EFFECTS OF ANGIOTENSIN CONVERTING ENZYME INHIBITORS, PEROXISOME PROLIFERATORS ACTIVATED RECEPTOR -α AND γ AGONISTS, AND STATINS ON A RAT MODEL OF HYPERTENSION

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ABSTRACT

Background: *Hypertension is a common and complex human disease that causes significant morbidity and mortality worldwide. The role of endothelial dysfunction as an early event of pathophysiologic importance has been recently delineated. Nitric oxide (NO) is an important vasodilator and a potent regulator of inflammation, and mitogenesis. Therefore, inhibition of NO synthesis provided an interesting model of hypertension with specific cardiovascular alterations in which different events of the disease process could be traced.*

Aim: *The aim of this work was to study the cardiovascular abnormalities in a rat model of hypertension induced by administration of the NO synthase inhibitor, N^{ω}-nitro-L-arginine methyl ester (L-NAME). In addition, the effects of short and long term administration of an angiotensin converting enzyme inhibitor (Lisinopril), a peroxisome proliferators activated receptor (PPAR)-α agonist (Fenofibrate), a PPAR-γ agonist (Pioglitazone) and a statin (Atorvastatin) were studied.*

Methods: *The study was conducted on 128 rats divided into groups and received the studied drugs. Arterial blood pressure and TGF-β1 were recorded. The animals were then sacrificed and the heart weight/body weight (HW/BW) ratio was calculated. Routinely processed hearts and aortae were examined to assess degree of inflammation or fibrosis. This was followed by immunohistochemical detection of CD68 and PCNA and histochemical staining using Masson trichrome stain. Evaluation was done by computerized image analysis.*

Results: *Short term administration of L-NAME was associated with a significant increase in blood pressure associated with marked myocardial inflammatory response (mostly CD68 positives macrophages) and increased proliferative activity. Long term administration of L-NAME resulted in a significant aggravation of hypertension accompanied by myocardial hypertrophy and significant increase in HW/BW ratio. Extensive fibrosis and a significant increase in plasma TGF-β1 level were noted.*

Short term administration of the studied drugs showed that only Lisinopril showed a significant decrease in blood pressure. The inflammatory and proliferative changes were attenuated by short term administration of the studied drugs, yet with different degrees. Long term administration of all drugs except Fenofibrate led to a significant reduction of blood pressure, HW/BW ratio, fibrosis and plasma TGF-β1.

Conclusions: *Disturbances of NO production are likely to be major determinants of endothelial dysfunction, hypertension and their pathological consequences; hence, directing therapy towards conserving endothelial NO bioavailability seems to be of paramount importance. The tested drugs were capable of modulating the different aspects of the disease whether the early or late changes, though their profiles were different. Among the studied classes of drugs, Lisinopril was superior in modulating alterations associated with hypertension.*

Key words: *Rat Model of Hypertension, Angiotensin Converting Enzyme Inhibitors, PPAR-α and γ Agonists, Statins.*

INTRODUCTION

Hypertension is a common and complex human disease that causes significant morbidity and mortality worldwide.[1-3] A major challenge is to identify the key determinants of hypertension and to evaluate how these critical pathways can best be modified to reduce blood pressure and disease risk. [4] Decades of work in diverse areas have identified a large number of interrelated factors that are altered in the setting of hypertension.[3] The role of endothelial dysfunction as an early event of pathophysiologic importance linking hypertension, cardiovascular inflammation and late cardiovascular structural abnormalities, has been recently delineated.[4]

According to the traditional view, hypertension acts as a major determinant of endothelial dysfunction and vascular damage, promoting activation of endothelial cells, recruitment of inflammatory cells in the arterial wall and activation of vascular resident elements. On the other hand, the recent epidemiological observation, that a systemic low inflammatory status precedes the onset of essential hypertension, suggests a link between endothelial dysfunction, vascular inflammation and high blood pressure.[4-6]

Endothelial dysfunction is characterized by a shift of the actions of the endothelium towards reduced

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vasodilation, a pro-inflammatory state, and pro-thrombic properties. This shift is attributed to imbalance between vasodilator and vasoconstrictor substances produced by the endothelium. Mechanisms that participate in the reduced vasodilatory responses in endothelial dysfunction include reduced nitric oxide (NO) generation. NO is not only one of the most important vasodilator substances released by the endothelium but also is a potent regulator of inflammation, mitogenesis, angiogenesis, vascular permeability, and fluid balance.[7-9]

Therefore, inhibition of NO synthesis by administration of the NO synthase inhibitor, N^{ω}-nitro-L-arginine methyl ester (L-NAME) provided an interesting model of hypertension with specific cardiovascular alterations in which different events of the disease process could be traced.[10]

The concept that vascular inflammation and hypertension share some pathophysiological mechanisms in both human essential hypertension and in L-NAME induced hypertension, [11] makes this experimental model a convenient one to investigate an important raised question of whether the treatment of one of the two conditions could have some impact on the other. Another point is that, since drugs are most efficient if they are effective in preventing and reversing early as well as late vascular alterations in hypertension, this model seems suitable to test the cardiovascular protective effects of drugs claimed to affect cardiovascular homeostasis and remodeling.[12]

In this respect, the present study assessed some of the cardiovascular effects of Lisinopril [an angiotensin converting enzyme (ACE) inhibitor], Fenofibrate [a peroxisome proliferator activated receptor (PPAR)-α-agonist], Pioglitazone [a PPAR-γ-agonist] and Atorvastatin [a statin] on a rat model of L-NAME induced hypertension.

Aim of the work:

The aim of the present work was to study the cardiovascular abnormalities in a rat model of hypertension induced by inhibition of NO synthesis. In addition, the effects of short and long term administration of an ACE inhibitor (Lisinopril), a PPAR-α agonist (Fenofibrate), a PPAR-γ agonist (Pioglitazone), combined PPAR-α and γ, and a statin (Atorvastatin) on the cardiovascular abnormalities in the rat model of hypertension were studied.

METHODS

I. Experimental animals:

The present study was carried out on 128 male albino rats weighing 200-250 grams. Animals were kept in metal cages in the animal house with free access to food and water.

II. Experimental design:

After a period of acclimatization, animals were randomly divided into eight groups, each of 16 rats. Then, each group was further subdivided into two subgroups (A & B), eight animals each, according to the duration of the study.

The first group (group I) consisted of untreated normal control rats, the second group (group II) was vehicle treated (A and B subgroups received 2% gum acacia, 1 ml/day orally for 7 and 32 days, respectively.), and the other six groups (group III-VIII) were subjected to L-NAME induced hypertension by administration of L-NAME (Sigma Chemical Co.) at a dose of 40 mg/kg/day orally.[13] Group III was only L-NAME treated and served as control for the next five drug treated groups.

Drug administration in groups IV-VIII started 4 days before further subdivision into A and B subgroups, which continued drug treatment with concomitant administration of L-NAME for 3 and 28 days, respectively.[14]

Animals in groups IV were treated with Lisinopril (Zestril ®, Estra/ Sedico.) in a dose of 20 mg/kg/day orally. Animals in groups V were treated with Fenofibrate (Lipanthyl ®, Minapharm.) in a dose of 100 mg/kg/day orally. Animals in groups VI were treated with Pioglitazone (Glusten ®, Pfizer) in a dose of 10 mg/kg/day orally. Animals in groups VII were treated with combined Fenofibrate / Pioglitazone. Animals in groups VIII were treated with Atorvastatin (Ator ®, Epico) in a dose of 30 mg/kg/day orally.

III. Experimental Procedures:

For animals in all groups, arterial blood pressure was recorded by a mercury manometer, using a blood pressure transducer (Letica®-Panlab.s.l.) connected to catheterized femoral artery.[14,15]

Blood samples were collected for biochemical analysis which included:

- Plasma total cholesterol (Diagnostic International Company (Diasystems)-Germany) and plasma triglycerides (Biosystem for reagents and instruments. Costa brava, Barcelona, Spain) for animals of all subgroups.[16]
- Plasma transforming growth factor-β1 (TGF-β1) level in animals of the B subgroups was assayed using the multispecies TGF-β1 ELISA kit (BioSource International, Inc.) [17]

The animals were then sacrificed and the following was done:

Hearts and aortae were excised and placed in formalin. Heart weight/body weight (HW/BW) ratio was calculated for animals in the B subgroups.

Pathological Study:

Formalin-fixed specimens were routinely processed and embedded in paraffin. From paraffin-embedded tissue blocks, 5 µm-thick sections were cut and stained with the conventional Haematoxylin and Eosin (H&E) stain. Each section was carefully

examined by the light microscope to assess degree of inflammation in specimens of all (A) subgroups and degree of fibrosis in specimens of all (B) subgroups. The preliminary histological examination was followed by immunohistochemical detection of CD68[14] and PCNA[10] in all (A) subgroups and histochemical staining using Masson trichrome stain (Sigma Chemical Co.) in all (B) subgroups.[10]

Immunohistochemistry:

Immunohistochemical staining for CD68 and was performed using an avidin-biotinylated immunoperoxidase methodology. The staining conditions were adjusted by including positive and negative controls in all runs. As regards antigen retrieval, For PCNA immunostaining, sections were microwaved in 10mM citrate buffer (pH 6.0). For CD68 immunostaining, antigen retrieval was performed by enzymatic protein digestion using Chymotrypsin (α chymotrypsin ®) (5 mg/150 ml PBS) for 30 minutes at room temperature. The primary antibodies (CD68, clone ED1 monoclonal antibody, HyCult biotechnology b.v., Netherlands; and Anti-PCNA, Monoclonal mouse anti-rat proliferating cell nuclear antigen [PCNA], Clone: PC10, Ready-to-Use; DakoCytomation, Denmark) were then applied in appropriate concentration; 1:50 for the CD68 and as provided; ready-to-use for PCNA. The reaction was visualized by using the DakoCytomation EnVision®+ System-HRP [DAB] kit (DakoCytomation). This system is based on a horseradish peroxidase (HRP) labeled polymer which is conjugated with secondary antibodies.[18]

Computerized Image Analysis:

Evaluation of Immunostaining:

Following immunostaining, areas with the most intense CD68 or PCNA immunostaining were chosen at low power magnification. The selected areas were subjected to further analysis using computerized image analysis software (Pixcavator). Binary images for measurement were generated and counting of positive immunostained cells was done in 10 non-overlapping high power fields for each section. The total number of CD68-positive cells and the percentage of PCNA-positive cells were calculated and the data were expressed as mean values.[10,14]

Evaluation of fibrosis highlighted by Masson trichrome stain:

Following staining by Masson trichrome stain, areas of fibrosis were highlighted by a blue-green color. The areas with the most pronounced fibrosis were chosen at low power magnification. The selected areas were subjected to further analysis using computerized image analysis software (ImageScope). Binary images were generated and quantitative estimation of the areas of fibrosis was done in 10 non-overlapping high power fields for each section. The percentage of areas of fibrosis was calculated and the data were expressed as mean values.[19]

IV. Statistical Methodology

Data were fed to Microsoft computer program (SPSS/win), version 10. Descriptive statistics were done using number, percent, arithmetic mean and standard deviation (S.D.). Analysis of data was conducted using the Kruskal-Wallis and Mann-Whitney tests. A 5% level of significance was chosen in all statistical significance tests used.

RESULTS

Effects of short term (3 days) oral administration of L-NAME on blood pressure, plasma lipids and cardiovascular inflammatory and proliferative activity in male albino rats:

The results of the present study revealed that short term inhibition of NO synthesis by oral administration of L-NAME was associated with a significant increase in the mean arterial blood pressure in animals of the L-NAME treated subgroup in comparison to control subgroups ($p= 0.0004$). There was no significant difference between animals of the L-NAME treated subgroup and control subgroups regarding the plasma lipids (total cholesterol and triglycerides) levels ($p=0.736$, and $P=0.409$ respectively). Table I

Histopathologically, L-NAME treated subgroup exhibited marked infiltration by mononuclear cells into the myocardial interstitial spaces. Mild infiltration into the perivascular areas around the coronaries was also noted. No inflammatory infiltrate was detected in aortic sections.

Immunohistochemical staining for CD68 showed that a considerable proportion of inflammatory cells were CD68-positive macrophages with a mean value of 82.075 ± 9.821 cells/ field. Table II.

Immunohistochemical staining for PCNA showed marked positive nuclear staining in the myocardium and mild to moderate staining of smooth muscles of the coronaries and aorta. A considerable number of the infiltrating inflammatory cells were shown to be immunopositive. The mean value of the percentage of PCNA immunopositive cells in the heart sections was 62.14 ± 6.169. Table III.

Effects of short term (7 days) oral administration of Lisinopril, Fenofibrate, Pioglitazone, combined Fenofibrate/Pioglitazone, and Atorvastatin on blood pressure, plasma lipids and cardiovascular inflammatory and proliferative activity in L-NAME treated male albino rats:

Among the studied drugs, only Lisinopril showed a significant decrease in the mean arterial blood pressure in comparison to the L-NAME treated subgroup ($p=0.0001$).

Short term oral administration of the used drugs did not significantly alter the plasma

total cholesterol level or plasma triglyceride levels (p=0.7430, and P=0.3123 respectively) in comparison to the L-NAME treated subgroup or to either the untreated or the vehicle treated control subgroups

The effect of short term drug administration to L-NAME treated rats on cardiovascular abnormalities are illustrated in tables II and III as well as in figure (1).

Lisinopril administration significantly decreased the L-NAME induced mononuclear cellular recruitment into the vascular and myocardial interstitial spaces. The early proliferative activity in recruited and resident cells was also attenuated by Lisinopril.

On the other hand, Fenofibrate and Pioglitazone were partially able to reduce the L-NAME induced cardiovascular abnormalities.

The combined use of PPAR-agonists, Fenofibrate and Pioglitazone in the current study revealed that they greatly aborted the early inflammatory and proliferative activity induced by concomitant L - NAME administration. The effect on both inflammation and proliferation was more pronounced than what was detected by either drug alone.

Atorvastatin significantly reduced mononuclear cellular recruitment into cardiac and vascular tissues together with decreasing the percentage of PCNA immunopositive cells in cardiac, vascular and infiltrating inflammatory cells.

Effects of long term (28 days) oral administration of L-NAME on blood pressure, plasma lipids, TGF-β1 and cardiovascular remodeling in male albino rats:

Persistent inhibition of NO synthesis by continuous administration of L-NAME for 28 days resulted in a significant aggravation of hypertension in comparison to control subgroups (p= 0.0004)

This persistent pressure overload was accompanied by cardiac and vascular remodeling changes manifested by myocardial hypertrophy (evidenced by increase in myocardial thickness with narrowing of the lumen) with significant increase in HW/BW ratio in comparison to control subgroups (p= 0.0004).

The wide areas of early inflammatory infiltrate seen on short term L-NAME administration were replaced by areas of extensive fibrosis highlighted by Masson's trichrome staining with a blue-green color. Wide areas of fibrosis were noted within the myocardium together with fibrous thickening of the coronaries and perivascular fibrosis. Computerized image analysis showed that fibrotic areas represented about 33.06%±3.04 of the scanned heart sections.

In comparison to control subgroups, there was a significant increase in plasma TGF-β1 level (p= 0.0004). On the other hand, no statistically significant change in plasma lipids (total cholesterol and triglycerides) levels was observed by long term L-NAME administration (p= 0.772 and p= 0.861 respectively).

Effects of long term (32 days) oral administration of Lisinopril, Fenofibrate, Pioglitazone, combined Fenofibrate/Pioglitazone, and Atorvastatin on blood pressure, plasma lipids, TGF-β1 and cardiovascular remodeling in male albino rats:

Long term administration of Lisinopril, Pioglitazone, Fenofibrate/ Pioglitazone, Atorvastatin led to significant decrease in mean arterial blood pressure (p= 0.0001) with decrease in cardiac hypertrophy and a significant reduction of HW/BW ratio (p= 0.0001). On the other hand, the effects of Fenofibrate were not significant.

A positive significant correlation between the mean arterial blood pressure and the HW/BW ratio in animals of the B subgroups was found (P = 0.000).

Histopathological examination of cross sections obtained from animals in the Lisinopril treated subgroup revealed no evidence of myocardial hypertrophy. Treatment with Lisinopril greatly prevented the L-NAME induced cardiac and vascular wall fibrosis as observed on examination with the Masson's trichrome stain. Computerized image analysis revealed significant reduction in the percentage of fibrotic areas in comparison to the L-NAME treated subgroup.

On the other hand, oral administration of Fenofibrate did not affect the L-NAME induced myocardial hypertrophy, although it partially prevented myocardial fibrotic changes as evident from modest blue-green reaction seen upon staining with the Masson's trichrome stain and the significant reduction in the percentage of fibrotic areas detected by software imaging in comparison to the L-NAME treated subgroup.

Results of the present study revealed that Pioglitazone administration to L-NAME treated animals prevented pressure overload induced cardiac hypertrophy as shown from normal cardiac and vascular wall thickness in the H&E examined sections. It was also associated with significant decrease in percentage of areas of fibrosis in comparison to L-NAME treated subgroup.

The combined administration of Fenofibrate and Pioglitazone abrogated the L-NAME induced cardiac hypertrophy and coronary thickening. Tissue sections stained with the Masson's Trichrome stain showed significant reduction in areas of fibrosis in comparison to what seen with either drug alone.

Treatment with Atorvastatin was found to inhibit the L-NAME induced hypertrophic changes in the

myocardium as well as coronary vascular wall thickening. Minimal blue-green staining with the Masson trichrome stain was observed consistent with the significant decrease in the percentage of fibrotic areas in comparison to the L-NAME treated subgroups.

The effects of long term oral administration of the studied drugs on the cardiovascular abnormalities are summarized in table IV and figure 2.

Long term oral administration of the used drugs did not significantly alter the plasma total cholesterol level or plasma triglyceride levels (p=0.6244, and P=0.9422respectively) in comparison to the L-NAME treated subgroup or to either the untreated or the vehicle treated control subgroups.

On the other hand, the study revealed that long term oral administration of the studied drugs to L-NAME treated rats was associated with significant reduction in the plasma TGF-β1 in comparison to the L-NAME treated subgroup (p= 0.0001).

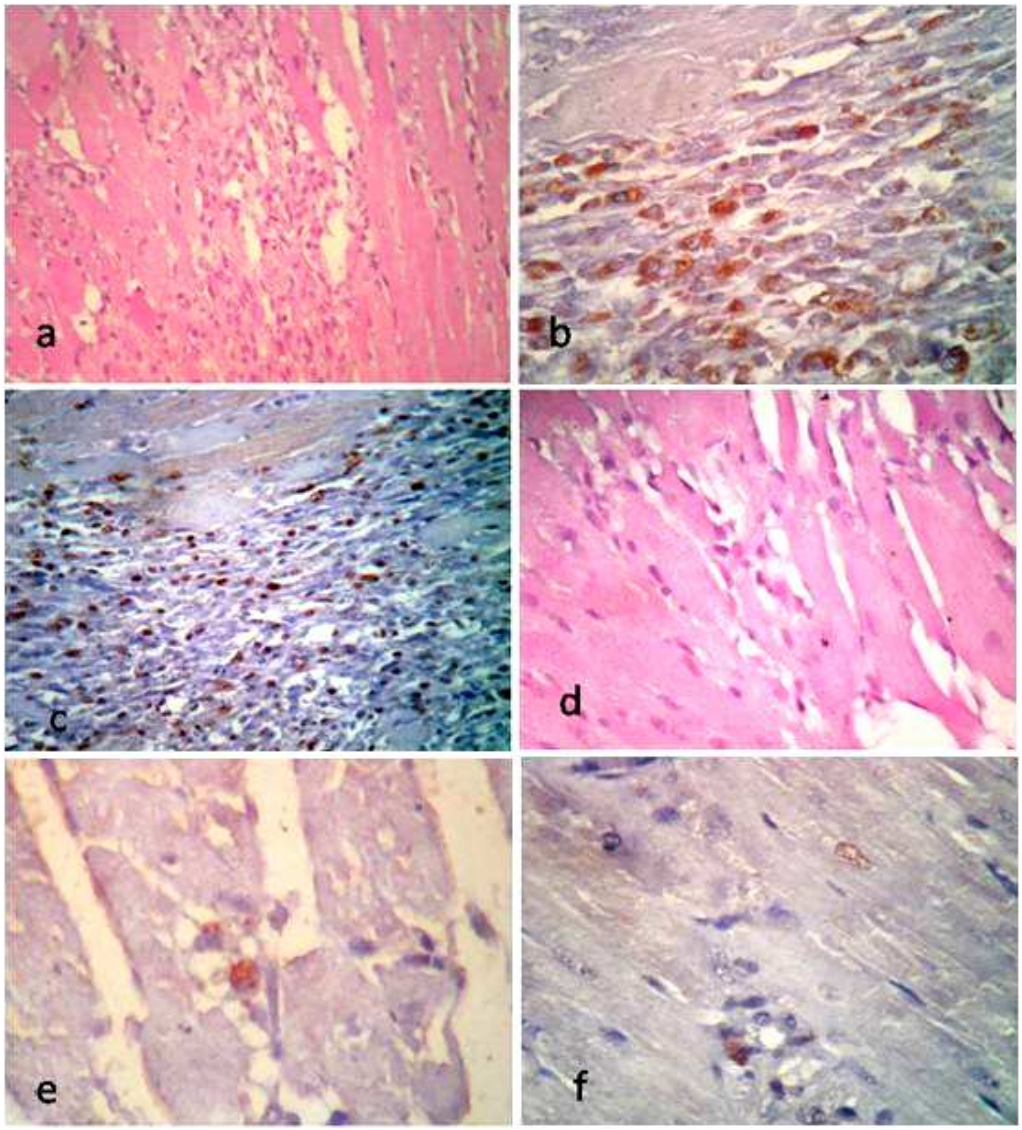

Fig 1: ***The effect of short term drug administration on cardiovascular inflammatory and proliferative activity***
(a) Heart section of L-NAME treated rat (III-A), showing marked infiltration by mononuclear inflammatory cells expanding the myocardial interstitial spaces and replacing some of the myocardial fibers. (H&E x200). **(b)** Heart section of L-NAME treated rat (III-A) stained with anti-CD68 antibody, showing positive staining of most of the infiltrating inflammatory cells with anti-CD68 antibody. The positive macrophages show intense granular cytoplasmic reaction. (x400). **(c)** Heart section of L-NAME treated rat (III-A) stained with anti-PCNA antibody. Most of the inflammatory infiltrate shows intense positive nuclear reaction. Some of the myocardial cells are also positively stained. (x200). **(d)** Lisinopril treated subgroup; shows scarce inflammatory infiltrate. (H&E x200). **(e)** Lisinopril treated subgroup; shows rare positive cells among a small cluster of inflammatory cells. (CD68, x200). **(f)** Lisinopril treated subgroup; shows rare positive cells among a mild inflammatory cell infiltrate. A single myocardial cell shows positive nuclear staining. (PCNA, x200)

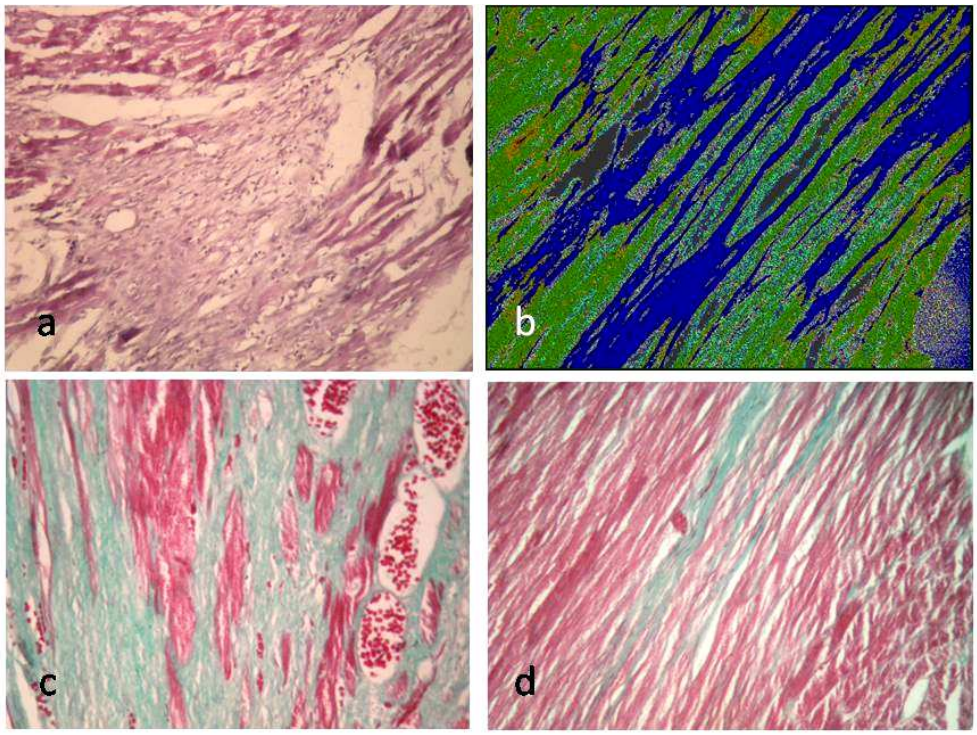

Fig 2*: The effect of long term drug administration on cardiovascular fibrosis.*
(a) H&E stained section of the heart of L-NAME treated rat (III-B), showing marked fibrosis replacing the myocardial cells. (H&E, x200). **(b)** Binary image selecting areas of fibrosis in blue color. **(c)** L-NAME treated subgroup; shows marked fibrosis replacing the myocardial cells. (Masson's trichrome stain, x200). **(d)** Lisinopril treated subgroup; shows mild fibrous tissue formation. (Masson's trichrome stain, x200)

Table I: Effect of short term (3 days) oral administration of L-NAME on mean arterial blood pressure (mm Hg), plasma total cholesterol and triglycerides (mg/dl) in male albino rats

Parameters	**Mean arterial blood pressure**			**Plasma total cholesterol**			**Plasma triglycerides**		
Subgroups	**(I-A) Untreated control**	**(II-A) Vehicle treated**	**(III-A) L-NAME treated**	**(I-A) Untreated control**	**(II-A) Vehicle treated**	**(III-A) L-NAME treated**	**(I-A) Untreated control**	**(II-A) Vehicle treated**	**(III-A) L-NAME treated**
n	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**8**	**8**
Minimum values	97.33	97.33	140.67	63.00	62.00	63.00	25.00	23.00	27.00
Maximum values	120.67	117.33	161.33	89.00	98.00	94.00	45.00	40.00	44.00
Mean	105.63	104.79	149.83 *	78.63	75.38	78.00	36.25	32.38	35.38
S.D.	7.41	6.92	6.55	7.84	12.63	11.64	7.21	6.57	6.44
S.E.	2.62	2.45	2.32	2.77	4.47	4.12	2.55	2.32	2.28
P value		0.0004			0.736			0.409	

* Significant increase in comparison to both control subgroups

Table II: Effect of short term (7 days) oral administration of the studied drugs on the number of L-NAME induced infiltration of ED-1 immunopositive cells into the cardiac tissues of male albino rats

Parameter	Number of ED-1 immunopositive cells							
	(I-A)	(II-A)	(III-A)	(IV-A)	(V-A)	(VI-A)	(VII-A)	(VIII-A)
Subgroups	Untreated control	Vehicle treated	L-NAME treated	Lisinopril & L-NAME treated	Fenofibrate & L-NAME treated	Pioglitazone & L-NAME treated	Fenofibrate/ Pioglitazone & L-NAME treated	Atorvastatin & L-NAME treated
n	8	8	8	8	8	8	8	8
Minimum values	0.00	0.00	96.8	5.5	69.4	57.8	10.3	18.9
Maximum values	0.00	0.00	70.8	2.2	49.9	39.8	7.8	12.7
Mean	0.00	0.00	82.075	3.650 †§	58.013 †	49.738 †	8.713 †♠	15.338 †♠
S.D.	0.00	0.00	9.821	1.262	5.973	5.657	0.868	2.233
S.E.	0.00	0.00	3.472	0.446	2.112	2.000	0.307	0.789
P value	0.000							

† Significant decrease in comparison to the L-NAME treated subgroup. § Significant decrease in comparison to all other drug treated subgroups.
♠ Significant decrease in comparison to both Fenofibrate and Pioglitazone treated subgroups.

Table III: Effect of short term (7 days) oral administration of the studied drugs on the percentage (%) of PCNA immunopositive cells in heart sections of L-NAME treated male albino rats

Parameter	Percentage (%) of PCNA immunopositive cells							
	(I-A)	(II-A)	(III-A)	(IV-A)	(V-A)	(VI-A)	(VII-A)	(VIII-A)
Subgroups	Untreated control	Vehicle treated	L-NAME treated	Lisinopril & L-NAME treated	Fenofibrate & L-NAME treated	Pioglitazone & L-NAME treated	Fenofibrate/ Pioglitazone & L-NAME treated	Atorvastatin & L-NAME treated
n	8	8	8	8	8	8	8	8
Minimum values	0.00	0.00	70.45	6.90	45.10	43.85	27.41	30.90
Maximum values	0.00	0.00	55.00	3.32	26.90	29.99	18.95	15.70
Mean	0.00	0.00	62.14	4.91 †§	38.45 †	36.74 †	23.44 †♠	23 †♠
S.D.	0.00	0.00	6.169	1.400	5.845	4.543	3.376	3.376
S.E.	0.00	0.00	2.181	0.495	2.066	1.606	1.194	2.083
P value	0.000							

† Significant decrease in comparison to the L-NAME treated subgroup. § Significant decrease in comparison to all other drug treated subgroups.
♠ Significant decrease in comparison to both Fenofibrate and Pioglitazone treated subgroups.

Table IV: Effect of long term (32 days) oral administration of the studied drugs on the percentage (%) of areas of L-NAME induced fibrosis in heart sections of male albino rats

Parameter	**Percentage (%) of areas of fibrosis**							
Subgroups	**(I-B)**	**(II-B)**	**(III-B)**	**(IV-B)**	**(V-B)**	**(VI-B)**	**(VII-B)**	**(VIII-B)**
	Untreated control	**Vehicle treated**	**L-NAME treated**	**Lisinopril** & **L-NAME treated**	**Fenofibrate** & **L-NAME treated**	**Pioglitazone** & **L-NAME treated**	**Fenofibrate/ Pioglitazone** & **L-NAME treated**	**Atorvastatin** & **L-NAME treated**
n	8	8	8	8	8	8	8	8
Minimum values	0.061	0.057	38.114	4.938	24.430	23.58	13.57	4.135
Maximum values	0.014	0.028	29.570	2.830	18.476	15.47	7.01	1.050
Mean	0.0379	0.0420	33.0655	3.4539 †§	20.8129 †	19.7716 †	10.8570 †♠	2.4656 †§
S.D.	0.0169	0.0107	3.0423	0.7456	2.0745	2.9430	2.0492	1.1295
S.E.	0.0058	0.0038	1.0756	0.2636	0.7335	1.0405	0.7245	0.3993
P value	0.000							

† Significant decrease in comparison to the L-NAME treated subgroup.

§ Significant decrease in comparison to V-B, VI-B and VII-B subgroups.

♠ Significant decrease in comparison to both Fenofibrate and Pioglitazone treated subgroups

DISCUSSION

Essential hypertension is the most common cardiovascular disorder. It is associated with functional and morphological alterations of the vascular endothelium which is thought to be both target and mediator of arterial hypertension.[20]

Since endothelial nitric oxide (NO) has vasodilating, anti-inflammatory, antithrombotic and antiproliferative effects, decreased production or bioavailability of NO not only increases the peripheral resistance, but also plays a fundamental role in the development of cardiovascular structural changes in hypertension.[21]

Modulation of endothelial function is therefore an attractive therapeutic option in attempting to minimize some of the important complications of hypertension.[22]

In this respect, the experimental model of inhibition of NO production by L-NAME was chosen in this study to detect the cardiovascular alterations produced by this type of hypertension, which are similar to those found in human hypertension.[23]

The use of this model helped to address two questions: the first is whether L-NAME induced inhibition of NO synthesis induces the infiltration of inflammatory cells and remodeling changes in both the cardiac and vascular tissues, and the second question is whether inhibition of the early cardiovascular inflammatory and proliferative changes can abort the development of late structural alteration in the setting of L-NAME induced hypertension.

The findings of the present study regarding the effects of short term administration of L-NAME were in accordance with previous reports.[10, 24-27] The increase in mean arterial blood pressure noted in this study may be explained by loss of the substantial and functionally significant NO-mediated vasodilator tone that may be the main determinant of blood flow and on which local or systemic vasoconstrictor influences act.[28]

The inflammatory changes induced by inhibition of NO synthesis have been attributed to increased monocyte chemoattractant protein-1 (MCP-1) expression in the heart and vessels. This also explains the predominance of CD68 positive macrophages among the noted inflammatory infiltrate.[10,29]

Moreover, it was found that L-NAME administration results in overexpression of factors involved in the recruitment of inflammatory cells such as COX II, iNOS type II, ICAM-1 and VCAM-1.[30,31]

The observed proliferative effect of L-NAME could be attributed to an imbalance between proliferative and antiproliferative factors.[13]

The findings noted following long term administration of L-NAME were in accordance to those obtained from previous reports.[32-36] Bell et al[36] attributed the noted hypertrophic responses to both the persistent pressure overload and to loss of the inhibitory effect of NO on cellular growth with upregulation of hypertrophic growth factors as endothelin-1 and re-induction of hypertrophic genes as α-smooth muscle actin.

On the other hand, Bartunek et al[37] reported unchanged heart weight/body weight ratio in L-NAME treated rats. However, it may be possible that the administration of L-NAME for a longer duration and in higher dose in their study provoked much more fibrotic changes that compensated for the pressure overload induced increase in cardiac mass.[38]

The mechanism of L-NAME-induced hypertension and cardiovascular alterations involves more than removal of the vasodilator action of NO.[39] There is evidence for contributions from the renin-angiotensin system (RAS),[23] sympathetic nervous system,[40] prostaglandins, COX-2,[31] mineralocorticoids,[41] and superoxide anions to this process.[23]

Koshikawa et al[42] have recently suggested a novel link between NO inhibition and the L-NAME induced inflammatory and fibrotic abnormalities. Their findings supported the early postulation of Kataoka et al [43] that the small GTPase Rho and the Rho kinase pathway play a crucial role in early transcription of genes encoding for MCP-1 and later for TGF-β1 leading to early vascular inflammation and late cardiovascular remodeling.

From the results of the present study as well as other studies,[19,27,44] it can be suggested that, in case of prolonged NO deficiency, the early inflammatory and proliferative changes observed after 3 days of L-NAME administration are replaced by late cardiac and vascular structural abnormalities.

The currently observed lack of effect of either short or long term L-NAME administration on plasma lipids is in line with that shown by Navarro et al [45] and Nakaya et al.[46]

The next objective of the present study was to examine the role of different therapies that may contribute to control of elevated blood pressure and/or prevention of the cardiovascular abnormalities.

The blood pressure lowering effect of ACE inhibitors was extensively studied and has been documented in many previous studies using other models [47] as well as this model.[48] These data together with the present data strongly support the assumed role of the RAS in the L-NAME induced hypertension and the earlier findings that NO inhibition increases renin activity.[37]

The currently presented anti-inflammatory and anti-proliferative effects of ACE inhibitors are consistent with those obtained by Peng et al.[49]

The link between RAS and monocyte recruitment had been elucidated to a great extent. Ang II may be involved in different steps in the onset and progression of inflammation. [50] The expression of monocytes integrins is dependent on labile zinc activation of Protein Kinase C. Recently; it was found that this activation is largely attenuated by ACE inhibitors.[51]

Hara et al[52] demonstrated that myocardial remodeling in L-NAME-induced hypertensive rats was significantly ameliorated even by a subdepressor dose of the ACE inhibitor, Imidapril. Brilla et al [53] reported similar ameliorative effect on myocardial remodeling with the use of two different ACE inhibitors. Moreover, Liang et al [54] reported that treatment with either Lisinopril or Trandolapril for four weeks could completely prevent cardiac fibrosis, reduce plasma TGF-β1 level and partially prevent cardiac hypertrophy and hypertension.

While the findings of Peng et al[49] regarding the antifibrotic effect of ACE inhibitors on collagen deposition and TGF-β1 level are consistent with this study, they yielded different results concerning their effect on cardiac hypertrophy and HW/ BW ratio. This controversy may be attributed to the different model (angiotensin II induced hypertension) they used as well as to the use of a subdepressor dose of Captopril which did not alter the angiotensin II induced pressure overload and hence the hypertrophic response.

Taken together, these findings of different studies conducted with different ACE inhibitors lead to the conclusion that the cardiovascular protective effects of Lisinopril observed in this study are class effect rather than a single drug effect and the mechanisms involved in their beneficial role include hemodynamic, anti-inflammatory, anti-proliferative and anti-fibrotic effects.

A growing body of published data suggests the ability of fibrates to prevent atherosclerosis and to exert cardiovascular protective effects which is not related to their lipid lowering effects but to other 'pleiotropic effects', such as anti-inflammatory, antioxidant and antithrombotic activity, as well as the ability to improve endothelial function.[55,56] Therefore, the purpose of this study was to examine the effects of the PPAR-α agonist, Fenofibrate on the L-NAME model of endothelial dysfunction.

The present study shows that Fenofibrate did not significantly alter the L-NAME induced pressure overload whether by short or longer term administration, which may explain the absence of late anti-hypertrophic effect. On the other hand it exerted early anti-inflammatory and anti-proliferative activity that was reflected on decreased cardiac fibrosis and remodeling responses. The mechanism underneath these actions may be attributed to inhibition of expression of target genes involved in inflammation, proliferation and fibrosis. It was observed that these effects were independent of the well known Fenofibrate lipid lowering activity. Chen et al[57] and Lebrasseur et al[58] reported similar results.

In addition, the anti-inflammatory effect of Fenofibrate observed in our study is in line with the results of the study of Maruyama et al[59] who attributed this to increased expression of IL-10 by Fenofibrate. However, in contrast to our result, the ratio of ventricular weight/body weight was decreased by Fenofibrate.

In another model of hypertension, the Dahl salt-sensitive (DS) rats, Ichihara et al [60] confirmed the inhibitory effect of Fenofibrate on hypertension induced inflammatory response. A reduction in fibrosis in this model was also noted.[61]

While the results of Diep et al[62] regarding the effect of Fenofibrate on macrophage infiltration are consistent with the present study, they yielded different results in relation to blood pressure.

This discrepancy in the effect of Fenofibrate on blood pressure may be attributed to the different mechanisms of induction of hypertension in the different models and the variable levels of blood pressure obtained in each model. The postulation that the blood pressure lowering effect of Fenofibrate may be related to increase in eNOS expression [63] may explain why Fenofibrate induced non significant decrease in blood pressure in this study as the activity of the eNOS was inhibited by the potent NOS inhibitor, L-NAME, while remaining unopposed in other models of pressure overload.

On comparing the results of various studies, it seems that the effect of Fenofibrate on cardiac hypertrophy is controversial. While the present study revealed insignificant decrease in HW/BW ratio, Li et al[64] and Lebrasseur et al[58] reported anti-hypertrophic activity in two different models of pressure overload. Conversely, other study reported increase in Fenofibrate-induced ventricular hypertrophy.[65]

Taken together, these observations lead to the conclusion that the favorable effects of Fenofibrate in cardiovascular disease are due to their PPAR-α agonistic activity on target genes involved in inflammation, proliferation and remodeling rather than a PPAR-α dependent metabolic activity.[66]

The relationship among hypertension, diabetes mellitus (DM), and endothelial function is complex, and many evidences point to the fact that the association of hypertension and DM results in more severe cardiomyopathy than would be anticipated with each condition alone. Since the changes

observed with hypertensive-diabetic cardiomyopathy mimics the alterations seen after inhibition of NO by administration of L-NAME in rats,[67] this study examined the possible effects of the anti-diabetic drug, Pioglitazone, on these L-NAME induced alterations. Given the wide spread use of PPAR-γ agonists in diabetic patients who are at high risk for cardiovascular disease, investigating their effects is not only of basic interest but also may have important clinical implications.

The results of the present study revealed that Pioglitazone had the potentiality to lower blood pressure with its continuous administration rather than with short term use. Also it could partially ameliorate all the studied cardiovascular abnormalities induced by L-NAME administration in both durations of the study.

Nakamura et al,[68] and Namikoshi et al [69] reported results consistent with the present findings. On the other hand, Ishibashi et al[19] disproved the blood pressure lowering effect of Pioglitazone in the same model of L-NAME induced pressure overload which might be explained by the higher dose of L-NAME (100 mg/kg) used in their study in comparison to 40 mg/kg in this study. However, they reported similar results in relation to the metabolic, anti-inflammatory and anti-proliferative effects of Pioglitazone and related the decrease in monocyte infiltration to an inhibitory effect of Pioglitazone on chemokine receptor 2 (CCR2) expression. Also, in contrast to our data, Majithiya et al[70] reported lack of blood pressure lowering effect of Pioglitazone[71] upon its administration to L-NAME treated diabetic rats.

Several postulations were suggested to explain the mechanisms by which Pioglitazone exhibited its depressor, anti-inflammatory, anti-proliferative and anti-fibrotic activities. For example, a reduction in insulin resistance, modulation of release of vasodilator substances such as prostaglandins or vasoconstrictors as endothelin-1, and a regulatory effect on vascular sodium-angiotensin II relationship in the kidney.[72] Maintenance of contractile phenotype in vascular SMCs could also be considered, down regulation of pro-inflammatory and pro-fibrotic gene expression, prevention of growth factor-induced degradation of the cyclin dependent kinase inhibitor (cdkI), p27.[73]

The common association between dyslipidemia and insulin resistance in many patients and the fact that both are risk factors associated with hypertension and subsequent cardiovascular disease necessitates a combined approach to concomitantly increase insulin sensitivity while decreasing dyslipidemia and thus cardiovascular risk. In this respect, compounds with dual PPAR-α/PPAR-γ activity appear well-suited for the treatment of such patients.[74]

Therefore, one of the aims of this study was to assess the additive effect of dual PPAR-α/γ activation achieved by the combined use of Fenofibrate and Pioglitazone on the L-NAME induced cardiovascular alterations.

In the current study, the combined use of PPAR-α and γ agonists did not significantly affect blood pressure with the short term administration, while on the long run, they were associated with more blood pressure lowering effect. They also had significant anti-inflammatory and anti-proliferative effects more pronounced than what was detected by either drug alone. Similarly, Cardiac hypertrophy, fibrosis and plasma TGF-β1 level were also significantly reduced by this combination.

Throughout the literature reviewed, limited studies addressed the effect of dual PPAR stimulation on cardiovascular abnormalities in the setting of hypertension. Of these, supporting data were provided by the study of De Ciuceis et al [75] the present findings are also in accordance with that reported by Mamnoor et al. [76]

The data provided in this study and those of the other limited publications available,[76] raise the interest in the use of PPAR-α/γ co-agonism as a potential tool for therapeutic intervention in cardiovascular high risk patients.

Since hypercholesterolemia is an important risk factor for hypertension and cardiovascular disease, and is commonly an initiating factor of endothelial dysfunction, lipid lowering therapies have been included in treatment regimens to reduce atherosclerosis and cardiovascular events.[77]

One of the earliest recognizable benefits of statin therapy is the improvement in endothelial function, which in some instances occurs before significant reduction in serum cholesterol levels.[77] The present study was therefore, designed to investigate these cholesterol independent effects of statins in the L-NAME model of endothelial dysfunction which exhibits normal lipid profile.[35]

The present study showed that Atorvastatin was found to exert pleiotropic cardiovascular protective effects including lowering mean arterial blood pressure, early anti-inflammatory and anti-proliferative effects as well as late anti-fibrotic effect.

The results of the present study are in agreement with those retrieved by Ni et al[78] Using the same model of L-NAME induced hypertension, Terata et al[79] reported that Pitavastatin partially inhibited hypertension. On the other hand, Zhao et al [80] showed an insignificant decrease in systolic blood pressure.

Rajamannan et al[81] demonstrated anti-inflammatory and anti-proliferative effects of statin therapy in a model of hypercholesterolemic aortic

valve stenosis.

Different mechanisms were proposed to explain the anti-inflammatory and anti-atherosclerotic activity of statins. Their possible modulatory effect on inflammatory cell signaling pathways seems to be critical in resolution of the atherosclerotic microenvironment. Down regulation of MCP-1, NF-κB and other pro-inflammatory cytokines by statins was also suggested. In addition, statins were reported to inhibit the expression of specific cell surface receptors on monocytes, adhesion molecules and also integrin-dependent leukocyte adhesion. [82] Some evidences point to upregulation of PPAR-α by statins as a participating mechanism in their anti-inflammatory activity.[83]

The ability of statins to decrease cardiovascular hypertrophy and fibrosis observed in the present study are supported by previous studies.[84,85]

Several mechanisms have been presented concerning the antihypertrophic effects of statins on the cardiovascular tissues. For example, decrease in Ang II type 1 receptor expression or myocardial angiotensin-converting enzyme activity, and increase in cardiovascular NO production which can potentially decrease blood pressure and attenuate the hypertrophic processes.[86]

The data presented in this study as well as in other studies conducted on different members of statins document an ameliorative effect of HMG CoA reductase inhibitors on cardiovascular inflammatory, proliferative and fibrotic alterations induced by L-NAME administration with subsequent hypertension and endothelial dysfunction in rats. These data could be of great relevance in designing future clinical trials to test new therapeutic strategies in patients at risk of development of these cardiovascular abnormalities.

Conclusions:

From the results of the present study, it could be concluded that disturbances of NO production or availability are likely to be major determinants of endothelial dysfunction, hypertension and their pathological consequences; hence, directing therapy towards conserving endothelial NO synthase activity and NO bioavailability in the cardiovascular system seems to be of paramount importance.

The tested drugs were capable of modulating the different aspects of the disease whether the early or late changes, though their profiles were different. Among the studied classes of drugs, ACE-inhibitors represented by Lisinopril were superior in modulating both the structural and functional alteration associated with hypertension.

Suppression of the early inflammatory and proliferative activity by the studied drugs is fundamental to abrogate the late structural fibrotic responses.

Dual PPAR-α/γ activation by the combined use of Fenofibrate and Pioglitazone provided superior effects over that produced by activation of PPAR-γ alone. Further support should be provided by clinical studies in this area.

The pleiotropic effects of statins represent an area of great interest in prevention and therapy of cardiovascular diseases. Early statin therapy in high risk cardiovascular patients may help to normalize endothelial function and prevent hypertensive induced changes, even in normolipidemic patients.

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