Comparative study of the cardioprotective effect of probiotics and symbiotics in 5/6th nephrectomized rats

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Background
Chronic kidney disease (CKD) is a well-known world health problem. Patients with CKD rapidly progress to end-stage renal disease with cardiovascular complications. CKD is characterized by retention of toxic substances that contribute to progression of uremia. Indoxyl sulfate is a known toxin that increases in the blood in patients with CKD and is related to uremic cardiomyopathy. Probiotics and prebiotics are reported to have health benefits to the host when supplemented in an adequate amount. We aimed to explore the pathogenesis of uremic cardiomyopathy and the possible protective effects of probiotics and symbiotic.
Materials and methods
A total of 48 white albino rats were divided into six groups: sham group (group 1), 5/6th nephrectomy group (group 2), probiotic-treated 5/6th nephrectomy rats (group 3 and group 4), and symbiotic-treated 5/6th nephrectomy rats (group 5 and group 6). Treatments were initiated either immediately or 2 weeks after the performed operation. Heart was exposed to 30-min ischemia followed by 30-min reperfusion. Systolic and diastolic functions and myocardial flow rate were measured at 15 and 30 min of reperfusion. Blood pressure measurement was done 1 day before killing. Biochemical studies including serum levels of creatinine, urea, and indoxyl sulfate, and transforming growth factor-β1 (TGF-β1) and NADPH oxidase in heart tissue were done.
Results
Creatinine, urea, TGF-β1, and NADPH oxidase were significantly elevated in all groups compared with sham group. In symbiotic-treated groups, these parameters significantly decreased compared with nephrectomized rats. Systolic and diastolic functions of the heart at 30-min reperfusion were decreased in nephrectomized rats compared with sham one, whereas these functions were attenuated in treated rats. Blood pressure was increased significantly in nephrectomized rats but decreased significantly in symbiotic-treated rats.
Conclusion
Use of symbiotic and not probiotic is cardioprotective in CKD rats. Symbiotics protect the heart by decreasing uremic toxins and oxidative stress and improve internal milieu.

Keywords:
cardioprotective, chronic kidney disease, indoxyl sulfate, probiotics, symbiotics

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Introduction

The increased risk of patients with chronic kidney disease (CKD) to develop cardiovascular complications is not only owing to traditional causes like diabetes mellitus, high blood pressure, and dyslipidemia [1] but also owing to the uremic toxins that play a vital role in development of cardiovascular complications [2]. Recently in a study of oxidative stress in hemodialysis patients, patients were divided according to history of cardiovascular events, and the authors found that hemodialysis patients with previous cardiovascular events had higher values of oxidative stress and antioxidative barriers. So they concluded that oxidative stress in hemodialysis patients is a risk signal for cardiovascular events [3].

CKD is characterized by retention of toxic substances that contribute to progression of uremia [4]. These toxins are classified according to their origin into endogenous, microbial, and exogenous [5]. Although most of these toxins are endogenous, coming from body metabolism, like dimethylarginine, homocysteine, and oxalate, the intestinal microbiota metabolism is a valuable source of these toxins [6].

In patients with CKD, gut microbes hydrolyze urea releasing large quantities of ammonia, which cause dysbiosis with overgrowth of pathogenic bacteria [7,8]. Many factors contribute to this dysbiosis, including decreased consumption of dietary fibers,

frequent use of antibiotics, intestinal wall congestion, and edema [9–11]. This is evidenced by decreased plasma level of uremic solutes in hemodialysis patients with colectomy compared with intact colon ones [12]. In addition, compared with healthy controls, hemodialysis patients had significant expansion of intestinal bacteria possessing urease, uricase, and enzymes forming indole and p-cresol [8,13].

In CKD, intestinal microbiota ferment the amino acids tyrosine and tryptophan, generating p-cresol and indole, respectively. After absorption, both substances are metabolized in the liver generating p-cresol sulfate and p-indoxyl sulfate, which circulate in the blood in free form or bound to albumin [14]. These toxins are excreted by tubular secretion in the kidneys, but during advanced CKD, the kidney is unable to excrete these toxins which accumulate in the blood triggering inflammatory and oxidative responses [6]. Not only accumulating in blood, uremic toxin concentrations also increase in the intestine, disrupt the intestinal barrier integrity, translocate bacteria and metabolites to blood, and consequently accelerate the progression of CKD and cardiovascular disease (CVD) [6,15].

Uremic cardiomyopathy is pathologically composed of hypertrophied heart with interstitial fibrosis, intramyocardial arteriolar wall thickening, and microvessel disease [16]. Lin *et al.* [17] found that p-indoxyl sulfate level can predict cardiovascular events in patients with advanced CKD. Indoxyl sulfate measured in CKD rats was suggested to induce oxidative stress in renal cells and cardiomyocytes, mediate cardiac hypertrophy and fibrosis, and decrease the antioxidative defense in hypertensive rats [18,19]. Recently, Cao *et al.* [20] investigated the relationship between p-indoxyl sulfate and first heart failure event in patients with end-stage renal disease on hemodialysis. The patients were divided into two groups according to p-indoxyl sulfate level and followed up for 48 months. The authors found that high risk of first heart failure event was found in hemodialysis patients with increased plasma p-indoxyl sulfate.

Moreover, patients with CKD are known to develop hypertension, atherosclerosis, and arteriosclerosis [21]. Wu *et al.* [22] demonstrated increased incidence of arteriovenous graft thrombosis in hemodialysis patients with increased level of p-indoxyl sulfate.

In an in-vitro study using vascular smooth muscle cells isolated from rats, Yamamoto *et al.* [23] reported that p-indoxyl sulfate induces proliferation in vascular smooth muscle and activates mitogen-activated protein kinase. In a similar study on human aortic smooth muscle, p-indoxyl sulfate increased smooth muscle proliferation that was concentration dependent and induced free radicals release by upregulating NADPH oxidase [24,25]. Vascular smooth muscle proliferation causes hyperplasia of the intima and arterial stenosis that progress to atherosclerosis [26]. Moreover, p-indoxyl sulfate is one of the uremic toxins that were positively and significantly associated with aortic calcification in patients with different stages of CKD [27].

Probiotics are living microorganisms that have health benefits to the host when supplemented in adequate amount. Patients with CKD supplemented with probiotics showed lower proinflammatory markers and increased IL-10 significantly [28]. In a recent study, the authors reported that frequent use of yogurt and/or probiotic cause decreased risk of proteinuria in kidney diseases according to National Health and Nutrition Survey Data [29].

Prebiotics are nondigestible food ingredients that contribute to well-being of the host by affecting the growth or activity of microorganisms. Prebiotics include inulin, fructo-oligosaccharides, galacto-oligosaccharides, soya-oligosaccharides, xylo-oligosaccharides, and pyrodextrins. Symbiotic are probiotics enriched with prebiotics [30]. Rossi *et al.* [31] found that symbiotic therapy in patients with CKD reduced level of p-cresol sulfate and altered intestinal microbes but did not significantly reduce serum p-indoxyl sulfate levels. This reduction of indoxyl sulfate became significant after exclusion of patients who received antibiotics during the experimental period. So we aimed to study the pathogenesis of remote effect of CKD on the heart and possible protection of heart from uremic toxins by using probiotics and symbiotics as a treatment.

Materials and methods

Animals

This study was performed on 48 male adult albino rats initially weighing 150–250 g. The rats were purchased from Research Institute of Ophthalmology (Giza, Egypt). They were maintained in animal cages (three rats/cage) under controlled conditions of temperature (25±2°C) and relative humidity (50–70% RH). The rats were allowed standard pelleted chow and tap water ad libitum with 12-h duration of light and dark cycle. They were acclimatized to the laboratory conditions for a week before start of experimental procedures to decrease the

possible discomfort of animals. Animals were not exposed to unnecessary pain or stress, and animal manipulation was performed with maximal care and hygiene.

Ethical consideration

All animal experiments were performed according to the Ethics Committee of Faculty of Medicine, Ain Shams University, and according to the National Institutes of Health guide for the care and use of laboratory animals (NIH publication no. 8023, revised 1978) [32].

Probiotic treatment

Colonies of *Lactobacillus acidophilus* were prepared by Dairy and Food Microbiology Department of National Research Center, Cairo, Egypt. The colonies were cultured in yogurt; each 1 ml contained 1×10^{10} CFU. They were prepared weekly and stored in refrigerator. They were then supplied by gavage daily in the morning at a dose of 1×10^{10} CFU=1 ml/kg/day.

Symbiotic treatment

The probiotic formulation was enriched with inulin fructo-oligosaccharides 2% of yogurt as a prebiotic [33] prepared by Dairy and Food Microbiology Department of National Research Center, Cairo, Egypt.

5/6th Nephrectomy

It was done according to Sugano *et al.* [34]. The rat was put in the prone position, and a small incision was made on the back of the rat to expose the kidney. Removal of right kidney and two-third of the left kidney was done with 1 week apart under ether anesthesia. The incisions were closed using 2–0 chromic catgut for the muscle and silk thread for the skin. Asepsis using Baneocin antibiotic powder (bacitracin+neomycin; Pharco Pharmaceuticals Co., Cairo, Egypt) was ensured during the operation and daily after that till wound healing.

Study design

Animals were randomly allocated into six experimental groups as follows:

(1) Group 1 included sham-operated control rats (n=8). Rats in this group were subjected to all the procedures of 5/6th nephrectomy operation without the removal of the kidneys and were killed after 6 weeks.
(2) Group 2 included 5/6th nephrectomy rats (n=8). Rats in this group were subjected to 5/6th nephrectomy operation [34] and sacrificed after 6 weeks.
(3) Group 3 included early probiotic-treated 5/6th nephrectomy rats (n=8). Rats in this group were subjected to 5/6th nephrectomy operation and received probiotic treatment immediately after operation. The treatment was continued for 6 weeks, and then the rats were killed.
(4) Group 4 included late probiotic-treated 5/6th nephrectomy rats (n=8). Rats in this group were subjected to 5/6th nephrectomy operation and received probiotic treatment 2 weeks after operation. The treatment was continued for 4 weeks, and then the rats were killed.
(5) Group 5 included early symbiotic-treated 5/6th nephrectomy rats (n=8). Rats in this group were subjected to 5/6th nephrectomy operation and received symbiotic treatment immediately after operation. The treatment was continued for 6 weeks, and then the rats were killed.
(6) Group 6 included late symbiotic-treated 5/6th nephrectomy rats (n=8). Rats in this group were subjected to 5/6th nephrectomy operation and received symbiotic treatment 2 weeks after operation. The treatment was continued for 4 weeks, and then the rats were killed.

Methods

Body weight and arterial blood pressure were determined for all groups initially and 1 day before killing. Arterial blood pressure [systolic blood pressure (SBP), diastolic blood pressure (DBP), and mean arterial blood pressure (MBP)] was measured using the noninvasive small animal tail blood pressure system (NIBP200A; Biopac Systems Inc., USA). On the day of killing, overnight fasted rats were anesthetized with intraperitoneal injection of pentobarbital, in a dose of 40 mg/kg, body weight. Then, the rats were subjected to the following studies:

(1) Blood samples were collected for determination of serum level of creatinine according to the method of Bartleset *et al.* [35] and urea according to Fawcett and Soctt [36] by calorimetric method using kits supplied by Bio-Diagnostic (Cairo, Egypt), in addition to the determination of indoxyl sulfate by ELISA technique using kits supplied by Shanghai YL Biotechnology Co. Ltd (Shanghai, China).
(2) Isolated Perfused Heart Study:
After blood samples collection, the heart was isolated and immediately placed in ice-cold modified Krebs–Henseleit bicarbonate buffer solution for fast cardioplegia. Isolated hearts were perfused according to the ordinary technique of Langendorff [37], as modified by Ayobe and

Tarazi [38]. The isolated heart was then attached by a clip to an isometric force transducer (model 7004-F, serial no. 101014, data EVO 14543; UGO BASILE S.R.L., Italy) and adjusted vertically to the heart. The transducer was connected with a USB cable to a recorder (Apparatus 21036, model 17304, serial no. 448A15; UGO BASILE S.R.L. Biological Research, Italy) and to a computer provided with iWorx LabScribe2 Data Recording and Analysis software. The heart was left to stabilize for 10 min.

The isolated perfused hearts were used to record heart rate (HR, b/min), peak developed tension (PT, g), time to peak tension (TPT, ms), half relaxation time (HRT, ms), and myocardial flow rate (MFR, ml/min). Both PT and MFR were also calculated per 100 mg left ventricular (LV) weight. These records were measured at baseline condition and 15 and 30 min of reperfusion following 30 min of total global ischemia [38].

(3) Heart tissue was weighed and kept at −80°C till determination of TGF-β1 and NADPH oxidase levels by ELISA technique using kits supplied by CUSABIO Technology LLC. (Houston, USA).

(4) Regarding histological studies, samples of left atrium, LV, and right atrium were fixed in 10% formalin, embedded in paraffin, and cut into 5-μm sections. Masson trichrome staining was used to evaluate interstitial fibrosis [39].

Statistical analysis

All obtained results in this study were expressed as mean±SEM. Statistical package for the social sciences (SPSS Inc., Chicago, Illinois, USA) program, version 20.0, was used to compare significance between each two groups. One-way analysis of variance for difference between means of different groups was performed on results obtained in the study. Differences were considered significant by least significance difference when P up to 0.05. Correlation coefficient and line of regression were calculated by Pearson's correlation (two-tailed).

Results

Serum creatinine, urea, and indoxyl sulfate

The data presented in Table 1 indicated that serum creatinine and urea in group 2 significantly increased compared with group 1. Upon treatment, the levels of both creatinine and urea were decreased significantly compared with group 2, but they were still significantly higher than the levels obtained in group 1.

Serum level of indoxyl sulfate was elevated significantly in group 2 compared with group 1. In comparison with group 2, group 3 and 4 had nonsignificant decrease in indoxyl sulfate level, whereas groups 5 and 6 had significant decrease in indoxyl sulfate level, which was nonsignificantly changed from group 1.

Heart tissue levels of TGF-β1 and NADPH oxidase

Table 1 shows significant elevation in levels of both TGF-β1and NADPH oxidase in group 2 compared with group 1. In treated groups, groups 3 and 4 showed nonsignificant change in levels of both TGF-β1 and NADPH oxidase compared with group 2. However, in other treated groups, levels of both TGF-β1 and NADPH oxidase were decreased significantly compared with group 2 but were still significantly higher than group 1.

Arterial blood pressure changes

As in Table 2, SBP, DBP, and MBP were significantly elevated in groups 2, 3, and 4 compared with group 1. However, groups 5 and 6 showed significant reduction of SBP, DBP, and MBP compared with group 2, and it was insignificantly changed from group 1.

Isolated heart studies

In Table 3, the peak developed tension calculated per 100-mg tissue left ventricular weight (PT/LVW) was significantly decreased in group 2 compared with group 1 at all assessed times. In either group 3 or 4, PT/LVW at baseline and 30 min reperfusion was significantly decreased compared with group 1, whereas PT/LVW was increased nonsignificantly relative to group 2. Both groups 5 and 6 showed nonsignificant changes in PT/

Table 1 Serum level of creatinine, urea, and indoxyl sulfate and levels of transforming growth factor β1 and NADPH oxidase in heart tissue in all groups

	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6
Creatinine (mg/dl)	0.6±0.04^{a}	2.2±0.1^{b}	1.5±0.07^{c}	1.6±0.06^{c}	1.6±0.07^{c}	1.6±0.08^{c}
Urea (mg/dl)	32±2^{a}	119±13.7^{b}	76±5.8^{c}	86±4.3^{c}	82±5.5^{c}	99±5.6^{d}
Indoxyl sulfate (μg/ml)	2.9±0.4^{a}	5.5±0.4^{b}	5±0.3^{b}	4.8±0.6^{b}	2.9±0.4^{a}	3.5±0.3^{c}
TGF-β1 (pg/ml)	1.3±0.08^{a}	3±0.09^{b}	3.8±0.2^{b}	3.8±0.3^{b}	1.9±0.1^{c}	1.9±0.1^{c}
NADPH oxidase (ng/ml)	156±15.1^{a}	530±22^{b}	465±30^{b}	526±44^{b}	274±11^{c}	263±7.6^{c}

All data are expressed as mean±SE. All data in the same row with different letters ^{a,b,c,d}significant at $P \leq 0.05$, using one-way analysis of variance.

Table 2 Systolic blood pressure, diastolic blood pressure, and mean arterial blood pressure in all groups

	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6
SBP (mmHg)	120±2.9[a]	130±1.7[b]	127±0.6[b]	126±2.2[b]	123±1.1[a]	122±1.1[a]
DBP (mmHg)	87±1.2[a]	96±2.3[b]	98±0.8[b]	98±2[b]	88±1.2[a]	87±2[a]
MBP (mmHg)	98±1.1[a]	101±1.7[b]	108±0.4[b]	107±0.7[b]	100±1[a]	99±1.3[a]

All data are expressed as mean±SE. All data in the same row with different letters [a,b,c]significant at $P \leq 0.05$, using one-way analysis of variance. DBP, diastolic blood pressure; MBP, mean arterial blood pressure; SBP, systolic blood pressure.

Table 3 Peak tension/left ventricle and time to peak tension in all groups

	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6
PT/LV (g/100 mg)						
Basal	0.7±0.04[a]	0.45±0.03[b]	0.56±0.03[b]	0.53±0.03[b]	0.75±0.05[a]	0.77±0.03[a]
15 min	0.56±0.08[a]	0.35±0.03[b]	0.5±0.02[a]	0.48±0.05[a]	0.62±0.05[c]	0.52±0.05[a]
30 min	0.63±0.04[a]	0.35±0.03[b]	0.46±0.04[b]	0.47±0.03[b]	0.75±0.06[a]	0.62±0.06[a]
TPT (ms)						
Basal	104±1.7[a]	116±5.7[b]	113±1.4[b]	115±1.3[b]	98±1.7[a]	97±1.2[a]
15 min	115±5.9[a]	129±4.4[b]	116±2.7[a]	118±1.3[c]	104±5.2[c]	112±3.7[c]
30 min	115±1.5[a]	135±4.6[b]	128±1.2[b]	129±2.1[b]	113±5.8[a]	110±6.3[a]

All data are expressed as mean±SE. All data in the same row with different letters [a,b,c]significant at $P \leq 0.05$, using one-way analysis of variance. LV, left ventricular; PT, peak developed tension; TPT, time to peak tension.

LVW compared with group 1 but significantly increased compared with group 2 at all assessed times.

Moreover, Table 3 shows that TPT was significantly prolonged in groups 2, 3, and 4 at basal and 30 min reperfusion compared with group 1. In groups 5 and 6, TPT was significantly shortened at all assessed times compared with group 2 but did not reach the level of significance compared with group 1.

Data presented in Table 4 show that basal and 30-min HRT was significantly elongated in groups 2, 3, and 4 compared with group 1. Upon symbiotic treatment in group 5 and 6, basal and 30 min HRT was shortened significantly compared with group 2 but nonsignificantly compared with group 1.

As in Table 4, HR was nonsignificantly changed at basal level among different studied groups. At 30 min reperfusion, HR was significantly increased in group 2 compared to group 1. Groups 4, 5 and 6 showed elevated HR at 30 min reperfusion compared with both groups 1 and 2.

Data presented in Table 5 show that MFR was significantly decreased in groups 2, 3, and 4 compared with group 1 at all measured timings. In groups 5 and 6, MFR was nonsignificantly changed compared with group 1 but significantly increased in comparison with group 2.

Body and heart weight changes

As in Table 5, % change in body weight (BW%) was significantly decreased in group 2 compared with group 1. Nonsignificant changes were found in groups 3 and 4, whereas groups 5 and 6 showed significant increase in body weight compared with group 1.

Heart/body weight ratio (Ht/BW%) was increased significantly in groups 2, 3, and 4 compared with group 1. Groups 5 and 6 had decreased Ht/BW% compared with group 2.

LV/heart weight ratio was significantly increased in group 2 compared with group 1. Symbiotic treatment significantly decreased the ratio in groups 5 and 6 compared with group 2.

Histopathological examination

Histopathological examination of sections of heart of group 1 revealed normal thickness of myocardium with unremarkable intramyocardial arterioles. No evidence of interstitial fibrosis was detected by Masson trichrome stain (Fig. 1a and b).

Sections of heart in group 2 revealed significant hypertrophied myocardium with significant thickening of intramyocardial arterioles walls and mild interstitial fibrosis highlighted by blue staining using Masson trichrome stain (Fig. 2a and b).

Histopathological examination of sections of heart in groups 3 and 4 showed a moderately hypertrophied myocardium with mild thickening of intramyocardial arteriole walls and minimal interstitial fibrosis highlighted by blue staining using Masson trichrome stain (Fig. 3a and b).

Table 4 Half relaxation time and heart rate in all groups

	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6
HRT (min)						
Basal	79±3.5[a]	103±3.4[b]	105±3.4[b]	110±3.4[b]	81±3.9[a]	82±3[a]
15 min	80±2.6[a]	110±1.4[b]	115±1.3[b]	118±1.7[c]	94±2.7[d]	90±3.1[d]
30 min	107±3.6[a]	123±1.3[b]	123±1[b]	123±0.9[b]	106±1.9[a]	105±1.9[a]
HR (bpm)						
Basal	176±1.5[a]	174±1.6[a]	181±3[a]	182±3.9[a]	182±2.7[a]	180±5[a]
15 min	183±2.6[a]	190±2.5[a]	193±3.7[a]	215±5[b]	220±2.7[b]	211±5.3[b]
30 min	140±3.4[a]	187±4.2[b]	217±12.3[c]	243±3.7[d]	213±5.5[c]	190±6[b]

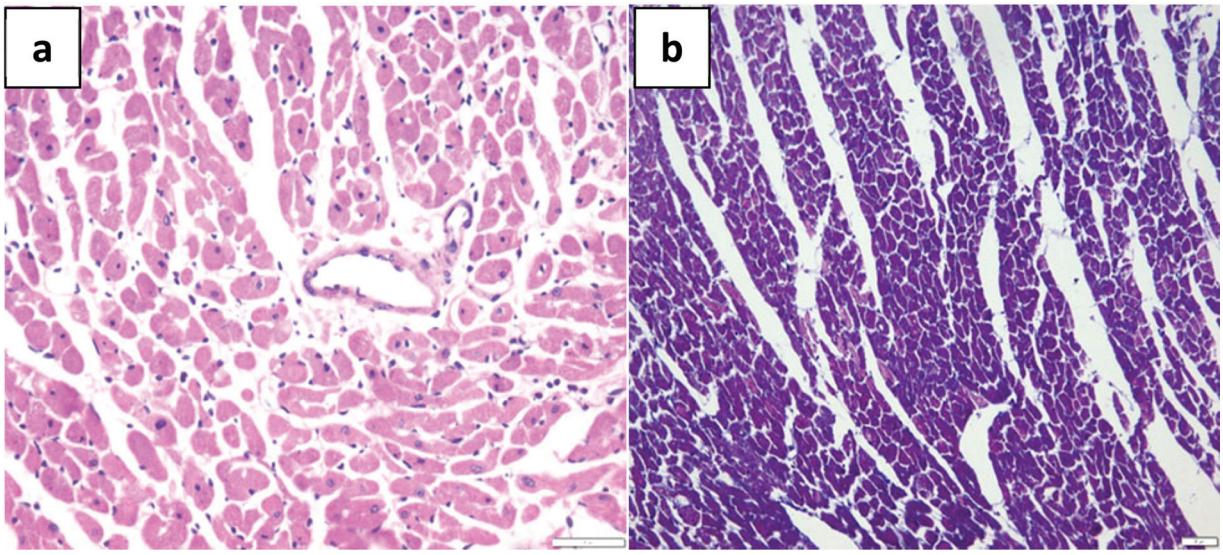
All data are expressed as mean±SE. All data in the same row with different letters [a,b,c,d]significant at $P \leq 0.05$, using one-way analysis of variance. HR, heart rate; HRT, half relaxation time.

Table 5 Myocardial flow rate, body weight percent change, heart to body weight ratio, and left ventricle to heart weight ratio in all groups

	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6
MFR/LV (ml/100 mg)						
Basal	1.6±0.08[a]	1.1±0.08[b]	1.3±0.05[b]	1.2±0.1[b]	1.5±0.05[a]	1.5±0.07[a]
15 min	1.4±0.1[a]	1.1±0.03[b]	1±0.08[b]	1.1±7[b]	1.2±0.1[a]	1.3±0.05[a]
30 min	1.3±0.07[a]	0.95±0.01[b]	0.97±0.09[b]	1±0.03[b]	1.1±0.06[c]	1.1±0.05[c]
BW percent (g% change)	14±1.1[a]	12±0.8[b]	13.5±0.7[b]	13±0.2[b]	16±0.7[a]	14±0.4[a]
Ht/BW (g/g)	0.33±0.01[a]	0.47±0.02[b]	0.47±0.01[b]	0.42±0.01[b]	0.38±0.02[a]	0.41±0.01[c]
Vent/Ht (g/g)	63±0.31[a]	65±0.36[b]	64±0.55[b]	64±0.31[b]	63±0.47[a]	62±1.7[a]

All data are expressed as mean±SE. All data in the same row with different letters [a,b,c]significant at $P \leq 0.05$, using one-way analysis of variance. BW, body weight; Ht/BW, heart/body weight ratio; LV, left ventricular; MFR, myocardial flow rate; Vent/Ht, left ventricular/heart weight ratio.

Figure 1



(a) Sections of heart of group 1 showing normal thickness of myocardium with unremarkable intramyocardial arterioles (×400, H&E). (b) No evidence of interstitial fibrosis detected by Masson trichrome stain (×200, MT stain).

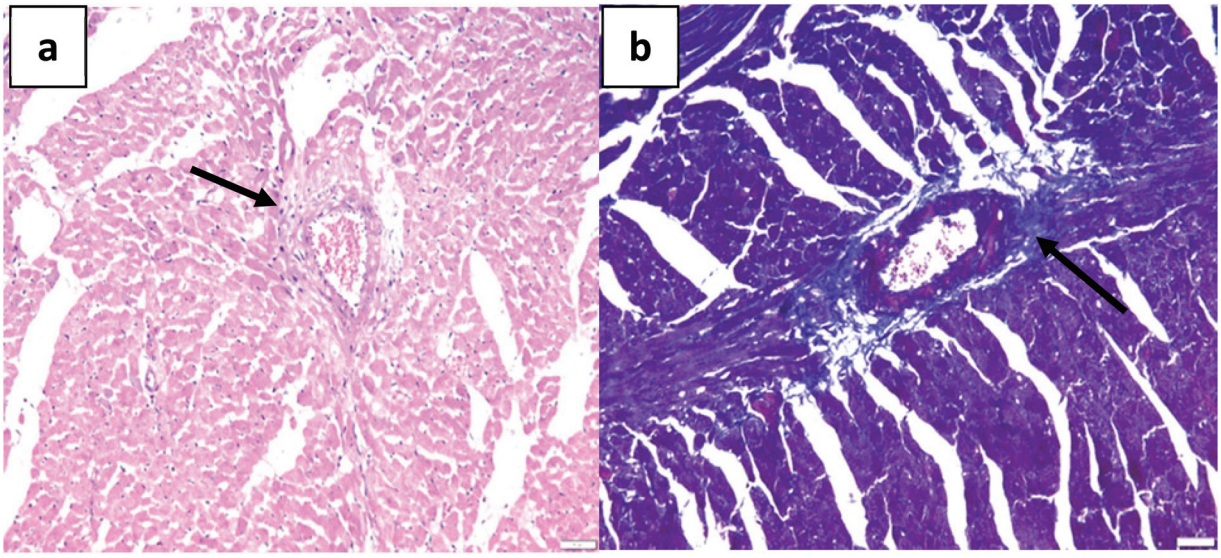
Histopathological examination of sections of heart in group 5 revealed insignificant hypertrophy of the myocardium with almost unremarkable intramyocardial arterioles. No evidence of interstitial fibrosis was detected by Masson trichrome stain (Fig. 4a and b).

Sections of heart in group 6 revealed minimally hypertrophied myocardium with minimal thickening of intramyocardial arteriole walls and minimal interstitial fibrosis highlighted by blue staining using Masson trichrome stain (Fig. 5a and b).

Correlation studies

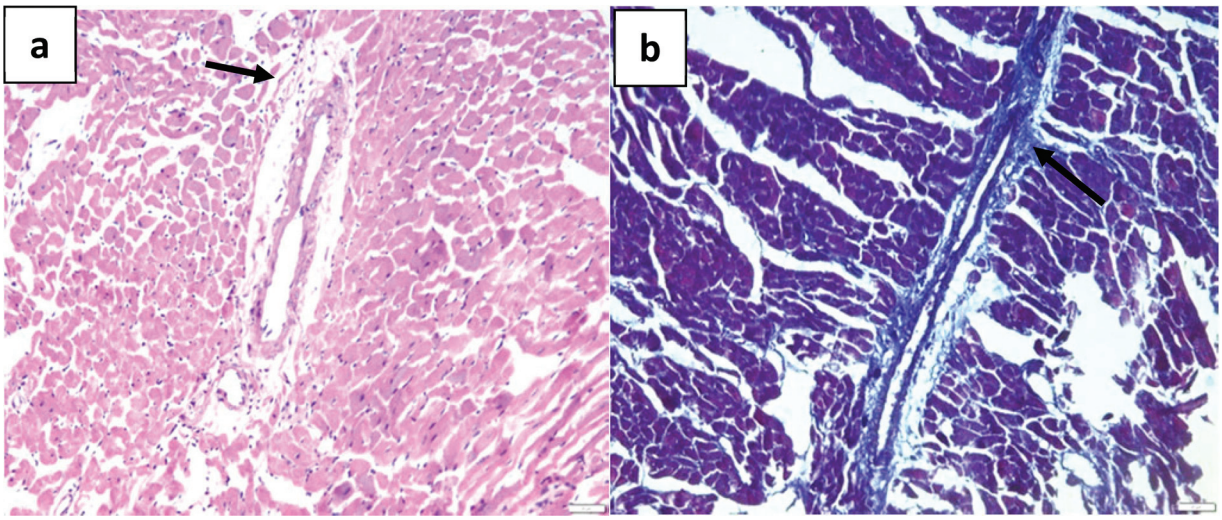
PT at 30 min reperfusion had significant negative correlation with indoxyl sulfate and TGF-β1 among all groups (Fig. 6a and b; R=0.01). However, indoxyl

Figure 2



(a) Sections of heart of group 2 reveal significantly hypertrophied myocardium with significant thickening of intramyocardial arteriole walls (×200, H&E stain). (b) Mild interstitial fibrosis highlighted by blue staining using Masson trichrome stain (×200, MT).

Figure 3



(a) Sections of heart of group 3 and 4 reveal moderately hypertrophied myocardium with mild thickening of intramyocardial arteriolar walls (×200, H&E stain). (b) Minimal interstitial fibrosis highlighted by blue staining using Masson trichrome stain (×200, MT).

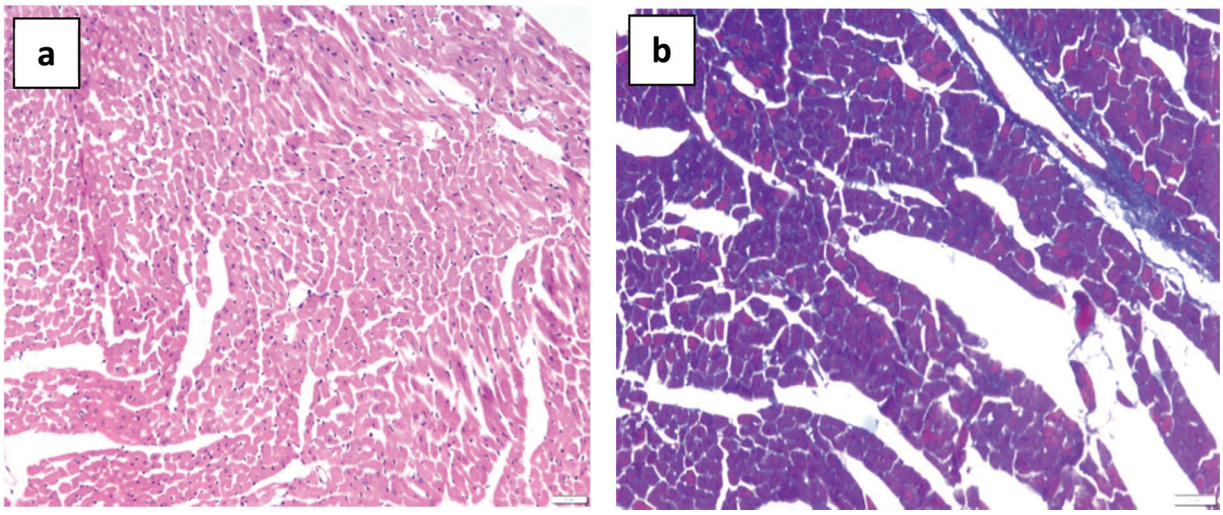
sulfate had significant positive correlation with TGF-β1 and NADPH oxidase among all groups (Fig. 7a and b; R=0.01). MBP had significant positive correlation with indoxyl sulfate and creatinine among all groups (Fig. 8a and b; R=0.01).

Discussion

In this study, induction of CKD by 5/6th nephrectomy was evidenced by significant increase in the levels of uremic toxins (creatinine, urea, and indoxyl sulfate) in group 2 compared with group 1.

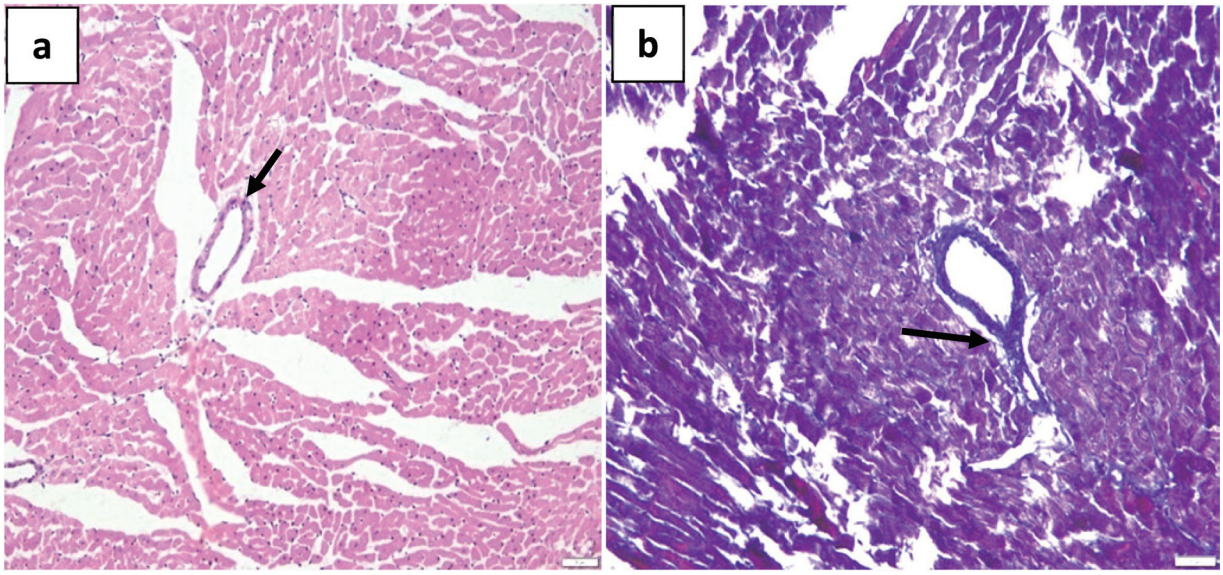
CKD is characterized by retention of toxic substances that contribute to progression of uremia [4]. Intestinal microbiota metabolism is a valuable source of these toxins [6]. Delivery of undigested protein to the colon contributes to the proliferation of proteolytic bacteria that ferment proteins and amino acids to generate toxins. Impaired gut barrier function allows translocation of these toxins into systemic circulation, contributing to CKD progression and CVD [40]. Vaziri [41] reported presence of gastrointestinal barrier dysfunction in uremic humans and animal models. Urea and its product ammonia were identified as a major

Figure 4



(a) Sections of heart of group 5 reveal insignificantly hypertrophied myocardium with almost unremarkable intramyocardial arterioles (×200, H&E stain). (b) No evidence of interstitial fibrosis detected by Masson trichrome stain (×200, MT).

Figure 5



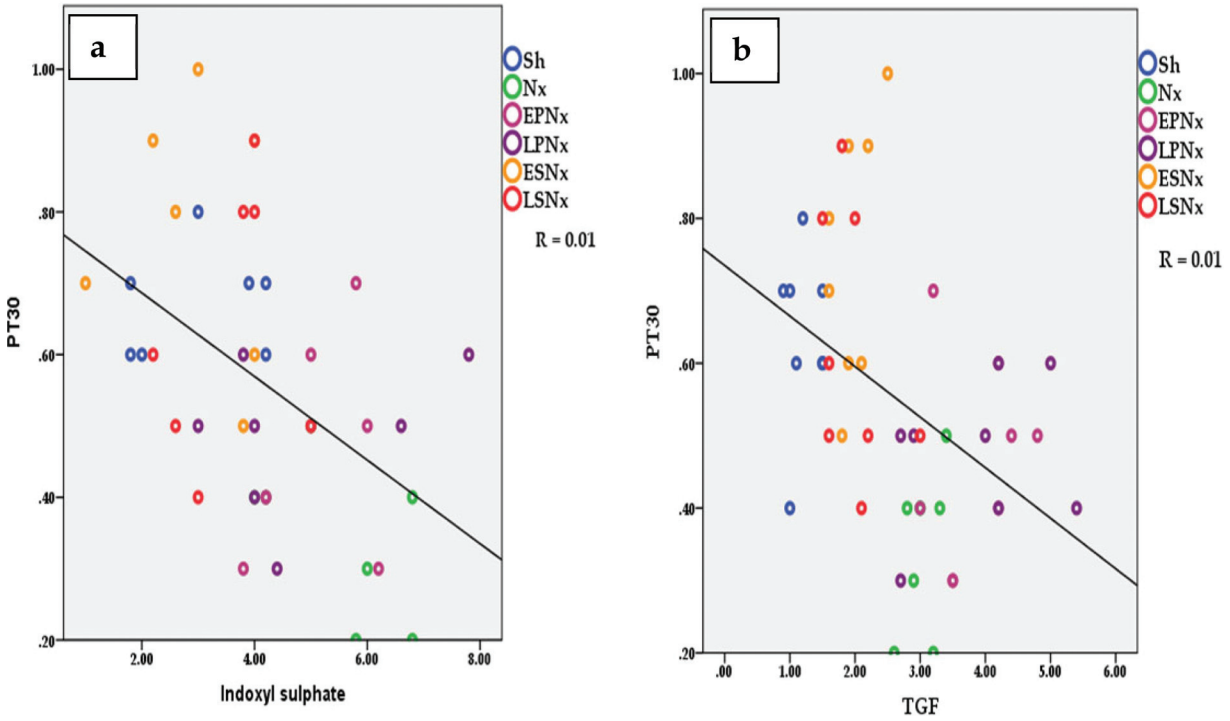
(a) Sections of heart of group 6 reveal minimally hypertrophied myocardium with minimal thickening of intramyocardial arteriole walls (×200, H&E stain). (b) Minimal interstitial fibrosis highlighted by blue staining using Masson trichrome stain (×200, MT).

mediator of disrupted intestinal barrier [42]. Indoxyl sulfate is an intestinal microbiota product, generated from tryptophan, which accumulates in the blood of patients with CKD [43], is involved in CKD progression [44] and contributes to CVD in patients with uremia [45].

Applied probiotic treatment was able to partially lower both creatinine and urea significantly but not indoxyl sulfate. Symbiotic treatment succeeded to lower both creatinine and urea significantly compared with group 2 and decreased indoxyl sulfate down to the levels obtained in the control group.

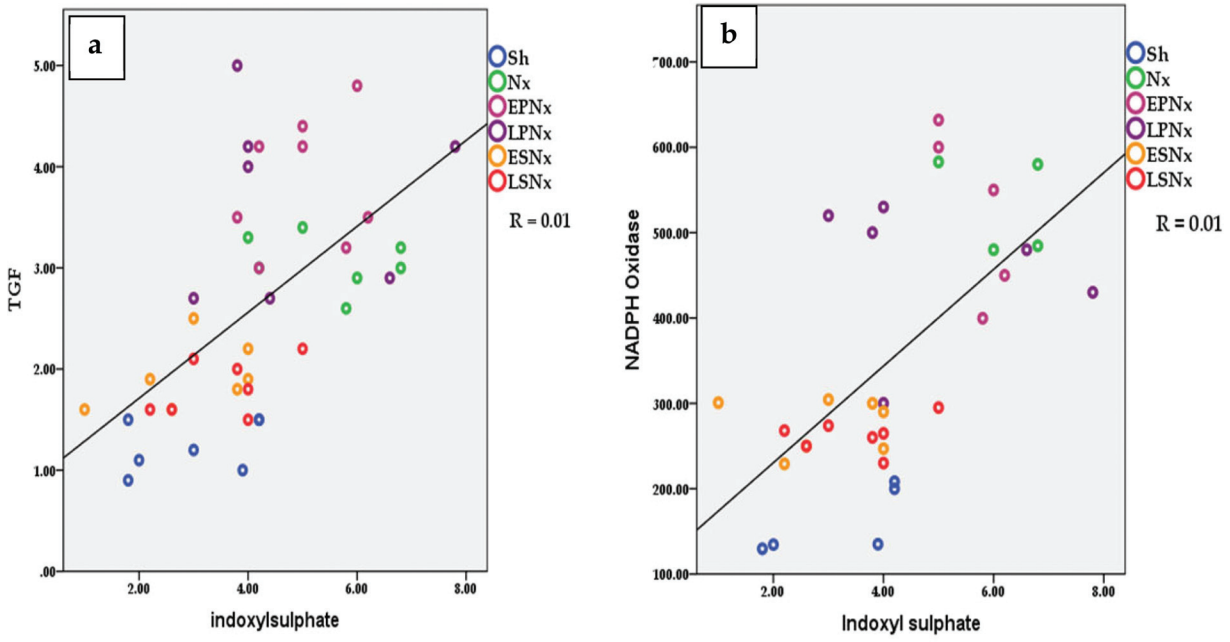
Probiotics are live bacterial cultures – supplemented by addition to food – that can improve the health of the host [46]. The probiotic bacteria may operate by decreasing the growth of pathogenic bacteria in the gastrointestinal tract (GIT), strengthening the epithelial gut barrier function and mucus production, and affecting the systemic immune system [47]. Clinical research trials achieved effective outcomes when administered probiotic strains with at least 10^9 CFU/dose with multiple strains [48]. However, we used probiotics in a concentration of 1×10^{10} CFU in this study. Probiotic administration had conflicting results, as it lowered only levels of urea and creatinine but not the level of indoxyl sulfate. A similar

Figure 6



Negative correlation between peak developed tension at 30 min reperfusion and both indoxyl sulfate (a) and TGF-β1 (b) among all groups.

Figure 7

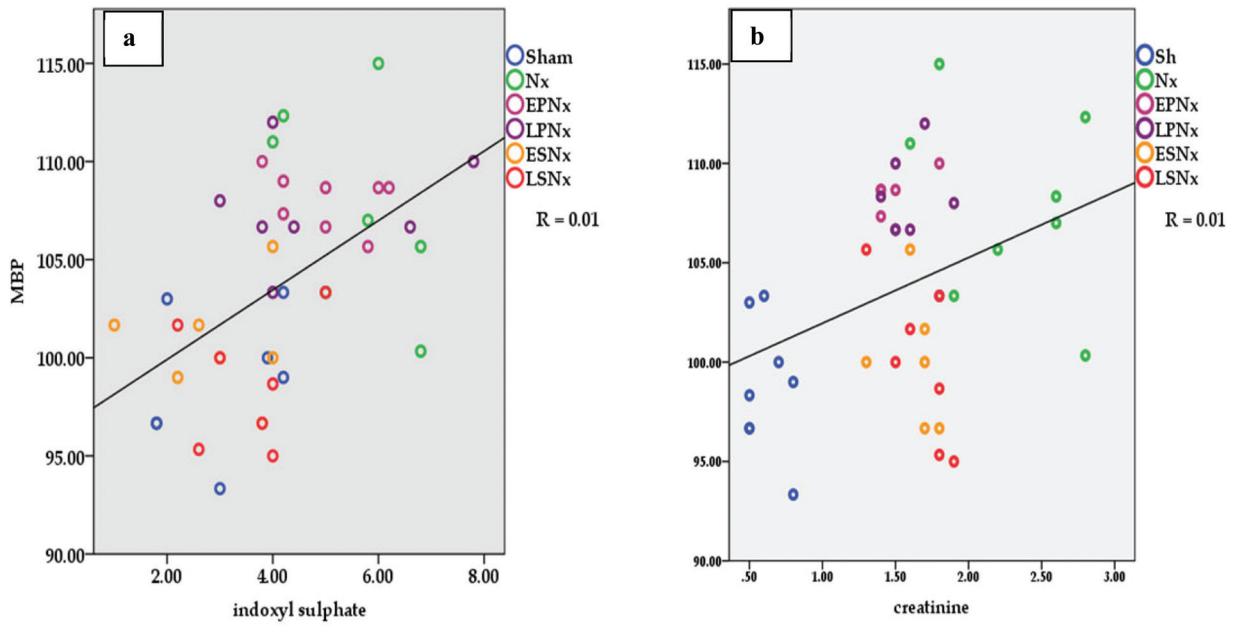


Positive correlation between indoxyl sulfate and TGF-β1 (a) and NADPH oxidase (b) among all groups.

outcome was found in patients with CKD supplemented with probiotics. These patient had lowered level of TNF-α, IL-5, and IL-6, whereas IL-10 level was elevated [28].

The efficacy of probiotics to reduce uremic toxins is improved when combined with prebiotics. In addition, selecting probiotic species in a multiple strain matrix and choosing species types that are capable of metabolizing urea such as a nitrogen growth source could contribute to reduction of uremia, like *Streptococcus thermophilus* [49,50]. In this study, we used only one strain *L. acidophilus* in concentration of 1×10^{10} CFU.

Figure 8



Positive correlation between mean arterial blood pressure and indoxyl sulfate (a) and creatinine (b) among all groups.

Prebiotics are mostly carbohydrates present in natural products such as fruits, vegetables, and cereals. It can increase the growth and/or activity of beneficial bacteria selectively [51]. Symbiotic are probiotics enriched with prebiotics. In this study, symbiotic treatment succeed to improve the internal milieu by decreasing creatinine, urea, and indoxyl sulfate. Similarly, in a randomized controlled trial in hemodialysis patients supplemented with prebiotic in the form of resistant starch for 6 weeks, plasma levels of indoxyl sulfate and p-cresol were reduced by 27 and 24% from their basal levels, respectively, with less intestinal symptoms of flatulence compared with the use of normal starch [52]. Moreover, Meijers *et al.* [53] demonstrated the effect of increasing fiber supplementation in the form of oligofructose-enriched inulin for 4 weeks in hemodialysis patients. The patients supplemented with the fibers had reduced production of p-cresol and plasma level of p-cresol sulfate by 20%.

In contrary to our results, Rossi *et al.* [31] evaluated the effect of symbiotic therapy on gut microbiota and serum concentrations of indoxyl sulfate and p-cresol sulfate in predialysis patients with CKD. According to the authors, symbiotic therapy did not significantly reduce serum indoxyl sulfate levels but reduced levels of p-cresol sulfate and favorably altered the stool microbiome, particularly with enrichment of Bifidobacterium and depletion of Ruminococcaceae.

The isolated perfused heart in this study showed impaired systolic and diastolic functions in response to ischemia in group 2. This was evidenced by decreased PT/LVW and prolonged TPT and HRT significantly. Administration of probiotic treatments did not improve the heart functions, as these parameters were nonsignificantly changed relative to group 2. Symbiotic treatment succeeded to improve both systolic and diastolic functions as PT/LVW increased whereas TPT and HRT shortened significantly in relation to group 2 and nonsignificantly changed compared with sham group.

In addition, negative significant correlation was found between PT/LVW at basal and 30-min reperfusion and indoxyl sulfate, urea, creatinine, TGF-β1, and NADPH oxidase in heart tissue. From these results, we can confirm that indoxyl sulfate has toxic effect on the heart, and lowering its level can protect the heart in patients with CKD. This is in agreement with other studies that suggested indoxyl sulfate to be the main toxin that predicts cardiovascular events in CKD [17,21].

Ischemia reperfusion initiates an inflammatory response and releases oxygen free radicals, resulting in microvascular and endothelial cell dysfunction, vasospasm, vascular thrombosis, and accelerated atherosclerosis [54]. Lekawanvijit *et al.* [55] demonstrated in-vitro that indoxyl sulfate has proinflammatory, profibrotic, and prohypertrophic effects, indicating that indoxyl sulfate contributes to cardiac remodeling in CKD. Moreover, antagonists of intracellular indoxyl sulfate transporters have been demonstrated to decrease its profibrotic and

prohypertrophic effects in cardiac fibroblasts and cardiac myocytes in *in vitro* [56].

In a study in subtotal nephrectomised rats, reduction of indoxyl sulfate was associated with decreased LV fibrosis, level of TGF-β1, and protein expression of phosphorylated nuclear factor kappa B [57]. Earlier, Tumur *et al.* [58] have shown in-vitro that indoxyl sulfate induces NADPH oxidase in human vascular endothelial cells, releasing free radicals and inhibiting NO production and cell viability. The same results were obtained in our study as indoxyl sulfate was significantly and positively correlated with NADPH oxidase and TGF-β1.

Moreover, arterial blood pressure was increased significantly in nephrectomized and probiotics-treated rats compared with control rats. In addition, MBP had positive significant correlation with indoxyl sulfate and creatinine. However, symbiotic treatment lowered the arterial blood pressure significantly compared with nephrectomized group but nonsignificantly compared with control group.

CKD is characterized by development of two distinct arterial diseases, which is atherosclerosis and arteriosclerosis with predominant calcification [59]. Intimal calcification is associated with ischemic heart disease, whereas medial calcification increases vascular stiffness, systolic hypertension, and LV hypertrophy [60,61]. Recently, Li *et al.* [62] demonstrated that transferring fecal material from hypertensive patients to germ-free mice leads to elevation of systolic, diastolic, and mean blood pressures. The authors attributed this effect owing to absorption of microbes product that increased systemic inflammatory condition, helping occurrence of hypertension.Atherosclerosis is known to be the major cause of CVD in patients with CKD and is accelerated by the uremic state [63]. Indoxyl sulfate is suggested to be partially responsible for atherosclerosis in CKD by induction of inflammation and endothelial dysfunction. Tumur *et al.* [64] reported that indoxyl sulfate upregulated adhesion molecule expression by oxidative stress and leukocyte endothelial interaction *in vitro* and *in vivo* [65].

Arteriosclerosis in CKD is caused by premature arterial aging and diffuse thickening and stiffening of arterial walls [66]. LV hypertrophy and altered coronary perfusion are main consequences of arteriosclerosis [26]. Adijiang *et al.* [67] demonstrated in-vivo that indoxyl sulfate promotes aortic calcification and aortic wall thickening in hypertensive rats. Indoxyl sulfate was reported to inhibit endothelial proliferation and wound repair by increasing free radical production [68]. Moreover, indoxyl sulfate suppresses circulating endothelial progenitor cells derived from bone marrow (essential cells for neovascularization) by inhibiting hypoxia-induced HIF-1a activation and IL-10 and VEGF synthesis [69].

In this study, administration of symbiotic treatment was associated with lowering of indoxyl sulfate and arterial blood pressure to nearly normal values seen in control group. Markowiak *et al.* [70] found that mice supplementation with prebiotic (inulin) leads to reduction of atherosclerotic lesion decrease by 35% compared with mice fed a control diet. In addition, probiotic and prebiotic administration was known to modulate lipid metabolism and indirectly had beneficial effects in CVD [71,72].

In this study, improved arterial blood pressure and MFR on exposure to ischemia indicate decreased atherosclerosis and arteriosclerosis in symbiotic-treated groups. In addition, a negative significant correlation between indoxyl sulfate and both blood pressure and myocardial flow rate existed, indicating the role of indoxyl sulfate in controlling the arterial blood pressure.

Conclusion

This study concluded that the use of probiotic and symbiotic in reno-cardiac syndrome can protect the heart by improving the intestinal barrier, antioxidant effects, and decreasing indoxyl sulfate level. Symbiotic had better outcomes than probiotic. Early use of the treatment is more beneficial than late use.

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Conflicts of interest

There are no conflicts of interest.

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