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RESEARCH

Rutin Nanoparticles Alleviate Cadmium-Induced Oxidative and Immune Damage in Broilers' Bursa of Fabricius via Modulating Hsp70/TLR4/NF-κB Signaling Pathway

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Abstract

Cadmium (Cd) is a serious environmental pollutant affecting various tissues/organs in broilers and compromising their immunological function and productivity. Therefore, the current study aimed to investigate Cd-induced immunotoxicity and potential immunoprotective effect of rutin nanoparticles (RNPs) in the bursal tissue of broilers. A total number of 150 chicks from the Hubbard breed were randomly divided into 5 groups. Group I was fed on standard basal diet (SD) with normal drinking water (DW), Group II received SD containing RNPs (50 mg/kg feed) with DW, Group III fed on SD and DW containing Cd (150 mg/L), Group IV co-treated with rutin-enforced SD (50 mg/kg diet) and DW containing Cd (150 mg/L), and finally, Group V co-supplemented with RNP-enhanced SD (50 mg/kg diet) DW containing Cd (150 mg/L). Productive performance, economic efficiency, oxidative biomarkers, histopathological changes, and the expression level of TLR-4, HSP-70, caspase 3, NF-κB, Bcl-2, and Bax were assessed in the BF tissue. Cd led to severe production and economic losses in exposed birds with a marked surge of oxidative biomarkers, pro-inflammatory cytokines, and histopathological changes in the bursal tissue which could be explained through upregulation of the Hsp70/TLR4/NF-κB molecular pathway in the BF tissue. Meanwhile, RNPs could alleviate most of these changes and prevail optimistic immunomodulatory properties which subsequently could enhance broilers' productivity when incorporated in their diets.

Keywords Cd · Rutin · Nanoparticles · Immunotoxicity · TLR4 · Hsp70 · Broilers

Introduction

Cadmium (Cd) is a poisonous heavy metal and released into the environment continuously from natural and industrial sources [1]. Exposure to small amounts of Cd over an extended period may produce serious effects in human and animal health [2]. The dietary intake of Cd in birds is implicated in various deleterious effects, including anemia [3], cardiotoxicity [4], hepatotoxicity [5], nephrotoxicity [6], and immunotoxicity [7]. Cd exerts its toxicity through excessive generation of free radicals with subsequent damage to various organs and tissue resulting in higher mortality rates and critical impacts upon animal productivity and economic efficiency [8, 9].

The immune system is a nexus between the organism and its environment, so environmental pollutants, including Cd, can cause serious damage to different components of immune system in broilers [10, 11]. Previous reports have indicated that Cd is an immunotoxic substance which not only affects innate

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immunity but also suppresses induced immunity leading to alteration of the overall immune function [12, 13]. Cd can also trigger apoptosis in the bursa of Fabricius (BF), which is the main immune organ in chickens, disrupt cytokine production, and provoke severe histopathological damage in the bursal tissue through excessive production of reactive oxygen species (ROS) leading to oxidative damage and several chronic illnesses [14–17]. Although Cd immunotoxicity in broilers has drawn more attention recently, the underlying molecular mechanism and interaction between Cd and chicken BF is still vague [13].

Various attempts have been made to combat the hazardous effects of Cd including dietary supplementation with various antioxidants, vitamins, and herbal substances [18]. Flavonoids are natural polyphenolic phytochemicals extracted from various vegetables, fruits, and grains and display a vast range of biological and pharmacological activities such as antioxidant, anti-inflammatory, antimutagenic, and antibacterial effect [19, 20]. As one of the best known dietary flavonoids, rutin has several pharmacological activities including high antioxidant capacity and radical scavenging activity [21, 22]. However, rutin's poor bioavailability, easy degradation, and low water solubility could restrict its therapeutic effects, and numerous efforts have been made to improve the physicochemical properties and oral availability of rutin via various drug delivery strategies such as nanoparticles, solvates, and cyclodextrin complexes [23–25].

The promising features of nanoparticles can be employed to prepare sustained and targeted delivery system for various therapeutic applications [26]. Chitosan alginate is considered one of the best known natural and biocompatible polymeric nanoparticles which was extensively employed recently to enhance water solubility, bioavailability, absorption, and biological efficacy of hydrophobic drugs such as rutin [27, 28]. Chitosan is derived from deacetylation of natural chitin which is a component of crustacean exoskeleton, while alginate is a natural anionic polymer extracted from brown seaweed, and both have numerous biomedical and pharmaceutical applications [29–31].

Therefore, the current study aimed to synthesize RNPs in a chitosan-alginate core–shell structure to improve its antioxidant activity, broilers' profitability, and productivity, in addition to evaluation of its immunoprotective effect against Cd-induced immunotoxicity in broiler bursal tissue, finally, addressing the gaps in our understanding of the molecular interaction between Cd and component of the immune system in birds.

Materials and Methods

Materials

Rutin trihydrate 95% (CAS: 250,249–75-3, MW: 664.57) was purchased from Alfa Aesar (Thermo Fisher Scientific, Haverhill, USA), and chitosan (low molecular weight and 90% DA) and sodium alginate were obtained from SRL (Mumbai, India). Anhydrous calcium chloride and acetic acid were provided by Piochem (Giza, Egypt). All the used chemicals were of laboratory grade.

Synthesis of RNPs

Rutin (20 mg/mL) was dissolved in an ethanolic solution (50%, v/v), and this solution was added in a dropwise manner into 100 mL of sodium alginate solution (0.06% w/v, pH = 4.9), then stirred for 2 h, followed by a dropwise addition of 7 mL of calcium chloride solution (0.2%, w/v) into sodium alginate with continuous stirring (500 rpm for 30 min.), and then sonicated in ultrasonic bath (Crest Ultrasonics Corp., USA) at a frequency of 25 kHz for 10 min; 100 mL of chitosan solution (0.15% in 1% acetic acid, w/v) was then added into the calcium alginate pregel with mechanical stirring (1000 rpm for 1 h). The resulting suspension was equilibrated overnight then washed and ultracentrifuged at 14,000 rpm for 30 min for further analysis [32].

Characterization of RNPs

Transmission Electron Microscopy (TEM)

The morphology of RNPs was examined through TEM, whereas the drop of sample solution was dispersed on a carbon-coated copper grid and then dried before examination [33].

Fourier Transform Infrared (FT-IR) Spectroscopy

The FTIR of samples (chitosan alginate, rutin, and RNPs) were measured on a FTIR spectrophotometer (Perkin-Elmer, Norwalk, CT, USA). Samples were mixed with KBr, and then, the spectra were recorded in the range of 4000–400 cm^{-1} resolution [34].

Experimental Design, Birds' Diet, and Management

A total of 150 newly hatched Hubbard breed female chicks were obtained from a commercial hatchery (Alwatania Poultry Co., Mansoura, Egypt). On the 1st week, the initial temperature was maintained at around 32 °C and then reduced by 2 °C every 6–7 days until it reached 20 °C on day 35 till slaughtering. During the first few days, a lighting schedule of 23–24 h/day was applied to stimulate feeding and drinking, which later transitioned into a program of 12 h of light followed by 2 h of darkness. The basic diets as outlined in

Table 1 (starter, grower, and finisher) were prepared based on the guidelines of the National Research Council [35]. After being adapted for a week, the birds were weighed, had their wings banded, and then, were distributed into five groups with three replicates each (30 birds/group, 10 birds/replicate) as illustrated in (Fig. 1). The five dietary treatments were as follows: Control group fed on SD (standard basal diet) and normal DW (drinking water), RNP-treated group fed on SD supplemented with 50 mg/kg RNPs based on previous studies [36, 37] and normal DW, Cd group fed on SD and 150 ppm cadmium chloride in DW according to previous reports [5, 9, 38, 39], rutin co-treated with Cd group fed on SD supplemented with rutin at 50 mg/kg and 150 ppm cadmium chloride in DW, and RNP co-treated with Cd group fed on SD enhanced with RNPs at 50 mg/kg and 150 ppm cadmium chloride in DW. All the birds were provided with unlimited access to feed and water, housed in well-ventilated rooms at a density of 10 birds/m^2. Experimental protocol and methodology used in this study were approved in accordance with Mansoura University guidelines of the Animal Care and Use Committee (MU-ACUC), which strictly comply with the ARRIVE guidelines, under the approval No. VM.R.24.01.149. All efforts were made to reduce the number of animals and minimize their suffering.

Table 1 Ingredients and chemical composition of basal diets

Ingredients	Starter (0–10 ds)	Grower (11–26 ds)	Finisher (27–40 ds)
Yellow corn	54.61	58.82	62
Soybean meal (46%)	36	33.9	29.93
Soybean oil	2.5	3.5	4
Corn gluten meal	2	0	0
Dicalcium phosphate	1.8	1.75	1.4
Limestone	1.46	1.36	1.2
L_Lysine	0.32	0.29	0.2
Sodium chloride	0.32	0.31	0.25
Vitamins and mineral premix	0.3	0.31	0.3
DL-methionine	0.25	0.26	0.27
Sodium bicarbonate	0.18	0.19	0.15
Anti-coccidian	0.05	0.05	0.05
Anti-mycotoxin	0.05	0.05	0.05
Anti-clostridia	0.03	0.03	0.03
L_Threonine	0.03	0.04	0
Energy enzyme	0.02	0.01	0.01
Lysomax	0.01	0.03	0.03
Choline chloride	0.05	0.05	0.05
Protease	0.01	0.04	0.02
Phytase enzyme	0.01	0.01	0.01
Chemical composition			
Dry matter (%)	89.64	89.59	89.51
Crude fiber (%)	2.28	2.23	2.17
Crude protein (%)	23.12	21.01	19.04
Crude fat (%)	4.94	5.99	7.01
ME (kcal/kg)	3050.95	3130.02	3210.87
Ash (%)	3.19	3.45	2.80
Lysine (%)	1.35	1.25	1.09
Sodium (%)	0.18	0.17	0.17
Methionine (%)	0.63	0.60	0.52
Calcium (%)	1.05	0.96	0.85
Available phosphorus (%)	0.50	0.46	0.42

Diet calculated according to National Research Council guidelines, supplied per kilogram of diet: vitamin D3, 2200 IU; vitamin E, 26 IU; vitamin A, 12,000 IU; vitamin K3, 6.25 mg; vitamin B2, 6.6 mg; vitamin B6, 1.5 g; vitamin B1, 3.75 mg; pantothenic acid, 18.8 mg; folic acid, 1.25 mg; biotin, 0.6 mg; vitamin B12, 0.31 mg; niacin, 30 mg; Se, 0.20 mg; Zn, 50 mg; Mn, 60 mg; Cu, 6 mg; Fe, 50 mg; I, 1 mg; and Co, 1 mg

ME, metabolic energy

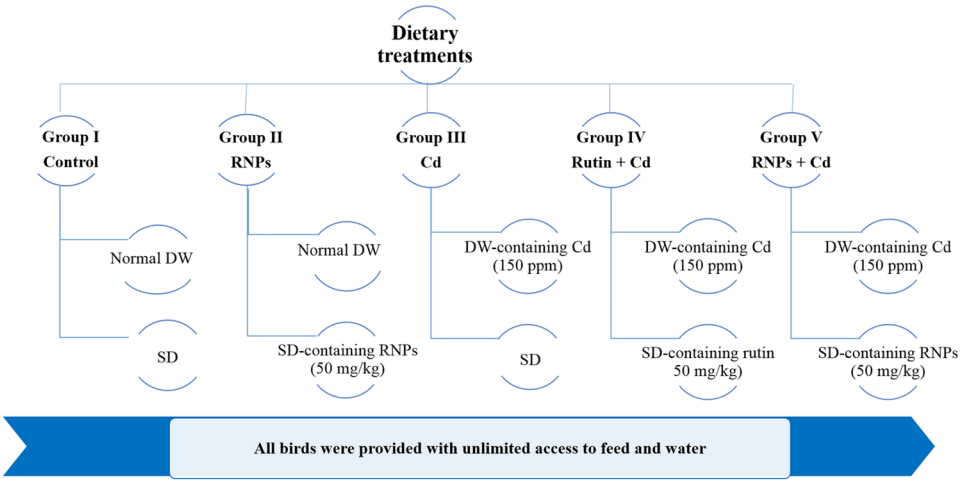

Fig. 1 Schematic diagram for the dietary treatment

All experimental animals were euthanized by decapitation which was performed by a trained individual to ensure complete bleeding out of birds and minimize the birds' suffering based on [40]. All chicks received vaccinations for Gumboro and Newcastle diseases in accordance with the guidelines provided by the manufacturer.

Productive and Economic Performance Evaluation

Productive Performance

The average body weight and feed intake (FI) of chicks were recorded throughout the study. The initial BW was measured after a week of adaptation, and the final weight was recorded at the end of the study. Furthermore, the feed conversion ratio (FCR) and mortality rates were also measured based on [41, 42].

Economic Evaluation Measures

The economic evaluation was conducted using the prevailed market price of chicks, diets, and live BW during the experiment in Egyptian pounds (LE). Total feed cost (FC), total cost of production (TC), total returns (TR), and net profit (NP) parameters were estimated based on previous studies [43, 44].

Tissue Sample Collection

At the end of the experiment (40 D), 45 birds were randomly selected from five groups as nine birds per treatment (three birds per replicate), then weighed individually and slaughtered. BF tissue samples were washed and divided into three parts for evaluation of histopathological changes (fixed in 10% formaldehyde solution, pH 7.2), oxidative biomarkers (homogenized in 10% ice-cold phosphate buffer saline, pH 7.4), and molecular analysis (stored at − 80 °C). Furthermore, blood samples were collected from the jugular vein for hematological and biochemical studies, then centrifuged (3000 rpm for 10 min) and sera collected in sterile tubes to be processed for the respective biochemical investigations.

Assessment of Oxidative Stress Biomarkers

BF tissue homogenate was centrifuged for 10 min at 3000 rpm, and then, the supernatant was collected and stored at − 80 °C for further biochemical analysis. The levels of MDA, GSH, CAT, SOD, and GPx were estimated via commercial diagnostic kits (Biodiagnostic, Egypt) following the manufacturer's protocols with a UV-1240 Spectrophotometer (Shimadzu, Japan).

Return Function (Relation Between Total Return and Oxidative Stress Activity)

To evaluate the impact of oxidative stress system on broiler production TR, a return function was utilized. The logarithmic model of the function, as proposed by [45, 46], was employed for this purpose. Statistical tests, including the *t*-test, were used to determine the significance of relationships between TR and its influencing variables [47]. Additionally, the adjusted regression coefficient R^2 was also calculated [48, 49].

Biochemical and Hematological Analysis

The serum concentrations of corticosterone (CORT), interleukin-6 (IL-6), and tumor necrosis factor α (TNF-α) were quantified with commercial ELISA kits (CSB-EQ027342CH, CSB-E08549Ch, CSB-E11231Ch, respectively) (Cusabio Biotech Co., Wuhan, Hubei, China) based on the manufacturer's protocol. Samples were measured in triplicates according to [50]; briefly, 100 µL of anti-chicken CORT, IL-6, and TNF-α monoclonal antibodies were added to microwell plate overnight at 4 °C. After incubation, standards and samples were added at room temperature and shaken for 2 h, followed by addition of biotinylated

anti-chicken CORT, IL-6, and TNF-α antibodies for 1 h. Then, wells were washed, and avidin-HRP conjugate was incubated for 30 min before adding TMB substrate for 15 min. The optical density value was recorded at 450 nm on a microplate reader (Thermo Fisher, USA), then expressed as pg/mL. The detection range is between 15.6 and 1000 pg/mL for IL-6 and 0.27–200 pg/mL for TNF-α.

Concerning hematological parameters, blood samples were collected in EDTA tubes, and then, the total leukocytic count (TLC) was analyzed via a hematology analyzer (Bayer-Advia 120). For differential leukocytic count, a drop of blood was smeared on a glass slide for each bird and then stained through May-Grünwald and Giemsa stains. One hundred leukocytes including heterophils, lymphocytes, eosinophils, and monocytes were counted on the slide, and the heterophil-to-lymphocyte (H/L) ratio was also calculated based on [51, 52].

Molecular Analysis

RNA Extraction and cDNA Synthesis

RNA was isolated from the BF tissue using the RNeasy Mini Kit (iNtRON Biotechnology, Seongnam, Korea) following the guidelines of the manufacturer's procedures. The quality of the extracted RNA was qualified by 1.5% agarose gel electrophoresis. The extracted RNA was subsequently reverse transcribed using the Reverse Transcription kit (Qiagen, Heidelberg, Germany) from each sample based on the manufacturer's guidelines. Then, cDNA samples were stored at − 20 °C until qRT-PCR.

qRT-PCR Analysis

Specific primer sequences of TLR4, HSP70, and caspase 3 genes beside their appropriate NCBI GenBank accession numbers are presented in Table 2. In brief, the qPCR investigation was performed in a Rotor-Gene Q apparatus with a QuantiTect® SYBR® Green PCR kit (SensiFast™ SYBR Lo-Rox kit, London, UK). The thermal cycling conditions of Rotor-Gene Q apparatus were 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, 60 °C for 15 s, and 72 °C for 15 s. The melting-curve analysis was complete to confirm the specificity of the qPCR. The relative expression profile of the target genes was measured through the comparative $2-\Delta\Delta Ct$ method using GAPDH as a housekeeping gene for target gene standardization [53].

Histopathological and Immunohistochemical Examination

BF tissues were fixed in neutral buffered formalin, then paraffin-embedded and sectioned (4 µm thickness). The sections were stained following deparaffinization with hematoxylin–eosin (H&E) method for microscopic examination [54]. Evaluation of immunohistochemical expression of nuclear factor-kappa B (NF-κB), Bax, and Bcl-2 in the bursal tissue was carried out following the indirect avidin–biotin-peroxidase staining technique based on [55]. Sections were incubated overnight at 4 ℃ with diluted primary antibodies against NF-κB (Cat# sc-8008), Bcl-2 (#sc-7382), and Bax (#sc-20067) (Santa Cruz Biotechnology, CA, USA). After the addition of the avidin–biotin complex, antibody binding was visualized with diaminobenzidine (DAB). All slides were observed and photographed through BX51 Olympus microscope with a built-in camera (Olympus optical LTD, Japan). Random five fields per slide were evaluated by mean of optical density using Image-Pro Plus 6.0 software (Media Cybernetics, MD, USA).

Statistical Analysis

All data were displayed as mean ± standard error mean (SEM) and then analyzed using one-way ANOVA followed by Tukey's post hoc test through SPSS statistical program software version 22 (SPSS Inc., Chicago, USA) in order to establish multiple comparisons between the control and treated groups at the same points of time [56]. Statistical significances between control, Cd, and RNP-treated groups were indicated at $p \leq 0.01$.

Table 2 Forward and reverse primer sequences used for RT-qPCR analysis

Gene	Primer sequence	Genbank accession no	Product size (bp)	Reference
TLR-4	F:5′-GTTCCTGCTGAAATCCCAAA-3′ R:5′-TATGGATGTGGCACCTTGAA-3′	NM_001030693	133	Lu et al. (2014)
HSP70	F:5′-CCAAGAACCAAGTGGCAATGAA-3′ R:5′-CATACTTGCGGCCGATGAGA-3′	EU747335	72	Abdo et al. (2017)
Caspase 3	F:5′-AAAGATGGACCACGCTCAGG-3′ R:5′-TCCGGTATCTCGGTGGAAGT-3′	NM_204725.1	189	Reno et al. (2022)
GAPDH	F:5′- TCTTCACCACCGCTCAGTTC-3′ R:5′-TATCAGCCTCTCCCACCTCC-3′	NM_204305.1	114	Lu et al. (2014)

Results

Characterization of RNPs

RNP morphology was investigated by TEM analysis which revealed nearly spherical particles with average particle size between 30 and 50 nm (Fig. 2).

FTIR analysis of RNPs and its individual components, rutin and chitosan alginate polymer, could provide further information about RNP synthesis (Fig. 3). The FTIR spectra of chitosan alginate polymer revealed its characteristic peaks through an interaction between amino groups of chitosan (positively charged) and carboxyl groups of alginate (negatively charged) at 1626 (N–H bending of primary amine and symmetric COO – stretching), 1417 (C-N stretching of amide group and asymmetric COO – stretching), 1022 (C-O stretching), and 3351 (O–H stretching vibration). Pure rutin, a polyphenolic compound, in the FTIR spectrum exhibited spectral peaks at 3337 and 2907 cm^{-1} (O–H and C-H stretching of phenols, respectively), 1651 and 1502 cm^{-1}

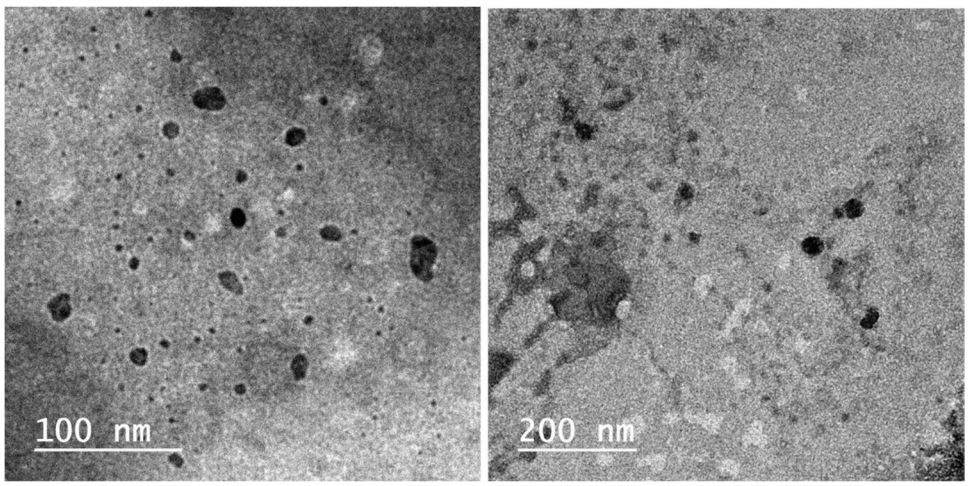

Fig. 2 TEM images of RNPs (100 and 200 nm)

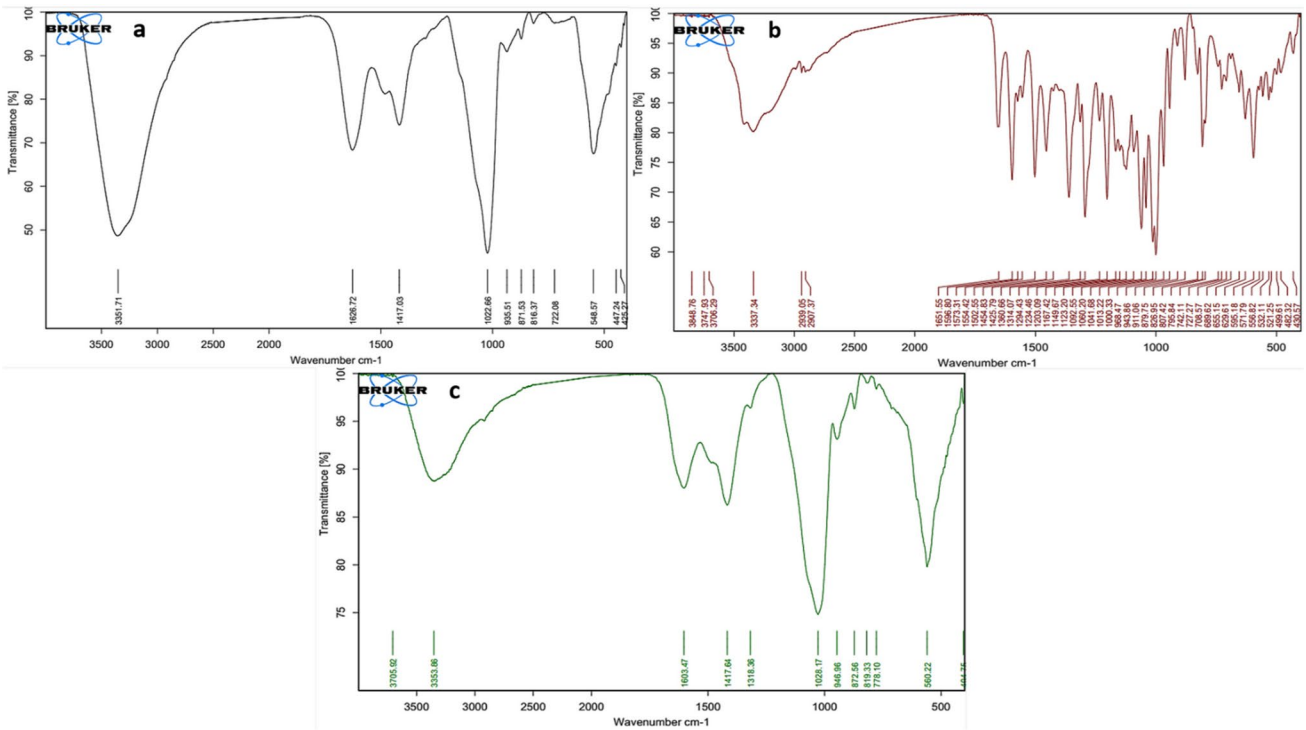

Fig. 3 FTIR spectra of chitosan alginate biopolymer (**a**), rutin (**b**), and RNPs (**c**)

(C = O and C = C stretching of carboxyl groups), and 1360 and 1294 cm^{-1} (C-O stretching of phenolic groups). Other characteristic peaks were displayed between 1203 and 1000 cm^{-1} (C–O–C and C–OH stretching) which supported the presence of phenolic and carboxylic groups of rutin. In the case of RNPs, the IR spectrum revealed certain changes in intensity and position of peaks compared with other IR spectra. For example, the characteristic peak of chitosan alginate polymer at 1626 broadened and shifted from 1626 to 1603, besides the appearance of a new peak at 1318 which confirms the successful loading of rutin into chitosan alginate nanocomposite.

Productive Performance

Table 3 illustrates the effect of rutin and RNPs on growth performance against Cd-induced immunological and oxidative stress in broiler chickens. Broilers treated with RNPs showed significantly better growth performance compared to the other groups since the final BW and BWG increased by 7.17% and 9.63%, respectively, in comparison to the control group. In addition, those broilers showed the best FCR, which decreased by 9.32% compared to the control group. On the contrary, the Cd-intoxicated group exhibited the poorest productive performance based on the final BW and BWG, which decreased by 39.17% and 53.49%, respectively, compared to the control group. Additionally, FCR of this group was increased by 112.05% compared to the control group.

Regarding co-treatment with rutin and RNPs after Cd intoxication, the rutin-co-treated group showed an increase in the final BW and BWG by 59.31% and 105.58%, respectively, when compared to the Cd-intoxicated group. Similarly, the RNP-co-treated group exhibited a significant rise in the final BW and BWG values by 61.63% and 109.97%, respectively, when compared to the Cd-intoxicated group. Furthermore, the FCR was markedly declined in rutin and RNP-co-treated groups by 50.95% and 52.21%, respectively, in comparison to the Cd-intoxicated group, while there was no significant change in the mortality rates between the control and different treated groups.

Economic Evaluation

Table 3 demonstrates the economic evaluation of rutin and RNP treatment against Cd-induced immunological and oxidative stress in broiler chickens. Concerning feed cost, a significant difference ($P < 0.01$) between groups was recorded since the feed cost declined by 1.37% in the Cd-intoxicated group but increased by 5.7% in the group treated with RNPs only when compared to the control group. However, the feed costs markedly were elevated by 6.99% and 7.03% in rutin and RNP-co-treated groups, respectively, in comparison to the Cd-intoxicated group.

In terms of TC, the RNP-supplemented group revealed a notable rise in TC by 4.44% while the Cd-intoxicated group showed a relative decline in TC by 1.1% when compared to the control group. However, rutin and RNP-co-treated groups following Cd exposure showed a marked rise in TC by 5.46% and 5.36%, respectively, when compared to the Cd-intoxicated group.

Based on TR data, the group that received RNPs alone had the highest TR (209.79 LE/bird) with an increase in rate of 4.2% compared to the control group. In contrast, the Cd-intoxicated group had the lowest reported TR (126.17 LE/bird) with a lower proportion of 39.42% compared to the control group. Additionally, the groups co-treated with rutin and RNPs displayed enhanced TR by 60.01% and 62.32%, respectively compared to the Cd-intoxicated group.

Table 3 Effect of rutin and RNPs on growth performance and economic parameters against Cd-induced immunological stress in broiler chickens

Item/bird	Control	RNPs	Cd	Cd + rutin	Cd + RNPs
Initial BW (g)	794.17 ± 4.74	797.50 ± 3.36	795.50 ± 4.21	798.00 ± 2.79	795.50 ± 1.73
Final BW (g)	$2975.0^{b} \pm 10.17$	$3188.3^{a} \pm 3.59$	$1809.7^{e} \pm 5.23$	$2883.0^{d} \pm 7.02$	$2925.0^{c} \pm 13.47$
BWG (g)	$2180.8^{b} \pm 12.26$	$2390.8^{a} \pm 4.37$	$1014.2^{e} \pm 5.47$	$2085.0^{d} \pm 9.39$	$2129.5^{c} \pm 13.84$
Total FI (g)	4870.0 ± 17.15	4836.7 ± 33.64	4803.3 ± 31.6	4855.0 ± 27.68	4825.0 ± 36.05
FCR	$2.24^{cd} \pm 0.06$	$2.02^{c} \pm 0.05$	$4.75^{a} \pm 0.22$	$2.33^{b} \pm 0.07$	$2.27^{bc} \pm 0.06$
Mortality (%)	13.33 ± 3.33	6.67 ± 3.33	16.67 ± 3.33	10.00 ± 5.77	6.67 ± 3.33
FC (LE)	$102.27^{b} \pm 0.36$	$108.1^{a} \pm 0.71$	$100.87^{b} \pm 0.67$	$107.96^{a} \pm 0.58$	$107.83^{a} \pm 0.76$
TC (LE)	$131.27^{b} \pm 0.36$	$137.1^{a} \pm 0.71$	$129.87^{b} \pm 0.67$	$136.96^{a} \pm 0.58$	$136.83^{a} \pm 0.76$
TR (LE)	$201.33^{a} \pm 0.51$	$209.79^{a} \pm 0.18$	$121.97^{b} \pm 0.26$	$195.16^{a} \pm 0.35$	$197.98^{a} \pm 0.67$
NP (LE)	$70.1^{a} \pm 0.53$	$72.69^{a} \pm 0.56$	$-7.9^{b} \pm 0.8$	$58.21^{a} \pm 0.34$	$61.15^{a} \pm 0.99$

Values are displayed as mean ± SEM. The mean values with different small superscript letters within the same row differ significantly at $P < 0.01$

BW, body weight; *BWG*, body weight gain; *FI*, feed intake; *FCR*, feed conversion ratio; *FC*, feed cost; *TC*, total cost; *TR*, total return; *NP*, net profit; *LE*, Egyptian pound

Regarding NP, our data showed a significant difference ($P < 0.01$) between groups since the RNP-treated group experienced the highest NP value (72.69 LE/bird) which increased by 3.69% compared to the control group. In contrast, the Cd-intoxicated group showed the lowest NP value (7.9 LE/bird) that declined by 111.27% when compared to the control group. In contrast, the NP was significantly improved by about 836.84% and 874.05% in groups co-treated with rutin and RNPs, respectively, in contrary to the Cd-intoxicated group.

Evaluation of Oxidative Stress Biomarkers

Cd-treated birds, as illustrated in Table 4, exhibited a significant rise in MDA level by 158% with a marked decline in non-enzymatic antioxidant GSH by 39.27% and enzymatic antioxidants including SOD, CAT, and GPx by 52.1%, 59.75%, and 48.25%, respectively, in BF tissue samples compared with the control group ($P < 0.01$). However, in contrary to the Cd-intoxicated group, rutin and RNP-co-treated groups displayed a notable decline in MDA level by 38.57% and 56.2%, respectively ($P < 0.01$). Moreover, there is an outstanding elevation of GSH, SOD, CAT, and GPx levels by 16.18%, 54.04%, 37.95%, and 27.96% in rutin-co-treated broilers and by 51.52%, 96.48%, 129.09%, and 75.88% in RNP-co-supplemented birds in comparison to the Cd-treated group ($P < 0.01$).

The return function which emphasized the relationship between TR and oxidative stress system, as displayed in Table 4, revealed that a 1% increase in GSH led to an approximate 0.67% increase in TR. Similarly, a 1% increase in SOD, CAT, and GPx enzymes corresponded to about 0.61%, 0.43%, and 0.59% increase in TR, respectively. Conversely, a 1% rise in MDA level caused a decline of approximately 0.44% in TR. Furthermore, the analysis of the return function revealed that approximately 92% of variations in the broiler farm's TR can be attributed to alterations in oxidative stress system in broilers.

Biochemical and Hematological Analysis

Cd-intoxicated birds revealed a substantial rise of serum corticosterone, TNF-α and IL-6 levels ($P < 0.01$) by 140.14%, 158.87%, and 50.72%, respectively, when compared to the control birds as summarized in Table 5. Meanwhile, co-treatment with rutin and RNPs significantly reduced CORT and TNF-α and IL-6 levels ($P < 0.01$) by 21.53%, 20.01%, and 16.4%, respectively, for rutin and by 44.19%, 49.25% and 29.64% correspondingly for RNPs when compared to the Cd-intoxicated group.

For hematological biomarkers, Cd-intoxicated broilers also showed a significant decline in the TLC by 13.92% with a notable reduction in the lymphocyte percentage while the heterophile percentage and the H/L ratio were significantly elevated in comparison to the control group ($P < 0.01$) (Table 5). On the other side, co-treatment with rutin and RNPs enhanced the TLC by 10.43% and 14.89%, respectively, when compared to the Cd-intoxicated group ($P < 0.01$). The lymphocyte percentage was relatively increased, particularly in the RNP-co-treated group, beside a marked decline in the heterophile percentage and the H/L ratio in contrast to the Cd-intoxicated group.

Quantitative RT-PCR Analysis

The relative gene expression levels of TLR4, HSP70, and caspase 3 genes in BF tissue were illustrated in Fig. 4. Our data displayed that Cd treatment dramatically upregulated expression levels of TLR4, HSP70, and caspase 3 gene

Table 4 Effect of rutin and RNPs on oxidative stress biomarkers, antioxidant enzymes, and return function against Cd-induced immunological stress in broiler chickens

Group	Control	RNPs	Cd	Cd + rutin	Cd + RNPs
MDA	$33.33^{d} \pm 0.88$	$30.17^{d} \pm 0.48$	$86^{a} \pm 0.52$	$52.83^{b} \pm 0.7$	$37.67^{c} \pm 0.61$
GSH	$27.17^{ab} \pm 0.6$	$28.17^{a} \pm 0.48$	$16.5^{d} \pm 0.43$	$19.17^{c} \pm 0.48$	$25^{b} \pm 0.58$
SOD	$99.17^{b} \pm 0.6$	$104.33^{a} \pm 1.02$	$47.5^{e} \pm 0.76$	$73.17^{d} \pm 0.79$	$93.33^{c} \pm 0.67$
CAT	$83.67^{a} \pm 0.84$	$85.17^{a} \pm 0.7$	$33.83^{d} \pm 0.87$	$46.67^{c} \pm 0.67$	$77.5^{b} \pm 0.76$
GPx	$122.33^{b} \pm 0.84$	$128.83^{a} \pm 0.79$	$63.3^{e} \pm 0.88$	$81^{d} \pm 0.97$	$111.33^{c} \pm 0.8$
Return function (relation between TR and antioxidant enzymes as well as oxidative stress biomarkers)					
Log TR =	0.75 + 0.59 Log GPX + 0.61 log SOD + 0.43 log CAT + 0.67log GSH-0.44log MDA				
t-value	0.78^{Ns}, 7.67**, 11.8**, 7.59**, 5.91**, −0.87**				
R^2	0.92				
F	66.33**				

Values are displayed as mean ± SEM; the mean values with different small superscript letters within the same row differ significantly at $P < 0.01$

Ns, non-significant; *TR*, total return

**Significant at $P < 0.01$

Table 5 Effect of rutin and RNPs on biochemical and hematological biomarkers against Cd-induced immunological stress in broiler chickens

Group	Control	RNPs	Cd	Cd + rutin	Cd + RNPs
CORT (ng/mL)	$1.47^{cd} \pm 0.15$	$1.4^{d} \pm 0.12$	$3.53^{a} \pm 0.0.15$	$2.77^{b} \pm 0.09$	$1.97^{c} \pm 0.09$
TNF-α (pg/mL)	$41.23^{d} \pm 2.19$	$39.37^{d} \pm 1.16$	$106.73^{a} \pm 1.9$	$85.37^{b} \pm 1.27$	$54.17^{c} \pm 1.55$
IL-6 (pg/mL)	$181.6^{d} \pm 1.74$	$180.1^{d} \pm 1.74$	$273.7^{a} \pm 2.03$	$228.8^{b} \pm 1.14$	$192.57^{c} \pm 1.72$
TLC (10^3/μL)	$20.83^{ab} \pm 0.77$	$21.37^{a} \pm 0.52$	$17.93^{b} \pm 0.96$	$19.80^{ab} \pm 0.35$	$20.60^{ab} \pm 0.47$
Heterophils (%)	$23.1^{c} \pm 1.28$	$22.1^{c} \pm 1.23$	$37.23^{a} \pm 0.78$	$31.83^{b} \pm 0.83$	$25^{c} \pm 1.44$
Lymphocytes (%)	$75.27^{a} \pm 0.55$	$76.13^{a} \pm 0.18$	$61.33^{c} \pm 0.64$	$67.7^{b} \pm 0.35$	$73.53^{a} \pm 1.01$
Monocytes (%)	$1.83^{a} \pm 0.09$	$1.9^{a} \pm 0.06$	$2.1^{a} \pm 0.12$	$1.87^{a} \pm 0.15$	$1.9^{a} \pm 0.12$
H/L ratio	$0.31^{c} \pm 0.02$	$0.29^{c} \pm 0.02$	$0.61^{a} \pm 0.01$	$0.47^{b} \pm 0.01$	$0.34^{a} \pm 0.02$

Values are displayed as mean ± SEM. The mean values with different small superscript letters within the same row differ significantly at $P < 0.01$

CORT: corticosterone, *TNF-α*: tumor necrosis factor α, *IL-6*: interleukin-6, *TLC*: total leukocytic count, *H/L ratio*: heterophil-to-lymphocyte ratio

in the BF tissue by 316%, 342%, and 276%, respectively, when compared to the control group ($P < 0.01$). On the contrary, rutin and RNP co-supplementation significantly downregulated mRNA expression levels of TLR4, HSP70, and caspase 3 gene in the BF tissue by 6.73%, 10.18%, and 12.77%, respectively, for rutin and by 30.53%, 28.28%, and 23.4%, respectively, for RNPs in opposition to Cd-treated birds ($P < 0.01$).

Histopathological and Immunohistochemical Evaluation

The control and RNP groups showed a normal histological structure of the BF with normal-sized follicles. Each follicle exhibited two distinct regions (cortex and medulla) separated by a clear corticomedullary junction and had a normal lymphocytic intensity (Fig. 5a, a', b, b'). In contrast, the Cd-treated group revealed a marked reduction in the number and size of follicles in the BF tissue with clear vacuolation and lymphocytic depletion (Fig. 5c, c'). The group co-treated with rutin showed a relative reduction of the lymphocytic populations (Fig. 5d, d'). Meanwhile, the birds co-supplemented with RNPs revealed nearly similar histological architecture to that of the control group (Fig. 5e, e').

Additionally, the follicle number and size of the BF tissue were counted in five random fields. Data emphasized that the Cd-treated group displayed a significant decrease in the number and size of follicles compared to the control group (Fig. 7a, b). On the other hand, the group co-treated with RNPs exhibited a significant increase in follicular number and size compared to the Cd-treated group.

Immunohistochemical Expression of NF-κB, Bax, and Bcl-2 in the BF Tissue

The immunohistochemical staining was performed to evaluate the expression of NF-κB, Bax, and Bcl-2 in bursal cells.

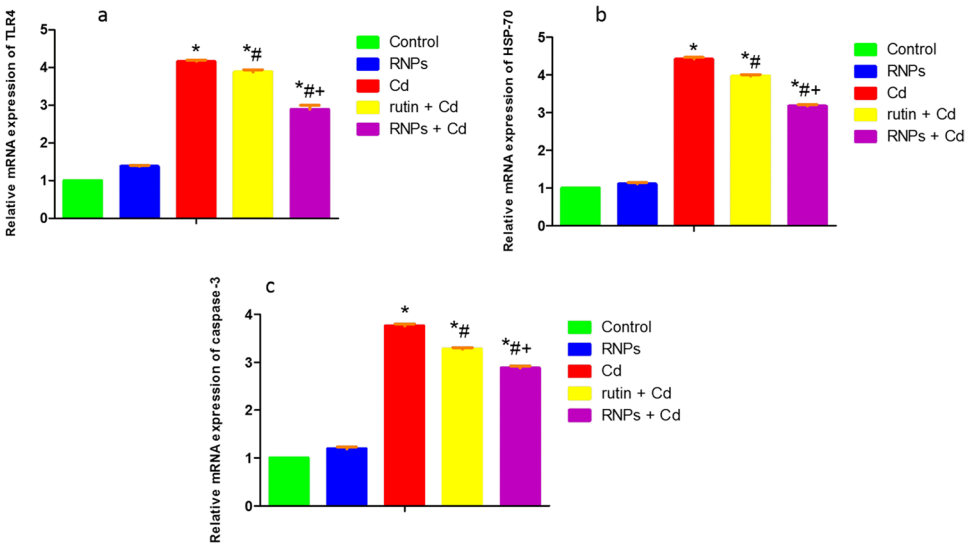

Fig. 4 Relative mRNA expression of **a** TLR4, **b** HSP70, and **c** caspase 3 genes in the BF tissue of broilers treated with rutin and RNPs against Cd-induced immunological stress. Data expressed as mean ± SEM ($n = 45$) were analyzed via one-way ANOVA followed by Tukey's test, and different letters indicate statistical significance at $P < 0.01$

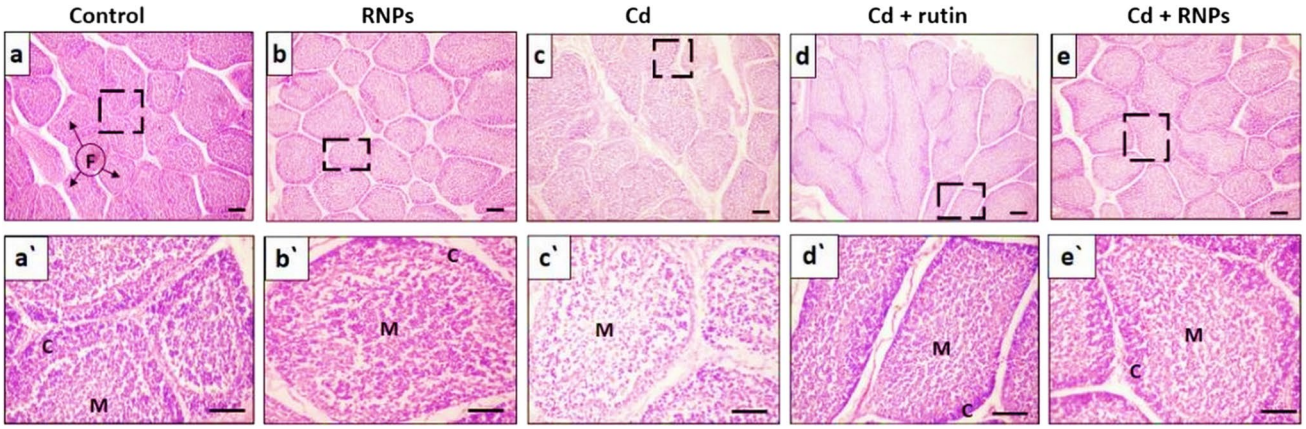

Fig. 5 Photomicrograph of H&E-stained sections of BF tissue showing the control group with normal medullar and cortical structure (**a**, a'), RNPs group showed normal-sized follicles (**b**, b'), Cd group showed follicular atrophy with vacuolation, lymphocytic depletion, and interstitial connective tissue hyperplasia (**c**, c'), Cd + rutin group (**d**, d') with relative lymphocytic depletion with reduction of the lymphocytic populations, and Cd + RNP group (**e**, e') revealed a normal histological structure of the lymphoid follicles and nearly similar histological architecture to that of the control group. F, follicles; C, cortex; M, medulla. Scale bars = 100 µm and 50 µm for the magnified insets

Examination of the BF sections of the control and RNPs groups showed a marked decline of NF-κB and Bax immunoreaction in the bursal cells (Fig. 6a, b, f, g), respectively. Meanwhile, the BF sections of the Cd-treated group showed a notable rise of NF-κB and Bax immunoreaction in contrary to the control group (Fig. 6c, h), respectively. Rutin co-treated with Cd showed less prominent NF-κB and Bax immunohistochemical staining in comparison to the Cd-exposed group (Fig. 6d, i), respectively, while RNPs co-supplemented with the Cd group showed relatively similar immunoreaction to that of the control group (Figs. 6e, j and 7c, d). On the other hand, BF sections of the control and RNP groups revealed a notable increase in the Bcl-2 immunoreactivity when compared with the other treated groups (Fig. 6k, l), while the Cd-exposed group showed a substantial decrease of Bcl-2 immunoreaction in BF cells in opposition to the control group (Fig. 6m). The BF cells of the group co-supplemented with rutin and Cd displayed an increase in positive immunoreaction of Bcl-2 when compared with the Cd-treated group (Fig. 6n), while the group co-treated with RNPs showed a significant increase in Bcl-2 immunoreaction which resemble that of the control group (Figs. 6o and 7e).

Discussion

The current study evaluates the antioxidant effect of RNPs against Cd-induced oxidative damage and immunosuppression in broiler chickens. Experimentally, our findings revealed that Cd could have a critical immunotoxic effect in broilers through substantial elevation of oxidative biomarkers and pro-inflammatory cytokines which may provoke degenerative and pathological changes in the BF tissue with marked reduction in the lymphocytic population. However, rutin mitigated some of those changes, and RNP displayed promising results with complete restoration of the normal function and antioxidant activity in the BF tissue.

Cd is a nonessential and toxic element that is released into the environment through extensive usage of industrial fertilizers, pigments, and plastics [57]. Cd could bioaccumulate among various food chain levels, including poultry, resulting in severe malfunction of multiple organs such as the liver, kidneys, and nervous and immune systems [58, 59]. Concerning the catastrophic drawbacks of Cd in the poultry industry, it leads to significant productive and economic losses, and therefore, a growing concern should be paid to such pollutants emerging in the production systems [60]. The main exposure source of Cd in broilers is through ingestion of contaminated drinking water or food which subsequently upsurges the free radical production causing oxidative lesions in various tissues [61].

Various therapeutic interventions against heavy metals are gaining great attention recently [62]. Rutin, as a phytochemical, has proven to have a powerful antioxidant activity to alleviate oxidative stress and could strengthen the birds' immunity [63]. Rutin also has mucosal protection and anti-ulcer functions which in turn improve the broilers' growth performance and intestinal function; however, the poor aqueous solubility of rutin could limit their efficacy [64–66]. Therefore, rutin was efficiently loaded on chitosan alginate polymeric nanoparticles which are natural, biocompatible, biodegradable, and safe drug delivery vehicle to improve its bioavailability, solubility, stability. and efficacy [32, 67].

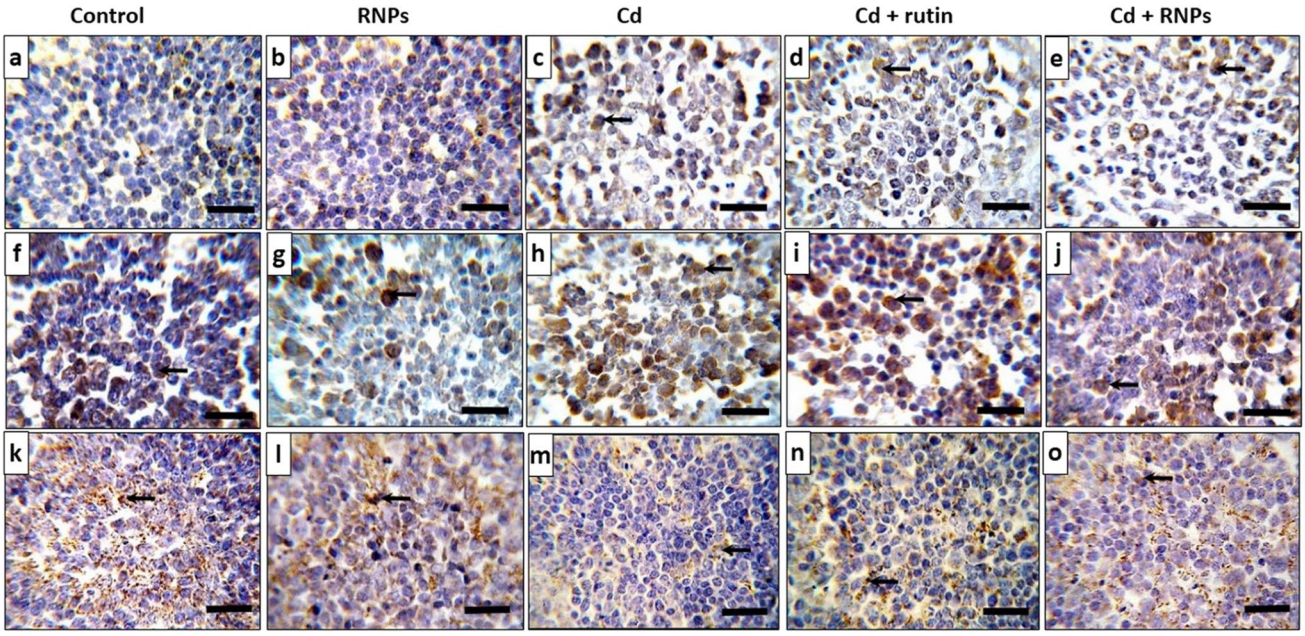

Fig. 6 Photomicrograph of immunohistochemical staining of the BF tissue with anti-NF-κB (**a**–**e**) and anti-Bax (**f**–**j**) that revealed the minimal density of immunohistochemical staining in the control- and RNP-treated groups, while there was a marked increase in immunostaining in the BF tissue of Cd-treated birds. However, anti-Bcl-2 (**k**–**o**) showed a significant rise in the immunohistochemical staining in the control- and RNP-treated groups, and the density of immunohistochemical staining was minimal in Cd-treated groups. Positive cells show a brown color (arrows). Scale bars = 30 µm

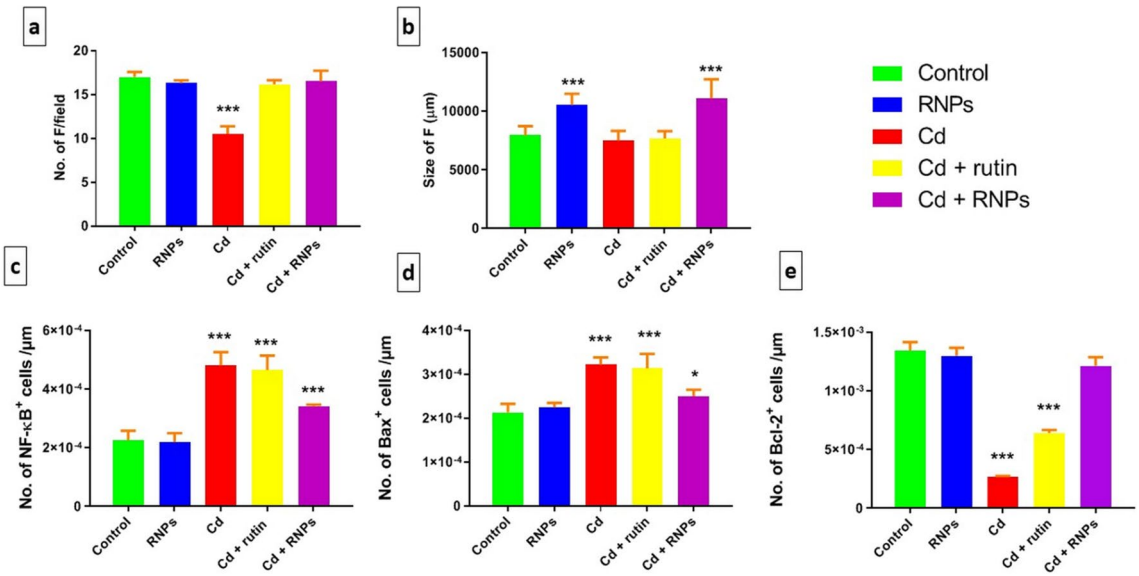

Fig. 7 Graphs showing the estimated results of the number of the bursal follicles of all groups (**a**), size of follicles (**b**), number of NF-κB + cells (**c**) and number of Bax + cells (**d**), and number of Bcl-2 + cells (**e**) in the BF tissue following treatment with rutin and RNPs against Cd-induced immunotoxicity. Significant results are represented by asterisks (*$P \leq 0.05$, ***$P \leq 0.01$)

TEM displayed that RNPs had spherical shapes, smooth surfaces, and small particle sizes which could enhance its permeability across the GIT, and these findings come in agreement with previous studies [68]. Furthermore, FTIR data confirmed the efficient loading of rutin upon the polymeric nanoparticles since the characteristic peak of chitosan alginate polymer at 1626 cm^{-1} was broadened and shifted to 1603 cm^{-1} beside the appearance of a new peak at

1318 cm.$^{-1}$, and these findings are consistent with previous reports [26, 69].

Concerning productive and economic performance, our findings exhibited that Cd significantly reduced the BW of exposed chickens, possibly due to a decrease in the FI and appetite beside an increase in the breakdown of proteins and fats associated with Cd toxicity [70]. Additionally, the negative impact of Cd on performance may be linked to its toxic effects on various systems within the animal body [71]. On the contrary, a notable rise in the FCR was observed in Cd-intoxicated chickens which may be linked to the reduction in BWG. These findings were consistent with [72] who reported that Cd-exposed broilers at 100 ppm in drinking water for 28 days showed a marked decline in the BW, BWG, and FI. Similarly [73], concluded that Cobb chickens treated with Cd chloride at a dosage of 50 mg/kg displayed a substantial elevation in the FCR beside a noticeable decline in the BWG and FI.

On the flip side, dietary addition of rutin and RNPs could potentially enhance the productive performance in broilers which comes in agreement with [74] who indicated that flavonoids such as rutin could protect the intestinal mucus membrane and prevent ulcers via blocking the gastric proton pump which subsequently stimulate the birds' appetite beside promoting a healthy balance of intestinal microenvironment [75]. Furthermore, our findings matched with previous studies as [76, 77] which suggested that rutin supplementation in broiler diets could significantly improve the BWG and FCR.

Concurrently, some varieties of flavonoids are degraded in the intestinal fluid and have limited absorption capability through the intestinal membranes, so encapsulation of these types of flavonoids in nanoparticulate form could enhance their absorption capacity and availability [78]. Chitosan is considered a favorable choice for drug delivery due to its mucoadhesive properties and its ability to enhance permeation with sustained drug release [79]. This may explain the comparable improvement of productive performance in RNP-co-treated birds than birds supplemented with rutin alone following Cd exposure which coincided with [80] who confirmed that rutin nanoparticles displayed a notable enhancement in the antioxidant properties than rutin alone.

Regarding the economic evaluation, our findings indicated that exposure to Cd in broilers triggers significant economic losses. Our data is consistent with [81] who demonstrated that Cd-contaminated feed could accumulate in tissues and disrupt the metabolic and physiological functions which evoked higher mortality rates and ultimately influence the economic efficiency. Furthermore [82], revealed that dietary Cd supplementation at a dose level of 120 ppm had a critical impact on broiler performance, health parameters, and economic efficiency.

Our findings exhibited a relative increase in feed cost and TC in rutin and RNP-co-treated groups which may be attributed to the additional cost of rutin in diets. This finding aligns with [83] who observed that incorporating herbal additives into broiler diets raised the production costs of broilers. However, from an economic point of view, optimizing feed efficiency, enhancing FCR, and managing the stress risk effectively are essential for minimizing the production expenses and providing a financial benefit through enhancing the live BWG [84]. This has elevated the quest for cost-effective feed additive that could enhance the productivity via preserving gut health [85].

Improvement in return economic parameters, such as TR and NP, after co-treatment with rutin and RNPs was attributed to enhanced feed utilization, increased BWG, and improved FCR since rutin is regarded as a natural feed additive that could enhance feed efficiency via promoting digestive secretions and nutrient absorption, maintaining gut health, exhibiting antioxidant properties, and reducing the microbial load on the animal immune system [86–88]. Moreover, the additional costs of incorporating rutin or RNPs into the broilers' diet were found to be negligible. Interestingly, broilers co-treated with RNPs showed a greater improvement in economic performance compared to the rutin co-treated group which may be attributed to the favorable effects of chitosan NP encapsulation on feed utilization, nutrient digestibility, growth performance, productivity, and immune responses [89, 90].

Overproduction of reactive oxygen species (ROS) resulted in compromising the antioxidant defense system including enzymatic biomarkers such as SOD and GPx which leads to oxidative stress [91]. The current study showed that the Cd-intoxicated group significantly raised MDA level with a marked reduction in antioxidant biomarkers such as SOD, GSH, CAT, and GPx in the BF tissue. These findings coincided with [92] who reported that dietary intake of Cd at a dose level of 100 mg/kg diet resulted in a significant elevation of MDA in the BF tissue, and also [93], displayed that a broiler diet containing Cd at a dose of 140 mg/kg provoked marked oxidative damage in the BF tissue with a notable decline in SOD and GPx. The ability of Cd to trigger oxidative damage may be due to its high affinity to thiol groups in the antioxidant enzymes with subsequent malfunctioning of their activity [94], and also, inhibition of NADPH oxidase is described as a possible mechanism of Cd-induced oxidative damage [95]. Furthermore, Cd could induce substantial alterations in the mitochondrial structure and mitochondrial permeability via blockage of the respiratory chain complexes [96].

Alternatively, rutin and RNP supplementation significantly raised the antioxidant enzyme activity with a marked reduction in MDA level in the BF tissue. These results come in agreement with [97] who reported that rutin at a dose

level of 500 mg/kg diet could counteract oxidative damage via suppressing the MAPK pathway in the liver tissue of broilers. Rutin, also a well-known antioxidant, could boost the intestinal antioxidant status of laying hens exposed to oxidized protein of soybean meal [98]. Return function provides important insights into the impact of oxidative stress on the overall outcome of the production process since oxidative damage could provoke various pathophysiological changes in birds' immune organs at the cellular level which is further associated with increased susceptibility to pathogens, compromised gut health, and reduced feed intake, weight gain, and economic efficiency [99]. Thus, our findings revealed that Cd-induced oxidative stress has a detrimental effect on broiler farm productivity and TR. In contrast, rutin and RNPs mitigated Cd-induced oxidative stress through enhancing the antioxidant enzyme activity that acts as ROS scavengers resulting in improved performance and return rates [100]. These findings are aligned with [37], who concluded that rutin supplementation could alleviate oxidative damage and improve productivity and TR in broilers.

Oxidative damage triggers excessive production of pro-inflammatory cytokines and CORT in broilers which is considered an important biomarker of stress and tissue damage in immune organs, particularly BF. The current study displayed that Cd-intoxicated birds displayed marked elevation of serum IL-6, TNF-α, and CORT hormone. Indeed, Cd could substantially elevate the expression levels of cytokines including IL-6 and TNF-α in central immune organs of poisoned chickens via the Toll-like receptor 4 (TLR4) pathway [7]. Moreover [93], revealed that Cd induced a complex inflammatory response in chicken splenic lymphocytes with significant elevation of various cytokines through upregulation of NF-κB expression level which appeared as a detrimental factor prompting the cytokine expression [101].

CORT or stress hormone is the main moderator of allostasis in broilers which is released following exposure to stressful conditions through stimulation of hypothalamic–pituitary–adrenal (HPA) axis resulting in numerous physiological changes to restore homeostasis [102]. Previous reports suggested that Cd triggers corticosteroid-induced immuno-regulatory circuit via activation of the HPA axis besides upregulation of glucocorticoid receptor (GR) expression, leading to a substantial rise in the glucocorticoid levels [103, 104]. Elevation of CORT hormone in broilers has a negative influence upon the hematopoietic system, particularly the TLC and differential leukocytic count, driving the dissolution of lymphocytes and inhibiting neutrophil apoptosis [105, 106]. The current study revealed that the Cd-intoxicated group showed a notable increase in the heterophil percentage and H/L ratio with a reduction of the lymphocyte percentage which may be due to the glucocorticoid influence. Similar findings were reported by [107] who concluded that Cd has marked immuno-toxic effects on the hematopoietic stem cells via increasing the myeloid progenitors and decreasing the lymphoid progenitors in exposed quails. Furthermore [108], documented that broilers experienced thermal stress-released excessive glucocorticoids resulting in severe leukopenia and lymphopenia with marked shift in the H/L ratio which regarded as an index of stress in broilers.

On contrary, rutin-co-treated birds showed a marked reduction of serum IL-6, TNF-α, and CORT hormone while RNP-co-treated broilers could efficiently restore the normal levels of such biomarkers. Similar findings from previous studies revealed that rutin had potent anti-inflammatory properties because of its inhibitory role in cytokine production, including TNF-α and IL6 [109, 110]. Furthermore, rutin effectively improved immunity and intestinal function via reducing pro-inflammatory cytokine content through inhibiting the Nrf2/HO-1 pathway in broilers [66, 111]. Additionally, our data revealed an increase in the lymphocytic population with a relative reduction of H/L ratio and heterophil percentage into normal levels. These findings coincided with [112, 113] who confirmed the immunomodulatory effect of rutin on hematopoietic tissue which may attributed to potent anti-oxidative potential of rutin. Furthermore, they hypothesized the capability of rutin to normalize the CORT homeostasis and HPA axis function following exposure to psychosocial stressful conditions which subsequently improve immunity and hematopoietic system [114].

Heat shock proteins (Hsp) are a part of the protein folding system in cells that are expressed intracellularly in all living organisms as a response to physiological stress, so they are regarded as stress-inducible and immunomodulants as they prompt the immune system response to adverse cellular conditions [115]. Notably, HSP70 plays an important role in proteostasis but, during stress, may be released to the extracellular matrix and triggers the pro-inflammatory response of immune cells which served as a hazard signal to the immune system [116, 117]. Extracellular Hsp70 initiates signal transduction facilitating the entry of NF-κB into the nucleus where it enhances the transcription of various pro-inflammatory cytokines as TNF-α and IL-6 [118]. Moreover, HSP70 can also trigger inflammatory responses via binding to TLRs, particularly TLR4, which play a vital role in the host innate immunity as a pattern recognition receptor superfamily [119, 120]. TLR4 can further activate various downstream inflammatory cascades such as the NF-κB pathway which results in the further production of various pro-inflammatory cytokines and chemokines [121, 122]. In the present study, our molecular and immunohistochemical findings revealed that the Cd-intoxicated group markedly upregulates the expression level of HSP70, TLR4, and NF-κB. These findings come in agreement with [123] who reported that Cd significantly promoted the expression level of the TLR4/NF-κB pathway in duck embryo hepatocytes, and also [124], exhibited that Cd enhanced the expression

of HMGB1 downstream mediators, including TLR-4 and NF-κB which exacerbated the damaging effects of Cd in exposed tissues via stimulating the release of several inflammatory cytokines. Interestingly [125], revealed that Cd could induce adrenal damage through activation of TLR4/NF-κB-mediated inflammatory responses which play an essential role in Cd-induced toxic lesions. Moreover [126], elucidated that Cd triggered oxidative damage in the hepatic tissue through a substantial elevation of NF-κB and HSP70 expression levels.

On the other hand, rutin and RNP-co-treated birds displayed a prominent decline in HSP70, TLR4, and NF-κB expression levels which suggested that the HSP70/TLR4/NF-κB pathway may be responsible for improving the anti-inflammatory properties of RNPs. Our data matched with previous studies which illustrated that rutin downregulated the MAPK/HSPs/NF-κB pathway as a protective mechanism against induced necroptosis in liver [36, 127]. Furthermore, rutin played an immuno-regulatory role and protected the immune organs through TLR4/MyD88/NF-κB signaling pathway against cyclophosphamide-induced immunological stress [128]. Therefore, this pathway was suggested to play a vital role in not only inflammation but also the immune regulation, tissue repair, and survival of cells.

Overproduction of ROS and pro-inflammatory mediators considered potent signal molecules plays a pivotal role in mitochondrial-dependent apoptosis through enhancing the release of pro-apoptotic molecules causing caspase cascade activation and induction of apoptosis [129]. The Bcl2 family, of which Bcl-2 and Bax are the most important members, plays an essential role in the regulation of apoptosis via controlling the outer mitochondrial membrane permeability [130]. The current study exhibited a marked elevation of the mRNA and protein expression levels of pro-apoptotic caspase 3 and Bax in Cd-intoxicated broilers besides a significant decline of the anti-apoptotic Bcl-2 expression levels. These findings confirmed that Cd could exacerbate apoptosis and tissue damage in immune organs and subsequently deteriorate organ function which coincided with previous studies [131, 132] which illustrated that Cd could induce apoptosis and tissue damage at a dose level of 150 mg/kg diet in chicken BF tissue. Furthermore, increased mRNA level of Bax, caspase-3, and cytochrome c and decreased Bcl-2 and CaM was observed following Cd exposure in chicken splenic lymphocytes [133]. In opposition, rutin and particularly RNP-co-treated birds showed a marked downregulation of the mRNA and protein expression levels of caspase 3 and Bax with a significant rise of the anti-apoptotic Bcl-2 level. Our data come in agreement with [134] who reported that rutin acts as a scavenger of ROS with a potent anti-apoptotic effect at a dose level of 50 mg/kg BW in liver and kidney of rats exposed to deltamethrin. Moreover, [111] reported that rutin boosted cell proliferation and suppressed apoptosis in laying hens through Nrf2/HO-1 pathway.

Morphological examination revealed that Cd-intoxicated birds showed excessive damage of the BF tissue with marked lymphopenia and follicular atrophy which agreed with [16] who showed that Cd exposure at a dose level of 100 ppm could provoke severe atrophic changes in the lymphoid follicles with cellular edema since excessive ROS production in the BF tissue promotes a cascade of tissue damage, apoptosis, and impaired lymphocytic proliferation with a marked reduction of the B-lymphocytes [135]. On the other hand, RNP-co-treated groups retained the normal medullar and cortical morphology of the BF tissue which confirmed the immunoprotective effect of RNPs based on its antioxidant and anti-inflammatory properties which matched with previous studies [112, 136]. However, further investigation of Cd immunotoxicity and RNP immunomodulatory mechanisms through Western blotting will be required in the follow-up studies and regarded as one of the limitations in this study. Additionally, our study design could not give information about the relationship between Cd bioaccumulation in the bursal tissue and RNPs, and if RNPs could reduce Cd bioaccumulation in the bursal tissue, so further investigations are needed to clarify this since we thought that RNPs may be a promising candidate in adsorption of Cd.

Conclusion

Cd treatment led to a severe decline in the productive performance of intoxicated broilers with marked economic losses. These findings were potentiated by marked oxidative damage in the BF tissue with a significant decline in the enzymatic and non-enzymatic antioxidant biomarkers such as CAT, SOD, GPx, and GSH with a substantial rise of MDA level which trigger excessive production of pro-inflammatory cytokines beside elevation of CORT level which in turn could rise the H/L ratio. Furthermore, the expression level of TLR4, HSP70, caspase3, NF-κB, and the pro-apoptotic Bax was significantly upregulated while the anti-apoptotic Bcl2 was downregulated in Cd-treated birds which suggested that HSP70/TLR4/NF-κB signaling pathway may be involved in the molecular mechanism of Cd immunotoxicity which is confirmed by the excessive damage of the BF tissue with marked lymphopenia, vacuolation, and follicular atrophy. Meanwhile, rutin and RNPs alleviated Cd-induced oxidative damage, suppressed release of pro-inflammatory cytokines, and modulated HSP70/TLR4/NF-κB molecular pathway which improved the morphological structure of the BF tissue. Our results also emphasized that RNPs were more effective against Cd-induced immunotoxicity than rutin alone since our nanoformulation could enhance solubility, bioavailability, targetability, and efficacy of rutin.

Thus, further investigations are still needed to demonstrate other potential action mechanisms and safety of RNPs in broiler diets to both broiler and human health.

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Author Contribution M. A. design this study, wrote the manuscript and analyzed oxidative biomarkers, B. H. performed RT-PCR analysis. Z. S. carried out the histopathological analysis. R. R. performed RNPs characterization, N. H. analyzed parameters. S. S. performed growth performance analysis. N. W. analyzed the economic efficiency measures and wrote the manuscript. All authors also read, revise and approved the article for publication.

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Data Availability No datasets were generated or analysed during the current study.

Declarations

Ethics Approval This study was approved by Mansoura University ethical committee, (MU-ACUC), No. (VM.R.24.01.149).

Consent to Participate Not applicable.

Consent for Publication Not applicable.

Competing Interests The authors declare no competing interests.

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