Quantitative Study of Myocardial Microcirculation in Arterial Hypertension due to Progressive Inhibition of NO Synthesis

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Objective *- To study the quantitative changes in intramyocardial blood vessels in rats in whom nitric oxide synthesis was inhibited.*

Methods *- Four groups of 10 rats were studied: control (C25 and C40) and L-NAME (L25 and L40). The animals L25 and L40 received L-NAME in the dosage of 50mg/kg/day for 25 and 40 days, respectively. On days 26 and 41 the animals in groups 25 and 40 were sacrificed. Analysis of the myocardium was performed using light microscopy and stereology.*

Results *- Arterial blood pressure and heart weight increased 74.5 and 57.8% after 25 days and 90.2 and 34.6% after 40 days, respectively. Comparing the L-NAME rats with the respective controls revealed that vessel volume density decreased 31.3% after 40 days, and the vessel length-density decreased 53.5% after 25 days and 25.7% after 40 days. The mean cross-sectional area of the vessels showed an important reduction of 154.6% after 25 days. The intramyocardial vessels decreased significantly in length-density in the L-NAME animals. The mean cross-sectional area of the vessels, which normally increases during heart growth between 25 and 40 days, showed a precocious increase by the 25th day in the L-NAME rats. This suggests an increase of the size of the heart, including blood vessels.*

Conclusion *- The inhibition of the NO synthesis provokes rarefaction in the intramyocardial vessels that progresses with the time of administration of L-NAME.*

Keywords: nitric oxide, myocardium, hypertension

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Adult mammalian cardiac myocytes are unable to replicate themselves [1]. Therefore, to resist pressure overload imposed by arterial hypertension, cardiac myocytes become hypertrophic, enlarging both in length and diameter. Hyperplasia of the cellular components of the connective tissue follows cardiac myocyte hypertrophy, for these cells do not loose their ability to replicate themselves [2]. Arterial hypertension is one of the risk factors for sudden cardiac death. This risk is even higher in patients who develop left ventricular hypertrophy in response to hypertension [3].

Nitric oxide (NO) is produced by vascular endothelium, and it acts as a potent vasodilator, controlling blood pressure and myocardial perfusion [4,5]. Endothelial cells synthesize and release NO, which in turn performs important regulatory functions for vascular tonus, platelet aggregation, leukocyte adhesion and vascular smooth muscle cell proliferation [6]. The activity of NO-synthase is inhibited by L-arginine (N^G-nitro-L-arginine-methyl-ester; L-NAME) [7,8] and this inhibition represents a very useful model for studying experimental hypertension [9].

Administration of L-NAME to rats promotes elevation of blood pressure and widespread reduction of peripheral blood flow, indicating that a continuous release of NO is important for controlling vascular tonus. Therefore, NO deficiency is associated with vascular relaxation deficit and vascular wall hypercontractility [10]. Nitric oxide seems to be a chemotactic factor for angiogenesis and NO-synthase inhibitors like L-NAME may impair neovascularization [11].

All the changes that occur in the cardiovascular system following NO-synthase inhibition and myocardial hypertrophy (as a result of systemic hypertension-caused NO inhibition), as well as fibrosis and coronary vascular remodeling have been reported in the medical literature [12-14].

A chronic increase in shear stress releases NO, inducing vasodilation and an increase in the vascular luminal diameter, normalizing wall stress. According to Laplace's law, vascular enlargement is associated with increasing wall stress that can stimulate protein synthesis in the extracellu-

lar matrix and produce smooth muscle cell hypertrophy. This vascular remodeling does not normalize wall stress but leads to recovery of dynamic mechanical properties and to adaptation of arterial dimensions to enhance blood flow [15].

Cardiac hypertrophy due to pressure overload also modifies the vascular structure in other organs and promotes changes in the extracellular space [16]. Myocyte degeneration and fibrosis occur in advanced stages of cardiac hypertrophy, because oxygen demand increases in the hypertrophic ventricle, exceeding the capability of vessels to supply adequate blood flow [17].

Myocardial microcirculatory vessels are adjacent to cardiac myocytes keeping a mean distance of 8µ m. This short distance suggests that vasoactive substances released by microvascular endothelium can affect the adjacent myocardium [18].

Recent studies in rats have demonstrated that the chronic administration of NO-synthase inhibitors can cause hypertension, reducing intracellular levels of GMP cyclic in smooth muscle cells, promoting structural modifications in microvascular circulation [12].

Methods

Forty normotensive adult male rats were used in this study (Wistar, body weight of 250±36g, and initial blood pressure under 120mmHg). For two weeks the animals were kept under observation and underwent weekly blood pressure determinations. After this initial observation period, the animals were divided into four groups housed under appropriate conditions of light and temperature.

Groups were separated as follows: a) 25-day control (C25); b) 40-day control (C40); c)L-NAME 25 days (L25); d) L-NAME 40 days (L40).

Animals in groups C25 and C40 received water and food *ad libitum*. Animals in groups L25 and L40 received L-NAME (N^G-nitro-L-arginine-methyl-ester, HCl, Sigma Co. batch 44:0102, 50mg/Kg per day) for 25 and 40 days, respectively [13]. This investigation was performed in accordance with the Guidelines for Care and Use of Laboratory Animals published by the United States National Institutes of Health (NIH Publication #85-23, revised in 1985).

At the end of the treatment period (26^{th} day for groups C25 and L25 and 41^{st} day for groups C40 and L40) the rats were anesthetized with inhaled ether and killed with an intracardiac injection of 10% potassium chloride, which induced a diastolic heart arrest. The weights and volumes of the hearts were measured (Scheler's method, hanging the hearts on a string and immersing them in a saline solution over an analytical precision scale) [19,20]. Afterwards, the hearts were immersion-fixed in a 4% formalin buffered phosphate solution (pH 7.2) for 48 hours.

The myocardium is an anisotropic tissue, but isotropic sections are needed to perform stereological studies, and for this reason the hearts were prepared in accordance with the orthtopic method [14]. The material was embedded in paraffin, cut in 5µ m-thick slices and stained with hematoxylin-eosin and Picro-sirius. A stereological analysis was performed in 15 random microscopic fields using an immersion lens, Leica microscopy video camera (model DMRBE), and test-system M42 calibrated to 1/100mm Leitz micrometer [19].

The volume-density of intramyocardial vessels (Vv-[vessels]) was determined by counting methods and calculated as: (Pp [vessels] / pt) % where *Pp[vessels]* is the number of points over the vessels and *Pt* is the total number of test points, in this case 42 points.

The length-density of intramyocardial vessels (Lv-[vessels]) was determined by:

$Lv\,[vasos] = 2.Q_A$ mm/mm^3 where *Qa* is the numerical vascular density by the myocardial area.

The cross-sectional area of vessels was assessed by:-[Aa/Qa mm^2] where *Aa* is the density of blood vessels by area.

Results

In rats from the control groups cardiac myocytes and intramyocardial vessels remained unchanged after 25 and 40 days. Rats that underwent NO-synthase inhibition had myocardial remodeling compatible with this model of arterial hypertension, showing perivascular and interstitial fibrosis and cardiac myocyte hypertrophy. These lesions were more intense as the time of NO inhibition increased (figures 1 to 4).

When compared with control animals, arterial blood

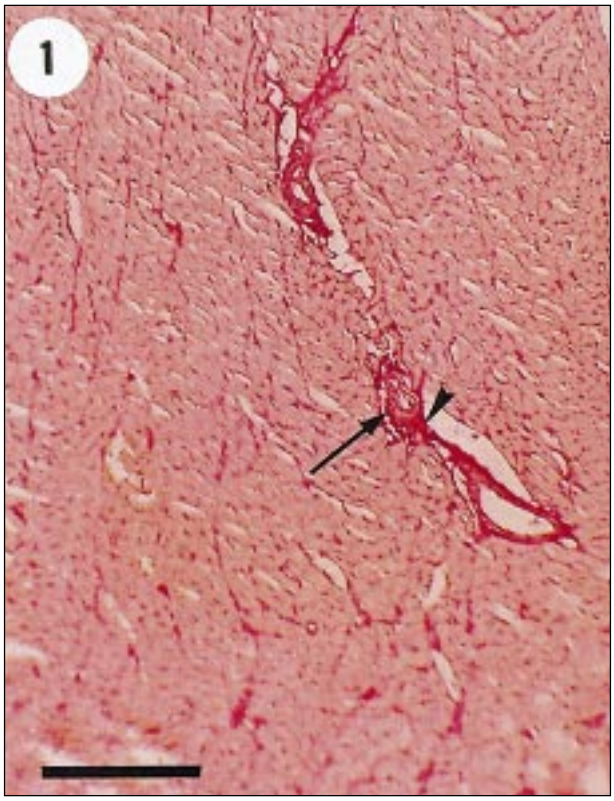


Fig. 1 – Rat myocardium after exposure to L-NAME for 25 days. Note mild tissue disorganization and the small arteries (arrow) presenting with perivascular fibrosis (arrow head). H-E (bar= 280µm)

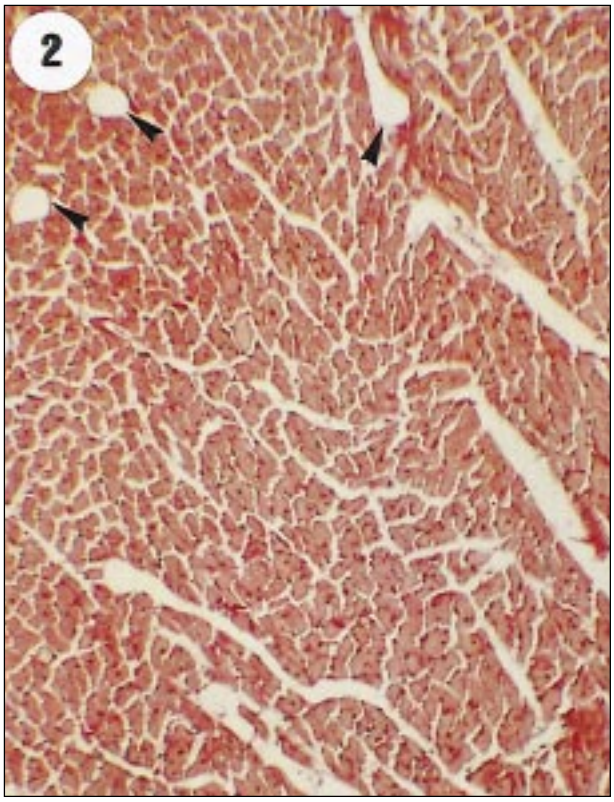


Fig. 2 – Photomicrography of control rat myocardium after 25 days. The myocardial tissue is well organized and the vessels have no lesions (arrow head). H-E (bar=-280μm)

pressure was 74.5% higher on the 25th day and 90.2% higher on the 40th day. Cardiac weight increased 57.8% in L25 rats and 34.6% in L40 rats when compared with their respective controls (fig. 5).

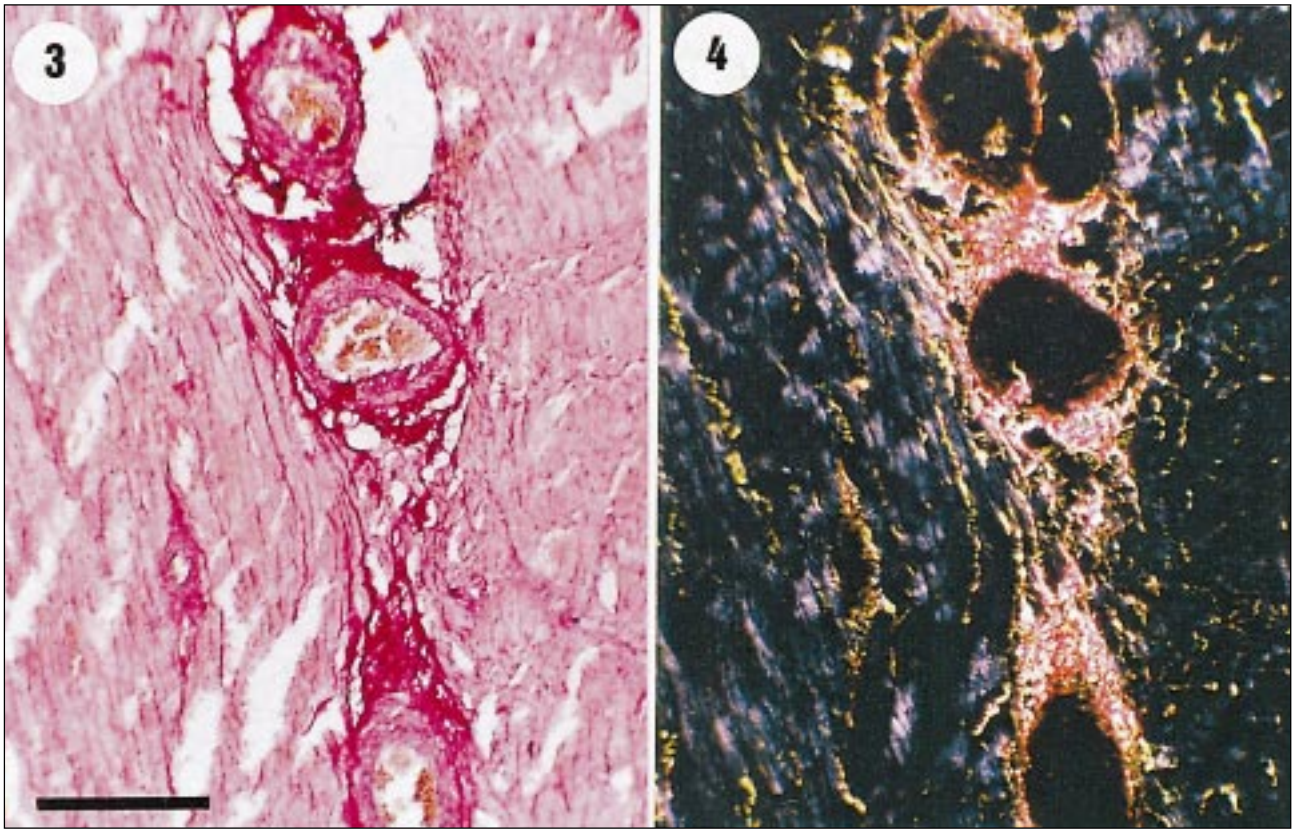
Significant stereological differences were revealed between control and L-NAME rats as shown in figures 2 to 4. Compared with their respective controls, Vv[vessels] decreased by 31.3% in rats from group L40 (fig. 6, median percent variation).

Lv[vessels] decreased 53.5% in group L25 and 25.7% in L40. However between groups C25 and C40 a decrease of 52.2% occurred in Lv[vessels] (fig. 7, median percent variation).

Aa[vessels] increased in two situations. It was 154.6% higher in L25 animals when compared with C25 animals, and it was also 198% higher in C40 animals when compared with C25 animals (fig. 8, median percent variation).

Discussion

The capillary to myocardial ratio in the normal adult myocardium is 1:1. In cardiac hypertrophy, the number of capillaries in the myocardium remains the same but the myocytes are enlarged, resulting in relative ischemia that is proportional to the degree of hypertrophy [21]. In addition, evidence exists that NO acts as a chemotactic factor for angiogenesis and that the inhibition of NO production impairs neovascularization [11].



Figs. 3 and 4 – Photomicrographies of a hipertensive rat that received L-NAME for 40 days. Note the intense perivascular fibrosis and thickning of the vessel wall. Picro-Sirius (bar= 85μm).

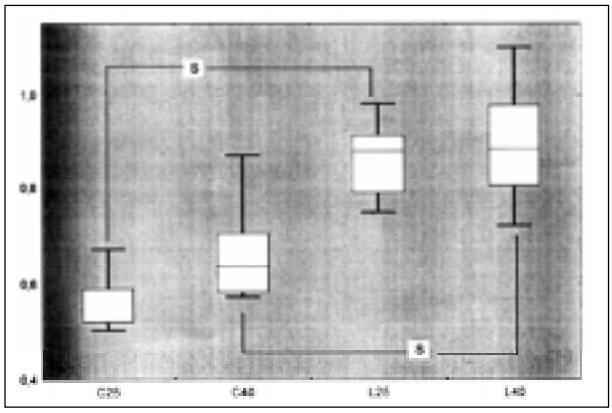


Fig. 5 – Box plot of the cardiac weight in the four groups of animals. C25 - control rats 25 days; C40 - control rats 40 days; L25 - L-NAME for 25 days; L40 - L-NAME for 40 days. S=p < 0.05.

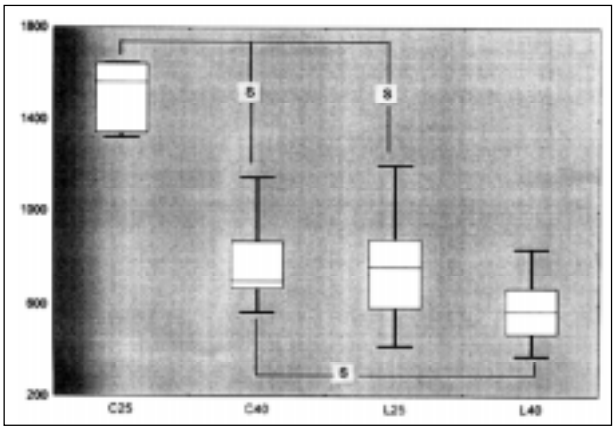


Fig. 7 – Box plot of the lengh density of the intra myocardial vessels.

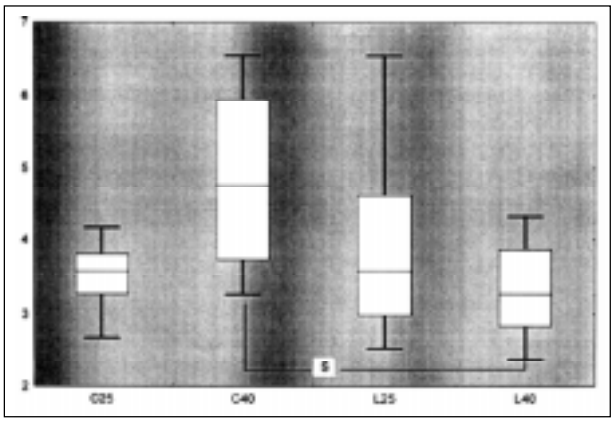


Fig. 6 – Box plot of the volume density of the intramyocardial vessels.

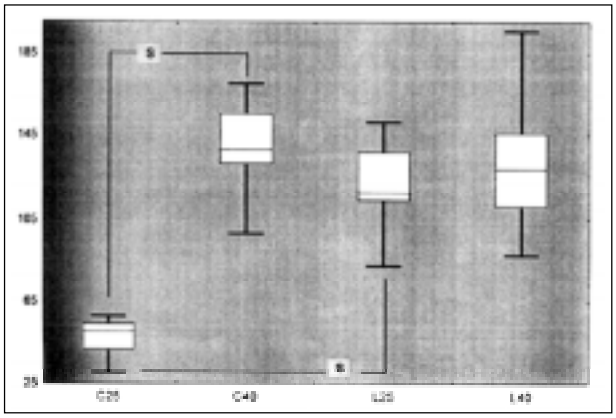


Fig. 8 – Box plot of the cross sectional area of the intra myocardial vessels.

In our study, the myocardium of animals that received L-NAME showed morphological modifications that resulted from the extension of time of NO synthesis inhibition. In this model of arterial hypertension there are descriptions of large areas of interstitial fibrosis mostly in endomysium and perimysium and also perivascular fibrosis around intramyocardial coronary branches and areas of ischemic lesions with inflammatory infiltrate. The intimal layer of small intramyocardial arteries is normally thick [13,14]. Administration of L-NAME at 12mg/kg per day for 15 weeks (105 days) caused myocyte hypertrophy and little interstitial and perivascular fibrosis; however, no significant quantitative modifications in microcirculation occurred [22]. In the present study, in which L-NAME was administered as daily doses of 50mg/kg for 25 or 40 days, we observed marked interstitial and perivascular fibrosis with significant modifications of myocardial microcirculation. These findings indicate that the modifications present in this model are dependent on the time and dose of NO inhibitor administration.

The present study revealed an important diminution in the length-density of myocardial vessels in rats that underwent NO inhibition for 25 and 40 days. Moreover, the volume-density of vessels also diminished in these animals after 40 days. The cross-sectional area of intramyocardial vessels that normally increases during cardiac growth between the 25th and 40th days, increased earlier in animals that underwent NO inhibition after 25 days, suggesting that these rats had an early enlargement of the heart (including vessels) due to pressure overload.

Capillary network increase parallel to the increase of the cross-sectional area of the muscular cells has been reported [23,24]. In a previous study, we observed a significant increase in the cross-sectional area of cardiac myocytes after 25 and 40 days of NO inhibition [14]. This factor may be responsible for the significant increase in cardiac weight at 25 and 40 days. The increment in cardiac weight was higher at 25 days then at 40 days in animals that received L-NAME, compared with their respective controls. It is known that the size of the heart is related to its collagen content and the size of the muscular cell [25,26]. Therefore, we believe that 25 days of ventricular overload is enough to induce significant myocyte hypertrophy (explaining the increased weight). Nevertheless, if the overload is extended for a few more days (*i.e.*, 40 days), the increment in cardiac weight is less marked because it is produced by connective tissue deposition (characterized by an increase in cardiac interstitium volume-density).

A recently published study shows that cardiac hyper-

trophy induced by angiotensin II in rats results in an 18% reduction in capillary density and a 54% increase in arteriolar density. Previous treatment with angiotensin antagonists (receptor subtypes 1 and 2) prevented an increase in arteriolar density, but only losartan prevented changes in capillary density. These findings suggest that angiotensin II dependent cardiac hypertrophy is associated with capillary rarefaction and arteriolar development and that these two processes are regulated independently [27]. Recent reports have suggested that angiotensin II may play an important role in cardiac hypertrophy, acting on muscle cells and fibroblasts both from the myocardium and vessels through insulin-like growth factor 1 that leads to myocyte hypertrophy and to a progressive deposit of collagen in the perivascular and interstitial space. Angiotensin II blocks the enzymatic action of collagenase that promotes the turnover of collagen and, because of that, the myocardial and vascular tissues may become more rigid [28,29].

In the present study, we analyzed stereologically the intramyocardial capillaries and arterioles in conjunction, using an experimental model for which the complete understanding is yet unknown but is probably angiotensin-dependent. Because of this uncertainty, new studies are needed to better clarify the consequences of NO action and inhibition of myocardial physiology.

The beginning and progression of myocyte necrosis may be related to functional and structural abnormalities in the vascular network [30]. The more that cardiac hypertrophy develops, the more heterogeneous is myocyte size and the less homogeneous is intercapillary distance, which may explain the ischemic necrosis that follows the imbalance of oxygen supply and demand [31]. There are several reports of extensive areas of fibrosis, necrosis and granulation tissue in models of arterial hypertension induced by NO inhibition [32]. Our results are in accordance to these reports.

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