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Original Article

Histopathological changes and biochemical analysis of sulfadiazine injected in egg in chicken embryo pectoral muscles

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ABSTRACT

This study aimed to investigate the effects of different doses of sulfadiazine on embryonic chicken pectoral muscles. In total, 100 fertile eggs were obtained and divided into five groups of control (no injection) and sulfadiazine injection at doses of 2, 10, 30, and 70 mg/kg. After hatching, pectoral muscle tissues were harvested from the newly hatched chickens for histopathological examination and measurement of oxidative stress parameters. Microscopic examination of pectoral muscle samples indicated that sulfadiazine administration changed the histopathological structure of chicken pectoral muscles only at very high doses (30 and 70 mg/kg). Major histopathologic events associated with sulfadiazine cytotoxicity were multifocal degeneration, necrotic tissue changes, and inflammatory cell infiltration (predominantly mononuclear cells) around degenerated and necrotic muscle fibers. Moreover, sulfadiazine at doses of 10, 30 and 70 mg/kg increased malondialdehyde level and decreased glutathione, ferric reducing antioxidant power, and total carotenoid, which indicated oxidative damage in broiler skeletal muscles. Therefore, it could be concluded that in-egg administration of up to 10 mg/kg of sulfadiazine is safe for chicken embryo, whereas dosage of 30 mg/kg (or above) is considered highly toxic.

Keywords: Sulfadiazine toxicity, Pectoral muscle, Chicken embryo

Analyses biochimiques et histopathologiques des changements induits par l'injection de sulfadiazine dans le muscle pectoral embryonnaire d'un œuf de poulet

Résumé: L'objectif de cette étude était l'étude des effets générés par l'injection de différentes doses de sulfadiazine dans le muscle pectoral embryonnaire de poulet. Au total, 100 œufs fertiles ont été collectés et répartis dans 5 groupes comprenant un groupe de contrôle (sans injection) et 4 groupes pour les injections de sulfadiazine à 2, 10, 30, et 70 mg/kg. Après éclosion, les tissus du muscle pectoral des poussins ont été prélevés et soumis à des examens d'histopathologies et de mesure des paramètres de stress oxydatif. Les observations microscopiques des prélèvements de muscle pectoral ont révélé des changements dans leurs structures histologiques suite à l'administration de sulfadiazine à très fortes doses (30 et 70 mg/kg). Les effets cytotoxiques majeurs du sulfadiazine comprenaient une dégénération multifocale, des changements dans les tissus nécrotiques et l'infiltration de cellules inflammatoires (particulièrement de cellules mononuclées) au niveau des fibres musculaires dégénérés et nécrotiques. De plus, les doses de sulfadiazine de 10, 30 et 70 mg/kg provoquaient une augmentation du taux de malondialdéhyde ainsi qu'une diminution du glutathionne, du FRAP (ferric reducing

antioxydant power) et du taux de caroténoïde total, signe de dommages oxydatifs au niveau des muscles squelettiques des poulets étudiés. En conclusion, l'administration dans l'œuf de jusqu'à 10 mg/kg de sulfadiazine n'entraine pas de risques pour l'embryon du poulet alors que les doses de plus de 30 mg/kg se sont avérées être fortement toxiques.
Mots clés : Toxicité du Sulfadiazine, Muscle pectoral, Embryon de poulet

INTRODUCTION

Sulfonamides (SNs) are synthetic antibiotics widely used against most gram-positive and many gram-negative organisms (Cheong et al., 2010). SNs inhibit the multiplication of bacteria as competitive inhibitors of para-aminobenzoic acid in folic acid metabolism (Baran et al., 2006; Accinelli et al., 2007). Administration of SNs at normal therapeutic doses is relatively nontoxic (Barragry, 1994). However, it has been reported that unwanted side effects occur in approximately 5% of human patients receiving SNs (Malik et al., 2013). SNs are associated with adverse reactions, especially in older populations; such examples are severe skin reactions, generalized bone marrow suppression, and thrombocytopenia. Moreover, SN administration may cause hypersensitivity and hematologic, renal and hepatic complications (Islam et al., 2012). On the other hand, administration of sulfaquinoxaline has been reported to cause keratoconjunctivitis sicca in dogs and hemorrhagic syndrome in chickens (Collins et al., 1986; Daft et al., 1989). In addition, SNs have been shown to exhibit carcinogenicity in mice, while further studies have suggested that carcinogenicity would not occur at normal or low doses of these drugs (Littlefield et al., 1990; Poirier et al., 1999). In poultry, SNs have been used for therapeutic, prophylactic, and growth-promoting purposes (Cheong et al., 2010). Extensive use of these drugs could be attributed to cost-effectiveness, ease of administration, and wide range of applications. However, use of SNs leads to the rapid rise of bacterial resistance, cross-resistance among SNs, and residues in animal products (Prescott, 2000). According to the literature, presence of SN residues in food products poses significant risks to the health of consumers (Malik et al., 2013). For instance, SN residues in meat (broiler chicken) and egg could increase antimicrobial resistance of microorganisms and decrease egg quality (Malik et al., 2013). SN residues may remain in animal products if adequate withdrawal times have not been observed or in case of improper administration (Cheong et al., 2010). To ensure the safety of human health, SN residue levels must be restricted to 0.1 μg/g in foods of animal origin (e.g., meat, milk and egg). Several epidemiological and experimental studies have denoted that in-utero exposure to some environmental chemicals and medication use during pregnancy could mediate various embryonic abnormalities, as well as complications associated with reactive oxygen species (ROS) generation, which damage cellular macromolecules, such as lipids. Therefore, polyunsaturated fatty acids are highly vulnerable to free radical damage. On the other hand, lipid oxidation is one of the mechanisms involved in the degradation of meat quality, which is determined based on the changed taste, color, and nutritional value of meat (Del Rio et al., 2005; Czauderna et al., 2011). Due to the high concentration of unsaturated fat and heme pigments, as well as the wide range of various oxidizing agents, muscle tissues are susceptible to oxidative deterioration. This study aimed to investigate the effects of sulfadiazine administration at various doses (low and high) on chicken embryonic pectoral muscles as an experimental model in order to detect histopathological changes, rate of oxidative stress, and antioxidant status. Despite the critical role of oxidative stress in animal growth, data is limited on the associations between antibiotic use, ROS production and its effect on meat quality. To the best of

our knowledge, this was the first study conducted in this regard.

MATERIALS AND METHODS

Study design. This study was performed in compliance with the principles of the Protection of Vertebrate Animals Used for Experimental and other Scientific Purposes. In total, 100 fertile eggs were obtained from a broiler breeder farm (Ross 308 strain), with mean weight of 63±1 grams. Samples were randomly divided into five groups and injected with different doses of sulfadiazine in the chorioallantoic membrane. Study groups were as follows: 1) control group (no injection); 2) injection of 2 mg/kg sulfadiazine; 3) injection of 10 mg/kg sulfadiazine; 4) injection of 30 mg/kg sulfadiazine and 5) injection of 70 mg/kg sulfadiazine. The eggs were incubated at the temperature of 37.5°C and 65% relative humidity. On the third day of incubation, the eggs were candled to remove dead embryos. On day four of incubation, sulfadiazine was injected into the chorioallantoic membrane of the experimental groups with 0.2 ml of the mentioned doses (10, 30, and 70 mg/kg), and the injection site was sealed with paraffin. On day 18 of incubation, fertile eggs were transferred to the hatchery and kept at the temperature of 37°C until hatching.

Sampling. During the incubation, dead embryos were removed (n=9). After hatching, embryos were washed with normal saline solution and examined for abnormalities and deformities on the external body surface. Afterwards, pectoral muscle samples (n=40) were provided from the newly hatched chicks and immediately fixed in 10% formalin for histopathological examination. In addition, pectoral muscle samples (n=60) were stored at the temperature of -70°C until the assessment of oxidative stress. Pectoral tissues were homogenized with 10% (w/v) sodium phosphate buffer (0.1 M, pH 7.4). The homogenate was centrifuged at 3000 rpm for 15 minutes, and the supernatant was used to estimate oxidative stress parameters.

Histopathology. After histological fixation, the sections (n=8 in each group) were processed and embedded in paraffin. Paraffin-embedded tissues were cut into 6-7 μm sections using a microtome and were stained using the hematoxylin and eosin (H&E) method.

Measurement of oxidative stress parameters. *Measurement of lipid peroxidation.* Formation of thiobarbituric acid (TBARS) in the samples was assessed for the measurement of lipid peroxidation based on an original method (Sicinska *et al.,* 2006). Briefly, the supernatant of the sample homogenate was mixed with 20% trichloroacetic acid, and the mixture was centrifuged. Following that, thiobarbituric acid was added to the supernatant and heated. Absorbance of the supernatant was measured at 532 nm, and TBARS concentration was calculated using the malondialdehyde (MDA) standard curve.

Measurement of total carotenoid of muscles. Total carotenoid of the muscles was measured using β-carotene standard curve and spectrophotometry at wavelength of 470 nm. Moreover, total carotenoid content of the samples was calculated based on β-carotene standard curve (Thaipong et al., 2006).

Measurement of total glutathione (GSH) assay. Glutathione (GSH) content was applied in accordance with the aforementioned method (Gibson et al., 1998). Samples were rinsed three times with phosphate-buffered saline and mixed with 20% trichloroacetic acid. After centrifugation of samples, the supernatant was mixed with vol of Tris. In the next stage, 1 mM DTNB [5,5′-dithiobis(2-nitrobenzoic acid)] was added to the samples and incubated for 30 minutes. Absorbance was read at 412 nm.

Ferric reducing capacity assay. Ferric reducing capacity assay is used to measure the ferric reducing capacity. Based on a redox reaction, an easily reduced antioxidant (Fe3) was employed in stoichiometric excess.

Statistical analysis. Overall significance of oxidative stress data was determined using one-way analysis of variance (ANOVA), and differences between the groups were considered statistically significant at the P value of less than 0.05 via

Bonferroni's post-hoc test. Obtained results of the study were presented as mean ± S.E.M.

RESULTS

Macroscopical findings. In this study, gross examination revealed no abnormalities in the internal organs and external body surfaces of all experimental groups.

Microscopical findings. Microscopic examination of pectoral muscle samples in the control group was indicative of no significant deviation from normal histological structures. Moreover, no histological evidence of muscle fiber damage was observed in experimental groups two and three (administered with 2 and 10 mg/kg sulfadiazine, respectively) (Figure 1).

In experimental group four (injected with 30 mg/kg sulfadiazine), slight degeneration of muscle fibers and mild mixed inflammatory cell infiltration were observed in some cases. In experimental group five, which was administered with the highest dose of sulfadiazine (70 mg/kg), we detected multifocal degeneration and necrotic changes marked by fragmented hypereosinophilic fibers, homogenization of fibers, loss of striations, and nuclear changes (i.e., pyknosis in skeletal muscles). Furthermore, inflammatory cell infiltration was mainly detected in mononuclear cells around degenerated and necrotic muscle fibers. In experimental group five, number of muscle fibers significantly reduced compared to other groups, and fragmented muscle fibers were separated by connective tissues (Figure 2). In addition, congestion was observed in the interstitial tissue, and more split fibers were detected in experimental group five compared to group four.

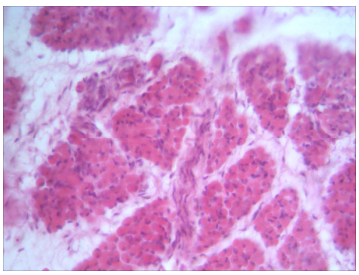

Figure 1. Transverse sections of chicken pectoralis muscle in experimental group three with normal morphology (H&E x200)

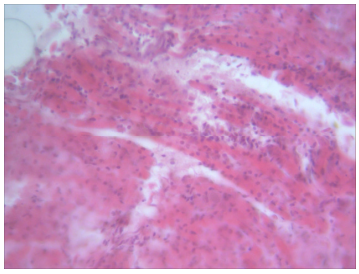

Figure 2. Note to muscle fiber fragmentation and necrotic fibers of experimental group five (H&E x200)

Evaluation of oxidative stress. Results of the evaluation of oxidative stress are presented in Table 1.

Concentration of carotenoid pigments in the pectoral muscle was determined using the standard curve obtained by a commercial β-carotene reagent via the

Table 1. Oxidative and antioxidant parameters of chicken embryo pectoral muscles exposed to different doses of sulfadiazine

Group / Parameters	Group 1 (control)	Group 2 (2 mg/kg)	Group 3 (10 mg/kg)	Group 4 (30 mg/kg)	Group 5 (70 mg/kg)
Ferric-reducing antioxidant power (mmol/g tissue)	1.41±0.46	1.00±0.25	0.79±0.29 a^{*}	0.61±0.30 a^{**}	0.37±0.27 a^{***} b^{*}
Total carotenoid (µg/g tissue)	0.13±0.01	0.10±0.02	0.07±0.02 a^{*}	0.06±0.03 a^{**}	0.06±0.03 a^{**}
Malondialdehyde (nmol/g tissue)	2.09±0.55	2.15±0.20	2.85±0.23 a^{*} e^{*}	3.27±0.33 a^{***} c^{***}	3.42±0.49 a^{***} b^{***}
Glutathione (µmol/g tissue)	0.05±0.03	0.03±0.01	0.01±0.00 a^{*}	0.01±0.00 a^{*}	0.01±0.00 a^{**}

Values presented as Mean ± SD

Statistical significance for differences between data of control group versus groups 3, 4 and 5; a*: *P<0.05*, a**: *P<0.01*, a***: *P<0.001*

Statistical significance for difference between data of group 2 versus group 5; b*: *P<0.05*, b***: *P<0.001*

Statistical significance for difference between data of group 2 versus group 4; c***: *P<0.001*

Statistical significance for difference between data of group 2 versus group 3; d*: *P<0.05*

following formula:

$Y=3240.7x + 0.1129; R^2=0.9974$

According to our findings, carotenoid content of embryonic pectoral muscles significantly decreased in experimental groups three, four and five compared to the control group ($P<0.05$ in group three versus control group, $P<0.01$ in groups four and five versus control group). Furthermore, muscle ferric reducing antioxidant power (FRAP) in experimental groups three and four had a significant difference with group one ($P<0.05$), while it was significantly higher compared to groups one and five ($P<0.001$). In this regard, experimental groups two and three showed no significant changes, while a significant difference was observed between groups two and five ($P<0.05$). According to the results of this study, MDA levels in muscles were higher in experimental groups three, four and five compared to the control group ($P<0.05$ in group three versus control group, $P<0.01$ in groups four and five versus control group). Additionally, muscle GSH in experimental groups three, four and five was significantly lower compared to the control group ($P<0.05$ in groups three and four versus control group, $P<0.01$ in group five versus control group).

DISCUSSION

Presence of antibiotic residues in the meat and edible viscera of food-producing animals has been a major concern of public health organizations across the world (Mahmoudi et al., 2014). If inadequate or excessive amount of antibiotics is used for livestock regardless of safety recommendations, harmful residues of these compounds are likely to remain in animal meat after slaughter. Residues of SNs in dairy products and foods of animal origin increase the risk of adverse health consequences, antibiotic resistance of pathogenic bacteria, allergic reactions, and carcinogenicity (Casella et al., 2012). Previous studies have focused on the measurement of SN residues in meat (Liu et al., 2009; Wang et al., 2011). According to the results of the current study, pathologic lesions occurred in embryonic pectoral muscles following the administration of sulfadiazine at high doses (70 mg/kg). On the other hand, gross examination revealed no significant abnormalities or deformities. In a study, Kato and Kitagawa (1974) evaluated the teratogenic effects of 3,6-Dimethoxy-4-sulfanilamidopyridazine (CS-61) on mice and rats. According to the results, cleft palate occurred in mouse fetuses, while rat fetuses had urogenital malformations in addition to cleft palate. In the mentioned study, non-teratogenic doses of CS-61 increased minor skeletal variations (i.e., ribs 7 and 14) in both species, the incidence of which was reported to be dose-dependent. In another research, Bass et al. (1951) denoted the reduction of offspring, while no teratogenic effects were reported in the mice treated with sulfadiazine during pregnancy. In the present study, there were nine dead embryos in the sample population, and the majority (n=5) belonged to the experimental groups administered with high doses of sulfadiazine. In a study in this regard, Paget and Thorpe (1964), reported that administration of sulfamoprine (sulfa-4,6-dimethoxypyrimidine) led to abnormalities in the incisor eruption and skull of the offspring of rats and mice. However, we observed no anomalies and/or teratogenic effects in newly hatched chicks administered with sulfadiazine in the embryonic stage.

Histopathological examination of samples revealed that sulfadiazine induces degeneration and necrotic changes at high dosages (30 and 70 mg/kg). Furthermore, high doses of this drug were found to cause more severe reversible and irreversible pathological changes compared to control and other experimental groups. However, irreversible injuries were detected in samples of experimental group five. In the present study, it was observed that sulfadiazine may continue to reduce oxidative stability and cause toxicity in muscle tissues. Moreover, sulfadiazine at doses of 10, 30 and 70 mg/kg increased the MDA level and decreased GSH, FRAP and total carotenoid, which was suggestive of oxidative damage in broiler skeletal muscles. Drug-induced oxidative stress is implicated as a mechanism of toxicity in numerous tissues (Devasagayam et al., 2004). Oxidative stability of meat and its products

depends on the balance of anti- and pro-oxidants and/or oxidation substrates, such as fatty acids, proteins and pigments (Guzik et al., 2002). Oxidative stress may damage the integrity of DNA, protein, and lipids (Halliwell; Nordberg and Arnér, 2001). Lipid oxidation could decrease meat quality through the formation of off-odor and off-flavor compounds and reducing its nutritional values. Furthermore, the secondary products of lipid peroxidation could react with proteins and cause protein oxidation. This process may lead to fragmentation and conformational changes in protein structures in order to modify their functions (Zhang et al., 2013). In the current study, level of lipid peroxidation was observed to change. Chicken meat is an abundant source of polyunsaturated fatty acids, and therefore, it is highly sensitive to oxidative stress. Consumption of foods containing MDA, which is a lipid peroxidation product, has been associated with several health problems. In the present research, MDA levels of muscles were higher in experimental groups three, four and five compared to the control group.Nutritional-qualitative factors of meat, including carotenoid content, have been shown to reduce the possibility of degenerative diseases. Moreover, the health-promoting functions of carotenoids have been well documented (Møller et al., 2000). Color of meat is an important factor in determining its quality (Sadighara et al., 2013). Carotenoids that are accumulated in muscles produce the color of meat and improve its quality and taste. As such, reduction of carotenoids is considered a negative issue in terms of the quality of meat (Lee et al., 2010). According to the findings of the current study, total carotenoid of pectoral muscles decreased after the administration of sulfadiazine 10 mg/kg and higher doses. Therefore, it could be concluded that co-administration of carotenoid as an antioxidant ameliorates the toxicity of oxidative stress caused by SNs at the mentioned dose. In another study, Archile-Contreras and Purslow (2011) stated that oxidative stress decreased collagen synthesis and solubility in some muscles, which ultimately led to the reduction of meat tenderness, elevated MDA levels, and reduction of GSH and FRAP in chicken breast meat. Findings of the current study suggested that oxidative stress is induced by sulfadiazine at doses of 10, 30 and 70 mg/kg, which might have been the cause of lower tenderness of poultry meat. GSH is one of the most important antioxidants found in animal tissues (Savary-Auzeloux et al., 2008). In the current research, GSH level was significantly affected by sulfadiazine administration at high doses. In a research conducted in this regard, Cheong et al. (2010) determined the level of four different SN compounds in chicken muscle and liver samples marketed in Malaysia using reversed-phase hydrophobic chromatography. According to the findings, level of SN residues in chicken liver was significantly higher compared to breast meat samples.

Drug residue concentrations vary noticeably in different tissues and are generally detected at higher levels in storage tissues, such as body fat or in organs that actively metabolize and excrete these residues (e.g., hepatic and renal tissues) (Booth, 1973). These findings are in congruence with previous studies in this regard (Sayrafi et al., 2015), which have suggested that sulfadiazine administration could significantly alter the histopathologic structure of chicken renal tissues in all treatment groups. Furthermore, it leads to the perturbation of the antioxidant defense mechanism through the marked increase of lipid peroxidation and reduction of GSH in a dose-dependent manner. However, in the present study, sulfadiazine administration at the dose of 70 mg/kg was observed to cause significant pathological lesions, which were mainly detected in muscle tissues, in experimental group five. In a study, Malik et al. (2013) assessed the effect of SN residues on egg quality traits and reported that this drug affected the external and internal quality of egg through decreasing the weight and thickness of egg shell, height and width of egg yolk, and yolk index. In this regard, it is suitable to escape eggs, selling and consumption during treatment and withdrawal periods. Presence of SN residues necessitates the application of biosecurity measures in poultry farming. In their research, Lebkowska-Wieruszewska and Kowalski

(2010) evaluated the residue depletion of healthy turkeys treated with sulfachloropyrazine, and the results showed sulfachloropyrazine to have long half-life and relatively high bioavailability. Moreover, this drug was detected in measurable edible tissues of turkeys 18 days after the cessation of treatment. Methods such as frying, canning process, and roasting are likely to eliminate several antibiotic residues (e.g., sulfonamide) in meats. Additionally, several cooking methods and freezer storage for different durations could reduce SN residues in meat tissues. Antibiotic residues in foods could be lowered to safe limits for human consumption through slow-cooking at high temperatures. However, it is possible that some metabolites emerge after cooking, the effects of which remain unclear on public health. Drug residues in animal tissues may vary depending on several factors, including age, disease state, drug solubility, sex, race, stability, pharmacokinetics, pharmaceutical formulations, route and time of drug administration, and duration and methods of cooking and storage (Fischer et al., 1992; Liman et al., 2015).

In conclusion, findings of the present study suggested that sulfadiazine administration alters the histopathologic structure of chicken pectoral muscle only at very high doses. Among the major histopathologic events associated with sulfadiazine cytotoxicity are multifocal degeneration, necrotic changes, and inflammatory cell infiltration, which occur mainly in the mononuclear cells around degenerated and necrotic muscle fibers. On the other hand, significant changes of oxidative stress parameters could be observed at sulfadiazine dosage of 10 mg/kg or higher. Therefore, it could be concluded that in-egg administration of up to 10 mg/kg of sulfadiazine is safe for chicken embryos, whereas dosage of 30 mg/kg (or above) could be highly toxic.

Ethics

I hereby declare all ethical standards have been respected in preparation of the submitted article.

Conflict of Interest

The authors declare that they have no conflict of interest.

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