Original Article

Journal of Food Safety and Hygiene

journal homepage: http://jfsh.tums.ac.ir

Involvement of oxidative stress in the sulfadiazine hepatotoxicity in chicken embryo model

Atefeh Araghi[a], Seyed Mohammad Hoseini[b], Reza Sayrafi[c], Parisa Sadighara[d*]

[a] Department of Food Hygiene, School of Veterinary Medicine, Amol University of Special Modern Technologies, Amol, Iran
[b] Department of Pathobiology, School of Veterinary Medicine, Babol Branch, Islamic Azad University, Babol, Iran
[c] Department of Pathobiology, School of Veterinary Medicine, Amol University of Special Modern Technologies, Amol, Iran
[d] Department of Environmental Health Engineer, Food Safety Division, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran

ARTICLE INFO

Article history:
Received 14 Mar. 2016
Received in revised form
12 May 2016
Accepted 04 June 2016

Keywords:
Sulfadiazine;
Toxic hepatitis;
Oxidative stress;
Chickens

ABSTRACT

Drug residues and its side effects are one of the major problems in global concerning for food contamination. Veterinary drugs in food-producing animals have potential to generate residues in animal-derived products and pose a health hazard to the customers. Sulfadiazine (SDZ) is a group of synthetic antibiotics with broad-spectrum effects. The present study was conducted to assess SDZ hepatotoxicity in chicken embryo models. SDZ was injected on the day 4 of chicken's incubation. Afterward, the livers and serum samples were collected after hatching. In addition, oxidative stress and biochemical parameters in organs and blood were measured, respectively. We found that there was a significant change in the liver's enzyme activities. Histopathological findings and liver enzyme activity indicated that SDZ is a hepatotoxic agent. There was a significant increase in lipid peroxidation, and also the same decrease was observed in glutathione level. Furthermore, a small reduction in ferric reducing/antioxidant power and total carotenoids were seen. Overall, the results of this study suggested the presence of oxidative stress in SDZ hepatotoxicity. These data might be useful in applying antioxidant components for protection of hepatotoxicity associated with SDZ therapy.

Citation: Araghi A, Hoseini SM, Sayrafi R, Sadighara P. **Involvement of oxidative stress in the sulfadiazine hepatotoxicity in chicken embryo model.** J Food Safe & Hyg 2016; 2(1-2): 42-6.

1. Introduction

The use of veterinary drugs in food-producing animals has potential to generate residues in animal-derived products and poses a health hazard to the customers. Veterinary drugs and feed additives, especially anticoccidials and antibacterials can be easily absorbed and distributed through the body of animals, accumulated in various tissues and transferred into their products (1,2). Sulfonamides are a group of synthetic antibiotics with broad-spectrum effects against most Gram-positive bacteria. They are frequently used in poultries for treatment of various types of infections in digestive and respiratory tracts (3). SDZ is a sulfonamide that is widely used as a veterinary and human drug to prevent and treat diarrhea and other infectious diseases. Treated animals excrete unbroken SDZ into the ecosystem through their excretory system (4). This drug infiltrates into agricultural soils during the fertilization. It is resistant to chemical degradation and biodegradation. SDZ was detected in the sea water with concentration of 2.5 µg/dry matter (dm), and also, different drugs of this group accumulate over a wide range: 3-41 µg/dm in sewage sludge, 0.48-2.64 µg/dm in cow's milk, and 16-39 µg/dm in poultry meat (5). The presence of sulfonamide residues in food can be critical for human consumption due to their potential carcinogenic character, and the possible development of antibiotic resistance (6).

Drug residues appear in both egg white and yolk after administration to laying hens. Intestinal absorption is a prerequisite for that, as transport

* Corresponding author. Tel.: +982188954914
E-mail address: sadighara@farabi.tums.ac.ir

through blood (plasma) is responsible for deposition of drugs in yolk in the ovary or egg white in the oviduct. Sulfonamides show appreciable levels in both egg white and yolk (1). Moreover, previous experimental studies showed that the residues in matrices of animal origin such as muscle and liver tissue in poultry were observed. Their feed contained 10% of the therapeutic dose of SDZ. Interestingly, this amount was more than maximum residue limits (7). To prevent consumers from potential health problems, European Community set a maximum residue limit for total sulfonamides of 100 µg/kg in edible tissue (8). Some drugs of this group including SDZ have been given high priority for risk assessment (9). In this study, the possible mechanism involved in hepatotoxicity of SDZ was surveyed in chicken embryo model.

2. Materials and methods

One hundred fertile eggs were obtained from a broiler breeder farm (Ross 308 strain). All eggs with mean weight of 63 ± 1 g were divided into five groups and received different amounts of SDZ by injection in chorioallantoic membrane according to our previous studies on chemical toxicity used chicken embryo model (10,11). Eggs were randomly assigned to one of the subsequent groups: (i) control group without any injection and (ii) four experimental groups receiving different concentrations of SDZ (2, 10, 30, and 70 mg/kg).

Eggs were incubated at 37.5 °C and 65% relative humidity. On the 3rd day of incubation, eggs were candled, clear eggs and dead embryos were removed from the experiment. On the 4th day of incubation, the experimental groups received SDZ into the chorioallantoic membrane with 0.2 ml of mentioned doses. To avoid contamination, all injections were carried out in a clean room, and all equipment was sterilized. The injection site was sealed with paraffin, and the eggs were returned into the hatchery and kept at a temperature of 37 °C until they hatched.

After hatching, liver samples were taken out and fixed in 10% formalin-saline solution for histopathological examinations. Moreover, blood and liver samples were taken from all hatched chicks. Blood samples were allowed to clot and were kept for about 1 hour at room temperature. After that, serum of each sample was separated, centrifuged, and transferred to sterile microtubes then kept at −20 °C until analyzed.

The liver was rinsed 3 times with phosphate-buffered saline (PBS). Then, the tissues were homogenized in PBS by the Teflon homogenizer. The homogenate was used to determine lipid peroxidation, glutathione (GSH) content, and antioxidant power, and carotenoids level.

The formation of thiobarbituric acid in organ samples was assessed to measure the level of lipid peroxidation according to an original method (12). Briefly, the supernatant of homogenate was mixed with 20% trichloroacetic acid, and the mixture was centrifuged. Then, thiobarbituric acid was added to the supernatant and heated. Subsequently, the absorbance of the supernatant was measured at 532 nm. The values were expressed in nmoles malondialdehyde (MDA), using a molar extinction coefficient of $1.56 \times 10^5\ M^{-1}\ cm^{-1}$.

The GSH content was applied according to the previous method (13). The supernatant of the liver homogenate mixed with 20% trichloroacetic acid. The samples were centrifuged and shortly after supernatant was mixed with 4 vol of Tris (hydroxymethyl) aminomethane. Then, 1 mM 5,5'-dithio-bis-(2-nitrobenzoic acid) was added to the sample and incubated for 30 minutes, and, the absorbance was read at 412 nm.

The ferric reducing capacity assay measures the ferric reducing capacity. The method was based on a redox reaction in which an easily reduced antioxidant (Fe^3) was employed in stoichiometric excess.

Total carotenoids of supernatant of homogenate were determined by β carotene standard curve and by spectrophotometric method at 470 nm. Total carotenoids content was calculated on the basis of the standard curve of β carotene (14).

Alanine transaminase (ALT), serum ALT, and aspartate aminotransferase (AST) activities in serum were measured using commercial kits.

Following histological fixation, tissues were processed through a standard alcohol dehydration-xylene sequence and embedded in paraffin. A rotary microtome was used to make eight cuts that were 6-7 µm, and they were stained with hematoxylin-eosin. The sections were observed under light microscope. Histopathologic finding was graded (−) showing no changes, (+), (++), and (+++) indicated mild, moderate, and sever changes, respectively.

The evaluation was made by one-way variance analysis in SPSS software (SPSS Inc., Chicago, IL, USA). The difference more than 95% ($P \leq 0.050$) was considered significant. The data values were expressed as mean ± standard deviation.

3. Results

3.1. Measurement of oxidative stress parameters in liver

The oxidative stress parameters including ferric reducing/antioxidant power (FRAP), GSH, MDA, and total carotenoid were measured. The results are shown in table 1.

Table 1. Level of oxidative stress parameters

Groups	Level of lipid peroxidation (nmol/g tissue)	GSH µmol/g tissue	Ferric reducing capacity mmol/g tissue	Total carotenoid µg/g tissue
Group 1 (Control)	0.26 ± 0.03	0.09 ± 0.01	1.70 ± 0.20	0.96 ± 0.12
Group 2 (2 mg)	0.35 ± 0.09	0.07 ± 0.02	1.59 ± 0.20	0.85 ± 0.13
Group 3 (10 mg)	0.39 ± 0.05*	0.05 ± 0.04	1.34 ± 0.23	0.80 ± 0.10
Group 4 (30 mg)	0.59 ± 0.06*	0.06 ± 0.02	1.18 ± 0.20*	0.63 ± 0.12*
Group 5 (70 mg)	0.77 ± 0.07*	0.02 ± 0.01*	1.05 ± 0.10*	0.46 ± 0.09*

Each value represents the mean ± SD per group *P < 0.050. SD: Standard deviation, GSH: Glutathione

The level of lipid oxidation is significantly different between the control compared to Group 3 (P ≤ 0.050) and also the control compared to Groups 4 and 5 (P = 0.001). The p value between Groups 3-5 is < 0.001. Concerning the liver GSH factor, it was found to be significantly different between Groups 1 and 5 (P < 0.050). The results of ferric reducing capacity are significantly different between all groups except Group 2 comparing Group 3. In addition, differences are highly significant in all groups (P < 0.001).

The changes in carotenoids level are markedly observed between groups. There is a significant difference between the Group 1 in comparison to the Groups 4 and 5. There are also significant changes between Group 2 comparing Groups 4 and 5. Although difference in Group 3 comparing Group 4 is not significant, difference between Groups 3 and 5 is considerably significant (P < 0.001).

3.2. Measurement of liver enzymes

The results of liver enzymes (alkaline phosphatase [ALP], AST, and ALT) are shown in table 2.

Table 2. Effect of SDZ treatment on enzyme activity of liver

Groups	The mean of enzyme activity (U/L)		
	AST	ALT	ALP
Group 1 (Control)	172.50 ± 10.75	5.2 ± 2.8	2049.00 ± 503.29
Group 2 (2 mg)	205.00 ± 17.08	8.0 ± 0.7	2694.80 ± 937.67
Group 3 (10 mg)	215.00 ± 25.00	9.0 ± 0.7	3194.00 ± 667.77*
Group 4 (30 mg)	287.60 ± 12.60*	10.0 ± 0.7*	4507.60 ± 623.39*
Group 5 (70 mg)	296.00 ± 82.64*	12.0 ± 1.1*	4908.0 ± 393.3*

Each value represents the mean ± SD per group *P < 0.050. SD: Standard deviation; AST: Aspartate aminotransferase; ALT: Alanine transaminase; ALP: Alkaline phosphatase

The general signs of hepatotoxicity can be affected by changed liver's enzyme activity. The enzyme activity is considerably different between groups. Surprisingly, the enzyme activities are elevated due to increasing level of SDZ. Changes in serum ALT are significant in the Group 1 compared to the Groups 4 and 5. The activity of serum AST shows a significant difference between Group 1 and the two Groups 4 and 5. Furthermore, there are significant differences between Group 2 when compared with Group 5, and also in the case of comparing Groups 3 and 5.

3.3. Histopathology finding

In this study, no lesion in Group 1 (control), 2 (2 mg) was observed. In Group 3 (10 mg) mild lesion was recorded including vacuolar degeneration, necrosis, hyperemia, and increased sinusoidal space (Figure 1).

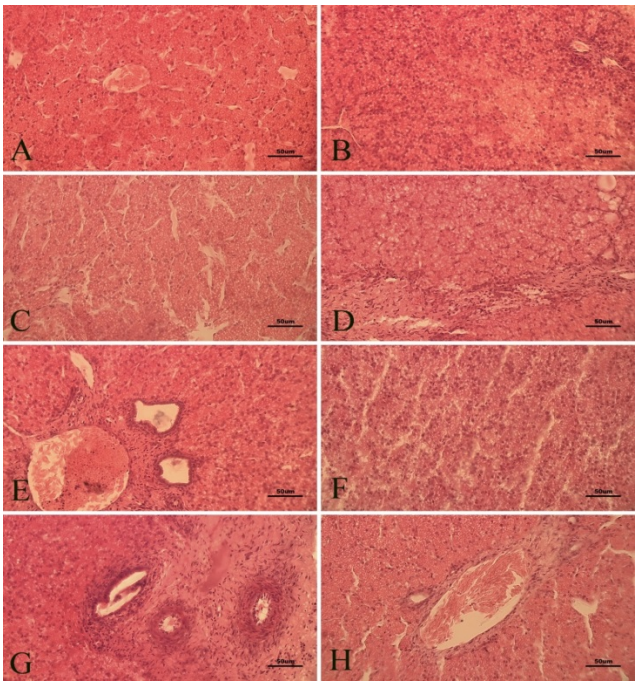

Figure 1. (a) 10 mg group, mild necrosis, hyperemia, (b) 10 mg group, vacuolar degeneration, (c) 30 mg group, necrosis, vacuolar degeneration, increased sinusoidal space, (d) 30 mg group, fibrosis and in sum degrees of cirrhosis, inflammatory cell infiltration, (e) 30 mg group, hyperemia, bile duct hyperplasia, (f) 70 mg group, vacuolar degeneration, necrosis, increased sinusoidal space, (g) 70 mg group, inflammatory cell infiltration, bile duct hyperplasia, fibrosis and in sum degrees of cirrhosis, (h) 70 mg group, hyperemia (H and E staining ×40 magnification)

The histopathological studies of liver in Groups 4 (30 mg) and 5 (70 mg) revealed vacuolar degeneration, hyperemia, bile duct hyperplasia, necrosis, inflammatory cell infiltration, increased sinusoidal space, fibrosis, and some degrees of cirrhosis were happened (Figure 1 and Table 3). The current study demonstrates that *in vivo* injection of SDZ can lead to different lesions in liver.

4. Discussion

We have demonstrated that different histopathological alterations were induced by *in vivo* administration of SDZ.

Table 3. The histopathology findings: 10, 30, and 70 mg/kg doses of SDZ induced hepatotoxicity

Groups	Inflammatory cell infiltration	Fibrosis and 4in sum degrees of cirrhosis	Increased sinusoidal space	Necrosis	Hyperemia	Vacuolar degeneration	Bile duct hyperplasia
Control	-	-	-	-	-	-	-
2 mg	-	-	-	-	-	-	-
10 mg	-	-	+	+	+	+	-
30 mg	+	+	++	+	++	++	++
70 mg	++	++	++	++	++	++	++

-: Showing no changes, +, ++, and +++ indicated mild, moderate, and sever changes, respectively

These alterations occurred in a dose-related manner. The histological changes were found at 10, 30, and 70 mg. Based on histopathological examinations of liver tissues, these lesions include vacuolar degeneration, hyperemia, bile duct hyperplasia, necrosis, inflammatory cell infiltration, increased sinusoidal space, and fibrosis (Figure 1). These hepatic histopathology findings indicate that sulfadiazine can be as a hepatotoxic drug. Present study revealed that dose-dependent changes have been seen in liver tissue samples. In Group 2 taken 2 mg/kg SDZ; changes were pretty mild. In Group 3 with administration of 10 mg/kg SDZ, changes were more severe though reversible yet. Groups 4 and 5 received 30 and 70 mg/kg SDZ, respectively, and histopathologic changes were more severe. It should also be noted that a high dose of SDZ causes irreversible changes in liver tissues. An investigation was done by Majeed et al. (15), to evaluate toxicological effect of sulfonamide in domestic pigeons by oral administration, histopathological results demonstrated, a periportal and septal fibrosis found in groups with intermediate dose (40 mg/kg/day sulfonamide). The high dose (80 mg/kg/day sulfonamide) exerted parenchyma foci with inflammatory cells, a minimal diffuse vacuolation of hepatocytes, and a periportal fibrosis with several lobules as compared with control group. Previous studies have shown that pre-exposure prophylactic consumption of sulfonamide drugs in albino Wistar rats caused evidence of inflammatory cell infiltration, distortion of bile duct, with slight hepatocellular necrosis (16). These findings were compatible with this study.

Furthermore, there were significant changes in serological parameters of liver. Enzymes such as ALT, AST, and ALP were also measured to assess liver toxicity. Liver enzyme concentration is a useful indicator of liver injury. Moreover, serum AST activity is the laboratory indicator of hepatotoxic effects. Damaged hepatocytes release AST and ALT into serum. ALT activity is the most frequently relied biomarker of hepatotoxicity. Furthermore; it is a liver enzyme that plays an important role in amino acid metabolism and gluconeogenesis. The estimation of this enzyme requires more specific tests for detecting liver abnormalities. This enzyme detects hepatocellular necrosis. AST is another liver enzyme that aids in producing proteins. It also helps in detecting hepatocellular necrosis (17).

ALP activity is additional conventional biomarker of liver function. In this study, changes in serum ALP were significant in groups receiving different amount of sulfadiazine. These changes are in accordance with previously done research that shows feeding of sulfamethoxypyridazine, a long-acting sulfonamide, to rats at dietary levels of 800, 1600, and 3200 ppm and daily oral administration of 30, 67, and 150 mg/kg to dogs resulting increased serum alkaline phosphatase (18).

The evaluation of biochemical and histological changes in liver are important tools to monitor hepatotoxicity. These results confirm sulfadiazine is a hepatotoxic drug. The clinical cases of hepatotoxicity of SDZ were also reported by Khalili et al. (19). This case of severe hepatotoxicity was observed in the patient, and also her skin appeared jaundiced. The high AST and ALT levels, hyperbilirubinemia, abnormal prothrombin time, and clinical evidence of acute fulminant hepatitis were reported of its hepatotoxicity.

Oxidative stress is induced by excessive levels of reactive oxygen species (ROS) and damages macromolecules such as DNA, lipids, proteins, and carbohydrates. ROS attacks polyunsaturated fatty acids and causes lipid peroxidation. MDA is a product of lipid peroxidation that has been used as an indicator in oxidative damages. The level of MDA was significantly different between control compared treated groups. Furthermore, FRAP, GSH, and total carotenoid level altered in between groups. The reduction in the total GSH content of the liver tissue was observed. The GSH is a necessary component of the natural antioxidant system and is not entirely replaceable (20). FRAP is a measure of the antioxidant power, based on the reduction of ferrous ions by the effect of the reducing power of samples, and contributed by low molecular weight antioxidants (21). We found a significant decrease in the FRAP levels.

A significant increase in MDA and a significant decrease in total antioxidant capacity, carotenoids, and GSH level suggest that SDZ induced oxidative stress.

In modern systems of livestock breeding, sulfonamide antibiotics are broadly employed to food-producing chicken for prophylactic or

therapeutic purposes, because of their inexpensiveness and wide-spectrum antimicrobial activity (8,22). The activities of AST, ALT and ALP were usually considered in serum as the liver function. The increase in activities of AST, ALT, and ALP in serum samples suggests that these changes are due to release of the enzymes from damaged liver. Furthermore, according to these findings and histopathology evidence, SDZ induced hepatotoxicity.

5. Conclusion

The liver is an important organ for the detoxification of exogenous and endogenous components, and also liver dysfunction can affect the metabolism of food and the production of proteins and vitamins. Therefore, liver dysfunction should be prevented. Presence of antioxidants plays an important role on the prevention oxidative changes. Antioxidants delay or inhibit cellular damages mainly through ROS scavenging (23). Therefore, antioxidants can lower the occurrence of oxidative stress. Together, these data might be useful in applying antioxidant components for protection of hepatotoxicity associated with SDZ therapy.

Conflict of Interests

Authors have no conflict of interest.

Acknowledgments

This study was funded by the Faculty of Veterinary Medicine, Amol University of Special Modern Technologies, Amol, Iran.

References

1. Kan CA, Petz M. Residues of veterinary drugs in eggs and their distribution between yolk and white. J Agric Food Chem 2000; 48: 6397-6403.
2. Weiss C, Conte A, Milandri C, et al. Veterinary drugs residue monitoring in Italian poultry: Current strategies and possible developments. Food Control 2007; 18: 1068-1076.
3. Furusawa N, Hanabusa R. Cooking effects on sulfonamide residues in chicken thigh muscle. Food Research International 2002; 35: 37-42.
4. Mulla SI, Sun Q, Hu A, et al. Evaluation of sulfadiazine degradation in three newly isolated pure bacterial cultures. PLoS One 2016; 11: e0165013.
5. Lemanska-Malinowska N, Felis E, Surmacz-Gorska J Photochemical degradation of sulfadiazine. Arch Environ Prot 2013: 39: 79-91.
6. Forti AF, Scortichini G. Determination of ten sulphonamides in egg by liquid chromatography-tandem mass spectrometry. Anal Chim Acta 2009; 637: 214-219.
7. Vandenberge V, Delezie E, Huyghebaert G, et al. Residues of sulfadiazine and doxycycline in broiler liver and muscle tissue due to cross-contamination of feed. Food Addit Contam Part A Chem Anal Control Expo Risk Assess 2012; 29: 180-188.
8. Wang H, Xu Y, Song W, et al. Automatic sample preparation of sulfonamide antibiotic residues in chicken breast muscle by using dynamic microwave-assisted extraction coupled with solid-phase extraction. J Sep Sci 2011; 34: 2489-2497.
9. Dalla Bona M, Di Leva V, De Liguoro M. The sensitivity of Daphnia magna and Daphnia curvirostris to 10 veterinary antibacterials and to some of their binary mixtures. Chemosphere 2014; 115: 67-74.
10. Gharibi S, Dilmaghanian A, Sadighara P, et al. The effect of bisphenol a on oxidative stress indices and pathological changes in the brain of chicken embryos. World Appl Sci 2015; 26: 345-351.
11. Sadighara P, Mohammadpour I, Jahanbakhsh M, et al. The effect of stevia on the chicken embryo heart. Cardiovasc Hematol Disord Drug Targets 2016; 16: 38-40.
12. Sicinska P, Bukowska B, Michalowicz J, et al. Damage of cell membrane and antioxidative system in human erythrocytes incubated with microcystin-LR in vitro. Toxicon 2006; 47: 387-397.
13. Gibson XA, Shartava A, McIntyre J, et al. The efficacy of reducing agents or antioxidants in blocking the formation of dense cells and irreversibly sickled cells in vitro. Blood 1998; 91: 4373-4378.
14. Thaipong K, Boonprakob U, Cisneros-Zevallos L, et al. Hydrophilic and lipophilic antioxidant activities of guava fruits. Southeast Asian J Trop Med Public Health 2005; 36: 254-257.
15. Majeed SK, Al-Sereah BA, Essa IM. The sulfonamide exposure and the histopathological alterations of domestic pigeon. Bas J Vet Res 2014; 1: 82-93.
16. Odigie BE. Histological effects of pre-exposure prophylactic consumption of sulfa drugs on Liver and Kidney of albino Wister rats (Rattus novergicus). IOSR J Pharm Biol Sci 2013; 5: 14-19.
17. Singh A, Bhat TK, Sharma OP. Clinical biochemistry of hepatotoxicity. J Clinic Toxicol 2011; S4: 1-19.
18. Ribelin WE, Owen G, Rubin LF, et al. Development of cataracts in dogs and rats from prolonged feeding of sulfaethoxypyridazine. Toxicol Appl Pharmacol 1967; 10: 557-564.
19. Khalili H, Soudbakhsh A, Talasaz AH. Severe hepatotoxicity and probable hepatorenal syndrome associated with sulfadiazine. Am J Health Syst Pharm 2011; 68: 888-892.
20. Vaziri ND, Wang XQ, Oveisi F, et al. Induction of oxidative stress by glutathione depletion causes severe hypertension in normal rats. Hypertension 2000; 36: 142-146.
21. Reddy PE, Manohar SM, Reddy SV, et al. Ferric reducing ability of plasma and lipid peroxidation in hemodialysis patients: Intradialytic Changes. Int J Nephrol Urol 2010; 2: 414-421.
22. Zotou A, Vasiliadou C. LC of sulfonamide residues in poultry muscle and eggs extracts using fluorescence pre-column derivatization and monolithic silica column. J Sep Sci 2010; 33: 11-22.
23. Lobo V, Patil A, Phatak A, et al. Free radicals, antioxidants and functional foods: Impact on human health. Pharmacogn Rev 2010; 4: 118-26.