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Synergistic protective activity of sodium hydrosulfide and L-arginine against cisplatin-induced nephrotoxicity even during nitric oxide synthase inhibition

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Highlights

- Cisplatin treatment significantly impaired renal function and increased creatinine, BUN, and oxidative stress.
- Administration of L-NAME exacerbated the cisplatin-induced nephrotoxicity.
- Co-administration of NaHS and L-arginine improved renal function parameters, reduced oxidative stress, and mitigated biochemical deterioration.
- These protective effects persisted even when NO synthesis was inhibited (via L-NAME), suggesting an alternative mechanism involving H_2S.

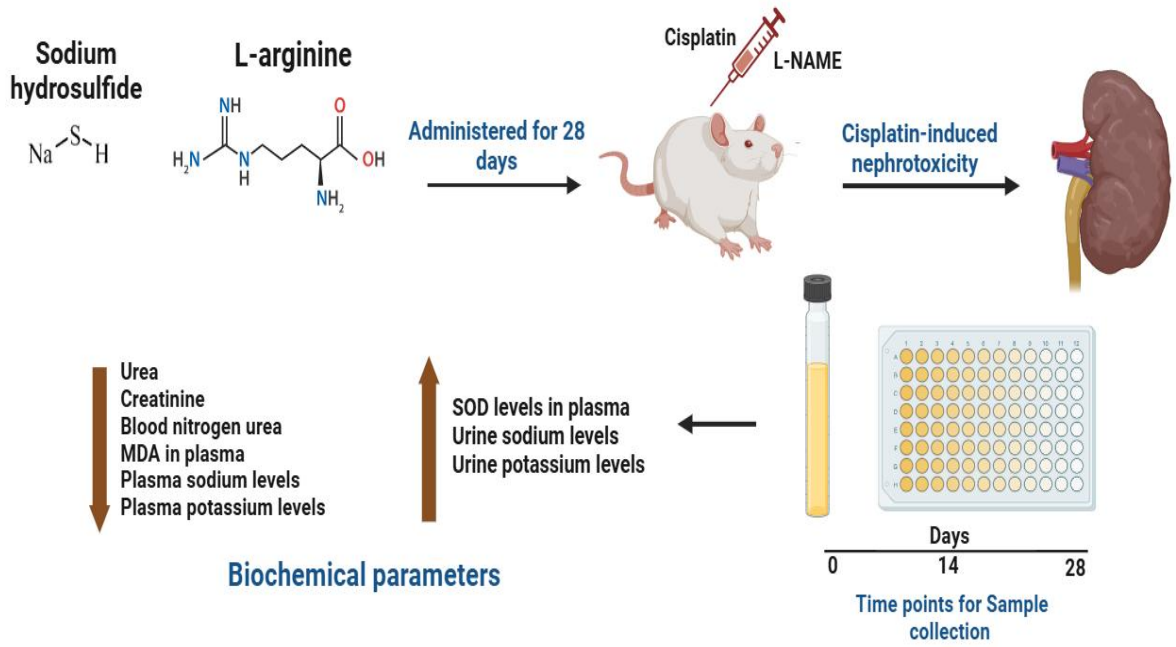
Abstract

Background: Cisplatin is a widely used chemotherapeutic drug known to induce nephrotoxicity through inflammation and oxidative stress. Hydrogen sulfide (H_2S) and nitric oxide (NO) are gaseous signaling molecules with cytoprotective potential in renal damage. The current study aimed to evaluate the nephroprotective potential of sodium hydrosulfide (NaHS), an H_2S donor, and L-arginine, a NO precursor, against cisplatin-induced nephrotoxicity, even under nitric oxide synthase (NOS) inhibition.

Methods: The Wistar rats were intraperitoneally treated with cisplatin (5 mg/kg) to induce nephrotoxicity while administered with L-NAME, sodium hydrosulfide (NaHS), a donor of H_2S, and L-arginine, a source of nitric oxide (NO), either alone or in combination for 28 days. Renal function was assessed by monitoring the various parameters, including body weight and urinary flow. Moreover, H_2S, NO, creatinine level and clearance, blood urea nitrogen (BUN), electrolytes (sodium and potassium) levels, and oxidative stress were monitored in body fluids.

Results: The findings revealed that L-NAME exacerbated the cisplatin-induced nephrotoxicity which was evident from reduced weights ($P<0.0001$) and elevated urine output ($P<0.01$), H_2S ($P<0.0001$), NO ($P<0.0001$) and creatinine ($P<0.01$), BUN ($P<0.0001$) levels along with reduced, sodium and potassium ($P<0.0001$) and elevated oxidative stress markers ($P<0.001$) in plasma, compared to healthy rats. The treatment of rats with NaHS and L-arginine markedly protected from L+NAME + cisplatin-induced nephrotoxicity as the parameters were reinstated including urine output ($P<0.01$), H_2S ($P<0.01$), NO ($P<0.0001$) and creatinine ($P<0.05$), BUN ($P<0.01$) levels, compared to L+NAME + cisplatin rats. Moreover, the co-administration of NaHS + L-arginine restored the sodium ($P<0.0001$) and potassium ($P<0.01$) levels in plasma and mitigated oxidative stress ($P<0.05$).

Conclusion: The results suggested that H_2S and NO mitigated L-NAME + cisplatin-induced nephrotoxicity by ameliorating the L-NAME + cisplatin-induced oxidative stress.



Keywords: Cisplatin, nephrotoxicity, hydrosulfide, nitric oxide, LNAME (Nitro-L arginine methyl ester).

1. Introduction

Nephrotoxicity and renal disorders affect millions of people globally, resulting in the overburdening of the healthcare system[1] (Luyckx *et al.*, 2018). Acute kidney injury often results from the use of non-steroidal anti-inflammatory drugs (NSAIDs), aminoglycosides, and cisplatin and may involve impaired elimination of metabolic waste products, a reduced capacity to maintain pH, fluid and electrolyte balance, and a decreased glomerular filtration rate[2] (Perazella and Rosner, 2022). The acute condition invariably has a sudden, unexpected onset and may progress to chronic kidney disease. The prolonged hospitalization and increased dependence on dialysis exert a significant financial strain in developing countries[3] (Jha *et al.*, 2023). Despite the progress, there is a need to explore the novel nephroprotective strategies to counter drug-induced nephrotoxicity, one of the challenges faced in patients undergoing chemotherapy and other essential treatments.

Hydrogen sulfide (H_2S) has emerged as an important gastrotransmitter, and it is endogenously produced by three enzymes, namely, cystathionine γ-lyase (CSE), cystathionine β-synthase (CBS), and 3-mercapto-pyruvate sulfurtransferase[4] (Li *et al.*, 2021). H_2S exerts cytoprotection by modulating the cytokine secretion and mitigating the inflammation and regulating cellular autophagy[5] (Wallace *et al.*, 2015). In this context, NaHS, a donor of H_2S, has also been known to decrease autophagy and enhance cell survival[6] (Hu *et al.*, 2020). In the renal system, H_2S plays a crucial role in vasodilation and glomerular filtration through the inhibition of tubular sodium/potassium ATPase and the sodium/potassium/chloride cotransporter, thereby instigating diuresis. H_2S production is extensively decreased during renal pathophysiology, and its

supplementation may have beneficial activity in hypertension and reduced kidney function[7] (Bełtowski, 2010). Baseline renal H_2S is important to sustain the renin-angiotensin system partly by modifying cAMP production and via regulation of reactive oxygen species[8] (Xue *et al.*, 2013). The enzymes implicated in the production of H_2S are lacking in the epithelial cells of glomeruli and are present in vascular endothelial cells and renal proximal tubule cells. The production is sufficient to fulfill the requirements of glomerular epithelial cells[9] (Lee *et al.*, 2012).

Nitric oxide (NO) is synthesized from L-arginine and plays a crucial role in renal physiology.[10](Lee, 2008). NO is biosynthesized by nitric oxide synthase (NOS) by neuronal NOS endothelial NOS (eNOS), neuronal NOS, and inducible NOS (iNOS)[11] (Förstermann and Sessa, 2011). NO exerts a nephroprotective role as it is a potent vasodilator and maintains the renal perfusion and oxygen supply as well as improves the glomerular filtration rate[12] (Blantz *et al.*, 2002). The literature broadly reports its significance in preventing acute renal damage by countering the hypoxia and oxidative stress while preserving mitochondrial health and endothelial function[13] (Araujo and Welch, 2006). The L-arginine analogue, L-N^G-nitro-L-arginine methyl (L-NAME), is a non-specific NOS inhibitor and prevents synthesis of NO by all three isoforms. The inhibition of NOS by L-NAME exacerbates the cisplatin-induced nephrotoxicity by reducing renal blood flow while increasing oxidative stress and inflammation[14] (Pfeiffer *et al.*, 1996).

In the current study, the nephroprotective potential of sodium hydrosulfide (NaHS), an H_2S donor, and L-arginine, a NO precursor, was tested in a rat model of cisplatin-induced nephrotoxicity, which was exacerbated by co-administration of L-NAME. The Wistar rats were subjected to test treatments followed by the collection of urine and blood samples to estimate H_2S and NO levels along with other biochemical parameters to understand the impact of test treatments on renal function.

2. Material and methods

2.1.Chemicals

Analytical grade chemicals were obtained from the following sources: cisplatin (Pharmax Life Sciences), 2,2-diphenyl-1-picrylhydrazyl (DPPH), sodium hydrosulfide, L-arginine, L-NAME, ferric chloride, N, N-2-dimethyl-p-phenylene-diamine sulfate (DMPD), trichloroacetic acid (TCA), thiobarbituric acid (TBA), butylated hydroxytoluene (BHT), potassium ferricyanide, sodium nitrite, linoleic acid, NADPH, sodium nitrate, and all other chemicals were obtained from Sigma-Aldrich USA). Ketamine from Global Pharmaceuticals (Pakistan), and xylazine from Prix Pharmaceuticals (Pakistan) were obtained.

2.2.Animals and their housing

A total of 36 male Wistar rats weighing 200-250g were used in this study. The rats were randomly assigned to six groups, each comprising 6 animals. The rats were accommodated under hygienic housing conditions maintained at 24.0 ± 4.0°C with a humidity >66% in the vivarium of the Department of Pharmacology, Faculty of Pharmacy, the Islamia University of Bahawalpur. All

animals were provided with a standard diet and water *ad libitum,* under a 12h-12h light-dark cycle. The animals were treated according to the Guide for the Care and Use of Laboratory Animals of the National Institute of Health, and the study design was reviewed and approved by the Pharmacy Animal Ethics Committee, the Islamia University of Bahawalpur, with approval number PAEC/2020/31.

2.3. In-Vitro Assays

2.3.1. DPPH assay

The radical scavenging potential of NaHS (3.125 – 100 µM) and L-arginine (0.075 – 2.4 mg/mL) was determined using DPPH assay[15] (Ahmad *et al.*, 2015). An equal volume of test compound was mixed with DPPH solution (0.2 mM in ethanol) followed by incubation at 37 ºC for 30 min. The absorbance was checked at 517nm, and % inhibition was calculated through the following formula using BHT as standard compound.

% inhibition = [1 - (Absorbance of sample − Absorbance of blank)/Absorbance of control] x 100.

2.3.2. Ferric thiocyanate assay

The anti-peroxidative potential of NaHS and L-arginine was performed through linoleic acid peroxidation assay[16] (Chapman and Mackay, 1949). The mixtures containing different concentrations of NaHS and L-arginine were incubated for 3 min at 37°C in the absence of light, and absorbance was measured at 500 nm to determine peroxide in the ferric thiocyanate assay. The % peroxide values (% POV) were calculated using the following formula:

$$\% \text{ POV} = \frac{\text{Absorbance of sample}}{\text{Absorbance of Control}} \times 100$$

2.3.3. Ferric reducing antioxidant power (FRAP) assay

The reducing power of NaHS and L-arginine was assessed by the reduction of ferric reducing antioxidant power assay based on the reduction of Fe^{3+} to $Fe^{2+,}$ forming Perl's Prussian blue complex[15] (Ahmad *et al.*, 2015). The intensity of the blue color was noted using a spectrophotometer and the results were expressed as ascorbic acid equivalents (µg/mg).

2.4. In-vivo experimentaion

The 28-day experimental protocol involved the following treatments:

Group 1: **Control**; received intraperitoneal (i.p.) normal saline once on the first day of study.
Group-2: **L-NAME**; received L-NAME (0.4 gm/litre) daily in the drinking water.
Group-3: **L-NAME + cisplatin**; received L-NAME (0.4 gm/litre) daily in the drinking water and administered with cisplatin (5.0 mg/kg; i.p.) on the first day of study.

Group-4: **L-NAME + cisplatin + NaHS**; received L-NAME (0.4 gm/litre), cisplatin (5.0 mg/kg; i.p.) and NaHS (56 µmol/kg i.p.) as H_2S donor,
Group-5: **L-NAME + cisplatin + L-arginine**: received L-NAME (0.4 gm/litre), cisplatin (5.0 mg/kg; i.p.) and L-arginine (1.25 g/liter in drinking water) as NO donor.
Group-6: **L-NAME + cisplatin + NaHS + L-arginine**; received L-NAME (0.4 gm/litre), cisplatin (5.0 mg/kg; i.p.), NaHS (56 µmol/kg i.p.) as H_2S donor and L-arginine (1.25 g/liter in drinking water) as NO donor.

The animals of groups 2-6 were given L-NAME (0.4 gm/litre daily in the drinking water). The animals of groups 3-6 were administered with cisplatin (5.0 mg/kg; i.p.) on the first day of study.
The animals of groups 4 and 6 were administered with NaHS (56 µmol/kg i.p.) as H_2S donor.
The animals of groups 5 and 6 were administered with L-arginine (1.25 g/liter in drinking water) as an NO donor.

On the 28th day, rats were euthanized after anesthetizing them with a xylazine: ketamine (10:1) mixture[17] (Mehdi *et al.*, 2022). The kidneys were excised, washed with normal saline and stored in 10% formalin solution for further examination.

2.4.1. Body weights measurement and Sample collection

The rats were monitored for body weights on day 0 (before the administration of any agent), day 14 and day 28. The blood samples were collected on these three time points i.e. day 0, day 14 and day 28. To collect blood samples, retro-orbital puncture was performed utilizing a hematocrit capillary tube. The blood was collected in heparinized centrifuge tubes and centrifuged (3,000 rpm x 15 min), after which the supernatant was collected and kept at -20 ^{0}C[18] (Lindstrom *et al.*, 2015). Moreover, the urine samples were collected at these time points to evaluate the urine flow rates.

2.4.2. Evaluation of H_2S Level in body fluids

To either urine or plasma samples (100 µL) distilled water (50 µL), zinc acetate (30 µL, 1% w/v), 20 mM of DMPD and $FeCl_3$ (30 mM in 1.2M HCl) were added followed by addition of TCA (150 µL, 10% w/v) after 20 min. The mixture was centrifuged at 10,000 rpm for 10 minutes and absorbance was noted at 670nm (IRMECO UV-visible U2020) and H_2S concentrations were determined through NaHS calibration curve[19] (Ahmad *et al.*, 2012).

2.4.3. Evaluation of Nitric Oxide in Plasma

Serum (100 µL) was incubated with nitrate reductase (vanadium chloride 8 mg/ml) and NADPH in 20 mmol/L of Tris buffer. The plasma (100 µL) was incubated with a methanol:di-ethylether mixture (900 ml, 3:1 v/v for 12 h) and centrifuged (10,000 rpm x 10 min). The supernatant was mixed with HCl (6.5 mol/L) and sulphanilic acid (37.5 mmol/L) and N-(1-napthyl) ethylene diamine (12.5 mmol/L). After 30 min of incubating the mixture at 48 ^{o}C, the mixture was centrifuged (10,000 rpm,

for 10 min) and absorbance was noted at 540 nm by a microplate reader (Lab-systems)[20] (Sun *et al.*, 2003).

2.4.4. Measurement of creatinine in plasma

To plasma samples (100 µL), sodium hydroxide (0.2 mL, 0.75N) and picric acid (1%) were added. After incubating the mixture at 30ºC, the absorbance at 515nm was measured and the concentration was calculated against a standard calibration curve using the known concentrations of creatinine[21] (Toora B D and Rajgopal G, 2002).

2.4.5. Measurement of blood urea nitrogen (BUN)

BUN was measured by taking serum (0.2 mL) plus distilled water (1.0 mL) and trichloroacetic acid (0.1 mL), followed by centrifugation (10,000 rpm x 10 min). Afterwards, 3.0 mL of reagent was added to the supernatant (0.2 mL) and it was maintained for 20 min on a boiling water bath, then cooled. Spectrophotometric absorbance was subsequently measured at 520 µm within 15 minutes[22] (Rajalingam *et al.*, 2009).

2.4.6. Evaluation of potassium and sodium in urine and plasma

Flame photometry was used to evaluate sodium or potassium concentrations in urine and serum. The samples were diluted as 1 in 200 for urinary or plasma sodium and potassium and as 1:1000 for potassium in urine[23] (Holiday and Preedy, 1953). The photometer was calibrated with standard concentrations of sodium or potassium chloride, and determinations were performed in duplicate.

2.5.Antioxidant assays

The SOD activity was measured by mixing the plasma samples with sodium carbonate (50 mM), EDTA (0.1 mM), and NBT (24 µM) followed by addition of HAC solution (1 mM). The absorbance was noted at 570 nm[24] (Javaid *et al.*, 2023).

To measure the MDA levels, 100 µl of plasma samples were mixed with 100 µl of TCA and TBA followed by heating and cooling the mixture respectively. The mixture was centrifuged (3500 rpm) for 10 min and absorbance was noted at 532 nm[24] (Javaid *et al.*, 2023).

2.6. Statistical Analysis

All data were expressed as mean ± SD (n=6). Statistical analysis was performed using GraphPad Prism software employing one-way analysis of variance (ANOVA) with post hoc Bonferroni test. Statistical significance was assumed at $p < 0.05$.

3. Results

3.1. In-vitro assays

3.1.1. DPPH assay

The activities of NaHS and L-arginine were measured by the ability to scavenge the free radical DPPH at different concentrations. Thus, NaHS produced an inhibition of DPPH formation by 77.99% at 100 µM, L-arginine inhibited by 65.11% at 2.4 mg/ml while BHT (standard) inhibited by 81% at 100µM (**Fig. 1A, 1B and 1C**).

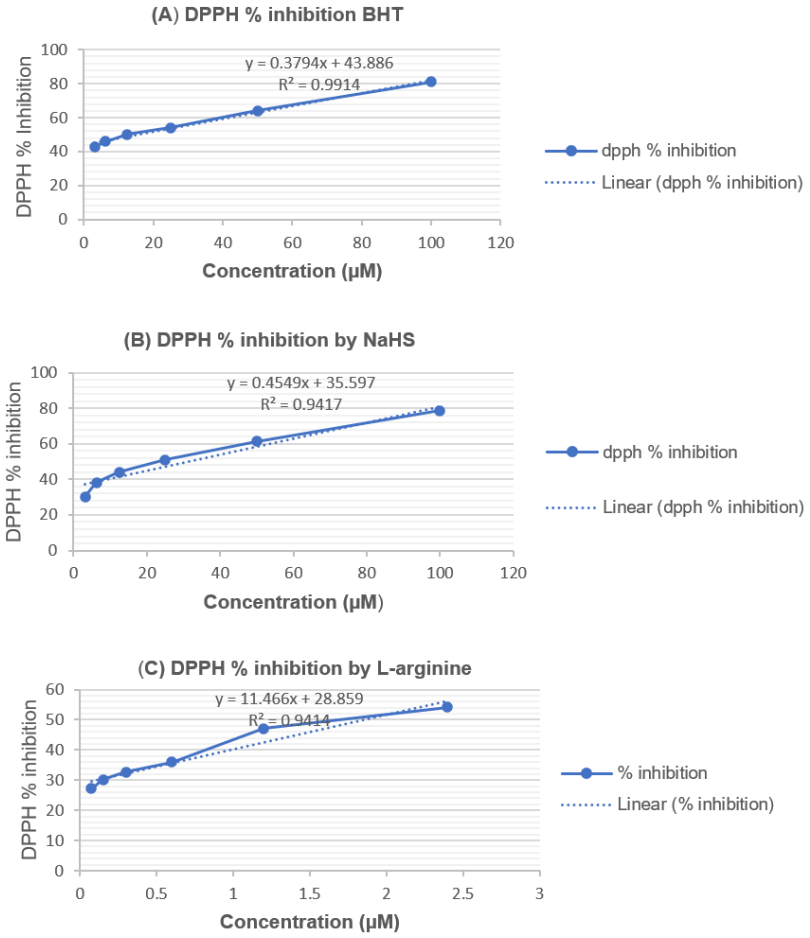


Figure 1. The representation of % inhibition of DPPH formation by **(A)** BHT, **(B)** NaHS, and **(C)** L-arginine.

3.1.2. Ferric thiocyanate assay

Thiocyanate (100 µM) as a standard generated an inhibitory peroxidation value (POV) of 71.11 % while NaHS (100µM) produced a POV of 47.78%, and L-arginine (2.4 mg/ml) yielded a value of 15.76% POV (**Fig. 2A, 2B and 2C**).

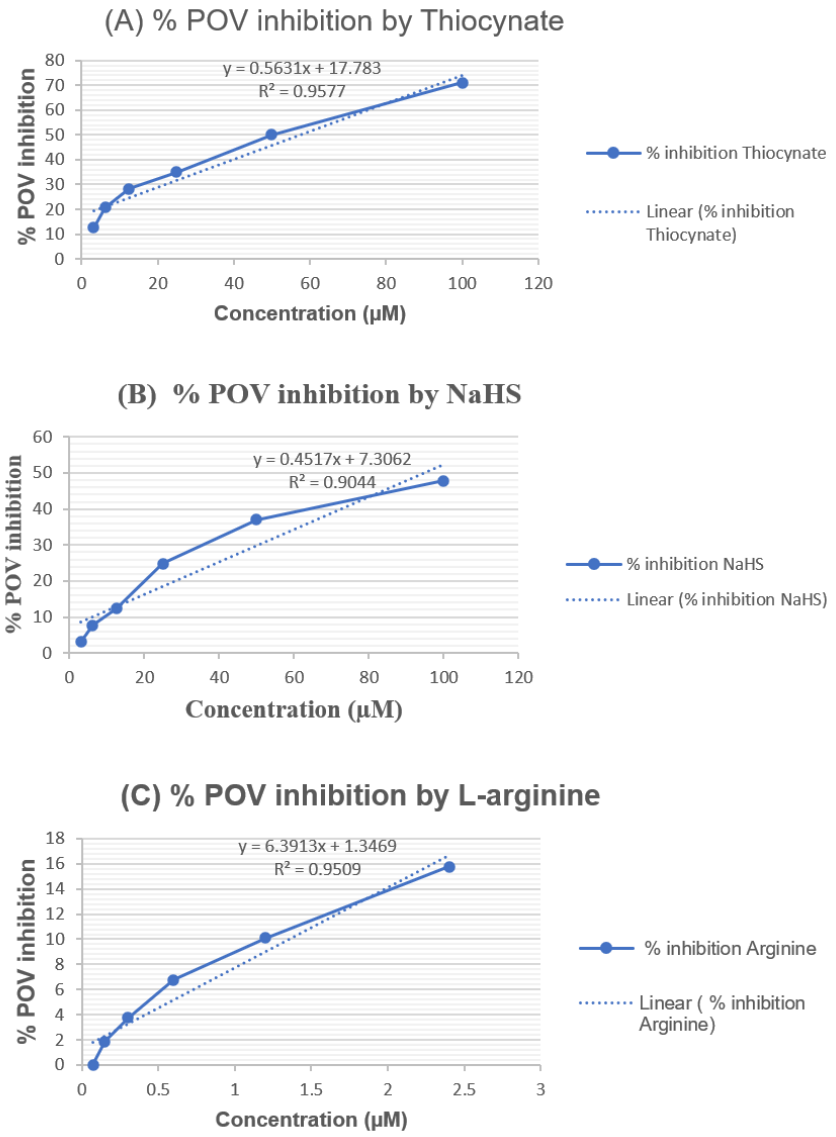


Figure 2. Inhibitory peroxidation values (% POV) of (**A**) thiocyanate (standard), (**B**) NaHS and (**C**) L-arginine.

3.1.3. Ferric reducing antioxidant power (FRAP) assay

The reducing power of a compound is thought to reflect its potential antioxidant capability. Consequently, NaHS and L-arginine reducing power was determined over a range of concentrations versus a standard comparator (ascorbic acid). Thus, at the highest concentration of 100 µg/ml, ascorbic acid yielded an absorbance of 0.411 at 700nm while NaHS (100 µM) revealed an

absorbance of 0.099, while L-arginine (2.4 mg/ml) produced an absorbance of 0.078 absorbance. (**Fig. 3A, 3B and 3C**).

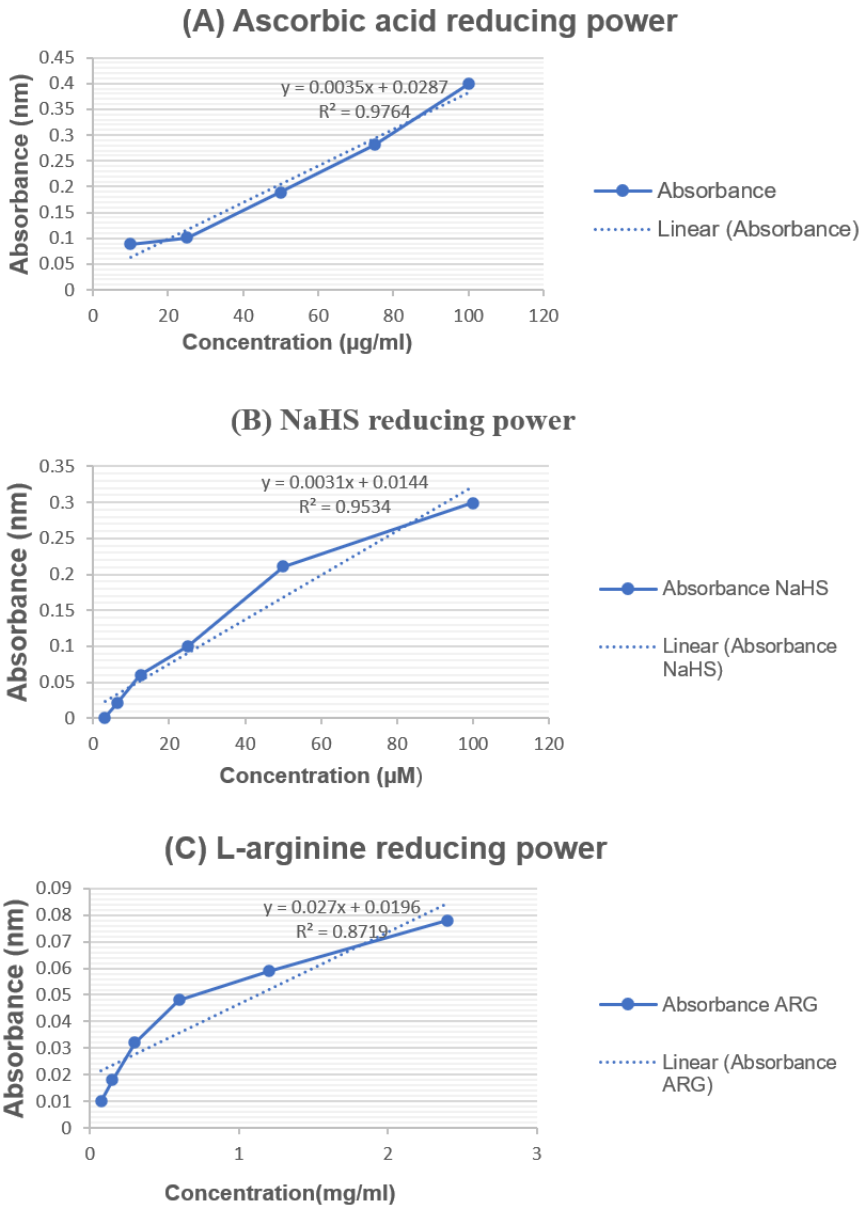


Figure 3. Ferric (Fe3+) to Ferrous (Fe 2+) reducing power (A) Ascorbic acid, (B) NaHS and (C) L-arginine.

3.2. *In-vivo* studies

3.2.1. Body weight and urinary output

The one-way ANOVA showed no significant variation in body weights on day 0 with [F (5, 30) = 0.57, P=0.72]. By the 14th day, the variation was significant with [F (5, 30) = 9.62, P<0.0001]. Compared to the control group, there was a substantial decrease in body weights of all the treated

animals ($P<0.01$ – $P<0.001$) except those that received L-NAME only. Likewise, the difference was notable when body weights were compared on day 28 with [$F (5, 30) = 53.96$, $P<0.0001$]. In detail, the body weights were markedly reduced ($P<0.01$ – $P<0.0001$) in all treatment groups by day 28, as presented in **Fig. 4A.**

Likewise, the rats were monitored for urinary flow rate on three time points and no significant inter-group difference was noted on day 0 with [$F (5, 30) = 0.75$, $P=0.58$]. However, the difference was notable on day 14 [$F (5, 30) = 7.40$, $P=0.0001$] and day 28 [$F (5, 30) = 7.39$, $P=0.0001$]. On day 14, in comparison to the control group, the urine flow rate was significantly increased in rats treated with L-NAME + Cisplatin ($P<0.001$) and L-NAME + Cisplatin + L-Arginine ($P<0.05$). Moreover, when outcomes were evaluated on day 28, cisplatin + L-NAME cotreatment did induce an increase in the flow rate ($P<0.01$) as did cisplatin + L-NAME + L-arginine combined administration ($P<0.01$) as presented in **Fig. 4B**.

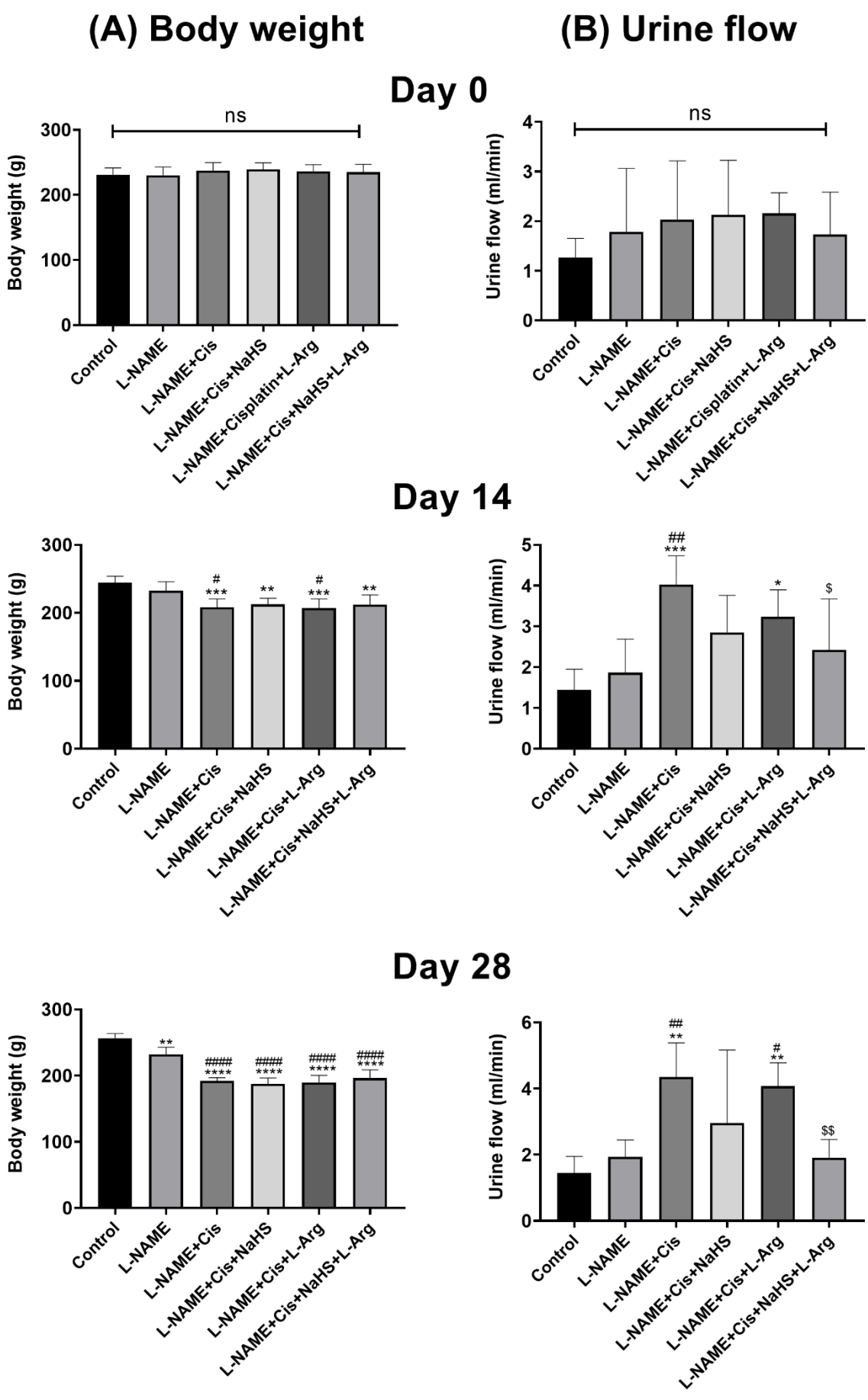

(A) Body weight
(B) Urine flow
Day 0
Day 14
Day 28
Body weight (g)
Urine flow (ml/min)
ns
Control
L-NAME
L-NAME+Cis
L-NAME+Cis+NaHS
L-NAME+Cisplatin+L-Arg
L-NAME+Cis+L-Arg
L-NAME+Cis+NaHS+L-Arg

Figure 4: The rats were treated with L-NAME, cisplatin, NaHS and L-arginine for 28 days, followed by evaluation of the test treatments on body weights and urine flow on days 0, 14 and 28. The results have been expressed as mean ± SD (n=6). *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 show comparison with control group, $^{\#}$P<0.05, $^{\#\#}$P<0.01, $^{\#\#\#\#}$P<0.0001 show comparison with L-NAME group, $^{\$}$P<0.05, $^{\$\$}$P<0.01, show comparison with L-NAME + Cisplatin group and ns shows non-significant difference.

3.2.2. Effects of NaHS and L-Arginine on H_2S levels in plasma and urine samples

The results of one-way ANOVA showed no marked difference in levels of H_2S in plasma on day 0 with [F (5, 30) = 0.26, P=0.93]. When these parameters were monitored on day 14, a decrease in plasma H_2S concentration was noted in the animals administered with L-NAME + cisplatin (P<0.001) and L-NAME + cisplatin + L-arginine (P<0.01), as compared to control rats. However, levels of H_2S were increased in rats receiving L-NAME + cisplatin + NaHS (P<0.001). On day 28, no difference in H_2S plasma levels was recorded in the groups treated with L-NAME or L-NAME + cisplatin. Moreover, a substantial rise in H_2S concentration was revealed in the animal group cotreated with L-NAME + cisplatin + NaHS, but no change was detected following cotreatment with cisplatin + L-NAME + L-arginine, though in the L-NAME + cisplatin + NaHS+ L-arginine group, a considerable augmentation in H_2S plasma level was noted **(Fig. 5A).**

Urinary H_2S levels were comparable among all experimental groups on day 0 with [F (5, 30) = 0.53, P=0.74]. On day 14, there was no modification of H_2S level following L-NAME treatment, while in the L-NAME + cisplatin, L-NAME + cisplatin+ NaHS and L-NAME + cisplatin + L-arginine groups, a sizeable decline in urinary H_2S was observed versus the control, and similar outcomes were found on day 28 **(Fig. 5B).**

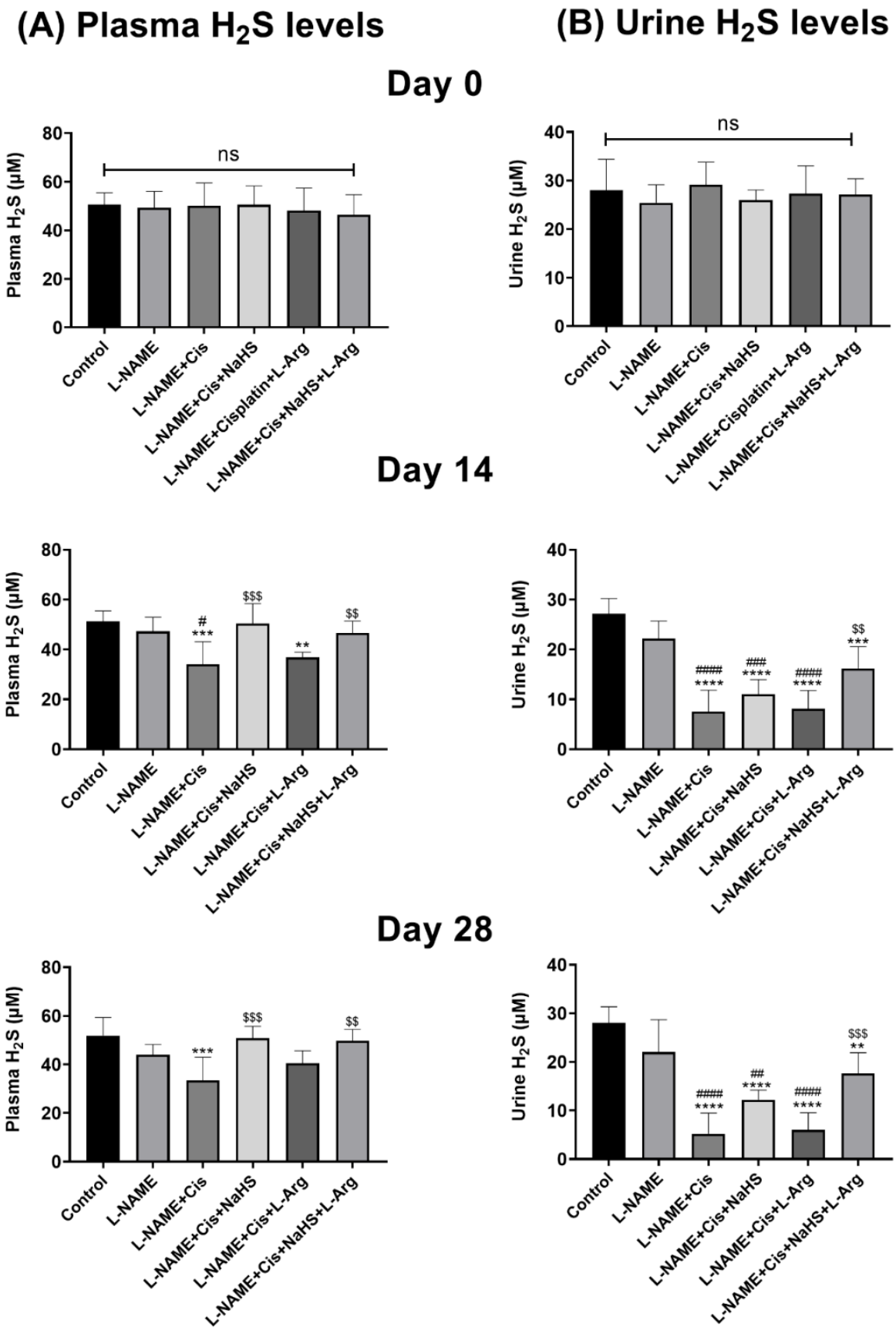

(A) Plasma H2S levels
(B) Urine H2S levels
Day 0
Day 14
Day 28
Plasma H2S (μM)
Urine H2S (μM)
ns
Control
L-NAME
L-NAME+Cis
L-NAME+Cis+NaHS
L-NAME+Cisplatin+L-Arg
L-NAME+Cis+L-Arg
L-NAME+Cis+NaHS+L-Arg

Figure 5: The effects of NaHS and L-arginine on H_2S levels in (**A**) plasma and (**B**) urine were noted on days 0, 14 and 28 in rats exposed to L-NAME and cisplatin-induced nephrotoxicity. The results have been expressed as mean ± SD (n=6). $^{**}P<0.01$, $^{***}P<0.001$, $^{****}P<0.0001$ show comparison with control group, $^{\#}P<0.05$, $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$, $^{\#\#\#\#}P<0.0001$ show comparison with L-NAME group, $^{\$\$}P<0.01$, $^{\$\$\$}P<0.001$, show comparison with L-NAME + Cisplatin group and ns shows non-significant difference.

3.2.3. Effects of NaHS and L-Arginine on nitric oxide levels

When tested by one-way ANOVA, the NO concentration in plasma varied non-significantly amongst all animal groups with [F (5, 30) = 2.188, P=0.819] on day 0 of the study. But the inter-group difference was notable on day 14 [F (5, 30) = 1.115, P=0.0001] and day 28 [F (5, 30) = 87.65, P=0.0001]. In detail, when compared to healthy control, a notable reduction in plasma NO levels was noted in the L-NAME group (P<0.0001) and L-NAME + Cis group (P<0.0001) on day 14 and day 28. The NO levels were increased in animals treated with L-NAME + Cis + NaHS on day 14 (P<0.01) and day 28 (P<0.0001), revealing the protective effects of NaHS. Likewise, the beneficial effects were noted in rats treated with L-NAME + Cis + L-Arg as NO levels were significantly elevated (P<0.0001) on day 14 and 28, in comparison to L-NAME + Cis group. The co-administration of L-NAME + Cis + NaHS + L-Arg resulted in the highest NO levels, revealing the additive protective effects of this combination therapy as presented in **Fig. 6**.

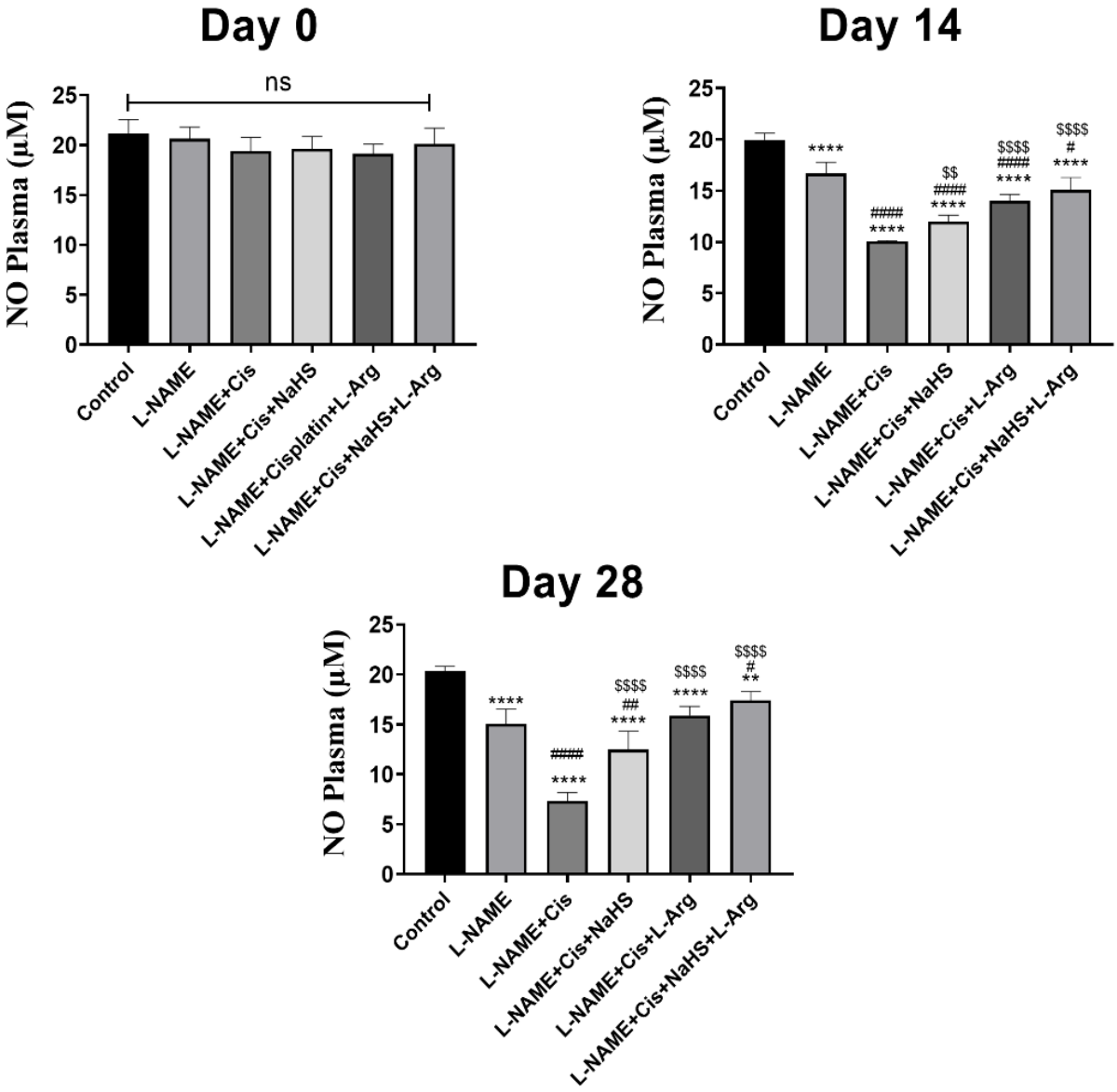


Figure 6: The effects of NaHS and L-arginine on NO levels in plasma were noted on days 0, 14 and 28 in rats exposed to L-NAME and cisplatin-induced nephrotoxicity. The results have been expressed as mean ± SD (n=6). **P<0.01, ****P<0.0001 show comparison with control group, $^{\#}$P<0.05, $^{\#\#}$P<0.01, $^{\#\#\#\#}$P<0.0001 show comparison with L-NAME group, $^{\$\$}$P<0.01, $^{\$\$\$\$}$P<0.0001, show comparison with L-NAME + Cisplatin group and ns shows non-significant difference.

3.2.4. Effects of NaHS and L-Arginine on creatinine concentration and clearance

At the beginning of the study, the plasma creatinine concentration was comparable on day 0 [F (5, 30) = 0.337, P=0.887]. But the difference was notable on day 14 [F (5, 30) = 4.097, P=0.01] and 28 [F (5, 30) = 5.226, P=0.001]. In detail, the L-NAME treatment resulted in the elevation of creatinine levels on day 14 and day 28, but the difference was statistically non-significant. However, the levels

of creatinine became significantly high on day ($P<0.01$) 14 and day 28 ($P<0.01$) in animals treated with L-NAME + Cis. The treatment with NaHS resulted in creatinine reduction ($P<0.01$), compared to L-NAME + Cis, but the L-Arg alone exerted a modest effect only. However, their combination resulted in substantial improvement ($P<0.05$), compared to L-NAME + Cis group (**Table 1**).

Table 1. The effects of NaHS and L-arginine on creatinine levels in plasma were noted on days 0, 14 and 28 in rats exposed to L-NAME and cisplatin-induced nephrotoxicity. The results have been expressed as mean ± SD (n=6). **P<0.01 shows comparison with control group, $^{\$}$P<0.05, $^{\$\$}$P<0.01, show comparison with L-NAME + Cisplatin group.

	Mean creatinine plasma concentration (mg/dL)		
Treatment	**Day 0**	**Day 14**	**Day 28**
Control	0.63 ± 0.07	0.58 ± 0.05	0.70 ± 0.06
L-NAME	0.63 ± 0.09	1.37 ± 0.17	1.53 ± 0.48
L-NAME + Cis	0.68 ± 0.07	2.92 ± 0.16**	2.95 ± 0.27**
L-NAME + Cis + NaHS	0.69 ± 0.14	1.31 ± 0.82	0.97 ± 0.19$^{\$\$}$
L-NAME + Cis + L-Arg	0.53 ± 0.10	1.59 ± 0.18	1.71 ± 0.55
L-NAME + Cis + NaHS + L-Arg	0.62 ± 0.06	1.18 ± 0.36	1.27 ± 0.247$^{\$}$

Likewise, the creatinine clearance was also monitored on all time points. There was no difference in creatinine clearance on day 0 [$F(5, 30) = 0.042$, $P=0.998$]. But the parameter varied notably on day 14 and day 28 with [$F(5, 30) = 3.34$, $P=0.015$] and [$F(5, 30) = 4.95$, $P=0.002$], respectively. On day 14, the treatment with L-NAME resulted in reduced creatinine clearance, but the renal damage was further exacerbated in animal receiving L-NAME + Cis ($P<0.05$), as compared to healthy control. The treatment with NaHS improved the creatinine clearance while only a mild benefit was noted in rates treated with L-Arg on day 14. However, the combination of NaHS and L-Arg exerted the highest protection ($P<0.01$) and nearly restored the creatinine clearance levels, as noted in healthy rats, on day 28 (**Table 2**).

Table 2. The effects of NaHS and L-arginine on creatinine clearance were noted on days 0, 14 and 28 in rats exposed to L-NAME and cisplatin-induced nephrotoxicity. The results have been expressed as mean ± SD (n=6). *P<0.05, **P<0.01 show comparison with control group, $^{\$\$}$P<0.01, show comparison with L-NAME + Cisplatin group.

	Mean creatinine clearance (mL/min)		
Treatment	**Day 0**	**Day 14**	**Day 28**
Control	1.94 ± 0.18	2.00 ± 0.17	1.9 ± 0.16
L-NAME	1.88 ± 0.32	1.32 ± 0.29	1.15 ± 0.30
L-NAME + Cis	1.87 ± 0.27	0.72 ± 0.21*	0.46 ± 0.07**
L-NAME + Cis + NaHS	1.85± 0.21	1.00 ± 0.17	1.31 ± 0.27
L-NAME+ Cis + L-Arg	1.96 ± 0.16	0.86 ± 0.26	1.18 ± 0.24

L-NAME + Cis + NaHS + L-Arg	1.97 ± 0.25	1.26 ± 0.24	1.65 ± 0.20$^{\$\$}$

3.2.5. Effects of NaHS and L-Arginine on blood urea nitrogen (BUN) and urinary urea in L-NAME and Cisplatin-induced nephrotoxic rats

The one-way ANOVA revealed no inter-group difference for BUN on day 0 [F (5, 30) = 0.314, P=0.9005]. The levels varied notably among all groups on day 14 [F (5, 30) = 3.673, P=0.010] and day 28 [F (5, 30) = 24.79, P=0.0001] of the study. In detail, there were substantial increases in BUN levels in rats administered with L-NAME and this change was further pronounced (P<0.001) in rats treated with L-NAME + Cis compared to health control. NaHS markedly protected this renal toxicity exerted by L-NAME + Cis administration as BUN levels were notably reduced on day 14 (P<0.001) and day 28 (P<0.001). L-Arg alone did not significantly ameliorated these nephrotoxic effects of L-NAME + Cis but combining it with NaHS caused a marked benefit as significant reduction in BUN was noted in L-NAME + Cis + NaHS + L-Arg rats on day 14 and 28 (P<0.01) as presented in **Fig. 7A**.

The animals did not vary in urinary urea concentrations on day 0 [F (5, 30) = 2.42, P=0.058]. However, the outcomes varied among the differently-treated rats on day 14 [F (5, 30) = 20.82, P=0.0001] and day 28[F (5, 30) = 105.1, P=0.0001]. Compared to heathy rats, the urinary urea concentration was reduced in rats treated with L-NAME on day 14 and day 28 (P<0.001), revealing the L-NAME-induced impairment in urea clearance in rats. This damage was further exaggerated when rats were treated with L-NAME + Cis as urea concentration was notably low on day 14 (P<0.0001) and day 28 (P<0.0001). On day 14, the L-Arg worked effectively in restoring the urea clearance as concentration was significantly high (P<0.05), in comparison to L-NAME + Cis group. But the benefits were evident with NaHS (P<0.0001) and L-Arg (P<0.0001) alone on day 28. However, the co-administration of both exerted earlier beneficial outcomes as urea clearnce was prominently high on day 14 (P<0.001) and day 28 (P<0.0001) as depicted in **Fig. 7B**.

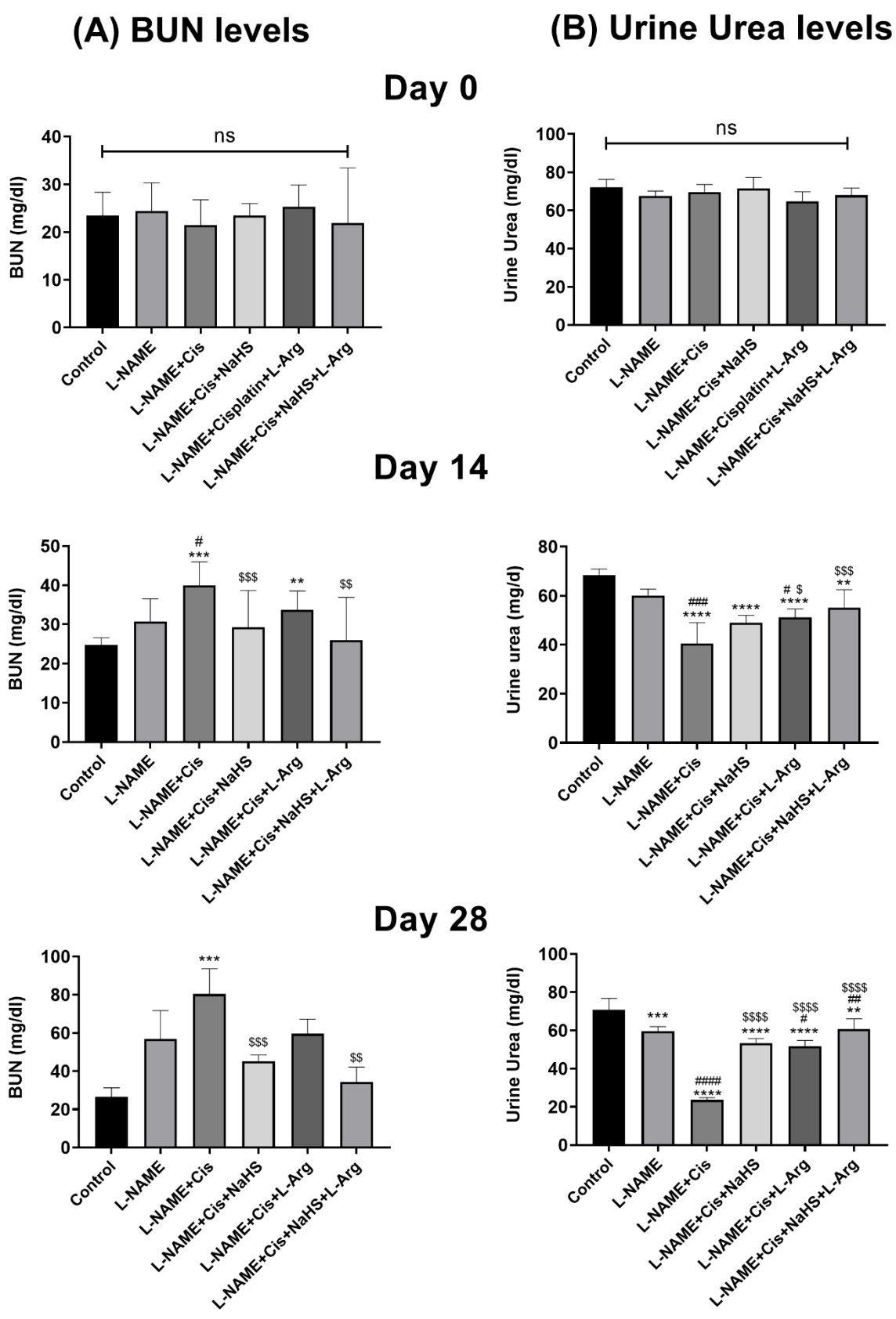

(A) BUN levels
(B) Urine Urea levels
Day 0
BUN (mg/dl)
Urine Urea (mg/dl)
ns
Day 14
Day 28
Control
L-NAME
L-NAME+Cis
L-NAME+Cis+NaHS
L-NAME+Cisplatin+L-Arg
L-NAME+Cis+L-Arg
L-NAME+Cis+NaHS+L-Arg

Figure 7: The effects of NaHS and L-arginine on (**A**) BUN and (**B**) Urea levels in urine were noted on days 0, 14 and 28 in rats exposed to L-NAME and cisplatin-induced nephrotoxicity. The results have been expressed as mean ± SD (n=6). **P<0.01, ***P<0.001, ****P<0.0001 show comparison with control group, $^{\#}$P<0.05, $^{\#\#\#}$P<0.001, $^{\#\#\#\#}$P<0.0001 show comparison with L-NAME group, $^{\$}$P<0.05, $^{\$\$}$P<0.01, $^{\$\$\$}$P<0.001, $^{\$\$\$\$}$P<0.0001, show comparison with L-NAME + Cisplatin group and ns shows non-significant difference.

3.2.6. Effect of NaHS and L-Arginine on sodium and potassium concentrations in the plasma and urine

The plasma concentration of sodium varied non-significantly among differently treated rats on day 0 [F (5, 30) = 1.53, P=0.21]. However, the inter-group difference was evident on day 14 [F (5, 30) = 184, P=0.0001] and day 28 [F (5, 30) = 148.1, P=0.0001]. Similarly, the baseline potassium noted on day 0 [F (5, 30) = 0.385, P=0.85] were changed among groups on day 14 [F (5, 30) = 130.6, P=0.0001] and day 28 [F (5, 30) = 130.6, P=0.0001].

In detail, the plasma concentrations of sodium and potassium fluctuated negligibly in control rats during 28 days, but the administration of L-NAME resulted in reduced sodium levels in plasma on day 14 (P<0.05) and day 28 (P<0.01). Likewise, the potassium levels were also reduced in plasma on day 14 and 28 (P<0.01), in comparison to control rats. Moreover, the co-administration of L-NAME + Cis resulted in further reduction of sodium (P<0.0001) and potassium (P<0.0001) levels at both time points. The treatment with NaHS and L-arginine resulted in mitigation of the sodium loss as sodium and potassium levels were elevated (P<0.0001) on day 14 and day 28 (**Table 3**).

Table 3. The effects of NaHS and L-arginine on plasma sodium and potassium levels were noted on days 0, 14 and 28 in rats exposed to L-NAME and cisplatin-induced nephrotoxicity. The results have been expressed as mean ± SD (n=6). **P<0.01, ****P<0.0001 show comparison with control group, $^{\#\#\#}$P<0.001, $^{\#\#\#\#}$P<0.0001 show comparison with L-NAME group, $^{\$\$}$P<0.01, $^{\$\$\$}$P<0.001, $^{\$\$\$\$}$P<0.0001, show comparison with L-NAME + Cisplatin group.

Treatment	Mean sodium plasma level (mEq/L)			Mean potassium plasma level (mEq/L)		
	Day 0	Day 14	Day 28	Day 0	Day 14	Day 28
Control	144.5 ± 1.71	148.7 ± 2.16	146.7 ± 2.28	6.04 ± 0.37	6.05 ± 0.27	5.98 ± 0.25
L-NAME	147.7 ± 2.03	138.0 ± 0.58*	136.0 ± 1.13**	6.23 ± 0.38	5.20 ± 0.11**	5.19 ± 0.11**
L-NAME + Cis	144.8 ± 2.09	99.0 ± 1.87s****	87.3 ± 2.8$^{****\#\#\#\#}$	5.98 ± 0.37	2.00 ± 0.08$^{****\#\#\#\#}$	1.80 ± 0.06$^{****\#\#\#}$
L-NAME + Cis + NaHS	150.0 ± 2.31	114.7 ± 0.67$^{****\#\#\#\$\$\$\$}$	123.5 ± 0.56$^{****\#\#\#\$\$\$\$}$	5.67 ± 0.42	2.40 ± 0.13$^{****\#\#\#\#}$	3.83 ± 0.042$^{****\#\#\#}$
L-NAME+ Cis + L-Arg	142.7 ± 4.15	112.5 ± 0.81$^{****\#\#\#\#\$\$\$\$}$	122.5 ± 1.31$^{****\#\#\#\#\$\$\$\$}$	6.00 ± 0.26	2.50 ± 0.06$^{****\#\#\#\#}$	3.48 ± 0.10$^{****\#\#\#}$
L-NAME + Cis + NaHS + L-Arg	142.2 ± 1.25	124.5±0.99$^{****\#\#\#\#\$\$\$\$}$	131.7 ± 0.80$^{****\$\$\$\$}$	5.67 ± 0.33	2.93 ± 0.13$^{****\#\#\#\#\$\$}$	4.10 ± 0.22$^{****\#\#\#\#\$\$}$

When evaluated by one-way ANOVA, the sodium concentration in urine was non-significantly different among groups on day 0 [F (5, 30) = 1.406, P=0.250] but the difference became notable on day 14 [F (5, 30) = 489.3, P=0.0001] and day 28 [F (5, 30) = 626.0, P=0.0001]. Similarly, urinary potassium varied non-significantly among all groups on day 0 [F (5, 30) = 0.505, P=0.766] but fluctuated significantly on day 14 [F (5, 30) = 30.84, P=0.0001] and day 28 [F (5, 30) = 23.45, P=0.0001].

In control rats, the urinary excretion of both sodium and potassium did not change during 28 days, but the treatment with L-NAME resulted in a significant increase in sodium (P<0.05) and potassium (P<0.05) excretion by the end of 28 days. This change was exacerbated in rats co-treated with L-NAME and cisplatin as sodium and potassium excretion in urine was markedly increased (P<0.0001), compared to healthy rats. However, these changes were restored in rats treated with NaHS and L-arginine as sodium and potassium excretion was normalized (P<0.0001), compared to the L-NAME + Cis group (**Table 4**).

Table 4. The effects of NaHS and L-arginine on sodium and potassium excretion in urine were noted on days 0, 14 and 28 in rats exposed to L-NAME and cisplatin-induced nephrotoxicity. The results have been expressed as mean ± SD (n=6). *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 show comparison with control group, $^{\#\#\#\#}$P<0.0001 show comparison with L-NAME group, $^{\$\$\$}$P<0.001, $^{\$\$\$\$}$P<0.0001, show comparison with L-NAME + Cisplatin group.

Name of Group	Mean sodium urine level (mEq/L)			Mean potassium urine level (mEq/L)		
	Day 0	Day 14	Day 28	Day 0	Day 14	Day 28
Control	168.2 ± 1.17	171.7 ± 2.80	171.7 ± 3.61	3.50 ± 0.41	3.83 ± 0.17	4.17 ± 0.31
L-NAME	167.0 ± 3.10	177.3 ± 0.62	185.5 ± 1.50*	3.87 ± 0.41	4.83 ± 0.11**	5.13 ± 0.22*
L-NAME + Cis	164.5 ± 3.91	310.2 ± 2.92$^{****\#\#\#\#}$	353.3 ± 4.02$^{****\#\#\#\#}$	4.17 ± 0.31	7.00 ± 0.37****	7.25 ± 0.31$^{****\#\#\#\#}$
L-NAME + Cis + NaHS	175.3 ± 1.28	248.2 ± 2.12$^{****\#\#\#\#}$	270.8 ± 1.87$^{****\#\#\#\#\$\$\$\$}$	3.83 ± 0.40	5.18 ± 0.08$^{****\#\#\#\#\$\$\$\$}$	5.66 ± 0.09$^{***\$\$\$}$
L-NAME+ Cis + L-Arg	166.5 ± 4.52	258.5 ± 2.14$^{****\#\#\#\#}$	272.2 ± 1.76$^{****\#\#\#\#\$\$\$\$s}$	4.00 ± 0.52	5.43 ± 0.12$^{****\$\$\$\$}$	5.67 ± 0.07$^{***\$\$\$}$
L-NAME + Cis + NaHS + L-Arg	165.0 ± 4.27	212.2 ± 2.94$^{****\#\#\#\#\$\$\$\$}$	240.2 ± 2.02$^{****\#\#\#\#\$\$\$\$}$	3.50 ± 0.34	5.20 ± 0.11$^{***\$\$\$\$}$	5.19 ± 0.11$^{*\$\$\$}$

3.2.7. Effects of NaHS and L-Arginine on SOD activity and MDA levels in plasma

When analyzed for antioxidant potential of test treatments, rats disclosed comparable plasma SOD levels day 0 [F (5, 30) = 0.305, P=0.905] but the difference was notable on day 14 [F (5, 30) = 15.89, P=0.0001] and day 28 [F (5, 30) = 12.13, P=0.0001]. The treatment with L-NAME resulted in reduced SOD activity on day 14 and day 28 but the difference remained non-significantly different from healthy control. However, the oxidative stress was markedly high in rats administered with L-NAME + Cis as SOD activity was significantly low on day 14 (P<0.0001) and day 28 (P<0.001). When rats were treated with NaHS and L-Arg alone and combination, the SOD activity was noted to be significantly increased on day 28 with P<0.01 and P<0.001, respectively (**Fig. 8A**)

Likewise, MDA levels were measured in the plasma of differently treated rats. The one-way ANOVA showed no inter-treatment difference on day 0 [$F(5, 30) = 0.157$, $P=0.976$]. But the inter-group difference was evident on day 14 [$F(5, 30) = 7.176$, $P=0.002$] and day 28 [$F(5, 30) = 5.035$, $P=0.001$]). A significant increase in the concentration of MDA was found in the rats administered with L-NAME + Cis ($P<0.001$), compared to healthy rats. The combination therapy with NaHS L-Arg protected the rats from nephrotoxicity-induced oxidative stress as MDA levels were notably less ($P<0.05$), compared to the L-NAME + Cis group (**Fig. 8B**).

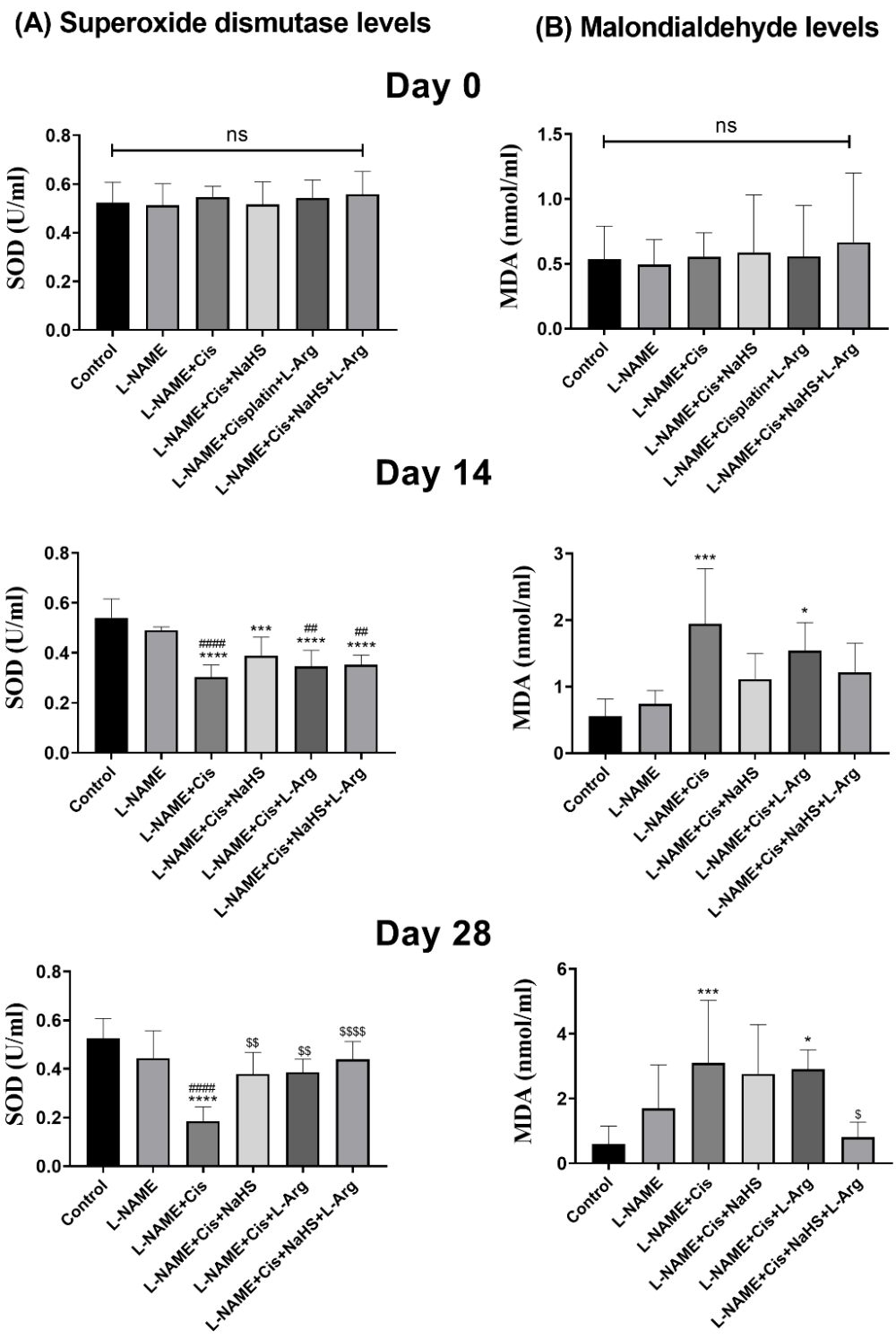


Figure 8: The effects of NaHS and L-arginine on (**A**) SOD and (**B**) MDA levels were noted on days 0, 14 and 28 in rats exposed to L-NAME and cisplatin-induced nephrotoxicity. The results have been expressed as mean ± SD (n=6). *P<0.0%, ***P<0.001, ****P<0.0001 show comparison with control

group, $^{\#\#}P<0.01$, $^{\#\#\#\#}P<0.0001$ show comparison with L-NAME group, $^{\$}P<0.05$, $^{\$\$}P<0.01$, $^{\$\$\$\$}P<0.0001$, show comparison with L-NAME + Cisplatin group and ns shows non-significant difference.

4. Discussion

The current study aimed to explore the nephroprotective and antioxidant potential of NaHS and L-arginine. In-vivo experimentation was carried out in which DPPH assay exposed the radical scavenging potential of NaHS and L-arginine as absorbance was reduced when compared to standard BHT. Moreover, the linoleic acid peroxidation inhibition in ferric thiocyanate assay and reducing potential in and the FRAP assay further validated the antioxidant capacity of NaHS and L-arginine, which has also been established by previous study[15] (Ahmad *et al.*, 2015), validating the potential of these compounds to mitigate oxidative stress.

Cisplatin is broadly used as a cytotoxic drug in chemotherapy, but nephrotoxicity is a significant limitation associated with its use. In the present study, cisplatin administration resulted in nephrotoxicity in rats, characterized by elevated blood urea nitrogen and serum creatinine, accompanied by tissue pathology, including glomerular inflammation and renal cortical hemorrhage[25,26] (Perše and Večerić-Haler, 2018; Jana *et al.*, 2023). Moreover, the cisplatin caused marked increase in oxidative stress was increased by cisplatin as SOD activity and lipid peroxidation was evident by elevated MDA levels in plasma. These findings were in accordance with the previous study in which a single dose of cisplatin (7 mg CP/kg) resulted in elevated oxidative stress in the kidneys of Wistar albino rats[27] (Ognjanović *et al.*, 2012).

Previous studies have revealed that L-NAME exacerbates cisplatin-induced nephrotoxicity, and its long-term oral administration induces nephropathy through nitric oxide depletion, presented by elevated serum creatinine and a decrease in urinary creatinine[28] (Moslemi *et al.*, 2013). The current study also confirmed that plasma concentrations of BUN and plasma creatinine were increased and urinary creatinine and urea were decreased in rats treated with L+NAME + Cisplatin. Cisplatin is known to escalate nitrite concentrations, with inducible nitric oxide synthase (iNOS)-derived NO contributing to its nephrotoxicity[28] (Moslemi *et al.*, 2013). However, NOS inhibition caused by L-NAME did not mitigate cisplatin-induced nephrotoxicity. In our experiments, a decreased NO level was noted in rats treated with L-NAME + cisplatin.

In present study, the H_2S levels remained unchanged by L-NAME, but the co-administration of L-NAME + cisplatin resulted in the reduction in plasma H_2S. H_2S has been implicated in modulating the NO levels as H_2S increases the availability of NO by elevating the eNOS activity and reducing the degradation of NO[29] (Munteanu *et al.*, 2024). Hence, the elevated H_2S may sustain the production of NO by preserving the eNOS function even under the eNOS inhibition caused by L-NAME[30] (Citi *et al.*, 2021). Our study supported this mechanism, showing that L-NAME and cisplatin-induced nephrotoxicity led to decreased nitrite/nitrate levels in kidney homogenates. Similar outcomes were noted when plasma nitrite levels were evaluated that might be ascribed to a pathological impairment

of glomerular endothelial cells. Cisplatin has been known to reduce renal blood flow and glomerular filtration rate[31] (McSweeney *et al.*, 2021), while L-arginine protects by improving these parameters[32] (Cernadas *et al.*, 1992). Our findings are in line as cotreatment with L-NAME + cisplatin + L-arginine improved creatinine clearance and urea concentration in urine.

In our research, L-NAME + cisplatin + NaHS treatment enhanced plasma SOD levels considerably but reduced MDA concentrations, which points towards a possible NO-dependent antioxidant mechanism. As oxidative stress is one of the key initiators of nephrotoxicity[33] (Hosohata, 2016), the alterations observed in SOD, MDA, and NO levels reflect the significance of NO and H_2S in renoprotection. MDA, one of the products of lipid peroxidation, is a well-established marker of oxidative damage[34] (Cordiano *et al.*, 2023), and its decrease following NaHS treatment also supports the nephroprotective action of H_2S.

NO acts in two contrasting ways, being both protective and cytotoxic[35] (Wink and Mitchell, 1998). In acute renal failure, NOS inhibition potentiates nephrotoxicity[36] (Chirino *et al.*, 2008), while L-arginine, being an NO precursor, shows renoprotection. The present results vindicate this protective action, as L-arginine co-administration with cisplatin and L-NAME enhanced markers of renal function. In addition, L-arginine and NaHS co-treatment better decreased nephrotoxicity markers, i.e., plasma creatinine and BUN, than either drug individually, reflecting a synergistic protective action. The interaction between NO and H_2S is well established. H_2S has been shown to upregulate NOS to increase the production of NO. In the current study, L-NAME + cisplatin + L-arginine co-treatment caused a moderate rise in plasma levels of NO.

The changes in NO synthesis in tissues exert an impact on the production of H_2S, revealing the bidirectional relationship of both transmitters in regulating the physiological processes[37] (Wu *et al.*, 2018). Our study revealed the synergism even under conditions of NOS inhibition as SOD activity was elevated and MDA levels were reduced in rats treated with L-NAME + cisplatin + NaHS + L-argnine.

Our findings demonstrate that NaHS and L-arginine exert nephroprotective effects in a rat model of cisplatin-induced nephrotoxicity. However, before considering these doses and translating these responses in humans, we must consider the challenges imposed by interspecies differences in pharmacokinetics and metabolism. In the future, further pre-clinical experimentations must be carried out to determine safe and effective dosing strategies for clinical use.

5. Conclusion

This study showed that L-NAME exacerbates cisplatin-induced nephrotoxicity, possibly by suppressing the NO protective pathways and elevating oxidative stress. Administration of NaHS, donor of H_2S, and L-arginine, source of NO, exerted an ameliorative effect on L-NAME + cisplatin-induced renal injury but the combined treatment with both NaHS and L-arginine resulted in greater effectiveness, possibly by diminishing the L-NAME + cisplatin-induced oxidative stress. However,

the current preliminary study has the limitation of a small group size and lack of histopathological studies that might be considered in future experimentation to provide more comprehensive understanding of underlying mechanisms.

Author Contributions:

All authors contributed equally to Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data Curation, Writing Original Draft, Software, and Visualization.

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Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability statement

The data presented in this study are available on request from the corresponding authors.

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