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A comparative evaluation of sacubitril-valsartan and nebivolol-valsartan in left ventricular remodeling following chronic myocardial infarction in female Wistar rats

Evaluación comparativa de sacubitril-valsartán y nebivolol-valsartán en la remodelación ventricular izquierda tras un infarto de miocardio crónico en ratas Wistar hembras

Erika Pérez-García,* Ignacio Valencia-Hernández,‡ Diego Lezama-Martínez,§ Diana Ramírez-Hernández,¶ Germán Isauro Garrido-Fariña,‖ César Ramírez-Hernández,** Karla Reyes-Alvarado,‡‡ Isabel Hidalgo,§§ Jazmín Flores-Monroy¶¶

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Palabras clave: *remodelación cardiaca, fibrosis, sacubitril-valsartán, histología, ratas hembras.*

ABSTRACT

Cardiac fibrosis following a myocardial infarction (MI) leads to adverse left ventricular remodeling and heart failure, with distinct patterns observed in women. Despite having smaller infarcts and less profibrotic activity, women have a higher risk of post-MI mortality and heart failure. Since on therapies currently target fibrosis directly, studying these mechanisms is essential for developing and testing treatments, including approved heart failure drugs such as sacubitril–valsartan. This study aimed to compare and evaluate the combination of nebivolol-valsartan (NV) vs sacubitril-valsartan (SV) as a known treatment for chronic infarction in female rats; 26-weeks-old Wistar rats were used. The animals were divided into four groups (n = 6): 1) control (SHAM); 2) myocardial infarction (LADL); 3) LADL + sacubitril 30 mg/kg/day + valsartan 28 mg/kg/day (LADL + SV); 4) LADL + valsartan 30 mg/kg/day + nebivolol 5 mg/kg/day (LADL + NV). Infarct induction was performed by permanent ligation of the left anterior descending coronary artery. The treated groups received their treatments right after infarct induction for two weeks. The rats were euthanized by cervical dislocation and hearts and lungs were obtained from all groups for histology using Van Gieson and HE staining. The NV combination resulted in 50% mortality in animals, promoting pulmonary congestion and pleural effusion. Therefore, the administration of the NV combination at different times was proposed, after three and seven days post-infarction. This

RESUMEN

La fibrosis cardiaca tras un infarto de miocardio (IM) provoca una remodelación ventricular izquierda adversa e insuficiencia cardiaca, observándose patrones distintos en las mujeres. A pesar de presentar infartos de menor tamaño y una menor actividad profibrótica, las mujeres tienen un mayor riesgo de mortalidad tras un IM y de insuficiencia cardiaca. Dado que las terapias actuales se dirigen directamente a la fibrosis, el estudio de estos mecanismos es esencial para desarrollar y evaluar tratamientos, incluidos los fármacos aprobados para la insuficiencia cardiaca, como el sacubitril-valsartán. El objetivo de este estudio fue comparar y evaluar la combinación de nebivolol-valsartán (NV) frente a sacubitril-valsartán (SV) como tratamiento conocido para el infarto crónico en ratas hembras. Se utilizaron ratas Wistar de 26 semanas de edad. Los animales se dividieron en cuatro grupos (n = 6): 1) control (SHAM); 2) infarto de miocardio (LADL); 3) LADL + sacubitril 30 mg/kg/día + valsartán 28 mg/kg/día (LADL + SV); 4) LADL + valsartán 30 mg/kg/día + nebivolol 5 mg/kg/día (LADL + NV). La inducción del infarto se realizó mediante ligadura permanente de la arteria coronaria descendente anterior izquierda. Los grupos tratados recibieron sus tratamientos inmediatamente después de la inducción del infarto durante dos semanas. Las ratas fueron sacrificadas mediante dislocación cervical y se extrajeron los corazones y los pulmones de todos los grupos para su análisis histológico mediante tinción de Van Gieson y HE. La combinación de

* MS, Laboratory of Myocardial Pharmacology, Facultad de Estudios Superiores Cuautitlán (FES-C), Universidad Nacional Autónoma de Mexico (UNAM). Cuautitlán Izcalli, Estado de México, México. ORCID: 0009-0007-3578-3817
‡ PhD, College of Medicine, Instituto Politécnico Nacional. Mexico City, Mexico. ORCID: 0000-0001-7526-3524
§ PhD, Laboratory of Myocardial Pharmacology, FES-C,

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UNAM. Cuautitlán Izcalli, Estado de México. ORCID: 0000-0003-1899-3736
¶ PhD, Laboratory of Myocardial Pharmacology, FES-C, UNAM. Cuautitlán Izcalli, Estado de México. ORCID: 0000-0002-5624-1693
‖ PhD, Histology and Biology Support Laboratory. FES-C, UNAM. Cuautitlán Izcalli, Estado de México. ORCID: 0000-0003-2137-9315
** Bachelor´s degree, Laboratory of Myocardial Pharmacology, FES-C, UNAM. Cuautitlán Izcalli, Estado de México. ORCID: 0009-0004-8125-3137
‡‡ Bachelor´s degree, Laboratory of Myocardial Pharmacology, FES-C, UNAM. Cuautitlán Izcalli, Estado de México. ORCID: 0009-0006-6504-1606
§§ PhD, Immunology and Public Health Research Laboratory. FES-C, UNAM. Cuautitlán Izcalli, Estado de México. ORCID: 0000-0003-0276-9414
¶¶ PhD, Laboratory of Myocardial Pharmacology, FES-C, UNAM. Cuautitlán Izcalli, Estado de México. ORCID: 0000-0002-0431-5427

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resulted in six experimental groups. There was a reduction in the mortality rate, reduction of hypertrophy and cardiac fibrosis when the NV combination was administered seven days after ligation. In conclusion, sacubitril–valsartan appears to be a safe and effective strategy to attenuate cardiac and pulmonary fibrosis after infarction female rats, however, based on the results, early administration of nebivolol-valsartan is not recommended, as it appears to increase the risk of post-infarction complications.

NV provocó una mortalidad de 50% en los animales, lo que favoreció la congestión pulmonar y el derrame pleural. Por lo tanto, se propuso la administración de la combinación de NV en diferentes momentos, a los tres y a los siete días tras el infarto. Esto dio lugar a seis grupos experimentales. Se observó una reducción de la tasa de mortalidad, así como de la hipertrofia y la fibrosis cardiaca, cuando la combinación de NV se administró siete días después de la ligadura. En conclusión, el sacubitril-valsartán parece ser una estrategia segura y eficaz para atenuar la fibrosis cardiaca y pulmonar tras el infarto en ratas hembras, mientras que la administración temprana de nebivolol-valsartán no se recomendaría de acuerdo a los resultados, ya que aparentemente podría generar más complicaciones postinfarto.

Abbreviation:
CVDs = Cardiovascular Diseases
HF = Heart Failure
HFrEF = Heart Failure with Reduced Ejection Fraction
LADL = Left Anterior Descending Artery Ligation
MI = Myocardial Infarction
NV = Nebivolol-Valsartan
RAAS = Renin Angiotensin Aldosterone System
SNS = Sympathetic Nervous System
SV = Sacubitril-Valsartan

INTRODUCTION

Cardiovascular Diseases (CVDs) are the leading cause of morbidity and mortality in women worldwide, surpassing even the impact of other pathologies such as breast cancer.[1] Despite their high incidence, women are notably under in clinical and preclinical studies. This underrepresentation has limited the development of effective and safe therapeutic strategies specifically targeted at this population. To reduce this knowledge gap and improve the diagnosis and treatment of CVDs in women, various health campaigns have promoted the study of these diseases and their complications from a gender perspective has been promoted through various health campaigns.[2]

Cardiac fibrosis following Myocardial Infarction (MI) plays a central role in adverse left ventricular remodeling and the progression to heart failure, a major cause of morbidity and mortality worldwide.[3] Because mammalian hearts have a limited capacity to regenerate after injury, dead tissue must be replaced with collagen, which decreases the function of the repaired organ.[4] In the case of the heart, this process can be divided into three phases. The first phase involves tissue damage from ischemia. The second phase involves scarring, which takes between four and six weeks in humans and three to five days in rodents. The third phase involves the complete replacement of tissue with collagen, which takes up to eight weeks in humans and seven to nine days in a rodent.[5,6] However, evidence shows that MI characteristics, remodeling patterns, and clinical outcomes differ significantly between men and women.[7] Women are generally less likely to develop spherical left ventricular geometry and severe dysfunction. This is partly due to smaller infarct size, reduced microvascular obstruction, and attenuated activation of inflammatory and profibrotic pathways. These factors are potentially influenced by estrogen, even after menopause.[8] However, despite having a lower prevalence of obstructive coronary artery disease and Heart Failure with Reduced Ejection Fraction (HFrEF), women paradoxically have higher mortality rates after myocardial infarction, as well as higher rates of reinfarction and hospitalization for Heart Failure (HF), which are often linked to microvascular dysfunction and impaired myocardial perfusion.[9] Studying mechanisms of cardiac fibrosis and remodeling in females is crucial for identifying therapeutic strategies tailored to women's needs and improving their outcomes. Heart complications and acute HF after MI are potentially serious complications affecting the lungs and kidneys. Increased concentrations of natriuretic peptides promote pulmonary capillary

wedge pressure generating lung crackles; which can lead to congestion and edema.[10]

Currently, there are no drugs that are specifically approved for directly reversing or reducing myocardial fibrosis,[11] although some HF therapies have demonstrated indirect antifibrotic effects. In this regard, Sacubitril-Valsartan (SV), a combination of a neprilysin inhibitor (sacubitril) and an angiotensin II receptor antagonist (valsartan), has been shown to provide clinical benefits to patients with HFrEF,[12] including reduced mortality and hospitalizations. Conversely, therapeutic combinations such as Nebivolol-Valsartan (NV) have shown promising results in models of arterial hypertension in male Wistar rats, primarily due to nebivolol's vasodilatory and oxidative stress-modulating properties of nebivolol, combined with valsartan's AT1 receptor blocking effect.[13] However, most studies supporting these effects have been conducted in predominantly male cohorts or have not included sex-specific analysis, so its effectiveness and safety in women remain uncertain.[14] Furthermore, the impact of this combination on models of post-infarction myocardial injury, particularly in female participants, has not been widely explored.[15]

Against this backdrop, the present study evaluated the pharmacological combination of valsartan and nebivolol (NV) in an experimental model of chronic myocardial infarction in female Wistar rats. The aim was to determine its impact on functional and structural heart parameters, such as myocardial fibrosis, cardiac hypertrophy, and mortality rate. Furthermore, the efficacy and tolerability of this combination were compared with those of the standard SV treatment in post-ischemic conditions.

MATERIAL AND METHODS

Thirty female Wistar rats, aged 26 weeks, were used. All the animals were fed LabDiet 5001 and given access to tap water ad libitum. The rats were obtained from the Faculty of Higher Studies Cuautitlán (Facultad de Estudios Superiores Cuautitlán [FES-Cuautitlán]) animal facility. The protocol was carried out in two phases. In the first phase the animals were divided into four groups (n = 6) as follows: 1) sham control (SHAM); 2) Left Anterior Descending Artery Ligation (LADL); 3) LADL + sacubitril-valsartan; 4) LADL + nebivolol-valsartan. The combined drug doses were: sacubitril (calcium salt) + valsartan (free base): 58 mg/kg/day (30 mg of valsartan and 28 mg of sacubitril calcium salt, equivalent to 26.7 mg of sacubitril free base), nebivolol (hydrochloride) + valsartan 35 mg/kg/day (5 mg of nebivolol hydrochloride, equivalent to 4.6 mg of nebivolol free base and 30 mg of valsartan). The doses were administered orally on the day of surgery using a gastric tube, dissolved in a small volume of water. The treatment lasted two weeks. Since a high mortality index was found with NV treatment, two additional groups were included in a second phase of the study: 5) LADL + nebivolol-valsartan administered three days after LADL (LADL + NV 3D); 6) LADL + nebivolol-valsartan administered seven days after LADL (LADL + NV 7D) as shown in *Figure 1*. These timings were chosen because nebivolol, being a beta-blocker, can have a rebound effect in the first few hours after a myocardial infarction. This allowed post-infarction compensatory mechanisms to activate, thus preventing a malcompensatory effect with Nebivolol. At the end of the treatment period, the animals were euthanized by cervical dislocation and heart and lung tissue samples were obtained. In cardiac tissue, the infarct area was measured using tetrazolium blue staining. Histology was performed on left ventricle and lung tissue using hematoxylin and eosin and van Gieson staining. The care and use of the animals were conducted in strict accordance with the Guide for the Care and Use of Laboratory Animals (8th edition, National Academies Press). All procedures described herein were approved by the Internal Bioethics Committee of our institution (protocol number FES-01/16-05-2017) and complied with the Mexican Official Standard (NOM-062-ZOO-1999), which establishes the technical specifications for the production, care, and use of laboratory animals. The Ethics Committee on the Care of Experimental Animals (CICUAE-FESC by its spanish meaning) approved this protocol under registration number CICUAE-FESC C 24_08.

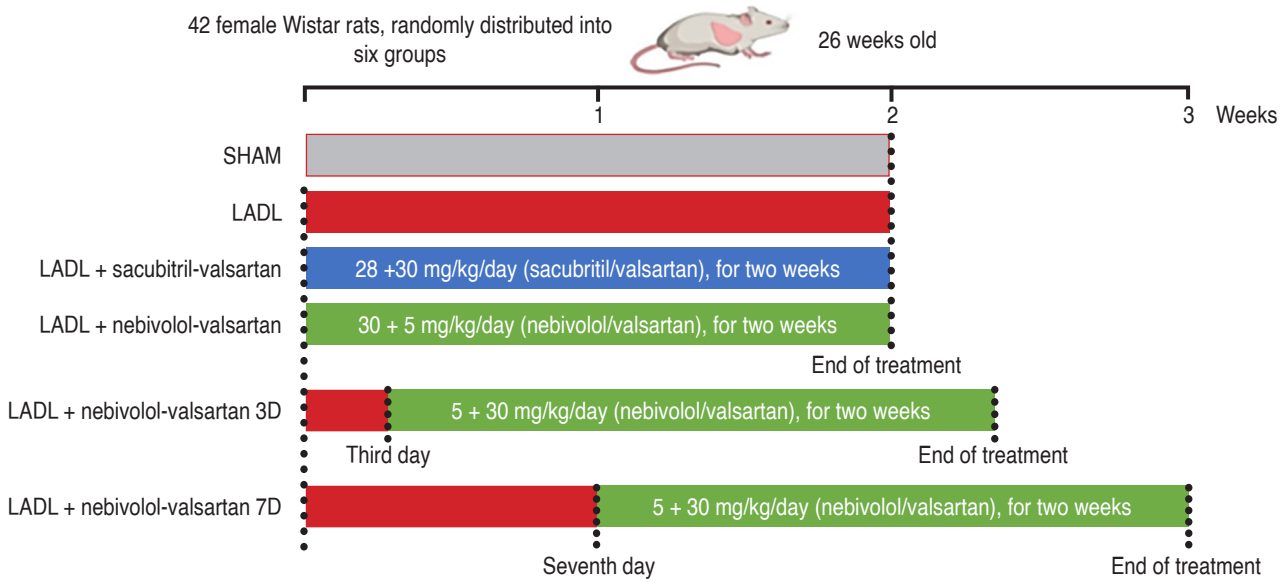

Figure 1: Experimental design.
LADL = Left Anterior Descending Artery Ligation.

Drugs. Valsartan free base, pure reagent from Sigma Aldrich®, sacubitril calcium salt, pure reagent from Sigma Aldrich®, and nebivolol hydrochloride, pure reagent from Sigma Aldrich®, were used. The drugs were dispersed in a solution of purified water containing 0.5% Poloxamer 401® for subsequent administration to the rats via gastric tube.

Left Anterior Descending Artery Ligation (LADL). The LADL surgery was performed during the estrous stage of the rats, since a previous study conducted by our team found that infarct progression is more severe at lower estrogen levels during the estrous cycle.[16] Prior to surgery, the rats were anaesthetized with an intraperitoneal injection of 20 mg/kg of ketamine + 9 mg/kg of xylazine. Once under surgical anesthesia, an incision was made through the third left intercostal space to allow exteriorization of the heart and location of the left anterior coronary artery, which was ligated with a 5-0 suture. The heart was the returned to the thoracic cavity, and the muscle and skin were sutured with 3-0 sutures. To allow for postoperative recovery, tramadol (6 mg/kg) and antibiotic cream (gentamicin) were administered. As our group has analyzed the progression of infarction according to the estrous cycle, the phases were only determined at the time of ligation surgery.

Samples for histology. To obtain cardiac and lung tissue samples, the Wistar rats were euthanized by cervical dislocation. A 100 mg portion of the left ventricle penumbra and 100 mg of the apex of the lung were removed and placed in a Petri dish containing physiological saline (0.9% NaCl), before being perfused with saline solution. The samples were then transferred to a 4% buffered formalin solution in Phosphate-Buffered Saline (PBS) at pH 7.4. The samples were stored in a refrigerator at 4 ºC for later use.

Mortality rate. To calculate the mortality rate, the number of deaths in each experimental group was quantified, and the ratio of live to dead individuals was obtained to determine the percentage.

Quantification of the infarct area. Once extracted, the heart was perfused with a physiological saline solution, wrapped in parafilm, frozen at −20 ºC for approximately one hour, and then cut into transverse slices of approximately 3 mm. These slices were placed in a 10 mL Falcon tube wrapped in aluminum foil and containing a 1% tetrazolium blue solution. The tube was then incubated at

37 ºC for 20 minutes with shaking. Finally, the stained tissue was placed between two glass slides. Photographs were captured, and the infarct area was quantified using Motic Images Plus 3.0 software.

Pulmonary and cardiac hypertrophy. This was calculated by measuring the length of each rat's tibia and weighing its heart or lung. The ratio of organ weight to tibia length was then obtained.

Histology of cardiac and lung tissue. Samples of left ventricle penumbra and lung tissue were dehydrated and embedded in paraffin. These samples were then cut into 10 µm-thick sections using a microtome. These sections were fixed on a slide. To clearly observe the collagen deposition, the van Gieson staining technique was used. The samples were be deparaffinized and hydrated by immersing the slides first in a xylene solution, then in a 1:1 mixture of ethanol and xylene, and finally in ethanol solutions, ranging from 100 to 50%. The deparaffinized samples were stained with Weigert's haematoxylin for 10 minutes. Finally, they are rinsed with distilled water and stained with Van Gieson's solution (acid fuchsin and picric acid) for three minutes. Finally, the sample is dehydrated.

Quantification of collagen in the myocardium. The percentage of collagen deposition will be measured in the left ventricular area of the samples stained with Van Gieson stain. First, a microscopic scan will be performed to identify the areas of high collagen content in the septum and the free wall of the left ventricle. These areas were then observed and images captured using AmScope software (version 4.11.20131.20220108). This procedure involves measuring three different fields for the same sample. Then, the amount of collagen present in each image will be measured using Motic Images Plus 3.0 software. Finally, the percentage of collagen deposition will be obtained by calculating the ratio of collagen-containing tissue to total tissue.

Statistical analysis. Data processing was performed using the mean ± standard error for each group (n = 6). Two-way ANOVA was used, with treatment and the gender of the Wistar rats as the two factors. Tukey's test was used as a *post hoc* test. Significant differences were considered when $p < 0.05$ and the statistical power was greater than 0.8. Mortality rates were analyzed using a contingency table and analyzed using Fisher's exact test.

RESULTS

Figure 2A shows a mortality rate of 16.6% in the untreated myocardial infarction group. In contrast, nebivolol-valsartan treatment administered right after the infarction resulted in a mortality rate of 50%. When Nebivolol-valsartan treatment was administered three days after the infarction, the mortality rate was also 16.6% notably, most of these deaths occurred during the first week of treatment. On the other hand, neither SHAM, nor sacubitril-valsartan treatment administered immediately after the infarction resulted in any deaths, nor did nebivolol-valsartan treatment administer seven days after the infarction. *Figure 2B* shows the percentage of infarct area according to the experimental group. The LADL group had an infarct area of 33%, which decreased to 22% with nebivolol-valsartan treatment administered immediately after the infarction. However, the treatments administered three and seven days after the infarction did not decrease the infarct area vs the LADL group. On the other hand, sacubitril-valsartan treatment administered immediately after the infarction decreased the infarct area by 15%, making it the most effective treatment for reducing the damaged area. *Figure 2C* shows the cardiac hypertrophy index graph, represented as a ratio of heart weight to tibial length, according to the experimental groups. There were no significant differences between groups.

Figure 3 (left), shows the graph of cardiomyocyte area according to the experimental groups. The cardiomyocyte area of the SHAM group was 129 μm^2. Following infarction, the size of the cardiomyocytes increased to 175 μm^2. The same result was observed following nebivolol-valsartan administration immediately after the infarction, with an area of 184 μm^2. This was no different to the infarcted group. Furthermore, the treatments administered three and seven days post-infarction produced similar results, with values of 192 and 175 μm^2, respectively. Sacubitril-

valsartan treatment, reversed this effect, significantly decreasing the cardiomyocyte area compared to the infarct, with a value of 148 μm^2, and showing no difference compared to the SHAM group. *Figure 3* (right) shows the morphology of the cardiomyocytes as seen with H&E staining. *Figure 3A* shows the myocytes of the SHAM group, which have a well-defined oval shape (black circles) with well-defined nuclei marked in purple (black arrows). *Figure 3B*, shows the cardiomyocytes corresponding to the infarcted group. Here, cell growth and loss of structure are notable and the cardiomyocytes have lost their characteristic oval shape (black circles). Furthermore, the nuclei appear diffuse and show loss of structure, as they appear elongated (black arrows), which could indicate cell necrosis. *Figure 3C* shows cardiomyocytes from the nebivolol-valsartan treatment group. These have a structure similar to the SHAM group, with oval/circular myocytes (black circles) and a preserved structure. However, some cells have absent nuclei and others have elongated nuclei (black arrows), which could indicate myocyte necrosis. *Figure 3D*, shows myocytes from the nebivolol-valsartan treatment group where the treatment was initiated three days after the infarction. The cells are more organized and have their characteristic oval or circular shape. The presence of nuclei in most cells, with a characteristic round shape, is also evident. *Figure 3E* shows myocytes from the nebivolol-valsartan treatment group where the treatment was initiated seven days after the infarction. The cells are very similar to those from treatment that was initiated three days after the infarction. They are organized and have their usual shape (oval/circular). Furthermore, the nuclei are circular, and are present in most cells, ruling out cell necrosis. Finally, *Figure 3F* shows the cardiomyocytes from the sacubitril-valsartan group, which were administered immediately after infarction. These cardiomyocytes are elongated and rectangular in shape (black circles) and organized, with the nuclei located in the center of most cells and retaining their usual circular shape.

Figure 4 (left) shows the collagen content graph for each experimental group. It shows that the infarction increases collagen content

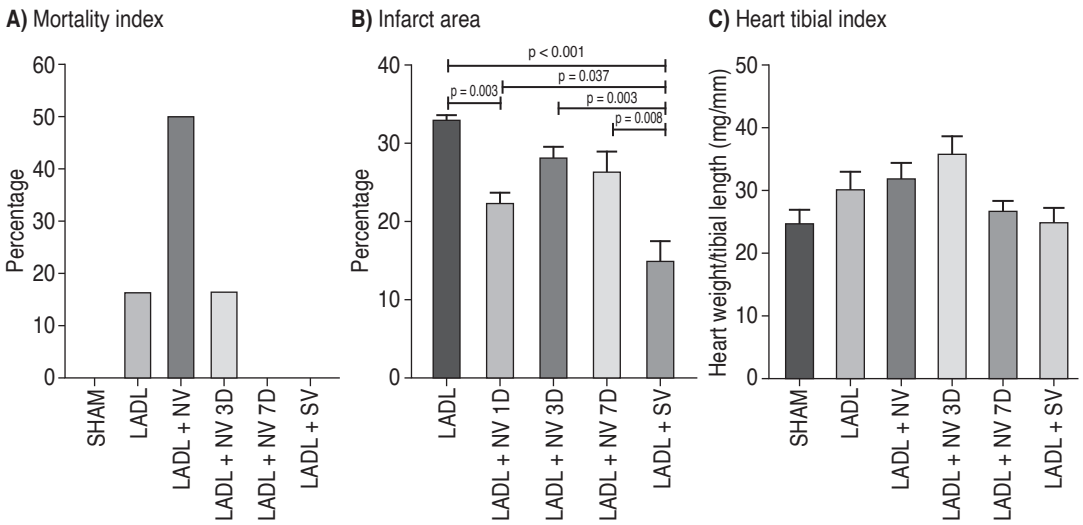

Figure 2: Physiological effects of the treatments.
A) Shows mortality rates for the different experimental groups. The results are presented as a percentage of total deaths, with n = 6 rats per experimental group. The mortality rate was analyzed using Fisher's exact test. No significant differences were found. **B)** Indicates the infarct area during the estrous stage of female Wistar rats in the different experimental groups. **C)** Points cardiac hypertrophy index, ratio of heart weight to tibial length for each experimental group. Results are presented as mean ± SE, n = 6 rats per experimental group. Analysis was performed using a one-way ANOVA, followed by a Tukey *post hoc* test. Significant differences were obtained when $p < 0.05$.

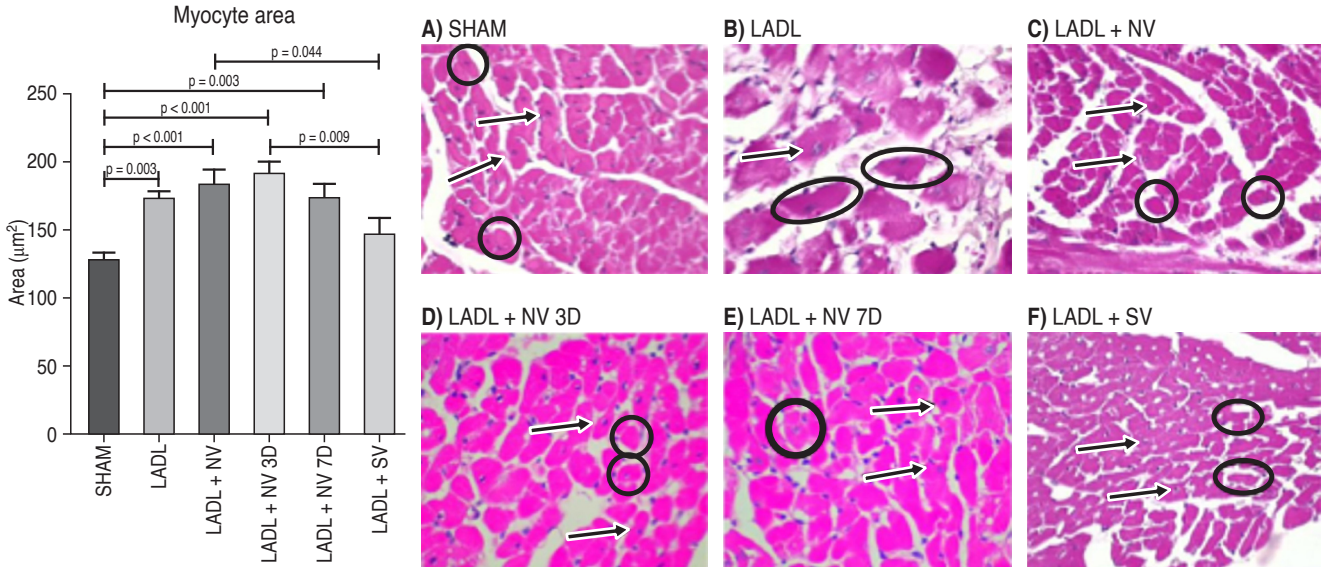

Figure 3: Cardiac tissue integrity evaluated by Hematoxylin-eosin staining.
Left shows cardiomyocyte area of female Wistar rats in the different experimental groups. Results are presented as mean ± SD, n = 6 rats per experimental group. Analysis was performed using a one-way ANOVA, followed by a Tukey post hoc test. Significant differences were obtained when $p < 0.05$. **Right** point cardiomyocytes seen with H&E staining at 40× magnification in the experimental groups: **A)** SHAM, **B)** LADL, **C)** LADL + NV, **D)** LADL + NV3D, **E)** LADL + NV7D, **F)** LADL + SV.

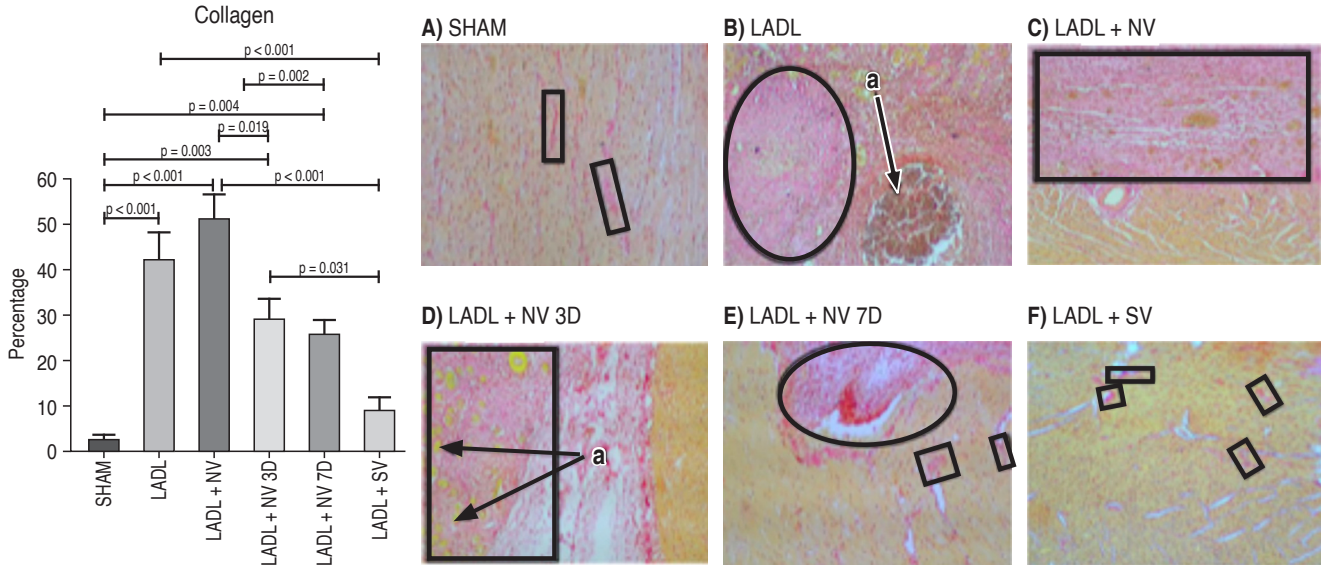

Figure 4: Collagen deposition in the heart by van Giesson staining.
Left shows Percentage of collagen in cardiac tissue of female Wistar rats from each experimental group. Results are presented as mean ± SD, n = 6 rats per experimental group. Analysis was performed using a one-way ANOVA followed by a Tukey *post hoc* test. Significant differences were obtained when $p < 0.05$. **Right** shows Collagen present in the cardiac tissue of a female Wistar rat seen with Van Gieson staining at 40× magnification in the experimental groups: **A)** SHAM, **B)** LADL, **C)** LADL + NV, **D)** LADL + NV3D, **E)** LADL + NV7D, **F)** LADL + SV.

in cardiac tissue by 40%, whereas the SHAM group has a collagen content of just 3%. The nebivolol-valsartan treatment shows a collagen content of 51%. Meanwhile, the treatments administered after three and seven days showed a decrease in collagen content of 29 and 26%, respectively. Finally, the sacubitril-valsartan treatment was observed to significantly decrease collagen content by 9%, showing no difference to the SHAM group. *Figure 4* (right) shows collagen deposition in the cardiac tissue of the experimental groups. *Figure 4A* shows the SHAM group, which exhibits small areas of interstitial collagen deposition (black rectangle), though cardiac tissue predominates. *Figure 4B* shows the infarcted group. The infarct suture is observed, and the infarcted area (black circle) surrounding it are visible, as well as a large amount of collagen, which indicates the size of the infarct. *Figure 4C* shows the nebivolol-valsartan treatment. A large area of collagen (black rectangle) is visible at the top, indicating the area affected by the infarct. *Figure 4D* shows the nebivolol-valsartan treatment administered three days after infarction. Large areas of collagen (black rectangle) deposition are still evident, although remnants of cardiac tissue are presented within the collagen (arrows labelled «a»). *Figure 4E* shows the nebivolol-valsartan treatment administered seven days after the infarction. There is a decrease in the amount of collagen in the cardiac tissue. Collagen is present in the upper portion (black circle). The rest of the tissue appears normal with slight interstitial collagen deposition (black rectangle). Finally, *Figure 4F* shows the sacubitril-valsartan treatment. Small areas of interstitial collagen deposition are observed (black rectangles), mostly in the cardiac tissue. This indicates that the treatment effectively reduces cardiac tissue death.

Figure 5 (left) shows the pulmonary hypertrophy index for each experimental group. No differences were observed between the experimental groups, indicating that neither the infarction nor the treatments administered immediately after the infarction or three or seven days afterwards modified lung size. *Figure 5* (right) shows lung tissue samples stained with haematoxylin and eosin at 40× magnification. *Figure 5A* shows lung tissue from the SHAM group and reveals a well-defined lung structure is observed, including the alveolar septum («a» arrow) and the alveolar sacs («b» arrow), both of which are a defined size. *Figure 5B* representing the infarcted group, shows a complete loss of alveolar structure, with the alveolar septum and alveolar sacs no longer distinguishable. This is indicative of lung damage. Furthermore, in the area enclosed by the black circle, shows a massive infiltration of leukocytes (black circle), indicating an inflammatory process. *Figure 5C*, representing the nebivolol-valsartan treatment, show that the alveolar structure has slightly recovered. The alveolar septum and alveolar sacs can now be distinguished. There is no leukocyte infiltration. However, the alveolar septum appears quite thick, and the alveolar sacs are very small. *Figure 5D* shows the nebivolol-valsartan treatment administered three days post-infarction, where thickening of the alveolar septum is evident. *Figure 5E*, shows nebivolol-valsartan treatment administered seven days post-infarction. Recovery of the lung structure is evident, with decreased thickness of the alveolar septum and increased in the size of the alveolar sac. Finally, *Figure 5F*, shows the sacubitril-valsartan treatment. Here, the lung structure is more defined, with a thinner alveolar septum and a larger alveolar sac and a well-defined bronchus («C» arrow).

Figure 6 (left) shows the extent of pulmonary fibrosis in each experimental group. It can be seen that treatment with nebivolol-valsartan does not reduce fibrosis caused by LADL surgery at any administration time point, but sacubitril-valsartan treatment does decrease fibrosis to baseline values. *Figure 6* (right) shows collagen deposition in the lung tissue of the experimental groups. *Figure 6A* shows collagen deposition in the SHAM group, where the lung structure remains unchanged and the alveoli are clearly visible. The thin alveolar septa and wide alveolar lumen are marked with circles. No interstitial collagen deposition is observed, with only the cellular cytoplasm (black rectangles) visible. *Figure 6B* shows the infarcted group, where loss of lung structure is evident, with disappearance of the alveolar lumen and sharp increase in the alveolar septa (black circles). An increase in interstitial collagen deposition is also evident (black rectangles). *Figure 6C*

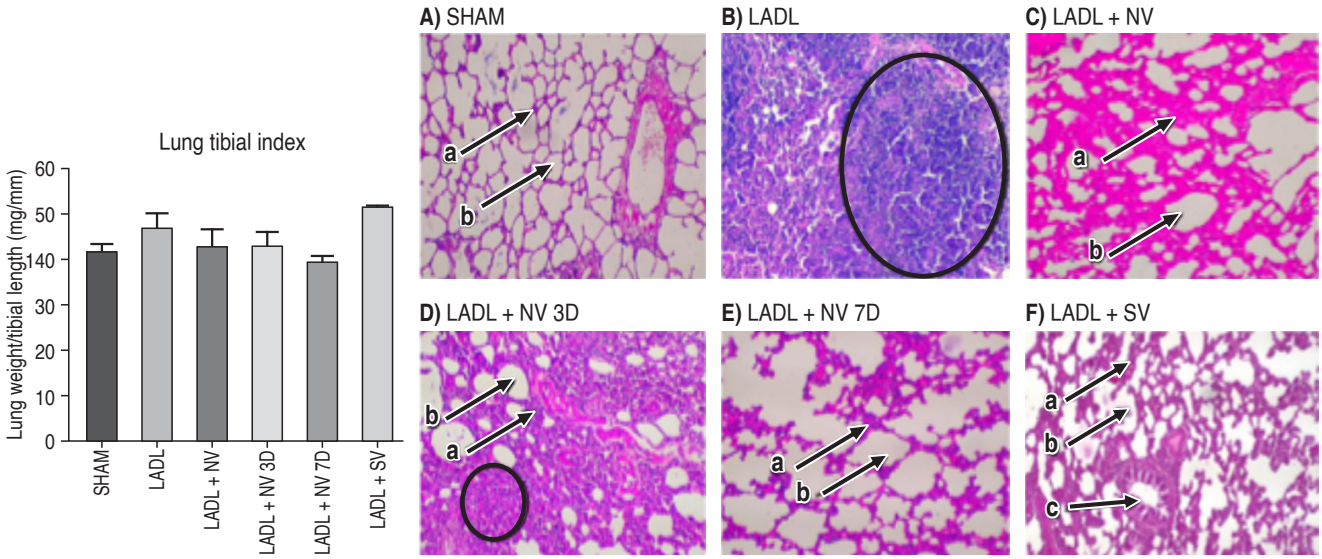

Figure 5: Lung tissue integrity evaluated by Hematoxylin-eosin staining of apex lung tissue.
Left indicate the pulmonary hypertrophy index, ratio of lung weight to tibial length for each experimental group. Results are presented as mean ± SD, n = 6 rats per experimental group. Analysis was performed using a one-way ANOVA. No significant differences were found. **Right** shows Female Wistar rat lung tissue seen with H&E staining at 10× magnification from the experimental groups: **A)** SHAM, **B)** LADL, **C)** LADL + NV, **D)** LADL + NV3D, **E)** LADL + NV7D, **F)** LADL + SV.

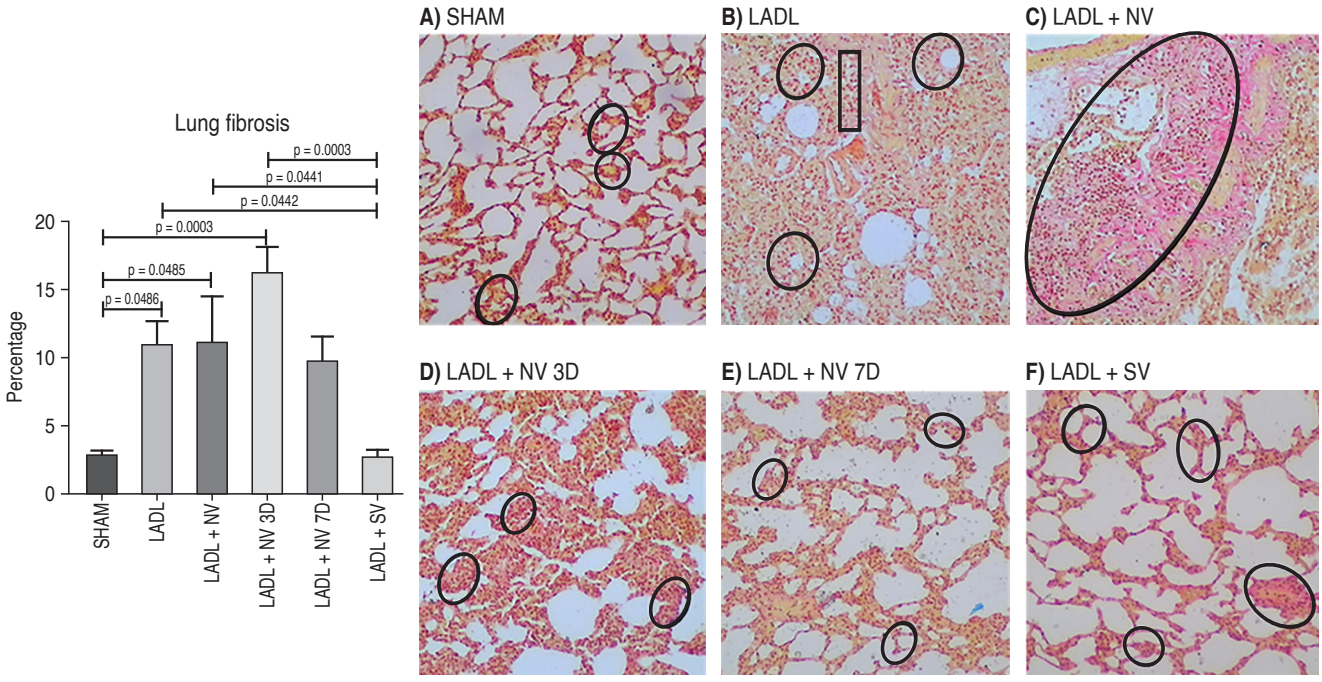

Figure 6: Collagen deposition in the lung apex by van Giesson staining.
Left indicates the collagen in lung tissue of female Wistar rats from each experimental group. The results are presented as mean ± SD, n = 6 rats per experimental group. Data were analyzed using one-way ANOVA followed by Tukey's *post hoc* test. **Right** shows collagen present in the lung tissue of a female Wistar rat seen with Van Gieson staining at 40× magnification in the experimental groups: **A)** SHAM, **B)** LADL, **C)** LADL + NV, **D)** LADL + NV3D, **E)** LADL + NV7D, **F)** LADL + SV.

shows collagen deposition in the nebivolol-valsartan treatment group. Loss of lung structure is evident here, with the alveolar septa and alveolar lumen no longer clearly visible. There is massive interstitial collagen deposition (black circle), which is even greater than that seen with the LADL. *Figure 6D* shows collagen deposition in the nebivolol-valsartan group administered three days after the infarction. Evident loose of lung structure is observed, while collagen deposition shows large areas of interstitial collagen (black circles) throughout all parts of the panel. *Figure 6E* shows collagen deposition in the nebivolol-valsartan group, which was administered seven days after the infarction. Although interstitial collagen deposition is still evident in various regions of the panel (black circles) a slight recovery of lung structure is observed. Finally, in *Figure 6F* shows that the sacubitril-valsartan group has recovered lung structure, with the alveolar septa and alveolar lumen visible. Regarding collagen deposition, the presence of interstitial fibrosis is evident (black circles), indicating that although treatment restores lung structure, it does not reduce pulmonary fibrosis.

DISCUSSION

In the present study, we evaluated the effects of two pharmacological combinations, sacubitril-valsartan (SV) and nebivolol-valsartan (NV), on cardiac and pulmonary remodeling following myocardial infarction in female Wistar rats. Our results demonstrated that these two treatments have markedly different effects on survival, myocardial structure and fibrosis. SV treatment was associated with reduced mortality, preserved cardiomyocyte morphology and reduced cardiac and pulmonary fibrosis. In contrast, early administration of NV resulted in increased mortality and more severe tissue damage. Delayed administration of NV partially improved some of these outcomes, suggesting that timing plays a critical role in the response to this therapy.

Cardiac fibrosis is the excessive deposition of extracellular matrix proteins,[5] most notably collagen. It occurs in most cardiovascular diseases, such as myocardial infarction. Initially, in this disease, fibrosis presents as a reparative process that maintains the structural integrity of the necrotic myocardium. However, as the disease progresses, it becomes a maladaptive process that increases the stiffness of cardiac tissue and puts stress on the left ventricular wall. This contributes to diastolic and systolic dysfunction, ultimately leading to heart failure,[17] which carries a poor prognosis for patients who develop it. In Wistar rats, the acute phase of infarction corresponds to the first seven days post-infarction, then the chronic phase begins, where fibroblast proliferation and collagen deposition for scar formation are observed.[18]

Animal mortality was due to lung complications and massive necrosis, as confirmed by measuring the infarct area and the staining of the lungs. In our study, we found that the infarcted group had areas of more than 30%. According to Pfeffer et al.,[19] large infarcts with areas greater than 30% increase mortality. However, the group treated with nebivolol-valsartan administered immediately after the infarction had a mortality rate of 50%. This contrasts with the results of various clinical studies, such as that of Puymirat et al.,[20] which found that administering beta-blockers such as atenolol, propranolol, and acebutolol early after the infarction improved patient survival within 30 days. It should be noted that the aforementioned clinical studies reported results based on the administration of a single drug, whereas our study involved a combination of therapy. When nebivolol-valsartan was administered for three days post-infarction, mortality was reduced, and no mortality was observed when it was administered seven days later. This suggests that at three days, the heart is still in the acute phase, and that blocking the initial compensatory systems may continue to affect cardiac function and lead to death. However, by seven days, the heart is in a chronic phase. Combined blockade of the Sympathetic Nervous System (SNS) and Renin Angiotensin Aldosterone System (RAAS) could therefore prevent or reverse the maladaptive changes caused by neurohumoral overstimulation. These results are similar to those observed by Xia et al.,[21] who administered fensartan three, 24, and 72 hours after myocardial infarction in male Wistar rats. They found that the infarct

area decreased compared to the infarcted group at three hours, 24 hours and seven days.

On the other hand, the sacubitril-valsartan treatment, did not cause mortality, and the infarcted area was very small, which is consistent with the observations of Liu et al.,[22] who administered sacubitril-valsartan to SHR rats with myocardial infarction. Similarly, Torrado et al.[23] used infarcted New Zealand white rabbits with myocardial infarction that were administered sacubitril-valsartan. In both studies, it was observed that the combined therapy decreased the infarct area compared to the infarcted group, with no deaths were reported during treatment. This can be attributed to the fact that SNS activation in the acute phase of the infarction is not blocked, and therefore, it carries out its adaptive effects of increasing blood pressure, ejection fraction, and contraction force, leading to the restoration of cardiac function.[24] Conversely, the sacubitril-valsartan combination has been shown to have an anti-inflammatory effect. Xiao et al.[25] found that administering this therapy to male C57BL/6 mice infarcted with reperfusion 30 minutes after infarction inhibited the NF-κB pathway. This could slow the initial inflammation, thereby limit myocardial necrosis and reduce the infarct area.

Collagen deposition in the left ventricle was measured to evaluate cardiac fibrosis and its progression following myocardial infarction. Treatment with nebivolol-valsartan did not reverse the effect generated by permanent ischemia. While there are no studies on the effects of this drug combination on fibrosis caused by MI, there is evidence supporting the antifibrotic effects of the drugs when used separately. Sui et al.[26] used infarcted Sprague-Dawley rats treated with valsartan and observed a decrease in collagen percentage compared to the infarcted group. Meanwhile, Zhang et al.[27] demonstrated that administering nebivolol via osmotic pumps to infarcted adult C57BL6/J mice reversed the fibrosis generated by LADL. Therefore, the results obtained with this drug combination contrast with those obtained experimentally. This finding could be explained, first, by the early onset of sympathetic blockade, which prevented the activation of the necessary compensatory mechanisms in the acute phase, such as RAAS and SNS. This exacerbated hypertrophy and, consequently, mechanical stress on the myocardium. This stress, in turn, can activate profibrotic pathways such as TGF-β/SMAD. Although nebivolol acts on β 3 receptors, promoting the production of nitric oxide (NO), with potential antioxidant and antifibrotic effects, this mechanism may not have been sufficient to counteract the intensity of the infarction and accentuated hypertrophy-induced damage. Additionally, simultaneously blocking the RAAS and the SNS could have generated a compensatory regulatory environment in which other pathways, such as the inflammatory or alternative fibroblastic pathways, took center stage. One such pathway is Endothelin-1, which is a strong fibrotic inducer.[28] Similarly, the increased necrosis observed in this group, reflected in higher mortality, could have induced a more intense inflammatory response, favoring the activation of profibrotic pathways such as NF-κB and SMAD.[11,25] Taking together, these results suggest that nebivolol-valsartan could have worsened fibrosis in this model and timeframe.

In contrast, sacubitril-valsartan treatment achieved the desired effect of reducing cardiac fibrosis. This finding is consistent with that reported by Vaskova et al.,[29] who used a chronic infarction model in Sprague-Dawley rats that were subsequently treated with sacubitril-valsartan. They observed that this treatment was effective in attenuating cardiac fibrosis and cardiac hypertrophy, suggesting a beneficial effect on post-infarction remodeling. This can be explained by the inhibition of neprilysin, which accumulates vasodilatory peptides such as bradykinin, and by blocking the AT-1 receptor. These actions both reduce the load on the ventricular wall and prevent the activation of fibrotic signaling pathways. Furthermore, other peptides such as natriuretic peptides, can inhibit tTGF-β/SMAD signaling via cGMP/PKG, as demonstrated by Burke et al.[30] Together, these findings demonstrate that sacubitril-valsartan administered immediately after myocardial infarction can effectively reverse induced cardiac fibrosis.

Finally, with regard to lung tissue histology, the LADL completely alters lung morphology and causes signific inflammation. These findings

are consistent with those of Chen et al.[31] in a pressure overload model in C57B6J mice, who demonstrated that left ventricular heart failure causes lung inflammation and thickening of the alveolar septum. These changes may have occurred due to the heart failure caused by the infarct, which decreases left ventricle end diastolic volume and generates increased pulmonary pressure, leading to fluid accumulation in the lung (increased alveolar septum thickness and leukocyte infiltration.[31] With nebivolol-valsartan treatment, however, no improvement in lung morphology was observed: the alveolar septa remained thickened, and the alveoli showed reduced lumens. However, a decrease in lung inflammation was observed. These results contrast with the beneficial effects attributed to these drugs, as demonstrated by Perros et al.[32] in a model of pulmonary hypertension model in male Wistar rats treated with monocrotaline. They found that Nebivolol improved cardiac function, lung remodeling, and inflammation. This could be explained by timing, since, when the combination was administered both immediately after the infarction and three days after LADL, the early blockade of the SNS with nebivolol could have worsened the depression of already compromised cardiac function. This would lead to a decrease in LVEDV, an increase in pulmonary pressure, and fluid accumulation in the alveoli. However, it seems that the anti-inflammatory effects of valsartan reduced the inflammation, as valsartan has been shown to modulate the MAPK and NF-kB pathways in the lung.[33] After seven days, considerable improvement in lung morphology was o, probably due to the restoration of cardiac function and the reduction in pulmonary pressure reported by Perros et al,[32] which helped reverse lung morphology.

Sacubitril-valsartan treatment showed improvements in lung morphology with thin alveolar septa and wide lumens observed. While there are currently no studies directly evaluating these effects on post-infarction lung tissue, the observed benefits could be explained by the previously described cardiovascular effects. Unlike the nebivolol-valsartan group, the sacubitril-valsartan group does not experience early sympathetic response blockades, which could allow transient cardiac function recovery and prevent LVEDV decrease and sustained pulmonary circulation pressure increase, leading to fluid accumulation in the lungs. This effect could also be potentiated by accumulated natriuretic peptides, since atrial natriuretic peptide was shown by Jin et al.[34] to decrease pulmonary pressure through its vasodilatory effect in hypoxic rats. Together with the anti-inflammatory effect of valsartan, this contributes to the reversal of the morphological and functional lung damage observed in this group.

Based on these results, we propose that the sacubitril-valsartan combination is a promising candidate for treating cardiac fibrosis in chronic myocardial infarction in females, as it was able to control the infarct area, reverse both cardiac hypertrophy and fibrosis, and restore pulmonary morphology. The alteration of this morphology contributes to the functional deterioration of the lungs. In contrast, nebivolol-valsartan, when administered immediately after the infarction, was not safe or effective in reversing fibrosis, hypertrophy, and post-myocardial infarction pulmonary damage, although improvements were seen when administered after seven days. We propose that this result is mainly due to the early blockade of the sympathetic response, the adverse effects of which have been discussed previously.

CONCLUSIONS

Our results suggest that sacubitril-valsartan represents a safer and more effective option for limiting post-infarction remodeling in females, whereas early nebivolol-valsartan administration may be detrimental.

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Correspondence:
Jazmín Flores-Monroy PhD
E-mail: jfmqfb@cuautitlan.unam.mx