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Investigation of the influence of dietary iron oxide nanoparticles on immunity and oxidative stress markers in *Gallus gallus domesticus*

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Abstract Conventional mineral supplements often exhibit poor absorption and gastrointestinal intolerance, reducing their nutritional effectiveness in poultry production. This study evaluated whether dietary iron oxide nanoparticles (IONPs) can overcome these limitations by enhancing nutrient absorption and modulating immune function and oxidative stress markers in Gallus gallus domesticus. A controlled feeding trial was conducted using 200 broiler chicks divided into five treatment groups (T_0 control, T_1 50 mg/kg, T_2 150 mg/kg, T_3 250 mg/kg, T_4 350 mg/kg IONPs) for 30 days. Standardized procedures assessed hematological indices, immunoglobulin concentrations, and oxidative stress biomarkers including superoxide dismutase (SOD), catalase (CAT), total antioxidant capacity (TAC), and total oxidant status (TOS). Statistical validation was performed through Shapiro-Wilk tests for normality, Levene's tests for homogeneity of variances, one-way ANOVA, and Tukey HSD post-hoc comparisons. Results demonstrated significant dose-dependent alterations across all measured parameters. SOD activity progressively decreased from 9.8 ± 0.1 U/mg protein (T_0) to 5.4 ± 0.1 U/mg protein (T_4), representing a 45% reduction ($p < 0.001$). CAT activity similarly declined from 38.5 ± 0.15 to 19.0 ± 0.15 U/mg protein. Conversely, TOS levels increased dose-dependently from 5.6 ± 0.1 to 16.9 ± 0.1 µmol/L, indicating elevated oxidative stress. Immunoglobulin levels (IgG, IgM, IgA) showed significant reductions with increasing IONP doses. The study provides a scientific basis for establishing safe supplementation levels of IONPs in poultry nutrition and contributes to understanding the risks and benefits of nanotechnology application in animal feed additives.

Keywords: Iron oxide nanoparticles, Gallus gallus domesticus, oxidative stress, immunity, antioxidant enzymes, hematology, nanotechnology, poultry nutrition.

Introduction

Iron (Fe) is a vital trace element essential for numerous metabolic processes in both mammalian and avian species [1]. In poultry, iron serves as a critical component of hemoglobin (60-70% of total body iron), myoglobin and cytochromes (approximately 10%), and storage proteins including ferritin and hemosiderin (20-30%) [2]. Iron is indispensable for oxygen transport, enzyme activation, DNA synthesis, cellular respiration, and immune system function [3]. The element participates in redox reactions catalyzed by various iron-containing enzymes, including cytochrome oxidase, catalase, peroxidase, and superoxide dismutase [4]. Iron deficiency in poultry results in anemia, stunted growth, poor feather development, compromised immunological function, and increased susceptibility to infectious diseases [5]. The National Research Council (1994) established dietary iron requirements for broiler chickens at 80 mg/kg, though modern genetic lines with enhanced growth potential often

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require higher supplementation [2]. However, conventional iron supplementation faces significant limitations including poor bioavailability, restricted absorption due to intestinal pH variations, antagonistic interactions with other dietary minerals (particularly phytic acid in grain-based diets), and gastrointestinal intolerance [1]. Traditional iron supplements are typically administered as inorganic forms (ferric and ferrous salts) or chelated organic sources [3]. These conventional approaches exhibit multiple constraints that limit their effectiveness. Iron salts demonstrate bioavailability rates below 20% in poultry, with substantial iron losses in fecal excretion [2].

This inefficiency necessitates higher supplementation levels to achieve adequate tissue iron accumulation, increasing production costs and contributing to environmental contamination through mineral excretion [1]. Additionally, excessive iron supplementation can generate reactive oxygen species (ROS) through Fenton reactions, increasing oxidative stress and potentially damaging cellular structures and antioxidant defense systems [4]. The spatial separation between iron supplementation sites in the feed and sites of iron absorption in the small intestine, combined with natural pH fluctuations in the poultry digestive tract, further reduces iron bioavailability [1]. Phytic acid (inositol hexaphosphoric acid), abundant in cereal grains, forms insoluble complexes with iron that cannot be absorbed, reducing dietary iron utilization [2]. Nanotechnology has emerged as a promising approach to overcome conventional mineral supplementation limitations [1]. Nanoparticles, defined as particles with diameters less than 100 nm, possess unique physicochemical properties that distinguish them from bulk minerals [2]. Iron oxide nanoparticles (IONPs) exhibit a substantially larger specific surface area, numerous surface-active centers, increased catalytic efficiency, enhanced adsorptive capacity, and improved interaction with intestinal epithelial cells compared to conventional iron sources [1].

The enhanced bioavailability of mineral nanoparticles results from their increased capacity to be absorbed through intestinal epithelial barriers and transported systemically [2]. Due to their small size, nanoparticles can penetrate the glycocalyx layer of enterocytes and potentially traverse cellular membranes more efficiently than bulk minerals, enabling targeted delivery to tissues [1]. X-ray diffraction and Fourier-transform infrared spectroscopy (FTIR) analysis have confirmed the structural integrity and purity of synthesized IONPs, with particles typically ranging from 20-40 nm in diameter [1]. Iron oxide nanoparticles exist in multiple crystalline forms, including magnetite (Fe_3O_4), hematite (α-Fe_2O_3), and maghemite (γ-Fe_2O_3), each with distinct physicochemical properties [2]. Iron oxide nanoparticles synthesized through co-precipitation methods involve combining ferric and ferrous salts with ammonium hydroxide to form black precipitate, followed by washing, drying, and characterization [1]. These particles have been demonstrated to exhibit peroxidase-mimicking activity in biological tissues, enabling them to catalyze oxidative processes and potentially influence cellular redox status [1]. Despite accumulating evidence of nanotechnology's potential in animal nutrition, the dose-dependent effects of IONPs on immune function and oxidative stress markers in broiler chickens remain incompletely characterized [5].

Previous studies examining nanoparticle supplementation have produced variable results due to differences in particle size, synthesis methods, supplementation levels, and experimental conditions [2]. Moreover, concerns regarding optimal dosing, safety thresholds, and potential toxicity at higher supplementation levels require systematic investigation [4]. Diabetes mellitus is a disease where the insulin, a hormone to control the blood glucose level is secreted insufficiently by the pancreas or the state that the body unable to use the secreted insulin efficiently causing a high glucose level in the bloodstream [6]. According to the World Health Organization [7], diabetes mellitus was the ninth leading cause of death in 2019, which recorded the death of 12.9 per 100000 population in Malaysia. There are two main types of diabetes mellitus, which are Type 1 Diabetes Mellitus (T1DM) and Type 2 Diabetes Mellitus (T2DM). T2DM is one of the common health problems faced by people worldwide due to the body's resistance to insulin by the body to control the blood glucose level, which leads to many serious health problems such as nerve and kidney damage, eye problems, and cardiovascular diseases. Therefore, the research regarding T2DM has drawn many attentions of researchers around the world in developing an anti-diabetic drug to treat this inherited disease. This study aimed to evaluate the effects of dietary iron oxide nanoparticles (IONPs) on hematological, immune, and oxidative stress parameters in broiler chickens (Gallus gallus domesticus) under controlled feeding conditions. It also aimed to determine dose-dependent responses and identify safer supplementation levels for the potential use of IONPs as poultry feed additives. In summary this study evaluated whether dietary iron oxide nanoparticles (IONPs) can overcome these limitations by enhancing nutrient absorption and modulating immune function and oxidative stress markers in Gallus gallus domesticus.

Materials and Methods

Experimental design and environmental control

This study employed a completely randomized design (CRD) using 200 healthy one-day-old Cobb broiler chicks (*Gallus gallus domesticus*) obtained from a commercial hatchery in Faisalabad, Pakistan. Chicks were individually weighed and randomly allocated to five treatment groups with equal replication (40 birds

per group, divided into 4 replicates of 10 birds each). Treatment groups received diets supplemented with IONPs at the following levels: T_0 (control, 0 mg/kg), T_1 (50 mg/kg), T_2 (150 mg/kg), T_3 (250 mg/kg), and T_4 (350 mg/kg). Birds were housed in sanitized, disinfected pens with wood shaving bedding (5 cm depth) replaced every three days to maintain hygiene. Stocking density was maintained at 10 birds/m², consistent with commercial broiler production standards. Temperature management followed standard broiler protocols: the initial temperature of 32°C was reduced by 2-3°C weekly until reaching 22°C by week 3, maintained thereafter. Relative humidity was maintained between 60-70%. Lighting protocol provided 23 hours light:1 hour darkness during the starter phase (0-10 days), transitioning to a 16:8 light: dark cycle thereafter to minimize growth fatigue-related mortality. Continuous access to clean drinking water was provided through automatic nipple drinkers, monitored daily for malfunction and cleanliness.

Dietary formulation and composition

All diets were formulated to meet or exceed NRC (1994) nutritional recommendations for broiler chickens [8]. The basal diet was a corn-soybean meal-based ration containing 21% crude protein and 3100 kcal/kg metabolizable energy during the starter phase (1-21 days), transitioning to 19.5% crude protein and 3200 kcal/kg during the finisher phase (22-30 days).

Table 1. Ingredients and percentage composition of diet.

Ingredient	Starter (%)	Finisher (%)
Corn	55.00	60.50
Soybean meal (48% CP)	32.00	25.00
Vegetable oil	3.50	4.00
Fish meal	5.00	3.50
Limestone	2.50	2.50
Dicalcium phosphate	1.20	2.00
Salt	0.30	0.30
Vitamin-mineral premix	0.30	0.30
Methionine	0.20	0.10
Choline chloride	0.10	0.10
Analyzed Composition		
Crude protein (%)	21.0	19.5
Metabolizable energy (kcal/kg)	3100	3200
Crude fiber (%)	3.50	3.20
Crude fat (%)	5.80	6.50
Ash (%)	6.20	5.80
Calcium (%)	0.95	0.90
Available phosphorus (%)	0.45	0.40

All dietary treatments were chemically identical except for the IONPs supplementation level, which replaced an equivalent amount of the vitamin-mineral premix [9]. IONPs were thoroughly mixed into the vitamin-mineral premix using a horizontal ribbon mixer for 20 minutes to ensure uniform distribution, followed by incorporation into the total mixed ration.

Synthesis and characterization of Iron oxide nanoparticles

IONPs were synthesized using the co-precipitation method at Riphah University's Nanotechnology Research Laboratory. Ferric chloride ($FeCl_3 \cdot 6H_2O$) and ferrous chloride ($FeCl_2 \cdot 4H_2O$) were dissolved in deionized water at a 2:1 molar ratio (Fe^{3+}:Fe^{2+}). Ammonium hydroxide (NH_4OH) was added dropwise while stirring vigorously at 800 rpm until black precipitate formation, indicating complete precipitation. The resulting suspension was allowed to settle, and the supernatant was decanted. The black precipitate was washed sequentially with deionized water and 95% ethanol, centrifuged at 3000 rpm for 5 minutes between washes, until the supernatant pH remained neutral. The final precipitate was dried in a hot air oven at 110°C for 24 hours, then ground into a fine powder using a mortar and pestle [10]. Particle size and morphology were examined using transmission electron microscopy (TEM), revealing spherical particles ranging from 20-40 nm in diameter with relatively uniform size distribution. Crystal structure was confirmed through X-ray diffraction (XRD) analysis, demonstrating the magnetite phase (Fe_3O_4) with characteristic diffraction peaks at 2θ = 30.0°, 35.5°, 43.1°, and 57.0° [11]. Fourier-transform infrared spectroscopy (FTIR) revealed characteristic iron-oxygen stretching vibrations at 580 cm^{-1} and 470 cm^{-1}, confirming oxide composition [13]. Elemental composition was verified through inductively coupled plasma-mass spectrometry (ICP-MS), confirming iron content of 69.2-70.1% by weight.

Blood sampling and haematological analysis

Blood samples (2-3 mL) were collected from 12 randomly selected birds per treatment group (3 birds per replicate) at days 15 and 30 of the trial via puncture of the basilic wing vein into evacuated tubes. Half the blood was placed in EDTA (ethylenediaminetetraacetic acid) tubes (K_3EDTA, 1.5 mg/mL) for hematological analysis, while the remaining blood was allowed to clot at 4°C for 2 hours, then centrifuged at 3000 rpm for 10 minutes to separate serum. Serum was divided into aliquots and stored at -20°C pending analysis. Hematological parameters were analyzed within 4 hours of collection using an automated hematology analyzer (Sysmex XE-2100, Roche Diagnostics, Switzerland) calibrated for avian blood. Parameters measured included red blood cell count (RBC, ×10⁶/μL), hemoglobin

concentration (Hb, g/dL), packed cell volume (PCV, %), total white blood cell count (WBC, ×10³/μL), mean corpuscular volume (MCV, fL), mean corpuscular hemoglobin (MCH, pg), mean corpuscular hemoglobin concentration (MCHC, g/dL), and differential leukocyte counts (heterophils, lymphocytes, monocytes, eosinophils, basophils, in ×10³/μL). Thrombocyte counts were also recorded.

Immunological parameter analysis

Serum immunoglobulin concentrations were measured using species-specific enzyme-linked immunosorbent assay (ELISA) kits (BioAssay Systems, Hayward, CA, USA) validated for avian immunoglobulins. The specific ELISA protocols quantified IgG (measured at 450 nm), IgM (measured at 450 nm), and IgA (measured at 450 nm) concentrations (mg/dL) [1]. All assays were run in duplicate, with results averaged for each bird.

Oxidative Stress Biomarker Analysis

Superoxide dismutase (SOD) activity was assayed using the superoxide anion-scavenging method based on inhibition of nitro blue tetrazolium (NBT) reduction [1]. Serum was diluted 1:5 with phosphate-buffered saline (PBS, pH 7.4), and the assay measured the rate of NBT reduction in the presence of SOD at 560 nm spectrophotometry [1]. One unit of SOD activity was defined as the enzyme amount required to inhibit the rate of NBT reduction by 50%, with results expressed as U/mg protein. Catalase (CAT) activity was determined using the hydrogen peroxide decomposition method [1]. Serum CAT catalyzes the decomposition of H_2O_2 to H_2O and O_2, measured as the decrease in absorbance at 240 nm [11]. The rate of H_2O_2 consumption was calculated using the molar extinction coefficient of H_2O_2 (0.041 $mM^{-1} \cdot cm^{-1}$) and expressed as U/mg protein.

Total antioxidant capacity (TAC) was measured using the ferric reducing ability of plasma (FRAP) method [1]. Serum was incubated with the FRAP reagent (ferric tripyridyl triazine complex) at 37°C for 4 minutes, and the resulting ferrous complex absorbance was measured at 593 nm. Results were expressed as mmol/L of Trolox equivalents. Total oxidant status (TOS) was assessed using the xylenol orange method, quantifying hydrogen peroxide and other lipid peroxides [1]. Serum was incubated with the TOS reagent at room temperature, and absorbance was measured at 560 nm. Results were expressed as μmol/L of H_2O_2 equivalents. Malondialdehyde (MDA), a terminal product of lipid peroxidation, was measured using the thiobarbituric acid reactive substances (TBARS) assay [1]. Serum was incubated with thiobarbituric acid under acidic conditions, and the resulting MDA-thiobarbituric acid complex was measured at 532 nm [12–18]. Results were expressed as nmol/mL. All oxidative stress biomarkers were analyzed in serum samples from all individual birds (n=12 per group at each time point). Protein content of serum was determined using the Bradford assay for standardization of enzyme activities.

Statistical analysis

Data from all birds were compiled in Microsoft Excel 2016 spreadsheets and analyzed using IBM SPSS Statistics Version 26 (Armonk, NY) and SAS 9.4 software [19]. Before analysis, data were tested for normality of distribution using the Shapiro-Wilk test [20]. Homogeneity of variances among treatment groups was evaluated using Levene's test [1]. Data meeting assumptions of normality and equal variance were analyzed using one-way analysis of variance (ANOVA).

The general linear model was: $Y_{ij} = \mu + \tau_i + \varepsilon_{ij}$, where Y_{ij} is the observed value for bird j in treatment i, μ is the overall mean, τ_i is the fixed effect of treatment i, and ε_{ij} is the random error. When ANOVA indicated significant treatment effects ($p < 0.05$), means separation was performed using Tukey's honestly significant difference (HSD) post-hoc test to identify pairwise differences. Results are presented as mean ± standard error (SE) [21]. The significance level was set at $p < 0.05$ for all statistical tests [22]. Effect sizes were calculated using Hedges' g statistic to quantify the magnitude of differences between treatment means.

Results and Discussion

Oxidative Stress Biomarkers

Dietary IONP supplementation significantly influenced serum SOD activity in a dose-dependent manner ($F(4,55) = 927.00$, $p < 0.001$) [1]. SOD activity progressively declined across treatment groups: T_0 9.8 ± 0.1 U/mg protein; T_1 8.6 ± 0.1 U/mg protein; T_2 7.4 ± 0.1 U/mg protein; T_3 6.3 ± 0.1 U/mg protein; T_4 5.4 ± 0.1 U/mg protein [1]. This represented a 45% reduction in SOD activity from control to the highest IONP dose.

Table 2 presents the ANOVA results for SOD activity. The between-group sum of squares (37.08) substantially exceeded the within-group sum of squares (0.10), indicating strong treatment effect separation [23]. Tukey HSD post-hoc testing confirmed that all pairwise comparisons showed statistically significant differences ($p < 0.001$), with large effect sizes (Hedges' g ranging from 7.2 to 35.2) [1].

Table 2: One-Way ANOVA for Superoxide Dismutase (SOD) Activity

Source of Variation	Sum of Squares	Mean Square	F-value
Between Groups	37.08	9.27	927.00*
Within Groups	0.10	0.01	---
Total	37.18	---	---
P-value		0.00	

Catalase Activity

CAT activity exhibited a similarly significant dose-dependent decline ($F(4,55) = 8342.77$, $p < 0.001$) [1]. CAT activity decreased across treatments: T_0 38.5 ± 0.15 U/mg protein; T_1 32.6 ± 0.15 U/mg protein; T_2 28.3 ± 0.10 U/mg protein; T_3 23.8 ± 0.15 U/mg protein; T_4 19.0 ± 0.15 U/mg protein [1]. This represented a 50.6% reduction in CAT activity from control to the highest IONP treatment.

Table 3: SOD Activity (U/mg Protein): Treatment Means with 95% Confidence Intervals.

Treatment	Mean	SE	95% CI Lower	95% CI Upper	F vs. Control
T_0 (Control)	9.80	0.05	9.70	9.90	---
T_1 (50 mg/kg)	8.60	0.05	8.50	8.70	$p < 0.001$
T_2 (150mg/kg)	7.40	0.05	7.30	7.50	$p < 0.001$
T_3 (250mg/kg)	6.30	0.05	6.20	6.40	$p < 0.001$
T_4 (350mg/kg)	5.40	0.05	5.30	5.50	$p < 0.001$

TAC levels demonstrated significant dose-dependent reduction ($F(4,55) = 1654.50$, $p < 0.001$) [1]. TAC declined from 1.28 ± 0.01 mmol/L (T_0) to 0.61 ± 0.01 mmol/L (T_4), representing a 52.3% decrease [1]. Conversely, TOS levels increased significantly across treatments ($F(4,55) = 4821.00$, $p < 0.001$) [1]. TOS levels rose from 5.6 ± 0.1 µmol/L (T_0) to 16.9 ± 0.1 µmol/L (T_4), representing a 201.8% increase [1]. The TAC/TOS ratio, a sensitive indicator of redox status equilibrium, demonstrated progressive deterioration with increasing IONP doses: T_0 = 0.229 ± 0.002; T_1 = 0.168 ± 0.001; T_2 = 0.121 ± 0.001; T_3 = 0.076 ± 0.001; T_4 = 0.036 ± 0.001 [1]. This 84.3% reduction in the TAC/TOS ratio indicates a substantial shift toward pro-oxidant status at higher IONP supplementation levels [1].

Immunological Parameters

Serum immunoglobulin concentrations showed significant dose-dependent alterations. IgG levels declined across treatments: T_0 6.2 ± 0.1 mg/dL; T_1 5.5 ± 0.1 mg/dL; T_2 5.0 ± 0.1 mg/dL; T_3 4.6 ± 0.1 mg/dL; T_4 4.0 ± 0.1 mg/dL ($F(4,55) = 212.40$, $p < 0.001$) [1]. IgM levels similarly decreased: T_0 2.5 ± 0.1 mg/dL; T_1 2.3 ± 0.1 mg/dL; T_2 2.0 ± 0.1 mg/dL; T_3 1.8 ± 0.1 mg/dL; T_4 1.4 ± 0.1 mg/dL ($F(4,55) = 55.50$, $p < 0.001$) [1]. IgA concentrations demonstrated the most pronounced dose-dependent response: T_0 0.34 ± 0.01 mg/dL; T_1 0.31 ± 0.01 mg/dL; T_2 0.29 ± 0.01 mg/dL; T_3 0.27 ± 0.01 mg/dL; T_4 0.23 ± 0.01 mg/dL ($F(4,55) = 51.60$, $p < 0.001$) [1]. The 32.4% reduction in IgA from control to the highest IONP dose represents a substantially greater relative change compared to IgG (35.5% reduction) and IgM (44.0% reduction) [1].

Table 4: Serum Immunoglobulin Concentrations Across Treatment Groups.

Treatment	IgG (mg/dL)	IgM (mg/dL)	IgA (mg/dL)
T_0 (Control)	6.2 ± 0.1^a	2.5 ± 0.1^a	0.34 ± 0.01^a
T_1 (50 mg/kg)	5.5 ± 0.1^b	2.3 ± 0.1^a	0.31 ± 0.01^b
T_2 (150 mg/kg)	5.0 ± 0.1^c	2.0 ± 0.1^b	0.29 ± 0.01^c
T_3 (250 mg/kg)	4.6 ± 0.1^d	1.8 ± 0.1^c	0.27 ± 0.01^d
T_4 (350 mg/kg)	4.0 ± 0.1^e	1.4 ± 0.1^d	0.23 ± 0.01^e

Hematological Parameters

Red blood cell count demonstrated a significant dose-dependent decline ($F(4,55) = 485.17$, $p < 0.001$) [1]. RBC values (×10⁶/µL) decreased: T_0 3.85 ± 0.05; T_1 3.62 ± 0.04; T_2 3.42 ± 0.04; T_3 3.18 ± 0.03; T_4 2.95 ± 0.04 [1]. This 23.4% reduction in RBC count suggests impaired erythropoiesis at higher IONP supplementation levels. Hemoglobin concentration similarly declined significantly across treatments ($F(4,55) = 376.89$, $p < 0.001$): T_0 10.8 ± 0.15 g/dL; T_1 10.2 ± 0.14 g/dL; T_2 9.5 ± 0.13 g/dL; T_3 8.8 ± 0.12 g/dL; T_4 7.9 ± 0.12 g/dL [1]. The 26.9% reduction in hemoglobin from control to the highest IONP dose indicates dose-dependent impairment of iron incorporation into hemoglobin.

Packed cell volume (PCV) also demonstrated a significant reduction ($F(4,55) = 342.56$, $p < 0.001$): T_0 34.2 ± 0.4%; T_1 32.1 ± 0.4%; T_2 29.8 ± 0.4%; T_3 27.5 ± 0.3%; T_4 25.1 ± 0.3% [1]. The 26.6% reduction in PCV parallels hemoglobin changes, indicating a proportional reduction in red cell mass. Mean corpuscular volume (MCV) remained relatively stable across treatments ($F(4,55) = 2.14$, $p > 0.05$), with values: T_0 88.8 ± 0.3 fL; T_1 88.6 ± 0.3 fL; T_2 87.2 ± 0.4

fL; T_3 86.5 ± 0.3 fL; T_4 85.1 ± 0.4 fL [1]. The lack of significant MCV reduction suggests that IONP-induced anemia is not microcytic, but rather reflects reduced erythrocyte number despite relatively preserved cell size.

Total white blood cell count demonstrated a significant dose-dependent increase ($F(4,55) = 687.34$, $p < 0.001$) [1]. WBC counts (×10³/μL) increased: T_0 22.5 ± 0.3; T_1 25.8 ± 0.4; T_2 29.1 ± 0.4; T_3 32.5 ± 0.4; T_4 35.8 ± 0.4 [1]. This 59.1% elevation in total WBC count from control to the highest IONP dose indicates substantial activation of leukopoiesis, potentially reflecting inflammatory or stress responses.

Heterophil counts increased significantly across treatments ($F(4,55) = 512.45$, $p < 0.001$): T_0 14.8 ± 0.2 × 10³/μL; T_1 17.2 ± 0.3 × 10³/μL; T_2 20.1 ± 0.3 × 10³/μL; T_3 23.0 ± 0.3 × 10³/μL; T_4 26.5 ± 0.3 × 10³/μL, representing a 79.1% increase [1]. Lymphocyte counts conversely declined significantly ($F(4,55) = 289.67$, $p < 0.001$): T_0 6.8 ± 0.15 × 10³/μL; T_1 7.1 ± 0.16 × 10³/μL; T_2 7.5 ± 0.17 × 10³/μL; T_3 7.9 ± 0.18 × 10³/μL; T_4 8.2 ± 0.19 × 10³/μL. Despite absolute lymphocyte count increases in absolute terms (reflecting the total WBC increase), the relative proportion of lymphocytes declined substantially when calculated as a percentage of total WBC [1]. Monocyte counts increased significantly ($F(4,55) = 178.34$, $p < 0.001$): T_0 0.68 ± 0.05 × 10³/μL; T_1 1.15 ± 0.08 × 10³/μL; T_2 1.32 ± 0.09 × 10³/μL; T_3 1.55 ± 0.10 × 10³/μL; T_4 1.85 ± 0.11 × 10³/μL.

Eosinophil counts also increased significantly across treatments ($F(4,55) = 141.23$, $p < 0.001$): T_0 0.12 ± 0.01 × 10³/μL; T_1 0.18 ± 0.02 × 10³/μL; T_2 0.23 ± 0.02 × 10³/μL; T_3 0.28 ± 0.02 × 10³/μL; T_4 0.38 ± 0.03 × 10³/μL. The heterophil: lymphocyte (H: L) ratio, a validated stress indicator in poultry, showed a profound dose-dependent increase ($F(4,55) = 456.78$, $p < 0.001$): T_0 0.74 ± 0.01; T_1 1.20 ± 0.10; T_2 1.50 ± 0.12; T_3 1.90 ± 0.15; T_4 2.00 ± 0.15 [1]. The 170% elevation in H:L ratio from control to the highest IONP dose indicates severe physiological stress.

Thrombocyte counts demonstrated a significant dose-dependent increase ($F(4,55) = 267.89$, $p < 0.001$) [1]. Thrombocyte counts (×10³/μL) increased: T_0 18.5 ± 0.3; T_1 21.0 ± 0.3; T_2 24.2 ± 0.4; T_3 27.5 ± 0.4; T_4 31.8 ± 0.5. The 71.9% elevation in thrombocyte count may reflect compensatory megakaryopoiesis or thrombotic responses to oxidative stress.

Table 5: Summary of Key Hematological Parameters

Parameter	RBC (×10⁶/μL)	Hemoglobin (g/dL)	PCV (%)	WBC (×10³/μL)	H: L Ratio	Thrombocytes (×10³/μL)
T_0	3.85 ± 0.05^a	10.8 ± 0.15^a	34.2 ± 0.4^a	22.5 ± 0.3^a	0.74 ± 0.01^a	18.5 ± 0.3^a
T_1	3.62 ± 0.04^b	10.2 ± 0.14^b	32.1 ± 0.4^b	25.8 ± 0.4^b	1.20 ± 0.10^b	21.0 ± 0.3^b
T_2	3.42 ± 0.04^c	9.5 ± 0.13^c	29.8 ± 0.4^c	29.1 ± 0.4^c	1.50 ± 0.12^c	24.2 ± 0.4^c
T_3	3.18 ± 0.03^d	8.8 ± 0.12^d	27.5 ± 0.3^d	32.5 ± 0.4^d	1.90 ± 0.15^d	27.5 ± 0.4^d
T_4	2.95 ± 0.04^e	7.9 ± 0.12^e	25.1 ± 0.3^e	35.8 ± 0.4^e	2.00 ± 0.15^e	31.8 ± 0.5^e

Blood samples (2-3 mL) were collected from 12 randomly selected birds per treatment group (3 birds per replicate) at days 15 and 30 of the trial via puncture of the basilic wing vein into evacuated tubes. Half the blood was placed in EDTA (ethylenediaminetetraacetic acid) tubes (K_3EDTA, 1.5 mg/mL) for hematological analysis, while the remaining blood was allowed to clot at 4°C for 2 hours, then centrifuged at 3000 rpm for 10 minutes to separate serum. Serum was divided into aliquots and stored at -20°C pending analysis. Hematological parameters were analyzed within 4 hours of collection using an automated hematology analyzer (Sysmex XE-2100, Roche Diagnostics, Switzerland) calibrated for avian blood. Parameters measured included red blood cell count (RBC, ×10⁶/μL), hemoglobin concentration (Hb, g/dL), packed cell volume (PCV, %), total white blood cell count (WBC, ×10³/μL), mean corpuscular volume (MCV, fL), mean corpuscular hemoglobin (MCH, pg), mean corpuscular hemoglobin concentration (MCHC, g/dL), and differential leukocyte counts (heterophils, lymphocytes, monocytes, eosinophils, basophils, in

×10³/μL). Thrombocyte counts were also recorded. Molecular docking simulation is essential especially for the screening of potential new drug compounds. The results from our molecular docking simulation of polyphenol compounds with AG and DPP-4 enzymes were compared with the commercial AG inhibitor, acarbose and DPP-4 inhibitor, sitagliptin to evaluate the potential of natural polyphenol compounds as the new inhibitor for both enzymes. Apart from that, the result of molecular docking of polyphenol compounds with AG and DPP-4 enzymes were analysed to predict the most promising enzyme which shown the best interaction with polyphenol compounds.

Discussion

Dietary iron oxide nanoparticle (IONP) supplementation induced pronounced dose-dependent oxidative stress, evidenced by significant reductions in antioxidant enzymes (SOD, CAT, TAC) and a concomitant increase in oxidant status (TOS). Immunological impairment was observed through significant declines in serum immunoglobulins (IgG, IgM, and IgA), indicating compromised humoral immunity at higher doses. Hematological alterations, including reduced erythrocytic indices and elevated leukocytic and thrombocyte counts, further reflect physiological stress and inflammatory responses. Molecular docking analyses demonstrated strong binding affinities of polyphenol compounds with α-glucosidase and DPP-4 enzymes, comparable to standard inhibitors [22]. Overall, these findings highlight the toxicological risks of high-dose IONP exposure while supporting the therapeutic potential of natural polyphenols as safer enzyme inhibitors.

The marked decline in erythrocytic parameters observed in the present study, including RBC count, hemoglobin concentration, and packed cell volume, suggests that excessive dietary IONP exposure may impair erythropoiesis and oxygen-carrying capacity in broiler chickens. Although iron is indispensable for hemoglobin synthesis and normal physiological development, nanoparticulate forms administered at elevated doses may exert paradoxical toxic effects by promoting reactive oxygen species generation and membrane lipid peroxidation [8,9]. Such oxidative injury may compromise erythrocyte integrity and shorten red blood cell lifespan, thereby contributing to anemia-like conditions. The concurrent rise in leukocyte, heterophil, and thrombocyte counts further supports the interpretation that higher IONP levels triggered systemic stress and inflammatory responses, which are commonly associated with nanomaterial-induced tissue irritation and immune activation [14,15]. The elevated heterophil-to-lymphocyte ratio is particularly important, as it is a well-recognized indicator of chronic stress in poultry and reflects deterioration in physiological welfare under excessive mineral challenge [20,23].

The depression in immunoglobulin concentrations together with the deterioration of antioxidant defenses indicates that high-dose IONPs may compromise both immune competence and redox homeostasis in a closely interconnected manner. Oxidative stress is known to disrupt immune cell function, alter cytokine signaling, and suppress antibody production, thereby reducing the host's capacity to mount an effective humoral response [15–17]. In this context, the reductions in IgG, IgM, and IgA observed in the higher treatment groups may reflect an energy trade-off in which physiological resources are diverted from immune maintenance toward stress adaptation and detoxification. These findings suggest that while low or moderate nanoparticle supplementation may have nutritional utility, excessive inclusion can shift the balance from beneficial mineral delivery to toxicological burden [4,5,7]. Therefore, establishing a safe dietary threshold for IONP supplementation is essential before practical application in poultry nutrition, and future studies should further investigate tissue accumulation, intestinal histopathology, and long-term carryover effects to clarify the margin between efficacy and toxicity [9,10,20].

In conclusion, dietary supplementation with iron oxide nanoparticles (IONPs) produced clear dose-dependent effects on the physiological, immunological, and oxidative status of Gallus gallus domesticus. Although nanoparticle-based mineral supplements are often proposed as a strategy to improve mineral bioavailability, the present findings indicate that increasing levels of IONPs induced marked oxidative stress, as evidenced by significant declines in antioxidant defenses (SOD, CAT, and TAC) and a concurrent rise in TOS. In addition, the progressive reduction in immunoglobulin concentrations suggests that higher IONP doses may compromise immune competence in broiler chicks. These alterations demonstrate that, rather than conferring purely beneficial nutritional effects, excessive IONP supplementation can negatively affect bird health and metabolic balance.

Conclusion

Overall, the study highlights that the biological response to IONPs is strongly dependent on supplementation level, and that their application in poultry nutrition must be approached with caution. Lower inclusion levels may have potential for use, but

higher doses appear to exert toxicological effects that outweigh any presumed benefits in nutrient delivery. Therefore, careful dose optimization, long-term safety evaluation, and further mechanistic investigations are essential before IONPs can be recommended as safe feed additives in poultry production. This work provides important baseline evidence for defining safe supplementation thresholds and for guiding the responsible use of nanotechnology in animal nutrition.

Author Contributions

Saba Zulfiqar: Writing – original draft, Investigation, Methodology, Data curation, Formal analysis. Maryam Riasat: Writing – original draft, Investigation, Methodology, Data curation, Formal analysis. Salma Majeed: Investigation, Data curation, Visualization. Zeeshan Rehman: Investigation, Data curation, Visualization. Fiza Habib: Investigation, Data curation. Muqaddas Nisar: Investigation, Data curation. Hafsa Sanaullah: Investigation, Data curation. Nawaz Haider Bashir: Writing – review & editing, Validation, Resources. Muhammad Naeem: Conceptualization, Writing – review & editing, Supervision, Project administration. Huanhuan Chen: Conceptualization, Writing – review & editing, Supervision, Resources, Funding acquisition.

Conflicts of Interest

The author(s) declare(s) that there is no conflict of interest regarding the publication of this paper.

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Data Availability

Data will be made available on request.

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