

RESEARCH PAPER

Direct In Vitro Effect of Zinc Oxide Nanoparticles on Serum ALT and AST Activities in Thalassemia Patients

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ABSTRACT

The present study evaluated the in vitro effect of chemically synthesized zinc oxide nanoparticles (ZnO NPs) on serum ALT and AST activities in transfusion-dependent β -thalassemia major patients (n = 30). Hexagonal wurtzite ZnO NPs (XRD crystallite size, 24.61 nm; SEM mean size, 58.23 nm) exhibited high hemocompatibility (< 2.0% hemolysis at 32-256 μ g/mL). Aliquots of Thalassemic serum were incubated with ZnO NPs (0–100 μ g/mL) for 6–24 h. Exposure caused a pronounced concentration- and time-dependent inhibition of transaminase activities. ALT exhibited extreme vulnerability, with reductions progressing from $51.6 \pm 9.1\%$ (6 h, 25 μ g/mL; GMR = 0.477) to $93.8 \pm 5.2\%$ (24 h, 100 μ g/mL; GMR = 0.050). However, AST was more stable with a maximum decrease of $30.3 \pm 8.5\%$ (GMR = 0.693). Two-way RM-ANOVA confirmed significant main and interaction effects of dose and duration (partial $\eta^2 > 0.75$, $p < 0.005$). Mechanistically, this inhibition is mediated by protein corona formation, active site masking, and optical scattering at 340 nm. These results demonstrate the danger of severe false-negative diagnostic underestimation of transaminase biomarkers during nanoparticle exposure.

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INTRODUCTION

β -Thalassemia is among the most common genetic hemoglobin production disorders that require regular blood transfusions to sustain patient survival [1, 2]. However, ongoing blood transfusions lead to iron overload in vital tissues, particularly the liver, causing oxidative stress and cellular injury, as evidenced by persistently elevated serum aminotransferase levels, including alanine aminotransferase (ALT) and aspartate aminotransferase (AST) [3]. Thus, these enzymes are the most important clinical biomarkers for assessing the extent of oxidative stress and hepatic damage in these patients [1]. Zinc oxide

nanoparticles (ZnO NPs) have unique optical and physicochemical properties, and their interactions with biological systems have led to a significant increase in their medical and biological applications [4, 5]. The high surface activity of these particles and their ability to release zinc ions upon entering or coming into contact with biological fluids such as blood and serum make them highly prone to binding to biomolecules [6, 7]. The direct in vitro interaction between nanoparticles and enzymes is governed by distinct biochemical mechanisms compared with the direct in vivo cytotoxic effect, which usually results in enzyme release and

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increased serum concentrations [8, 9]. When ZnO nanoparticles come into contact with serum, their surface is rapidly covered by a dynamic layer of adsorbed proteins, known as the protein corona [10]. This physicochemical interaction may alter the three-dimensional conformation of the enzymes or occlude their active sites, thereby directly inhibiting their enzymatic activity [11, 12]. Furthermore, the presence of nanoparticles in biological specimens can interfere with spectroscopic reagents used in routine laboratory assays, leading to false-negative results that prevent an accurate diagnostic evaluation [13–15]. Despite the critical diagnostic importance of ALT and AST enzymes in thalassemia patients, who possess a serum matrix characterized by specific biochemical properties such as elevated levels of ferritin and free iron, the impact of direct exposure to ZnO NPs on the activity of these enzymes in the patients' serum has not been adequately investigated. Accordingly, this study aims to systematically evaluate, *in vitro*, the concentration and time-dependent effects of ZnO NPs on the enzymatic activities of ALT and AST in the serum of thalassemia patients, while elucidating the kinetic variations in the enzymes' sensitivity to nanoparticle-induced inhibition and the underlying biochemical mechanisms.

MATERIALS AND METHODS

Synthesis of ZnO Nanoparticles

Zinc oxide nanoparticles (ZnO NPs) were prepared via chemical precipitation. Briefly, $\text{Zn}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$ (4.0 g, 0.54 M) and NaOH (1.0 g, 1.0 M) were dissolved separately in 25 mL of deionized water. The precursor solution was heated to 60 °C, and the NaOH solution was added dropwise over 30 min under continuous stirring until reaching pH 7. The mixture was ultrasonicated for 15 min, magnetically stirred at 90–100 °C for 1 h, and filtered under vacuum (Whatman No. 42). The precipitate was washed with absolute ethanol (2×) and deionized water (3×), dried at 120 °C for 6 h, and calcined at 600 °C for 4 h to yield ZnO NPs.

Study Design and Experimental Workflow

Serum samples were collected from 30 transfusion-dependent β -thalassemia major patients ($n = 30$) and 30 age- and sex-matched healthy controls ($n = 30$) recruited from Diyala Governorate, Iraq. Equal amounts of each donor were treated with ZnO NPs at concentrations of

0 (control), 25, 50, and 100 $\mu\text{g}/\text{mL}$ and incubated for 6, 12, 18, or 24 h. Samples were centrifuged after incubation to sediment nanoparticles and to reduce analytical interferences. The clarified supernatants were immediately analyzed for ALT and AST activity using a Mindray BS-120 auto-analyzer. All experimental assays were performed in triplicate (3×).

Ethical Approval and Patient Selection

Diyala Health Directorate, Iraq, and conducted in accordance with the Declaration of Helsinki. This study was approved by the Institutional Review Board (IRB). All participants or their legal guardians provided written informed consent. The cohort included 30 patients with iron overload and transfusion-dependent beta-thalassemia major, as well as 30 healthy controls. Exclusion criteria were active viral hepatitis (HBV/HCV), HIV, liver cirrhosis, or concurrent use of hepatotoxic drugs. Venous blood was drawn after an overnight fast, allowed to clot at room temperature, and centrifuged ($3000 \times g$, 10 min). Clarified serum aliquots were either analyzed immediately or stored at $-80\text{ }^\circ\text{C}$ [16].

Biochemical Measurement of Serum ALT and AST Activities

Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities were measured at 37 °C using an automatic clinical chemistry analyzer (Mindray BS-120, Shenzhen Mindray Bio-Medical Electronics Co., Ltd., China) and the corresponding commercial reagent kits, according to the IFCC standardized UV kinetic methods without pyridoxal phosphate activation [17–19]. Enzyme activity was determined in both assays by measuring the rate of decrease in NADH absorbance at 340 nm [17–19]. The measuring ranges were linear from 4 to 1000 U/L for ALT and 4 to 800 U/L for AST [17–19]. Samples exceeding upper linearity limits were diluted in 9 g/L NaCl saline, re-assayed, and multiplied by the corresponding dilution factor [17–19]. System calibration and analytical accuracy were regularly verified using Mindray Multi Sera Calibrator and two levels of Mindray ClinChem Multi Control materials according to the manufacturer's specified acceptable ranges [17–19].

In Vitro Hemocompatibility Evaluation

To evaluate cell membrane integrity and

establish the biosafety profile of ZnO nanoparticles prior to downstream serum-exposure assays, an *in vitro* hemolysis test was conducted across serial concentrations (32, 64, 128, and 256 µg/mL) [20]. Phosphate-buffered saline (PBS) and 1% Triton X-100 served as the negative control (baseline 0% lysis) and positive control (complete 100% cellular disruption), respectively [20, 21]. After incubating the RBC suspensions at 37 °C for 1 h, samples were centrifuged at 1500 × g for 10 min, and the released hemoglobin in the supernatant was quantified spectrophotometrically at 540 nm using a UV-Vis spectrophotometer. The quantitative percentage of hemolysis was calculated according to the

following standard equation [20, 21]:

$$\text{Hemolysis (\%)} = \frac{(A_{\text{sample}} - A_{\text{negative control}})}{(A_{\text{positive control}} - A_{\text{negative control}})} \times 100$$

Statistical Analysis

Data were analyzed using GraphPad Prism (v9.0) and SPSS (v26.0). Continuous metrics are expressed as mean ± SD or median (IQR), and percentage reductions are reported with 95% CIs. Enzymatic activities and treatment-to-control ratios were log-transformed to meet parametric assumptions and back-transformed to report geometric mean ratios (GMR). The main and

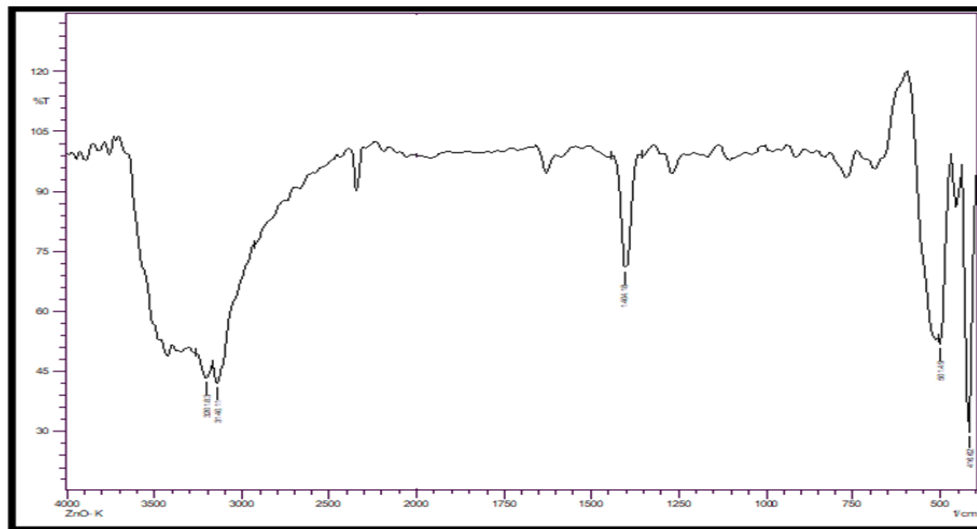


Fig. 1. FT-IR spectrum of the synthesized ZnO nanoparticles.

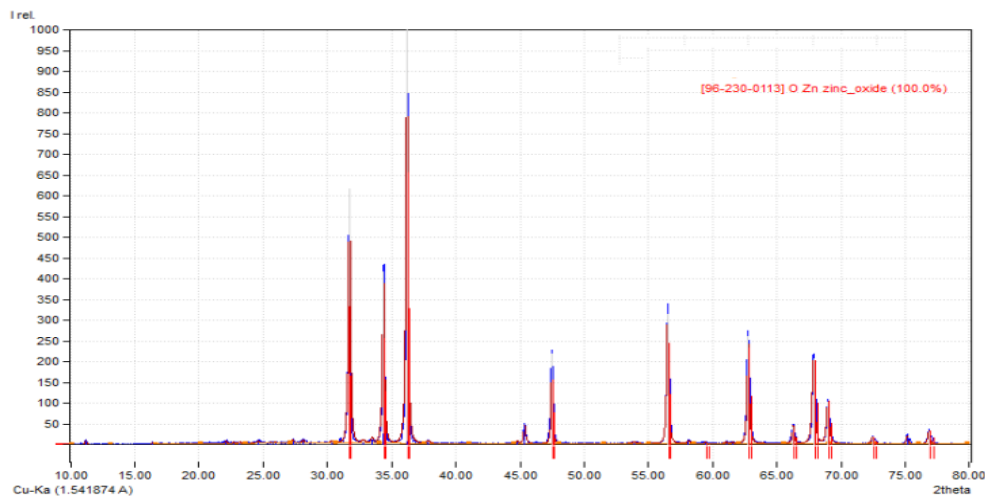


Fig. 2. XRD pattern of the synthesized ZnO nanoparticles.

interaction effects of ZnO NP concentration (0–100 $\mu\text{g/mL}$) and incubation duration (6–24 h) on ALT and AST activity were evaluated by two-way RM-ANOVA with Greenhouse-Geisser correction and partial η^2 effect sizes. Pairwise comparisons between treatment and control were conducted using planned contrasts with Holm's adjustment of p-values and Cohen's d effect sizes. Statistical significance was defined as two-tailed $p < 0.05$.

RESULTS AND DISCUSSION

FT-IR Spectroscopic Analysis of ZnO Nanoparticles

FT-IR spectroscopy (Fig. 1) of synthesized ZnO nanoparticles was used to validate the functional groups and phase formation. The characteristic absorption bands at 501.49 and 416.62 cm^{-1} are attributed to the Zn-O stretching vibrations, confirming the hexagonal wurtzite structure [22]. Symmetric C–O/C=O stretching or residual precursor nitrates (NO_3^-) [23] are assigned to a sharp band at 1404.18 cm^{-1} . Moreover, the broad high-frequency bands at 3201.83 cm^{-1} and 3140.11 cm^{-1} are attributed to O–H stretching of surface-adsorbed hydroxyl groups and moisture, respectively, thereby indicating the structural purity of the nanoparticles [24].

Structural Analysis of ZnO Nanoparticles by XRD

The crystalline phase purity and structural parameters of synthesized ZnO nanoparticles were determined by X-ray diffraction (XRD)

analysis (Fig. 2). The XRD pattern reveals sharp and highly intense diffraction peaks, which are in full agreement with the standard crystallographic reference (COD Entry No. 96-230-0113), indicating a single-phase hexagonal wurtzite structure with 100.0% phase purity and space group P63mc [25]. The refined lattice parameters were $a = 3.2494 \text{ \AA}$ and $c = 5.2054 \text{ \AA}$ with a calculated theoretical density of 5.677 g/cm^3 and a stoichiometric elemental distribution of 80.3 wt% Zn and 19.7 wt% O. No secondary phases or crystalline impurities from unreacted precursor materials were observed, indicating high phase purity. The average crystallite size (D) was calculated as 24.61 nm using the Debye–Scherrer equation across the characteristic Bragg reflection angles ($2\theta = 27.53^\circ$ – 68.18° , Cu-K α radiation, $\lambda = 1.54178 \text{ \AA}$, $K = 0.94$), which confirmed the successful fabrication of ultra-small nanoscale crystallites [26].

EDX Elemental Analysis of ZnO Nanoparticles

Fig. 3 shows the Energy-Dispersive X-ray (EDX) spectroscopy analysis of the elemental composition and chemical purity of synthesized ZnO nanoparticles. The EDX spectrum clearly shows the characteristic optical absorption peaks for Zn (Zn L α at $\sim 1.01 \text{ keV}$, Zn K α at $\sim 8.63 \text{ keV}$, and Zn K β at $\sim 9.57 \text{ keV}$) and O (O K α at $\sim 0.52 \text{ keV}$), confirming the synthesis of zinc oxide nanostructures unequivocally [27]. Quantitative elemental analysis demonstrated a

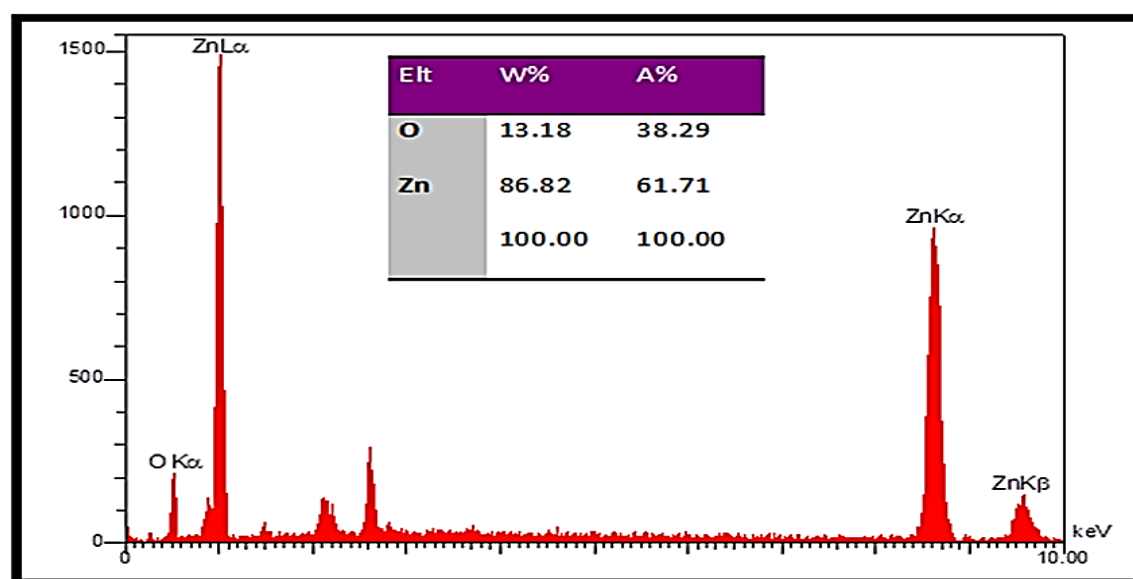


Fig. 3. EDX spectrum and elemental composition of the synthesized ZnO nanoparticles.

weight composition of 86.82 wt % Zn and 13.18 wt % O, corresponding to atomic percentages of 61.71 at % Zn and 38.29 at % O, respectively. The nonexistence of additional impurity peaks in the spectrum confirms the high chemical purity and phase stoichiometry of the prepared nanoparticles, therefore strongly supporting the XRD and FT-IR observations [28].

Morphological Analysis by SEM of the synthesized ZnO nanoparticles

Scanning Electron Microscopy (SEM) was used to characterize the surface morphology,

grain shape and particle size distribution of the synthesized ZnO nanoparticles (Fig. 4). The SEM micrograph shows quasi-spherical and faceted polyhedral nanoparticles with sharp boundaries. There is also significant agglomeration of the nanoparticles owing to high surface energy and cohesive electrostatic forces during the synthesis process [31]. The particle size distribution is shown in the inset histogram ranging from 35–75 nm and the calculated mean particle size is 58.23 nm [29]. The discrepancy between the SEM average particle size (58.23 nm) and the XRD crystallite size (24.61 nm) is explained by the aggregation of

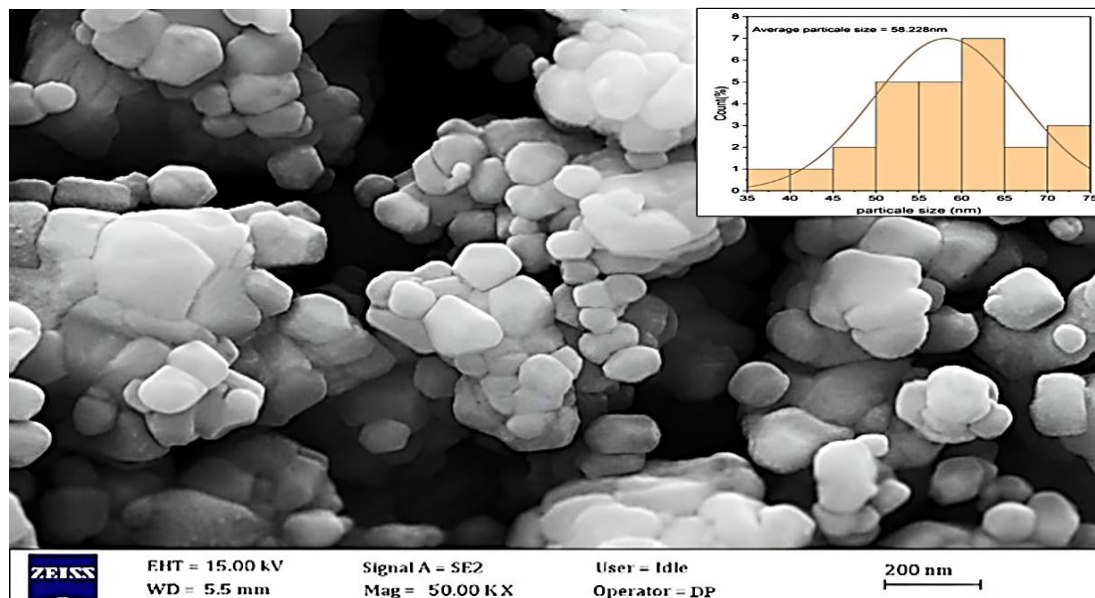


Fig. 4. SEM micrograph of the synthesized ZnO nanoparticles (magnification: 50.00 KX) with the inset showing the particle size distribution histogram (mean size: 58.23 nm).

Table 1. Serum ALT and AST activities across varying ZnO NP concentrations and incubation times.

Time (h)	ZnO (µg/mL)	ALT Mean ± SD	ALT Median (IQR)	AST Mean ± SD	AST Median (IQR)
6	Control	421.46 ± 712.91	106.30 (75.00–190.00)	416.90 ± 645.25	92.50 (59.00–323.00)
	25	205.72 ± 358.37	49.80 (32.80–73.00)	354.48 ± 541.87	76.40 (54.00–284.00)
	50	171.44 ± 301.94	39.20 (26.40–57.90)	333.48 ± 509.81	71.40 (51.70–265.00)
	100	166.72 ± 298.36	38.70 (22.90–50.70)	339.32 ± 523.32	72.40 (53.10–261.00)
12	Control	422.98 ± 721.54	102.80 (72.90–188.00)	415.08 ± 638.62	89.60 (58.40–331.00)
	25	150.32 ± 260.98	30.70 (20.60–65.00)	343.24 ± 521.60	69.40 (50.40–287.00)
	50	156.82 ± 286.49	33.40 (21.30–42.80)	349.56 ± 534.93	71.50 (51.20–285.00)
	100	108.22 ± 197.84	21.90 (16.20–27.50)	319.14 ± 490.02	64.60 (47.40–258.00)
18	Control	421.90 ± 715.19	106.90 (75.90–183.00)	415.08 ± 639.86	91.60 (60.40–326.00)
	25	162.90 ± 288.65	34.40 (29.30–48.00)	347.16 ± 530.30	73.60 (53.70–280.00)
	50	74.60 ± 129.74	13.20 (12.80–33.60)	305.98 ± 466.25	63.20 (49.60–248.00)
	100	53.14 ± 96.72	11.40 (11.00–14.10)	299.02 ± 444.81	61.50 (50.30–261.00)
24	Control	415.02 ± 711.26	101.70 (70.20–179.00)	410.14 ± 636.06	88.80 (56.50–318.00)
	25	57.38 ± 99.86	14.00 (11.60–14.60)	310.12 ± 476.09	61.60 (46.70–248.00)
	50	47.96 ± 86.12	9.30 (9.20–11.00)	300.02 ± 453.00	60.00 (44.60–258.00)
	100	24.88 ± 46.48	4.10 (3.40–6.10)	268.52 ± 401.42	51.70 (44.20–232.00)

several single sub-nanometer crystallites to form larger polycrystalline grains observed by electron microscopy [30].

Descriptive Statistics of Serum ALT and AST Activities

Table 1 shows the serum ALT and AST activities after in vitro exposure to ZnO NPs at different concentrations (0, 25, 50 and 100 µg/mL) and incubation times (6, 12, 18 and 24 h). The unexposed baseline control (0 µg/mL) showed increased enzyme activities (ALT: 415.02-422.98 U/L; AST: 410.14-416.90 U/L), which suggest baseline hepatic involvement and chronic iron overload in patients with β -thalassemia major [1, 3]. Incubation with ZnO NPs resulted in a progressive reduction of the measured transaminase activities, in a concentration and time dependent manner. At 6 h, exposure to 25 µg/mL ZnO NPs decreased mean ALT activity from 421.46 ± 712.91 U/L to 205.72 ± 358.37 U/L, reaching a minimum of 24.88 ± 46.48 U/L after 24 h at 100 µg/mL. Similarly, mean AST activity fell from 410.14 ± 636.06 U/L in baseline controls to 268.52 ± 401.42 U/L under maximum exposure conditions (24 h, 100 µg/mL). Unlike in vivo toxicological pathways, where enzyme leakage is caused by cell lysis, this direct in vitro suppression of activity is caused by physical interactions on

the surface such as protein corona formation, conformational changes, or catalytic site blockage [8, 10, 11].

Percentage Reduction in Serum Transaminase Activities

Table 2 shows the percent reductions in serum ALT and AST activities compared with the unexposed controls and indicates a significant difference in kinetic sensitivity to nanoparticles. The ALT had high susceptibility to ZnO NPs with mean reductions from $51.6 \pm 9.1\%$ (6 h, 25 µg/mL) to $93.8 \pm 5.2\%$ (24 h, 100 µg/mL). In contrast, AST was more stable, with decreases of $12.9 \pm 3.7\%$ to $30.3 \pm 8.5\%$ under the same conditions. This differential inhibition indicates differences in structural binding affinities, protein conformations or surface charge interactions with the nanoparticle protein corona [9, 12]. Furthermore, colloidal optical interference at 340 nm during UV kinetic measurements may result in lower detectable enzyme activity [13–15].

Repeated-Measures Analysis of Variance (RM-ANOVA)

As presented in Table 3, two-way repeated-measures ANOVA with Greenhouse-Geisser correction revealed significant main and interactive effects of ZnO NP concentration and

Table 2. Percentage reduction in serum ALT and AST activities relative to controls.

Time (h)	ZnO (µg/mL)	ALT reduction, Mean $\pm$ SD (%)	95% CI	AST reduction, Mean $\pm$ SD (%)	95% CI
6	25	51.6 ± 9.1	40.3–63.0	12.9 ± 3.7	8.3–17.5
	50	60.5 ± 8.5	50.0–71.1	17.5 ± 4.5	12.0–23.1
	100	63.1 ± 9.0	51.9–74.3	16.8 ± 4.7	11.0–22.6
12	25	64.6 ± 9.0	53.4–75.8	16.0 ± 4.3	10.6–21.4
	50	66.0 ± 11.9	51.2–80.7	15.1 ± 3.5	10.8–19.4
	100	75.8 ± 9.6	63.9–87.8	22.8 ± 3.3	18.8–26.9
18	25	62.2 ± 9.7	50.2–74.2	14.9 ± 3.5	10.6–19.2
	50	82.6 ± 7.4	73.4–91.8	24.4 ± 4.9	18.3–30.5
	100	87.0 ± 10.6	73.9–100.2	23.9 ± 7.1	15.1–32.7
24	25	83.9 ± 7.7	74.3–93.5	22.6 ± 5.5	15.8–29.4
	50	86.9 ± 8.4	76.4–97.4	24.8 ± 5.6	17.8–31.7
	100	93.8 ± 5.2	87.3–100.2	30.3 ± 8.5	19.7–40.8

Table 3. Two-way RM-ANOVA for serum ALT and AST activity dynamics.

Biomarker	Effect	F	GG-adjusted df	ϵ	Adjusted p	Partial η^2
ALT	Concentration	74.077	1.070, 4.281	0.357	<0.001	0.949
	Time	53.106	1.657, 6.627	0.552	<0.001	0.930
	Concentration $\times$ Time	22.432	2.676, 10.704	0.297	<0.001	0.849
	Concentration	68.625	1.016, 4.062	0.339	0.001	0.945
AST	Time	15.738	1.735, 6.939	0.578	0.003	0.797
	Concentration $\times$ Time	12.457	2.137, 8.550	0.237	0.003	0.757

incubation duration on ALT and AST activities. For ALT, highly significant main effects were observed for concentration ($F = 74.077$, $p < 0.001$, partial $\eta^2 = 0.949$) and incubation time ($F = 53.106$, $p < 0.001$, partial $\eta^2 = 0.930$), as well as their interaction ($F = 22.432$, $p < 0.001$, partial $\eta^2 = 0.849$). Similarly, AST showed significant main effects for concentration ($F = 68.625$, $p = 0.001$, partial $\eta^2 = 0.945$) and time ($F = 15.738$, $p = 0.003$, partial $\eta^2 = 0.797$), along with a significant interaction effect ($F = 12.457$, $p = 0.003$, partial $\eta^2 = 0.757$). High effect sizes across all factors (partial $\eta^2 > 0.75$) confirm that nanoparticle dose and exposure duration independently and synergistically drive transaminase inactivation in thalassemic serum [7, 11].

Pairwise Treatment-to-Control Comparisons for ALT

As shown in Table 4, pairwise contrast analyses of log-transformed treatment-to-control geometric mean ratios (GMRs) with Holm's correction confirmed significant reductions in ALT activity across all evaluated conditions (adjusted $p \leq 0.007$). Increasing ZnO NP concentration and exposure time caused a progressive decrease in GMR from 0.477 (52.3% reduction; 6 h, 25 $\mu\text{g}/\text{mL}$) to a minimum of 0.050 (95.0% reduction; 24 h, 100 $\mu\text{g}/\text{mL}$). Very large standardized effect sizes (absolute Cohen's d from 3.00 to 4.57) are

indicative of significant nanoparticle interference that may result in dramatic false-negative underestimation of serum ALT levels in routine automated clinical assays [13–15].

Pairwise Treatment-to-Control Comparisons for AST

Table 5 shows that pairwise contrast analyses of log-transformed treatment-to-control GMRs with Holm's correction confirmed statistically significant reductions in AST activity for all conditions evaluated (adjusted $p \leq 0.009$). The GMR gradually decreased from 0.870 (13.0% reduction; 6 h, 25 $\mu\text{g}/\text{mL}$) to a minimum of 0.693 (30.7% reduction; 24 h, 100 $\mu\text{g}/\text{mL}$) with increasing ZnO NP concentration and exposure time. Standardized effect sizes were large at all timepoints with a range of absolute Cohen's d from 2.92 to 6.05. The percentage of total reduction of AST was observed to be less than that of ALT, which further confirms the higher enzymatic stability of AST and its different structural binding dynamics with ZnO nanoparticles [9, 12, 13–15].

In Vitro Hemocompatibility Evaluation of ZnO Nanoparticles

An in vitro hemolysis evaluation was performed using serial ZnO NP concentrations (32, 64, 128 and 256 $\mu\text{g}/\text{mL}$) to evaluate the biosafety profile

Table 4. Pairwise GMR comparisons for serum ALT activity.

Time	Concentration	Treatment/Control GMR	ALT reduction	95% CI	Adjusted p	Cohen's dz
6 h	25	0.477	52.3%	40.2–61.9	0.007	4.07
	50	0.388	61.2%	49.9–70.0	0.006	4.57
	100	0.360	64.0%	51.2–73.4	0.007	4.18
12 h	25	0.346	65.4%	53.5–74.4	0.006	4.43
	50	0.325	67.5%	50.4–78.7	0.007	3.30
	100	0.228	77.2%	63.6–85.7	0.007	3.92
18 h	25	0.368	63.2%	49.4–73.2	0.007	3.90
	50	0.162	83.8%	72.7–90.4	0.006	4.32
	100	0.102	89.8%	73.7–96.0	0.007	3.00
24 h	25	0.147	85.3%	73.8–91.7	0.007	4.14
	50	0.113	88.7%	76.2–94.7	0.007	3.62
	100	0.050	95.0%	87.8–98.0	0.007	4.13

GMR: Geometric Mean Ratio; CI: Confidence Interval. The analysis was performed using the log-transformed treatment-to-control ratio, followed by back-transformation for interpretation. All p-values were adjusted for multiple comparisons using the Holm correction method.

and red blood cell (RBC) membrane integrity prior to downstream serum-exposure assays [20, 21]. As illustrated in Fig. 5, 1% Triton X-100 (positive control) treated erythrocytes were completely disrupted with marked release of hemoglobin in the supernatant (100% hemolysis). Conversely, PBS incubation (negative control) resulted in a clear supernatant with intact pelleted RBCs. The supernatants of all tested concentrations of ZnO NP (32–256 $\mu\text{g}/\text{mL}$) were clear with well-defined

RBC pellets with visual profiles comparable to that of the negative control (Fig. 5). Quantitative spectrophotometric analysis at 540 nm confirmed that the hemolysis was minimal ($< 2.0\%$ for all tested concentrations), which is much lower than the 5.0% hemolysis threshold for non-hemolytic biomaterials according to ASTM F756 [20, 21]. These results agree with the good hemocompatibility and structural integrity of RBC membranes in the presence of ZnO NPs and

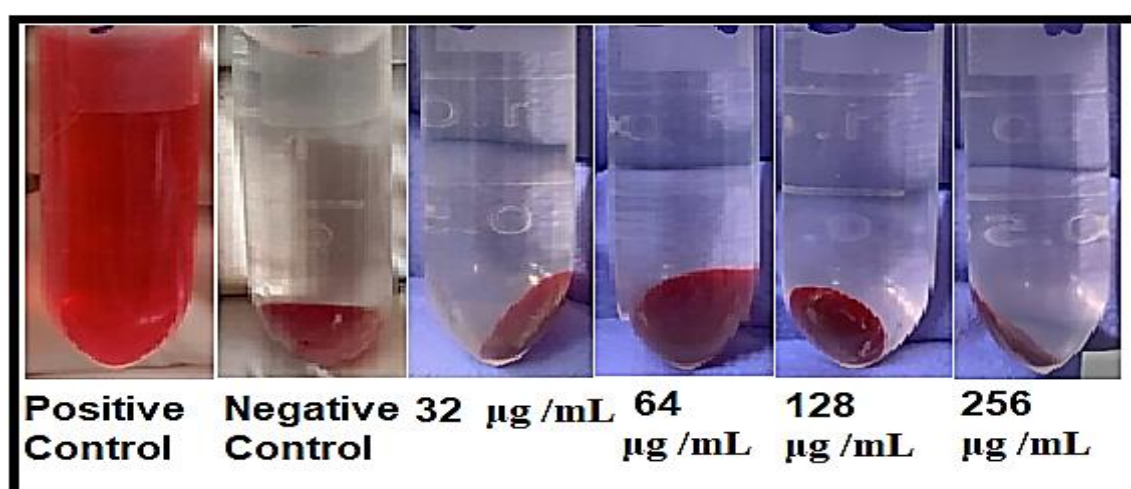


Fig. 5. In vitro hemocompatibility assay of red blood cells exposed to serial concentrations of synthesized ZnO nanoparticles (32, 64, 128, and 256 $\mu\text{g}/\text{mL}$), compared with negative control (PBS) and positive control (1% Triton X-100).

Table 5. Pairwise GMR comparisons for serum AST activity.

Time	Concentration	Treatment/Control GMR	AST reduction	95% CI	Adjusted p	Cohen's dz
6 h	25	0.870	13.0%	8.3–17.4	0.009	3.28
	50	0.824	17.6%	11.9–23.0	0.009	3.59
	100	0.831	16.9%	10.9–22.4	0.009	3.32
12 h	25	0.839	16.1%	10.4–21.4	0.009	3.35
	50	0.849	15.1%	10.7–19.3	0.009	3.99
	100	0.771	22.9%	18.7–26.9	0.002	6.05
18 h	25	0.851	14.9%	10.5–19.1	0.009	3.94
	50	0.755	24.5%	18.1–30.4	0.007	4.29
	100	0.758	24.2%	14.7–32.6	0.009	2.92
24 h	25	0.773	22.7%	15.5–29.4	0.009	3.57
	50	0.751	24.9%	17.5–31.7	0.009	3.79
	100	0.693	30.7%	19.0–40.7	0.009	2.92

GMR: Geometric Mean Ratio; CI: Confidence Interval. The analysis was performed using the log-transformed treatment-to-control ratio, followed by back-transformation for interpretation. All p-values were adjusted for multiple comparisons using the Holm correction method.

show that the decrease in activities of ALT and AST is due to direct physical interactions of enzymes with nanoparticles and not due to artifacts like membrane-disruption [8, 20].

CONCLUSION

The present study clearly demonstrates that the direct in-vitro exposure of synthesized hexagonal wurtzite ZnO nanoparticles significantly suppresses the serum ALT and AST activities in a dose and time-dependent manner in transfusion-dependent β -thalassemia major patients. The red blood cell hemolysis assays confirmed good hemocompatibility (< 2.0% lysis) and preservation of the membrane. However, a significant difference in the enzyme vulnerability kinetics was revealed. ALT was highly susceptible to nanoparticle-induced inactivation (up to 93.8% reduction), while AST was significantly more structurally stable (up to 30.3% reduction). Two-way RM-ANOVA revealed that nanoparticle concentration and incubation time are independent and synergetic factors for transaminase suppression (partial $\eta^2 > 0.75$). Mechanistically, loss of activity is caused by surface physical interactions including protein corona formation, masking of the catalytic active site, and colloidal light scattering that interferes with standard automated UV kinetic measurements at 340 nm. Therefore, clinical biochemists and hematologists should be careful in evaluating the liver function of thalassemic patients exposed to zinc-based nanomaterials, as direct nanoparticle-assay interactions could lead to profound false-negative diagnostic misinterpretations.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this manuscript.

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