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Evaluating the drug repurposing benefits of clopidogrel against adenine induced chronic renal failure in experimental animals

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ABSTRACT

Introduction: Chronic renal failure (CRF) is a life-threatening condition which can be defined as a progressive and irreversible loss of renal function associated with inflammation and oxidative stress having limited treatment options. The anti-platelet drug, clopidogrel showed nephroprotective action in 5/6 nephrectomy model, lithium induced nephrogenic diabetes insipidus and diabetes-induced renal fibrosis.

Objectives: In this study we evaluated the effect of clopidogrel on various serum, urine parameters linked with CRF, vascular calcification, electrolyte abnormalities, inflammatory markers and renal histology.

Materials and Methods: Seven groups of male Wistar rats were treated with saline normal control), adenine (50 mg/kg, disease control), clopidogrel (15, 30, 60 mg/kg) concomitantly with adenine (preventive regimen), clopidogrel 60 mg/kg from day 15th curative regimen) and acetylcysteine (50 mg/kg) in combination with taurine as standard drug (50 mg/kg) from day 15th in a 4-week model. Following the completion of the treatments, urine, blood, and kidneys were collected from all rats, and then various biochemical, oxidative, and histological parameters were estimated.

Results: Adenine administration decreased body weight and kidney function due to injury as indicated by increased markers like serum urea, uric acid, creatinine and potassium in all animals except normal control group. There was a significant difference between disease and test drug groups especially for 60 mg/kg of preventive and curative regimen for all the estimated parameters. Adenine administration caused histopathological alterations, significant reduction in antioxidant indices and initiated a fibrotic response in kidney by increasing the profibrotic protein like transforming growth factor-beta (TGF-β1).

Conclusion: The results suggest that clopidogrel treatment improves majority of the parameters in a dose-dependent manner and the renoprotective effect might be associated with its antioxidant and anti- fibrosis activity.

Implication for health policy/practice/research/medical education:
Chronic kidney disease is a progressive illness, becoming a significant concern of community and among the world's leading cause of mortality. Adenine elevates creatinine and urea level in the serum, causes proteinuria and fibrosis in animals. Furthermore, the model resulted in a substantial reduction in free amino acid and calcium concentrations, hyper-lipidaemia, and vascular calcification, all of which are characteristics of human CKD. Clopidogrel, an anti-platelet drug acts by inhibiting ADP- induced P2Y12 receptor activation and thus inhibits platelet activation and aggregation. Clopidogrel repurposed based on its lowering plasma levels of urea and creatinine, reduced blood glucose, collagen. Clopidogrel preventing a decrease in cellular cAMP levels, may have a significant ameliorative effect on adenine-induced CRF.

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Original Article

Introduction

Chronic kidney disease (CKD) characterized as renal damage that lasts longer than three months and has an average estimated glomerular filtration rate (eGFR) of less than 60 mL/min/1.73 m^2 (1). Cysts, cancers, malformations, and atrophy are examples of structural anomalies that can be seen on imaging. Kidney dysfunction, on the other hand, can cause hypertension, edema, changes in urine production or consistency, and development delays in juvenile. Almost all these changes are commonly identified by elevated serum levels of cystatin C, creatinine and urea (2). CKD is significant contributor to disease incidence across both established and emerging nations, affecting approximately 10% of several community and even exceeding 200 million people worldwide (3,4). Currently, there is no single treatment for patients with CKD who have impaired kidney function, while current therapeutic options are to delay the disease's progression that are restricted to maintenance of glucose, and blood pressure (5). As a result, innovative therapies to postpone or alleviate kidney function loss are urgently required. When stimulated with adenine, the adenine receptor is connected to the inhibitory G protein (G_i) and inhibits adenylyl cyclase (AC) activity, that results in reduction in intracellular cAMP output. The cAMP-dependent signaling pathway regulates numerous kidney water and electrolyte homeostatic purposes in several segments of nephron, especially proximal convoluted tubule (PCT), thick ascending limb, and collecting ducts (6). Clopidogrel, an anti-platelet drug acts by inhibiting ADP- induced P_2Y_{12} receptor activation and thus inhibits platelet activation and aggregation. The P_2Y_{12}-R is primarily found in blood platelets, as well as astrocytes and microglia in the brain. Moreover, P_2Y_{12} receptor is coupled to G_i/G_q; since, its activation by ADP inhibits AC and lowers cellular cAMP levels. Activation of this receptor also increases phosphoinositide signaling, which is essential for platelet activation and clot formation. Additionally, P2Y12 receptor mRNA expression was found in several regions of rodent kidney (7). Clopidogrel reduced histopathological score, decreased renal cell apoptosis and increased renal total antioxidant capacity (8-10). Based on the above results, we hypothesized that clopidogrel, by inhibiting the P_2Y_{12} receptor and preventing a decrease in cellular cAMP levels, may have a significant ameliorative effect on adenine induced chronic renal failure (CRF).

Acetylcysteine is most conducted as a mucolytic agent and to treat paracetamol poisoning. Since cysteine is a major component of glutathione, acetylcysteine helps to replenish glutathione stocks. Acetylcysteine with its antioxidant activity, aids to alleviate the effects of a wide range of diseases caused by reactive oxygen species (ROS). Acetylcysteine, for example, is often used to avoid the onset of acute kidney failure in people with renal impairment (11). Taurine is an amino acid found in a variety of organs that comes from the metabolism of methionine and cysteine. It acts as a human metabolite, an antioxidant, a glycine receptor agonist, a nutrient, and a radical scavenger (12). Various studies are reported for the administration of acetylcysteine and taurine alone or in combination that could have renoprotective effect against injuries. In animal model of fipronil induced injuries, combination of acetylcysteine and taurine prevented the oxidative damage caused by fipronil by normalizing the elevated malondialdehyde (MDA) and nitric oxide (NO) formation and reduced glutathione (GSH) concentration caused by fipronil in the renal and hepatic tissues (13). In a previous study of cisplatin induced nephrotoxicity, treatment with acetylcysteine and taurine preserved the kidneys from the changes in serum, urine, and tissue induced by cisplatin (14).

Objectives

In this study we evaluated the effect of clopidogrel on various serum, urine parameters linked with CRF, vascular calcification, electrolyte abnormalities, inflammatory markers and renal histology.

Materials and Methods

Chemicals

Adenine was purchased from Sigma-Aldrich, India. Deplatt (Clopidogrel tablets I.P.) and Mucomix (acetylcysteine tablets) were procured from CHARUSAT pharmacy, Gujarat, India. Taurine was purchased from Loba Chemie Pvt Ltd. Diagnostic kits for evaluation of serum creatinine, uric acid, urea, sodium, chloride, potassium, calcium, magnesium, SGPT (serum glutamic-pyruvic transaminase) and SGOT (serum glutamic-oxaloacetic transaminase) and gamma-glutamyl transferase (GGT) kits from serum were purchased from coral clinical systems - tulip diagnostics Pvt Ltd. ELISA kit for estimation of transforming growth factor-β (TGF-β1) from serum was purchased from Elabscience.

Animal

Male Wistar albino rats were procured from Jai Research Foundation, Vapi, India. All the animals were housed in polypropylene cage with three animals per cage and pelleted corn cob was used as the bedding material. Prior to commencement of treatment, all the animals were habituated to laboratory conditions for one week with temperature maintained at 25 ± 2°C and relative humidity at 30-70%. 12-hour light and 12-hour dark cycle was maintained. Standard laboratory rat pellet and pure drinking water was supplied.

Experimental design

Molecular docking

Molecular docking of clopidogrel was performed by using PDB code; 4NTJ which represents three-dimensional crystal structure of human P2Y12 receptor.

Acute oral toxicity study

The study was carried out by using male Wistar rats according to the OECD (organization for economic cooperation and development) guideline 423. A total of six male rats were conducted for the study and were divided into two groups, i.e. 1000 mg/kg and 2000 mg/kg with three animals in each group.

Induction of chronic renal failure

The animals were randomly assigned to two groups after the acclimatization stage was over; preventive regimen and curative regimen. The preventive regimen has been further grouped into:

- Group I- Normal control group (n=6): Animals in this group received normal saline orally once daily for 28 days.
- Group II- Disease control group (n=10): Animals in this group received 50 mg/kg adenine suspended in 0.5% sodium carboxymethyl cellulose (Na CMC) administered once daily for 28 days.
- Group III- Low dose clopidogrel (15 mg/kg): Animals in this group received 50 mg/kg adenine and clopidogrel (15 mg/kg) suspended in 0.5% Na CMC administered once daily for 28 days.
- Group IV- Medium dose clopidogrel (30 mg/kg): Animals in this group received 50 mg/kg adenine and clopidogrel (30 mg/kg) suspended in 0.5% Na CMC administered once daily for 28 days.
- Group V- High dose clopidogrel (60 mg/kg): Animals in this group received 50 mg/kg adenine and clopidogrel (60 mg/kg) suspended in 0.5% Na CMC administered once daily for 28 days.

Curative regimen was further divided into following groups:

- Group I- High dose clopidogrel (60 mg/kg): Animals in this group received 50 mg/kg adenine suspended in 0.5% Na CMC administered once daily for 28 days and clopidogrel (60 mg/kg) suspended in 0.5% Na CMC administered once daily from day 15th to 28th days.
- Group II- Standard control group: Animals in this group received 50 mg/kg adenine suspended in 0.5% Na CMC administered once daily for 28 days and acetylcysteine (50 mg/kg) suspended in 0.5% Na CMC and taurine (50 mg/kg) administered once daily from day 15th to 28th days.

Sample collection

On 15th day, retro-orbital blood collection was performed under the anesthetic condition from animals of group I and II of preventive regimen and curative regimen for estimating serum creatinine and urea for confirming the development of CKD. All the rats were individually kept in metabolic cages for 24 hours to collect urine at the end of the study (28 days) and had unlimited access to water during collection hours. After collection of urine, urine was centrifuged at 6000 rpm for 8 minutes and supernatant was collected, to which 1-2 drops of concentrated hydrochloric acid were added and was stored at 4°C for analysis of urinary parameters such as urine urea nitrogen and creatinine. Retro-orbital blood collection was conducted under the anesthetic condition for estimation of complete blood count and serum biochemical analysis. For serum separation, blood was centrifuged at 6000 rpm for 15 minutes and separated serum was stored at -20°C for analysis of serum biochemical parameters. After urine and blood collection, animals were sacrificed using the high dose of anesthetic agent and both the kidneys were isolated. One kidney was fixed in 10% formalin for histopathological evaluation and another kidney was crushed in phosphate buffer saline for preparation of tissue homogenate for estimation of antioxidant parameters.

Complete blood count

Complete blood count was measured by using the CELL-DYN ruby hematology analyzer.

Histopathology analysis

The kidney was fixed in 10% formalin solution and then it was dehydrated using the ethanol solution then it was cleared through xylene solution then it was embedded in paraffin. Accordingly, 2 µm section of kidney tissue cut from the paraffin block by microtome and then it was stained with hematoxylin and eosin (H&E).

Estimation of biochemical and oxidative parameters

Various biochemical parameters like creatinine (alkaline picrate method), blood urea nitrogen (modified Berthelot method), uric acid (uricase/PAP method), calcium (OCPC method), phosphorus (molybdate U.V. method), magnesium (Calmagite method), sodium, chloride, potassium, SGOT (Reitman & Frankel's method), SGPT (Reitman & Frankel's method), γ-glutamyl transferase (GGT) (carboxy substrate method) were estimated using coral clinical system kits as per the manufacture instructions. The TGF-β1 estimated with Elabscience ELISA kit. Different oxidative parameters like reduced GSH (15), MDA (16), superoxide dismutase (SOD) (17), and catalase were estimated as per the reported methodology.

Statistical analysis

All the data were analyzed by using GraphPad Prism version 8.4.2 and data are expressed as mean ± SEM (n=6). One-way ANOVA was used to compare disease control group with all the other groups and the *P* value of less than 0.05 was considered significant.

Results

Molecular docking

The amino acid in green indicates Van der Waals interaction with target (Figure 1). The other amino acid indicates Pi-Pi and Pi-alkyl interaction with target. Clopidogrel binds to the target with docking score of -6.37. Based on the binding energy we can say that clopidogrel binds to the target efficiently.

Acute oral toxicity study

In this study, single oral dose of clopidogrel at 1000 mg/kg, animals did not show any mortality whereas one animal administered with dose of 2000 mg/kg died on the 2nd day of the study and one animal showed diarrhea and secretion of porphyrin surrounding the nose. Any of the treated rats in 1000 mg/kg group did not show any detectable signs of toxicity or behavioral changes and were found to be normal across the 14-day study period. Body weight was constant during the study period.

Disease confirmation

To confirm the induction of disease, serum creatinine and urea were estimated on 15th day. On comparing with normal control, the serum levels were increased in all the other three groups (Table 1). These increase in critical renal indices indicated disease induction in animals.

Effect of clopidogrel on physiological parameters

A significant decrease in body weight (Table 2) and elevation in urine output (Figure 2) of rats in disease control group in comparison to normal control group. Treatment with C-60 was most effective and increased the weight and reduced urine output of rats significantly.

Effect of clopidogrel on various serum renal function indices

The adenine treated group showed significant elevation in serum creatinine, urea and uric acid levels (Figure 2). The clopidogrel treatment group showed dose dependent reduction in serum creatinine and uric acid levels and curative standard and clopidogrel treated group also showed significant decrease in above mentioned parameters.

Effect of clopidogrel on various urine renal function indices

Significantly low levels of urinary creatinine and urea were reported in DC on comparison with normal control group (Figure 2). Dose dependent elevation in urine creatinine and urea levels were found in clopidogrel treated group. Their levels were also significantly high in curative clopidogrel and standard treated group.

Effect of clopidogrel on serum electrolytes

The adenine treated group showed substantial elevation in serum sodium, potassium and chloride levels (Figure 3). The clopidogrel treatment group showed dose dependent decrease in serum potassium and chloride levels and curative standard and clopidogrel treated group also showed significant decrease in above mentioned parameters.

Effect of clopidogrel on serum vascular calcification parameters

There was substantial reduction in calcium levels and significant rise in phosphorus and magnesium levels of disease control group (Figure 3). Dose dependent increase in calcium and dose dependent decrease in phosphorus

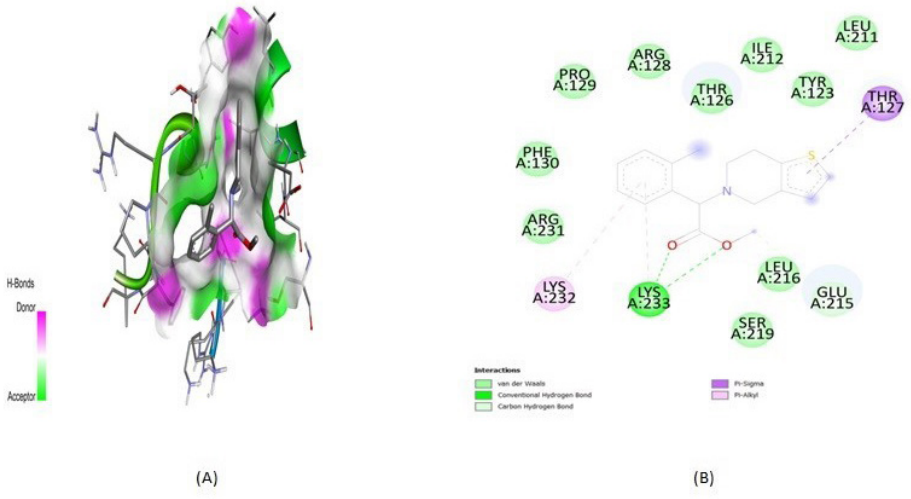

Figure 1. (A) 3-D interaction and (B) 2-D interaction.

Table 1. Confirmation of disease induction on 15th day

Parameters	Groups			
	NC	DC	CC-60	STD
Creatinine	0.57±0.03	1.19±0.08**	1.02±0.11**	1.03±0.04**
Urea	9.32±0.56	15.41±1.28**	15.64±0.68**	15.27±0.69**

Values in the table are mean ± SEM (n=6). * P<0.05 comparison of disease versus different treatment groups, ** P<0.001 comparison of disease versus different treatment groups, ## P<0.001 comparison of NC versus DC group.

Table 2. Effect of clopidogrel on body weight, liver function and anemia indicator

Parameters	Groups						
	NC	DC	C-15	C-30	C-60	CC-60	STD
Body weight	17.12 ± 2.57	-15.76 ± 2.83##	-4.32 ± 4.79	-12.64 ± 5.40	22.86 ± 3.4**	5.38 ± 1.32**	11.12 ± 2.01**
SGOT	43.58 ± 5.55	59.42 ± 9.35	53.33 ± 5.65	36.67 ± 4.74*	21.08 ± 2.96**	50.42 ± 4.25	31.82 ± 5.87*
SGPT	26.00 ± 2.78	35.33 ± 1.23	29.83 ± 3.27	28.67 ± 2.43	27.33 ± 1.80	31.50 ± 3.34	31.00 ± 1.69
GGT	0.69 ± 0.15	1.62 ± 0.39##	1.16 ± 0.41	0.81 ± 0.18*	0.93 ± 0.19*	0.93 ± 0.24*	1.27 ± 0.31
RBC	6.67 ± 0.08	5.08 ± 0.13##	7.96 ± 0.04**	9.18 ± 0.22**	7.16 ± 0.52**	6.52 ± 0.32*	7.97 ± 0.08**
HB	12.23 ± 0.16	9.21 ± 0.35##	14.19 ± 0.24**	15.62 ± 0.43**	13.02 ± 0.64**	11.43 ± 0.50*	14.22 ± 0.21**
HCT	34.08 ± 0.24	26.44 ± 0.97##	38.93 ± 0.69**	43.93 ± 1.53**	35.10 ± 2.27**	33.29 ± 1.45*	39.50 ± 0.74**

Values in the table are mean ± SEM (n=6). * P<0.05 comparison of disease versus different treatment groups, ** P<0.001 comparison of disease versus different treatment groups, ## P<0.001 comparison of NC versus DC group.
SGOT: Serum glutamic oxaloacetic transaminase, SGPT: Serum glutamic pyruvic transaminase, GGT: Gamma-glutamyl transferase, RBC: Red blood cell, HB: Hemoglobin, HCT: Hematocrit.

and magnesium levels was observed in clopidogrel treated group. Levels of these parameters were found near to normal group for curative clopidogrel and standard group.

Effect of clopidogrel on serum liver indices

No relevant difference between levels of SGOT and SGPT of disease group statistically, in comparison to normal

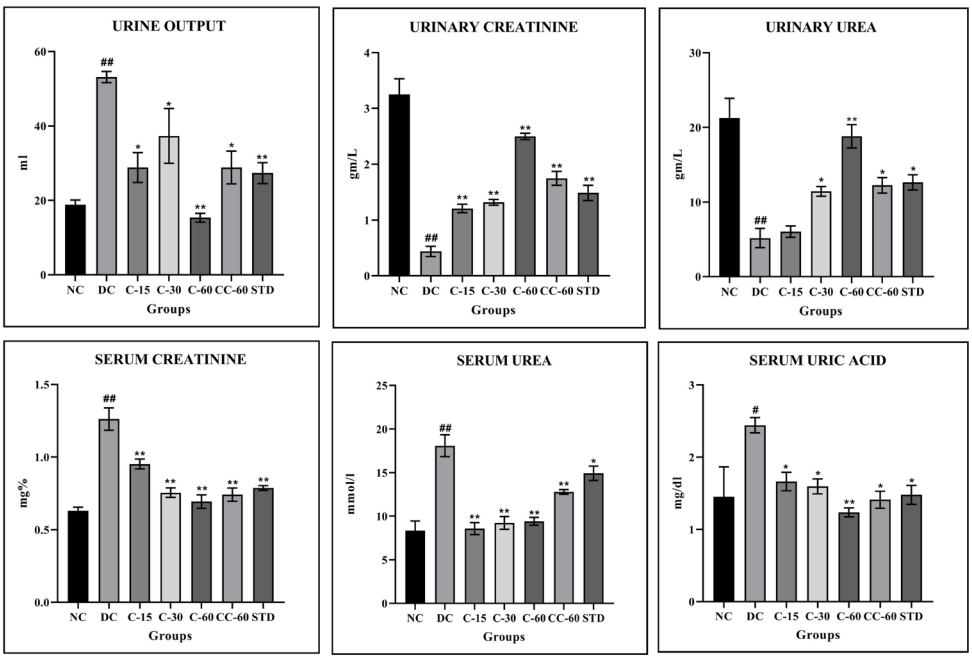

Figure 2. Effect of clopidogrel on serum and urinary renal function. Columns and vertical bars depict mean ± standard error of the mean (SEM) (n=6). NC: Normal Control, DC: Disease Control (50 mg/kg Adenine), C-15: 15 mg/kg clopidogrel + Adenine, C-30: 30 mg/kg Clopidogrel + Adenine, C-60: 60 mg/kg clopidogrel + Adenine, CC-60: 60 mg/kg clopidogrel from day 15 + Adenine, STD: 50 mg/kg acetylcysteine and 50 mg/kg taurine from day 15 + Adenine. * P<0.05 comparison of disease versus different treatment groups, ** P<0.001 comparison of disease versus different treatment groups, # P<0.05 comparison of NC versus DC group, ## P<0.001 comparison of NC versus DC group.

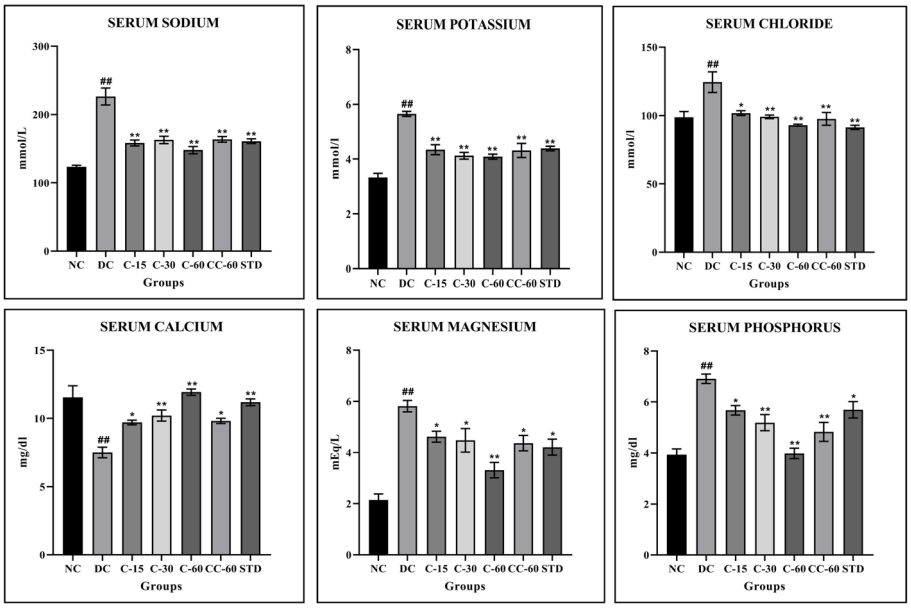

Figure 3. Effect of clopidogrel on serum electrolytes and serum vascular calcification. Columns and vertical bars depict mean ± standard error of the mean (SEM) (n=6). NC: Normal Control, DC: Disease Control (50 mg/kg Adenine), C-15: 15 mg/kg clopidogrel + Adenine, C-30: 30 mg/kg Clopidogrel + Adenine, C-60: 60 mg/kg clopidogrel + Adenine, CC-60: 60 mg/kg clopidogrel from day 15 + Adenine, STD: 50 mg/kg acetylcysteine and 50 mg/kg taurine from day 15 + Adenine. * P<0.05 comparison of disease versus different treatment groups, ** P<0.001 comparison of disease versus different treatment groups, # P<0.05 comparison of NC versus DC group, ## P<0.001 comparison of NC versus DC group.

group was observed. Significant elevation of GGT levels were reported in disease which were decreased significantly on treatment (Table 2).

Effect of clopidogrel on anemia indicator

Significantly low levels were reported in disease group on comparison with normal control group (Table 2). Significant increase in levels of red blood corpuscles, hemoglobin and hematocrit were found in clopidogrel treated group. Their levels were also significantly higher in curative clopidogrel and standard treated group.

Effect of clopidogrel on oxidative stress markers

Oxidative stress markers like catalase, GSH and SOD levels significantly reduced in disease control group in comparison with normal control group (Table 3). Clopidogrel at the dose of 60 mg/kg substantially elevated the levels of above-mentioned markers. Serum MDA levels were significantly high in disease control group and there was dose dependent decrease in MDA levels were observed.

Effect of clopidogrel on serum TGF-β1

Disease group showed substantial rise in serum TGF-β1 levels in comparison to NC group (Table 4). Dose dependent decrease in serum TGF-β1 levels was seen. Curative regimen also significantly decreased serum TGF-β1 levels.

Effect of clopidogrel on gross morphology and histopathology of kidney

Gross morphology of the kidney of normal rats appeared normal in size, shape and color. The kidney of rats in disease control group showed discoloration with white spots on kidney. Kidney at the treatment dose of C-60 and CC-60 appeared almost normal in color, size and

Table 3. Effect of clopidogrel on oxidative stress markers

Parameters	Groups						
	NC	DC	C-15	C-30	C-60	CC-60	STD
Catalase	398.63 ± 11.39	136.67 ± 8.82$^{\#}$	324.60 ± 11.67*	324.60 ± 7.64*	387.25 ± 22.78*	358.77 ± 22.92*	353.07 ± 25.97*
GSH	35.53 ± 1.38	9.41 ± 0.60$^{\#\#}$	17.50 ± 1.95	19.76 ± 3.70*	25.96 ± 3.68**	21.66 ± 2.00*	31.96 ± 1.51**
MDA	29.74 ± 3.91	76.96 ± 4.20$^{\#\#}$	33.51 ± 3.85**	16.59 ± 3.76**	13.51 ± 1.57**	31.78 ± 3.03**	12.77 ± 1.17**
SOD	154.90 ± 6.56	24.51 ± 1.81$^{\#\#}$	63.73 ± 17.34	66.67 ± 11.53*	82.35 ± 12.52*	46.08 ± 10.78	33.33 ± 6.38

Values in the table are mean ± SEM (n=6). * P<0.05 comparison of disease versus different treatment groups, ** P<0.001 comparison of disease versus different treatment groups, # P<0.05 comparison of NC versus DC group, ## P<0.001 comparison of NC versus DC group.

Table 4. Effect of clopidogrel on TGF-β1

Parameters	Groups						
	NC	DC	C-15	C-30	C-60	CC-60	STD
SOD	1.15 ± 0.06	5.20 ± 0.35##	3.75 ± 0.31*	2.58 ± 0.27**	1.50 ± 0.22**	2.73 ± 0.23**	2.43 ±0.08**

Values in the table are mean ± SEM (n=6). * P<0.05 comparison of disease versus different treatment groups, ** P<0.001 comparison of disease versus different treatment groups, ## P<0.001 comparison of NC versus DC group.

shape (Figure 4). Kidney section stained with H&E from normal control group showed normal histology of renal tissue such as the normal architecture of renal distal convoluted tubule (DCT), renal PCT and renal glomeruli. Kidney section from the disease control group showed larger area of renal injury and irregular architecture of renal tissue such as loss of brush border of proximal tubules, inflammatory cells infiltrations, adenine crystal deposition, several renal tubule dilation, tubular atrophy, and thickening of Bowman's capsules and degeneration of glomeruli. Treatment with clopidogrel reduced these abnormal histopathological renal changes that occurred in disease control group (Figure 4).

Discussion

The kidney has a crucial function in maintaining fluid and electrolyte balance as well as removing waste, releasing hormones to regulate blood pressure and stimulate red blood cell output, and activating vitamin D to preserve bone health (18). Since adenine and its metabolite 2,8-dihydroxyadenine (DHA) are less soluble, their precipitation occurs in the kidney tubules, occluding them and causing inflammation, oxidative stress, and kidney dysfunction (6). The adenine model is more reproducible, simpler to perform and causes structural and functional alterations which are close to human CKD than 5/6th nephrectomy model (19). In our study, the weight of rats in DC group was significantly lower in comparison to rats of NC group. These changes may be due to the accumulation of uremic toxins, which cause inflammation and activate protein catabolic pathways, resulting in protein degradation, in addition to polyuria and dehydration (20). Treatment with CC-60 remarkably prevented the lowering of body weight and polyuria but,

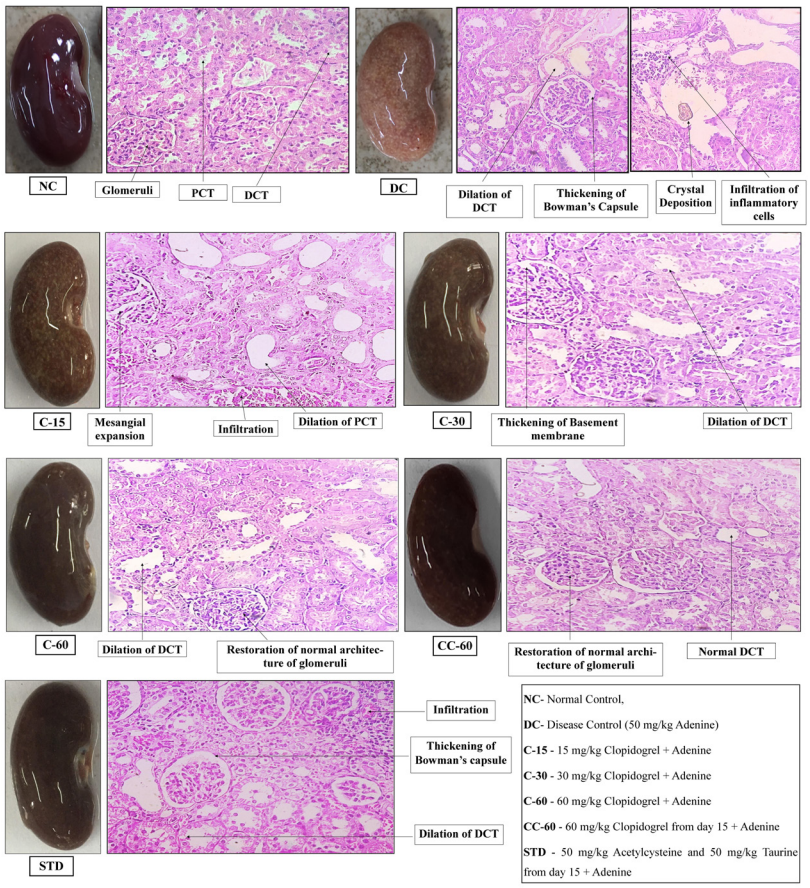

Figure 4. Effect of clopidogrel on renal morphology and renal histology.

was less effective as compared to standard. This protective effect of standard treatment can be due to its ability to reduce loss of water from the body.

Common renal function indices including serum urea, creatinine, and uric acid concentrations have long been established as markers of impaired kidney function. The enzyme xanthine oxidase converts adenine to uric acid, which raises uric acid levels in the bloodstream (21). As a result of impaired urea and creatinine renal excretion, adenine causes serum urea, creatinine, and uric acid elevations, suggesting glomerular filtration rate decrease. Treatment with clopidogrel reduced the elevations of these indices in a dose-dependent manner. Treatment with CC-60 showed similar effectiveness like standard in reducing the levels of creatinine and uric acid in serum while CC-60 was more effective than standard in reducing the levels of urea in serum.

Vascular calcification, a metabolic disorder characterized by hyperphosphatemia, hypocalcemia, and hypermagnesemia, is often associated with CKD (22). In hemodialysis patients, vascular calcification is higher risk factor for CRF and is linked to elevated risk of irregular cardiovascular events (23). In the current study, rats treated only with adenine exhibited hyperphosphatemia, hypermagnesemia with hypocalcemia. By reacting with bioavailable calcium to form CaHPO4, high serum phosphate levels lead to acidosis and promote more hypocalcemia. Furthermore, impaired renal function suppresses renal vitamin D activation and, as a result, reduce calcitriol production, resulting in no or insufficient calcium absorption in the intestine, as well as a lack of calcitriol effect on parathyroid hormone (PTH) actions on bone and kidney to boost plasma Ca levels. It is well known that CKD can cause an increase in circulating magnesium, which can cause cardiovascular problems, as well as a decrease in PTH secretion, which causes hypocalcemia (21). Treatment with clopidogrel improved indicators of vascular calcification in a dose dependent manner, suggesting a protective role in the associated complication of CKD. Treatment with CC-60 was less effective in reduction of calcium levels while it was more effective in reducing phosphorous levels as compared to standard.

In our research, significant elevations in the levels of serum sodium, chloride and potassium were reported in the adenine treated group in comparison to normal group. The inability of glomeruli to filter the electrolytes or increased tubular reabsorption could be the mechanism involved in elevated levels of these electrolytes. Treatment with C-60 significantly reduced the elevated serum levels of electrolytes. Also, no remarkable difference was detected between CC-60 and standard treatment in reducing levels of sodium, potassium and chloride. No remarkable difference in liver function indices was detected in adenine treated group in comparison to normal group.

Renal anemia occurs due to defective erythropoietin (EPO) production by renal EPO-producing cells in the interstitial, which occurs in conjunction with renal function decline (24). In our study, adenine administration substantially reduced red blood cell count, hemoglobin and hematocrit levels whereas clopidogrel therapy enhanced anemic markers, reducing adenine-induced renal anemia symptoms. Treatment with CC-60 significantly increased level of anemic markers near to NC group but was less effective than standard. The effects may be attributed to the renoprotective effect of clopidogrel thereby improving erythropoietin levels which accelerates erythropoiesis.

Increasing evidence suggests that upregulation of TGF-β and production of ROS, is common in CRF patients and may contribute to the disease's occurrence due to damage or death of renal cells (25). Moreover, DHA precipitated crystals, an adenine metabolite, can increase the development of ROS such as peroxides and superoxide anion radicals, resulting in oxidative stress. When ROS are produced because of adenine-induced tissue injury and are not removed, they attack various cell components such as DNA, RNA, proteins, lipids, and enzymes, causing glomerular disease, kidney ischemia, perfusion injury, and ultimately kidney failure (6). Adenine treated group showed decreased in levels of SOD, suggesting increase in superoxide radical presence in kidney. GSH levels were also decreased which acts as radical scavenger and MDA levels were elevated in adenine treated group. Treatment with clopidogrel provide dose dependent elevation in levels of catalase, SOD and GSH and dose dependent reduction in MDA. Treatment with CC-60 significantly increased level of catalase similarly as standard but for GSH it was less effective as compared to standard. No significant increase in SOD levels were observed by treatment with CC-60 and standard. Levels of MDA were significantly decreased by treatment with CC-60 as compared to DC group and was equally effective as standard. The protective effect by the standard treatment may be due to its ability to scavenging of free radicals, forming less reactive molecule or by increasing synthesis of GSH.

TGF-β1 is an essential and efficient fibrogenic factor that plays a role in each stage of renal interstitial fibrosis, according to various studies (25). All renal cell types, including proximal tubular cells, mesangial cells, podocytes, and interstitial fibroblasts, react to profibrotic triggers of TGF-β1 and lead to renal fibrosis by enhanced expression of ECM molecules, especially collagens and fibronectin. TGF-β1 is the most plentiful isoform detected and formed by all forms of renal resident cells, and it is well established as a key mediator in renal fibrosis. The

serum levels of TGF-β1 were substantially increased in adenine treated group and on treatment dose dependent decrease TGF-β1 levels were observed. Treatment with CC-60 significantly and equally reduced level of TGF-β1 as standard.

The kidney of the rats in normal control group showed regular histology and architecture. The loss of the brush border of proximal tubules, inflammatory cell infiltrations, adenine crystal deposition, many renal tubule dilation, tubular atrophy, thickening of bowman capsules, and glomeruli degeneration were all seen histologically in the kidneys of adenine-treated animals which were consistent with results of other studies (19). Excessive adenine crystal aggregation in the renal tubules has been linked to renal tubule damage and the activation of inflammatory responses (8). Animals treated with C-60, CC-60 and standard treatment showed good architecture of renal tissue in comparison to disease control which is further supported by improvement in various serum and urine parameters.

Conclusion

From the study, we can conclude that clopidogrel at the dose of 60 mg/kg ameliorated kidney damage caused by adenine which is evidenced from the physicochemical, biochemical, oxidative stress and histopathological examination. The mechanism for this protection can be due to reduction of oxidative stress and reduction of renal fibrosis by decreasing the expression of TGF-β. The drug repurposing of clopidogrel open the door for alternative option of chronic kidney failure in animal study. More detail work needs to be done to explore the extrapolation of this study results in the clinical setting.

Limitations of the study

Kidneys involved in multiple homeostatic mechanism of maintaining blood pressure, red blood cells population. Parameters to estimate influence on blood pressure and renal anaemia can need to be evaluated. Detailed investigation is needed to establish the molecular mechanism of clopidogrel's function in CRF. There is need to conduct cohort and retrospective studies to explore the potential of clopidogrel at clinical condition.

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Authors' contribution

Conceptualization: AP and SP.
Methodology: AP and SP.
Validation: RH and PP.
Formal analysis: RH and PP.
Investigation: AP, RH and PP.
Data curation: AP, RH and PP.
Writing—original draft preparation: AP and PP.
Writing—review and editing: RH.
Visualization: AP and PP.
Supervision: AP.
Project administration: AP.
Funding acquisition: RH.

Conflicts of interest

The authors declare that they have no competing interests.

Ethical issues

The research and the protocol of this study was in accordance with the guidelines of animal studies and was approved by institutional animal ethical committee (IAEC; Protocol No: RPCP/IAEC/2020-21/R1). Accordingly, we conducted animal experiments as per the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), 2018, India. Besides, ethical issues (including plagiarism, data fabrication, double publication) have been completely observed by the authors.

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