Cardio-protective effect of cilostazole in a rat model of cardiorenal syndrome type 4

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Abstract

The Chronic kidney disease (CKD) is a major health problem . Cardiorenal syndrome (chronic renocardiac syndrome) (CRS) type 4 is gaining a major health concern causing significant morbidity and mortality, putting major burdens on the healthcare system. This study was designed to explore the possible protective effect of cilostazol (phosphodiesterase III inhibitor) versus valsartan (angiotensin-II receptor blocker) against CRS type 4 induced by subtotal 5/6 nephrectomy in rats and to explore the underlying mechanisms. Just after surgery, cilostazole (50 and 100mg/kg/day) and valsartan (30mg/kg/day) were given daily by gavage for 10 weeks. Cilostazole treatment significantly improved kidney functions, decreased cardiac malondialdehyde (MDA), nuclear factor kappa B (NFκB), transforming growth factor beta (TGF-β1), heart weights, heart weight/body weight phosphorylated protein kinase A (p-AKT) , phosphorylated glycogen synthase kinase 3beta (p-GSK3β) levels and serum brain natriuretic peptide (BNP) in 5/6 nephrectomized rats, in addition to significant increase in cardiac levels of superoxide dismutase (SOD), cardiac expressions of peroxisome proliferation-activated receptor gamma (PPARϒ) and endothelial nitric oxide synthase (eNOS) compared to diseased group. Better results were obtained with cilostazole (100 mg/kg/day) pretreated group that were insignificantly different from valsartan pretreated group. Cilostazole has cardioprotective effects in a rat model of CRS type 4. These protective effects are mediated through exerting antiinflammaory, antioxidant , antifibrotic actions , increasing cardiac PPARϒ and eNOS expression as well as abating cardiac hypertrophic signaling pathway .

Keywords: Cilostazole, Valsartan, Cardiorenal syndrome, Chronic kidney disease , Nephrectomy

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Introduction:

The heart and kidneys are tightly interlinked with each other. So, primary disorder of one of these organs often results in secondary dysfunction of the other **(Suresh et al., 2017)** .Also, acute or

chronic impairment of the heart or the kidneys can cause acute or chronic impairment in the other organ **(Ronco et al. 2018).** Such interactions play a vital role in the pathogenesis of the clinical entity called cardio-renal syndrome (CRS). Currently, the most accepted classification is the one proposed by Consensus Conference of the Acute Dialysis Quality Initiative, which distinguishes between 5 distinct types of CRS based on the presumed initial trigger and acute or chronic systemic illness. CRS type 4 (also known as chronic renocardiac syndrome) is a subtype in which primary CKD, such as diabetic nephropathy, chronic glomerular disease or autosomal dominant polycystic kidney disease, promotes the progression of chronic heart failure with is characterized by left ventricular hypertrophy (LVH) and diastolic dysfunction interstitial fibrosis, capillary rarefaction , systolic dysfunction and later heart failure **(Rangaswami et al .,2019 Suresh et al., 2017).**Major cardiac events actually represent almost 50 % of the causes of death in CKD patients **(Sárközy et al.2018).**

The exact pathophysiology of type 4 CRS is not completely understood. The mechanisms that underlie CRS are probably multifactorial and may include neurohormonal activation and hemodynamic alterations, inflammation, oxidative stress, endothelial dysfunction, endoplasmic reticulum (ER) stress and renin angiotensin-aldosterone system (RAAS) and/or sympathetic system activation (**Kingma et al., 2015**).

One effective and well-studied approach in preventing chronic renal injury is blockade of RAAS. The salutary effects of RAAS blockade in slowing progression of CKD are not only caused by its antihypertensive and hemodynamic properties, but also by its ability to reduce oxidative stress, inflammation, and fibrosis **(Siragy and Carey,2010).** Therefore, RAAS blockade , including angiotensin-II receptor blockers (ARBs), are advocated and superior to other medication in patient with CKD based on their renoprotective properties as well as cardioprotective potential **(Sayer and Bhat ,2014).** Valsartan , an ARB, has non-hemodynamic cardiovascular and renal protective effects via the blockade of AT1R **(Suematsu et .,2018).**

Despite the broad availability of standard heart failure medications such as ARBs, cardiovascular morbidity and mortality among CKD patients remained high **(Herzog et al., ,2011)**. Therefore, using novel agents that ameliorate or prevent the progression of CRS type 4 is urgently needed.

Cilostazole a selective phosphodiesterase III inhibitor, has potent vasodilating and antiplatelet effects **(Jung et al., 2010).** The drug is approved for treatment of intermittent claudication in patients with peripheral vascular diseases also in coronary artery diseases **(Chen et al., 2016).** Cilostazol inhibits PDE III leading to decrease degradation of intracellular 3′-5′-cyclic adenosine monophosphate (cAMP), and 3′-5′-cyclic guanosine monophosphate (cGMP) (**Sholokh and Klussmann, 2021).**

Recently, studies have reported that cilostazole possess pharmacological potentials , such as anti-inflammatory (**da Motta and de Brito 2016**), and antioxidant **(Chen et al. 2016) ,** antifibrotic (**Han et al. 2019)** effects. The aim of the current study was to evaluate the possible

cardioprotective effects of cilostazol against CRS type 4 in rats and to evaluate the underlying mechanisms .

2. Materials and Methods

Experimental animals

Fifty six male Wistar rats weighting 200 to 250 grams were purchased from the Faculty of Veterinary Medicine, Zagazig University, Egypt. Rats were distributed three/cage and kept for one week as acclimatization period. Rats had free access to diet and water. Temperature, humidity and light/dark cycles were kept constant at the following values (23±2°C, 60±10% and 12/12 h respectively). The experimental design and animal handling performed in this study were in compliance with the National Institutes of Health for care and use of laboratory animals (NIH publications) "NO.ZU-IACUC/3/F/2/2020, revised 1996" and were approved by Zagazig University's Institutional Animal Care and Use Committee (ZU)

Drugs and chemical

Cilostazole was purchased from Sigma-Aldrich Chemical Company (St. Louis, MO, USA),valsartan was purchased from Novartis, Switzerland. All other chemicals utilized were of analytical grade obtained from Sigma-Aldrich.

Induction of renal failure by subtotal 5/6 nepherctomy of total renal mass

The rats left kidneys were exposed through a lateral dorsal incision and decapsulated after anesthetization with sodium pentobarbital (100 mg/kg, ip). Then, renal vessels were clamped and both poles (2/3 of the functional kidney mass) were dissected 2 weeks later, total right nephrectomy through lateral dorsal incision was done to achieve 5/6 reduction. Renal failure was significant after 10 days of surgery while heart failure and cardiac remodeling was significant after 10 weeks of surgery .Sham operated rats were anesthetized with just decapsulation of the intact kidneys bilaterally **(Švíglerová et al., 2010).**

Experimental Design:

Rats were randomly divided into seven groups; each group having eight rats as follows:

1.Control normal group

2. Sham group

3. Diseased group : (5/6 nephrectomy) **(Švíglerová et al., 2010)**

4. Diseased +vehicle group: 5/6 nephrectomized rats received carboxymethylcellulose at 0.1% (2.5 ml/kg) once daily by gavage, for 10 weeks, immediately after surgery.

5.Cilostazole 50mg group: 5/6 nephrectomized rats received cilostazole 50 mg/kg;dissolved 0.1% carboxymethylcellulose to enhance the solubility of cilostazole once daily by gavage, for 10 weeks, immediately after surgery**(El Awdanet al.,2019).**

6. Cilostazole 100mg group: 5/6 nephrectomized rats received cilostazole (100 mg/kg ;dissolved in 0.1% carboxymethylcellulose once daily by gavage, for 10 weeks, immediately after surgery**(El Awdanet al.,2019).**

7.Valsartan group :5/6 nephrectomized rats received valsartan (30 mg/kg once daily by gavage, for 10 weeks, immediately after surgery **(Suematsu et al., 2018).**

At the end of the experimental period, all animals were anesthetized with urethane (1.3 g/kg, ip). Body weights and heart weights (measured by digital weight scale) to assess heart /body weight ratio by the following equation [heart weight body weight ratio = heart wt. / body wt. × 100]. Blood samples were obtained from the orbital sinus of fasted rats. Blood samples were centrifuged at 3000 rpm for 10 minutes to get clear sera and the obtained serum was stored rapidly at - 20°c for later bioassay of serum BNP level, serum urea and creatinine. Serum urea and creatinine were also measured 10 days after the 5/6 subtotal nephrectomy where blood were obtained from rats tail vein. Hearts was divided into two parts one part was maintained in formalin 10% for histopathological examination, while the other part was frozen using liquid nitrogen and stored at -80°C until homogenised for biochemical evaluation of cardiac inflammatory mediator nuclear factor kappa B (NFκB), mediator of oxidative stress malondialdehyde (MDA) , antioxidant super oxide dismutase (SOD), fibrotic mediator transforming growth factor beta (TGF-β1) ,hypertrophy pathway phosphorylated protine kinase A (AKT) and phosphorylated glycogen synthase kinase 3beta (GSK3β),cardiac expressions of endothelial nitric oxide synthase (eNOS) and peroxisome proliferation-activated receptor gamma (PPARɤ) .

Measurement of blood pressure:

Blood pressure was monitored using a tail cuff blood pressure measuring system (Harvard Apparatus Ltd, Edenbridge, Kent, England).Systolic blood pressure (SBP) and diastolic blood pressure (DBP) were measured 10 days and 10 weeks post nephrectomy

Colorimetric assays for serum creatinine , serum urea ,cardiac SOD and MDA

Serum creatinine and serum urea were measured using commercially available quantitative colorimetric assay kits obtained from Bio-diagnostic Co. (Egypt). All procedures were performed according to the manufacturer's instructions. SOD and MDA colorimetric assay Kits (Bio-diagnostic company, Egypt) were used for measurement of SOD and MDA cardiac levels.

Enzyme linked immunosorbent assays (ELISA) for cardiac NFκB, TGF-β1, p- AKT , p-GSK3β and serum BNP

Cardiac level of NFκB (Bio-diagnostic company, Egypt), cardiac TGF-β1(Boster Biological Technology, Pleasanton CA, USA), cardiac phosphorylated AKT (Sandwich Elisa Kit, San Diego, USA) cardiac phosphorylated glycogen synthase kinase 3β (GSK3β) (Sandwich Elisa Kit, San Diego, USA) and serum level of BNP (Peninsula Laboratories, Bachem Group, USA) were measured using rat ELISA kits. All procedures were performed according to the manufacturer's instructions.

Quantitative real time PCR for eNOS and PPARɤ

Cardiac expressions of eNOS and PPARɤ were assessed by quantitative real time PCR in which total RNA were extracted from heart tissue using using quantitative real-time PCR according to the manufacturer's instructions using the Qiagen tissue extraction kit (Qiagen, USA) and were reverse transcribed into cDNA using a high-capacity cDNA reverse transcription kit (Fermentas, USA). Then, using Applied Biosystems with Step One TM software version 3.1 (USA), amplification and analysis of real-time qPCR product were conducted. The primer sequence of the gene under study include:

eNOS: **forward primer** 5′-CGA GAT ATC TTC AGT CCC AAG C-3′ , **reverse primer** 5′-GTG GAT TTG CTG CTC TCT AGG-3′ .

PPARɤ: **forward primer** 5'-TGATATCGACCAGCTGAACC-3'

reverse primer 5'-GTCCTCCAGCTGTTCGCCA-3'.

Histological analysis

Hearts were harvested, fixed in 10% paraformaldehyde, embedded in paraffin, and cut into 5-μm sections. Sections were stained with Masson's trichrome staining **(Polysciences , Inc.,USA).**

Statistical analysis

Data were expressed as mean ±standard error of the mean (SEM). Ordinary one-way analysis of variance (ANOVA) was used to detect statistical differences among groups followed by Post-Hoc (least significant difference "LSD") tests as designated by Armitage & Berry (1994). Differences were considered significant at a $P<0.05$. Data analysis was conducted using SPSS, Version 26 Software .

3.Results

Because there were no significant differences between the results coming from control normal group and sham group also those coming from diseased group and diseased group

receiving carboxymethylcellulose (cilostazole vehicle); only results obtained from sham group and diseased group were included in our study.

Effect of cilostazole (50 and 100mg/kg/day) on kidney functions at different time points

Baseline mean serum urea and creatinine levels were not significantly (P>0.05) different among all groups . Ten days after surgery, serum urea and creatinine levels were significantly (P< 0.05) higher in all groups compared to sham group .Ten weeks after surgery, serum urea and creatinine levels were significantly (P< 0.05) increased in the diseased group compared to sham group. Cilostazole treatment caused significant (P< 0.05) decrease in mean serum urea and creatinine levels in its two doses compared to diseased group . Better results were obtained with cilostazole (100 mg/kg/day) pretreated group, that was insignificantly different from valsartan (30 mg/kg/day) pretreated group as a standard therapy as presented in table 1

Table 1: Effect of cilostazole (50 and 100mg/kg/day) on kidney functions at different time points

Interval	Groups (n=8) / parameter	Sham- group	Diseased - group	Treated groups		
				Cilostazole-50mg/kg	Cilostazole-100 mg/kg	Valsartan 30mg/kg
Baseline	Serum urea (mg/dl)	29.22 ±1.08	29.97 ±1.13	28.25 ± 1.23	26.77 ±1.08	27.92 ± 1.41
	Serum creatinine	0.23 ±0.008	0.24 ±0.008	0.22 ± 0.006	0.23 ± 0.005	0.25 ± 0.01
10 days after surgery	Serum urea (mg/dl)	29.91 ±1.33	109.27±2.37 ^{A}	105.05±2.66 ^{B}	103.03 ± 2.54^{B}	102.82±2.83 ^{B}
	Serum creatinine	0.26 ±0.018	0.87 ±0.021 ^{A}	0.85 ±0.02 ^{B}	0.84 ±0.02 ^{B}	0.84 ±0.031 ^{B}
10 weeks after surgery	Serum urea (mg/dl)	27.67 ± 1.47	118.96±3.22 ^{A}	62.66 ±2.71 ^{B}	50.06 ± 3.20 ^{C}	43.83±1.40 ^{C}
	Serum creatinine (mg/dl)	0.24 ± 0.015	1.64 ± 0.022 ^{A}	0.58 ± 0.03 ^{B}	0.40 ± 0.02 ^{C}	0.40 ± 0.02 ^{C}

Data represent means ± SE; n, number of rats ; significance p< 0.05

[A] Significantly different from sham group

[B] Significantly different from diseased group

[C] Significantly different from cilostazole 50mg group

Values with in the same row with different superscript capital letters are significantly ($p<0.05$) different

Statistical comparisons were carried out using one-way ANOVA followed by post-hoc tests using LSD method

Effect of cilostazole (50 and 100mg/kg/day) on SBP and DBP at different time points

Baseline Mean SBP and DBP measurements were not significantly (**P>0.05)** different among all groups . Mean SBP and DBP were significantly(P**<0.05)** higher in the diseased group compared to sham group ten days and ten weeks after surgery.

Cilostazole treatment caused non significant **(P>0.05)** decrease in mean SBP and DBP in its two doses compared to the diseased group ten days and ten weeks after surgery as presented in table 2.

Table2: Effect of cilostazole (50 and 100mg/kg/day) on SBP and DBP at different time points

Interval	Groups (n=8) / parameter	Sham- group	Diseased - group	Treated groups		
				Cilostazole-50mg/kg	Cilostazole-100 mg/kg	Valsartan 30mg/kg
Baseline	SBP (mmHg)	125.57±2.25	127. 51±2.76	127.20±2.51	123.17±2.32	126.20±2.51
	DBP (mmHg)	77.67±1.79	78.77±1.71	75.07±1.79	76.37±2.40	73.77±2.59
	SBP (mmHg)	129.02 ± 3.02	164.27 ± 3.52 [A]	160.52 ±3.27 [A]	159.12 ±3.09 [A]	139.07±3.05 [B]

10 days after surgery	DBP (mmHg)	78.37 ±1.53	102.30 ±1.65 [A]	97.57 ±1.76 [A]	98.70 ±1.76 [A]	86.90±3.35 [B]
10 weeks after surgery	SBP (mmHg)	127.17± 2.97	172.27±3.10 [A]	168.45 ± 3.06 [A]	166.92±3.63 [A]	141.95± 2.98 [B]
	DBP (mmHg)	78.20 ±1.39	108.50 ±1.64 [A]	105.52 ±1.70 [A]	104.27±1.74 [A]	88.20±3.94 [B]

Data represent means ± SE; n, number of rats ; significance p< 0.05

SBP, systolic blood pressure; DBP, diastolic blood pressure

[A] Significantly different from sham group

[B] Significantly different from diseased group

Values with in the same row with different superscript capital letters are significantly (p<0.05) different

Statistical comparisons were carried out using one-way ANOVA followed by post-hoc tests using LSD method

Effect of cilostazole (50 and 100mg/kg/day) on HW ,BW and HW/ BW

Diseased group showed significant (p<0.05) increase in HW and HW/BW with decrease in BW values compared to sham group. Pretreatment with cilostazole (50 and 100 mg/kg/day) showed significant (p<0.05) decrease HW and HW/BW with non significant (p>0.05) increase in BW compared to diseased group. Better results were obtained with cilostazole 100 mg/kg/day as presented in table 3.

Table 3:Effect of cilostazole (50 and 100mg/kg/day) on HW ,BW and HW/ BW

Groups (n=8) / parameter	Sham- group	Diseased - group	Treated groups		
			Cilostazole-50mg group	Cilostazole-100 mg group	Valsartan 30mg-group
HW (g)	1.20 ±.019	1.87 ±.084 [A]	1.63 ±.067 [B]	1.43 ±.069 [C]	1.41±.052 [C]

BW (g)	394.16 ±5.18	350.0 ±3.56 [A]	356.0 ±3.65[A]	360.16 ±3.84 [A]	361.0 ±4.78[A]
HW/BW (mg/g)	3.11 ±0.052	3.95 ±0.048[A]	3.79 ± 0.043 [B]	3.35 ±0.037[C]	3.31±0.031[C]

Data represent means ± SE; n, number of rats ;significance p< 0.05

HW, heart weight; BW, body weight; HW/BW, heart weight/ body weight

[A] Significantly different from sham group

[B] Significantly different from diseased group

[C] Significantly different from cilostazole 50mg group

Values with in the same row with different superscript capital letters are significantly (p<0.05) different

Statistical comparisons were carried out using one-way ANOVA followed by post-hoc tests using LSD method

Effect of cilostazole (50 and 100mg/kg/day) on cardiac NF-κB and TGF-β1

Diseased group showed significant (p<0.05) increase in cardiac NF-κB and TGF-β1 compared to sham group. Pretreatment with cilostazole (50 and 100 mg/kg/day) showed significant (p<0.05) decrease in cardiac NF-κB and TGF-β1 by its two doses compared to diseased group. Better results were obtained with cilostazole (100 mg/kg/day) pretreated group, regarding NF-κB there was no significant difference (p>0.05) between cilostazole (100 mg/kg/day) pretreated group and valsartan (30 mg/kg/day) pretreated group as a standard therapy as presented in table 4.

Effect of cilostazole (50 and 100mg/kg/day) on cardiac MDA and SOD levels

Diseased group showed significant (p<0.05) increase in cardiac MDA and decrease in SOD levels compared to sham group. Rats pretreated with cilostazole (50 and 100 mg/kg/day) showed significant (p<0.05) decrease in cardiac MDA and increase in SOD values compared to diseased group. Better results were obtained with the cilostazole (100 mg/kg/day) pretreated group, there were no significant differences (p>0.05) between cilostazole (100 mg/kg/day)-pretreated group and valsartan (30 mg/kg/day) pretreated group as standard therapy as presented in table 4.

Table 4: Effect of cilostazole (50 and 100mg/kg/d) on cardiac inflammatory , fibrotic ,oxidant and antioxidant markers

Groups (n=8) / parameter	Sham- group	Diseased - group	Treated groups		
			Cilostazole-50mg group	Cilostazole-100 mg group	Valsartan 30mg-group
Cardiac MDA (nmol/mg protein)	23.40 ±1.22	123.90±2.31 ^{A}	66.935±3.26 ^{B}	40.975±1.126 ^{C}	41.025±.916 ^{C}
Cardiac SOD (U/mg protein)	18.86 ±.108	4.52 ±.276^{A}	8.70±.475 ^{B}	12.32 ±.279 ^{C}	12.45±.275 ^{C}
Cardiac NF-κB (pg/ mg protein)	123.25 ± 3.25	318.37± 5.11 ^{A}	188.80 ± 3.55 ^{B}	143.22 ± 4.34 ^{C}	144.60 ±5.22 ^{C}
Cardiac TGF-β1 (Pg/mg protein)	33.30 ± 2.20	128.20 ±1.32^{A}	71.67 ±1.93 ^{B}	56.07 ± 2.35 ^{C}	41.05 ± 2.60 ^{D}

Data represent means ± SE; n, number of rats ;significance p< 0.05

NF-κB, nuclear factor kappa B;**TGFβ-1**, transforming growth factor beta 1; **MDA,** Malondialdehyde; **SOD**, super oxide dismutase

^{A} Significantly different from sham group

^{B} Significantly different from diseased group

^{C}Significantly different from cilostazole 50mg group

^{D} Significantly different from cilostazole 100mg group

Values with in the same row with different superscript capital letters are significantly (p<0.05) different

Statistical comparisons were carried out using one-way ANOVA followed by post-hoc tests using LSD method

Effect of cilostazole (50 and 100 mg/kg/d) on cardiac p-AKT and p-GSK3β and serum BNP levels

Diseased group showed significant ($p<0.05$) increase in cardiac p-AKT p-GSK3β and serum BNP levels compared to sham group. Pretreatment with cilostazole (50 and 100 mg/kg/day) showed significant ($p<0.05$) decrease in cardiac p-AKT p-GSK3β and serum BNP by its two doses compared to diseased group . Better results were obtained with the cilostazole (100 mg/kg/day) pretreated group as presented in table 5.

Table 5: Effect of cilostazole (50 and 100 mg/kg/d) on cardiac hypertrophy markers p-AKT , p-GSK3β and serum BNP levels

Groups (n=8) / parameter	Sham- group	Diseased - group	Treated groups		
			Cilostazole-50mg group	Cilostazole-100 mg group	Valsartan 30mg-group
Cardiac P-AKT (ng/mg protein)	8.23 ±0.57	19.27±0.51 [A]	15.22 ±0.56 [B]	13.07 ±0.57 [C]	10.45 ±0.58 [D]
Cardiac P-GSK3β (ng/mg protein)	4.82 ± 0.27	13.12 ±0.18 [A]	10.02 ± 0.37 [B]	8.75 ± 0.26 [C]	6.50 ±0.22 [D]
Serum BNP (pg/ml)	209.20 ± 3.92	418.63 ± 5.91 [A]	299.36 ±4.76 [B]	254.13 ± 4.0 [C]	226.80 ±4.12 [D]

Data represent means ± SE; n, number of rats ;significance $p< 0.05$

P-AKT, phosphorylated protein kinase A ; **p-GSK3β** phosphorylated glycogen synthase kinase3beta ;**BNP**, brain natriuretic peptide

[A] Significantly different from sham group

[B] Significantly different from diseased group

[C] Significantly different from cilostazole 50mg group

[D] Significantly different from cilostazole 100mg group

Values with in the same row with different superscript capital letters are significantly ($p<0.05$) different

Statistical comparisons were carried out using one-way ANOVA followed by post-hoc tests using LSD method

Effect of cilostazole (50 and 100 mg/kg/d) on cardiac eNOS and PPARγ expressions

Diseased group showed significant ($p<0.05$) decrease in cardiac **eNOS** and **PPARγ** expressions compared to sham group. Pretreatment with cilostazole (50 and 100 mg/kg/day) showed significant ($p<0.05$) increase in their expressions in its both doses compared to diseased group. Better results were obtained with the cilostazole (100 mg/kg/day) pretreated group, there were no significant differences ($p>0.05$) between cilostazole (100 mg/kg/day) pretreated group and valsartan (30 mg/kg/day) pretreated group as standard therapy as presented in table 6.

Table 6:Effect of cilostazole (50 and 100 mg/kg/d) on cardiac eNOS and PPARγ expressions

Groups (n=8) / parameter	Sham- group	Diseased - group	Treated groups		
			Cilostazole-50mg/kg	Cilostazole-100 mg/kg	Valsartan 30mg/kg
Cardiac eNOS (relative expression)	1.056±0.017	0.256±0.024 [A]	0.650±.026 [B]	0.801±.025 [C]	0.830±.028 [C]
Cardiac PPARγ (relative expression)	1.070 ±0.019	0.192 ±0.023 [A]	0.537 ±0.021 [B]	0.755 ±0.024 [C]	0.780±0.019 [C]

eNOS,endothelial nitric oxide synthase; **PPARγ**, peroxisome proliferation-activated receptor gamma

Data represent means ± SE; n, number of rats ;significance $p< 0.05$

[A] Significantly different from sham group

[B] Significantly different from diseased group

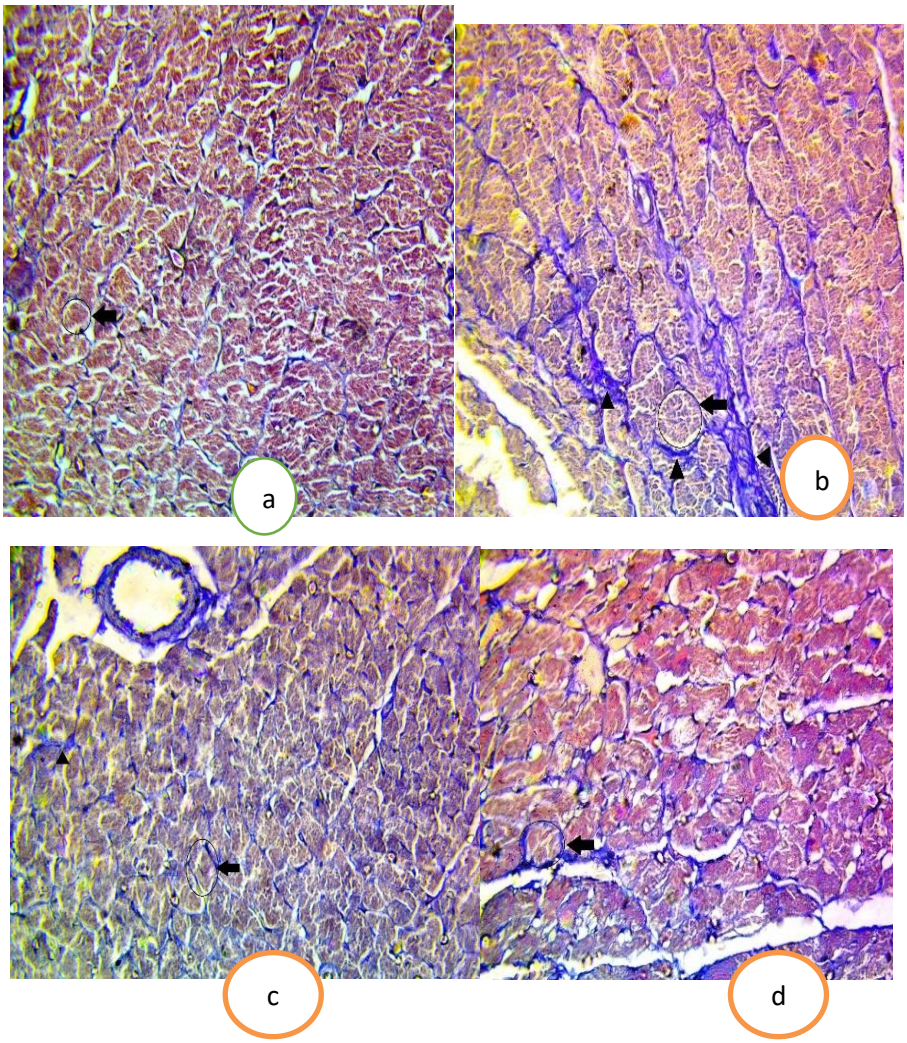
[C] Significantly different from cilostazole 50mg group

Values with in the same row with different superscript capital letters are significantly ($p<0.05$) different

Statistical comparisons were carried out using one-way ANOVA followed by post-hoc tests using LSD method

Histo-pathological Findings

Histopathological examination of M & T stained heart slices of diseased group showed marked hypertrophy of cardiac muscles with marked interstitial fibrosis in-between muscle fibers. While, treatment with cilostazole (50 and 100mg/kg/day) showed mild hypertrophy of cardiac muscles with no interstitial fibrosis in-between muscle fibers.



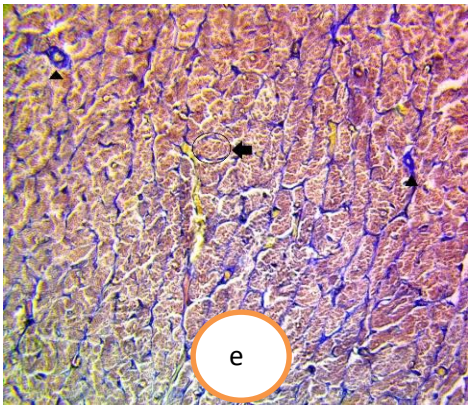


Figure 1. Photo micro-graph of myocardium a. sham normal group shows normal structure and thickness of cardiac muscles b.diseased group shows marked hypertrophy of cardiac muscles (shown by circle) with interstitial fibrosis in-between muscle fibers (arrowheads d. .diseased +vehicle group shows marked hypertrophy of cardiac muscles (shown by circle) with moderate perivascular fibrosis and interstitial fibrosis in-between muscle fibers (arrowheads).c.cilostazole 50 group shows moderate hypertrophic changes of cardiac muscles (shown by circle) with minimal perivascular fibrosis and interstitial fibrosis in-between muscle fibers (arrowhead). d.cilostazole 100 group shows mild hypertrophy of cardiac muscles (shown by circle) with no interstitial fibrosis in-between muscle fibers. e.valsartan group shows mild hypertrophy of cardiac muscles (shown by circle) with mild perivascular fibrosis (arrowheads) and no interstitial fibrosis in-between muscle fibers. (M &T x 400)

4.Discussion

Chronic kidney disease (CKD) is a public health problem with high economic burden, morbidity and mortality rates. Patients suffering from CKD are at high risk of cardiovascular complications, a condition known as cardiorenal syndrome (CRS) **(Suresh et al., 2017).** The current study showed significant elevations in serum creatinine and urea levels in the diseased group 10 weeks after 5/6 subtotal nephrectomy compared to sham group. In chronic renal disease there is a steady and continued decrease in renal clearance or glomerular filtration rate (GFR), which leads to the gathering of urea, creatinine and other chemicals in the blood**(McWilliam et al.,2017)**. Serum creatinine and urea are the principle indicators of renal dysfunction and nephrotoxicity **(Campos et al. 2018)**. Matching with our results **Švíglerová et al., 2010** reported significant increase in serum urea and creatinine levels 10 days and 10 weeks after 5/6 subtotal nephrectomy. **Gui et al. 2021** found that CKD induced by the 5/6 nephrectomy model in rats led to impaired kidney functions. Cilostazole successfully decreased serum urea and creatinine levels compared to diseased at 10 weeks after surgery. Better results were observed with cilostazole 100 mg/kg. In the same context **Abdelsameea et al. (2016)** demonstrated that administration of cilostazole 10mg/kg once daily for 8 days reduced creatinine, urea and uric acid level in nephrotoxicity induced by gentamycin. Histopathological changes of cardiac tissue in the present study, showed marked hypertrophy of cardiac muscle fibers with marked interstitial fibrosis in the diseased group

compared to sham group ,changes that was alleviated with cilostazol treatment in its two doses. In line with our results, **Sárközy et al. (2019)**; **Tyralla et al . (2011)** founded that at cross-sectional level cardiomyocyte diameters were significantly increased in the CKD group (subtotal 5/6 nephrectomy) as compared to the sham group proving the presence of LVH at the cellular level with sever interstitial fibrosis . **Zhao et al. (2020)** reported that cilostazol significantly decreased cardiac fibrosis and structural remodeling in a canine model of rapid atrial pacing .

The present study showed significant increases in both diastolic and systolic blood pressures compared to sham group 10 days and 10 weeks after surgery. Multiple pathways have been implicated in CKD induced hypertension including hyperactivity of the RAAS, sympathetic hyperactivity, arterial calcification ,reactive oxygen species, that induced modifications in large artery function and structure, that seriously increase the blood pressure in CKD patients **(Pateinakis and Papagiann, 2011)**. Our results are in accordance with **Švíglerová et al., 2010; Mohamed et al .,2021** they reported significant increases in rats blood pressure10 days and 10 weeks after 5/6 nephrectomy .Cilostazole non significantly decreased BP at 10 weeks after surgery ,our results matching with **Chancharoenthana et al. (2017)** who reported that cilostazole non significantly reduced the blood pressure in ischemia-reperfusion injury with unilateral nephrectomy mouse model (CKD model). In contrast to our results, **Reddy et al. (2018**) showed that cilostazole markedly suppressed the elevated SBP in obese-hypertensive mice 19 weeks after AngII infusion ,they attributed cilostazole vascular relaxant effect to protein kinase A-dependent decrease in [Ca2+] in vascular smooth muscle cells.

A robust association has been established between the decline in renal function and the risk of emerging functional and structural alterations of the heart **(Park et al. 2012; Gori et al. 2014)**. Left ventricular hypertrophy (LVH) which is characterized by myocyte hypertrophy and interstitial fibrosis is highly prevalent in CRS type 4 which causes progressive impairment in cardiac contractility and increasing stiffness of the myocardium leading to diastolic and systolic dysfunction and eventually heart failure **(Di Lullo et al., 2017)**. Increased heart weight (HW) and heart weight/ body weight ratio (HW /BW) are used as indicator of LVH.

The present study showed significant increase in HW , HW/BW in diseased group compared to sham group. LVH in CKD patients is secondary to both pressure and volume overload **(Taddei et al., 2011)**. Pressure overload is secondary to preexisting hypertension, loss of elasticity of the vessels and to vascular calcifications .Volume overload attributed to anemia and the retention of sodium and water secondary to decreased renal function It results in lengthening of the cardiac myofibers by serial addition of sarcomeres thus causing eccentric LVH **(Losi et al.,2010)**. Our results are in accordance with that obtained by **Uchida et al. (2022)** who showed significant elevations in HW and HW /BW in rat model of CKD induced by 5/6 subtotal nephrectomy 8 weeks post nephrectomy. **Tang et al. (2015**) showed significant increase LV and LV/BW, in the diseased group compared to treated groups after 4 weeks of CRS induction by 3/4 nephrectomy. In the current study, pretreatment with cilostazole 50mg and 100mg showed significant reduction

in both HW and HW/BW by its both doses compared to diseased group10 weeks post nephrectomy. Better results were observed with a dose of 100 mg/kg. In line with these results **Lee et al. (2020)** demonstrated that cilostazole significantly decreased HW and HW/BW ratio which was taken as index of LV mass in myocardial infraction rat model 5 weeks post surgery. **Hada et al.(2022)** reported that cilostazole significantly decreased HW, HW/BW and attenuated cardiac hypertrophy in AngII-induced cardiac fibrosis in apo E deficient mice ,also reduced AngII-induced increases in fibrotic and inflammatory gene expression .

In CRS type 4, there is an imbalance between ROS and NO toward oxidative stress, due to increased ROS production and decreased NO synthesis, causes alteration in permeability of myocytes and finally damage to cardiac tissues **(Radi, 2018)**. ROS have been recognized as key chemical mediators causing cellular aging, increased production of inflammatory cytokines such as NF-κB, TGF-β and both cardiac and renal disease dysfunction. ROS simultaneously being enhanced by the typical chronic inflammatory state of CRS patients.

Oxidative stress can be monitored with oxidative stress indicators as MDA, that formed as reactive oxygen species from lipid peroxidation end product also monitored with endogenous antioxidants produced in vivo SOD **(Du et al.,2014).** The present study showed significant increase in cardiac MDA level in addition to significant decrease in cardiac SOD level in the diseased group compared to sham group. Increased ROS production in CKD may be induced by inappropriate activation of the RAAS and, consequently, decreased NO availability (due to inactivation of NO by ROS), which impairs vasodilation, reduces renal perfusion and allows platelet aggregation and neutrophil adhesion **(Rajapakse et al.,2015).** In the same context, **Li et al .2020** demonstrated significant decrease in SOD level in rats cardiac tissue in 5/6 nephrectomy group 12 weeks after surgery compared to control group. **Liu et al. 2015** showed significant increase in cardiac MDA and NADPH oxidase activity which was significantly correlated with cardiac hypertrophy in type 4 CRS induced by 5/6 nephrectomy in rats 8 weeks after surgery compared to sham group. The present work demonstrated that pretreatment with cilostazole (50 and 100mg/kg) significantly decreased MDA and increased SOD levels in cardiac tissue as compared to diseased group that could be explained to its free radical scavenging property or by increasing the activity of the endogenous antioxidants.In the same context **Olivera Lopes et al. (2022)** reported that cilostazol attenuated cardiac oxidative stress and inflammation in hypercholesterolemic rat model, cilostazol treatment significantly increased cardiac SOD and decreased MDA levels .

PPARɤ is a nuclear receptor regulating transcription of several genes involved mainly in fatty acid and energy metabolism (**Aggarwal, 2011**), PPAR γ activation has a wide spectrum of biological functions, regulating metabolism, reducing inflammation, oxidative stress ,fibrosis and improving endothelial function **(Martin, 2010).** PPARγ expression and activity are regulated by inflammatory cytokines and ROS **(Kim et al., 2013).** The PPAR-γ effect in CRS depends on the modification of the various CKD related markers including NF-κB **(Wang et al., 2022).**

NF-κB functions as the master regulator of pro-inflammatory genes expression, such as cytokines and adhesion molecules. Chronic inflammation ,one of main pathophysiology in CRS type 4 is a complex biological response to cellular or tissue damage and it is associated with myocardial fibrosis, diastolic dysfunction and cardiac hypertrophy **(Shimizu and Minamino ,2016).** In chronic kidney disease ROS overproduction also with the decline in GFR, gradual accumulation of multiple toxins as phenols, indoles and aliphatic amines can occur, that contribute to the inflammatory process of progressive CKD **(Schiffrin et al., 2007&House, 2012).**

Results of the current study showed significant decrease in cardiac PPARγ gene expression with significant increase in cardiac NF-κB level in the diseased group compared to sham group. Over activation of the RAAS and sympathetic nervous system, due to decreased renal filtration capacity and excess ROS overproduction in CKD promote an inflammatory response. At the same time, reduced clearance of the inflammatory cytokines can itself also contribute to the characteristic inflammatory state of type 4 CRS **(Di Lullo et al .,2015).**Our results are in accordance with **Liu et al .2018** who observed a significant decrease in the expression of PPARγ in calcified arteries both in CKD patients and in a mouse model of CKD. **Watanable et al., 2016** showed significant increase in cardiac NF-κB expression in rats with 5/6 nephrectomy compared to sham group reporting that Ang II induced activation of NF-κB and ROS via AT1R, play a critical role in cardiac remodeling in CKD

The results of the present study showed that pretreatment with cilostazole (50 and 100mg/kg) significantly increased cardiac expression of PPARγ with decrease in NF-κB level 10 weeks post nephrectomy as compared to the diseased group. In accordance with our results **Li et al .(2017)** explained cilostazole cardiac protective effects in mice model of ischemia reperfusion injury via activating PPARɤ/JAK/STAT3 signaling pathway suppression myocardial apoptosis and proinflammatory reactions .

Our results are in match with **Ragab et . (2014)** who reported that pretreatment with cilostazole (50 and 100mg /kg) increased renal PPAR-ɤ transcription activity with significant decrease in NF-κB level in renal ischemia/reperfusion rat model. Their study has verified the strong correlation between cilostazole antioxidant and antiinflammatory effects and PPAR-ɤ transcription in renal tissues. **Da Motta and de Brito (2016)** demonstrated that cilostazol can markedly ameliorate hyperlipidemia-induced platelet activation through inhibiting the PKC-α and NF-κB pathway. **Elshazly et al .(2020)** showed that cilostazol significantly decreased NF-κB activity in acetic acid-induced colitis in rats.

TGF-β1 is a multifunctional peptide with a variety of functions that include increasing collagen and matrix protein formation, maintaining fibroblast viability, and inhibiting metalloproteinase production ,TGF-β1 has been discovered as a master regulator of cardiac remodeling and fibrosis in CKD **(Aihara et al., 2010 ;Kim et al.,2014).** TGF-β1 causes phosphorylation of Smad2/3 transcription factor, which mediates canonical signalling, by interacting with its receptor in the

plasma membrane. In the cytoplasm, phosphorylated Smad2/3 interacts with Smad4 and translocates to the nucleus, inducing transcription of fibrosis-related genes. Cardiac fibrosis is a maladaptive process that results in LV remodelling and dysfunction, as well as poor outcomes **(Ten Dijke & Arthur, 2007).**Results of the current study showed significant increase in cardiac level of TGF-β1 10 weeks after induction of cardiorenal syndrome type 4 in the diseased group compared to sham group**.**

LVH in CKD is a pathologic process and, unlike physiologic adaptations to increased workload (e.g., "athletes heart"), is accompanied by fibrosis, which is also attributed to conditions related to the uremic milieu, including increased levels of angiotensin II ,parathyroid hormone, endothelin, aldosterone and catecholamines **(Amann et al., 2006) . Prado-Uribe et al.2013; Zhang and Xu ,2021** showed significant increase in cardiac expression of TGF-β that was associated with left ventricular hypertrophy and interstitial fibrosis in CKD model induced by subtotal nephrectomy, suggesting that the existence of a deficient action of thyroid hormones at tissue level in 5/6Nx rats is a mechanism responsible for myocardial fibrosis. The present work demonstrates that pretreatment with cilostazole significantly decreased cardiac level of TGF-β1 in its both doses. **Chian et al.** (2020) reported that cilostazole deceleration of hyperglycemia-induced diabetic nephropathy in STZ induced diabetic rats is attributable down-regulation of TGF-β and NF-κB thereby maintain of the mitochondrial function, thus preventing TGF-β-stimulated hypertrophic growth and fibrosis. Cilostazol protects rats against alcohol-induced hepatic fibrosis via suppression of hepatic TGF-β1 activation **(Han et al., 2019).**

Endothelium-derived nitric oxide is a key molecule in vascular biology, promoting the proliferation of endothelial cells and reducing vascular tone and leukocyte adhesion **(Farah et al., 2018).** Decreased activity of endothelial NOS (eNOS) and nitric oxide (NO) bioavailability are considered major contributors to heart failure and uremic cardiomyopathy **(Van Heerebeek et .al .2012; Heinzel et al.2020).**

Our findings revealed significant downregulation of cardiac eNOS expression level in the diseased group compared to sham group. Advanced CRF results in accumulation of naturally occurring compounds as asymmetric dimethylarginine (ADMA), an endogenous NOS inhibitor, that are capable of inhibiting eNOS activity and NO production, also oxidative stress, inflammation depress the endothelium-dependent vasodilation through impairing the synthesis of eNOS **(Six et al., 2020). Zanetti** and colleagues reported similar observations in the aorta of CKD rats induced by subtotal nephrectomy (**Zanetti et al., 2017).** In the same context **Jing et al. 2018** showed that eNOS expression was downregulated in the brain cortex tissues of the CKD rats induced by 5/6 nephrectomy .Cilostazole treatment significantly increased cardiac expression of eNOS compared to diseased group. Better results were obtained with the cilostazole 100 mg/kg pretreated group**.** This results are in line with (**Wu et al ., 2021)** who reported that cilostazol induced eNOS expression in endothelial cells via activation of SIRT1.**Li et al. 2019**

demonstrated that cilostazol increased eNOS expression and enhanced cell proliferation in hypoxia-treated human vascular smooth muscle and vascular endothelial cells.

Cardiac hypertrophy is considered as an adaptive response of the heart to pressure overload, and is distinguished by an increase in myocardial mass and accumulation of extracellular matrix. Although the hypertrophic response is initially a compensatory mechanism that augments cardiac output, prolonged hypertrophy can lead to ventricular dilatation and heart failure with high rates of mortality and morbidity **(van Berlo et al., 2013**

; Schiattarella and Hill.,2015) .Hypertrophic stimuli as pressure over load , Ang II or β adrenergic stimulation induce chronic PI3K activation in the heart accentuates cardiac hypertrophy and myocardial dysfunction, the hypertrophic response following PI3K activation is associated with PI3K downstream AKT phosphorylation and activation. AKT is a serine/ threonine kinase that involved in the regulation of a variety of cellular functions including metabolism, glucose uptake, proliferation and protein synthesis **(Sugden et al., 2008).**Activated AKT subsequently stimulates cell protein synthetic machinery by phosphorylation inhibition of GSK3β (a negative regulator of calcineurin/nuclear factor of activated T cell signaling and hypertrophy), resulted in increased myocyte size and cardiac hypertrophy **(Braz et al.,2009;Gao et al., 2017).**

The current study revealed significant elevation in cardiac levels of both P-Akt and p-GSK3β in the diseased group compared to sham group. In accordance with our findings, **Chen et al. 2022** reported significant increase in p-Akt and p-Gsk3β expression in cardiac tissue of mice with myocardial hypertrophy induced by the trans-aortic constriction after 8 weeks through activation of Akt/GSK3β/mTOR signaling pathways. In the current work cilostazole significantly decrease cardiac p-Akt and p-Gsk3β levels, better results were obtained with the large dose. In agreement to the present results, a study conducted by **Reddy and coworkers (2018)** showed that cilostazole significantly reduced gene expression of hypertrophic and fibrotic markers Akt/mTOR and TGF-β1/SMAD3 in obese and non-obese hypertensive mice model. **Lee et al. 2014** reported that cilostazol significantly reduced P-GSK3β expression invitro in N2aSwe cells suppressed β-amyloid induced tau acetylation and phosphorylation in Alzheimer disease .

BNP is a natriuretic peptide that regulates a spectrum of physiological and pathophysiological activities by decreasing cardiorenal filling overload . In clinical settings, serum BNP levels act as an indicator for pathophysiological conditions associated with cardiac hypertrophy and failure **(Ibrahim et al. 2016; Ibrahim and Januzzi 2018).** The results of this work showed that, 5/6 subtotal nephrectomy induced CRS type 4 rat model caused significant elevations in serum level of BNP in the diseased group compared to sham group, that could be explained by volume overload or left ventricular dysfunction concomitant to CRS type 4 .This result is in accordance with that obtained by **Mohemed et al. (2021)** who showed significant elevations in serum level of BNP in rat model of CRS type 4 induced by 5/6 subtotal nephrectomy 10 weeks post

nephrectomy . The results of the present study showed that pretreatment with cilostazole significantly lower cardiac level of BNP when compared to the diseased group. In accordance with our results **Koh et al., 2015** demonstrated that cilostazole significantly decreased serum BNP level and enhanced the left ventricular systolic function in doxorubicin induced cardiomyopathy in mice attributed cilostazole cardioprotective effect to anti-oxidative , anti-inflammatory and anti-fibrotic actions. **Wang et al . (2020)** showed that cilostazole significantly decreased the expression of BNP induced by nicotine in neonatal rat ventricular myocytes in vitro. **Lee et al. (2020)** showed that cilostazole significantly decreased cardiac BNP expression and LV mass in post-myocardial infarction rat model compared to the control group.

In conclusion, our study results revealed that cilostazole cardioprotective effect in CRS type 4 are mediated through exerting antiinflammaory, antioxidant , antifibrotic actions and increasing cardiac PPARɤ expression as well as abating cardiac hypertrophic signaling pathway

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