS Elements v.5.50 software. The images were obtained with a 40x lens

(1.3 oil). The coloring used was excited in a sequential manner using a 405 nm (DAPI) and a 564 nm (Alexa 564) laser line. The corresponding fluorescence was acquired within the 401-472 nm (DAPI) and 570-620 nm (Alexa 564) ranges, using the filter kits provided by the manufacturer. The images were analyzed using the NIS Elements v 5.50 software.

Statistical methods

The expressions of TPH1 and TPH2, SERT and serotonergic receptors 5-HT_{2A}, 5-HT_{2B} and 5-HT₄ in PLVI and SIV are obtained through the fluorescence intensity of each of the molecules in each of the groups. First, the medians and standard deviations were obtained, followed by the normalized fluorescence intensity (NFI). Afterward, the groups were compared to the Student *t* test, accepting a significance level of *p* < 0.05.

Results

Figure 1A shows the TPH1 expression in cardiomyocytes in LVWL and SIV in both groups. NFI of the enzyme increased significantly in both structures of hearts with DMC compared to the control groups (LVWL *p* = 0.041,

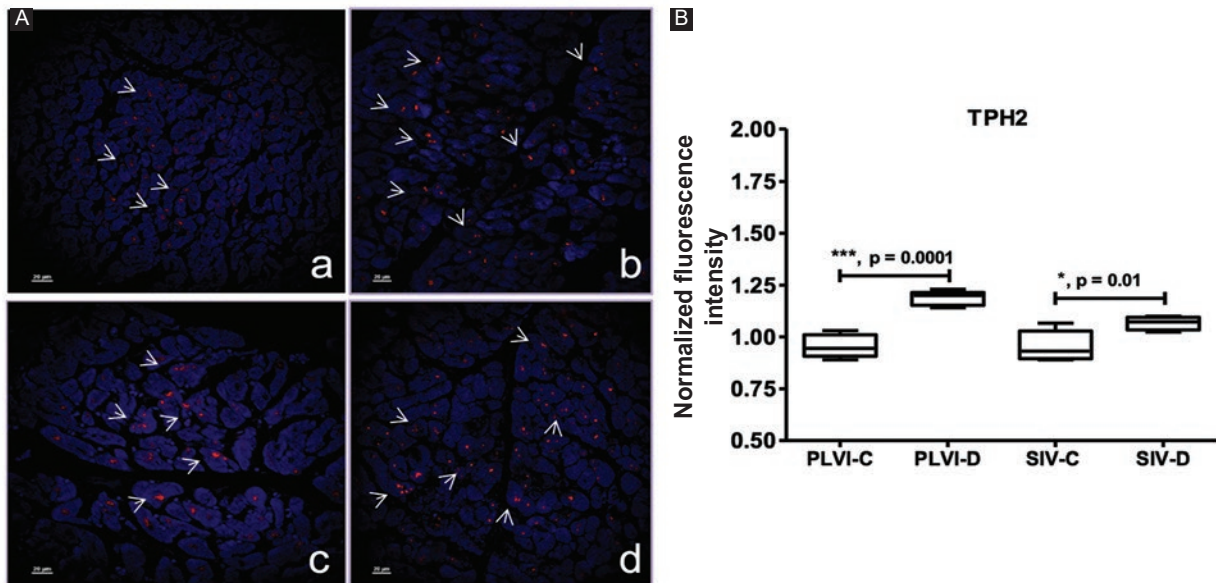


Figure 2. A: cross-transversal micrographs of PLVI (a, control; b, dilated) and SIV (c, control; d, dilated) showing TPH2 immunoreactive cardiomyocytes. The arrows point to cytoplasm immunoreactivity. Scale 40x, $-20\ \mu\text{M}$. **B:** IFN of PLVI and SIV. Each bar represents the medians $\pm$ standard deviation of five determinations in each group. The difference between groups was obtained by means of Student's *t* test. **p* < 0.05. ****p* < 0.001.

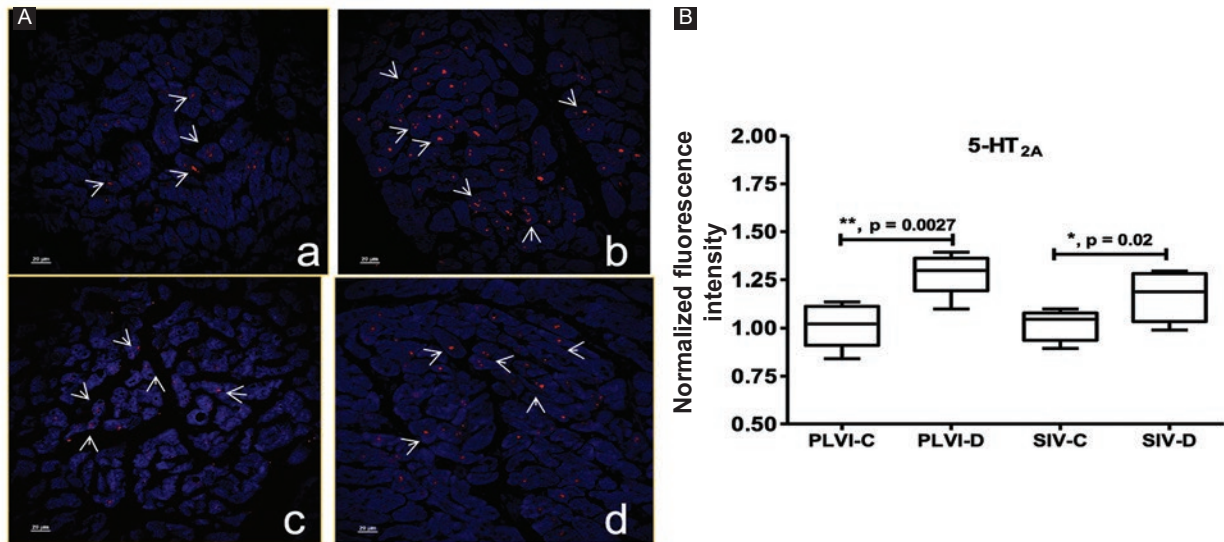


Figure 3. A: cross-transversal micrographs of PLVI (a, control; b, dilated) and SIV (c, control; d, dilated) showing 5-HT immunoreactive cardiomyocytes. The arrows point to cytoplasm immunoreactivity. Scale 40x, $-20\ \mu\text{M}$. **B:** IFN of PLVI and SIV. Each bar represents the medians $\pm$ standard deviation of five determinations in each group. The difference between groups was obtained by means of Student's *t* test. **p* < 0.05. ***p* < 0.01.

median differences (MD) -0.124 ± 0.063 , *p* < 0.05; SIV, *p* < 0.0006, DM -0.277 ± 0.048 , *p* < 0.001) (Fig. 1B). Regarding the TPH2 expression shown by cardiomyocytes in hearts with DMC and controls, it is shown in Figure 2 A. It must be noted that TPH2 expression increased in

both dilated structures compared to the controls (PLVI, *p* = 0.0001, DM -0.231 ± 0.029 , *p* < 0.001; SIV, *p* = 0.01, DM -0.111 ± 0.039 , *p* < 0.05) (Fig. 2B).

The expression of the 5-HT_{2A} in cardiomyocytes of LVWL and SIV in hearts of both groups is shown in

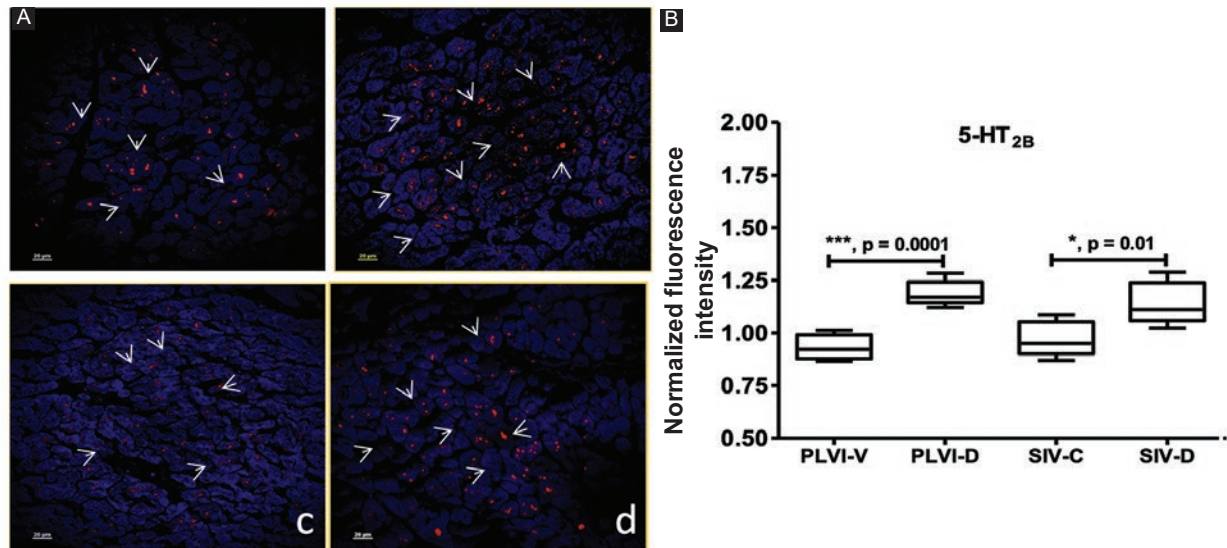


Figure 4. A: cross-transversal micrographs of PLVI (a, control; b, dilated) and SIV (c, control; d, dilated) showing 5-HT immunoreactive cardiomyocytes_{2B}. The arrows point to cytoplasm immunoreactivity. Scale 40 $\times$, $\sim$ 20 μ M. **B:** IFN of PLVI and SIV. Each bar represents the medians $\pm$ standard deviation of five determinations in each group. The difference between groups was obtained by means of Student's t test. * $p < 0.05$. *** $p < 0.001$.

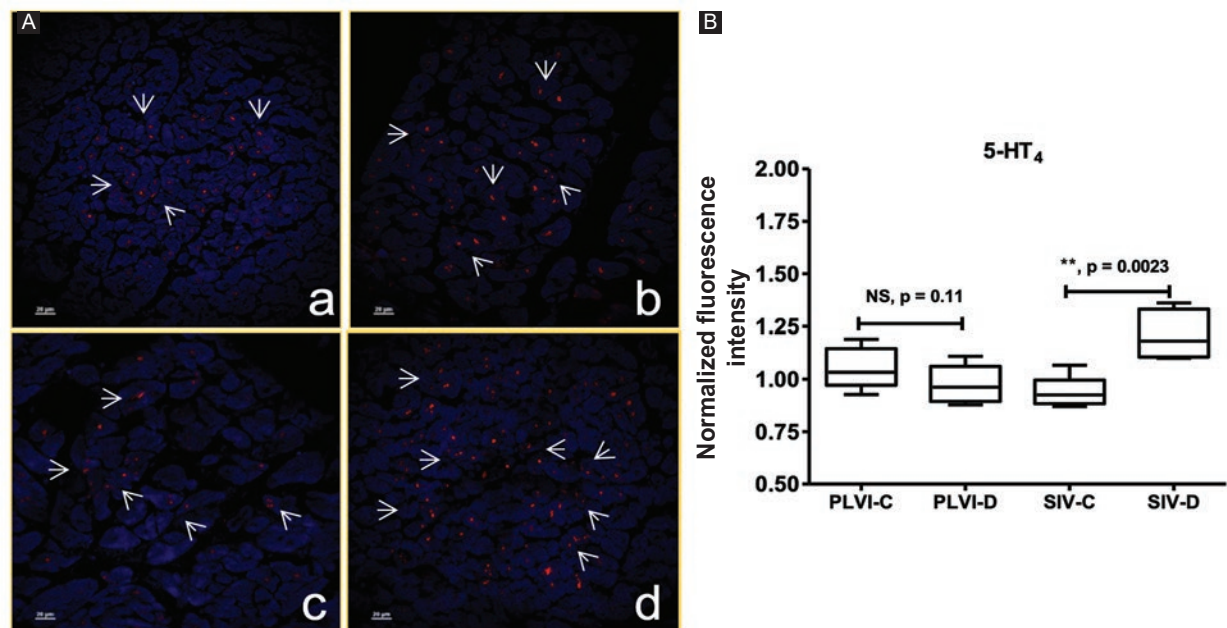


Figure 5. A: cross-transversal micrographs of PLVI (a, control; b, dilated) and SIV (c, control; d, dilated) showing 5-HT immunoreactive cardiomyocytes₄. The arrows point to cytoplasm immunoreactivity. Scale 40 $\times$, $\sim$ 20 μ M. **B:** IFN of PLVI and SIV. Each bar represents the medians $\pm$ standard deviation of five determinations in each group. The difference between groups was obtained by means of Student's t test. * $p < 0.001$. NS: not significant.

figure 3A. As shown in figure 3B, NFI shown by the 5-HT receptor_{2A} was significantly higher in both structures of hearts with DMC compared to controls (LVWL $p = 0.0027$, DM -0.268 ± 0.070 , $p < 0.01$; SIV, $p = 0.02$,

DM -0.148 ± 0.067 , $p < 0.05$). Figure 4A shows the expression of 5-HT receptor_{2B} in hearts of both groups; NFI significantly increased in cardiomyocytes of both dilated structures compared to controls (LVWL, $p = 0.0001$,

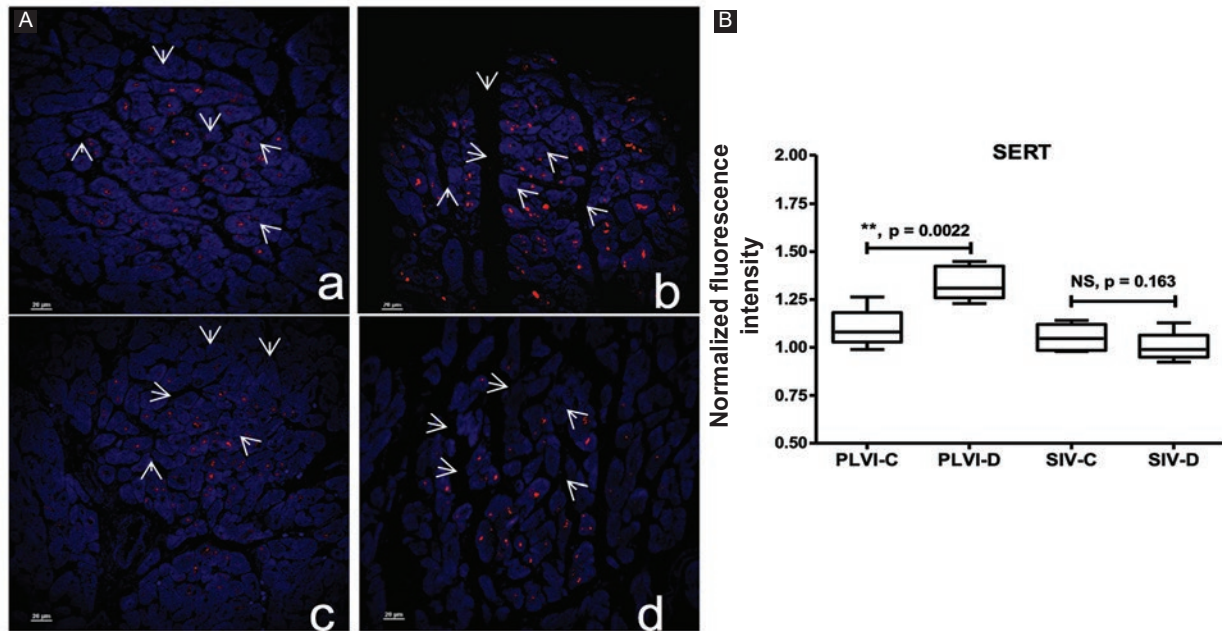


Figure 6. A: cross-transversal micrographs of PLVI (a, control; b, dilated) and SIV (c, control; d, dilated) showing SERT immunoreactive cardiomyocytes. The arrows point to cytoplasm immunoreactivity. Scale 40x, ~20 μ M. **B:** IFN of PLVI and SIV. Each bar represents the medians $\pm$ standard deviation of five determinations in each group. The difference between groups was obtained by means of Student's *t* test. ***p* < 0.01. NS: not significant.

DM -0.256 ± 0.037 , *p* < 0.001; SIV, *p* = 0.01, DM -0.169 ± 0.058 , *p* < 0.05) (Fig. 4B).

Figure 5A shows the expression of 5-HT receptor₄ in the cardiomyocytes of LVWL and SIV. The NFI of the LVWL receptor did not show significant differences in both groups; however, the dilated SIV increased significantly when compared to the control (*p* = 0.0023, DM -0.269 ± 0.065 , *p* < 0.01) (Fig. 5B). The expression of SERT in cardiomyocytes of both groups is shown in figure 6A. A significant increase in NFI in LVWL in hearts with DMC compared to controls (LVWL *p* = 0.0022, DM -0.235 ± 0.059 , *p* < 0.01) was found, and there were no differences in the SIV between both groups (Fig. 6B).

Discussion

The main purpose of our study was to determine the expression of TPH1 and TPH2, SERT, and 5-HT serotonergic receptors_{2A}, 5-HT_{2B} and 5-HT₄ in LVWL and SIV cardiomyocytes in hearts of patients who died from DMC compared to hearts of patients who died from non-cardiovascular conditions. The present work confirms previous results observed in cardiomyocytes located in the structures of normal hearts in rats and humans that express both isoforms

of TPH, SERT and serotonergic receptors 5-HT_{2A}, 5-HT_{2B} and 5-HT₄^{28,30}, and enlarges cardiomyocytes from hearts with MCD. It is important to point out that LVWL and SIV of hearts with DMC showed a significant increase of the expressions of both isoforms of TPH and 5-HT_{2A} and 5-HT_{2B}. The 5-HT receptor₄ only increased in the SIV and SERT in the LVWL compared to control hearts. The increase of the expression of the isoforms of TPH in dilated structures suggest that cardiomyocytes in these regions of the heart have the ability to synthesize serotonin, which would contribute to autocrine and paracrine modulation of cardiomyocyte activity³⁰.

In the same manner, the increases observed in the expressions of 5-HT_{2A} and 5-HT₄ receptors in hearts with DMC suggest that these receptors could contribute to the electrical activity of cardiomyocytes and the regulation of the ventricular contraction strength during the evolution of DMC, similarly to what was reported in an experimental model for acute ventricular failure²⁰. This study also observed a significant increase in the expression of 5-HT_{2B} receptor in both structures of hearts with DMC compared to control heart structures. It has been shown that this receptor regulates heart development and functions^{31,32}, and there is evidence showing that its overexpression in

mice cardiomyocytes induces a mitochondrial proliferation within the same molecular mechanisms that has been proposed in the transition of the hypertrophy of cardiomyocytes to heart failure³³⁻³⁵, which are commonly seen in the pathophysiology of DMC^{36,37}. In addition, a significant increase in SERT expression in LVWL in dilated hearts was shown compared to control hearts. The SERT increase in cardiomyocytes may actively contribute to the dynamic between the reuptake and catabolism of serotonin in human hearts with DMC^{2,3,38}.

Conclusions

With all available information and the results obtained, we can confirm that DMC causes significant increases in the expression of TPH, SERT, and serotonergic receptors in cardiomyocytes compared to control cardiomyocytes. Therefore, these increases as a whole could play an important role in the development of the pathophysiology of DMC in humans.

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Conflicts of interest

The authors state they have no conflicts of interest.

Ethical disclosures

Protection of people and animals. The authors declare that no experiments were carried out on humans or animals for this research.

Data confidentiality. The authors declare that no patient data appear in this article.

Right to privacy and informed consent. The authors have received the approval of the Ethics Committee for the analysis and publication of the clinical data obtained routinely. The informed consent of the patients was not required as this is a retrospective observational study.

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