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Mediating the cardiovascular and renal effects of hyperhomocysteinemia in rats: Role of angiotensin II type I receptor

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Abstract

Hyperhomocysteinemia is usually associated with cardiovascular and renal disorders. Hyperhomocysteinemia is proved to be a cause of cell cytotoxicity, lipid peroxidation, platelet aggregation, increased activation of the coagulation system and stimulation of vascular smooth muscle cell proliferation. Aim: To identify whether the pathological changes on cardiovascular and renal systems induced by hyperhomocysteinemia could be mediated through the activation of angiotensin II type 1 receptors (AT1R) in rats.Methods: Animals were randomized into 3 groups. Each group contained 5 rats. Hyperhomocysteinemia was induced by methionine delivered in drinking water in a concentration of 1.5 g/kg/day per rat based on average water intake for 12 weeks in group II and group III. Valsartan was administered orally in drinking water at a concentration to deliver 30 mg/kg/day per rat in group III. Withdrawal of blood samples for chemical and spectral assay of antioxidant markers was done .Animals were sacrificed, heart, kidney and blood vessels were processed for histopathology .Results: There was a significant improvement in the serum levels of blood urea nitrogen in methionine-valsartan-treated group (GIII) rather than methionine-induction group (GII). In addition, there was marked improvement in methionine-valsartan treated group (GIII) than in methionine-induction group (GII) regarding interstitial edema, focal degeneration of myocytes and congestion. When comparing vacuolar degeneration of the medial layer, focal endothelial injury and wall thickness, we found marked improvement in methionine-valsartan-treated group (GIII) than in methionine-induction group (GII).Conclusion: Blocking Ang II AT1- receptors by valsartan reduces cardiovascular and renal changes in rats with hyperhomocysteinemia.

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INTRODUCTION

Homocysteine is a homologue of the amino acid cysteine. It can be converted into cysteine or recycled into methionine with the aid of certain B-vitamins(1). Hyperhomocysteinemia is an independent risk factor for cardiovascular diseases such as arteriosclerosis, coronary arteries and cerebrovascular disorders (2,3,4,5).

Hyperhomocystienemia is a risk factor for vascular disease mostly in patients with chronic renal insufficiency and end-stage renal disease. In end-stage renal disease, median levels of homocysteine are markedly elevated which is a risk factor for vascular disease in both normal individuals and those with renal disease (6).

Hyperhomocystienemia activates the renin-angiotensin system (RAS) which plays an important role in elevation of arterial blood pressure. Angiotensin II type-1 receptor (AT1R) activation is also involved in vascular and cardiac hypertrophy, cardiac remodeling, endothelial dysfunction and processes leading to atherothrombosis (7,8,9). Therefore, valsartan,which is a selective AT1- receptor antagonist, has been used to prevent vascular and cardiac hypertrophy in models of hyperhomocyctienemia (10).

Animal studies examining the association between hyperhomocysteinemia and the RAS activation in the pathogenesis of ventricular hypertrophy suggest that vasomotor responsiveness of isolated vascular smooth muscle cells or carotid rings to angiotensin II is enhanced in the presence of high serum levels of homocystiene (11). In addition, Ang II promotes the stiffness and collagen deposition of small arteries in mice with mild hyperhomocysteinemia (12).

So, the aim of this work was to determine the Pathophysiological changes on cardiovascular and renal systems induced by hyperhomocysteinemia through studying functional, biochemical and histopathological cardiac and renal changes. Also, clarifying the role of angiotensin II type 1 receptors (AT1R) inmediating these changes .

Materials:

Animals:

All experimental protocols were approved by the Research Ethics Committee at the Faculty of Medicine, Suez Canal University. All efforts were made to minimize animal suffering and to reduce the number of animals used. Sixty adult male Albino rats weighing average of 200-250gm, 8 month old, were used in this study. Animals were purchased from the Ophthalmology Research Institute in Giza. Animals were housed in the Physiology department, Faculty of Medicine, Suez Canal University in spacious plastic cages at controlled room temperature and were kept with free access to standard rat chow diet and tap water. The rats were left for acclimatization for one week before the start of the study.

Study groups: Rats were randomly divided into 3 groups each containing 20 rats.Group (I) Normal group; received normal saline for 12 weeks.Group (II): Methionine group; concentration of methionine in water was adjusted to deliver 1.5 g/kg/day per rat based on average water intake for 12 weeks (13).Group (III): Methionine and valsartan group;concentration of methionine in

water was adjusted to deliver 1.5 g/kg/day per rat based on average water intake for 12 weeks.Valsartanwas administered orally in drinking water at a concentration to deliver 30 mg/kg/day per rat (14).

Methods

1-Spectrophotometric assay of antioxidant markers:at the end of the experiment, superoxide dismutase (SOD), lipid peroxide, Malondialdehyde (MDA), reduced glutathionewere measured in serum, heart, kidney and blood vessels

2- Kidney function test: serum creatinine and blood urea nitrogen levels were measured at the end of the experiment.

3-Assessment of cardiac parameters:

Arterial blood pressure and heart rate were measured in rats using BIOPAC system weekly.

4-Histopathological assessment:

Rats were sacrificed under general anesthesia,Right kidney, heart, aorta, left common carotid artery and the normal arteries were collected, fixed in 10% neutral buffered formalin and embedded in paraffin. Each paraffin block was cut into 5 μm sections and stained with Hematoxylin and Eosin (H&E).

- Kidney histopathology: Evaluation of renal glomerular and tubular damage was scored according to the method of *Uehara et al. (1994).*The scoring system for the degree of renal tubular damage was as follows: 0, no lesion, 1 + very mild focal dilatation, 2+ large number of dilated tubules with widening of the interstitium, 3+, fairly extensive dilatation of tubules with cystic formation and/or protein cast and widening of the interstitium, and 4+, complete atrophy of the tubules. Each animal was given a score (0 to 4+). Individual scores were then averaged for each group as previously described *Uehera et al. (1994).* Scoring system for evaluation of glomerular damage was from 0 to 4+ as follows:0 no sclerosis; (1+) 1-25% mesangial expansion and sclerosing glomerulus; (2+) 25-50% mesangial expansion and sclerosing glomerulus; (3+) 50-75% mesangial expansion and sclerosing glomerulus; (4+) 75-100% mesangial expansion and sclerosing glomerulus.

- Heart and blood vessels histopathology:

Interstitial edema, focal degeneration and congestion of cardiac myocytes were inspected by blind pathologist for histopathological changes. The luminal, neointimal, and adventitial areas were also assessed.

4- Quantitative assessment of collagen type 1 and AT1R by ELISA

Freshly isolated blood vessels, heart and kidney were homogenized in 1 mL sterile phosphate buffered saline (PBS; Sigma-Aldrich Ltd, cat. No. P7059) pH 7.4.(15).Resultant supernatants of the blood vessels, heart and kidneys were used for quantitative assessment of collagen type 1 and AT1R in rats using ELISA kits.(16).

Statistical analysis:

One-way analysis of variance test was used to test the differences between the study groups followed byBonferroni test as a post-hoc.$P < 0.05$ was considered significant.

Results:

1-Arterial blood pressure and heart rate in conscious rats: Systolic and diastolic blood pressure means were significantly higher in methionine group when compared either to control or valsartan group. Valsartan group blood pressure values showed no difference when compared to normal values. Heart rate is significantly higher in GII when compared with GI and GIII.(Shown in table 1).

2-Blood Chemistry: After 12 weeks of methionine induction,BUN level showed a significant increase when compared to normal group. Valsartan treated group showed significant improvement when compared to GII and GI.(Shown in table 2).

3-Spectrophotometric assay of oxidative stress markers in serum:

MDA levels showed significant increase in methionine group while SOD and reduced glutathione means showed significant increase in valsartan treated group (shown in table 3).

4-Spectrophotometric assay of oxidative stress markers in tissues:

-Kidney: Only MDA means showed significant increase in methionine group when compared to normal and valsartan groups. (Shown in table 3)

-Heart: heart MDA values weresignificantly higher in GII than in GI and GIII while means of reduced glutathione weresignificantly lower in GII than in GI and GIII.(Shown in table 3)

- blood vessels: MDAvalueswere significantly higher in GII than in GI and GIII. The blood vessels reduced glutathione weresignificantly higher in GIII than in GI and GII. (Shown in table 3)

4-Quantitative assessment of collagen type 1 in kidney, heart and blood vessels:

Collagen type 1 in kidney, heart and blood vessels showed a significant increase after 12 weeks of methionine induction. Valsartan treatment group showed significant decrease in collagen type I means when compared to methionine group.(Shown in fig. 1).

Table (1): Blood pressure and heart rate.

Groups	Systolic blood pressure (mm/Hg)	Diastolic blood pressure (mm/Hg)	Heart rate (beats/ minute)
GI	119±1.45	75.6±1.24	**168±2.51**
GII	198±4.53*	126±3.09*	**367±13.30***
GIII	116±2.41	84.8±2.30	**190±16.40**
P value	**0.000***	**0.000***	**0.000***

*There is homogeneity of the experimental groups. Results are expressed as mean± SEM and analyzed using one way ANOVA followed by post-hoc Bonferroni multiple comparison test. * $P \leq 0.05$ compared to normal group. $^{\Delta}P \leq 0.05$ compared to valsartan- treated group.*

Table (2) : Kidney function tests.

Groups	Serum creatinine (U/ml)	Blood urea nitrogen (U/ml)
GI	3.32 ± .048	**29.2 ±1.42**
GII	3.46± .037	**103.3 ±13.09***
GIII	3.36± .029	**33.20 ±2.15**
P value	**0.076**	**0.000***

*There is homogeneity of the experimental groups. Results are expressed as mean± SEM and analyzed using one way ANOVA followed by post-hoc Bonferroni multiple comparison test. * $P \leq 0.05$ compared to normal group. $^{\Delta}P \leq 0.05$ compared to valsartan- treated group*

Table (3) : Spectrophotometric assay of oxidative stress markers in serum and tissues.

Oxidative stress markers	SOD				MDA				Reduced glutathione			
	Serum (nmol/ml)	Kidney (nmol/g)	Heart (nmol/g)	Vessels (nmol/g)	Serum nmol/ml	Kidney nmol/g	Heart nmol/g	vessels nmol/g	Serum (nmol/ml)	kidney (nmol/g)	Heart (nmol/g)	vessels (nmol/g)
GI	.804 ±.032	.946 ±.350	.853 ±.049	.821 ±.046	.134 ±.052	.124 ±.008	.118 ±.018	.118 ±.008	.018 ±.006	.060 ±.116	.047 ±.006	.174 ±.016
GII	.836 ±.039	.810 ±.250	.840 ±.041	.810 ±.043	.296 ±.144*	.389± .113*	.350± .140*	.165± .019*	.008±.004	.039± .014	.007 ±.003*	.155± .033
GIII	.956±.153 $^{\pi}$	.928± .242	.861 ±.037	.831 ±.035	.189 ±.064	.126 ±.007	.124 ±.017	.124 ±.010	.033 ±.017$^{\pi}$	.071± .007	.085± .025	.330 ±.093$^{\pi}$
P value	0.001*	0.314	0.727	0.159	0.001*	0.000*	0.000*	0.000*	0.000*	0.152	0.000*	0.000 *

*There is homogeneity of the experimental groups. Results are expressed as mean± SEM and analyzed using one way ANOVA followed by post-hoc Bonferroni multiple comparison test. * $P \leq 0.05$ compared to normal group.$^{\pi}P \leq 0.05$ compared to methionine group.*

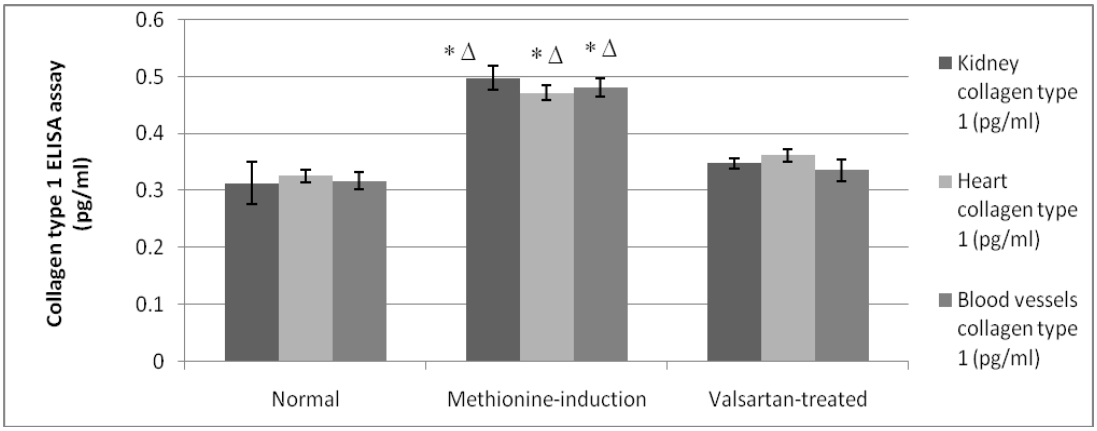

Fig. (1): *Collagen type 1 (in kidney, heart and blood vessels). There is homogeneity of the experimental groups. Results are expressed as mean± SEM and analyzed using one way ANOVA followed by post-hoc Bonferroni multiple comparison test. * $P \leq 0.05$ compared to normal group. $^{\Delta}P \leq 0.05$ compared to valsartan-treated group.*

5-Quantitative assessment of AT1R in kidney, heart and blood vessel

AT1R in kidney and blood vessels showed a significant decrease after 12 weeks of methionine induction. Significant high levels of AT1R in Valsartan group when compared to methionine group. (shown in fig.2).

6-Histopathology

Kidney tubular, glomerular and congestion score showed significant difference among the three groups.GIII (treated with valsartan) has significant amelioration and preservation of tubular and glomerular architecture when compared to GII (methionine group). Congestion score showed significant decrease inGIII when compared to GII. (Table 4-A) (Figure 3A-C).

Heart interstitial edema, focal degeneration of myocytes and congestion showed significant amelioration in GIII when compared to GII. GII cellular changes score showed significant increase when compared to GI scores of normal architecture.(Table 4-B) (Figure 4A-C)

Only focal endothelial injury scores have significant increase in GII. Vacuolar degeneration of media layer and wall thickness score showed no significant changes. (Table 4-C) (Figure 5A-C).

Table4: *comparison between all groups according to morphological change inkidney(4- A),heart 4-B), blood vessels(4-c).There is homogeneity of the groups.*

A

Kidney morphological changes		**GI**	**GII**	**GIII**	**P value**
Tubular score	Preserved architecture, no changes	100%	0%	25%	0.043*
	Preserved architecture, mild focal dilatation	0%	0%	75%	
	large number of dilated tubules with widening of the interstitium	0%	85%	0%	
	extensive dilatation of tubules with cystic formation and/or protein cast and widening of the interstitium	0%	15%	0 %	
	complete atrophy of the tubules	0%	0%	0 %	
Glomerular score	No mesangial expansion and no sclerosing glomerulus	100%	0%	50%	0.025*
	1-25% mesangial expansion and sclerosing glomerulus	0%	0%	50%	
	25-50% mesangial expansion and sclerosing glomerulus	0%	30%	0%	
	75% mesangial expansion and sclerosing glomerulus	0%	70%	0%	
Congestion	No Congestion	100%	0%	80%	0.055
	Mild Congestion	0%	0%	20%	
	Moderate Congestion	0%	28%	0%	
	Sever Congestion	0%	72%	0%	

B

Heart morphological changes		**GI**	**GII**	**GIII**	**P value**
interstitial edema	Preserved architecture, no changes	100%	0%	50%	0.072
	mild interstitial edema	0%	25%	30%	
	Moderate interstitial edema	0%	25%	20%	
	Marked interstitial edema	0%	50%	0 %	
focal degeneration of myocytes	No focal degeneration of myocytes	100%	0%	70%	0.001*
	Mild focal degeneration of myocytes	0%	50%	30%	
	Moderate focal degeneration of myocytes	0%	50%	0%	
	Sever focal degeneration of myocytes	0%	0%	0%	
Congestion	No Congestion	100%	0%	100%	0.210
	Mild Congestion	0%	75%	0%	
	Moderate Congestion	0%	25%	0%	
	Sever Congestion	0%	0%	0%	

C

Morphological changes		GI	GII	GIII	P value
Vacuolar degeneration of medial layer	Preserved architecture, no changes	100%	0%	75%	0.072
	mild vacuolar degeneration of medial layer	0%	25%	20%	
	Moderate vacuolar degeneration of medial layer	0%	25%	5%	
	Marked vacuolar degeneration of medial layer	0%	50%	0 %	
Focal endothelial injury	No focal endothelial injury	100%	0%	70%	0.001*
	Mild focal endothelial injury	0%	0%	30%	
	Moderate focal endothelial injury	0%	50%	0%	
	Sever focal endothelial injury	0%	50%	0%	
Wall thickness	average wall thickness	100%	0%	100%	0.210
	Mild increase in wall thickness	0%	25%	0%	
	Moderate increase in wall thickness	0%	75%	0%	
	Marked increase in wall thickness	0%	0%	0%	

Results are analyzed using one way ANOVA followed by post-hoc Bonferroni multiple comparison test.

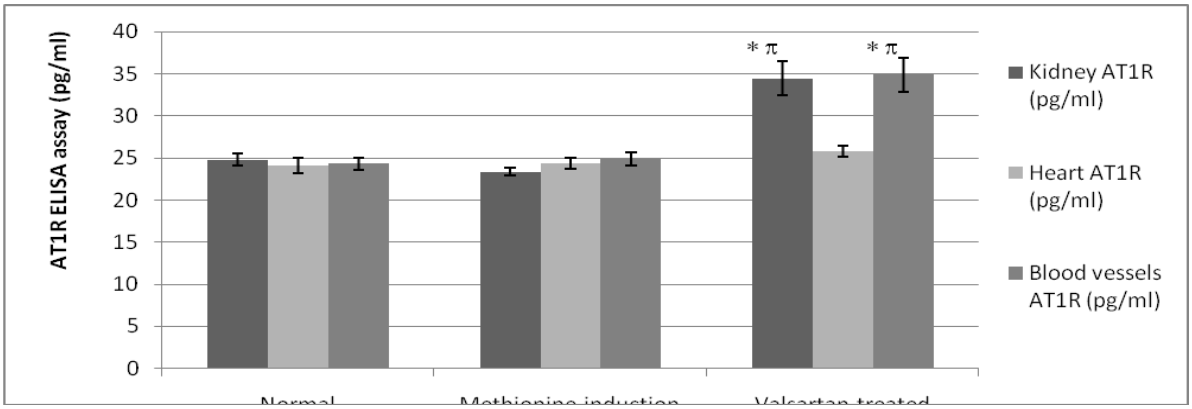

Fig. (2):*AT1R (in kidney, heart and blood vessels). There is homogeneity of the experimental groups. Results are expressed as mean± SEM and analyzed using one way ANOVA followed by post-hoc Bonferroni multiple comparison test. *P ≤ 0.05 compared to normal group. πP ≤ 0.05 compared to methionine- induction group.*

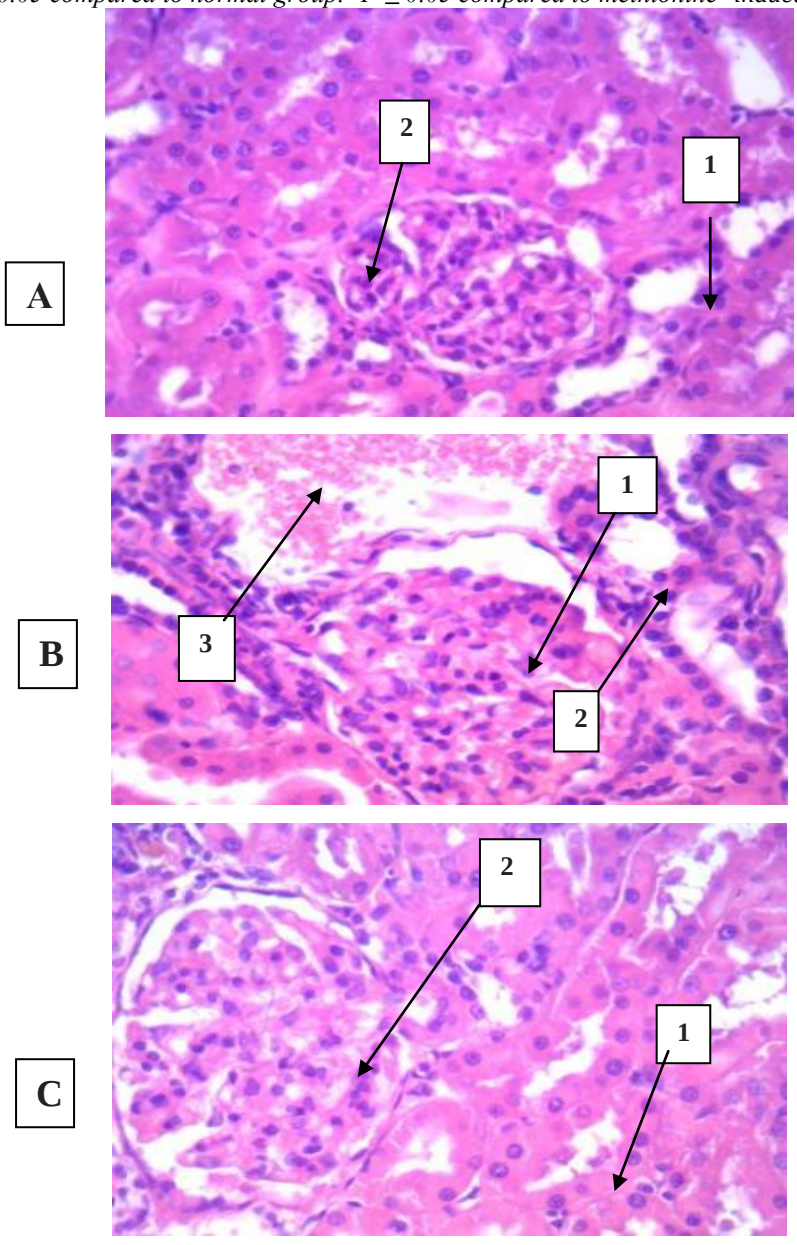

Fig. (3):*(A)Section from kidney tissue of GI showing normal kidney tissue; preserved architecture, No tubular changes (2) , No mesangial expansion and no sclerosing glomerulus (1) and no congestion (H&E X400). (B) Section from kidney tissue of GII showing large number of dilated tubules with widening of the*

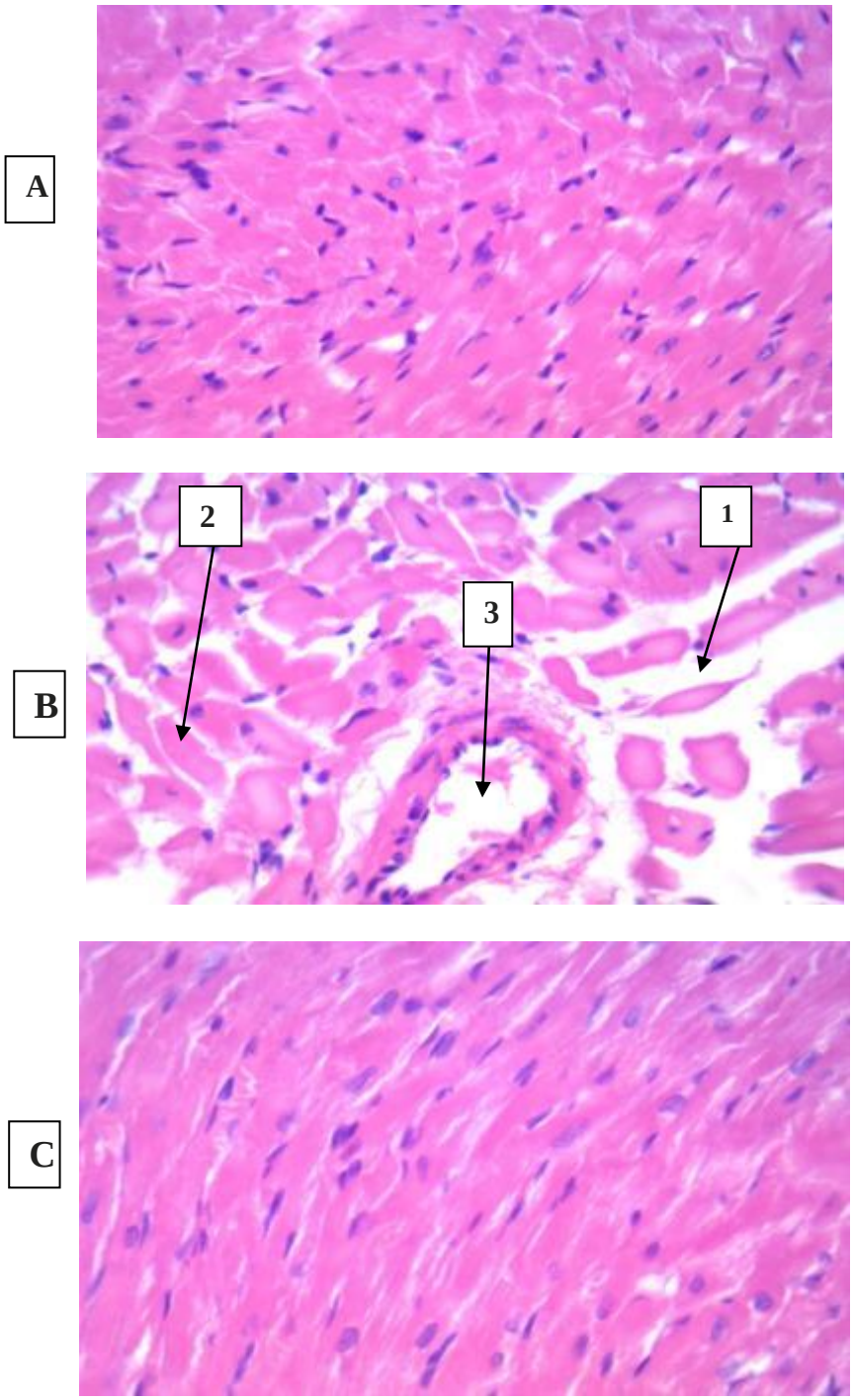

Fig. (4):*(A): Section from heart tissue of GI showing normal heart tissue; preserved architecture, No interstitial edema, no focal degeneration of myocytes and no congestion. (B): Section from heart tissue of GII showing marked interstitial edema (1), moderate focal degeneration of myocytes (2) and mild congestion (3). (C): Section from heart tissue of GI showing normal heart tissue; preserved architecture, No interstitial edema, no focal degeneration of myocytes and no congestion.*

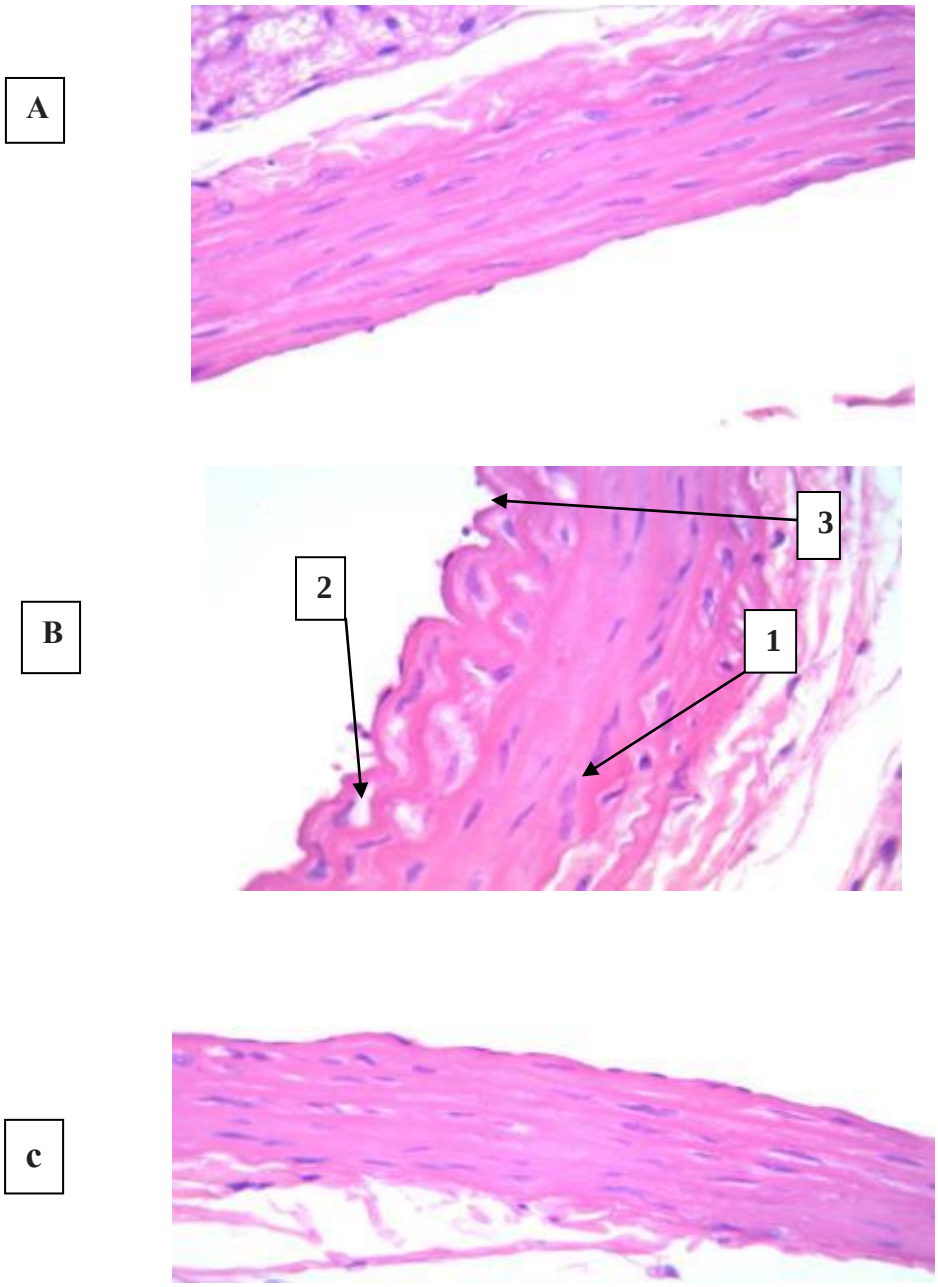

Fig.(5):*(A): Section from blood vessels tissue of GI showing normal tissue; preserved architecture, no vacuolar degeneration of medial layer, no focal endothelial injury and average wall thickness.(B): Section from blood vessels tissue of GII showing marked vacuolar degeneration of medial layer (1), moderate focal endothelial injury (2) and moderate increase in wall thickness (3). (C): Section from blood vessels tissue of GIII showing normal tissue; preserved architecture, no vacuolar degeneration of medial layer, No focal endothelial injury and average wall thickness.*

Discussion

In this study, serum homocysteine level was increased in both methionine- induced group (GII) and methionine-valsartan-treated group (GIII) after12 weeks of methionine induction as well as valsartan treated group(GIII). Methionine induction for 12 weeks was associated with a significant increase in arterial pressure, ECG changes, increased collagen type 1 deposition and oxidative stress response in methionine- induction non- treated rats (GII).

On the other hand, all these pathological changes were significantly attenuated in methionine-valsartan treated rats (GIII). We use this model of hyperhomocysteinemia because there is a significant correlation between hyperhomocysteinemia and cardiovascular disease and its complications as heart attacks and strokes *(17).*Our results are similar toprevious studies*(18,19,20, 14* and *21)* which reported that activation of angiotensin system is one of the mechanisms involved in homocysteine-mediated hypertension. However, other reported mechanisms indicated that homocysteine can promote hypertension by both nitrative and oxidative stress*(14).*Previous studies demonstrated thathomocysteine plays a role in the pathogenesis of essential hypertension mediated by arteriolar constriction, renal dysfunction and increased sodium reabsorption, and increases arterial stiffness*(22).*

We also found that heart rate decreasedin methionine-valsartan treated group (GIII) more than in methionine-induction group (GII). This result is concordant with other studies reporting that elevated plasma homocysteine level was associated with an increase in heart rate due to increased arterial stiffness which leads to hypertension and increased heart rate*(23).*

Elevation of serum SOD level in methionine-valsartan-treated group (GIII) means that there is a powerful anti-inflammatory activity done by SOD in this group due to treatment with valsartan. This effect decreases the oxidative stress done by homocysteineby blocking angiotensin II receptors on which homocysteineact and causes its pathology(*24*). Our results are comparable to a study which reported that SOD is a highly effective experimental treatment of chronic inflammation and treatment with SOD decreases reactive oxygen species generation, oxidative stress , inhibits endothelial cell activation, reduces free radical damage and fibrosis*(25).*

We also measured serum malondialdehyde (MDA) as another oxidative stress marker which was significantly higher in methionine-induction group (GII) than in methionine- valsartan-treated group (GIII) and normal group (GI). Measurement of malondialdehyde is widely used as an indicator of lipid peroxidation that has been associated with a wide variety of chronic diseases in both humans and animal models *(26).*

Decreased MDA serum level in methionine-valsartan treated group (GIII) may be explained that valsartan is a selective angiotensin II type 1 receptor antagonism thus decreasing the action of homocysteine on these receptors leading to increasing its oxidative stress action. This is consistent with another study which demonstrated a positive correlation between elevated plasma homocysteine level and lipid peroxidation mediated by MDA *(27).*

By measuring reduced serum glutathione as another oxidative stress marker, we found that it was significantly higher in methionine- valsartan-treated group (GIII) than in methionine-induction group (GII) and normal group (GI). It was found also by another study that the elevated level of homocysteine results in a decrease in the activity of cellular glutathione reduced which is an intracellular antioxidant enzyme that reduces hydrogen peroxide and lipid peroxides*(28).*

On the other hand, MDA was significantly higher in methionine-induction group (GII) than in methionine-valsartan-treated group (GIII) and normal group (GI) in all tissues (kidney, heart and blood vessels). It was explained previously by another studythat there is a positive correlation between elevated plasma homocysteine level and lipid peroxidation mediated by MDA, which may suggest the role of homocysteine in the release of reactive oxygen species and mediation of oxidative stress*(29).*

In current study regarding quantitative assessment of collagen type 1 deposition in cardiovascular and renal systems we found that the collagen type 1 deposition in heart, kidney and blood vessels were significantly higher in methionine-induction group (GII) rather than in methionine-valsartan-treated group(GIII). Our results agreed with another studystating that homocysteinehas growth promoting and collagen production-stimulating effects on vascular smooth musclecausing endothelial and structural vascular *(30).*

Marson et al. (2015) suggested that activation of Ang II, through AT1-receptors, appears to play a role in collagen deposition in cardiovascular system in hyperhomocysteinemic rats*(31).* However, an alternative explanation reported by *Undas et al. (2017)*who stated that the blunted blood pressure elevation observed in methionine-valsartan-treated group (GIII) is responsible for the attenuation of collagen type 1 deposition*(32).*

Regarding quantitative assessment of AT1R expression in cardiovascular and renal systems we found that the AT1R expression in kidney and blood vessels was significantly higher in valsartan-treated group (GIII) than in methionine –induction group (GII). *Jardine et al. (2012)* data reported that angiotensin II type 1 receptor (AT1R) blockade was associated with AngII type 1 receptor (AT1R) up-regulation and activation*(33).*

In the current study there was no significant difference in serum creatinine level between study groups. It is found that there was a significant improvement in the serum levels of blood urea nitrogen in methionine-valsartan-treated group (GIII) rather than methionine-induction group (GII). *Vyssoulis et al. (2011)*reported that there is an independent relationship between homocysteine and elevated BUN. Since BUN is excreted by glomerular filtration, which interpret the relationship between hypertension and BUN*(34).Kolluru et al. (2013) andCheug et al. (2015)*reported that hyperhomocyctienemia is strongly associated with elevation of serum BUN in methionine-treated rats (GII)*(35,36).*

*Boset al. (2013),*used *Uehera*scoring system for assessment of renal tissue damage. The histopathological results showed that renal tissue recovery from damage was much better in GIII, which was treated with valsartan*(37).*

Histopathological assessment of the extent of myocardial injury found marked improvement in methionine-valsartan-treated group (GIII) than in methionine-induction group (GII) regarding interstitial edema, focal degeneration of myocytes and congestion. Our results are Consistentwith the results of*Joseph et al. (2013), Cheng et al. (2016),* who reported that AT1-receptor antagonism significantly reduced the myocardial injury, congestion and collagen content in hyperhomocysteinemic rats*(38,39).*

Conclusion: In conclusion, the present study demonstrates that hyperhomocysteinemia causes hypertension, ECG changes, renal and cardiovascular changes. All these changes were significantly attenuated by blocking Angiotensin II AT1- receptors.

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