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A novel *in-vivo* drug delivery strategy against *Salmonella*: A case study of ciprofloxacin loaded chitosan nanoparticles for boiler birds

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Abstract :The present study aimed to evaluate the *in-vivo* efficacy of chitosan nanoparticles loaded with ciprofloxacin antibiotic (CSNPs-CIP) against *salmonella enteritidis* infection as a nanodrug delivery system. Our results revealed that this nanodrug delivery system exhibited a significant improvement in external clinical symptoms which appeared after infection as compared with the infected group. This can be expressed and shown in the recovery percentage of fresh weight and clinical indices measurements of ciprofloxacin-loaded chitosan nanoparticles which gives 73.9 % recovery compared with treatment with ciprofloxacin; 2.0 mg ml^{-1} singly or chitosan; 10% singly which give 54.3% and 58.7% percent recovery respectively. Meanwhile, gene expression levels and biochemical profile of hepatic and intestinal tumor necrosis factor- α (*TNF-α*) and superoxidase dismutase (*SOD*) activity were significantly different in the group treated with chitosan nanoparticles loaded with ciprofloxacin (2.0 mg ml^{-1}) compared with healthy and infected groups. This prevents the adverse effects of the pathogen, as showed in the corrected histo-pathological picture of both the intestine and liver which showed significant improvement in the tissues of treated groups compared with the control infected group. Therefore, we can conclude that treatment of diseased chickens with *Salmonella enteritidis* by oral drop technique with chitosan nanoparticles; 10% loaded with ciprofloxacin; 2.0 mg ml^{-1} exhibited controlled release of the antibiotic, which can prolong the antibacterial effect and it was the best novel strategy for recovery from salmonellosis disease in boiler birds.

Keywords: broilers, chitosan nanoparticles, ciprofloxacin, nanodrug delivery system, *Salmonella.*

Abbreviations: CSNPs; chitosan nanoparticles, CFU; colony forming unit, CSNPs-CIP; ciprofloxacin-loaded chitosan nanoparticles, PCR; polymerase chain reaction, *SOD;* superoxidase dismutase activity, *TNF-α;* Tumor necrosis factor- α.

1.Introduction

Salmonella is one of the most frequently reported bacterial pathogens associated with human disease. It causes salmonellosis disease which also known as typhoid, gastroenteritis, and paratyphoid in humans, and it can infect many hosts, including reptiles, birds, and mammals. Poultry is the main carrier of this pathogen [1], and its products have been associated with great outbreaks [2]. In poultry, when infected birds shed the bacteria in their feces, *Salmonella* can colonize in the gastrointestinal tract through horizontal transmission and consequently infect the environment and other birds living nearby. Young chicks are mostly susceptible to *Salmonella* colonization through horizontal transmission at the hatcheries during feeding, handling, and transportation [3, 4] Hence, *Salmonella* has been reported to be resistant to many antibiotics and this in turn poses a major

threat to public health and food safety [5, 6]. Some strains of *Salmonella* are resistant to at least one drug in three or more antimicrobial categories, showing multidrug-resistant strains [7].

Ciprofloxacin (CIP) is a member of the fluoroquinolone drug group of has broad-spectrum activity against Gram-ve bacteria, particularly *Salmonella* spp. infections [8]. During DNA replication, CIP has the ability to inhibit the activities of both bacterial DNA topoisomerase and DNA-gyrase [9]. Ciprofloxacin is recommended as the drug which treats salmonellosis; however, overuse of this antibiotic may cause antibiotic-resistance problems, failure of disease treatment, and subsequent severe clinical outcomes. *Salmonella* resistance to ciprofloxacin has been progressively reported worldwide due to plasmid-mediated-quinolone resistance in plasmids or chromosomes of bacteria [10, 11].

Recently, the emergence of ciprofloxacin resistance or even reduced susceptibility has resulted in treatment failures [12]. Prolonged free antibiotic use for more than 7 days can led to several limitations when delivered intracellulary and using extra-dose for treating *Salmonella* infection can led to severe adverse effects [13]. In addition, the world health organization stated that beside antimicrobial resistance, *Salmonella* has the ability to form biofilms of fimbriae components which represent a major threat to veterinary medicine and human [14]. These limitations emphasize the importance of developing new biocompatible drug delivery system with high loading capacity which can control drug release, allow reduced dosage without compromising therapeutic effect of intracellular pathogens [15]. Also, some benefits of developing drug delivery systems are summarized as improvement of the overall pharmacokinetics, reducing of antimicrobial resistance, increasing the solubility of some antibiotics, and over all clinical efficacy [16, 17].

Helal *et al.* [17] and Rathore and Mahesh [18] confirmed that nanotechnology is a new technology that stimulus the division of material at a nanoscale range from 1 to 100 nm which can led to change the properties of material and increase its utilization potential due to the increase in the surface area that makes them more usable in several applications such as drug delivery systems as it provides a convenient method for the delivery of small molecular weight drugs as well as macromolecules such as proteins, peptides and genes to different cells and tissues and also protect them from enzymatic degradation. Nanoparticles are considered effective for growth promotion, micronutrients delivery and production of feed amount per unit time during aquafeed development due to their biodegradability, non-toxicity, and sufficient stability over long periods [17, 19].

Chitosan nanoparticles (CSNPs) are very promising as biomedical applications. Due to they are manufactured at low temperatures, they have the ability to carry biologically active substances such as drug as a delivery system. Additionally, their special properties including good biocompatibility, non-toxicity and high surface area can led to high capacity of drug loading and delayed release of antibiotic resulting in more frequent dose reduction. As shown in several previous studies, CSNPs as delivery systems for antibiotic have been shown to improve the antimicrobial efficacy and provide a safety profile for ciprofloxacin by protecting it from chemical, enzymatic, and immunological degradation [17, 20, 21, 22]. The introduction of CSNPs into chicks feed can increase the quality of feed, nutritional availability and removal of pathogen [23].

This study provided a new hope for further validation of the *in-vivo* efficacy of a chitosan nanoparticles loaded with reduced doses of ciprofloxacin compared with conventional ciprofloxacin treatment in the evaluation of the gene expression profiles, biochemical parameters and the histopathological examination showing good biocompatibility and lower cytotoxicity.

2. Materials and methods

a- Preparation of chitosan nanoparticles (CSNPs) emulsion

Chitosan nanoparticles (CSNPs) were prepared according to the method of Hasaneen

et al. [21], through the polymerization of methacrylic acid in chitosan solution. Under magnetic stirring for 12 hours, approximately 200 mg of chitosan powder was dissolved in aqueous solution of methacrylic acid (0.5 %). Then, 0.05 g of potassium persulfate was added to the previous mixture with continued stirring till the solution became clear. At 70 °C, the mixture was heated with continuous stirring using magnetic stirrer for an hour to confirm the formation of chitosan nanoparticles. Finally, the solution was cooled in an ice bath to stop the reaction.

b- Loading of ciprofloxacin antibiotic on chitosan nanoparticles (CSNPs) emulsion

The loading of ciprofloxacin (2.0 mg ml^{-1}) on CSNPs solution was achieved by adding 20 cm^3 of ciprofloxacin solution into 30 cm^3 of CSNPs solution and stirred at room temperature for six hours under magnetic stirring [21, 24, 25].

c- Time course experiment

A large-scale experiment was conducted to compare growth performance, gene expression and biochemical profile induced by *Salmonella enteritidis* pathogenic bacteria in broiler chickens and efficacy of nano-chitosan either singly or loaded with ciprofloxacin antibiotic at optimum concentrations (2.0 mg ml^{-1}). The current investigation was carried out at a private poultry farm in Meet-khamis village, Mansoura, Dakahlia Governorate, Egypt during the period from first November to late December 2023. This experiment was repeated in the next year during the period from November to December 2024. A total of 25, seven-day-old Hubbard *Salmonella*-free broiler birds were purchased from a commercial hatchery and randomly allocated into five treatment groups; 5 birds in each group. The five groups were categorized throughout the entire period of experiment as following:

1- Control; healthy group

2- Control; infected group inoculated by *Salmonella enteritidis* (1.0 cm^3)

3- Infected group treated with ciprofloxacin antibiotic (CIP; 2.0 mg ml^{-1}) singly.

4- Infected group treated with chitosan nanoparticles (CSNPs; 10 %) singly.

5- Infected group treated with chitosan nanoparticles; 10% loaded with ciprofloxacin; 2.0 mg ml^{-1}.

All experimental chicks were housed in cleaned and disinfected pens and kept for approximately 6 weeks (experimental period) under complete observation. All birds were offered un-medicated broiler ration and had free access to water and feed. Diet was designed to meet the recommendation for broiler chickens [26] (Table 1).

After one week, each bird in the experimentally infected groups was orally inoculated with 1 cm^3 (1.0 × 109 CFU/ml) *Salmonella enteritidis* isolate by using a polyethylene tube attached to a syringe [27]. Chicks in the healthy control group were orally inoculated with the same volume of sterile phosphate buffer saline at 14 day-old. After complete appearance of the external disease symptoms at day 25 old of chicks, treatment of chicks was carried out orally with the same volume of ciprofloxacin antibiotic and chitosan nanoparticles either singly or in combination every day until 7 day (33 d-old diseased chicks), at day 35 old, chicks were harvested for clinical studies on liver and intestine. In the first week, the temperature was adjusted to 32 °C and gradually decreased to 25 °C at the end of the experiment. *Salmonella enteritidis* strain was isolated in microbiology laboratory, Faculty of Veterinary, Mansoura University and had been fully identified, classified and serotyped as previously performed by [28, 29]. *Salmonella enteritidis* was characterized by resistance to 2.0 mg ml^{-1} of ciprofloxacin. Experimental procedures were carried out in accordance with the recommendations of the Mansoura University Animal Care and Use Committee (MU-ACUC).

d- Clinical signs and mortalities rate

In every experimental group, the chicks were observed twice daily throughout the entire period of experiment to monitor and record clinical symptoms and mortality rates as well as recording the intact fresh weight of birds and the average percent decrease or increase throughout the entire period of experiment.

Table 1. Nutrient composition of the basal diet

Ingredients	Content	Chemical composition	Content
Yellow corn	62.13	ME (Kcal kg $^{-1}$)	3160
Soybean oil	1.80	Lysine (%)	1.35
Corn gluten meal (60%)	4.00	Methionine (%)	0.60
Soybean meal	28.12	Crude protein (%)	20.86
Vitamin and mineral mixture [a]	0.30	Nonphytate P (%)	0.45
Dicalcium phosphate	1.45	Calcium (%)	0.94
Limestone	1.15	Methionine+ Cysteine (%)	1.00
Salt	0.25		
DL-Methionine	0.25		
Sodium bicarbonate	0.25		
Choline chloride	0.10		
L-lysine HCl	0.20		

Nutrient Level in the diet was based on NRC (1994)

[a] Vitamin-mineral mixture supplied per kilogram of diet: Vit D_3: 2000 IU, Vit A: 15000 IU, Vit E: 20 mg, Vit B_2: 5 mg, Vit K_3: 5 mg, Vit B_1: 2 mg, Vit B_6: 2 mg, Vit B_{12}: 0.02 mg, Biotin: 0.1 mg, Pantothenic acid: 12 mg, Niacin, 25 mg, Folic acid: 1 mg, Zinc: 50 mg, Copper: 5 mg, Manganese: 70 mg, Iodine: 1 mg, Iron: 50 mg and Selenium: 0.1 mg.

e- RNA extraction, reverse transcription, quantitative real time PCR

At the end of experimental period, three chicks of each experimental group were randomly selected and sacrificed for collecting liver and intestine tissue samples. For quantification of gene expression, liver and intestine samples were sterile collected, washed by using phosphate buffer saline, snap-frozen in liquid nitrogen and stored at −80 °C.

By using Trizol reagent, total RNA was extracted from both liver and intestine according to the instructions of manufacturer (Direct-zol™ RNA MiniPrep, catalog No. R2050). While, the amount and purity were measured by using a Nanodrop (UV-Vis spectrophotometer Q5000/USA) and the integrity was assessed using gel electrophoresis.

The synthesis of cDNA was done following the protocol of manufacture (SensiFast™ cDNA synthesis kit, Bioline, catlog No. Bio-65053). The reaction mixture was run in a total volume of 20 µL containing up to 1 µg of total RNA, 4 µL of 5x Trans Amp buffer, 1 µL of reverse transcriptase and up to 20 µL of DNase free water. The final reaction mixture was placed in a thermal cycler and the following procedures were carried out; primer annealing at 25 °C for 10 min, reverse transcription at 42 °C for 15 min and finally inactivation at 85 °C for 5 min. The samples were stored at 4 °C.

Relative quantification of the levels of mRNA of *TNF-α*, and *SOD* in hepatic and intestinal tissues was carried out by RT-PCR using SYBR Green PCR Master Mix (2x SensiFast™ SYBR, Bioline, catlog No. Bio-98002). Both primer sequences and the size of each amplified PCR product are shown in Table (2). *β-actin* was used as housekeeping gene. The total volume of reaction mixture was 20 µL contained 10 µL of 2x SensiFast SYBR, 3 µL of cDNA, 5.4 µL of d.distilled water and 0.8 µL of each primer. The conditions of PCR cycling were as follows: 95 °C for 2 min followed by 40 cycles of 94 °C for 15 seconds, temperatures of annealing was shown in Table (2) for 30 seconds and 72 °C for 20 seconds. Amelting curve analysis was performed at the end of the amplification step to verify the specificity of the PCR product. As previously described by [30], the relative gene expression in each sample was compared with the control of *β-actin* gene and calculated according to the $2^{-\Delta\Delta Ct}$.

f- Biochemical analysis

The inflammatory *TNF-α* concentration and antioxidant *SOD* activity in liver and intestine were evaluated by enzymatic colorimetric technique using commercial kits from Bio-diagnostic Co., Egypt, following the instructions of manufacturer. About 500 mg of tested samples was homogenized in 5 cm^3 of phosphate buffer saline (0.1 M, pH 7.6) [31],

where tissue homogenates were centrifuged at 500 rpm for 10 minutes, and the supernatant was then used for biochemical determination [32].

Table 2. Real-time PCR primers made of oligonucleotides that are forward and reverse for investigated genes under study.

Investigated marker	Primer	Product size (bp)	Annealing Temperature (°C)	GenBank isolate	Origin
TNF-α	F5′- CACACTTCGGGCAGCTCTTA -3 R5′- AGGGTTATTTCAGCCCCGTG -3′	133	60	NM_001024578.2	Present Research
SOD	F5′- ACCCCTTTGGAGTGAACCAC -3 R5′- TGGATCACAACGGATCTGCC -3′	145	60	XM_040699307.2	
ß. actin	F5′- TGAATCCGGACCCTCCATTG -3′ R5′- AGACTGCTGCTGACACCTTC -3′	195	58	L08165.1	

g- Histopathological screening

The liver and intestine specimens were isolated from 3 chicks in each experimental group, collected and then fixed in 10% neutral buffered formalin for 24 hrs. The excised samples were placed in tissue cassettes, processed and embedded in paraffin wax. Embedded samples were sectioned at 5 µm using a microtome. The sections were stained using hematoxylin and eosin and then examined under a light microscope (Olympus CX 31) [33].

h- Statistical analysis

The full obtained experimental data of variously treated groups were analyzed using the SPSS statistical program software version 17.0 (SPSS Inc., Chicago, IL) through one-way analysis of variance (ANOVA). The results were further compared *via* Tukey's test. Comparisons were statistically significant at $P < 0.05$ [34].

3. Results and Discussion

a- Characterization of chitosan nanoparticles either singly or loaded with ciprofloxacin

Both size and shape of the prepared nanomaterials either singly or loaded with antibiotic were characterized using transmission electron microscopy (TEM). It is clear from TEM micrographs that both CSNPs and CSNPs-CIP have a semi-spherical shape. The minimum diameter of the CSNPs is range from 50.96 nm to 108.42 nm (Fig. 1a). The size of CSNPs exhibited significant increase with the addition of ciprofloxacin as antibacterial agent by a ratio of 145.5% as shown in Fig. 1b.

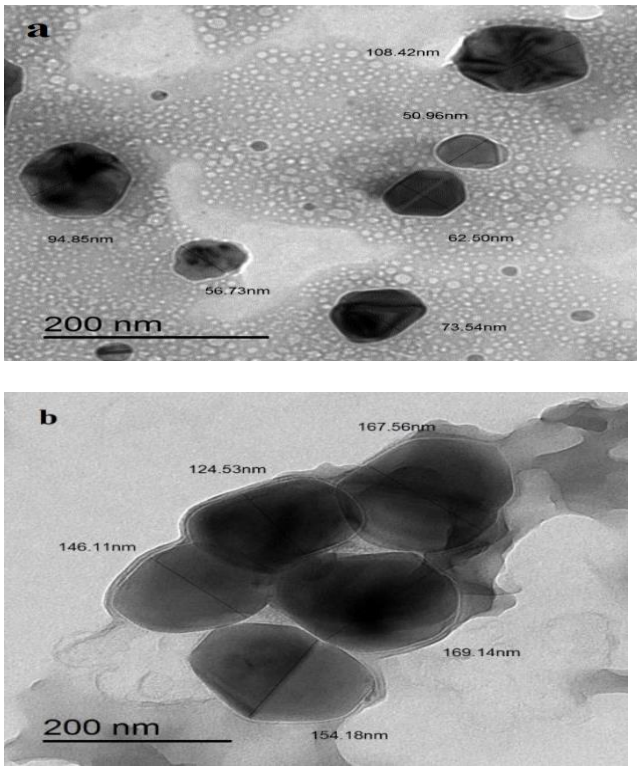

Fig. 1. TEM micrograph of **a**; CSNPs, **b**; CSNPs-CIP

Salmonella enteritidis is a major cause of persistent diseases as it has the ability to survive intracellularly, form biofilms, and evade the host's strong inflammatory response, causing reduced susceptibility to ciprofloxacin antibiotic. Thus, novel and ideal strategies must be applied to treat these diseases by delivering the drugs with sustained release from a single dose with high biocompatibility and low toxicity to improve and restore the antibiotic effect. Nanotechnology has prolonged the therapeutic options of current antibiotics by

creating drug delivery systems that can improve the antibiotic properties and/or facilitate drug administration [17, 35, 36]. Previous studies demonstrating mesoporous silica nanoparticles loaded with ciprofloxacin as a vehicle for antibiotic delivery were performed *in-vitro*. However, resistance to CIP has been observed at a high rate among *Salmonella* species associated with food and human infections, including Enteritidis [37, 38].

Several investigations have evaluated the effects of CSNPs against *Salmonella* in chickens. Recent studies suggested and demonstrated the immune response of broiler chickens to oral *Salmonella* vaccination based on chitosan nanoparticles [39]. Our results suggested that the small size of CSNPs loaded with CIP plays a vital role in the drug internalization into targeted cells. Accordingly, chitosan nanoparticles can regulate the rate of release of an antimicrobial agent.

The mechanism of chitosan nanoparticles antimicrobial activity has not yet been fully explained, and several hypotheses have been proposed. A common hypothesis is that the change in the permeability of cells due to the interaction between the negative charge of bacterial cell membrane and the positive charge of amino group in chitosan molecule [40]. Additionally, it has been hypothesized that chitosan nanoparticles can inhibit the bacterial growth due to its ability to chelate essential nutrients and metals [41]. Also, Zheng and Zhu [42] suggested that chitosan with high molecular weight may form a polymeric membrane around the bacterial cells preventing them from receiving nutrients.

Here, the prepared nano-chitosan particles exhibited spherical shapes and uniform sizes of approximately between 50.96 nm to 108.42 nm (Fig. 1a). According to the mechanism proposed by [21] and [43], the CSNPs formation can take place through both inter- and intramolecular bonds between the chitosan

The large surface area of nanomaterials due to the nanosize of particles leads to more drug release [17, 47]. When the drug is homogenously integrated in the lipid matrix, slow release of drug can be achieved depending on the type of nanomaterials and the drug encapsulation model [48].

amino groups and methacrylic acid carboxyl groups. During the polymethacrylic acid polymerization process by adding potassium persulfate as a monomer polymerization initiator to chitosan solution, the persulfate anion attacked and cut into the long chain of chitosan at a temperature of 70°C, causing it to become shorter. The reaction between chitosan and potassium persulfate occurred after one hour, and stopped when the solution was transferred immediately to an ice bath to cool [21] and [44].

As mentioned above in figure 1 b, the size of CSNPs was increased with the addition ciprofloxacin antibiotic. This is due to the presence of great content of both primary amino and hydroxyl groups of chitosan which combined with other molecules or ions through ion exchanges or simple chelation forming numerous chemical bonds with these ions thus improving the stability of nanoparticles and prevents aggregation [17] and [45]. In support of the present results, Corradini *et al*. [46] and Hasaneen *et al*. [21] reported that the size of CSNPs was increased by 53%, 32% and 13%, respectively when 60 ppm of phosphorus, 400 ppm of nitrogen and 400 ppm of potassium were added. Also, Hasaneen *et al.* [26] found that the diameter of CSNPs loaded with antibacterial compounds was increased approximately 55 upto 100 nm.

b- In-vitro drug release study

Membrane diffusion method is widely used to study the *in-vitro* release of drug incorporated in colloidal system. In this case, drug release from nanomaterial follows more than one mechanism. The release profile of ciprofloxacin from CSNPs is shown in figure 2. As it is clear in figure 2, the ciprofloxacin drug was released in sustained manner from CS nanoparticles by approximately 23.16 % of in the first six hours. Furthermore, about 15 % of ciprofloxacin drug were released from CSNPs at the longest time point measured (12 hrs).

The release of a drug from polymer can be controlled by one of the following mechanisms: (a) surface erosion of the polymer matrix, (b) breaking the bonds of the used polymer either at the surface or in the bulk of the matrix, or (c) diffusion of the loaded drug [49]. Nanoemulsions have several benefits, including

improved delivery efficiency, physical stability, and resistance to volatility and degradation [50]. The chitosan-induced muco-adhesion effect was limited to the surface of each individual droplet.

Due to the nanometric size of the droplets, which resulted in an increased surface/volume ratio, the encapsulated oil was able to achieve greater contact with the mucosal surface. By improving the passive transport process, the pharmaceuticals enclosed in emulsions enable absorption of medicinal components with increased particle uptake; moreover,

nanoemulsions are expected to pass through biological barriers without encountering any obstacles [51]. In order to transport ciprofloxacin-loaded nanoparticles to the site of inflammation and enhance epithelial permeability and retention effect [52], biomaterials composed of chitosan can be degraded in the colon without being damaged by the gastric environment [53, 54].

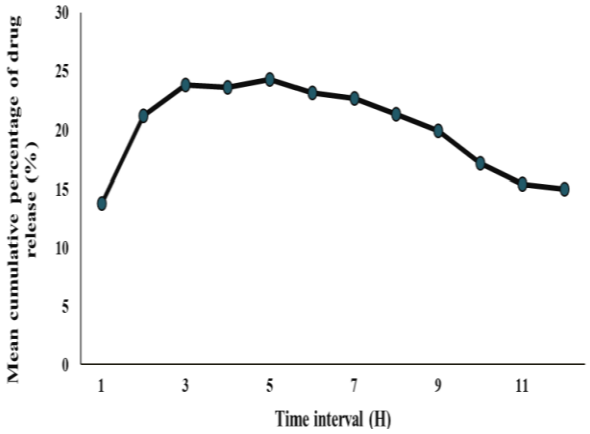

Fig. 2. Ciprofloxacin release profile from nanosuspension

c- Clinical external signs and mortality rate

After 1 week of experimental infection, external clinical signs were clearly observed as shown in table 3. In this respect, the external clinical symptoms which observed on the experimental birds were weakness, poor in growth, in-appetence, crowded close to heat sources, depression, weight loss and diarrhea. Table 3 showed the external symptoms of salmonellosis disease caused by *Salmonella enteritidis* appeared on diseased birds and the recovery of differently treated birds as affected by nanodrug delivery systems. Chicks in the treated groups with CIP and chitosan nanoparticles either singly or loaded with CIP had less external clinical signs when compared with infected group. No mortalities were recorded in groups throughout the experimental period.

Table 3. External clinical symptoms of salmonellosis disease caused by *Salmonella enteritidis* in boiler chicks at 24 d-old (+++; Severe symptom, ++; Strong symptom, +; Mild symptom)

External symptoms	Appearance	% change
Weakness	+++	100
Loss of appetite	+++	90
Poor growth	+	48
Closed eyes	Nill	-
Crowded close to heat sources	+	15
Sit with dropping	Nill	-
Watery diarrhea	+	51
Depression	+++	90
Dehydration	Nill	-
Weight loss	++	55

d- Changes in body weight

Examination of table 4 revealed that administration of CIP antibiotic and nanodrug delivery system; CSNPs-CIP showed significant increases in body weight of the experimental chicks treated with these strategies as compared to the infected group with notice that CSNPs; 10% + CIP; 2.0 mg ml^{-1} treated groups was the highest value.

In the present study, the percent change (decrease or increase) in apparent body weight of the experimental birds were increased as compared with the control infected birds. The following sequence of treatment: CSNPs-CIP> CIP > CSNPs was display with respect to control (infected or healthy) chicks.

Chitosan nanoparticles can enhance the growth performance by different mode of actions; they may form beneficial gut microflora and consequently, improve digestion and nutrients absorption [55, 56], increase ileal digestibility of nutrients [57], and improve the intestine architecture with hypertrophied intestinal villi and epithelial cells [58]. In addition, CSNPs can stimulate the secretion of digestive enzymes from the pancreas, stomach, and intestinal walls, as well as raise the levels of growth hormones and insulin-like growth

factor I in the serum, were noted after feeding with chitosan [59].

Tamara *et al*. [60] and Xu *et al.* [61] attributed these outcomes to the ability of CSNPs to promote the formation of a biofilm-like structure in coliforms bacteria and improve the colonization of lactobacilli in the gastrointestinal tract. Therefore, CSNPs contribute to health, productivity, and performance as they help in the digestion of carbohydrates, protein and fats in the diet.

Table 4. Effect of ciprofloxacin and chitosan nanoparticles either singly or in-combination as a nanodrug delivery system on the body weight of broiler chicks diseased by salmonellosis throughout the entire period of experiment. Mean values listed are given as gm per fresh chicken ± standard error.

Weight Treatment	Initial wt. (7-d-old)	Starting infection (14 -d-old)	Starting recovery (24-d-old)	Sampling (36-d-old)	Recovery % as control (healthy)	Recovery % as control (infected)
Control; Healthy	510±4.1	859±7.1	1520±6.3	2470±7.3	---	---
Control; Infected	513±4.2	865±7.2	1240± 6.2	1380±7.2	44.1	---
CIP; 2.0 mg ml^{-1}	517±4.1	871±7.1	1260±6.3	2190±7.1	88.7	58.7
CSNPs; 10%	505±4.0	851±7.2	1399±6.1	2150± 7.1	86.6	55.7
CSNPs; 10% + CIP; 2.0 mg ml^{-1}	512±4.1	870±7.1	1480±6.3	2400±7.3	97.2	73.9

e- Gene expression

Gene expression profile of hepatic and intestinal *TNF-α and SOD* is elucidated in table 4. Gene expression analysis showed that the CSNPs, CIP, and CSNPs-CIP treated groups significantly down-regulated the expression levels of *TNF-α* and *SOD* in liver and intestine compared with control infected group. CSNPs; 10% + CIP; 2.0 mg ml^{-1} experienced chickens produced non-significant differences compared to the control group.

The calculated percent recovery and percent change for relative gene expression of hepatic and intestinal *TNF-α and SOD* in response to treatment of diseased chicks with nanomaterial either singly or loaded with ciprofloxacin were recorded in table 4. The results revealed that nanodrug delivery system represented in CSNPs-CIP enhanced the gene expression of *TNF-α and SOD* as compared with control infected chickens.

f- Biochemical parameters

Results of our study revealed that nanodrug delivery system represented in CSNPs; 10% +

CIP; 2.0 mg ml^{-1} demonstrated a significant decrease of *TNF-α* concentration in liver and intestine in relation to the other groups. However, the hepatic and intestinal *SOD* enzyme activity elicited an opposite pattern.

CSNPs; 10% + CIP; 2.0 mg ml^{-1} experienced chicks produced non-significant differences compared to the control group (Table 5).

It is of interest to mention that the percent recovery of both *TNF-α* concentration and *SOD* activity of the variously treated chicks either healthy or recovered from illness, showed variable decreases above the control infected level as shown in table 5.

Regarding to the data of gene expression and biochemical parameters elucidated in table 4 and 5, Ali *et al.* [62] reported that oxidative stress index was significantly decreased in CSNPs-treated groups when compared with control group. In contrast with Chang *et al.* [63] findings who noticed an elevation in the expression of *SOD* after nano-chitosan treatment, which in turn increased the total antioxidant capacity in the serum of experimental broilers.

According to Shapiro *et al*. [64] inflammatory cytokines including *TNF-α* release a number of chemokines and promote chemotaxis. Scavenging ROS from the environment, preventing their synthesis, or protecting transition metals that are needed to produce free radicals antioxidants provide protection [65]. These mechanisms, known as endogenous antioxidant indicators, involve the body's own enzymatic and non-enzymatic

antioxidant defenses, such as superoxide dismutase (*SOD*) [66].

Nanochitosan can modulate immune of the host due to the presence of amino groups, which promote the production of serum antibodies, activate macrophages and natural killer cells, and steadily improve the immune response [67]. Supplementation of copper-chitosan nanoparticles to the broiler diet could increase the lysozyme content, immunoglobulins, and some complement system proteins [68]. In addition, chitosan nanoparticles can control the antigen-presenting cells activity and activate both transduction of inflammatory signal and expression of cytokines [69].

The antioxidant activity of CSNPs can be attributed to their interaction with free radicals due to the presence of active hydroxyl and amino groups in its chains. Both groups acted as a hydrogen donor for proxy unstable free radicals and subsequently scavenging superoxide anions and hydroxyl free radicals, thus protecting the cells from damage [61, 70] and reducing oxidative stress [71].

Table 5. Effect of ciprofloxacin and chitosan nanoparticles either singly or in-combination as a nanodrug delivery system on gene expression profile of hepatic and intestinal *TNF-α and SOD* of broiler chicks diseased by salmonellosis. Means of within the same row having different upper-case superscripts are significantly different at $p \leq 0.05$.

Treatment	*TNF-α* (Liver)	% Recov.	% change	*TNF-α* (intestine)	% Recov.	% change	*SOD* (Liver)	% Recov.	% change	*SOD* (intestine	% Recov	% change
Control (Healthy)	1 ± 0.15^{g}	-74.2	-	$1 \pm 0.06_{h}$	-75	-	1 ± 0.05^{i}	-74.6	-	1 ± 0.09^{f}	-66.5	-
Control (Infected)	3.89 ± 0.18^{a}	-	289	3.95 ± 0.13^{a}	-	295	3.95 ± 0.13^{a}	-	295	3.83 ± 0.07^{a}	-	283
CIP; 2.0 mg ml^{-1}	2.96 ± 0.16^{c}	-23.9	196	2.94 ± 0.08^{c}	-25	194	2.98 ± 0.15^{d}	-24.5	198	2.99 ± 0.14^{c}	- 21	199
CSNPs; 10%	3.48 ± 0.13^{b}	-10.5	248	3.58 ± 0.16^{b}	-9.3	258	3.95 ± 0.13^{b}	0.0	295	3.78 ± 0.04^{a}	-1.3	278
CSNPs; 10% + CIP; 2.0 mg ml^{-1}	1.21 ± 0.05^{fg}	-68.8	21	1.05 ± 0.08^{h}	-73.4	5	1.18 ± 0.09^{i}	-70.1	18	1.12 ± 0.13^{f}	-70.7	12

Table 6. Effect of ciprofloxacin and chitosan nanoparticles either singly or in-combination as a nanodrug delivery system on hepatic and intestinal *TNF-α* concentration (pg/mg protein) and *SOD* activity (u/gm tissue) of broiler chicks diseased by salmonellosis. Means of within the same row having different upper-case superscripts are significantly different at $p \leq 0.05$.

Treatment	*TNF-α* (Liver)	% Recov.	% change	*TNF-α* (intestine)	% Recov.	% change	*SOD* (Liver)	% Recov.	% change	*SOD* (intestine)	% Recov.	% change
Control (Healthy)	135.2 ± 0.14^{l}	- 58.6	-	104.6 ± 0.05^{l}	- 61.6	-	216.4 ± 0.09^{a}	196.0	-	178.3 ± 0.18^{a}	226.5	-
Control (Infected)	327.1 ± 0.05^{a}	-	141.9	272.7 ± 0.15^{a}	-	160.7	73.1 ± 0.13^{l}	-	- 66.2	54.6 ± 0.15^{k}	-	- 69.3
CIP; 2.0 mg ml^{-1}	284.3 ± 0.14^{d}	-13.0	110.2	235.3 ± 0.17^{c}	-13.7	124.9	87.1 ± 0.08^{j}	19.1	- 59.7	63.4 ± 0.04^{ij}	16.1	- 64.4
CSNPs; 10%	311.0 ± 0.08^{ab}	- 4.9	130.0	264.1 ± 0.14^{b}	- 3.1	152.4	81.6 ± 0.16^{k}	11.6	- 62.2	62.7 ± 0.13^{j}	14.8	- 64.8
CSNPs; 10% + CIP; 2.0 mg ml^{-1}	224.5 ± 0.16^{gh}	- 31.3	66.0	151.5 ± 0.12^{h}	- 44.4	44.8	159.5 ± 0.14^{e}	118.1	- 26.2	140.2 ± 0.18^{c}	156.7	- 21.3

g- Histo-pathological evaluation

Histo-pathological findings of liver tissues from different treatment groups were presented in figure 4. Representative photomicrograph of hepatic section revealed that liver in the control group showing normal histological appearance (Fig. 4 a), meanwhile, in infected group inoculated by *S. enteritidis*, paratyphoid nodules were observed that characterized by focal, extensive aggregations of numerous macrophages, epithelioid cells admixed with few lymphocytes (Fig. 4 b), at a high magnification power, this group showing proliferative cholangitis represented by luminal papillary proliferation of biliary epithelium and severe periductal aggregations of numerous lymphocytes and macrophages admixed with few fibroblast cells (Fig. 4 c). The group treated with CIP singly showed focal, coalescing biliary aggregations of cellular infiltrates admixed with moderate perivascular edema and fibroblast proliferations, edema extended in between the surrounding hepatocytes (Fig. 4 d). Additionally, in the group treated with CSNPs singly showed normal histoarchitecture of hepatocytes (Fig. 4 e). In the group treated with CSNPs; 10% + CIP; 2.0 mg ml^{-1} showed increase number of bile duct with mild, coalescing periductular aggregations of lymphocytes, macrophage and fibroblasts, the remaining hepatocytes appears normal (Fig. 4 f).

Moreover, histopathological findings of intestinal tissues from different treatment groups were presented also in figure 4. Representative photomicrograph of intestinal section from different treatment groups showed that in control group, normal histological architecture of intestinal layers was observed (Fig. 4 g). The group inoculated with *S. enteritidis* exhibited necrotic enteritidis characterized by desquamation of intestinal mucosa and severe leukocytic infiltrations (Fig. 4 h). Higher power of b showing cryptitis represented by extensive replacement of crypt with numerous cellular infiltrates that extended in lamina propria and widely separated crypts (Fig. 4 i). But, in the group treated with CSNPs singly, normal arrangement of intestinal mucosa was detected (Fig. 4 j). CIP group showed goblet cell hyperplasis with minimal to mild submucosal edema admixed with few inflammatory cells (Fig. 4 k). Meanwhile, in CSNPs; 10% + CIP; 2.0 mg ml^{-1} group, mild villous thickening was observed (Fig. 4 l).

Observations of histological changes intestine and liver tissues of boiler between the control infected group and the groups treated with CIP and CSNPs either singly or loaded with ciprofloxacin antibiotic indicated variable differences. The improvement in clinical symptoms and lack of undesirable histopathological changes in both intestine and liver confirmed the antibacterial efficacy of orally supplemented CSNPs either singly or loaded with ciprofloxacin antibiotic against *S. enteritidis* (Fig. 4 and 5). Our results agree with the i*n-vivo* study published by Zhao *et al*. [72] who found no pathological changes and no obvious toxicity when compared to the results of the control group, indicating a high level of safety of chitosan nanoparticles when used orally in chickens.

Talab *et al*. [73] reported that rats treated with high dose of CSNPs showed a nearly normal appearance of histological structure of the liver, normally blood sinusoids and nucleus, with slight dilatation of the central vein. Also, Alandiyjany *et al*. [38] found that after *Salmonella typhimurium* infection, the histopathological architecture of intestinal tissues showed a diffuse infiltration of inflammatory cell and complete desquamated epithelial tissues. Meanwhile, the severity of intestinal inflammation and liver damage was significantly reduced when CIP was administered. Moreover, the tissues were nearly restored to normal condition and more prominent in the group supplemented with mesoporous silica nanoparticles loaded with ciprofloxacin representing its better efficiency in treating infection.

In conclusion, we can said that novel nanodrug delivery system represented by chitosan nanoparticles either singly or loaded with ciprofloxacin antibiotic as a prebiotic substance which have antibacterial effects demonstrated its resistance to *Salmonella enteritidis* at concentrations (0.2 mg ml^{-1}) this fact was confirmed by various studies including molecular, biochemical and pathological ones, all proved their role in stimulation of immune

system of chicks and mitigate the negative effect of bacteria (S. *enteritidis*) that have been detected clinically and pathologically.

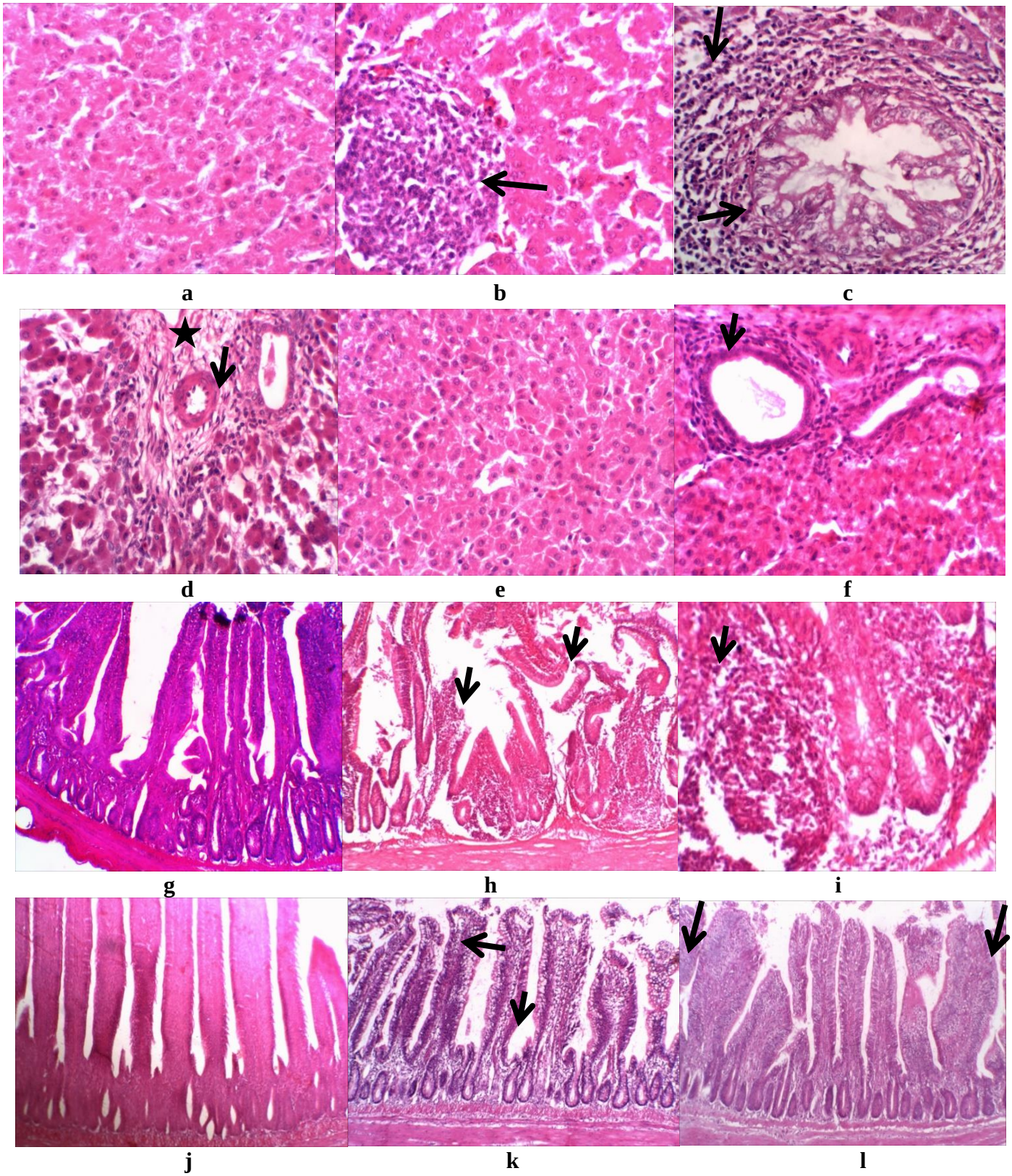

Fig. 4. Representative photomicrograph of hepatic and intestinal section of chickens of a& g; healthy group, b &h; infected group, c & i; a high magnification power of infected group, d& j; group treated with CIP, e& k; group treated with CSNPs singly, f& l; the group treated with CSNPs; 10% + CIP; 2.0 mg ml^{-1}.

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