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AT1-receptor mediated vascular damage in myocardium, kidneys and liver in rats[1]

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ABSTRACT.- Vailati M.C.F., Rocha N.S., Matsubara L.S., Padovani C.R., Schwartz D.S. & Matsubara B.B. 2010. **AT1-receptor mediated vascular damage in myocardium, kidneys and liver in rats**. *Pesquisa Veterinária Brasileira 30(7):605-611*. Departamento de Clínica Veterinária, Faculdade de Medicina Veterinária e Zootecnia, Universidade Estadual Paulista, Distrito de Rubião Júnior s/n, Botucatu, SP 18618-000, Brazil. E-mail: mfvailati@yahoo.com.br

The systemic aspect of vascular damage induced by angiotensin II (ANG II) has been poorly explored in the literature. Considering the presence of ANG II and its specific receptor AT1, in several organs, all tissues might be potentially affected by its effects. The aims of this study were: To evaluate the early histological changes in the heart, liver and kidneys, produced by ANG II infusion, to evaluate the protective effect of losartan. Wistar rats were distributed into three groups: control (no treatment), treated with ANG II, and treated with ANG II + losartan. ANG II was continuously infused over 72 hours by subcutaneous osmotic pumps. Histological sections of the myocardium, kidneys and liver were stained and observed for the presence of necrosis. There were ANG II-induced perivascular inflammation and necrosis of the arteriolar wall in the myocardium, kidney, and liver by, which were partially prevented by losartan. There was no significant correlation between heart and kidney damage. Tissue lesion severity was lower than that of vascular lesions, without statistical difference between groups. ANG II causes vascular injury in the heart, kidneys and liver, indicating a systemic vasculotoxic effect; the mechanisms of damage/protection vary depending on the target organ; perivascular lesions may occur even when anti-hypertensive doses of losartan are used.

INDEX TERMS: Angiotensin, arteries, hypertension, remodeling, necrosis.

RESUMO.- [**Lesão vascular mediada pelo receptor AT1 em miocárdio, rins e fígado de ratos.**] O aspecto sistêmico da lesão vascular induzida pela angiotensina II (ANG II) tem sido pouco explorada na literatura. Considerando a presença de ANG II e de seu receptor AT1 em diversos órgãos, todos os tecidos poderiam ser potencialmente afetados por esses efeitos. Os objetivos deste estudo foram: avaliar as alterações histológicas iniciais no coração, fígado e rins, produzidas pela infusão de ANG II, e avaliar o efeito protetor do losartan. Ratos Wistar foram divididos em três grupos: controle (sem tratamento), tratados com ANG II, e tratados com ANG II + losartan. A ANG II foi infundida continuamente por 72 horas por meio de mini-bombas osmóticas. Foram realizados cortes histológicos de miocárdio, rim e fígado para coloração e observação para a presença de necrose. Observou-se a presença de inflamação perivascular e necrose de parede arteriolar em miocárdio, rins e fígado, que foram parcialmente prevenidas pelo losartan. Não houve correlação significante entre as lesões observadas no coração e nos rins. A severidade da lesão tissular foi menor quando comparada às lesões vasculares, sem diferença estatística entre os grupos. A ANG II causa injúria vascular no coração, rins e fígado, sugerindo um efeito

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vasculotóxico sistêmico; os mecanismos de lesão/proteção variam dependendo do órgão afetado; as lesões perivasculares podem ocorrer mesmo quando doses anti-hipertensivas de losartan forem utilizadas.

TERMOS DE INDEXAÇÃO: Angiotensina, artérias, hipertensão, remodelamento, necrose.

INTRODUCTION

The renin-angiotensin-aldosterone system (RAAS) contributes to the regulation of the cardiovascular system, which maintains the hemodynamic balance of the body. RAAS is well known to play a leading role in the physiology of cardiovascular diseases (Yamagishi et al. 1993).

Angiotensin II (ANG II) is one of the most powerful known vasoconstrictors and its effects are mediated by specific receptors in vessels, the heart, adrenal gland, brain, liver and kidneys (Chiu et al. 1989, Johnston 1994). Most ANG II biological effects are mediated by the specific AT_1 receptor, although other subtypes have been identified (Timmermans et al. 1993).

Originally, losartan, an ANG II type-1 (AT_1)-receptor antagonist was used for the treatment of hypertension, and has been recently studied for its varied effects in different animal models (Hashimoto et al. 1999, Suzuki et al. 2001, Kumari et al. 2004, Silva et al., 2009).

Most of the studies in which ANG II effects on the myocardium were evaluated agree regarding severity and the early occurrence of lesions involving vessels and muscular tissue (Henegar et al. 1995, 1998, Vailati et al. 2010). These studies used subcutaneous continuous infusion of ANG II. An aspect, little explored in the literature, is the systemic pattern of the ANG II-induced vascular abnormalities. Theoretically, all tissues may be affected, as ANG II receptors have already been identified in several organs.

The overall objectives of the researches with this study were: (1) to assess the early histological changes in the heart, liver and kidneys produced by angiotensin II infusion, and (2) to evaluate the effects of losartan on morphologic changes in the heart, liver and kidney.

MATERIALS AND METHODS

Eight-week old male Wistar rats (250±20g, n=34) were obtained from the Central Vivarium of Botucatu Medical School, São Paulo State University, Brazil. The animals were housed under standard environmental conditions and maintained on commercial rat laboratory diet (Purina Labina®, São Paulo, Brazil) and tap water *ad libitum*. The experimental protocol herein was approved by our Institution's Animal Care and Use Committee and conforms with the NIH Guidelines on the Care and Use of Experimental Animals (NIH publication 85-23, Revised 1985).

ANG II (Sigma, A-9525, Germany) was delivered at a rate of 150 ηg/min by an osmotic minipump (model 2002, ALZA Corp., CA) implanted subcutaneously in the nape of the neck with the use of aseptic techniques over 72 hours. This ANG II dose is known to cause myocyte necrosis (Tan et al. 1991, Kabour et al. 1994, Henegar et al. 1995, 1998, Vailati et al. 2010). Surgical procedures were performed with rats under ketamine (50mg/kg) -xylazine (10mg/kg) anesthesia administered by intramuscular injection.

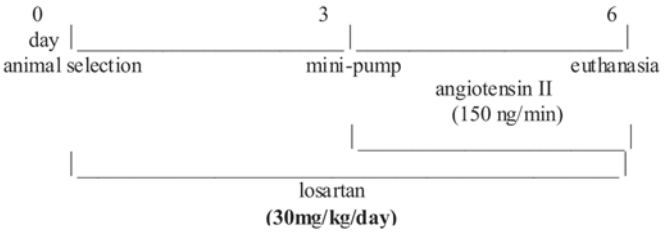

Fig.1. Protocol diagram.

Thirty-four rats were randomly divided into three groups: control rats (no drug interventions, n=12); ANG II rats (n=9) and ANG II plus losartan rats (n=13). In this group, losartan treatment (30 mg/kg/day, in drinking water) was started three days before the mini-pump was implanted. Rats drink approximately 16ml water/100-g body-weight/day. From this estimate, the concentration in the drinking water was adjusted to ensure correct dosing. This losartan dose is known to prevent cardiomyocyte necrosis and vascular damage (Kabour et al. 1994).

Tail cuff blood pressure and heart rate (HR) were monitored one day before the beginning of the treatment and immediately before sacrifice. The results were averaged from three consecutives measurements. Blood pressure evaluation was performed twice or three times before the beginning of the study in order to have the animals used for the procedure. Body weight was measured at three moments (Fig.1): day 0 (animal selection), day 3 (before mini-pump implantation) and on day 6 (euthanasia). At the end of each study period, the animals were killed by thoracotomy and heart excision under pentobarbital anesthesia.

Eight rats from each group were randomly selected for histological evaluation. After removal of the heart and before fixation in 10% buffered formalin, the left and right ventricles were separated and weighed. The interventricular septum was included with the left ventricle. A 3mm-thick coronal section taken from the middle portion of each ventricle and a sample of both the kidney and the liver were embedded in paraffin and stained with haematoxylin and eosin (HE) in order to delineate necrotic sites, areas of abnormal cellular infiltrate, and the cell types present in the areas of damage.

Damaged areas were measured under light microscopy (200x magnification). Each slide included ten fields randomly chosen with 200x magnification. Lesions were considered vascular or perivascular when found at a distance of up to five mm from the vessel; from that mark on, they were considered as tissue lesions. Lesions were defined whenever any of the following alterations were observed: cellular edema, esteatosis, necrosis, congestion, inflammatory infiltrate and apoptosis. Quantification of organ injury was independently performed by two pathologists blinded to the source of the tissue sections. The injury level in each field was classified according to a 0-3 score (0= no damage; 1= up to 25% of the field with lesions; 2= 25 to 50% of the field with lesions; 3= more than 50% of the field with lesions. At the end of the slide examination, the level of injury in the organ was determined by the sum of field scores. In general, the pathologists' assessments were similar. When there was marked disagreement, the slides in question were jointly re-analyzed in order to reach consensus.

Haematoxylin-eosin-stained tissue sections (left and right

ventricles) with higher scores were selected for macrophage identification. Immunolabelling was used to identify macrophages by using the avidin-biotin-peroxidase technique (mouse anti-rat macrophages, MCA 342; Serotec, UK) (Hsu 1981).

Haematoxylin-eosin-stained tissue sections (left and right ventricles) with higher scores were selected for the identification of mast cells and eosinophils using acidic Congo-Red and toluidine blue staining (Tarpley 1984).

The quantification of macrophages, mast cells and eosinophils was performed using an image analysis system (Image-Pro^{Ò} Program Plus-Media Cybernetics, Silver Spring, MD, USA) under a Leica microscope with an image captor chamber Leica MPS30, Germany. Ten fields without vessels and ten fields with vessels were selected at 400x magnification, to determine if these cells were perivascular and/or tissue located.

Values were expressed as means ± SD or median ± interquartile range. Body weight, heart rate, systolic blood pressure, left and right normalized ventricular weights were analyzed using one-way ANOVA, followed by Tukey's Test (Zar 1999). The scores of vascular and tissue lesions were analyzed by the nonparametric Kruskal-Wallis test, complemented with the Dunn´s test (Zar 1999). The same test was used for the analysis of the cell count of macrophages and mast cells in the perivascular and interstitial areas. In the ANG II group correlations were accomplished among the scores of the vascular lesions in different organs, using Spearman's correlation coefficient. Differences were considered significant when $P<0.05/k$, where k is the number of comparisons.

RESULTS

Body weight, heart rate, systolic arterial pressure and normalized ventricular weight data are shown in Table 1. The ANG II group showed a significant increase in arterial blood pressure, when compared to the control and ANG II

Table 2. Vascular lesions scores

Group	LV	RV	PP	TV	K
Control	0.0±0.0^a (0.0;0.0)	0.0±0.0^a (0.0;0.0)	0.0±0.0^a (0.0;0.0)	0.0±0.0^a (0.0;0.0)	0.0±0.0^a (0.0;0.0)
ANG II	9.5±1.8^b (4.0;13.0)	9.5±1.5^b (4.0;13.0)	3.0±3.0^b (0.0;8.0)	6.0±2.8^b (2.0;11.0)	8.5±3.5^b (3.0;13.0)
ANG II + Los	4.0±1.8^c (1.0;10.0)	4.0±1.3^c (2.0;10.0)	3.0±1.5^b (0.0;5.0)	2.0±1.3^c (1.0;5.0)	3.5±2.0^c (0.0;10.0)
P value	P < 0.05	P < 0.05	P < 0.05	P < 0.05	P < 0.05

Median and interquartile range. LV: left ventricle. RV: right ventricle. PP: peri-portal. TV: terminal vein. K: kidney. Values in parentheses correspond to the minimum and maximum scores lesion intensity, respectively; a, b and c indicate differences between the groups (P<0.05 by Kruskal-Wallis test, n=8/group).

Table 1. Morphometric and hemodynamic data

Group	BW (g)	HR (bpm)	BP (mmHg)	LVW/BW (mg/g)	RVW/BW (mg/g)
Control n = 12	276 18	388±40	128±5^a	2.47±0.34	0.85±0.18
ANG II n = 9	261±28	375±34	170±23^b	2.66±0.21	0.81±0.09
ANG II + Los n = 13	260±23	400±29	120±11^a	2.37±0.32	0.76±0.17
P value	P > 0.05	P > 0.05	P < 0.05	P > 0.05	P > 0.05

Mean ± standard deviation. BW: body weight. HR: heart rate. BP: blood pressure. LVW and RVW/BW: left and right normalized ventricular weights; a and b indicate statistical differences between the groups (P<0.05, ANOVA and Tukey test).

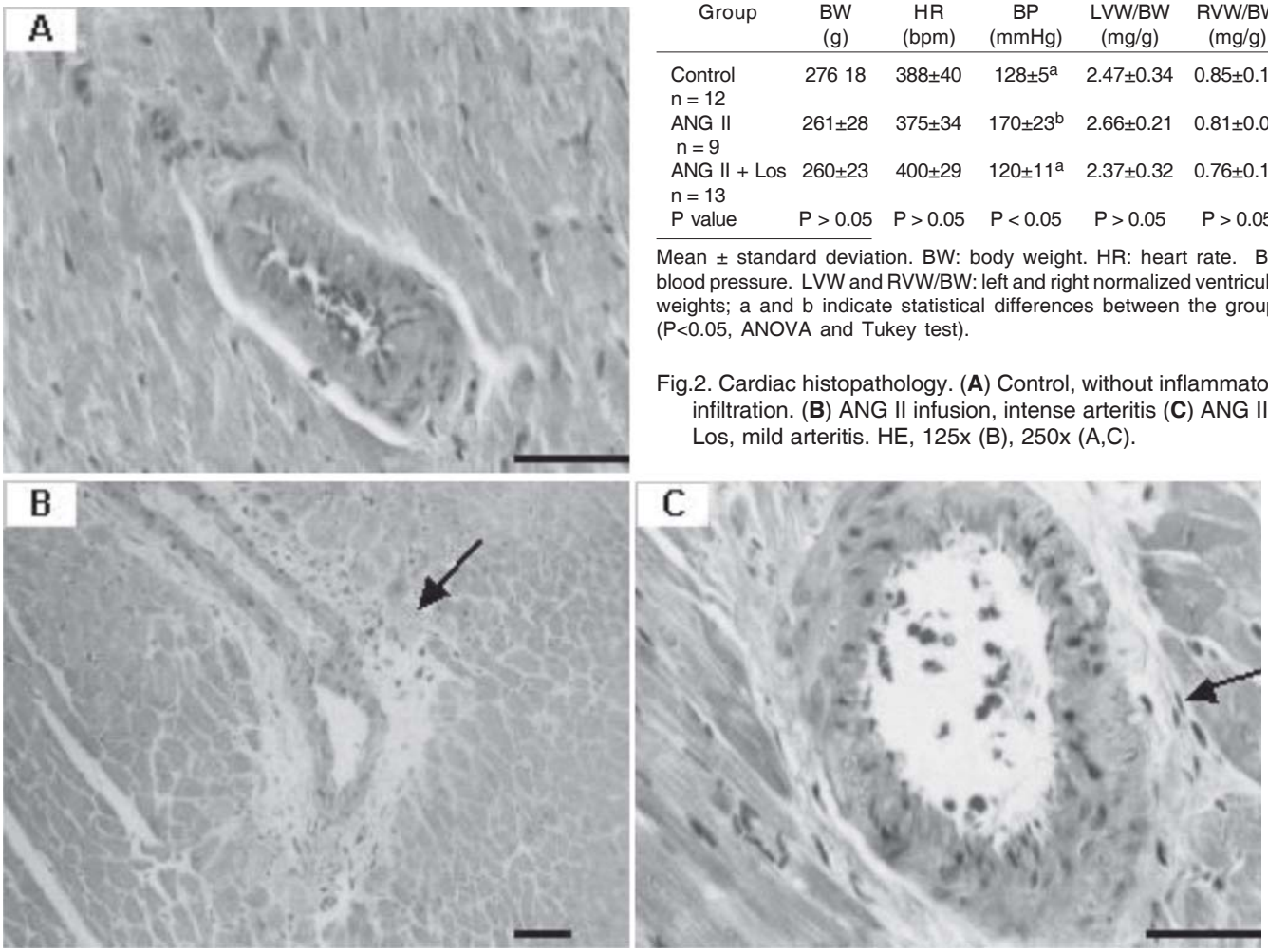

Fig.2. Cardiac histopathology. (**A**) Control, without inflammatory infiltration. (**B**) ANG II infusion, intense arteritis (**C**) ANG II + Los, mild arteritis. HE, 125x (B), 250x (A,C).

+ Los groups. There were no statistical differences in the other parameters among the groups.

Myocardium lesions were mainly vascular or perivascular and included cellular edema, steatosis, congestion and inflammatory infiltrate. Vascular lesion scores are listed on Table 2. The results are reported as median and interquartile range. In the control group, no relevant histological changes were observed in any of the organs studied. Perivascular

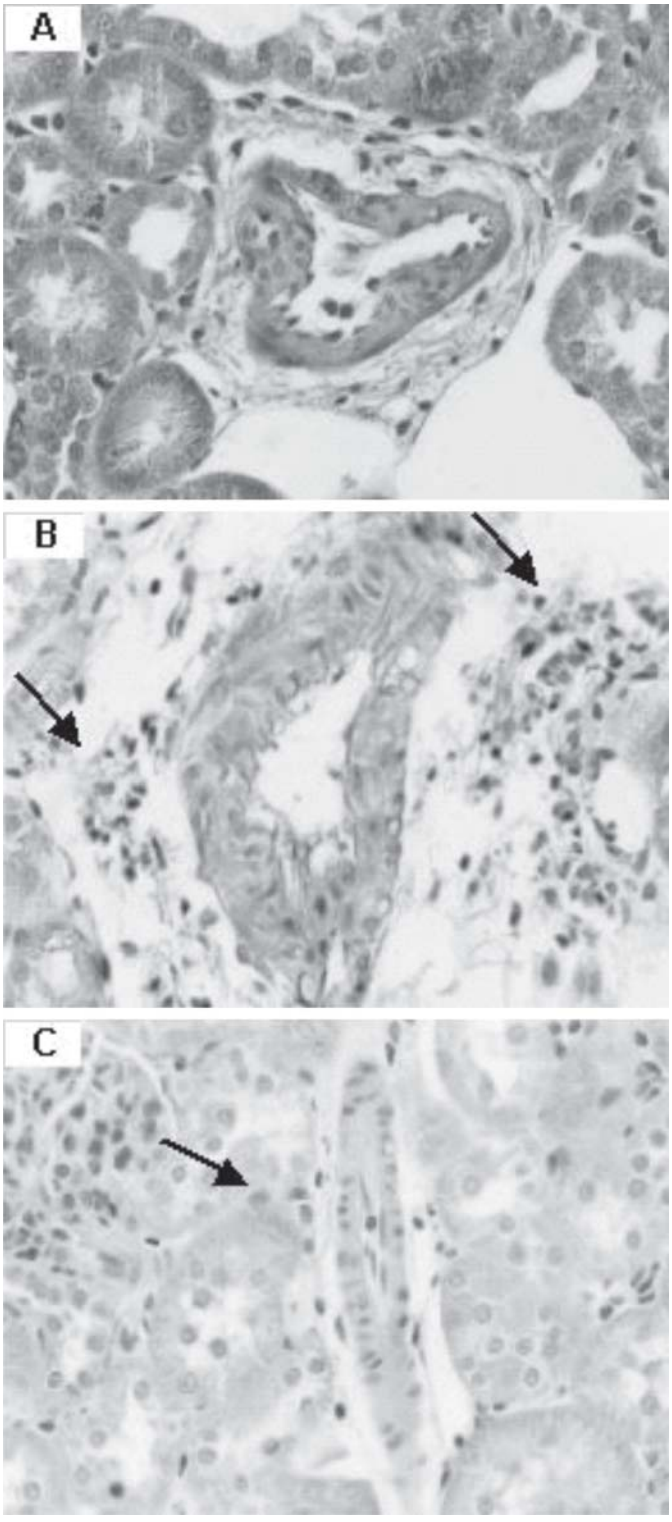

Fig.3. Kidney histopathology. (**A**) Control, without inflammatory infiltration. (**B**) ANG II infusion, intense arteritis. (**C**) ANG II + Los, mild arteritis. HE, 250x.

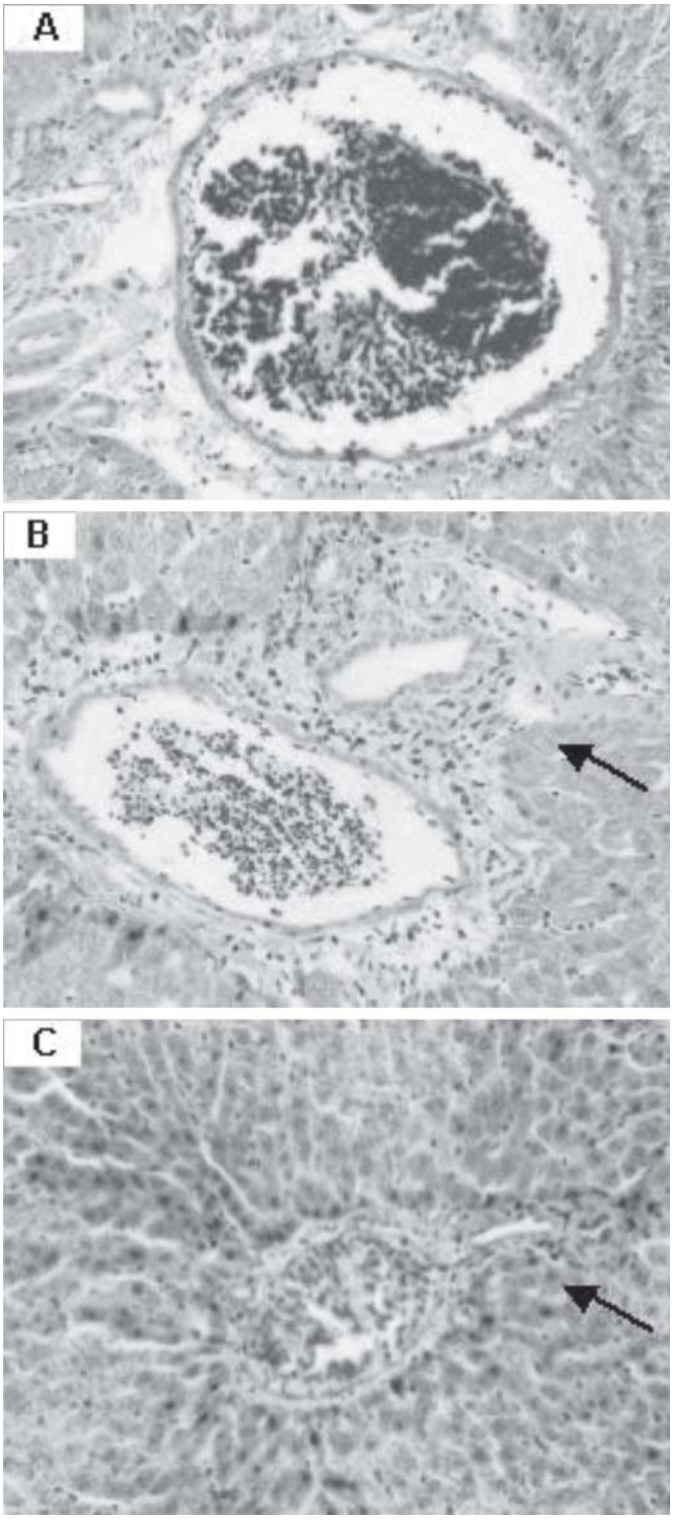

Fig 4. Liver histopathology (peri-portal space). (**A**) Control, without inflammatory infiltration. (**B,C**) ANG II and ANG II + Los, respectively, intense arteritis. HE, 200x.

lesion scores for the left ventricle were higher in the ANG II group than in the control group and ANG II group showed significantly more intense lesions than ANG II + Los. Similarly, right ventricle scores were higher in ANG II as compared to the control group. Losartan decreased but did not totally prevent perivascular damage. Examples of perivascular damage and the effects of losartan are shown in Figure 2A-C.

Renal perivascular lesions were significantly more severe in ANG II than in the control group. The observed lesions were similar to the lesions described in the myocardium. The lesion score for group ANG II + Los was statistically higher than that for the controls and lower than that of the ANG II group (Fig.3A-C).

In the peri-portal space, the perivascular lesion score for both ANG II and ANG II + Los groups was similar and significantly greater than that for the control group. Particularly in the terminal vein, the lesion score for the ANG II group was higher, as compared to that of the control group. Losartan treatment partially prevented the toxic effect of ANG II around the terminal vein (Table 2, Fig.4A-C).

In the ANG II group, the severity of tissue lesions was strikingly lower than that of perivascular lesions for any of the studied organs. Either mild myocardial or kidney damage was found in only three animals, including one rat with focal myocardial necrosis. There was no difference in lesion severity among the groups studied.

In the ANG II group, there was a close correlation between the perivascular lesion scores for the right and left ventricle ($r=1.00$, $P<0.001$), and the left ventricle and the peri-portal space ($r=0.77$, $P=0.02$).

Macrophage antibody labeling showed immunoreactive cells in the myocardium, the liver and kidneys (Fig.5A-B). In all the experimental groups, those cells were found in tissues. Mast cells were observed in the myocardium, the liver and kidneys of animals in all the groups, as shown in Figure 5C-D.

Eosinophils were rarely seen, regardless of the experimental group. There was no significant difference in the number of either macrophages or mast cells among the groups.

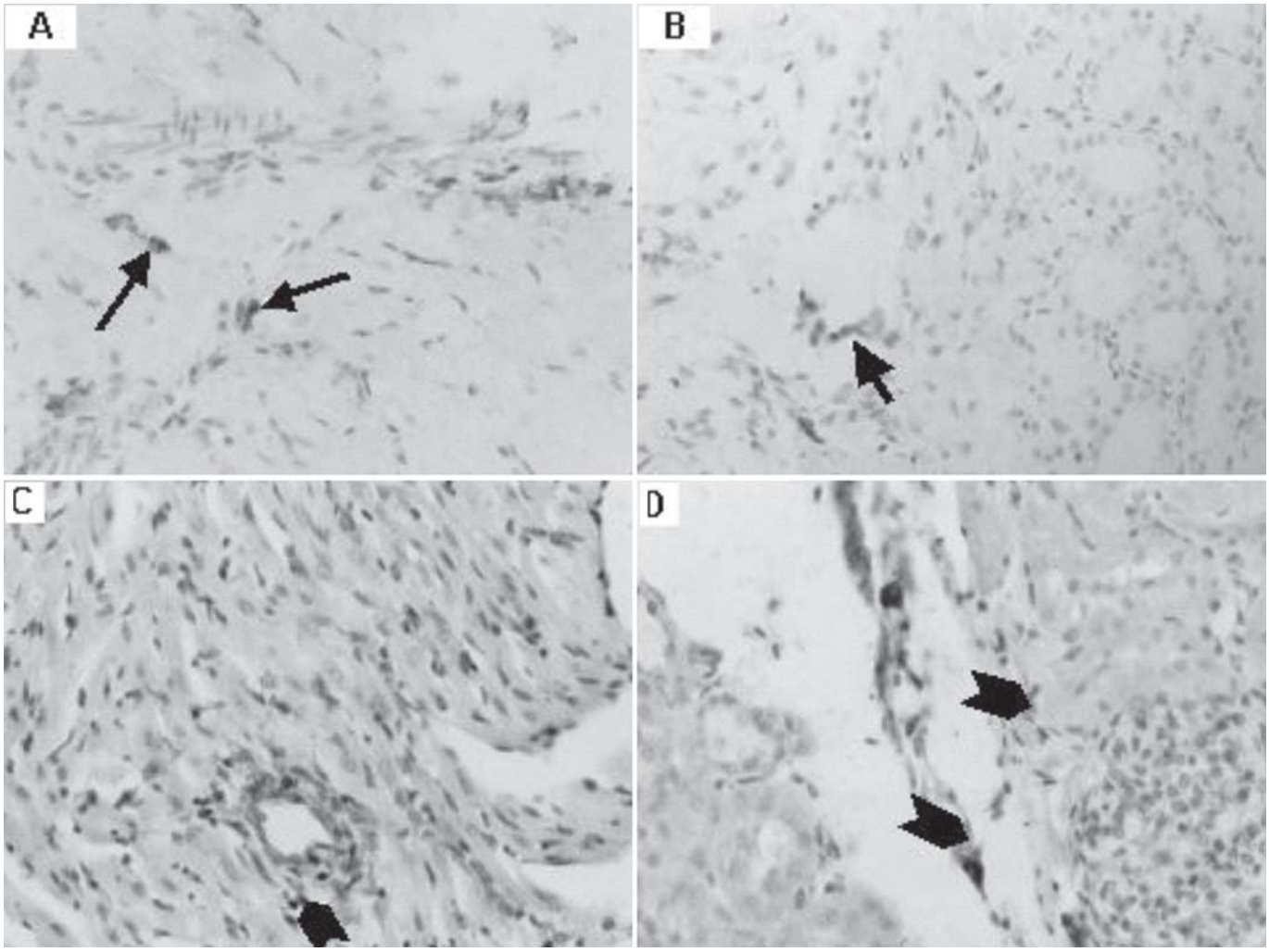

Fig.5. Immunohistochemistry (A,B) showing macrophages stained by avidin-biotin-peroxidase technique (arrow), and histochemistry (C,D) showing mastocytes, stained with acidicated Red-Congo, and Blue Toluidine (arrow), from ANG II-infused rats. (A,C) Myocardium. (B,D) Kidney. 125x.

DISCUSSION

In this study, the increase in blood pressure caused by 72-hour ANG II infusion was expected and has also been reported by others (Wiener et al. 1979, Tan et al. 1991). Losartan fully prevented pressure rise, suggesting that the AT_1 receptor blockade was effective. In fact, several investigators have reported the successful prevention of ANG II effects on the heart using the same dose used herein (Kabour et al. 1994).

The absence of myocardial hypertrophy was expected as we used a very short ANG II-infusion period, in order to detect the early effects of ANG II in the organs. In the literature, left ventricle hypertrophy in rats has been reported to be detectable within 10 days after endogenous or exogenous stimuli in experimental studies (Lever et al. 1992).

The ANG II group presented significant perivascular lesions regardless of the organ studied. These findings support the hypothesis that ANG II-induced perivascular damage appears in other than the classic target organs. Therefore, studies on ANG II effects and protection mechanisms should look beyond the myocardium and kidneys.

The significant, positive correlation observed between the lesion scores for both ventricles in the ANG II group is consistent with other studies (Weber & Brilla 1991). This finding has been used by others as the main evidence that myocardial and perivascular lesions are caused by the humoral effect of the hormone and not by the mechanical effects of hypertension (Weber & Brilla 1991). However, the vasculature in both ventricles was submitted to the same pressure. Therefore the mechanical effect should not be ruled out. The significant, positive correlation observed between the lesion scores for the left ventricle and the peri-portal space was noteworthy. This finding, never reported before, suggests that ANG II has a vasculotoxic systemic effect. Other authors have described mesenteric vascular hypertrophy in rats (Cao et al. 1999) and kidney lesion in rabbits (Gavras et al. 1971). The lack of a significant correlation between heart and kidney damages, suggests that there are distinctive mechanisms of protection/damage, depending on the target organ.

The hepatic terminal vein damages observed in the present study should be cautiously interpreted because although these lesions were classified as perivascular, it is important to emphasize that the hepatocytes were affected.

The hepatotoxic response induced by chemical agents depends on the concentration that reaches the hepatocytes and the localization of the cell in the acinus. Zone 1 hepatocytes are located closer to the afferent vessels, and, therefore, receive the largest amounts of oxygen and nutrients. The cells in zone 2 are located in the intermediary area between zone 1 and zone 3, close to the terminal vein, and receive smaller amounts of oxygen and nutrients (Rappaport 1973). The hepatocytes of both the afferent (zone 1) and the efferent (zone 3) zones differ in their metabolic capacity due to their differences in enzymes, especially cytochrome P450, that has its highest expression in the hepatocytes of zone 3 (Jungermann & Katz 1989). It may be concluded that the hepatocytes necroses observed in this study suggests a direct hepatotoxic effect of ANG II, which is more evident in zone 3 due to the anatomical and biochemical peculiarities of the hepatic acinus.

Losartan only partially reduced the ANG II-induced lesions (Kabour et al. 1994), demonstrating that the AT_1 blockade was not sufficient to avoid aggression. This could indicate either that the final vascular lesion involves mechanisms other than the ANG II direct toxic effects via AT_1 receptor or the dose of 30mg/kg/day was insufficient to completely block ANG II effects. However, this same dose has been used by others (Kabour et al.1995). Regardless of the mechanism underlying the lack of total protection, our overall results suggest that although losartan prevented ANG II-induced hypertension it did not prevent damage in target-organs. Therefore, the effective prevention of myocardial remodeling would require either higher doses of AT1 blocker or multiple neurohormone blockade rather than simply treating hypertension. This is consistent with the finding that subpressor doses of ANG II lead to necrosis regardless of ANG II hypertensive effects (Tan et al. 1991, Ratajska et al. 1993). Aside from knowing the limitations of extrapolating experimental work to clinical condition, our results would emphasize the findings that the use of higher doses of neurohormonal blockers have better effects on patients that present neurohormonal activation (Azizi et al. 1997, Brunner-La Rocca et al. 1999).

Tissue lesions were clearly less severe than perivascular lesions. However, the absence of statistical differences among the groups hindered a conclusion on the protecting effect of losartan, or the possible aggression injury mechanisms. In relation to the physiopathological mechanisms involved, one might suggest that tissue lesions are secondary to vascular damage and, therefore, depend on it. Another possibility is that tissue lesions were caused by a mild ANG II direct action, not demonstrable by the quantitative analysis used. The physiopathological mechanism underlying tissue damage has also been investigated by others. While some of them suggest that vascular lesion is secondary to ischemic or anoxic damage (Giacomelli et al. 1976, Rodrigues et al. 1992, Ratajska et al. 1993), others suggest a relation with ANG II adrenergic-mediated cardiotoxic effect (Ratajska et al. 1994, Henegar et al. 1995). Catecholamines effects may be involved, since sympathetic nervous system and renin-angiotensin-aldosterone are intimately related. However, we have not explored this effect in the present study.

The involvement of cellular inflammatory reaction caused by ANG II in the lesion mechanism has been studied by several authors (Giacomelli et al. 1976, Rodrigues et al. 1992, Kabour et al. 1994, Sun et al. 1994, Campbell et al. 1995, Okoshi et al. 1997).

The presence of macrophages and lymphocytes in the areas of ANGII-induced myocardial necrosis both in the parenchyma and in the areas surrounding the vessels, has been reported by others (Yamagishi et al. 1993). Perivascular macrophages and occasionally neutrophils, have been found in the myocardium of rats infused with ANG II (Ratajska

et al. 1994). In the present study the perivascular hypercellularity occurred due to lymphocytes and neutrophils.

In all the groups studied herein, macrophages and mast cells were found in the myocardium, the liver and kidneys of the animals, with no difference among groups. No differences in the number of these cells were observed. In contrast to the findings of others who suggest a toxic effect of ANG II mediated by these cells (Kabour et al. 1994). However, the fact that tissue lesions are caused by cell activation, regardless of the number of inflammatory cells should be taken into consideration. This cell activation cannot be detected by the immuno-reaction technique used herein.

In conclusion: (1) angiotensin II has a systemic vasculotoxic effect, (2) there are separate damage/protection mechanisms depending on the target organ, and (3) perivascular lesions may occur even if the antihypertensive losartan is used.

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