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INFLUENCE OF ENROFLOXACIN ADMINISTRATION ON OXIDATIVE STRESS AND ANTIOXIDANT ENZYME ACTIVITIES OF EXPERIMENTALLY INFECTED BROILERS WITH *SALMONELLA ENTERICA SEROVAR ENTERITIDIS*

ABSTRACT

The objective of this study was to assess influence of enrofloxacin administration on oxidative stress and the antioxidant enzyme activities of experimentally infected broilers with *S. enterica Serovar Enteritidis*. At the end of the experiment, blood and tissue samples were collected and malondialdehyde(MDA), nitric oxide (NO), glutathione peroxidase (GSH-Px) and catalase (CAT) activities were determined. Plasma MDA levels increased in all study groups compared to controls, being highest in antibiotic-administered group. Erythrocyte CAT activity and levels of plasma NO did not change among groups whereas erythrocyte GSH-Px activity decreased in all study groups compared to controls. There were no change levels of MDA and GSH-Px activity in liver. Activity of CAT decreased in all study groups compared to controls. Intestinal GSH-Px activity didn't change in all groups. As a result, to decrease oxidative damage taking place in regard to bacterial infection and using antibiotic, the substances making antioxidant system strong should be given.

Keywords: *S. enterica Serovar Enteritidis*, Enrofloxacin, MDA, NO, GSH-Px, CAT.

SALMONELLA ENTERICA SEROVAR ENTERITIDIS ILE DENEYSEL OLARAK ENFEKTE EDILMIŞ BROYLERLERIN OKSIDATIF STRES VE ANTIOKSIDAN ENZIM AKTIVITELERI ÜZERINE ENROFLOKSASININ ETKISI

ÖZET

Bu çalışmanın amacı, *S. enterica Serovar Enteritidis* deneysel olarak enfekte edilmiş broylerlerin oksidatif stres ve antioksidan enzim aktiviteleri üzerine enrofloksasinin etkisini araştırmaktır. Deneysel uygulamanın sonunda kan ve doku örnekleri alınarak malondialdehit (MDA), nitrik oksit (NO) düzeyleri ile glutatyon peroksidaz (GSH-Px) ve katalaz (KAT) aktiviteleri ölçüldü. Plazma MDA düzeyleri kontrole göre bütün gruplarda arttı, en fazla artış tek başına antibiyotik verilen grupta görüldü. Eritrosit KAT aktivitesi ve plazma NO düzeyi hiçbir grupta değişmezken, eritrosit GSH-Px aktivitesi her üç grupta da kontrole göre düştü. Karaciğer MDA düzeyi ve GSH-Px aktivitesi hiçbir grupta değişmedi. KAT aktivitesi kontrole göre bütün gruplarda düştü. Sonuç olarak, antibiyotik kullanımı ve bakteriyel enfeksiyona bağlı olarak oluşan oksidatif hasarı azaltmak için antioksidan sistemi kuvvetlendiren maddeler verilmelidir.

Anahtar Kelimeler: *S. enterica Serovar Enteritidis*, Enrofloksasin, MDA, NO, GSH-Px, KAT.

1. INTRODUCTION (GİRİŞ)

Salmonella enterica are facultative intracellular bacteria of which serovar Typhimurium (ST) and serovar Enteritidis (SE) have a broad host range, including the capacity to cause human infections [2]. Lipopolysaccharide (LPS, endotoxin), a major constituent of the outer membrane of gram-negative bacteria, is essential for bacteria, is essential for bacterial viability. LPS stimulates the immune cells and activates multiple mechanism involved in the cellular defense and killing of bacteria including: increased formation of radical oxygen species (ROS), the rise of cytokine production and nitric oxide (NO) generation. Macrophages play a crucial role in the defense against invading microorganisms, an done of their important bactericidal mechanisms is the production of ROS [32]. Intestinal colonization by Salmonella of animals, including poultry, induces intestinal infiltration of polymorphonuclear (PMN) leukocytes (neutrophils in mammals and heterophils in poultry) which confers a high level of resistance to bacterial invasion by virulent Salmonella as well as the pathological effects associated with infection, including gastroenteritis and localization in the reproductive tract [10, 16, 27 and 28].

Enrofloxacin, a synthetic fluoroquinolone, is used in veterinary medicine for the treatment of infections of the respiratory and alimentary tract. Enrofloxacin are oxidized by liver microsomal enzymes of the cytochrome P450 family. As a result of fluoroquinolone metabolism through cytochrome P450, free radical intermediates are generated, and these subsequently can cause lipid peroxidation [9].

NO is produced is numerous physiological and pathological conditions, including inflammation and host defense against microbes and parasites. It can be produced either constituvely or after stimulation of inducible nitric oxide synthase (iNOS) by, for example, pro inflammatory cytokines and bacterial LPS. The cytotoxic effects of ROS result mainly from the peroxidation of the lipid contents of cellular and mitochondrial membranes. A measure of the lipid peroxidation products such as malondialdehyde (MDA) is an indication of the extent of peroxidation.

The intestinal microvillus membrane is highly specialized to perform transport and enzymatic functions essential for normal digestion and absorption. Since it is believed that lipid and lipid-protein interactions in the membrane play a major role in their function, it is important to study the effect of free radicals on these dynamic features in membrane components.

2. RESEARCH SIGNIFICANCE (ÇALIŞMANIN ÖNEMİ)

The objective of this study was to assess influence of enrofloxacin administration on oxidative stress and the antioxidant enzyme activities of experimentally infected broilers with *S. enterica Serovar Enteritidis*.

3. MATERIALS AND METHODS (MATERYAL VE METOD)

Broiler chickens were divided into 4 groups, each consisting of 15 animals. Group 1 was used as control group; Group 2 orally received a single dose of *S. enterica Serovar Enteritidis* strain (10^9 bacteria in 0.1 ml); Group 3 orally received a single dose of *S. enterica Serovar Enteritidis* strain (10^9 bacteria in 0.1 ml) and enrofloxacin (10mg/kg/day) in drinking water for 9 days beginning from 15^{th} day post administration and Group 4 was administered enrofloxacin (10mg/kg/day) in drinking water for 9 days. Beginning from 3 day post infection, samples were collected and bacterial isolation was done. At the end of the experiment blood and tissue samples were collected and MDA, NO,

glutathione peroxidase (GSH-Px) and catalase (CAT) activities were determined.

- **Bacteriological Analysis:** For microbiological analysis, chicken liver and spleens specimens were directly streaked onto plates of MacConkey agar and incubated 37^0 C for 24-48 h. The intestine samples were inoculated to Rappaport- Vassiliadis broth and incubated for 18-24h at 43^0 Then, one loopful from Rappaport-Vassiliadis broth was streaked to onto plates of Brillant Green Agar and incubated 37^0C for 24-48 h [4 and 7]. Prepared preparat from pure cultures of suspected Salmonella colonies were stained with Gram staining. The identification of isolates was confirmed by the following conventional biochemical tests: Gram staining, biochemical and antigenic quality were controlled [7 and 18].
- **Nitric Oxide Assay:** NO measurement is very difficult in biological specimens, because it is easily oxidized to nitrite (NO_2) and subsequently to nitrate (NO_3) which serve as index parameters of NO production. Samples were initially deproteinized with NaOH and $ZnSO_4$. Total nitrite (NO_2 + NO_3) was measured by spectrophotometer at 545 nm after conversion of NO_2 to NO_3 by assay reactive [20]. A standard curve was established by a set of serial dilutions of sodium nitrite. Results were expressed as micromole per L and micromole per gram wet tissue.
- **TBA-RS Assay:** Serum MDA level was estimated according to the method of Yagi [34], tissue MDA level was estimated according to the method of Ohkawa [24] which is based on the coupling of MDA with thiobarbituric acid (TBA). The organic layer was taken and resulting pink stained TBA-RS was determined in a spectrophotometer at 535 nm. Calibration curve was performed using 1,1, 3,3-tetramethoxypropane. The results are reported as nmol/ml and nmol/g tissue.
- **CAT Activity Assay:** The eryrocyte and tissue CAT activities were measured according to the Aebi method [1]. The principle of the assay is based on the determination of the rate constant, k,(dimension: s-1, k) of hydrogen peroxide decomposition. By measuring the absorbance changes per 30 seconds, the rate constant of the enzyme was determined. Activities were expressed as k (rate constant)/g hemoglobin and k/g protein.
- **GSH-Px Activity Assay:** GSH-Px activity was measured by the method of Beutler [6]. Briefly, in the presence of glutathione reductase and nicotinamid adenin dinucleotid phosphate (NADPH), oxidized glutathione (GSSG) is immediately converted to its reduced form with a concomitant oxidation of NADPH to $NADP^+$. The decrease in absorbance at 340 nm is measured. GSH-Px activity was expressed as U/g hemoglobin and U/g protein.
  Hemoglobin concentration of erythrocyte lysate was measured by Drabkin's reagent [31]. Protein was measured by the method of Lowry et al. [19] using bovine serum albumin as standard.
  All assays were performed in duplicate and the mean was used for statistical analysis. Data were analyzed by one-way ANOVA followed by the Duncan multiple test. All analyses were performed using the Statistical Package for the Social Sciences (SPSS) software in PC-compatible computer. Values of $p< 0.05$ were considered to be significant.

4. RESULTS AND CONCLUSIONS (SONUÇLAR VE TARTIŞMA)

Macroscopically, lesions seen in chickens of group 2 and 3 were almost similar each other. There was fluid accumulation in the pericardium. The changes in the liver were mild to moderate enlargement and mottled with a few white foci. In the intestine, especially, ileum and cecum, the wall of the intestine was thick. The mucosa of the intestine was hyperemic, and a brownish red and copicous exudate were seen in the lumen. The kidney and spleen were of mild to middle congested.

Histopathologically, in the liver, lesions consisted of focal necrosis of hepatocytes with infiltration of heterophils and mononuclear inflammatory cells (Figure 1), and Kupffer cell proliferation. There were also vacuoler degeneration of hepatocytes in the pericentral area and sinusoidal congestion. In the heart, myocardial necrosis, and mononuclear and few heterophils inflammatory cells infiltration were seen (Figure 2). Pericardial thicking due to edema, inflammatory cells and congestion were found. In the intestine, there was mild to middle catarrhal or catarrhal hemorrhagic enteritis. Epithelial degeneration, necrosis, desquamation and villous atrophy were seen in some areas of mucosal surface. In the lamina propria, moderately to several infiltrated by heterophils and mononuclear inflammatory cells (Figure 3). There was also marked epithelial hyperplasie in the crypts of Liberkuhn and submucosal edema. Hemorrhage and congestion were seen in the parenchyma of the spleen, and aggregates of reticular cells were seen at certain places in the red pulp. Focal tubular necrosis and interstitial mononuclear cells infiltration were prominent findings seen in the kidney (Figure 4). Histopathologic findings were not found in the group 1 and 4 chickens. Similar findings between group 2 and 3 were observed as histopathology. This situation happened since antibiotic was given in 15 days after salmonella infection.

Plasma MDA levels increased in all study groups compared to controls, being highest in antibiotic-administered group. Erythrocyte CAT activity and levels of plasma NO did not change among groups whereas erythrocyte GSH-Px activity decreased in all study groups compared to controls (Table 1). There were no change levels of MDA and GSH-Px activity in liver. Levels of NO increased only groups given antibiotic (Salmonella+Antibiotic and Control+Antibiotic). Activity of CAT decreased in all study groups compared to controls (Table 2). Intestinal GSH-Px activity didn't change in all groups. Activity of CAT and levels of NO decreased in all groups compared the control group. Levels of MDA increased in control groups given antibiotic and Salmonella group (Table 3).

Table 1. The effect of Salmonella infection and using antibiotic on plasma MDA and nitrite levels, erythrocyte CAT and GSH-Px activities. (Tablo 1. Salmonella enfeksiyonunun ve antibiyotik kullanımının plazma MDA ve NO seviyeleri ile eritrosit KAT ve GSH-Px aktiviteleri üzerine etkisi)

	MDA (nmol/ml)	CAT (k/g Hb)	GSH-Px (U/g Hb)	Nitrite (µmol/L)
Control	3,04±0,58^{c}	0,045±0,01	3,43±0,72^{a}	36,85±0,93
Salmonellosis	5,31±2,69^{b}	0,041±0,01	2,15±0,34^{b}	37,98±1,37
Salmonellosis + Antibiotic	6,34±1,83^{ab}	0,038±0,02	1,61±0,20^{c}	38,80±0,85
Control + Antibiotic	7,99±2,93^{a}	0,045±0,01	2,16±0,46^{b}	37,51±1,20
p	<0.05	>0.05	<0.05	>0.05

Table 2. The effect of Salmonella infection and using antibiotic on MDA and nitrite levels, CAT and GSH-Px activities in liver.
(Tablo 2. Salmonella enfeksiyonunun ve antibiyotik kullanımının karaciğer MDA ve NO seviyeleri ile KAT ve GSH-Px aktiviteleri üzerine etkisi)

	MDA (nmol/g tissue)	CAT (k/g protein)	GSH-Px (U/g protein)	Nitrite (µmol/g tissue)
Control	2,76±0,19	57,00±4,00[a]	252,54±16,61	30,69±1,59[b]
Salmonellosis	2,10±0,20	47,10±5,33[b]	262,31±9,17	34,74±2,44[b]
Salmonellosis + Antibiotic	2,68±0,26	42,70±4,95[b]	267,34±16,90	42,85±1,74[a]
Control + Antibiotic	2,58±0,24	39,30±2,82[b]	260,50±14,95	42,18±2,00[a]
p	>0.05	<0.05	>0.05	<0.05

Tablo 3. The effect of Salmonella infection and using antibiotic on MDA and nitrite levels, CAT and GSH-Px activities in bowel.
(Tablo 3. Salmonella enfeksiyonunun ve antibiyotik kullanımının bağırsak MDA ve NO seviyeleri ile KAT ve GSH-Px aktiviteleri üzerine etkisi)

	MDA (nmol/g tissue)	CAT (k/g protein)	GSH-Px (U/g protein)	Nitrite (µmol/g tissue)
Control	0,63±0,06[b]	40,06±4,07[a]	847,89±96,06	74,65±0,50[a]
Salmonellosis	1,03±0,07[a]	31,28±2,64[b]	788,14±59,07	68,03±0,76[b]
Salmonellosis + Antibiotic	0,65±0,05[b]	26,30±2,19[b]	739,51±64,39	63,49±0,42[c]
Control + Antibiotic	1,09± 0,13[a]	28,38±1,87[b]	600,31±139,73	68,88±0,62[b]
p	<0.05	<0.05	>0.05	<0.05

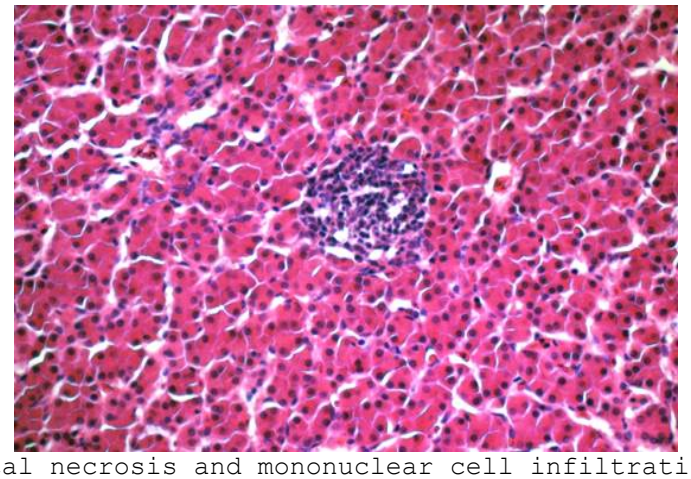

Figure 1. Focal necrosis and mononuclear cell infiltration at broiler liver inoculated with *S. enterica Serovar Enteritidis*. HE. X 200.
(Şekil 1. *S. enterica Serovar Enteritidis* ile inoküle edilmiş broyler karaciğerinde fokal nekroz ve mononüklear hücre infiltrasyonu. HE. X 200)

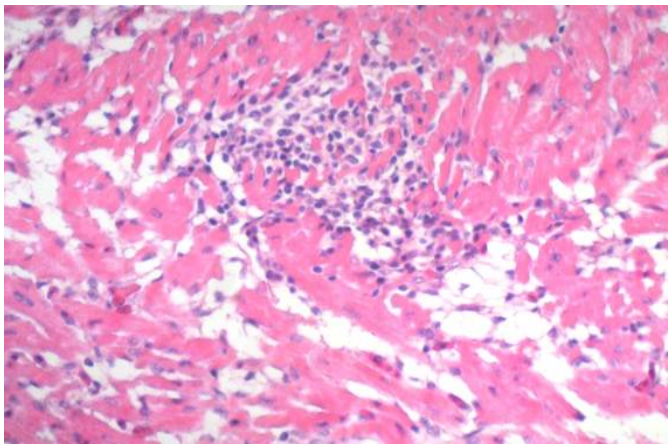

Figure 2. Inflammational cell infiltration at broiler myocard inoculated with *S. enterica Serovar Enteritidis*. HE. X 230.
(Şekil 2. *S. enterica Serovar Enteritidis* ile inoküle edilmiş broyler kalbinde yangısal hücre infiltrasyonu. HE. X 230)

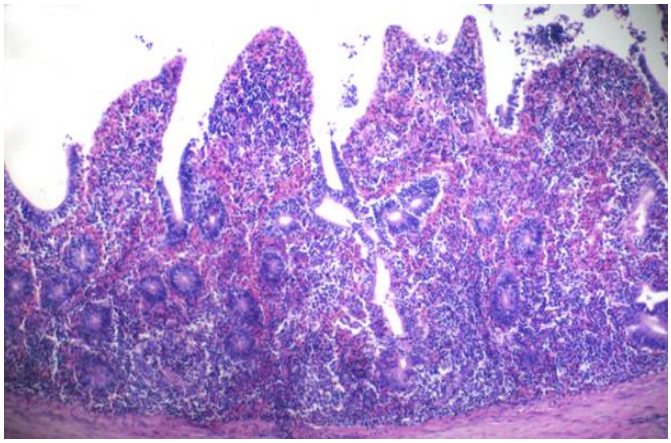

Figure 3. Epithelial necrosis, villoz atrophy and severely inflammational cell infiltration in propria mucosa at intestine inoculated with *S. enterica Serovar Enteritidis*. HE X 200.
(Şekil 3. *S. enterica Serovar Enteritidis* ile inoküle edilmiş broyler bağırsak propriya mukozasındaki epiteliyal nekroz, villöz atrofi ve şiddetli yangısal hücre infiltrasyonu. HE. X 200)

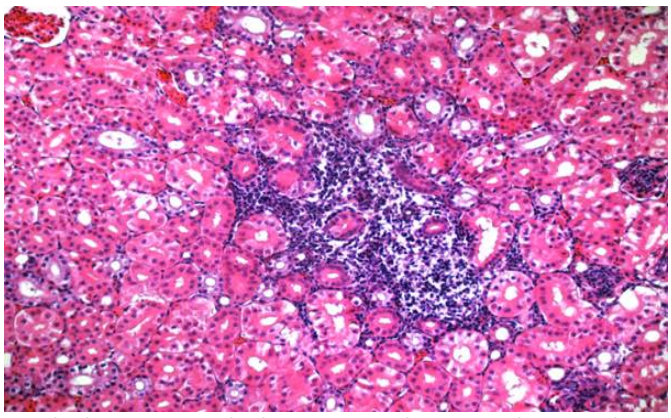

Figure 4. Interstitial nephritis at broilers kidney inoculated with *S. enterica Serovar Enteritidis*. HE X 200.
(Şekil 4. *S. enterica Serovar Enteritidis* ile inoküle edilmiş broyler böbreğinde interstitisyel nefritis. HE. X 200)

5. DISCUSSION (TARTIŞMA)

Bacterial LPS (endotoxin) induces extensive damage to a variety of organs, including liver, due to the increased production of reactive oxygen intermediates and a resultant rise in lipid peroxidation [17 and 22]. Endotoxin accumulates in tissues rich in cells of the reticuloendothelial system such as liver and spleen [30]. In the liver, Kupffer cells are the major targets of LPS, which produce excessive amounts of O.- on activation by LPS [3].The development of tissue injury and the outcome of the disease depend upon the balance between the generation of toxic radicals and tissue antioxidant status [33]. The cell has protective agents that guard against damage induced by oxygen-reactive species. GSH-Px, CAT and SOD constitute an antioxidant cellular enzymatic system [9].

LPS-induced increase in lipid peroxidation and nitric oxide levels and decrease in antioxidant activity in tissues, which are an index of oxidative stress, have been described in several studies [5, 14, 26 and 29]. In our study, LPO in liver had no change in regard to bacterial infections but capacity of antioxidant decreased. The reason for harmonies of MDA level in the liver with the studies which were done could be the style of giving bacteria. If agent was given to inside vessel or as intraperitoneal, this situation could affect seriously to severity of infection and damage of free radical.

It was observed increased level of serum MDA in regard to bacterial infections [13 and 15]. Serum MDA and SOD activities of the patients infected with H. Pylori increased more than the control groups, but level of plasma ascorbic acid decreased. Increasing MDA in serum was accepted as an indicator of increasing cell damage but increasing SOD activity was interpreted as a reply of the cell to recover to increasing MDA [15].

Martynenko et al. [21] suggested that experimental salmonella infection in rabbits was accompanied by activation of LPO not only in enterocytes, but also in blood serum. An increase in level of MDA led to decrease in the antioxidant capacity and in the peroxidation resistance of erytrocytic membranes. The severity of the pathological process was found related to the activity of LPO. Our study showed that *S. enterica Serovar Enteritidis* which was one of gram negative bacteria caused increasing at plasma MDA levels but it caused decreasing GSH-Px activity.

An important decreasing was observed at antioxidant enzyme activities in enterocytes at a study which was done in a rabbit intestine related with Vibrio cholera, one of gram negative bacteria and it was thought that this decreasing happened because of changing positive balance from antioxidant to oxidant [11]. Increasing LPO and decreasing alive cells were observed in the study which was searched effects of S. typhimurium enterotoxin on rat enterocytes by Mehta et al. [23]. Increasing MDA level and decreasing CAT activity in intense tissue in regard to bacterial infection in our study adjust the studies.

Flouroquinolones are one of the antimicrobial agents in human and animal medicine. Enrofloxacin is used in veterinary medicine for the treatment of infections of the respiratory and alimentary tract [8]. Enrofloxacin are oxidized by liver microsomal enzymes of the cytochrome P450 family. As a result of fluoroquinolone metabolism through cytochrome P450, free radical intermediates are generated, and these subsequently can cause lipid peroxidation [12].

Activities of GSH-Px, SOD and CAT enzyme in breast, leg and liver by supplemented with 50 mg/L enrofloxacin for 5 days were searched in the study of serum and plasma concentration of oxidant and antioxidants in patients of H. Pylori gastritis and its correlation

with gastric cancer. Supplementation of the diet with 50 mg/L did not have a significant effect on antioxidant activities in liver, breast and leg muscle samples [9].

In the study of influence of naringenin on oxytetracycline mediated oxidative damage in rat liver, intraperitoneal administration of oxyteracycline 200mg/kg for 15 days resulted a significant elevation in the levels of MDA. Oxytetracycline also caused a significant reduction in the antioxidant activities [25].

In our study, while application of antibiotic didn't change levels of MDA and activity of GSH-Px in liver, it caused decreasing CAT activity. However; while application of antibiotic increased level of plasma MDA, decreased GSH-Px activity. Decreasing CAT activity in liver, increasing MDA level in plasma and decreasing GSH-Px activity in blood were in a harmony with the studies.

While intraperitoneal *S. enterica Serovar Enteritidis* LPS injection caused increasing NO production in rat peritoneal macrophages; injection of intravenous LPS cause increasing NO in both plasma and liver of rats. In the study, a statistical change was not observed at plasma NO level; however there was an insignificant increasing depending on Salmonella in liver, it was observed an significant increasing upon antibiotic. However, there was decreasing in intestine tissue. The different results that are obtained may be resulted from producing radicals like peroxinitrite, besides NO being an antioxidant that make superoxide inactive.

6. CONCLUSSION AND SUGGESTS (SONUÇ VE TARTIŞMALAR)

As a result, both using antibiotic and bacterial infections cause oxidative stress. Using antioxidant substances with antibiotic can be suggested for broilers infected with *S. enterica Serovar Enteritidis*.

NOTICE (NOT)

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