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Full Length Research Paper

Diabetes Mellitus Causes Changes in the Heart Intrinsic Serotonergic System During the Development of the Cardiomyopathy

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The main objective of the study was to explore some of the intrinsic serotonergic system changes suffered during the development of diabetic cardiomyopathy (DCM). The diabetes mellitus model by streptozotocin (STZ) was used with their respective controls. Hearts were obtained at 7, 14 and 21 days after STZ to evaluate: tryptophan-5-hydroxylase (TPH) activity and serotonin (5-HT) concentration measured by high-resolution liquid chromatography. Besides, it was determined the expression of 5-HT_{1B}, 5-HT_{2A}, and 5-HT_{2B} receptors, as well as the serotonin transporter (SERT) expression by means of Western blot analyses. At day, 7, diabetic rats showed a remarkable decrease of activity and expression of the TPH1 and of the 5-HT concentration ($P < 0.001$), without any change on TPH2 expression. At day 14 it was also observed a decrease in the expression of SERT and 5-HT_{2B} receptors ($P < 0.001$), and an increase in 5-HT_{2A} receptors' expression ($P < 0.001$). No changes were observed of 5-HT_{1B} expression. These results suggest that the observed alterations may play an important role in the development of the DCM, which is frequently observed in diabetic patients.

Keywords: Diabetes, Cardiomyopathy, Heart, Serotonergic system.

INTRODUCTION

Cardiovascular disease is the foremost cause of morbidity and mortality in diabetic patients. In fact, diabetes mellitus type 2 increases the risk of coronary heart disease, leading to a 50% of the deaths. Meanwhile diabetes mellitus type 1 is the cause of death in 25% of patients (Cases, 2002; Taegtmeyer et al., 2002; Thierer, 2006). It has been observed that normotensive diabetic patients, without coronary artery disease, frequently present ventricular dysfunction (Frustaci et al., 2000; Nessler and Skrzypek, 2006; Bell, 2003; Galderisi, 2006; Álvarez and Lice, 1988; Shimoni and Rattner, 2001). These findings allow us to propose the possible involvement of some different type of pathophysiogenic mechanism in the diabetic cardiomyopathy (DCM)

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(Frustaci et al., 2000; Nessler and Skrzypek, 2006; Bell, 2003; Galderisi, 2006; Álvarez and Lice, 1988; Shimoni and Rattner, 2001).

The DCM is defined as a myocardial disease not related to atherosclerosis of coronary vessels. It is produced by cellular stiffness and loss of the ventricular compliance, secondary to myocardial fibrosis, microvasculature changes, structural collagen alterations and modifications in the cardiomyocytes' sarcoplasmic reticulum (Frustaci et al., 2000; Nessler and Skrzypek, 2006; Bell, 2003; Galderisi, 2006; Álvarez and Lice, 1988; Shimoni and Rattner, 2001). Recent scientific evidence support the existence of an intrinsic serotonergic system in the rat heart, which besides playing an important role in heart physiology, it is involved in the pathophysiology of different cardiovascular disorders (Manjarrez et al., 2015). In addition, it seems that serotonergic receptors 5-HT_{1A}, 5-HT_{2A}, 5-HT_3, 5-HT_4 and 5-HT_7 are also related to cardiovascular function, triggering bradycardia or tachycardia, hypotension or hypertension, vasodilation or vasoconstriction (Nebigil and Maroteaux, 2001; Frishman and Grewall, 2000; Ramage and Villalon, 2008; Cöte et al., 2003; Ovisgstad et al., 2005). These receptors are located in nerve terminals, but recently they have also been shown in cardiomyocytes (Nebigil and Maroteaux, 2001; Frishman and Grewall, 2000; Ramage and Villalon, 2008; Cöte et al., 2003; Ovisgstad et al., 2005). On the other hand, it has been previously demonstrated the presence of the serotonin transporter (SERT) in cardiomyocytes, and in the interstitial cells of the heart valves of fetal and adult rats (Mekontso et al., 2006; Gustafsson et al., 2005; Sari and Zhou, 2003; Pavone et al., 2007), and have also shown their involvement in the development and remodeling of heart tissue. Furthermore, heart 5-HT plays an important role in the pathophysiology of valvular dysfunction associated with the activation of SERT, as a result of an increased level of 5-HT in plasma, which is a characteristic of patients with Carcinoid Syndrome or in those receiving management with the 5-HT_{2B} receptor antagonistor with SERT inhibitors (Mekontso et al., 2006; Elangbam et al., 2005; Levy, 2006; Connolly et al., 1997; Fitzgerald et al., 2000; Robiolio et al., 1995). Likewise, it is known that the activation of the 5-HT_{2B} receptor in cardiac tissue produces fibroblastic mitogenesis and subendocardial fibrosis (Mekontso et al., 2006; Gustafsson et al., 2005; Sari and Zhou, 2003; Pavone et al., 2007; Elangbam et al., 2005) and also it has been shown that in heart failure in the rat, cardiomyocytes, are under the influence of 5-HT through the 5-HT_{2A} and 5-HT_4 receptors (Qvisgstad et al., 2005; Braattelid et al., 2012). In fact, the activation of the 5-HT_{1B} receptor on the surface of fibroblasts induces the production of collagen (Mekontso et al., 2006). Besides, the presence of cardiac mast cells, which express tryptophan-5-hydroxylase have been recently reported during cardiogenesis, suggesting that these cells are able to produce serotonin, also they have (Manjarrez et al., 2009) been found in the valve interstitial cells of the adult heart in rats (Disatian et al., 2010). On these bases, we propose that Diabetes Mellitus (DM) may produce a remarkable influence on the heart intrinsic serotonergic system, triggering the mechanisms that eventually lead to the development of cardiomyopathy in the diabetic rat.

MATERIALS AND METHODS

Seventy male Wistar rats, weighing 250 ± 10 g were kept for two weeks under the following environmental conditions: Temperature 22 ± 2°C, light and darkness cycles of 12 h, relative humidity, 50-60% and minimal noise and handling. In order to decrease any possible variations in the circadian rhythm, handling and dissection were carried out between 09.00 and 11.00 h, throughout this period, all the experimental models were fed with rodent diet lab pellets (Nutritional International, Brent Wood, MO, USA) and water *ad libitum*. After an adaptation period of 14 days, the animals were randomly divided into two groups: Group D or the diabetic group, which included 40 rats that were treated with 65 mg/Kg of STZ,i.p, (Sigma Chemical Co. St Louis, USA), diluted in citrate buffer 0.1 M, pH 4.5; and group C (the control group) which included 30 rats exclusively submitted to the vehicle by the same route. At days 7, 14 and 21 after treatments and after sedation with C02, rats were sacrificed by cervical dislocation, in accordance to health standards and ethics of the Internal CINVESTAV Committee for the Care and Use of Laboratory Animals; their hearts were then dissected out and washed three times with Hartman solution for biochemical and immunohistochemical assays. Rats were obtained from the animal facility of CINVESTAV-IPN (Mexico City). The research protocol was also previously approved by the Research Committee of the Cardiology Hospital, CMN-SXXI, IMSS. The management of the experimental animals was conducted in the Production Unit and Laboratory of Animal Experimentation (UPEAL, CINVESTAV-IPN), in accordance to the Official Mexican Norm (NOM-062-ZOO-1999).

Biochemical assays

TPH activity was measured by 5-hydroxytryptophan production using high-performance liquid chromatography (HPLC) with fluorometric detection as previously described (Johansen et al., 1995; Johansen et al., 1996). Briefly: a supernatant solution of heart homogenate (300 µg protein) was incubated with 0.05 M tris buffer, pH 7.40, 1.0 mM EGTA, 15 µg catalase, 200 µM 2-amine-4-hydroxy-l-methyl tetrahydrobiopterine ($6MPH_4$). The reaction was performed at 37 ^{0}C for 10 min and stopped by the addition of 10 µl of 6 M $HClO_4$ containing 5 mM EDTA and 0.1 % (wt/vol) ascorbic acid.

Twenty µL of this reaction mixture were injected into the HPLC (Waters and Waters model 474 scanning fluorescence detector). A symmetry C_{18} column (5 µm particle size), 3.9 x 150 mm (Waters) was used. The mobile phase was prepared with sodium acetate 40 mM, pH 3.30 and acetonitrile, in a relation 95:5 respectively at one ml/min.

Heart 5-HT was determined by HPLC and the fluorescence method of Peat and Gibb 1983 (Peat and Gibb 1983). Briefly; samples were homogenized in 0.1 M perchloric acid containing 4 mM sodium bisulfite. Homogenates were centrifuged at 15,000 g for 15 min. A 20 µL volume of supernatant were injected into the HPLC (Waters Corporation and Waters model 474 scanning fluorescence detector). A symmetry C_{18} column (5-µ particle size), 3.9 x 150 mm (Waters Corporation) was used. The mobile phase was prepared with potassium monobasic phosphate (2 mM, pH 3.50), containing 1 g/L of heptanosulfonic acid sodium salt and a mixture of methanol double-distilled deionized water (3:2).

Immunotransference of TPH1 and 2, SERT, 5-HT_{1B} 5-HT_{2A} and 5-HT_{2B} by Western blot

Hearts from each group were homogenized for 30 sec at 4 °C in a Tris-HCl 50 mM solution, pH 7.4, plus protease inhibitors (Protease Inhibitor Cocktail, Sigma-Aldrich). Afterwards, several samples were centrifuged at 10,000 rpm for 15 min at 4°C. The Bradford method was employed to quantify protein concentration; 30 µg of protein was then placed in each of the 1-mm-thick channels of 12% SDS-polyacrylamide gel. Electrophoretic run was done at 100 V for 2 h. For electrotransference, the gel was mounted on nitrocellulose membranes and the run was carried out at 10 V, 1.30 mA for 45 min. Nitrocellulose membranes with the transferred proteins were placed in a blocking solution (Millipore Chemiluminescent blocker) at 50% for 30 minutes to be incubated with a primary antibody, specific for TPH1 (Rabbit monoclonal IgG, Gene Tex Inc., Irvine, CA), TPH 2 (Rabbit polyclonal, Merck-Millipore) and antibodies for SERT (Rabbit polyclonal Santa Cruz Biotechnology, Santa Cruz, CA), 5-HT_{1B}, 5-HT_{2A} and 5-HT_{2B} receptors (Rabbit polyclonal, Santa Cruz Biotechnology, Santa Cruz, CA) at a dilution of 1:500 in the same blocking solution. The following day, membranes were incubated with Goat anti-rabbit IgG (H+L)-HRP conjugate secondary antibody (Bio Rad., Laboratories 2000. Alfred Nobel. Dr. Hércules, CA 94547) at a dilution of 1:5000 in the same blocking solution. Afterwards membranes were immediately incubated with the ABC Elite system (Vector Laboratories, Burlingame, CA) (Naish et al., 1989) for 1 h. Then, washed with PBS, and revealed with diaminobenzidine (DAB). The Internal control was glyceraldehyde-3-phosphate dehydrogenase (GADPH).

Statistical method

Data were evaluated and matching the results of serotonergic activity (5-HT and TPH activity). And the relative optical densities of bands TPH1, TPH2, SERT, 5-HT_{1B}, 5-HT_{2A} and 5-$HT_{2B,}$ obtained by Western, blot was determined. Averages and standard deviations of each group were first calculated, and once having demonstrated non-normal distributed variables, both groups were matched by means of Mann–Whitney U test; $P< 0.05$ was accepted as statistically significant.

RESULTS

Hearts obtained from group D at day 7, showed a remarkable decrement of TPH activity and of 5-HT concentration, when compared to the control group ($P< 0.001$) (Figure 1). TPH1 and TPH2 expression, measured by Western blot, is shown in Figure 2. Three immunoreactive bands appeared in both groups, D and C, at 7, 14 and 21 days after STZ administration, a 51 kDa band for TPH1, a 53 kDa for TPH2, and a 37 kDa band for GADPH, used as an internal control for each experiment (Figure 2A). It must be pointed out the remarkable decrease in TPH1 expression observed in group D at day 7 in contrast to controls ($P<0.01$); nevertheless, TPH2 expression was equivalent to that found in the control group (Figure 2B). Figure 3A illustrates SERT expression and GADPH. Two bands were observed, a 70-kDa band representing SERT, and the internal control band of 37-kDa. It can be noticed that group D showed a significant decrease in SERT expression at day 7, in contrast to controls ($P< 0.01$) (Figure 3B).

With regard 5-HT_{2A}, and 5-HT_{2B} receptors' expression, they can be analyzed in Figure 4, as expected, two immunoreactive bands, one of 53 kDa that represents the 5-HT_{2A} receptor expression, and other band of 55 kDa representing 5-HT_{2B} (Figure 4A). Figure 4B illustrates the immunopositivity expressed by these receptors in both groups. It is interesting to remark the increased immunoreactivity shown by the 5-HT_{2A} receptor in the diabetic heart tissue at day 14, in contrast to controls ($P<0.001$); whereas, the immunoreactivity shown by the 5-HT_{2B} receptor in group D at day 7, was significantly lower than in the control group ($P<0.01$).The expression of 5-HT_{1B}receptors is shown in Figure 5. There was a 47kDa band in diabetic animal's heart tissue compared to controls; its expression was similar in both groups throughout the study.

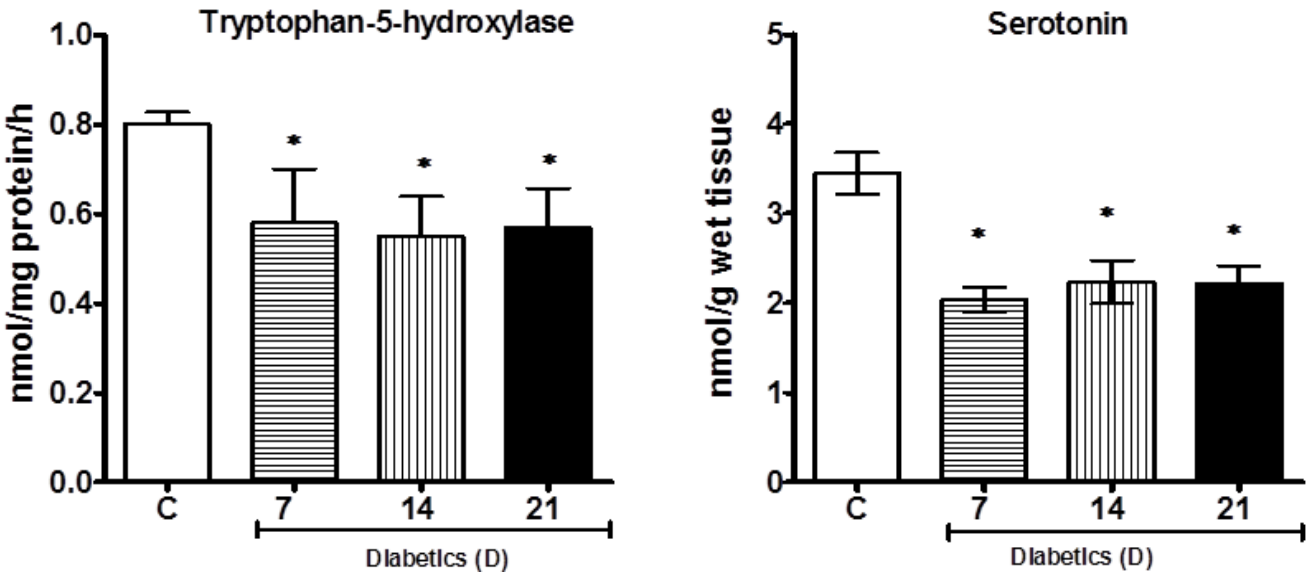

Figure 1. Serotonergic activity in the heart. C, controls and D, diabetics. Each bar represents the mean value ± SD from six experiments from in each group. All determinations were performed in duplicate samples. The difference between groups was obtained using Mann-Whitney U test. * P < 0.001

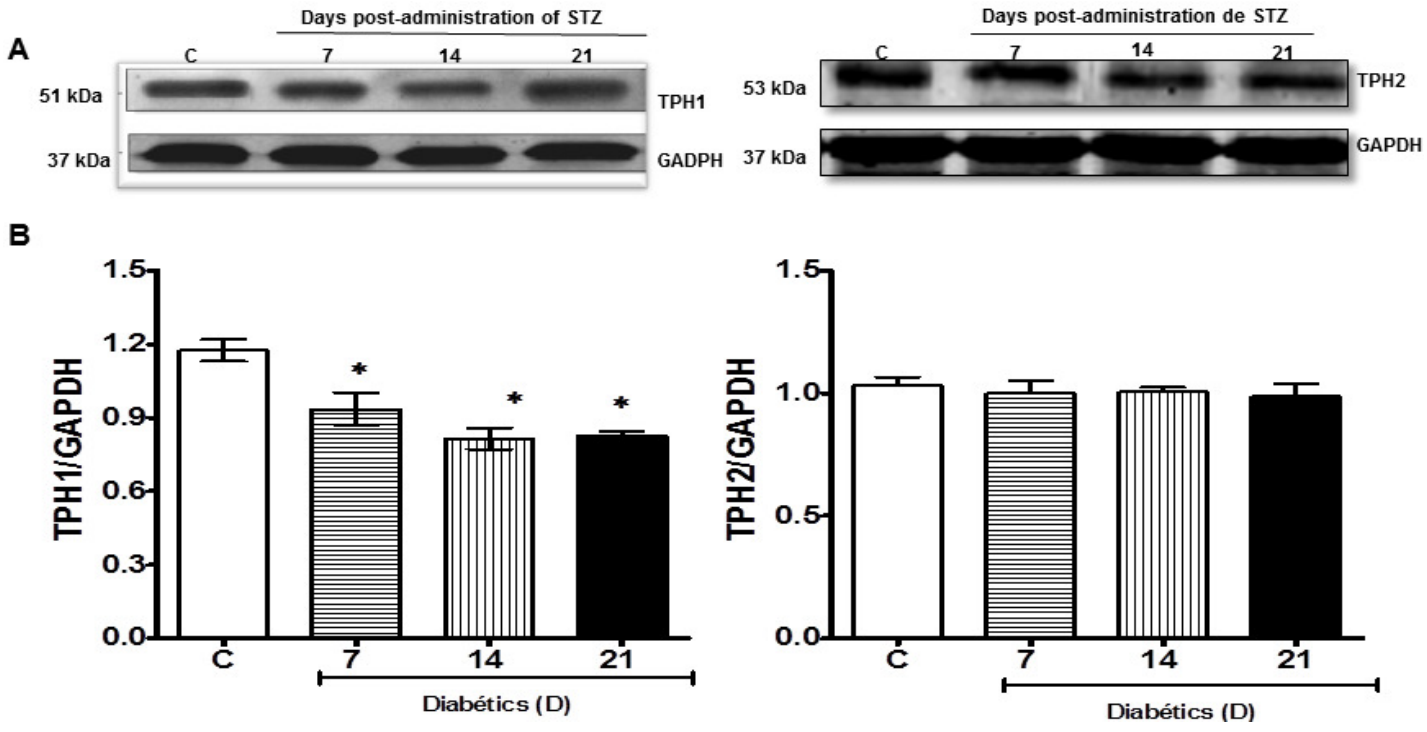

Figure 2. Expression of TPH 1 and 2 in the heart. C, controls and D, diabetics. A) Immunoreactivity detected by electrotransference, with specific antibodies for each isoform, two bands were observed, one 51 kDa (TPH1), other of 53 (TPH2) and 37 kDa to GAPH as an internal control. B) Relative optical density of each isoforms. Each bar corresponds to the mean value ± SD of six experiments in duplicate samples. The difference between groups was obtained by Mann-Whitney U test. * P < 0.01

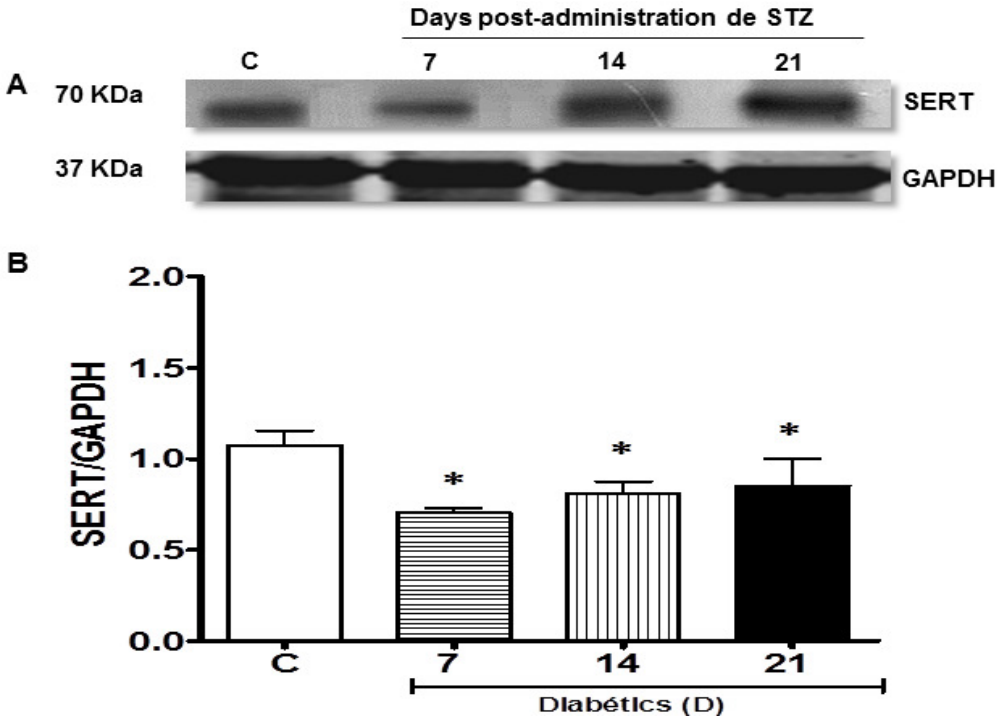

Figure 3. Expression of SERT in the heart. C, controls and D, diabetics. A) Immunoreactivity detected by electrotransference 7, 14 and 21 days post-administration of STZ, with specific antibodies, one band of 70 kDa (SERT) was observed and another of 37 kDa (GADPH) as an internal control. B) Relative optical density of SERT. Each bar corresponds to the mean value ± SD of six experiments in duplicate samples. The difference between groups was provided by Mann-Whitney U test * $P < 0.01$

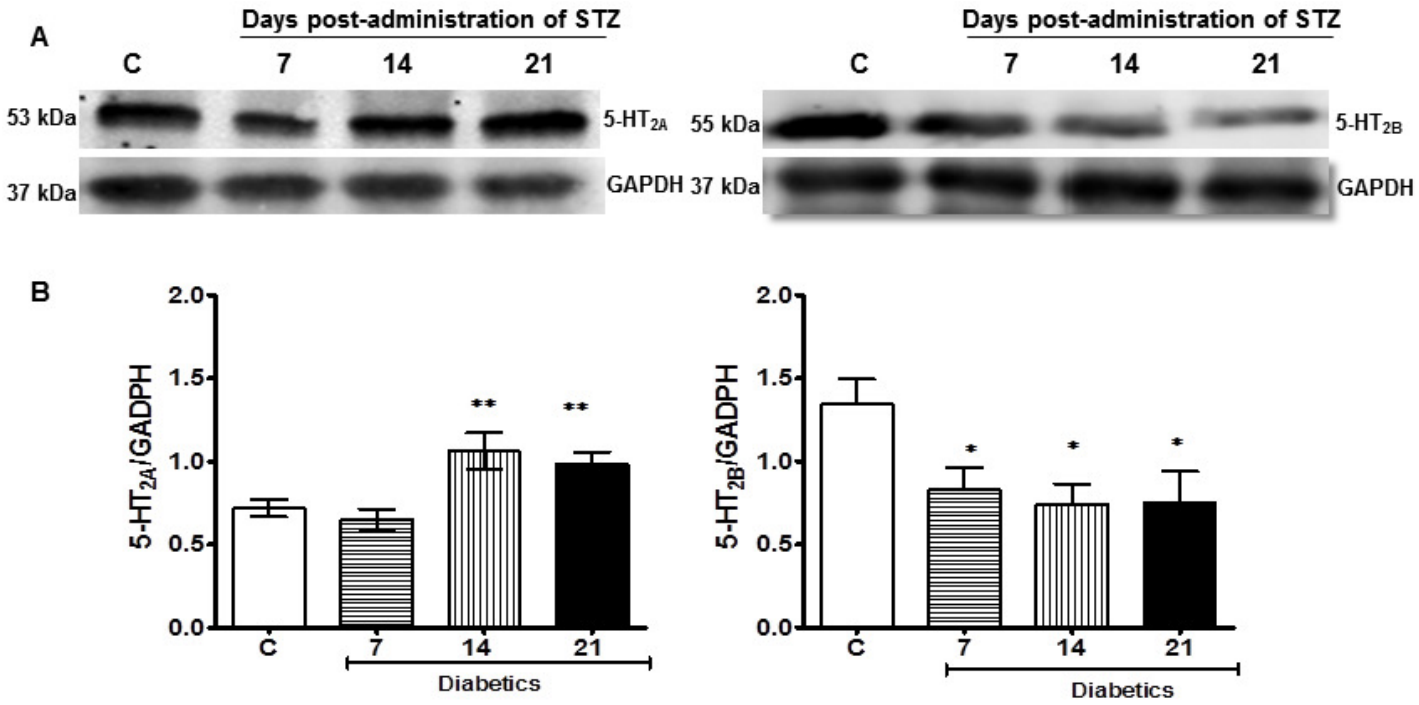

Figure 4. Expression of 5-HT_{2A} and 5-HT_{2B} receptors in the heart. C, controls and D, diabetics. A) Immunoreactivity detected by elctrotransference, with specific antibodies for each receptor, three bands were observed: 53 kDa (5-HT_{2A}), 55 kDa (5-HT_{2B}) and other of 37 kDa (GADPH). B) Relative optical density of receptors. Each bar corresponds to the mean value ± SD of six experiments in duplicate samples. The difference between groups was obtained by Mann-Whitney U test. *P< 0.01; **P< 0.001

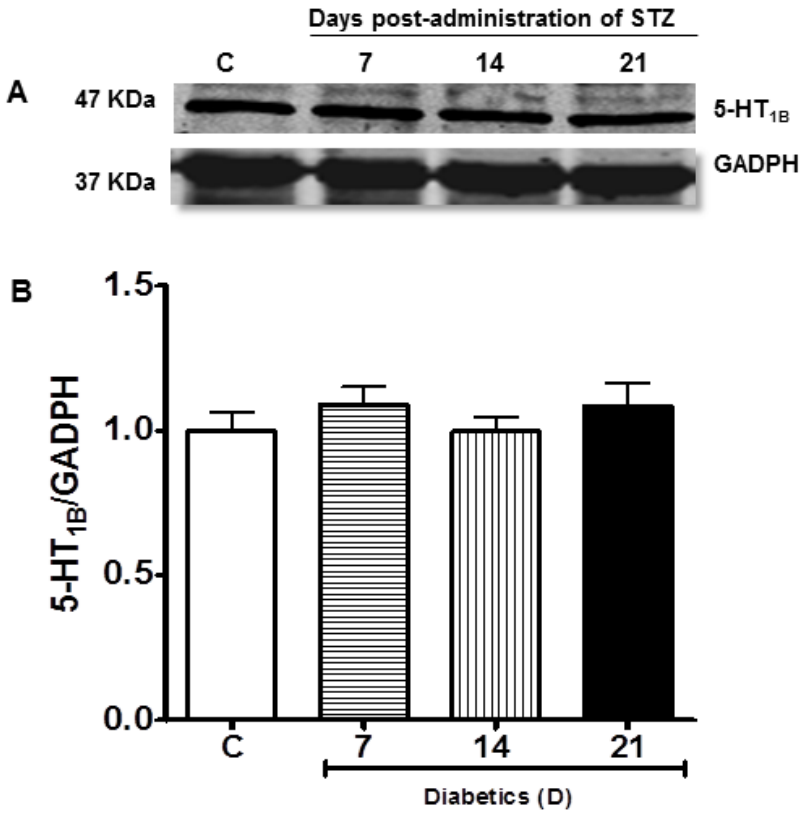

Figure 5. Expression of 5-HT_{1B} receptor in the heart. C, controls and D, diabetics. A) Immunoreactivity detected by electrotransference, with specific antibodies for 5-HT_{1B}receptor, two bands were observed, 47 kDa (5-HT_{1B}) and other of 37 kDa (GADPH). B) Relative optical density of 5-HT_{1B} receptor. Each bar corresponds to the mean value ± SD of six experiments in duplicate samples. There was no difference between groups by Mann-Whitney U test

DISCUSSION

It has been shown recently, that cardiomyocytes synthesize, use and recapture 5-HT, by means of an intrinsic serotonergic system actively involved in the equilibrium that must be held between autocrine and paracrine heart activity. (Manjarrez et al., 2015). The main objective of this study was to explore the intrinsic serotonergic system changes that cardiomyocytes suffer during the development of the diabetic cardiomyopathy. It is very interesting to notice that diabetic rat cardiomyocytes show a significant decrease in 5-HT concentration and in TPH activity, in accordance with a low expression of TPH1, and in contrast to TPH2, strongly suggesting a failure in 5-HT synthesis, just as it happens in TPH1 knockout rats, which also develop dilated cardiomyopathy (Cöte et al., 2003). In accordance too, it was also observed a significant decrease in 5-HT concentration in heart tissue of the diabetic rats, which let us propose a possible cardiomyocyte defect in the interactive relationship between actin and myosin by means of Transglutaminase 2.

It has previously been also demonstrated that low insulin levels lead to remarkable changes in the structure of actin, myosin and microtubules, giving rise to structural and functional cytoskeleton cardiomyocyte deterioration (Frustaci et al., 2000; Nessler and Skrzypek, 2006; Bell, 2003; Galderisi, 2006; Alvarez and Lice, 1988; Shimoni and Rattner, 2001). These type of mechanisms could explain the early diastolic dysfunction that diabetic patients show during the first stages of the disease (Herrera et al. 2014).

It has also been described that SERT, a key cytoplasmatic protein related to 5-HT dynamics, appears in cardiomyocites since the fetal life (Sari and Zhou, 2003; Pavone et al., 2007), and in the endothelial cells of capillaries and coronary vessels in the adult heart , where it plays an important role in the reuptake and catabolism of 5-HT (Manjarrez et al., 2015; Mekontso et al., 2006; Pavone, et al. 2007). Our results show the capability of controls to sustain 5-HT reuptake and as a consequence, to maintain myocardial function; however, in the experimental model there was a significant decrease of SERT expression during the evolution of the diabetic

state, just as it has been observed in other studies carried in 5-HTT-KO mice, in which the animals develop myocardial infarction, valvular fibrosis, myocardial dilation and left ventricular dysfunction. These facts also support the role that 5-HT plays in the development of several cardiovascular diseases in adult diabetics (Mekontso et al., 2006; Noorlander et al., 2008).

In the present work there were other interesting findings; such as the variable expression of 5-HT_{1B}, 5-HT_{2A} and 5-HT_{2B} heart receptors in both groups. For instance, taking into account how the 5-HT_{2A} receptor is related to the triggering of the electrical cardiomyocyte activity, and to the regulation of the ventricular myocardial force contraction in acute heart failure (Qvisgstad et al., 2005), it is possible to support that the significant increase of 5-HT_{2A} receptor expression in the hearts of the diabetic animals, could probably explain the compensatory positive inotropic mechanism, described during the initial states of the diabetic cardiomyopathy, which could appears also in the diabetic patient (Nessler and Skrzypek, 2006; Bell, 2003; Galderisi, 2006; Álvarez and Lice, 1988). Likewise, there are some reports about the structural and functional changes linked to dilated cardiomyopathy, developed during cardiogenesis as a result of the ablation of the 5-HT_{2B} receptor; (Choi et al., 1997; Nebigil et al., 2001) whereas, its over-expression during the adult life leads to cardiac hypertrophy (Nebigil et al., 2003). Thus, the significant decrease of the 5-HT_{2B} receptor expression here observed, in the diabetic group may as well, have a role in the pathophysiology of the diabetic dilated cardiomyopathy. There are some reports about the influence that the 5-HT_{1B} receptor exerts in the subendocardium of the heart valves triggering fibroblastic proliferation, (Seuwen et al., 1988) in this work no change in its expression was detected, during the development of the diabetic cardiomyopathy.

On the whole, this research study shows the significant changes in the intrinsic serotonergic system during the development of the diabetic cardiomyopathy. Moreover, it was demonstrated a remarkable decrease in TPH1 activity and in 5-HT concentration, possibly related to the decrease in SERT and in 5-HT_{2B} receptor expression, and to the increased expression of 5-HT_{2A}. These findings provide new knowledge about the mechanisms of action of 5-HT in the heart under the influence of a serious metabolic disease such as diabetes mellitus; therefore, the facts found in this study strengthen the knowledge on the pathophysiology of the diabetic cardiomyopathy.

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