International Journal of Science and Research (IJSR)
ISSN (Online): 2319-7064
Index Copernicus Value (2013): 6.14 | Impact Factor (2013): 4.438

Potential Role of Vitamin D_3 Compared with Rho-Kinase Inhibitor (Fasudil) on L-NAME – Induced Hypertension in Rabbits

Nageh A. El.Mahdy[1], Samia S. Sokar[2], Karima I. El-Desouky[2], Omyma A. Nassar[4]

[1, 2, 4]Department of Pharmacology& Toxicology, Faculty of Pharmacy ,Tanta University, Egypt

[3]Department of Pathology, Faculty of Medicine, Tanta University , Egypt

Abstract: ***Cardiovascular function is mainly controlled by the renin- angiotensin system, which affects blood vessel tone. Inappropriate activation of the renin-angiotensin system can lead to hypertension. In light of emerging evidence of a widespread global problem of vitamin D deficiency, there has been increasing interest concerning the role of vitamin D in hypertension. Little attention has been paid to the effect of Rho-kinase inhibitor on increased Angiotensin II and the vascular dysfunction of nitric oxide-deficient hypertension. This study aimed to investigate whether the Rho-kinase inhibitor showed beneficial effect on the vascular dysfunction of the NG-Nitro-L-arginine methyl ester (L-NAME) induced hypertension in rabbits, as well as to compare the differential effects of fasudil and Vitamin D on vascular function. Rabbits were divided into five groups; Control, L-NAME, Fasudil, Vitamin D and combination of fasudil and vitamin D. Blood pressure, Biochemical analysis including; NO, GSH, MDA, HDL, LDL, TG, Total cholesterol and LDH were estimated. Pathological study including; Ventricular/Total body weigh ratio, histopathological study of the aorta and ventricular muscles was done. It was found that chronic NOS inhibition not only induced hypertension and cardiac hypertrophy, but also impaird the vascular function. Vitamin D exhibited higher antihypertensive, antihyperlipedimic and antioxidant activity, improved the attenuated vasoconstriction, and possessed a greater beneficial effect on aortic thickness than rho-kinase inhibitor.***

Keywords: Vitamin D, rho-kinase inhibitor, Angiotensin II, vascular dysfunction, hypertension.

1. Introduction

Hypertension is one of the most common worldwide eleven diseases affecting humans. Because of the associated morbidity, mortality and the cost to society, hypertension is an important public health challenge, and also the most important modifiable risk factor for coronary heart diseases, stroke (the third leading cause of death), congestive heart failure, end-stage renal disease, and peripheral vascular disease. Therefore, health care professionals must not only identify and treat patients with hypertension but also promote a healthy lifestyle and preventive strategies to decrease the prevalence of hypertension in the general population [1] .

Left ventricular hypertrophy are common finding in hypertensive patient that increase the incidence of cardiac-related deaths [46].

ROCK activity has also been investigated in the pathogenesis of hypertensive vascular disease. Short and long term administration of a ROCK inhibitor decreased blood pressure in several animal models of systemic hypertension through inhibition of angiotensin II-induced cardiac hypertrophy. Also, non-hypotensive doses of fasudil have been shown to suppress coronary vascular lesion formation in spontaneously hypertensive rats.[22]

Although RAS inhibitors, including angiotensin converting enzyme inhibitors (ACEIs) and angiotensin receptor blockers, are widely used in the therapy of renal and cardiovascular diseases, the major problem of these drugs is the compensatory renin rise due to the disruption of the feedback inhibition of renin production [55].

The discovery of the vitamin D endocrine system and a receptor for the hormonal form, lα 25-dihydroxyvitamin D_3, has brought a new understanding of the relationship between vitamin D and metabolic bone diseases, and has also established the functions of vitamin D beyond the skeleton. This has ushered in many investigations into the possible roles of vitamin D in autoimmune diseases, cardiovascular disorders, infectious diseases, cancers and granuloma-forming diseases.[36]

2. Materials and Methods

2.1. Animals and Treatment

Thirty Adult male Newzeland rabbits obtained from the animal house of the National Research Center (NRC), Egypt, weighing from 1000-1500 g were used in the present study. They were fed on standard pellet chow (El-Nasr Chemical Company, Cairo, Egypt) and allowed for free access to water. Animals were housed in the same conditions for one week prior to the experiment for acclimatization. Then they were randomly divided into two groups: control group (n=6) and L-NAME treated group (n=24). Rabbits in the L-NAME group received an intake of L-NAME (40mg/kg/day) and those in the control group received distilled water intragastrically at the same time.

After four weeks, animals in the L-NAME treated group were randomly subdivided into four groups (six animals each): (**L-NAME group**), in which rabbits continued administration of L-NAME (40mg/kg/day) in drinking water and intragastrically for further 6 weeks [20]. (**Alfacalcidol-treated group)** :rabbits have been treated with Alfacalcidol (0.06 ug/kg) i.p. every other day for 6 weeks. [17]. **,** (**Fasudil-**

treated group) :rabbits have been treated with Fasudil (30 mg/kg/day) in water intragastrically for 6 weeks [5]. and (**Alfacalcidol and Fasudil combination group**: rabbits have been treated with Fasudil (30 mg/kg/day) added on distilled water and given intragastrically and Alfacalcidol (0.06ug/kg every other day i.p.) for 6 weeks.

2.2. Drugs and Reagents

L-NAME, N-(1-naphthyl) ethylenediamine Dihydrochloride, Vanadium (III) chloride ,and Bis-(3-carboxy-4-nitrophenyl) disulphide (Sigma , U.S.A) , Fasudil (Tianjin,China), Alfacalcidol (LEO ,Denemark), Sulfanilamide and Trichloroacetic acid (Winlab, England), Sodium nitrite (El-Nasr, Egypt), Sulfosalicylic acid (Fluka Biochemica, Switzerland), Reduced Glutathione (GSH) (BDH, England), Cholesterol,Cholesterol LDL precipitating agent, Lactate Dehydrogenase, Triglycerides, Cholesterol HDL precipitating agent (BioSystems S.A., Barcelona (Spain).All chemicals used were of high analytical grade and solutions used in the present study were all prepared freshly and protected from light.

2.3. Measurement of Arterial Blood Pressure

At the end of the experiment (10 weeks) Blood pressure of all studied groups was measured using Harvard Apparatus, Student Oscillograph (Ser.No.K22514) by inserting needles acutely into the distal part of the central artery of the ear three successive days and the mean readings for each animal was recorded. [40]

2.4. Determining the Arterial Diameter

The seimens x300 , superficial transducer (Seimens-Germany) were used for ultrasound imaging. The ultrasound frequency was 4–12 MHz. Coupling gel was applied to the rabbit's neck. Before using colour Doppler , B-mode gray scale US was performed. Data from ultrasonography analysis for each rabbit were registered separately and processed in a blinded manner for statistical evaluation.

2.5. Biochemical Analysis

All animals were weighd ,then, anaesthetized with urethane (1.2 gm / kg i.p.).The chest of each rabbit was rapidly opened; blood sample was taken immediately by intracardiac puncture, centrifuged and serum was kept frozen at -20° C for the following **biochemical analysis**: Lipid peroxides (measured as MDA) [53]., Nitric oxide (measured as nitrate/nitrite)[31]., Glotathione (GSH) content [Reduced glutathione (GSH) was determined spectrophotometrically . [11], Lipid profile (cholesterol [30] , LDL cholesterol [2]., HDL cholesterol[8] ,Triglycerides [7]), and Lactate Dehydrogenase [44].

2.6. Pathological Study

The heart was excised, washed off the blood with physiological saline, then the atria was removed, the ventricles were weighed and the ventricles weight /Body weight ratio was determined[37]. The thoracic aorta was dissected, the heart and thoracic aorta were excised and immediately fixed in 10% neutral buffered formaldehyde for 24 hours.

Tissue samples were taken from the heart at the midventricular level, which was confirmed by the presence of papillary muscle in the cavity and semicircular shape of the right ventricular free wall, also transverse sections of thoracic aorta were taken. Sections were processed in ascending grades of alcohol cleared in xylene and paraffin embedded serial sections were prepared on glass slides in 3-5µm thickness for routine Hematoxylin and Eosin stain (H&E) ,masson trichrome stain to assess cardiac pathological changes.[52]

2.7. Morphometric Study

The mean diameter of 50 cardiomyocytes per field and total aortic ring thickness with maximum & minimum diameter of 50 individual smooth muscle cells per field in the vessel wall was measured in 10 fields in different groups for morphmetrical analysis, microphotographs were taken at 400X magnification, using a LEICA Qwin microscope. [52]

2.8. Statistical Analysis

In all series of experiments, (n) was the number of rabbits from which the tissues were obtained. Results are given as mean ± SD. All data were analyzed by one-way ANOVA followed by Post Hoc test (LSD) for multiple comparisons. The percentage of change in mean between groups was obtained. A two-tailed value of $P \leq 0.05$ was regarded as statistically significant.

3. Results

At the end of 4-week administration of L-NAME (30mg/kg/day), the rabbits in NO-deficiency developed sustained higher blood pressure compared to the control rabbits(117.12 ± 2.24mmHg), indicating an established hypertensive state in those animals. As shown in Table(1), the SBP and aortic diameter of rabbits in the control group remained at the basal level during the 10-week treatment period; whereas the blood pressure of rabbits in the L-NAME group displayed an increase, reaching its maximum within 4 weeks(191.65 ± 5.27mmHg).

Both vitamin D (0.06µg/kg i.p. every other day) and Fasudil (30 mg/kg/day intragastrically) significantly decreased systolic blood pressure L-NAME group, but interestingly vitamin D treatment was more efficient in blood pressure reduction(121.40 ± 4.29mmHg) with41.15%decrease compared to L-NAME group .

Compared to L-NAME group; Vitamin D also produced a higher increase in Nitric Oxide, GSH, HDL concentrations than fasudil with percent increase of (6047.06%, 306.22%, 129.22%) respectively, and a higher decrease in LDL and TG concentrations than fasudil with percent decrease of (70.22%, 6.57% respectively) compared to L-NAME.Tables(3,4)

These results were proved pathologically by the significant decrease in aortic thickness that vitamin D produced (↓41.04

%)compard to L-NAME (Fig.8), and by the augmented action that vitamin D produced with fasudil in reduction of aortic smooth muscle thickness (↓39.27%) (Fig.9). Table(5). The pathological results reported also that fasudil treatment alone (30mg/kg/day) was more efficient in the reduction of myocardial muscle thickness(↓44.87%) than vitamin D(↓33.02%) compared to L-NAME group .(Fig.3,4),Table (5).

The addition of vitamin D to Rho-kinase inhibitor like fasudil produced, augmented action in, increase in aortic diameter(↑392.22%) (Table 2) and GSH concentration(↑425.84%), also a significant decrease in blood pressure(↓40.43%), aortic smooth muscle thickness (↓39.27%), TG, MDA concentration(↓96.64%) (Table 3) were reported.

Table 1: Comparison between the mean systolic blood pressure in the different studied groups and % of change in it between groups

Groups	Mean ± SD	% of change in mean of systolic blood pressure compared to: Control (%)	L.NAME (%)	Fasudil (%)	Vitamin D treatment (%)
Control	117.12 ± 2.24				
L.NAME	191.65 ± 5.27	↑63.64**			
Fasudil	127.60 ± 4.52	↑8.95**	↓33.42**		
Vitamin D treatment	121.40 ± 4.29	↑3.66	↓36.66**	↓4.86*	
Fasudil+ vitamin D	114.17 ± 6.67	↓2.52	↓40.43**	↓10.53**	↓5.96*

*: Statistically significant at $p \leq 0.05$
**: Statistically significant at $p \leq 0.01$ In table above: L-NAME (NO inhibitor) used in dose 40 mg/kg/day for 4 weeks,Fasudil (Rho-kinase inhibitor) 30mg/kg/day intragastrically by gastric gavage and alfacalcidol 0.06µg/kg i.p. every other day were used as treatment of induced hypertension for 6 weeks (either alone or in combination with each other).

Table 2: Comparison between the mean aortic diameter(radius) in different studied groups and % of change in it between

Groups	*Mean ± SD*	% of change in mean of aortic diameter compared to: *Control (%)*	*L.NAME (%)*	*Fasudil (%)*	*Vitamin D treatment (%)*
Control	1.88 ± 0.18				
L.NAME	0.90 ± 0.14	↓52.13**			
Fasudil	4.30 ± 0.42	↑128.72**	↑377.78 **		
Vitamin D treatment	2.27 ± 0.55	↑20.74	↑152.22 **	↓47.21**	
Fasudil+ vitamin D	4.43 ± 0.29	↑135.64*	↑392.22 **	↑3.02	↑95.15**

*: Statistically significant at $p \leq 0.05$ **: Statistically significant at $p \leq 0.01$
#:compard to control. *:compared to L-NAME.

In table above: L-NAME (NO inhibitor) used to induce hypertension in dose 40 mg/kg/day for 4 weeks, Fasudil (Rho-kinase inhibitor) 30mg/kg/day intragastrically by gastric gavage and Alfacalcidol 0.06µg/kg i.p. every other day were used as a treatment of induced hypertension for 6 weeks (either alone or in combination with each other).

Table 3: Mean concentrations± SD of NO, GSH, MDA (µmol/1ml serum) and LDH (U/L) in different studied groups

Groups	Mean concentration ± SD of: NO (µmol/1ml serum)	GSH (µmol/1ml serum)	MDA (µmol/1ml serum)	LDH (U/L)
Control	9.54 ± 7.32	90.13 ± 29.14	3.90 ± 0.75	351.80±360.36
L.NAME	0.34 ± 0.17 (↓96.44%##)	22.33 ± 11.88 ↓75.22%##	32.78 ± 34.12 ↑740.51%##	923.60±202.95 ↑162.54%##
Fasudil	12.96 ± 3.60 ↑3711.76%**	54.29 ± 38.76 ↑143.13%	4.19 ± 4.43 ↓87.22%**	308.0 ± 36.35 ↓66.65%**
Vitamin D treatment	20.90 ± 3.43 ↑6047.06%**	90.71 ± 36.94 ↑306.22%**	5.59 ± 4.44 ↓82.95%**	231.50 ± 47.09 ↓74.94%**
Fasudil+ vitamin D	15.01 ± 4.08 ↑4314.71%**	117.42 ± 37.19 ↑425.84%**	1.10 ± 0.48 ↓96.64%**	315.33 ± 25.42 ↓65.86%**

*: Statistically significant at $p \leq 0.05$ ‘ **: Statistically significant at $p \leq 0.01$
#:compard to control. *:compared to L-NAME.
In tables above: L-NAME (NO inhibitor) used to induce hypertension in dose 40 mg/kg/day for 4 weeks, Fasudil (Rho-kinase inhibitor)30mg/kg/day intragastrically and alfacalcidol 0.06µg/kg i.p. every other day were used as treatment of induced hypertension for 6 weeks (either alone or in combination with each other).

Table 4: The mean concentrations ± SD of total cholesterol, TG,HDL and LDL(mg/dl serum) and LDH (U/L) indifferent studied groups

	Mean concentration ± SD(mg/dl) of			
Groups	**Total cholesterol**	**TG**	**HDL**	**LDL**
Control	69.94 ± 9.19	74.28 ± 17.28	32.31 ± 11.02	127.0 ± 19.96
L.NAME	74.94 ± 21.32 ↑7.15%	193.66 ± 7.85 ↑160.72%##	20.12 ± 10.71 ↓37.73%#	257.05 ± 35.36 ↑102.40%##
Fasudil	142.74 ± 46.45 ↑90.47%**	199.01 ± 0.81 ↑2.76%	39.07 ± 8.39 ↑94.18%**	136.94 ± 49.32 ↓46.73%**
Vitamin D treatment	80.17 ± 13.22 ↑6.98%	180.94 ± 12.45 ↓6.57%	46.12 ± 8.98 ↑129.22%**	76.56 ± 18.81 ↓70.22%**
Fasudil+ vitamin D	69.39 ± 15.06 ↓7.41%	131.71 ± 10.18 ↓31.99%**	36.33 ± 8.71 ↑80.57%*	181.97 ± 53.70 ↓29.21%**

*: Statistically significant at $p \leq 0.05$ **: Statistically significant at $p \leq 0.01$
#:compard to control. *:compared to L-NAME.
In tables above: L-NAME (NO inhibitor) used to induce hypertension in dose 40 mg/kg/day for 4 weeks, Fasudil (Rho-kinase inhibitor)30mg/kg/day intragastrically and alfacalcidol 0.06µg/kg i.p. every other day were used as treatment of induced hypertension for 6 weeks (either alone or in combination with each other).

Table 5: the mean thickness ± SD of aorta,aortic smooth muscles and myocardial muscles in different studied groups:

	Mean concentration ± SD(µm) of		
Groups	Aortic thichness	Aortic sm.ms.thickness	myocardial muscle thickness
Control	234.55 ± 7.21	6.70 ± 1.24	14.44 ± 2.12
L.NAME	372.82 ± 19.27 ↑58.95%##	10.08 ± 1.26 ↑50.51%##	15.78 ± 2.64 ↑9.28%
Fasudil	316.90 ± 16.38 ↓15.0%**	7.56 ± 0.93 ↓25.07%**	9.35 ± 0.91 ↓44.87%**
Vitamin D treatment	219.81 ± 8.11 ↓41.04%**	9.56 ± 1.56 ↓5.18%	10.57 ± 0.85 ↓33.02%**
Fasudil+ vitamin D	242.33 ± 12.53 ↓35.0%**	6.12 ± 0.49 ↓39.27%**	10.16 ± 1.32 ↓35.61%**

*: Statistically significant at $p \leq 0.05$ **: Statistically significant at $p \leq 0.01$
#:compard to control. *:compared to L-NAME.
In tables above: L-NAME (NO inhibitor) used to induce hypertension in dose 40 mg/kg/day for 4 weeks, Fasudil (Rho-kinase inhibitor)30mg/kg/day intragastrically and alfacalcidol 0.06µg/kg i.p. every other day were used as treatment of induced hypertension for 6 weeks (either alone or in combination with each other).

Table 6: Comparison between the mean ± SD of Ventricle weight/body weight ratio(g/kg) different studied groups and % of change in it between groups

Groups	*Body weight (kg)*	*Ventricle weight (g)*	*Ventricle weight/ body weight ratio(g/kg)*	*% of change in mean of Ventricle weight/body weight ratio(g/kg) compared to*			
	Mean ± SD	Mean ± SD	Mean ± SD	Control	L-NAME	Fasudil	Vitamin D
Control	2.46 ± 0.10	7.07 ± 0.19	2.87 ± 0.09				
L-NAME	1.95 ± 0.18	6.41 ± 0.58	3.30 ± 0.17	↑14.98**			
Fasudil	2.08 ± 0.07	8.07 ± 0.25	3.88 ± 0.20	↑35.19**	↑17.58**		
Vitamin D treatment	2.20 ± 0.16	7.32 ± 0.29	3.33 ± 0.13	↑16.03**	↑0.91	↓14.18**	
Fasudil + vitamin D	1.98 ± 0.13	7.64 ± 0.40	3.87 ± 0.06	↑34.84**	↑17.27**	↓0.26**	↑16.22**

*: Statistically significant at $p \leq 0.05$ **: Statistically significant at $p \leq 0.01$
In tables above :L-NAME (NO inhibitor) used to induce hypertension in dose 40 mg/kg/day for 4 weeks, Fasudil (Rho-kinase inhibitor) 30mg/kg/day intragastrically by gastric gavage and alfacalcidol 0.06µg/kg i.p. every other day were used as treatment of induced hypertension for 6 weeks (either alone or in combination with each other).

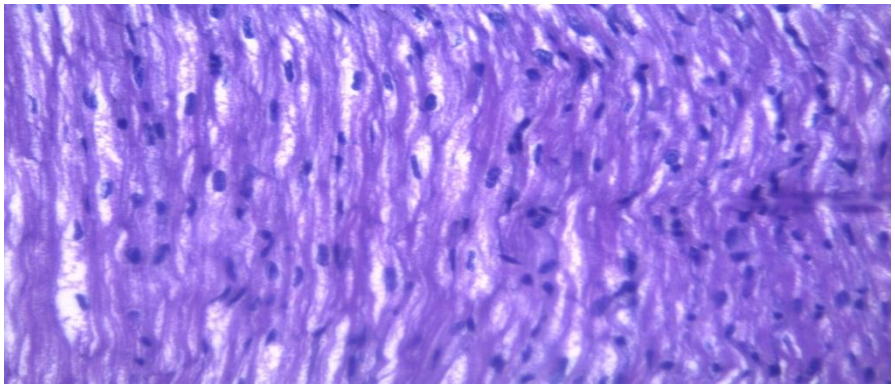


Figure 1: Control group showing normal myocardial sencytial fibers (branched anastomosing fibers of uniform diameters.

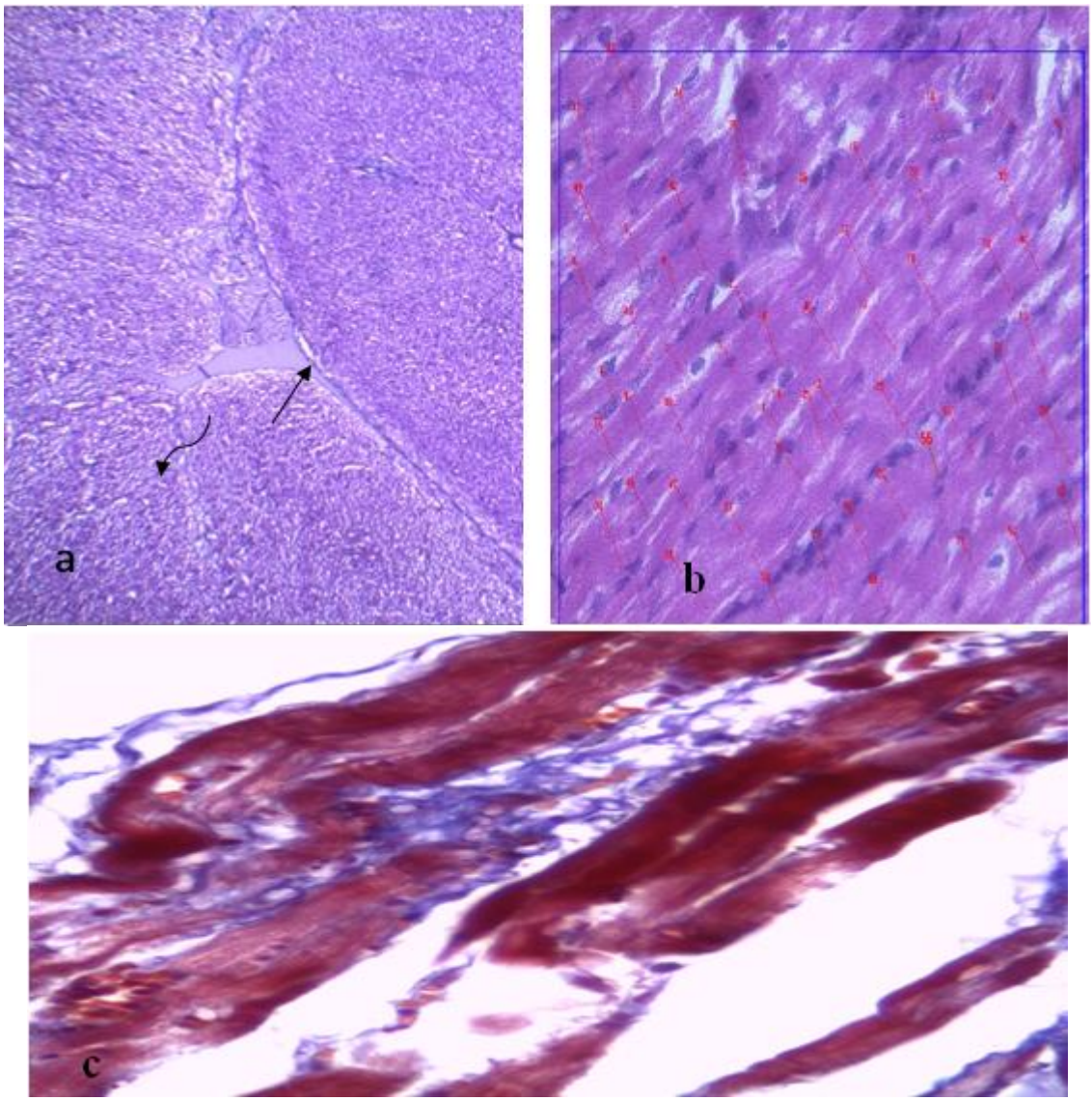
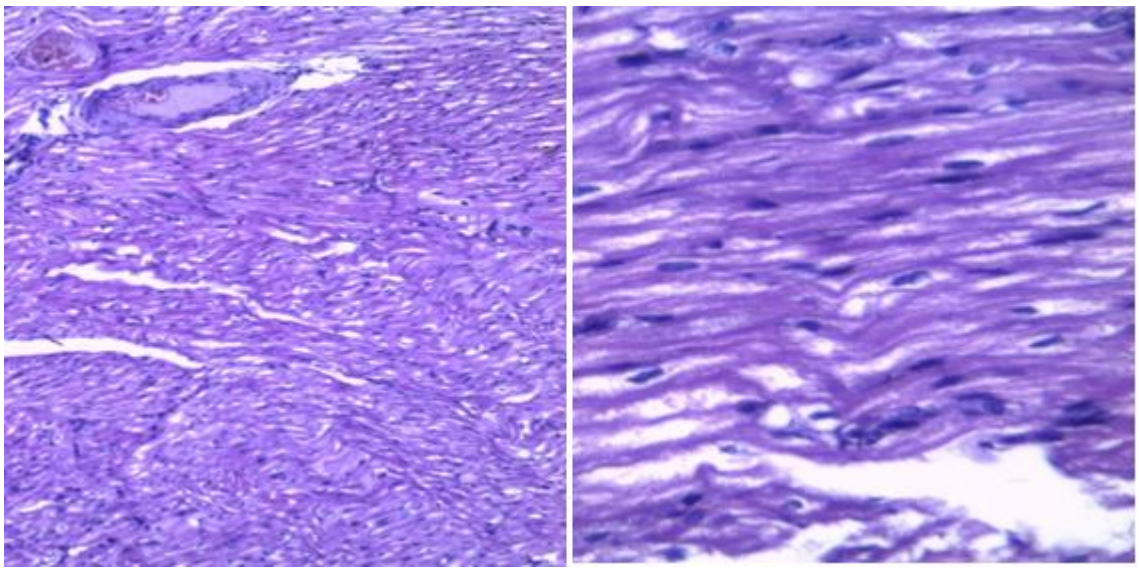


Figure 2 : Chronic L-NAME (30mg/kg/day)for 10 weeks. (a) Showing hypertrophied myocardial muscle fibers with severely hypertrophied papillary muscle (straight arrow), with increasing interstitial fibrosis in between muscle fibers .(curved arrow)(HX &E X 40). (b)Morph metric illustration of the same group (HX &E X 400).(c)masson trichrome stain illustrating fibrosis of myocardial muscle fibers (blue stain) X 400.



(a) **(b)**

Figure 3 a,b: : Fasudil-treated group showing thin myocardial muscle fibers with wide interstitial vascular channels.(a):(Hx&E X40),(b):(Hx&E X400).

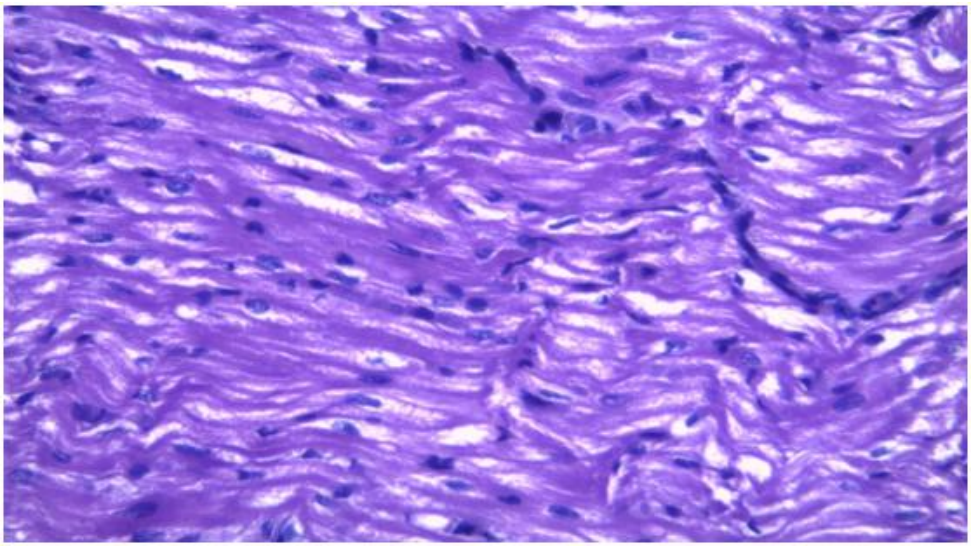


Figure 4: 1-alpha-(OH)D_3 showing wavy apparently normal muscle fibers with slight disarrangement with decreased myocardial muscle thickness.(Hx&E X400).

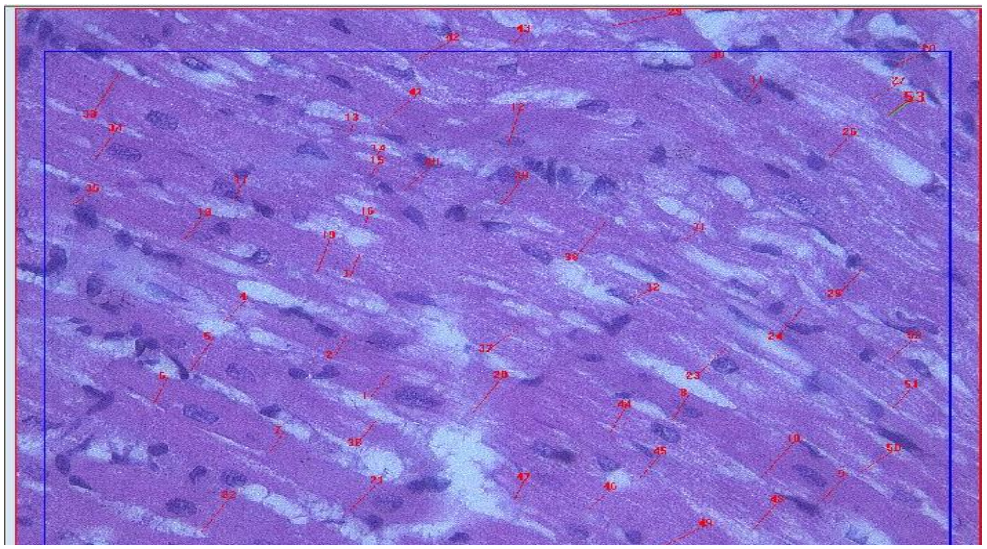


Figure 5: Morphmetric figure of Fasudil(30mg/kg/day i.g.) +1-alpha-(OH)D_3 (0.06µg/kg every other dayi.p.) group showing normal arrangement with reduction in muscle thickness of myocardial muscle thickness (Hx&E X400).

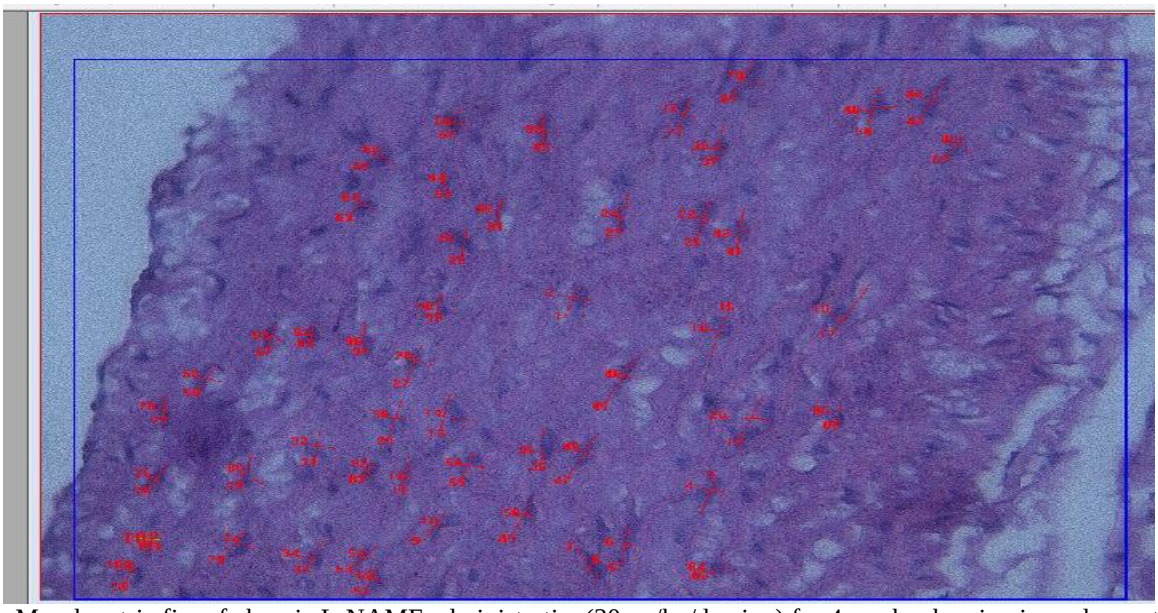


Figure 6: Morphmetric fig. of chronic L-NAME administration(30mg/kg/day i.g.) for 4 weeks showing irregular aortic layers with prominent thickening of aortic smooth muscle fibers (Hx & E X400).

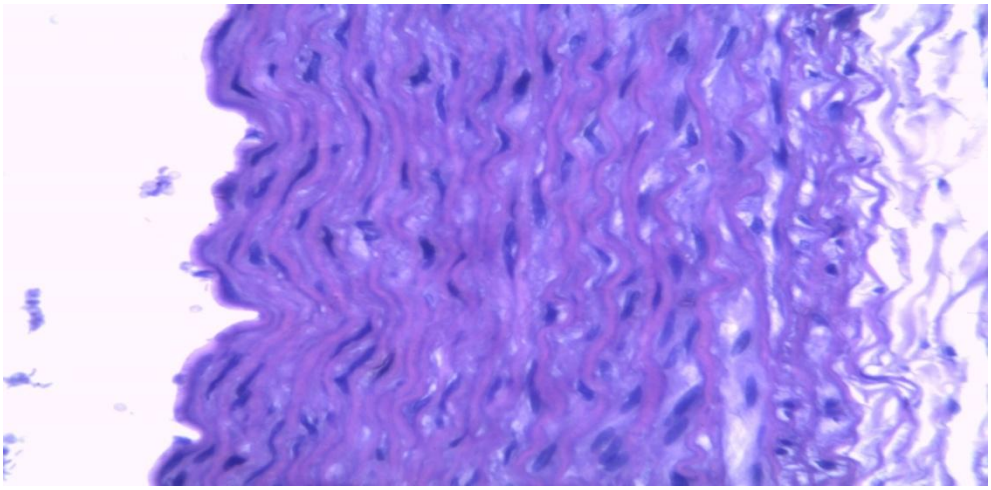


Figure 7: Fasudil treated group (30mg/kg/day i.g.) showing partially irregular wavy smooth muscle and elastic fibers of aortic media with apparently decreased thickness (Hx&E X400).

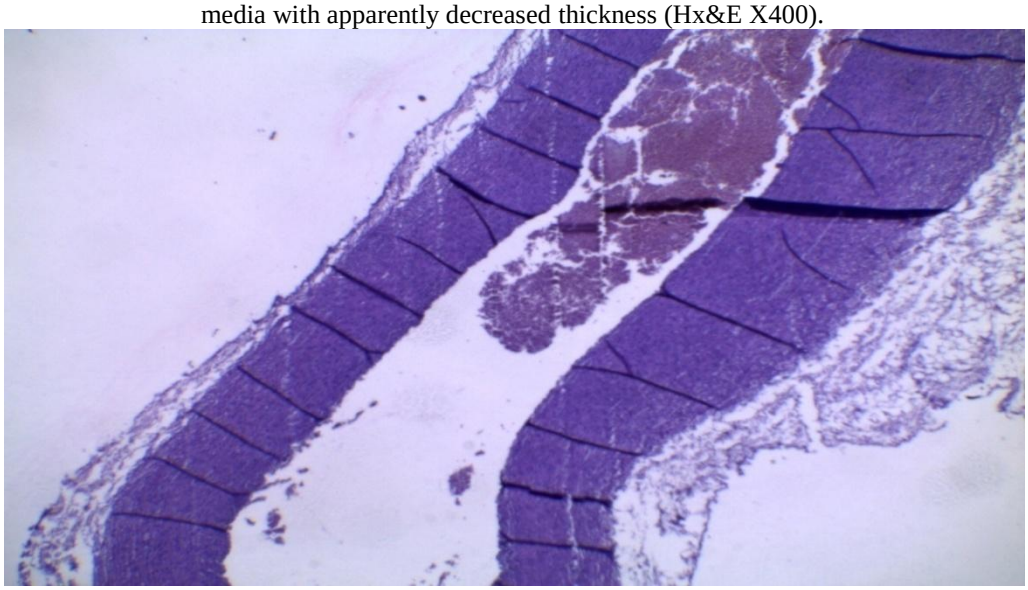
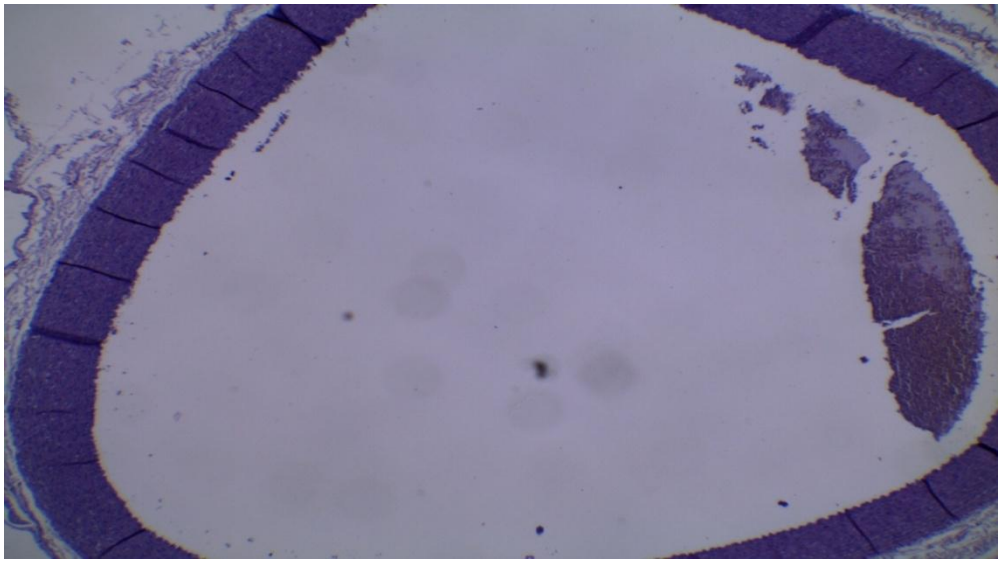


Figure 8:1-alpha-(OH)D_3 (0.06µg/kg every other dayi.p.) showing apparently normal aortic ring less in thickness than L-NAME treated group (Hx& E X 40).



Firure 9: Fasudil(30mg/kg/day i.g.) +1-alpha-(OH)D_3 (0.06 µg/kg every other day i.p.)showing thin walled aorta with wavy smooth muscle cells.(Hx &E X40).

4. Discussion

The development of left ventricular hypertrophy and the stiffening of the left ventricular chamber results in diastolic dysfunction secondary to chronic hypertension ,and facilitate heart failure progression and increase risk of death. [4] The structural rearrangement that occurs in the left ventricular wall due to chronic pressure overload determines the degree of ventricular stiffening, thus affecting the systolic and diastolic function of the heart chambers .[43]

It is already known that RAS induces left ventricular remodeling. [50]. Moreover, both angiotensin¬ converting enzyme inhibitor and/or AT-I receptor blockers are able to prevent the development of cardiac hypertrophy in hypertensive rats .[39] as well as reduce cardiac hypertrophy in humans. [3].

Given that the efficacy of the classic RAS inhibitors is often compromised by the compensatory increase in renin production and that Vitamin D3 down regulates renin gene transcription . [29].There is growing evidence that vitamin D either directly or indirectly affects cardiac structure and function. The present study was designed to block the compensatory renin increase with l-alpha-(OH)D_3 used alone or in combination with Fasudil. [32]

In the present study, treatment with alfacalcidol either alone or in combination with fasudil produced highly significant ($P \leq 0.01$) decrease in the elevated systolic blood pressure produced by L-NAME, but the combination of vitamin D_3 (0.06µg/kg every other day),and fasudil (30mg/kg/day)was the most significant in decreasing systolic blood pressure and this may be attributed to the endothelial vasodilator function of vitamin D proved in work of Harris et al.and Jablonski et al.who recorded the ability of Vitamin D to ameliorate the high blood pressure in spontaneous hypertensive rats and increase flow-mediated vasodilatation. [14,18,6]

Studies in knockout mice confirm that the absence of vitamin D receptor activation leads to tonic up regulation of RAS, with the development of hypertension and Left Ventricular Hypertrophy (LVH) [29].

In the last two decades, clinical studies have revealed at inverse relationship between the plasma 1, 25$(OH)_2$D concentration and the blood pressure and/or the plasma rennin activity in both normotensive men and patients with essential hypertension [24]. Furthermore, it has been reported that vitamin D_3 supplementation reduces blood pressure in patients with essential hypertension[38] , and 1, 25$(OH)_2D_3$. treatment reduces blood pressure, plasma renin activity, and Ang II levels in hyperparathyroidism patients .[23].

Fasudil, a selective Rho-kinase inhibitor, has been widely used for prevention and treatment of cerebral vasospasm after subarachnoid hemorrhage and pulmonary artery hypertension since a decade ago.[28]

Bainian et al. demonstrated that fasudil showed antihypertensive effect on the long-term NO-deficient rats and beneficial effect on the endothelium-dependent vasorelaxation , whereas chronic fasudil treatment leads to a marked elevation in the heart rate. Therefore, fasudil therapy for the patients with compromised cardiac function should be pursued with caution at the clinical situation due to this unwanted adverse event[5].

In the present study ,Alfacalcidol and fasudil (30 mg /kg /day) was the most significant combination in increasing aortic diameter.

This was approved morphmetrically by the highly significant decrease in the minimum diameter (thickness) of aortic smooth muscle fibers compared with L-NAME group, and the highly significant increase in nitric oxide (NO) concentration produced by this combination, this may be due to that in the aorta, nitric oxide inhibits the activity of Rho kinase and the sensitization of the tissue to calcium, leading to the hypothesis that nitric oxide-mediated vasodilatation occurs through inhibition of the vasoconstrictor activity of Rho kinase[9]

These results were in accordance with results obtained from prvious studies of Sauzeau et al.and Kataoka et al.who found that NO induces vasodilatation through cyclic GMP and that protein kinase G phosphorylates Rho a and inhibits its activity. [42,21].

In hypertensive patients, fasudil, a specific inhibitor of Rho kinase , was seen to induce a stronger vasodilatory response in the forearm of hypertensive than control subjects, while the response to sodium nitroprusside was similar in both patient groups. This result constitutes the first evidence that the Rho/Rho kinase pathway participates in the pathogenesis of increased systemic vascular resistance in hypertensive patients.[42]

Kataoka et al. found that in rat model of hypertension induced by NO synthesis inhibition ,the activity of Rho A/ROCK pathway was increased and long term blockade of ROCK suppresses vascular lesion formation such as medial hypertrophy and perivascular fibrosis in small coronary arteries. [21]
Also, 1, 25- dihydroxyvitamin D_3 has favorable effects on endothelial cells of aorta ,this may be by protecting them against the deleterious effects of glycation end products and increasing the activity of endothelial NO.

This was in line with previous in vitro studies of Wang et al. and Norman et al. who showed that 1, 25-dihydroxyvitamin D_3 acutely reduce endothelium-dependent contractions in the spontaneous hypertensive rats' aorta and a statistically significant reduction in the number of elastic lamellae with increasing vitamin D consumption in Sprague-Dawley rats.[47,34]

In this study , L-NAME group(hypertensive rabbits), significantly reduced NO and increased both MDA and GSH concentrations. Malondialdehyde (MDA) and reduced glutathione (GSH) contents were used as indices of oxidative stress , which is the main culprit in hypertension. The occurrence of oxidative stress may arise from a primary decrease in the antioxidant defense system activity or from an elevation of ROS concentration. This derangement leads

to oxidative damage to the structure of biomolecules, likely involving the antioxidant enzymes, thus contributing to the oxidative stress found in hypertensives, but not in normotensives. As a consequence of increased ROS, a reduction in the endothelium-dependent vasodilation of the vascular smooth muscle cells of hypertensives could be expected. In turn, elevations of blood pressure could also contribute to the increase of ROS, thereby enhancing the mechanism of ROS-mediated hypertension through a complex interdependency [45]

These results were supported with previous studies of Zimmerman and Granger who reported that MDA is the end product of lipid peroxidation and used for determining the increased free radicals formation in tissues. Determination of lipid peroxidation was found to be a practical method to evaluate the factors causing cellular injury. The ability of hydroxyl radicals to initiate lipid peroxidation resulted in the formation of lipid derived free radicals such as conjugated dienes, lipid peroxide radicals, and lipid hydroperoxides. [56]

Both superoxide anion and hydrogen peroxide production by leukocytes and the plasma levels of lipid peroxides were higher and plasma nitrite levels were lower in patients with uncontrolled essential hypertension compared with normal control subjects; ANG II stimulated free radical generation in normal leukocytes, suggesting that an increase in free radical generation and a simultaneous decrease in the production of NO and antioxidants occurs in essential hypertension . [25]

Wolf suggested that drugs interfering with ANG II effects may serve as antioxidants, preventing vascular and renal changes. [49]

In this study, Nitric Oxide was highly increased by treating hypertensive rabbits with vitamin D_3 alone or in combination with Fasudil , and also, reduced MDA and GSH concentrations compared to L-NAME group.These data are in agreement with the previous indirect observation of Ni et al.and Zhihong et al.who found in their studies on high-cholesterol diet-induced hypercholestrimic rats that the anti-inflammatory and antiarteriosclerotic properties of statins are mediated, at least in part, by inhibition of Rho protein isoprenylation, preventing the activation of downstream Rho targets such as ROCKs., they found that administration of fasudil (30mg/kg/day) or Simvastatin (10mg/kg/day) to hyperchlolesterolemic rats for 2 weeks suppressed the activation of ROCK and NF-κB in thoracic aorta and status of dyslipidemia were improved and inflammatory markers lowered. [33,54]

These results suggested that fasudil-induced inhibition of ROCK improved lipid metabolism and has anti-inflammatory effect.
Higashi et al. also,deduced that treatment with non hypotensive doses of fasudil significantly suppressed the coronary vascular lesion formation in hypertensive rat model along with normalization of endothelial NAD(P)H oxidaze activity aand endothelial production of superoxide anions and resultant improvement of endothelial vasodilator function. [15]

Calcitriol has been reported to exert a receptor-mediated effect on human monocyte hydrogen peroxide secretion . [10] Human monocytes in culture gradually lose their capability to produce superoxide when stimulated, but the addition of calcitriol, lipopolysaccharide, or lipoteichoic acid (LTA) restores this and increases their oxidative capacity compared with unstimulated monocytes .[27]

Vitamin D could reduce the extent of lipid peroxidation and induce superoxide dismutase (SOD) activity in the hepatic antioxidant system in rats [41,13] In addition, calcitriol also enhances intracellular glutathione (GSH) pools and significantly reduces LPS-induced nitrite production [12]

In the present study treatment withVitamin D elicited significant reduction in cardiomyocyte diameter and myocardial damage compared to L-NAME treated group. The association between vitamin D and cardiovascular diseases could be explained by lipid-lowering effect of vitamin D . In studies of Hypponen et al., Lee et al. and Jorde et al.,a negative association between serum 25(OH)D and HDL-C concentrations tended to be higher than those with lower serum 25(OH)D concentration . A negative relation between serum 25(OH)D and both TC and LDL-C was also reported in a study on 126 subjects .[16,26,19]

Xiang et al., reported that the size of left ventricular cardiomyocytes in VDR knockout mice was markedly increased compared with wild-type mice . This is consistent with the results of(Park et al,1999) who showed regression of LV hypertrophy in hemodialysis patients treated with vitaminD.[51]

Additionally, Weishaar et al. has shown that rats fed a vitamin D–deficient diet have increased amounts of collagen by measuring hydroxyproline per gram of heart tissue and collagen deposition in the extracellular space of the myocardium. [48] These previous results about the ability of vitamin D to reduce cardiomyocyte diameter and myocardial damage support and explain the highly significant reduction in lactate dehydrogenase level (LDH) that vitamin D had done .

In conclusion,these results suggest that chronic NOS inhibition not only induces high blood pressure and cardiac hypertrophy, but also impairs the vascular function. Both Vitamin D and Rho-kinase inhibition exhibit an antihypertensive effect,while onlyRho-kinase inhibitor shows antihypertrophic potential on cardiomyocytes.Vitamin D exerted antihyperlipedimic and antioxidant activity and also improves the attenuated vasoconstriction, where as the Vitamin D exerted greater beneficial effect on endothelium-dependent vasorelaxation . This work provides the first evidence that both Vitamin D and fasudil decrease the augmented RhoA/ ROCK signaling pathway in the vasculature of hypertensive rabbit, which may be one of the molecular mechanisms for their blood pressure-lowering activity.

5. Acknowledgments

Special thanks to *Dr. Ibrahim Abbas Nassar* ,lecturer of radiodiagnosis, Faculty of Medicine, Tanta University for

his great effort in doplex imaging technique in this work, and we also would like to extend our thanks to *Dr. Mohamed El-Kahwagy* ,lecturer of cardiosurgery, Faculty of Medicine,Tanta University for his help in learning arterial catheterization technique.

Refrences

[1] Albert WD (2010), Sharma S and Kortas C. Hypertension on e-medicine specialities. http://emedicine .medscape.
[2] Assmann G, Jabs HU, Kohnert U, Nolte W and Schriewer H.(1984). LDL-cholesterol determination in blood serum following precipitation of LDL with polyvinylsulfate. Clin Chim Acta ; 140: 77-83.
[3] Avanza AC Jr, El Aouar LM and Mill JG (2000). Reduction in left ventricular hypertrophy in hypertensive patients treated with enalapril ,losartan or the combination of enalapril and losartan. Arq Bras Cardiol. ;74:111-117.
[4] Baldo MP, Forenchi L, Morra EA, Machado RC, Lunz W et al.(2011). Long term use of low dose spironolactone in spontaneously hypertensive rats: effects of left ventricular hypertrophy and stiffness. Pharmacol Rep.; 63(4):975-982.
[5] Bainian C, Lili S,Xiaoyan Y, Jialin S, Hengai Z, Shoubao W, Lianhua F,Guanhua D (2012).Effects of Rho-kinase inhibitor and angiotensin II type-1 receptor antagonist on the hypertensive rats . Acta Pharmaceutica Sinica B.2(5):450–458.
[6] Borgs ACR , Feres T, Vianna LM and Paiva TB(1999). Effect of cholecalciferol treatment on the relaxant responses of spontaneously hypertensive rat arteries to acetylcholine. Hypertension.;34:847-901
[7] Bucolo G and David H .(1973). Quantitative determination of serum triglycerides by use of enzymes. Clin Chem; 19: 476-482.
[8] Burstein M, Scholnick HR and Morfin R.(1980). Rapid method for the isolation of lipoproteins from human serum by precipitation with polyanions. Scand J Clin Lab Invest; 40: 583-595.
[9] Chitaley K, Webb RC. Nitric oxide induces dilation of rat aorta via inhibition of rho-kinase signaling. Hypertension. 2002;39: 438-42.
[10] Cohen, M.S. et al. (1986). 1,25-dihydroxyvitamin D3 activates secretion of hydrogen peroxide by human monocytes. J Immunol,136, 1049-1053.
[11] Ellman GL.(1959). Tissue sulfahydryl groups. Arch Biochem Biophys.; 82: 70-7.
[12] Garcion, E. et al. (1999). 1,25-dihydroxyvitamin D3 regulates the synthesis of γ-glutamyl transpeptidase and glutathione levels in rat primary astrocytes. J Neurochem, 73, 859-866.
[13] George, N. et al. (2012). Effect of vitamin D3 in reducing metabolic and oxidative stress in the liver of streptozotocin-induced diabetic rats. Br J Nutr. 2012 Jan 6, 1-9.
[14] Harris RA,Pedersen-White J, Guo DH,Stallmann-Jorgensen IS,Keeton D et al. (2011).Vitamin D3 supplementation for 16 weeks improve flow-mediated dilation in overweight African –American adults. Am J Hypertens.24:557-562.
[15] Higashi M, Shimokawa H, Hattori T, Hiroki J, Mukai Y, Morikawa K, Ichiki T, Takahashi S, Takeshita A.(2003). Long-term inhibition of Rho-kinase suppresses angiotensin II-induced cardiovascular hypertrophy in rats in vivo: effect on endothelial NAD(P)H oxidase system. *Circ Res.*;93:767–775.
[16] Hyppo¨nen E, Boucher BJ, Berry DJ, Power C (2008). 25-Hydroxyvitamin D, IGF-1, and metabolic syndrome at 45 years of age: a cross-sectional study in the 1958 British Birth Cohort. Diabetes 57, 298–305.
[17] Iseki K,Tatsuta M,Uehara H,Iishi H,Yano H et al.:Inhibition of angiogenesis as amechanism for inhibition by 1-alpha-hydroxy vitamin D3 and 1,25-dihydroxy vitamin D3 of colon carcinogens induced by azoxymethane in wistar rats.Int.J.Cancer.81:730-733.
[18] Jablonski KL, Choncbol M, Pierce GL, Walker AE and Seals DR .(1999).25-Hydroxyvitamin D deficiency is associated with inflammation-linked vascular endothelial dysfunction in middle-aged and older adults. Hypertension.2011; 57:63-69.
[19] Jorde R, Sneve M, Torjesen P, Figenschau Y (2009). No improvement in cardiovascular risk factors in overweight and obese subjects after supplementation with vitamin D for 1 year. J Intern Med 267, 462–472.
[20] Kasal DA,Neves MF,Oigman W and Mandarim-de-Lacerdo CA.(2008). Allopurinol attenuates L-NAME induced cardiomyopathy comparable to blockade of angiotensin receptor. Histol Histopatology.23(10):12418.
[21] Kataoka C, Egashira K, Inoue S, Takemoto M, Ni W, Koyanagi M, Kitamoto S, Usui M, Kaibuchi K, Shimokawa H, Takeshita A.(2002). Important role of Rho-kinase in the pathogenesis of cardiovascular inflammation and remodeling induced by long-term blockade of nitric oxide synthesis in rats. *Hypertension.*39:245–250.
[22] Kishi T, Hirooka Y, Masumoto A, Ito K, Kimura Y, Inokuchi K, Tagawa T, Shimokawa H, Takeshita A, Sunagawa K.(2005). Rho-kinase inhibitor improves increased vascular resistance and impaired vasodilation of the forearm in patients with heart failure. *Circulation.*111: 2741–2747.
[23] Kimura Y, Kawamura M, Owada M, Oshima T, Murooka M et al. (1999). Effectiveness of 1, 25-dihydroxyvitamin D supplementation on blood pressure reduction in a pseudohypoparathyroidism patient with high renin activity. Intern Med. 38:31-35.
[24] Kristal-Boneh E, Froom P, Harri G and Ribak J.(1997). Association of calcitrol and blood pressure in normotensive men. Hypertension . 30: 1289-1294.
[25] Kumar KV and Das UN (1993) Are free radicals involved in the pathobiology of human essential hypertension? Free Radic Res Commun 19:59–66.
[26] Lee DM, Rutter MK, O'Neill TW, Boonen S, Vanderschueren D, Bouillon R et al. (2009). European Male Ageing Study Group. Vitamin D, parathyroid hormone and the metabolic syndrome in middle-aged and older European men. Eur J Endocrinol 161, 947–954.
[27] Levy, R., Malech, H.L. (1991). Effect of 1,25-dihydroxyvitamin D3, lipopolysaccharide, or lipoteichoic acid on the expression of NADPH oxidase

components in cultured human monocytes. J Immunol, 147, 3066-3071.
[28] Liao JK ,Seto M , Noma K. (2007) Rho-kinase (ROCK) inhibitors. J Cardiovasc Pharmacol;50:17–24.
[29] Li YC, Kong J, Wei M, Chen ZF, Liu SQ et al.(2002).1,25 dihydroxyvitamin D3 is a negative endocrine indicator of the rennin- angiotensin system . J Clin Invest. 110:229-238.
[30] Meiattini F, Prencipe L, Bardelli F, Giannini G and Tarli P.(1978) The 4-hydroxybenzoate/4-aminophenazone chromogenic system used in the enzymic determination of serum cholesterol. Clin Chem; 24: 2161-2165.
[31] Miranda K, Espey M and Wink D. A.(2001) rapid, simple spectrophotometric method for simultaneous detection of nitrate and nitrite. Nitric oxide. 5(1): 62-71.
[32] Neutel JM and Smith DHG .(1998). Dose response and antihypertensive efficacy of AT1 receptor antagonist telmisartan in patients with mild to moderate hypertension . Ada Ther.15:206-217.
[33] Ni W, Egashira K, Kataoka C, Kitamoto S, Koyanagi M, Inoue S, Takeshita A.(2001). Antiinflammatory and antiarteriosclerotic actions of HMG-CoA reductase inhibitors in a rat model of chronic inhibition of nitric oxide synthesis. Circ Res.89:415–421.
[34] Norman P, Mossb I, Sian M, Gosling M and Powell J . (2002). Maternal and postnatal vitamin D ingestion influences rat aortic structure, function and elastin content. Oxford Journals.; 55(2):369-374.
[35] PARK CW, OH YS, SHIN YS, et al.(1999). Intravenous calcitriol regresses myocardial hypertrophy in hemodialysis patients with secondary hyperparathyroidism. Am J Kidney Dis.;33:73–81.
[36] Plum LA and De luca HF .(2010).Vitamin D ,disease and therapeutic opportunities . Nat Rev Drug Discov. 9(12):941-55.
[37] Pojoga LH, Romero JR, Yao TM, Loutraris PC, Ricchiuti V et al. (2010).Caveolin-1 Ablation reduces the adverse cardiovascular effects of Nω-Nitro-L-arginine methyl ester and Angiotensin II. Endocrinology. 151(3):1236-1246.
[38] Pfeifer M, Begerow B, Minne HW, Nachtigall D and Hansen C.(2001). Effects of a short-term vitamin D3 and calcium supplementation on blood pressure and parathyroid hormone levels in elderly women. J Clin Endocrinol Metab.86:1633-1637.
[39] Rocha WA, Lunz W, Baldo MP, Pimentel EB, Dantas EM et al.(2010).: Kinetics of cardiac and vascular remodeling by spontaneously hypertensive rats after discontinuation of long term captopril treatment . Braz J Med Biol Res.43:390-396.
[40] Romero J. C., Ott. C. E., Aguilo J. J., Torres V. E. &Strong C. G. (1975).Role of prostaglandins in the reversal of one-kidney hypertension in the rabbit. *Circulation Research*..37,683-689.
[41] Sardar, S. et al.(1996).Comparative effectiveness of vitamin D3 and dietary vitamin E on peroxidation of lipids and enzymes of the hepatic antioxidant system in Sprague- Dawley rats. Int J Vitam Nutr Res. 66, 39-45.
[42] Sauzeau V, Rolli-Derkinderen M, Marionneau C, Loirand G, Pacaud P. (2003).RhoA expression is controlled by nitric oxide through cGMP-dependent protein kinase activation. J Biol Chem.;278:9472-80.
[43] Swynghedauw B.(1999). Molecular mechanisms of myocardial remodeling.79 :215-262.
[44] Tietz Textbook of Clinical Chemistry and Molecular Diagnostics, 4th ed. Burtis CA, Ashwood ER, Bruns DE. WB Saunders Co, 2005.
[45] Touyz RM, Schiffrin EL.(2000). Signal transduction mechanisms mediating the physiological and pathophysiological actions of angiotensin II in vascular smooth muscle cells. *Pharmacol Rev*.52:639–672.
[46] Valdivelso JM and Ayus JC.(2008). Role of vitamin D receptor activators on cardiovascular risk. Kidney Int Supp.111:S44-49.
[47] Wang TJ, Pencina MJ, Booth SL, Jacques PF, Ingesson E et al.(2008) Vitamin D deficiency and risk of cardiovascular disease .Circulation . 117:503-511.
[48] Weishaar, kim SN, Saunders D, Simpson RU.(1990). Involvement of vitamin D3 with cardiovascular function III. Effects on physical and morphological properties. Am J Physiol. 258:W134–E142.
[49] Wolf G.(2000).Free radical production and angiotensin. Curr Hypertens Rep. 2:167–173.
[50] Wrigh JW, Mizotani S and Harding JW.(2008) Pathways involved in transition from hypertension to hypertrophy to heart failure .Treatment strategies. Heart Fail Rev. 13:367-375.
[51] Xiang W, Kong I, Chen 5, et al.(2005). Cardiac hypertrophy in vitamin D receptor knockout mice: role of the systemic and cardiac renin- angiotensin systems. Am I Physiol. Endocrinol. Metab.288:E1 25-132.
[52] Yang HY, Cheng CF, Djoko B, Lian WS, Tu CF et al.(2007). Transgenic over expression of the secreted ,extracellular EGF-CUB domain-containing protein SCUBE3 induces cardiac hypertrophy in mice . Cardiovascular Research .75:139-147.
[53] Yoshioka T, Kawada K, Shimada T and Mori M.(1979). Lipid peroxidation in maternal and cord blood and protective mechanism against activated oxygen toxicity in blood. *Am J Obstet Gynecol*. 135: 372-76.
[54] Zhihong Ma , Jianping Zhang , Rongpin Du, Ensheng Ji, Li Chu. (2011). Rho Kinase Inhibition by Fasudil Has Anti-inflammatory Effects in Hypercholesterolemic Rats. Biol Pharm Bull. 34(11):1684-9.
[55] Zhang Z, Sun L, Wang Y, Ning G, Minto AW, Kong J, Quigg RJ, Li YC.(2008). Renoprotective role of the vitamin D receptor in diabetic nephropathy. Kidney Int. 73:163–171.
[56] Zimmerman BJ, Granger DN. Reperfusion injury.(1992). Surg Clin North Am. 72: 65-83.

Author Profile

Omyma Abbas Nassar, Department Of Pharmacology& Toxicology, Faculty of Pharmacy, Tanta University, Egypt.