Myocardial changes after spironolactone in spontaneous hypertensive rats. A laser scanning confocal microscopy study¹

Leila Maria Meirelles Pereira, C. A. Mandarin-de-Lacerda *

Laboratory of Morphometry & Cardiovascular Morphology, Biomedical Centre, Institute of Biology, State University of Rio de Janeiro, Brazil

Received: December 28, 2001; Accepted: January 16, 2002

Abstract

The present study aims to objectivate by laser scanning confocal microscopy, the cardiac structure of the spontaneously hypertensive rats (SHR) treated with different doses of spironolactone, either alone or in combination with an angiotensin converting enzyme inhibitor or with a calcium channel blocker. Thirty SHRs were divided into six groups and treated during 13 weeks as follows: control, spironolactone (5, 10 and 30 mg/kg/day), spironolactone + enalapril and spironolactone + verapamil. Spironolactone action on the SHR blood pressure (BP) was dose-dependent. The cardiac hypertrophy was affected by the treatment with spironolactone (high dose) or a combination of spironolactone and Enalapril. The myocardial structure was less affected by the spironolactone monotherapy (at all doses) showing hypertrophied cardiac myocytes, focal areas of the reactive fibrosis, inflammatory infiltrate. The treatment with spironolactone in combination with enalapril or verapamil prevented these alterations. In conclusion, the monotherapy with spironolactone had only a limited effect in the preservation of the myocardial structure and in the attenuation of the interstitial fibrosis in SHRs, even after high dose. This action on the myocardium is more efficient when the spironolactone (even in medium dose) was associated with enalapril or verapamil.

Keywords: spironolactone • enalapril • verapamil • hypertension • confocal microscopy

Introduction

Hormones of the circulating renin-angiotensin-aldosterone system (RAAS) may contribute to a structural remodelling of the myocardium [1-2]. The RAAS has emerged as a potential candidate

mediating the regulation of the cardiac myocyte growth (myocardial hypertrophy) and the accumulation of collagen in cardiac fibroblasts [3-4]. Modulation of the RAAS by angiotensin-converting enzyme (ACE) inhibitors, angiotensin II (AngII) receptor antagonists or aldosterone (ALDO) antagonists, can alter the progression of myocardial fibrosis, although the mechanisms involved are poorly defined [5].

The ALDO-antagonist spironolactone was considered able to prevent [6-9], or partially reverse [10], the cardiac fibrosis associated with hypertension

* Mail to: Carlos Alberto MANDARIM-DE-LACERDA, MD, PhD
Laboratório de Morfometria e Morfologia Cardiovascular,
Centro Biomédico, Instituto de Biologia, Universidade do
Estado do Rio de Janeiro, Av. 28 de Setembro, 87 (fds),
20551-030 – Rio de Janeiro, RJ, Brasil.
Tel/Fax: [+55.21] 2587-6416

Email: mandarin@uerj.br. [Http://www2.uerj.br/~lmme](http://www2.uerj.br/~lmme)

¹ Research supported by Brazilian agencies CNPq and FAPERJ

in rats. When rats were long-term treated with ALDO, a small dose of spironolactone did not prevent the development of hypertension nor the left ventricular hypertrophy, but the interstitial and perivascular fibrosis did not occur [6].

The prevention of arterial hypertension and elevation in circulating Ang II and ALDO appear as logical targets for the prevention of myocardial fibrosis [5]. In antihypertensive therapy, a revival of the use of spironolactone combined with ACE inhibitors is the result of the cardioprotective effects of both drugs in severe hypertension [11]. Potentiation by lowering side effects is expected, due to the combination therapy. The rationale behind using, in conjunction with spironolactone, ACE inhibitors or calcium channel blockers (CCB) is; - the combined beneficial effects on blood pressure; - the prevention of the left ventricular hypertrophy; - protective effects on the kidneys, resulting in reduction of proteinuria. Side effects are expected to be smaller than monotherapy: the required dosage of spironolactone for an effective antifibrotic regimen is not sufficient to satisfactorily decrease blood pressure in man in many cases, and is accompanied by the well-known side effects of ALDO antagonists [12]. However, treatment with ALDO antagonist in conjunction with an ACE inhibitor is relatively contraindicated due to the potentially serious hyperkalemia [13].

The ALDO-antagonist treatment of heart failure associated with ACE inhibitors and loop diuretic has been proposed more recently [11, 14]. The cardioprotective effects of spironolactone may explain the prognostic value of anti-ALDO therapy in patients with severe chronic heart failure evaluated in the RALES mortality trial [8].

The aim of this study was to observe using laser scanning confocal microscopy the cardiac structure of the *spontaneously hypertensive rats* (SHR) treated with different doses of spironolactone, or with spironolactone in combination with an ACE inhibitor or with a CCB.

Materials and methods

Experiments were performed using male SHR obtained from the colony maintained in the Laboratory of Morphometry & Cardiovascular Morphology at Rio de Janeiro (www2.uerj.br/~lmmc), with free access to stan-

dard rat chow and fresh water. At the beginning of the study, animals were 20 weeks old, had body mass of 302 ± 28 g (mean $\pm$ SD). The BP was weekly verified in conscious SHRs using the non-invasive method of the tail-cuff plethysmography (RTBP1007, Kent Scientific Co, Litchfield, USA). At the beginning of this study animals had a BP of 151 ± 2 mmHg (no difference among the groups). The animals were maintained in individual cages at a temperature of $23 \pm 1^\circ\text{C}$ with a constant humidity of $55 \pm 5\%$ and a cycle of 12-h light/dark.

All protocols were approved by the Animal Experimentation Ethics Committee of The State University of Rio de Janeiro. Animal care conformed to the "Guide for the Care Use of laboratory Animals" published by the US National Institutes of Health (NIH Publication No.85-23, revised 1996).

After one week acclimatization, the animals were treated for thirteen weeks. They were distributed among five experimental groups as described below. Each group was composed by five animals.

a) Control group (C) – SHRs were manipulated and sacrificed as the animals of the experimental groups, but they only received water and food *ad libitum*.

b) Spironolactone, low dose group (SL) - SHRs received spironolactone, 5mg/kg/day dissolved in the drinking water (7a-(Acetylthio)-17a-hydroxy-3-oxopregn-4-ene-21-carboxylic acid g-lactone) (Sigma Chemical Co., St. Louis, Lot 77H1291).

c) Spironolactone, medium dose group (SM) - SHRs received spironolactone, 10mg/kg/day dissolved in the drinking water.

d) Spironolactone, high dose group (SH) - SHRs received spironolactone, 30mg/kg/day dissolved in the drinking water.

e) Spironolactone+enalapril group (SE) - SHRs received spironolactone, in the medium dose and, simultaneously, enalapril maleate ((S)-N-(1-(Ethoxycarbonyl)-3-phenylpropyl)-Ala-Pro maleate) (Sigma Chemical Co., St. Louis, Lot 38H0500) 15mg/kg/day dissolved in the drinking water.

f) Spironolactone+verapamil group (SV) - SHRs received spironolactone in the medium dose and, simultaneously, verapamil hydrochloride ($\text{C}_{27}\text{H}_{38}\text{N}_2\text{O}_4 \cdot \text{HCl}$) (Sigma Chemical Co., St. Louis, Lot 56H0925) 15mg/kg/day dissolved in the drinking water.

In the morning of the 91st day the animals were deeply anesthetized, weighted and sacrificed (heart injection of 3ml of KCl at 10%), the hearts were then removed by cutting the vessels shortly and left ventricles were separated and weighted.

The fragments of left ventricle were placed 48h at room temperature in fixative (freshly prepared 4% w/v formaldehyde in 0.1M phosphate buffer pH 7.2), embedded in Paraplast plus and sectioned with 10-15 μm

of thickness to be observed in laser scanning confocal microscopy (Zeiss, model LCM 410). The analysis used a laser of 543nm for exciting the picro-sirius red modified stain [15-16] and getting images in the band of 580nm. Fifteen random fields per group (three fields per animal, five animals per group) were analysed using the Image Pro Plus software (Media Cybernetics). The areal fraction of the connective tissue (connective tissue fraction in the myocardium, CTF) was calculated. The image analysis automatically selected the connective tissue pixel range after calibrating and classifying all images.

The weekly difference of the BP in a group was tested with the paired t-test. The BP and the cardiac quantitative data differences among groups were tested with the analysis of variance and the Newman-Keuls multiple comparison test. The significant level of 0.05 was considered for statistical significance [17].

Results

Fig. 1 and Tables 1, 2 summarize results regarding the BP evolution in the different groups. Table 3 shows the cardiac quantitative data among the groups. The Fig. 2 shows confocal laser scanning microscopy of the myocardium in all groups.

The young adult SHR showed a moderate hypertension at the beginning of the study and no significant differences were recorded among the

groups at this time (mean $\pm$ SD was 151 $\pm$ 2mmHg). In the C group, the BP increased after the first four weeks of experimentation, reaching the value of 177 $\pm$ 2.7mmHg after 10 weeks when it stabilised. However, this habitual evolution of the BP in young adult SHR was significantly altered by the spironolactone treatment, with either attenuation or reversion of the BP increasing trend ($p<0.05$).

In the SL group, the BP decreased slowly reaching a level of 140 $\pm$ 0.9mmHg in the last two weeks of experimentation. In the SM and SH groups the BP has a more deep decrease. This was more evident after the 7th week of experimentation, when the BP values in SH animals were significantly lower than that of the SM animals. Spironolactone administration in combination with enalapril or verapamil caused a BP reduction even more pronounced than that using spironolactone monotherapy (all doses) mainly in the last two weeks of experimentation (Tables 1 and 2).

The left ventricular weight (LVW) was more than 15% smaller in the SH and SE animals than in the control ones. The CTF was significantly low in SH, SE and SV groups in relation to the other groups. The CTF decrease was of great magnitude, more than 60% than the other groups (Table 3). No difference was found to the CTF comparing the SH, SE and SV groups.

Fig. 1 Evolution of the blood pressure (BP) during the time of experimentation (mean $\pm$ SD).

Groups:

C – control,
SL – spironolactone low dose,
SM – spironolactone mean dose,
SH – spironolactone high dose,
SE – spironolactone + enalapril,
SV – spironolactone + verapamil.

Fig. 2 Laser scanning confocal micrographs of the left ventricle myocardium stained with collagen-specific dye picro Sirius red (same magnification for all photomicrographs, bar in **A** = 140µm). The intercellular and perivascular collagen distribution is marked in yellow color. *Control SHR* (**A**) - the myocardium had hypertrophied cardiac myocytes; a thick network of collagen surrounding intramyocardial vessels connects with perimysium and endomysium. *Spironolactone monotherapy groups* (**B-D**) – no significant structural changes were observed in the myocardium of the groups, hypertrophied cardiac myocytes and reactive fibrosis are seen in all spironolactone doses (**B** – low dose, **C** – medium dose, and **D** – high dose). *Spironolactone + enalapril group* (**E**) and *spironolactone + verapamil group* (**F**) – in these SHR the myocardium was considerably preserved, the myocytes are almost normal in size, scarce collagen interstitial network and well-preserved intramyocardial branches of the coronary arteries were mainly observed in spironolactone + enalapril group.

Table 1 Correlation matrix in the different weeks (W) of the experimentation. Differences among groups were tested with analysis of variance and the multiple comparison test of Newman-Keuls. NS is not significant. See legend in Table 1.

Groups	SL	SM	SH	SE	SV
C	W1 – NS	W1 – NS	W1 – NS	W1 – NS	W1 – NS
	W4 – NS	W4 – NS	W4 – NS	W4 – 0.003	W4 – NS
	W7 – 0.0002	W7 – 0.0001	W7 – 0.0002	W7 – 0.0001	W7 – 0.0001
	W10 – 0.0002	W10 – 0.0001	W10 – 0.0001	W10 – 0.0001	W10 – 0.0001
	W12 – 0.0002	W12 – 0.0001	W12 – 0.0001	W12 – 0.0001	W12 – 0.0001
SL		W1 – NS	W1 – NS	W1 – NS	W1 – NS
		W4 – NS	W4 – NS	W4 – NS	W4 – NS
		W7 – NS	W7 – 0.03	W7 – 0.0001	W7 – NS
		W10 – 0.0003	W10 – 0.0001	W10 – 0.0001	W10 – 0.0002
		W12 – 0.0002	W12 – 0.0001	W12 – 0.0001	W12 – 0.0002
SM			W1 – NS	W1 – NS	W1 – NS
			W4 – NS	W4 – 0.003	W4 – NS
			W7 – 0.05	W7 – 0.0002	W7 – NS
			W10 – 0.0002	W10 – 0.0002	W10 – 0.0001
			W12 – 0.005	W12 – 0.0001	W12 – 0.0001
SH				W1 – NS	W1 – NS
				W4 – NS	W4 – NS
				W7 – 0.0001	W7 – NS
				W10 – 0.0003	W10 – 0.0002
				W12 – 0.002	W12 – 0.002
SE					W1 – NS
					W4 – NS
					W7 – 0.0002
					W10 – NS
					W12 – NS

Laser scanning confocal micrographs of the SHR myocardium were taken preferentially in the cross-sectioned sections facilitating observations on the myocyte and the intercellular/perivascular collagen distribution analysis (Fig. 2). The general myocardial aspect was similar in SHR controls and SHR treated with spironolactone monotherapy (all doses). In these 33-weeks-old SHR, the cardiac myocytes had characteristics of moderate to severe hypertrophy and several focal areas with reactive fibrosis and inflammatory infiltrate. However, the hypertrophy of the cardiac myocytes was practically not present and the intercellular collagen distribution suggested no reactive fibrosis, in SHR treated with the combination of spironolactone and enalapril or verapamil. Consequently, the

intramyocardial branches of the coronary arteries in these treated SHR were preserved, especially in the SE group.

Discussion

A dose dependent effect of the spironolactone on the typical SHR BP increasing tendency and on the myocardial structural alteration was observed. This effect was modest with a spironolactone dose of 5 mg/kg/day, but it was pronounced using a dose of 30mg/kg/day. However, the SHR BP decreased efficiently using the association of spironolactone and enalapril or verapamil. The use of spironolactone monotherapy (low and medium

Table 2 Correlation matrix analyzing the intragroup blood pressure variation during the experimentation. Differences were tested with the paired t-test. NS is not significant. See legend in Table 1.

Groups	Weeks	4	7	10	12
C	1	NS	0.0003	<0.0001	<0.0001
	4		0.0004	<0.0001	<0.0001
	7			0.01	0.02
	10				NS
SL	1	NS	0.02	0.0003	0.0004
	4		NS	0.006	0.03
	7			NS	NS
	10				NS
SM	1	NS	0.01	0.0005	<0.0001
	4		<0.0001	<0.0001	<0.0001
	7			0.0003	0.0001
	10				0.0006
SH	1	0.002	<0.0001	<0.0001	<0.0001
	4		0.009	0.0002	<0.0001
	7			0.0001	0.0001
	10				NS
SE	1	0.01	0.0002	<0.0001	<0.0001
	4		0.001	0.0001	0.0001
	7			0.001	0.0002
	10				0.02
SV	1	0.03	0.006	<0.0001	<0.0001
	4		NS	<0.0001	<0.0001
	7			0.0004	0.0003
	10				NS

dose) did not show significant alteration in the typical SHR myocardial structure. It did not prevent myocyte hypertrophy nor cardiac interstitial and perivascular fibrosis. The administration of combined therapy (spironolactone and enalapril or verapamil) was effective in preventing these SHR myocardial alterations. The cardiac fibrosis was prevented (SE group) or attenuated (SV group); the myocyte hypertrophy was prevented in both

groups. The intramyocardial arteries were also maintained in good condition.

Some previous studies considered that monotherapy with spironolactone is effective to prevent or to reduce the cardiac fibrosis even without simultaneous BP reduction. These studies used different spironolactone dose, regimens and different experimental models (or human individuals) to corroborate their data.

Table 3 Myocardial quantitative data (mean±SD): left ventricular weight (LVW) and connective tissue areal fraction (CTF). Differences among groups were tested using the analysis of variance and the multiple comparison test of Newman-Keuls. See legend in Table 1.

Groups	LVW (gm)	CTF %
C	885.8±59.2	7.8±2.8
SL	757.2±94.3	5.2±2.0
SM	795.2±101.2	5.0±3.1
SH	726.4±91.0	2.0±1.2
SE	715.2±57.1	0.9±0.4
SV	756.5±40.1	1.0±0.5
ANOVA and Newman-Keuls test		
C x SL	NS	NS
C x SM	NS	NS
C x SH	<0.05	<0.05
C x SE	<0.05	<0.05
C x SV	NS	<0.05
SL x SM	NS	NS
SL x SH	NS	<0.05
SL x SE	NS	<0.05
SL x SV	NS	<0.05
SM x SH	NS	<0.05
SM x SE	NS	<0.05
SM x SV	NS	<0.05
SH x SE	NS	NS
SH x SV	NS	NS
SE x SV	NS	NS

Spironolactone dose normally varied from 0.37 to 50.0mg/kg/day (present study used 5.0 to 30.0mg/kg/day), but Brilla [8] and Lacolley and co-workers [18] used an extreme dose of 200mg/kg/day.

Different possibilities of cardiac responses were found after spironolactone treatment, varying from prevention [6, 8, 18-19] to reversion of the increased deposition of perivascular and interstitial

collagen [20-21]. Usually, these spironolactone effects were irrespective of the development of cardiac hypertrophy and hypertension [6, 8]. Contrarily to previous reports using other models of experimental hypertension [6, 8], the decrease of the BP in SHR with spironolactone was dose-dependent, and was not associated with an expected prevention of the cardiac myocyte hypertrophy, even with the long-term treatment (13 weeks). The

high dose of spironolactone monotherapy had a positive effect on the myocardial structure, more than the medium dose had. Therefore, the association of the medium dose with enalapril or verapamil should determine the efficacy of these associations with spironolactone. In comparison with the control SHR, the left ventricular hypertrophy was only observed in the SH and SE groups. This probably was not only due to the overload reduction in these animals, because the SV group had a similar BP reduction but not a reduction in the LVW.

The rationale of combining an ACE inhibitor and spironolactone is to achieve a more complete inhibition of the whole RAAS. This is particularly important in studies using SHRs because the rat heart produces ALDO and endogenous cardiac ALDO may affect cardiac function and hypertrophy in hypertension in rats [19]. There is also an advantage associating spironolactone and calcium channels blockers, because CCB can prevent myocardial fibrosis by attenuating the number of extracellular matrix producing myofibroblasts at sites of repair [22]. This is possible because Ang II and ALDO may both operate on intracellular calcium levels [2], and increased intracellular free calcium levels are associated with proliferation of myofibroblast [23]. The present study showed similar results on SHR BP reduction and prevention of cardiac fibrosis and myocyte hypertrophy using spironolactone in combination with enalapril and in combination with verapamil.

Previous reports in the literature [24-25] have shown that treatment of hypertensive rats with ACE inhibitors may lead to enhanced left ventricle, even at a low dose not affecting blood pressure. The left ventricular hypertrophy, and the significant decrease of the left ventricular cardiac myocyte number in hypertensive rats submitted to nitric oxide synthesis blockade, were prevented with the use of enalapril and verapamil [26]. In the present study, the monotherapy with spironolactone (low and medium dose) was not efficient to preserve the myocardium in SHRs. In high dose, the spironolactone showed a partial effect in the attenuation of the interstitial fibrosis and a positive effect in the left ventricle hypertrophy. This action on the myocardium was pronounced when the spironolactone was combined with the enalapril more than with verapamil.

Acknowledgements

The authors would like to thank *Patricia A. M. Martinez* for her technical assistance in the confocal microscopy use and *Mario S. Pereira* for his expertise in the image analysis. The authors would like to thank *Professor Henrique Lenzi*, Head of the Department of Pathology, Oswaldo Cruz Institute (Rio de Janeiro) for his kind permission to use his laboratory and the confocal microscopy facilities.

References

1. **Brilla C.G., Pick R., Tan L.B., Janick J.S., Weber K.T.**, Remodeling of the rat right and left ventricle in experimental hypertension, *Circ. Res.*, **67**: 1355-1364, 1990
2. **Sun Y., Weber K.T.**, Cardiac remodelling by fibrous tissue: role of local factors and circulating hormones, *Ann. Med.*, **30**:(Suppl. 1), 3-8, 1998
3. **Lijnen P., Petrov V.**, Antagonism of the renin-angiotensin-ALDO system and collagen metabolism in cardiac fibroblasts, *Method. Find. Exp. Clin. Pharmacol.*, **21**: 215-227, 1999
4. **Lijnen P., Petrov V.**, Induction of cardiac fibrosis by aldosterone, *J. Mol. Cell. Cardiol.*, **32**: 865-879, 2000
5. **Dixon I.M., Ju H., Reid N.L., Scammell-La Fleur T., Werner J.P., Jasmin G.**, Cardiac collagen remodelling in the cardiomyopathic Syrian hamster and the effect of losartan, *J. Mol. Cell. Cardiol.*, **29**: 1837-1850, 1997
6. **Brilla C.G., Matsubara L.S., Weber K.T.**, Anti-ALDO treatment and prevention of myocardial fibrosis in primary and secondary hyperaldosteronism, *J. Mol. Cell. Cardiol.*, **25**: 563-575, 1993
7. **Robert V., Silvestre J.S., Charlemagne D., et al.**, Biological determinants of aldosterone-induced cardiac fibrosis in rats, *Hypertension*, **26**[Part 1]: 971-978, 1995
8. **Brilla C.G.**, Aldosterone and myocardial fibrosis in heart failure, *Herz*, **25**: 299-306, 2000
9. **Brilla C.G., Weber K.T.**, Reactive and reparative myocardial fibrosis in arterial hypertension in the rat, *Cardiovasc. Res.*, **26**: 671-677, 1992
10. **Young M., Head G., Funder J.**, Determinants of cardiac fibrosis in experimental hypermineralocorticoid states, *Am. J. Physiol.*, **269**: E657-E662, 1995
11. **Pitt B., Zannad F., Remme W., et al.**, The effect of spironolactone on morbidity and mortality in patients with severe heart failure, *New Engl. J. Med.*, **341**: 709-717, 1999

12. **Maish B., Brilla C., Kruse T.,** Directions in antihypertensive treatment – our future from the past, *Eur. Heart J.*, **16: (Suppl C)**, 74-83, 1995
13. **Weber K.T.,** Cardioreparation in hypertensive heart disease, *Hypertension*, **38: (Part 2)**, 588-591, 2001
14. **R.A.L.E.S. Study,** Effectiveness of spironolactone added to an angiotensin-converting enzyme inhibitor and a loop diuretic for severe chronic congestive heart failure (the randomized aldactone evaluation study [RALES]), *Am. J. Cardiol.*, **78:** 902-907, 1996
15. **Dolber P.C., Spach M.S.,** Picrosirius red staining of cardiac muscle following phosmolybdc acid treatment, *Stain Tech.*, **62:** 23-26, 1987
16. **Dolber P.C., Spach M.S.,** Conventional and confocal fluorescence microscopy of collagen fibers in the heart, *J. Histotechnol. Cytochem.*, **41:** 465-469, 1993
17. **Zar J.H.,** *Biostatistical analysis*, Upper Saddle River: Prentice Hall, 1999
18. **Lacolley P., Safar M.R., Lucet B., Ledudal K., Labat C., Benetos A.,** Prevention of aortic and cardiac fibrosis by spironolactone in old normotensive rats, *J. Am. Coll. Cardiol.*, **37:** 662-667, 2001
19. **Takeda Y., Yoneda T., Demura M., Miyamori I., Mabuchi H.,** Cardiac aldosterone production in genetically hypertensive rats, *Hypertension*, **36:** 495-500, 2000
20. **Brown L., Duce B., Miric G., Sernia C.,** Reversal of cardiac fibrosis in deoxycorticosterone acetate-salt hypertensive rats by inhibition of the rennin-angiotensin system, *J. Am. Soc. Nephrol.*, **10:** S143-S148, 1999
21. **Miric G., Dallemagne C., Endre Z., Margolin S., Taylor S.M., Brown L.,** Reversal of cardiac and renal fibrosis by pirfenidone and spironolactone in streptozotocin-diabetic rats, *Br. J. Pharmacol.*, **133:** 687-694, 2001
22. **Ramires F.J.A., Sun Y., Weber K.T.,** Myocardial fibrosis associated with ALDO or angiotensin II administration: attenuation by calcium channel blockade, *J. Mol. Cell. Cardiol.*, **30:** 475-483, 1998
23. **Sadoshima J., Izumo S.,** Molecular characterization of angiotensin II-induced hypertrophy of cardiac myocytes and hyperplasia of cardiac fibroblasts. Critical role of the AT1 receptor subtype, *Cir. Res.*, **73:** 413-423, 1993
24. **Gohlke P., Kuwer I., Schnell A., Amann K., Mall G., Unger T.,** Blockade of bradykinin B₂ receptors prevents the increase in capillary density induced by chronic angiotensin-converting enzyme treatment in stroke-prone spontaneously hypertensive rats, *Hypertension*, **29:** 478-482, 1997
25. **Unger T., Mattfeld T., Lamberty V., Bock P., Mall G., Linz W.,** Effect of early onset angiotensin converting enzyme inhibition on myocardial capillaries, *Hypertension*, **20:** 478-482, 1992
26. **Pereira L.M.M., Mandarim-de-Lacerda C.A.,** The effect of enalapril and verapamil on the left ventricular hypertrophy and the left ventricular cardiomyocyte numerical density in rats submitted to nitric oxide inhibition, *Int. J. Exp. Path.*, **82:** 115-122, 2001