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RESEARCH ARTICLE

Hepatoprotective Effects of Thiamine and Zinc Oxide Nanoparticles against L-Arginine-Induced hepatic Injury

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ABSTRACT

Objective(s): This study examined the hepatoprotective effects of thiamine and zinc oxide nanoparticles (ZnO NPs) in a murine model of L-arginine-induced hepatic damage.

Methods: Eighty-four adult male BALB/c mice were randomly assigned to seven groups (n = 12 per group): control, thiamine (30 mg/kg), ZnO NPs (1 mg/kg), L-arginine (6 g/kg), L-arginine + thiamine (6 g/kg + 30 mg/kg), L-arginine + ZnO NPs (6 g/kg + 1 mg/kg), and L-arginine + thiamine + ZnO NPs (6 g/kg + 30 mg/kg + 1 mg/kg). All treatments were administered intraperitoneally daily for seven consecutive days. Serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), and malondialdehyde (MDA) levels, alongside superoxide dismutase (SOD) and catalase (CAT) activities and transforming growth factor-β (TGF-β), were assessed. Hepatic histostereological analysis was conducted to evaluate tissue architecture.

Results: L-arginine significantly increased ALT, AST, ALP, MDA, and TGF-β levels while reducing SOD and CAT activities ($p < 0.05$). Histological analysis showed hepatocyte cytoplasmic degeneration, nuclear abnormalities, decreased sinusoidal and interstitial volumes, and disrupted lobular organization. Treatment with thiamine alone or combined with ZnO NPs significantly attenuated biochemical markers ($p < 0.05$), restored antioxidant enzyme activities ($p < 0.05$), decreased TGF-β expression, and preserved hepatic structure and stereological parameters.

Conclusions: High-dose L-arginine induces liver injury through oxidative stress and inflammation. Thiamine and ZnO nanoparticles, especially combined, protect the liver by enhancing antioxidants, reducing inflammation, and inhibiting fibrogenic signaling.

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INTRODUCTION

As a semi-essential amino acid, L-arginine is critical in preserving cellular integrity and regulating inflammatory responses in hepatic tissue [1]. In experimental models of acute cholestasis, it effectively reduces parenchymal damage, normalizes serum liver enzymes, and mitigates histological abnormalities such as necrosis and leukocyte infiltration. These protective outcomes largely rely on inducible nitric oxide synthase (iNOS)-mediated nitric oxide (NO) production, thereby mitigating oxidative stress, stimulating parenchymal regeneration, and boosting endogenous cellular defenses [2–4].

Under physiological conditions, L-arginine acts as an essential precursor for nitrogenous metabolism and nitric oxide (NO) formation. Moderate doses (~100–300 mg/kg) sustain metabolic balance in experimental animals without causing cytotoxic effects. In contrast, supraphysiological levels provoke severe cellular distress and liver pathology. Acute exposure to high concentrations (2.5–3.0 g/kg) in rodents inflicts

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significant injury on both pancreatic exocrine tissue and hepatic parenchyma. This toxicity is driven by mitochondrial dysfunction, excessive reactive oxygen species (ROS) generation, aberrant NO signaling, and triggered inflammatory pathways. In the liver, such overdose induces acute degenerative changes in hepatocytes, while recurrent exposure results in chronic architectural lesions reflecting persistent inflammation and initial fibrogenesis. Crucially, the parallel induction of inflammatory and fibrogenic factors, notably interleukin-1β (IL-1β) and transforming growth factor-beta 1 (TGF-β1), mirrors the pathological expression patterns characteristic of liver cirrhosis and chronic viral hepatitis [5–7].

Considerable interest has centered on zinc oxide nanoparticles (ZnO NPs) owing to their hepatoprotective properties, which stem mainly from modulating apoptotic signaling, quenching oxidative stress, and dampening inflammatory cascades (8). Administration of ZnO NPs alleviates hepatocellular injury and normalizes circulating liver biomarkers, including alkaline phosphatase (ALP), aspartate aminotransferase (AST), and alanine aminotransferase (ALT). By downregulating pro-apoptotic factors and upregulating anti-apoptotic genes, these nanoparticles modulate cell viability and promote hepatic renewal. Moreover, their nanoscale profile allows preferential accumulation within liver tissue, improving targeted therapeutic outcomes while reducing systemic adverse effects. Nonetheless, dosage must be meticulously calibrated, given that excessive amounts may elicit paradoxical hepatotoxic responses [9].

Thiamine (vitamin B1) represents an indispensable micronutrient for metabolic equilibrium; its depletion precipitates heightened oxidative stress, lactic acidosis associated with parenteral nutrition, impaired hepatic cholinergic pathways, and cardiac dysfunction [10, 11]. In animal studies, preventive administration of thiamine protects against cholestasis and endotoxemia-induced hepatic damage through macrophage suppression and the modulation of galactose metabolism [12]. Furthermore, administering high doses of thiamine counteracts diet-associated hepatic steatosis [13].

While the distinct biological actions of thiamine, ZnO NPs, and L-arginine have been documented individually, the hepatoprotective capacity of thiamine against hepatic damage provoked by combined exposure to L-arginine and ZnO NPs has not yet been elucidated. Addressing this gap, the current investigation was designed to explore the protective influence of thiamine on histostereological modifications and serum biochemical markers in a murine model of liver injury induced by L-arginine and ZnO NPs.

MATERIALS AND METHODS

L-arginine

L-arginine hydrochloride (≥98% purity; Sigma-Aldrich, USA) was dissolved in sterile normal saline, and the pH was adjusted to 7.4 ± 0.1 by titration with 0.1 M NaOH.

ZnO NPs

Commercial-grade zinc oxide nanoparticles (ZnO NPs; Catalog No. 721077, Sigma-Aldrich, St. Louis, MO, USA) were utilized in this study. As reported by the manufacturer, the material exhibits a hexagonal wurtzite crystalline phase, a primary particle size spanning 10–30 nm, and a purity of 99% on a trace metals basis, with a density of 5.606 g/cm^3 and a molecular weight of 81.38 g/mol. Prior to daily intraperitoneal administration, the nanoparticles were suspended in sterile phosphate-buffered saline (PBS, pH 7.0–7.4; Sigma-Aldrich, USA) at a stock concentration of 25 mg/mL. To disrupt agglomerates and yield a homogeneous suspension, the mixture was subjected to probe sonication in an ice bath for 15 min (20–40 kHz, 100–200 W), ensuring the temperature remained strictly below 25°C

Animal Experimental design and protocols

Eighty-four adult male BALB/c mice, weighing 22–27 g, were included in this study in accordance with institutional and international guidelines for the care and use of laboratory animals. Animals were maintained in standard cages under controlled conditions, including a 12-hour light/dark cycle, a temperature of 22 ± 2°C, relative humidity of 55–60%, and unrestricted access to standard chow and fresh water [14].

Following a one-week acclimation period, mice were randomly allocated into seven equal groups (n=12). Random allocation was performed using the Random Number Generators function in IBM SPSS Statistics software version 28.0 (IBM Corp., Armonk, NY, USA) to minimize selection bias.

The sample size was selected based on previous similar experimental hepatoprotective,

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biochemical, and stereological studies using comparable experimental designs and outcome measures. A total of 12 animals per group was considered sufficient to provide reliable biochemical and histostereological measurements while minimizing the number of animals used in accordance with ethical principles [10, 16].

The animals were assigned to the following seven groups. The control group received intraperitoneal injections of normal saline. Groups 2 and 3 were administered thiamine at 30 mg/kg and ZnO NPs at 1 mg/kg, respectively, based on a previous stereological and biochemical study using thiamine and ZnO NPs in mice [10]. Hepatic injury was induced in groups 4, 5, 6, and 7 by two intraperitoneal injections of L-arginine hydrochloride at 3000 mg/kg per injection, administered at a 2-hour interval, resulting in a total dose of 6 g/kg [2]. After liver injury induction, groups 5, 6, and 7 received intraperitoneal treatments with thiamine at 30 mg/kg, ZnO NPs at 1 mg/kg, and a combination of thiamine and ZnO NPs, respectively. All administrations were performed intraperitoneally in a final volume of 1 mL once daily for 7 consecutive days.

Animals were monitored twice daily for any clinical signs of distress, abnormal behavior, or mortality during the experimental period. No adverse events or unexpected deaths were observed during the study.

Prior to blood collection via cardiac puncture, mice were deeply anesthetized using an intraperitoneal injection of a combination of ketamine hydrochloride (100 mg/kg) and xylazine (10 mg/kg). The depth of anesthesia was confirmed by the loss of the pedal withdrawal reflex. All efforts were made to minimize animal suffering during the procedure. Blood samples were collected from all groups via cardiac puncture on days 0 and 7 for biochemical analysis.

At the end of the experiment, mice were euthanized by intraperitoneal administration of sodium pentobarbital (150 mg/kg; Sigma-Aldrich, USA). Livers were carefully excised, fixed in 10% neutral buffered formalin, and processed using a Leica TP 1020 tissue processor (Leica, Germany). Tissue sections were stained with hematoxylin and eosin (H&E) and examined using stereological methods to assess histological changes.

To minimize observer bias, histostereological, immunohistochemical, and biochemical assessments were performed by investigators blinded to the treatment group allocation.

Stereological Procedure

Serial sections of entire liver lobes were prepared using systematic uniform random (SUR) sampling. Each lobe was randomly oriented, and a random starting point was selected to obtain parallel sections at uniform intervals across the lobular thickness. Sections (5 µm) were stained with hematoxylin and eosin (H&E) and examined under an Olympus BX41 microscope (Japan) equipped with stereology software to ensure measurement precision.

For quantitative analysis, a calibrated point grid (3–4 cm spacing) was superimposed on the sections. Systematic random sampling was applied to 10–14 microscopic fields per section, and the point-counting method was used to quantify hepatic parenchyma, sinusoidal spaces, connective tissue, central veins, and portal triad components. Volume densities (Vv) were calculated as the ratio of structure-specific points (Ps) to total reference points (Pt) within the grid:

$$V_v = \frac{P_{structure}}{P_{reference}}$$

Where Vv represents the volume density, Ps denotes the number of points intersecting the structure of interest, and Pt corresponds to the total points in the reference space (15, 16).

TGF- β Immunohistochemistry assessment

Liver sections embedded in paraffin were first deparaffinized in xylene and rehydrated through a graded ethanol series. Antigen retrieval was performed by heating the sections in 10 mM citrate buffer (pH 6.0) at 95°C for 20 minutes. Endogenous peroxidase activity was inhibited using 3% hydrogen peroxide in methanol for 15 minutes at room temperature, followed by blocking of non-specific sites with 10% normal goat serum (Vector Laboratories, USA) in PBS for 30 minutes.

Sections were incubated overnight at 4°C with rabbit anti-TGF-β primary antibody (Abcam, UK) diluted 1:200 in PBS containing 1% BSA. After PBS washes, a biotinylated goat anti-rabbit secondary antibody (Vector Laboratories, USA; 1:500) was applied for 1 hour at 37°C, followed by incubation with ABC reagent (Vector Laboratories, USA) for 30 minutes. Visualization was achieved with 3,3′-diaminobenzidine (Agilent, USA) for 5

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minutes, after which sections were counterstained with Mayer's hematoxylin (Sigma-Aldrich, USA), dehydrated, and permanently mounted using Permount (Fisher Scientific, USA).

Biochemical Parameters

Serum enzyme activities were assessed using Pars Azmoon commercial kits. ALT activity was determined by the transfer of an amino group from L-alanine to α-ketoglutarate, forming pyruvate, which was subsequently reduced to lactate with concomitant oxidation of NADH to NAD^+; the decrease in NADH absorbance at 340 nm was measured spectrophotometrically and expressed in units per liter (U/L).

AST activity was evaluated through the transamination of aspartate and α-ketoglutarate to generate glutamate and oxaloacetate, which was then converted to malate by malate dehydrogenase with NADH oxidation; the decline in absorbance at 340 nm reflected AST activity in U/L.

ALP levels were measured via the enzymatic hydrolysis of p-nitrophenylphosphate to p-nitrophenol, and the resulting increase in absorbance at 405 nm was directly proportional to ALP activity, reported in U/L.

Serum antioxidant enzymes

Serum antioxidant enzyme activities were assessed using commercial kits (Navand Salamat, Iran) and spectrophotometric methods. SOD activity was measured with the Nasdox kit based on its capacity to scavenge superoxide radicals (O_2^-), thereby inhibiting pyrogallol autoxidation; the resulting decrease in autoxidation rate was used to calculate SOD activity in units per liter (U/L).

CAT activity was determined with the Nactaz kit by monitoring the decomposition of hydrogen peroxide (H_2O_2) in a mixture containing 1 mL of 30 mM H_2O_2, 50 µL of serum, and 1.95 mL of 50 mM phosphate buffer (pH 7.0). The decrease in absorbance at 240 nm was recorded kinetically, and H_2O_2 concentration was calculated using the formula:

H_2O_2 (mM) = (absorbance × 1000) / molar extinction coefficient (43.6 M^{-1} cm^{-1}).

Catalase activity was expressed in U/L, where one unit corresponds to the amount of enzyme decomposing 1 µmol of H_2O_2 per minute under the assay conditions.

MDA

Serum lipid peroxidation was quantified by measuring MDA levels. In this assay, MDA forms a colored complex with thiobarbituric acid, generating thiobarbituric acid reactive substances (TBARS), which were detected spectrophotometrically at 535 nm. Concentrations of MDA were calculated using a molar extinction coefficient of 1.56×10^5 M^{-1} cm^{-1} and expressed as nanomoles per liter (nmol/L).

Statistical analysis

Results are expressed as mean ± SD. One-way ANOVA with LSD post hoc analysis was conducted using SPSS, and significance was defined as $P < 0.05$.

RESULTS

No animals or data points were excluded from the analysis. All 84 mice completed the 7-day study period.

Body and Liver Weight

At day 0, no significant differences were observed in liver weight or body weight among the experimental groups ($p > 0.05$). By day 7, the L-arginine plus thiamine group and the thiamine + ZnO NPs + L-arginine group showed significantly higher values compared with the L-arginine–only group ($p<0.05$), (Table 1).

Histological Evaluation

Day 0: Across all experimental groups, hepatic architecture was fully preserved. Hepatocytes were organized in tightly packed cords, displaying uniform cytoplasmic staining and centrally positioned nuclei. Sinusoidal spaces retained normal fenestration, and Kupffer cells were evenly distributed throughout the lobules. No evidence of cytoplasmic vacuolation, nuclear abnormalities, or inflammatory cell infiltration was observed. The proportion of binucleated hepatocytes (3–5%) and the structural integrity of portal triads, including both vascular and biliary components, remained within physiological limits. Overall, sinusoidal structures were well maintained, with no signs of congestion or inflammation. (Fig. 1A and B).

Day 7: Hepatic histology in the control, thiamine, and ZnO NPs groups largely maintained normal architecture. In contrast, discernible histopathological alterations were observed in the other experimental groups (Figs. 2A; 3A, B).

Hepatocytes in the L-arginine group exhibited mild to moderate degenerative changes.

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Table 1. Liver and body weight measurements in control and experimantal groups

Parameters	Weight (g)			
	mice		liver	
days	0	7	0	7
Control	25.3 ± 0.7 [a]	25.5 ± 0.6 [a]	1.3 ± 0.2 [a]	1.2 ± 0.1 [a]
Thiamine	25.3 ± 0.9 [a]	24.6 ± 0.7 [a]	1.3 ± 0.1 [a]	1.4 ± 0.2 [a]
ZnONPs	23.3 ± 0.8 [a]	25.3 ± 0.7 [a]	1.4 ± 0.1 [a]	1.3 ± 0.2 [a]
L-arginine	24.4 ± 0.5 [a]	20.2 ± 0.6 [b]	1.2 ± 0.5 [a]	0.66 ± 0.1 [b]
Thiamine + L-arginine	24.6 ± 0.8 [a]	23.6 ± 0.4 [a]	1.3 ± 0.1 [a]	0.99 ± 0.1 [a]
ZnONPs+L-arginine	24.5 ± 0.6 [a]	21.3 ± 0.9 [b]	1.3 ± 0.2 [a]	0.77 ± 0.1 [b]
Thiamine +ZnONPs +L-arginine	24.9 ± 0.4 [a]	23.8 ± 0.3 [a]	1.4 ± 0.2 [a]	1.17 ± 0.2 [a]

Different letters (a&b) indicate significant differences between different treatment groups (P< 0.05); data are presented as Mean ± SEM.

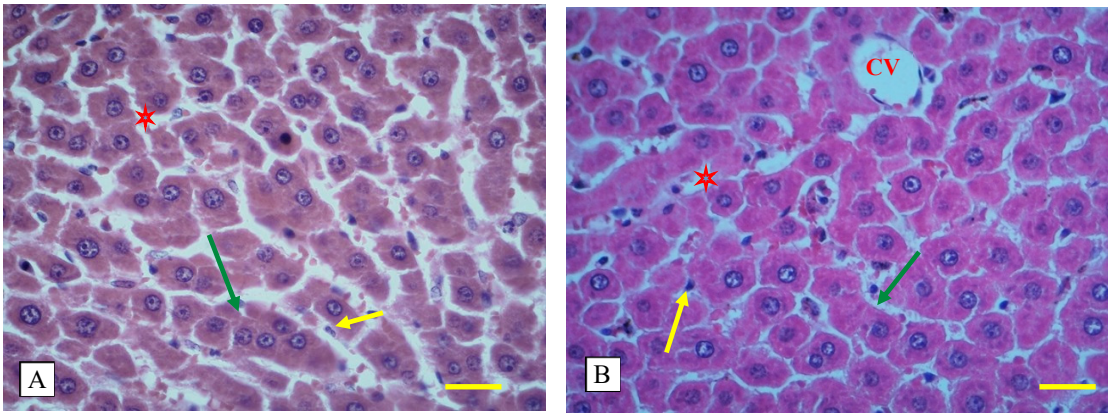


Fig. 1. A photomicrograph illustrating the hepatic histoarchitecture of the control group (A) and L-arginine-treated group (B) at baseline (day 0). The micrograph demonstrates well-organized hepatocyte cords (green arrows) arranged in conjunction with hepatic sinusoids (red stars), which are lined by Kupffer cells (yellow arrows). The central vein (CV) is also clearly identifiable; (H&E staining, ×400 magnification, scale bar=50µm).

Cytoplasmic alterations were frequent, with cells showing granular, eosinophilic, or vacuolated appearances. In some hepatocytes, cytoplasmic swelling resulted in partial membrane distension and cellular lysis. Nuclear abnormalities, including chromatin condensation, pleomorphism, and multinucleation, were also present. In addition, hepatocyte density was decreased, and intercellular spaces were widened, reflecting ongoing degenerative processes (Figs. 2B; 3C).

In the thiamine + L-arginine group, hepatocyte nuclei were generally well preserved, although occasional cytoplasmic vacuolation was noted. Some reduction in tissue damage was observed; however, thiamine alone did not fully prevent the hepatocellular changes associated with L-arginine exposure (Fig. 3D).

In the ZnO NPs + L-arginine group, hepatocyte cytoplasm was largely preserved and appeared intact. Mild nuclear alterations, including chromatin condensation and irregular nuclear membranes, were observed, suggesting that ZnO NPs provided only a limited protective effect against L-arginine–induced hepatic changes (Fig. 3E).

Hepatic architecture was fully preserved in the thiamine + ZnO NPs + L-arginine group. Hepatocytes displayed uniform cytoplasm without vacuolation, and nuclei maintained normal morphology, including even chromatin distribution and distinct nucleoli. No signs of inflammatory cell infiltration, sinusoidal congestion, bile duct dilation, or portal fibrosis were detected. Hepatic plates were well organized, and Kupffer cells remained in a quiescent state, collectively indicating a pronounced protective effect of the combined thiamine and ZnO NPs treatment against L-arginine–induced liver injury (Fig. 3F).

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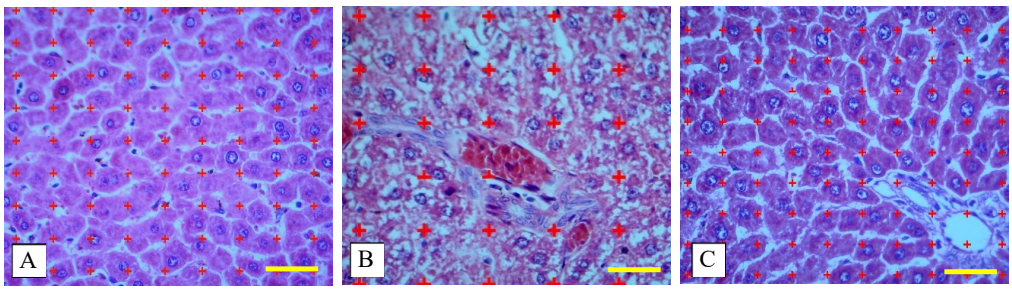


Fig. 2. Photomicrographs illustrating the hepatic histoarchitecture of the control group (A), L-arginine (B) Thiamine + ZnO NPs + L-arginine group (C) on day 7. The L-arginine group (B) exhibited significant hepatic tissue damage, characterized by disrupted cellular architecture and compromised tissue integrity. The Thiamine + ZnO NPs + L-arginine group (C) demonstrated marked histological recovery, with restoration of normal hepatic morphology; (H&E staining, ×400 magnification, scale bar=50µm).

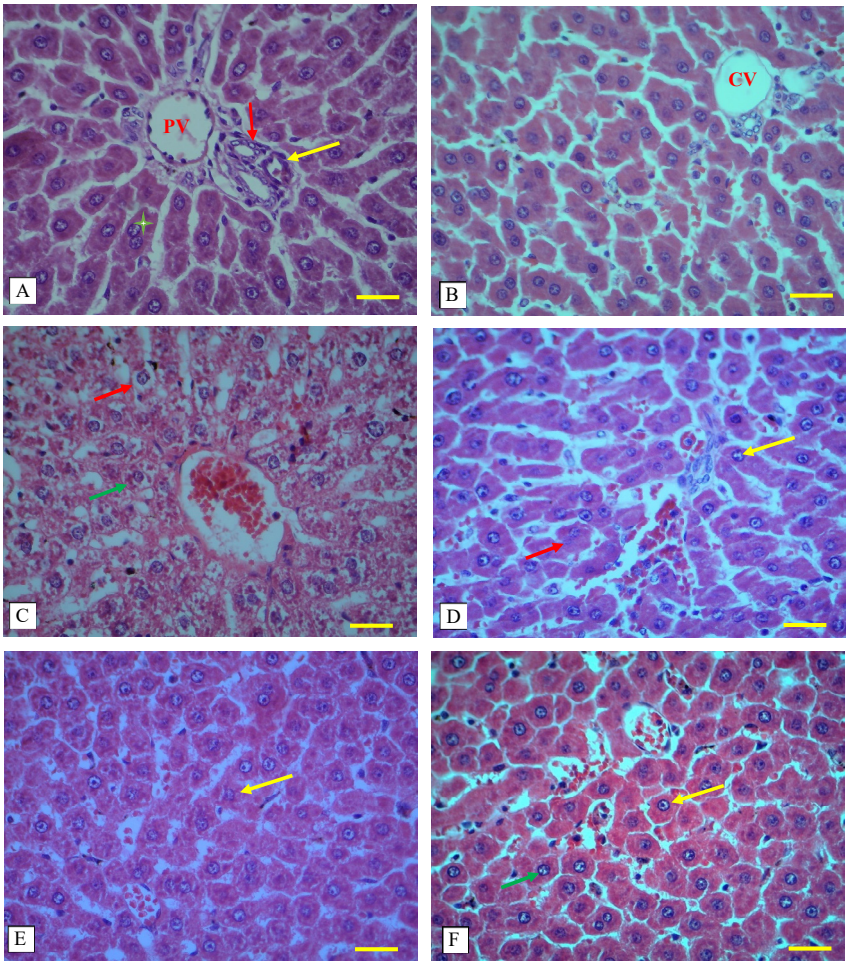


Fig. 3. Photomicrographs of the hepatic structure groups in the control (A) & ZnO NPs (B) groups. The normal hepatic structure consists of lobules made up of hepatocytes (star), arranged around a central vein (CV) and bordered by portal triads (PV), bile duct (red arrow) and hepatic capillaries (yellow arrow). L-arginine group (C): mild to moderate vacuolation (red arrow) and evident disorganization of the hepatic nuclear (green arrow), disrupting the normal lobular architecture into the hepatic parenchyma. The thiamine + L-arginine group (D) showed largely improved nuclei (yellow arrow) but occasional cytoplasmic changes (red arrow), and the ZnO NPs + L-arginine group (E) showed mild cytoplasmic irregularities (yellow arrow). Thiamine + ZnO NPs + L-arginine group (F) showed largely preservation of hepatic architecture, with hepatocytes displaying normal cytoplasmic structure (lack of vacuoles, yellow arrow) and consistently maintaining nuclear morphology, uniform chromatin distribution, distinct nucleoli, (green arrow) ; (H&E staining, ×400 magnification, scale bar=50µm).

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Serological evaluation

At baseline (day 0), no statistically significant differences were observed among the experimental groups ($p>0.05$).

On day 7, hepatocyte volume was significantly higher in the L-arginine group compared with the control group ($p < 0.05$). Treatment with thiamine, ZnO NPs, and their combination markedly reduced hepatocyte volume relative to the L-arginine group ($p < 0.05$).

Additionally, L-arginine administration led to a significant reduction in sinusoidal and interstitial tissue volumes compared with controls ($p < 0.05$). Administration of thiamine alone or together with ZnO NPs significantly increased these volumes relative to the L-arginine group ($p < 0.05$). Total hepatic volume was also significantly decreased following L-arginine treatment, whereas co-administration of thiamine and ZnO NPs significantly restored total liver volume ($p<0.05$; Table 2).

TGF-β Expression (Immunohistochemistry)

Immunohistochemical examination of control hepatic tissue showed only minimal TGF-β expression, consistent with normal physiological levels. Weak staining was detected in isolated hepatocytes and occasional non-parenchymal cells, without any evidence of pathological overexpression. The labeling was primarily cytoplasmic and diffuse, with occasional faint nuclear signals, and no focal intensification was observed. Sinusoidal and periportal regions largely lacked reactivity. Quantitative and spatial evaluations confirmed the absence of abnormal upregulation, underscoring the tightly controlled physiological expression of TGF-β in healthy hepatic tissue. (Fig. 4A).

Administration of L-arginine resulted in a marked increase in TGF-β expression compared with the control group. Immunoreactivity was primarily observed in the cytoplasm of hepatocytes, with occasional nuclear labeling. TGF-β immunoreactivity exhibited moderate to strong intensity and was broadly distributed across hepatic periportal and centrilobular zones. Focal zones of heightened TGF-β immunoreactivity were evident, suggesting localized activation of fibrogenic pathways. This upregulation of TGF-β reflects an active inflammatory response and the early onset of fibrotic changes in hepatic tissue following L-arginine exposure. (Fig. 4 B).

The combined administration of thiamine and ZnO NPs markedly reduced L-arginine–induced TGF-β expression in both intensity and distribution. Immunoreactivity was largely confined to isolated hepatocytes showing weak to moderate cytoplasmic staining. Minimal labeling was observed in periportal and centrilobular regions, with no focal areas of intensified expression. These results suggest that the thiamine and ZnO NPs combination effectively suppresses TGF-β upregulation, likely by modulating inflammatory and fibrogenic pathways, thereby mitigating hepatic injury induced by L-arginine (Fig. 4C).

Biochemical Parameters

L-arginine administration caused a significant elevation in ALT levels compared with the control group ($P < 0.05$). In contrast, all combination treatments including thiamine, ZnO NPs, or their co-administration with L-arginine, produced significant reductions in ALT by day 7 relative to baseline ($P < 0.05$). These groups also displayed significantly lower ALT than the L-arginine group at both time points ($P < 0.05$). Notably, by day 7, ALT levels in the thiamine + ZnO NPs + L-arginine group were comparable to those of the control group ($P > 0.05$, Table 3).

AST levels remained stable and statistically similar in the control, thiamine, and ZnO NPs groups throughout the study, with no significant differences between them at any time point ($P > 0.05$). By contrast, the L-arginine group exhibited a marked increase in AST on days 0 and 7, reflecting significant hepatic injury compared with controls ($P < 0.05$). Administration of thiamine, ZnO NPs, and their combination with L-arginine reduced AST levels by day 7 relative to the L-arginine group, with the most pronounced decrease observed in the thiamine + ZnO NPs + L-arginine group, which approached control values ($P < 0.05$). These findings suggest that thiamine and ZnO NPs, particularly when combined, mitigate L-arginine–induced hepatic injury (Table 3).

ALP levels were stable and statistically comparable among the control, thiamine, and ZnO NPs groups on days 0 and 7 ($P > 0.05$). L-arginine treatment caused a significant increase in ALP compared with control ($P < 0.05$). Co-administration of thiamine or ZnO NPs with L-arginine partially attenuated this elevation, although ALP values remained significantly higher than control ($P < 0.05$). Importantly, the group

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Table 2. volumetric parameters (μm) in the experimental groups

	Parameters							
	Total Liver Volume (mL)		Hepatocyte Volume (mL)		Sinusoidal Volume (mL)		Interstitial Tissue Volume (mL)	
Group	**0**	**7**	**0**	**7**	**0**	**7**	**0**	**7**
Control	1.52 ± 0.18 [a]	1.50 ± 0.22 [a]	0.97 ± 0.12 [a]	1.09 ± 0.12 [a]	0.22 ± 0.06 [a]	0.23 ± 0.02 [a]	0.12 ± 0.04 [a]	0.15 ± 0.01 [a]
Thiamine	1.54 ± 0.21 [a]	1.56 ± 0.25 [a]	0.95 ± 0.14 [a]	1.05 ± 0.14 [a]	0.21 ± 0.02 [a]	0.22 ± 0.03 [a]	0.12 ± 0.03 [a]	0.13 ± 0.01 [a]
ZnONPs	1.49 ± 0.19 [a]	1.55 ± 0.23 [a]	0.99 ± 0.11 [a]	1.08 ± 0.13 [a]	0.20 ± 0.04 [a]	0.24 ± 0.02 [a]	0.14 ± 0.04 [a]	0.14 ± 0.01 [a]
L-arginine	1.50 ± 0.23 [a]	0.72 ± 0.20 [b]	1.05 ± 0.15 [a]	1.67 ± 0.10 [b]	0.24 ± 0.06 [a]	0.11 ± 0.03 [b]	0.16 ± 0.04 [a]	0.05 ± 0.02 [b]
Thiamine +L-arginine	1.51 ± 0.25 [a]	0.87 ± 0.14 [b]	1.12 ± 0.16 [a]	1.32 ± 0.15 [b]	0.23 ± 0.07 [a]	0.18 ± 0.02 [b]	0.18 ± 0.03 [a]	0.08 ± 0.01 [b]
ZnONPs+L-arginine	1.53 ± 0.22 [a]	0.76 ± 0.12 [b]	1.02 ± 0.14 [a]	1.39 ± 0.12 [b]	0.22 ± 0.03 [a]	0.14 ± 0.03 [b]	0.13 ± 0.04 [a]	0.06 ± 0.01 [b]
Thiam+ZnONPs+ L-arginine	1.47 ± 0.27 [a]	1.13 ± 0.25 [a]	1.18 ± 0.17 [a]	1.10 ± 0.15 [a]	0.20 ± 0.07 [a]	0.21 ± 0.01 [a]	0.14 ± 0.02 [a]	0.12 ± 0.01 [a]

Different letters (a&b) indicate significant differences between different treatment groups (P< 0.05); data are presented as Mean ± SEM.

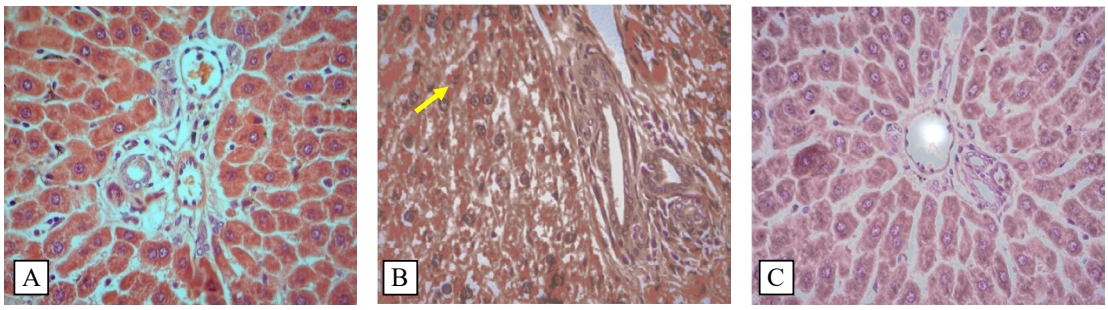


Fig. 4. Immunohistochemical staining for TGF-β in liver tissues from control (A), L-arginine (B), and thiamine + ZnO NPs + L-arginine (C) groups. Extensive brown or dark staining indicates increased TGF-β expression, notably elevated in the L-arginine-treated (yellow arrow), reflecting the effect of high-dose L-arginine administration.

Table 3. ALT, AST and ALP changes (U/L) in the experimental groups

Factors	ALT		AST		ALP	
Groups	0	7	0	7	0	7
Control	69.43±2.51 [a]	67.42±3.73 [a]	54.22±2.36 [a]	56.47±3.08 [a]	304.74±43.1 [a]	322.61±40.5 [a]
Thiamine	64.13±3.37 [a]	66.74±3.45 [a]	53.48±3.21 [a]	51.91±4.27 [a]	327.18±38.9 [a]	316.74±34.2 [a]
ZnONPs	70.08±3.47 [a]	69.27±3.52 [a]	54.61±2.79 [a]	58.82±5.67 [a]	328.82±39.4 [a]	319.87±40.1 [a]
L-arginine	178.61±4.83 [b]	169.76±5.61 [b]	112.84±5.93 [b]	109.69±4.61 [b]	562.13±57.2 [b]	523.44±60.2 [b]
Thiamine +L-arginine	165.38±4.12 [c]	87.98±4.31 [d]	95.63±5.11 [c]	80.27±4.47 [d]	456.61±52.7 [b]	441.47±50.6 [b]
ZnONPs+L-arginine	169.29±4.83 [e]	101.34±5.06 [f]	98.28±4.16 [e]	89.15±4.68 [f]	468.29±48.1 [b]	453.71±50.3 [b]
Thiam+ZnONPs+L-arginine	153.61±4.83 [g]	75.26±4.38 [h,a]	81.76±4.39 [g]	60.37±4.29 [h,a]	331.25±38.2 [a]	329.67±34.1 [a]

Different letters indicate significant differences between days and L-arginine and control groups (P< 0.05); data are presented as Mean ± SEM.

receiving thiamine, ZnO NPs, and L-arginine together showed ALP levels on day 7 that did not differ significantly from control (P > 0.05), indicating complete normalization of ALP activity. This demonstrates that the combined treatment effectively counteracts L-arginine–induced hepatic dysfunction (Table 3).

Serum antioxidant enzymes

SOD activity remained stable and statistically comparable in the control, thiamine, and ZnO NPs groups at days 0 and 7 (P > 0.05), indicating no evidence of oxidative stress. By contrast, L-arginine treatment caused a significant decrease in SOD activity relative to control (P < 0.05), reflecting enhanced oxidative stress. Co-administration of either thiamine or ZnO NPs partially restored SOD levels, whereas the combined treatment of thiamine and ZnO NPs fully reinstated SOD activity to values statistically indistinguishable from controls at both time points (P > 0.05, Table 4).

Similarly, CAT activity in the control, thiamine, and ZnO NPs groups remained stable and statistically similar at days 0 and 7 (P > 0.05). L-arginine administration significantly reduced CAT activity compared with the control group (P < 0.05), indicating increased oxidative stress. Co-treatment with thiamine or ZnO NPs partially improved CAT levels relative to L-arginine alone, but full restoration to control-equivalent activity was achieved only with the combined administration of thiamine and ZnO NPs (P > 0.05). These results suggest a synergistic protective effect of thiamine and ZnO NPs against L-arginine–induced oxidative stress (Table 4).

MDA

MDA levels remained stable and statistically comparable in the control, thiamine, and ZnO NPs groups at both days 0 and 7 (P > 0.05). In contrast, L-arginine administration caused a significant increase in MDA compared with control (P < 0.05), reflecting enhanced oxidative stress and lipid peroxidation. Co-treatment with either thiamine or ZnO NPs partially mitigated this effect; however, full restoration of MDA levels to values statistically indistinguishable from the control group was achieved only with the combined administration of thiamine and ZnO NPs at both time points (P > 0.05). These findings indicate that the

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Table 4. SOD and CAT changes (U/L) in control and experimental groups

Factors	SOD		CAT		MDA (nmol/ml)	
Groups	0	7	0	7	0	7
Control	28.32±3.71[a]	29.26±4.43[a]	18.68±3.25[a]	18.72±3.95[a]	2.42±0.95[a]	2.57±0.59[a]
Thiamine	28.65±5.36[a]	31.47±4.22[a]	19.22±3.47[a]	17.89±2.72[a]	3.01±0.32[a]	2.27±0.31[a]
ZnONPs	30.38±3.28[a]	28.59±5.81[a]	18.56±2.32[a]	19.18±2.69[a]	2.18±0.23[a]	3.36±0.22[a]
L-arginine	13.64±2.78[b]	12.28±3.52[b]	10.12±1.03[b]	9.34±1.62[b]	5.82±0.16[b]	6.68±0.43[b]
Thiamine +L-arginine	23.48±3.91[a]	24.84±3.75[a]	15.17±1.87[a]	16.20±1.77[a]	4.68±0.83[b]	3.42±0.27[a]
ZnONPs+L-arginine	17.75±2.46[b]	19.43±2.18[b]	12.43±1.29[b]	14.23±2.64[b]	4.97±0.78[b]	4.62±0.62[b]
Thiam+ZnONPs+ L-arginine	26.61±3.52[a]	27.76±3.65[a]	16.73±2.19[a]	17.02±2.15[a]	2.64±0.37[a]	2.61±0.94[a]

Different letters indicate significant differences between days and L-arginine and control groups (P< 0.05); data are presented as Mean ± SEM.

combination of thiamine and ZnO NPs effectively prevents L-arginine–induced lipid peroxidation, as demonstrated by normalized MDA levels (Table 4).

DISCUSSION

Our results reveal that high-dose L-arginine exposure creates significant histostereological damage in hepatic tissues, marked by cytoplasmic degeneration, nuclear abnormalities, and decreased cellular density. These findings align with prior data indicating that L-arginine induces liver impairment by stimulating inflammatory cascades and oxidative stress [1]. It is established that L-arginine influences nitric oxide synthase (NOS) activity, impacting liver function in cases of cholestasis and traumatic injury [1, 17]. The hepatic toxicity reported here is linked to excessive ROS production and the activation of fibrogenic factors like IL-1β and TGF-β1, which parallels the pathophysiology observed in liver injury models [5].

Thiamine supplementation yielded distinct improvements in liver architecture. This supports earlier findings showing that thiamine, particularly when combined with ZnO NPs, mitigates metabolic and histopathological disturbances in diabetes models [10]. The protective capacity of thiamine is primarily associated with its role as a cofactor in carbohydrate metabolism and its ability to bolster NADPH levels and glutathione production, which enhances ROS neutralization [13]. Moreover, thiamine prevents hepatic injury in endotoxemia by dampening macrophage activation [12].

ZnO NPs also demonstrated potent anti-inflammatory and antioxidant activities, contributing to the preservation of hepatic integrity. They effectively normalized serum liver markers (ALT, AST, ALP) and modulated apoptotic pathways, preventing hepatocyte death through the upregulation of anti-apoptotic genes like Bcl-2 and downregulation of pro-apoptotic factors such as Bax [8, 9].

Co-administration of thiamine and ZnO NPs provided the most robust protection against L-arginine-induced injury, with no detectable histostereological abnormalities. This aligns with prior evidence that combination therapy effectively neutralizes oxidative stress and protects liver architecture [15]. Stereological analysis showed that this regimen reduced hepatocyte swelling while enhancing interstitial and sinusoidal volumes, reflecting improved microcirculation. This combination fortifies antioxidant defenses by increasing glutathione and NADPH levels via thiamine, while ZnO NPs inhibit apoptotic and inflammatory signaling. These synergistic effects curb hepatocyte death and promote tissue regeneration [8, 13].

L-arginine administration upregulated TGF-β expression, consistent with previous reports [6]. Elevated serum TGF-β and IL-3 levels have been documented in hepatitis B and cirrhosis patients, correlating with disease progression [6]. Our study shows that this combined treatment markedly reduced TGF-β expression, thereby mitigating fibrosis and inflammation [10].

Furthermore, L-arginine treatment significantly increased hepatic injury markers, including MDA, ALP, AST, and ALT, while depressing SOD and CAT activity. Administration of thiamine and/or ZnO NPs restored these parameters to near-control levels, supporting their anti-inflammatory role. ZnO NPs enhance antioxidant defenses by upregulating glutathione peroxidase, glutathione-S-transferase, and key enzymes [10, 13, 18]. Additionally, they boost proteins like superoxide

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dismutase and metallothionein to efficiently scavenge ROS and diminish oxidative stress. Concurrently, ZnO NPs reduce lipid peroxidation biomarkers, including TBARS and MDA, thereby maintaining hepatocyte integrity. They also lower pro-inflammatory cytokines such as IL-6 and TNF-α, indirectly bolstering antioxidant function by suppressing inflammatory-induced oxidative stress [19, 20].

Thiamine diphosphate, a vital coenzyme, facilitates energy metabolism and maintains redox homeostasis via aerobic respiration. It lowers ROS generation and boosts endogenous antioxidant defenses, whereas thiamine deficiency impairs these processes, inducing oxidative damage [21–24]. Overall, combination therapy with thiamine and ZnO NPs provides additive or synergistic hepatoprotection, as evidenced by more pronounced normalization of hepatic enzymes and oxidative stress biomarkers compared with monotherapy. This enhanced protection likely arises from the suppression of oxidative stress, modulation of apoptotic signaling, and regulation of cellular energy metabolism [8, 9, 12].

CONCLUSION

High doses of L-arginine induce hepatic injury primarily via oxidative stress and inflammatory mechanisms, as indicated by elevated hepatic enzyme levels, increased lipid peroxidation, depletion of antioxidant enzymes, and histostereological alterations in hepatic tissue. Administration of thiamine and ZnO NPs effectively counteracts these adverse effects by strengthening antioxidant defenses (SOD and CAT), lowering oxidative damage (MDA), and suppressing fibrogenic signaling (TGF-β), thereby maintaining hepatocellular integrity and normal tissue architecture. Thiamine supports mitochondrial activity and redox balance, while ZnO NPs enhance hepatic antioxidant capacity by upregulating key enzymes, mitigating oxidative stress, and attenuating inflammatory responses.

AUTHORS' CONTRIBUTIONS

R. Fatahian Dehkordi conceptualized and designed the study, supervised the experimental procedures, and performed the histostereological analysis and immunohistochemistry (TGF-β) assessments. J. Kaboutari contributed to the biochemical assays, interpreted the serological data, and provided critical revisions to the manuscript. A. A. Azadi was responsible for the animal handling, and statistical analysis. All authors participated in drafting the manuscript and have read and approved the final version for submission.

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ETHICAL APPROVAL

The study protocol was reviewed and approved by the Research Vice-Chancellorship of Shahrekord University (IR.SKU.REC.1404.096), in accordance with international guidelines for the care and use of laboratory animals.

CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest regarding the publication of this paper.

DATA AVAILABILITY

The biochemical, stereological, and immunohistochemical data used to support the findings of this study are available from the corresponding author upon request.

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