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EFFECT OF METHIONINE DEFICIENCY ON OXIDATIVE STRESS AND APOPTOSIS IN THE SMALL INTESTINE OF BROILERS

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The aim of this study was to investigate the effects of methionine (Met) deficiency on antioxidant functions (in the duodenal, jejunal and ileal mucosa) and apoptosis in the duodenum, jejunum and ileum of broiler chickens. A total of 120 one-day-old Cobb broilers were divided into two groups and fed a Met-deficient diet and a control diet, respectively, for six weeks. The activities of superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSH-Px), the ability to inhibit hydroxyl radicals, and glutathione (GSH) content were significantly decreased in the Met-deficient group compared to the control. In contrast, malondialdehyde (MDA) content was significantly higher in the Met-deficient group. As measured by terminal deoxynucleotidyl transferase 2'-deoxyuridine 5'-triphosphate dUTP nick end-labelling (TUNEL) and flow cytometry (FCM), the percentages of apoptotic cells were significantly increased. In conclusion, dietary Met deficiency can cause oxidative stress and then induce increased apoptosis in the intestine. Oxidative stress contributes to intestinal apoptosis. This results in the impairment of local intestinal mucosal immunity due to oxidative stress and apoptosis in the small intestine. The results of this study provide new experimental evidence for understanding the negative effects of Met deficiency on mucosal immunity or the functions of other immune tissues.

Key words: Methionine deficiency, small intestine, oxidative stress, apoptosis, broilers

As a sulphur-containing amino acid, methionine (Met) performs a wide variety of biological functions (Avila et al., 2000; Yang et al., 2004; Oz et al., 2008) including growth promotion (Yen et al., 2002; Mirzaaghatabar et al., 2011), detoxification (Kim et al., 2006), antitumour function (Horvat et al., 2006; Li et al., 2009), resistance to coccidial infection (Rama Rao et al., 2003), involvement in methyl transfer (Stadtman, 2002; Waterland et al., 2006; Sanchez-

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Roman et al., 2011), influence on protein synthesis (Waterlow, 1996; Brosnan and Brosnan, 2006; Tesseraud et al., 2011; Li et al., 2014) and impact on the oxidative status of cells (Tesseraud et al., 2009; Tang et al., 2015). Apart from these functions, Met is also involved in avian immune function (Swain and Johri, 2000; Wu et al., 2013) and nutrient digestibility in the intestine of chickens (Adeniji et al., 2015).

The small intestine serves as the principal site for the digestion and absorption of nutrients, electrolytes and water from the intestinal lumen, and for the secretion of enzymes or transporters. The mucosal immune system is very important for host defence as well (Hecht, 2003; Ahmad et al., 2012). The intestine has another important function, intestinal immunity (Jeurissen et al., 2002). The intestinal mucosa displays complex defence mechanisms and plays a unique key role in mucosal immunity (Vervelde and Jeurissen, 2003; Deitch, 1990; Halliwell et al., 2000). Our previous studies proved that dietary Met deficiency can induce damage of the immune organs in broilers (Wu et al., 2012*a, b,* 2013). However, the effects of Met on gut function remain poorly documented. Thus, the aims of the present study were to investigate the role of Met in oxidative stress and cellular apoptosis in the intestine of broilers, and to provide new experimental evidence on the influence of Met deficiency on small intestinal functions in broilers. The present research could also provide useful insights for similar studies in both humans and in animal species other than chickens in the future.

Materials and methods

Animals and diets

One hundred and twenty one-day-old healthy Cobb broilers were allotted by body weight to two separate treatment groups of 60 broilers each. The broilers were fed either a control or a Met-deficient diet (as shown in Table 1). They were housed in cages with electrical heaters and were provided feed and water *ad libitum* for 42 days. The experimental diets were formulated according to the NRC recommendations (1994). The research and the use of animals complied with the guidelines of China West Normal University Animal Care Committee, and the environment and facilities of the laboratory animals were in line with the national standard (Chinese, GB4925-2010).

Table 1

Components of the basal diets for broilers (%)

Ingredients[1] (%)	Methionine-deficient diet		Control diet	
	Starter diet 1 to 21 d	Grower diet 22 to 42 d	Starter diet 1 to 21 d	Grower diet 22 to 42 d
Ground yellow corn	56	59.5	56	59.5
Soybean meal	37	32.85	37	32.85
Soybean oil	3.66	4.7	3.66	4.7
Ground limestone	0.57	0.5	0.57	0.5
Dicalcium phosphate	1.8	1.6	1.8	1.6
Salt	0.3	0.3	0.3	0.3
Choline chloride	0.1	0.1	0.1	0.1
DL-Met	0.0	0.0	0.24	0.12
Bentonite	0.24	0.12	0.0	0.0
Micronutrients[2]	0.33	0.33	0.33	0.33
Calculated nutrient levels (%)				
ME, MJ/kg	12.39	12.79	12.39	12.79
CP	21.17	19.72	21.17	19.72
Lys	1.19	1.08	1.19	1.08
Met	0.26	0.28	0.50	0.40
Met+Cys	0.62	0.50	0.74	0.62
Ca	0.85	0.77	0.85	0.77
Nonphytate P	0.44	0.40	0.44	0.40

[1]Ingredients and nutrient composition are reported on as-fed basis. [2]For the diet of d 1–21 and d 22–42, provided per kilogram of diet: vitamin A (all-*trans* retinol acetate), 12,500 IU; cholecalciferol, 2,500 IU; vitamin E (all-*rac*-α-tocopherol acetate), 18.75 IU; vitamin K (menadione Na bisulfate), 5.0 mg; thiamin (thiamin mononitrate), 2.5 mg; riboflavin, 7.5 mg; vitamin B_6, 5.0 mg; vitamin B_{12}, 0.0025 mg; pantothenate, 15 mg; niacin, 50 mg; folic acid, 1.25 mg; biotin, 0.12 mg; Cu ($CuSO_4 \times 5H_2O$), 10 mg; Mn ($MnSO_4 \times H_2O$), 100 mg; Zn ($ZnSO_4 \times 7H_2O$), 100 mg; Fe ($FeSO_4 \times 7H_2O$), 100 mg; I (KI), 0.4 mg; Se (Na_2SeO_3), 0.2 mg

Determination of oxidative stress parameters in the small intestine by biochemical methods

On days 14, 28 and 42, six broilers each in both groups were humanely sacrificed for observations and measurements. The small intestines were immediately removed and chilled to 0 °C in 0.85% sodium chloride (NaCl) solution, and divided into duodenum, jejunum, and ileum. An approximately 4-cm-long intestinal segment was collected from the middle section of each intestinal region, and then dissected and thoroughly cleaned with 0.85% NaCl solution. The mucosa was carefully scraped from the luminal face of the collected intestinal segments and stored at –80 °C prior to the measurements. Total protein quantity in the supernatant (per milligram or gram) of the intestinal homogenate was determined using Bradford's method (Bradford, 1976). The activities of superoxide

dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSH-Px), the ability to inhibit hydroxyl radicals (AIHR), and the contents of glutathione (GSH) and malondialdehyde (MDA) in the supernatant were detected by biochemical methods following the instructions of the reagent kits (SOD assay kit, Cat. No.: A001-1; CAT assay kit, Cat. No.: A007-2; GSH-Px assay kit, Cat. No.: A005; AIHR assay kit, Cat. No.: A018; GSH assay kit, Cat. No.: A006-1; MDA assay kit, Cat. No.: A003-2, purchased from Nanjing Jiancheng Bioengineering Institute of China, Nanjing, China). The absorbance of SOD, CAT, GSH-Px, the abilities to inhibit hydroxyl radical, GSH and MDA were measured by hydroxylamine method, ultraviolet, colorimetric method, hydroxylamine method, spectrophotometric method and thiobarbituric acid (TBA) method, respectively, and at 550 nm, 240 nm, 412 nm, 550 nm, 532 nm and 420 nm, respectively, under a microtitre plate reader (Thermo, Varioskan Flash, USA).

Detection of intestinal apoptosis by TUNEL and flow cytometry (FCM)

TUNEL method. The middle section of the duodenum, jejunum and ileum were collected and fixed in 10% neutral buffered formalin after postmortem examination, and then processed, trimmed, and embedded in paraffin. The terminal deoxynucleotidyl transferase 2'-deoxyuridine 5'-triphosphate dUTP nick end-labelling (TUNEL) assay was performed in 5 μm thick dewaxed sections with an Apoptosis Detection Kit (Cat. No.: QIA33, Merck, Germany) according to the manufacturer's instructions, as described by Peng et al. (2009).

Flow cytometry (FCM method). The mucosa was carefully scraped from the luminal face of the collected intestinal segments as described above, and ground to form a cell suspension that was filtered through a 300-mesh nylon screen. The cells were washed twice with cold phosphate-buffered saline solution (PBS, pH 7.2–7.4) and suspended in 1× binding buffer (Cat. No.: 51-66121E) at a concentration of 1×10^6 cells/mL. Subsequently, 100 μL of the cell suspension was transferred to 5-mL culture tubes, followed by the addition of 5 μL of Annexin V-FITC (Cat. No.: 51-65874X) and 5 μL of propidium iodide (Cat. No.: 51-66211E). The mixture was gently vortexed and incubated at 25 °C in the dark for 15 min. Four hundred μL of 1 × binding buffer was added to each tube, and finally analysis by flow cytometry on a BD FACSCalibur platform (BD Biosciences, Singapore) was conducted within 1 h.

Statistical analysis

Data (the mean data of the two groups) of the control and the Met-deficient groups were statistically evaluated with independent-samples *t*-test using SPSS 11.0 software package program for Windows, first using the general linear model and next the single variable, then the dependent variable was used to analyse the statistics. All results were expressed as means ± standard devia-

tion, representing six broilers in each group. Differences between means were assessed, and values of $P < 0.05$ and $P < 0.01$ were considered significant and highly significant, respectively.

Results

Changes of oxidative stress parameters

Changes in SOD activity. As shown in Table 2, no significant changes in SOD activity were observed in the Met-deficient group in the duodenum, jejunum and ileum at 14 days of age. However, SOD activities were significantly or highly significantly lower ($P < 0.05$ or $P < 0.01$) in the Met-deficient group than in the control at 28 days of age, and highly significantly lower ($P < 0.01$) in all of the three intestinal segments at 42 days of age.

Table 2

Changes of superoxide dismutase (SOD) activities (U/mg protein) in the duodenum, jejunum and ileum of broiler chickens

Intestine	Groups	14 days	28 days	42 days
Duodenum	Control	61.06 ± 5.23	67.13 ± 4.86	64.77 ± 3.96
	Met-deficient	61.97 ± 4.54	60.03 ± 3.97*	55.95 ± 3.48**
Jejunum	Control	57.25 ± 4.66	58.67 ± 3.78	58.04 ± 4.26
	Met-deficient	56.27 ± 4.51	52.81 ± 1.89*	46.43 ± 4.39**
Ileum	Control	58.71 ± 4.53	61.53 ± 3.71	59.98 ± 3.87
	Met-deficient	56.12 ± 3.36	55.34 ± 4.33**	51.35 ± 4.21**

Data are presented as means ± standard deviation (n = 6); Met = methionine; *P < 0.05 compared to the control group; **P < 0.01 compared to the control group

Changes in CAT activity. At 14 days of age, the activities of CAT in the jejunum were reduced ($P < 0.05$) in the Met-deficient group compared to those in the control, but there were no significant changes in the duodenum and ileum. The activities of CAT in the duodenum, jejunum and ileum were significantly reduced ($P < 0.05$) in the Met-deficient group at 28 days of age, and were significantly or highly significantly reduced ($P < 0.05$ or $P < 0.01$) at 42 days of age, as shown in Table 3.

Changes in GSH-Px activity. Compared to the GSH-Px activities found in the duodenum, jejunum and ileum in the control group, there were no significant changes in GSH-Px activity in the Met-deficient group at 14 days of age. However, the GSH-Px activities in all of the three segments of the small intestine in the Met-deficient group were significantly lower ($P < 0.05$ or $P < 0.01$) at 28 days of age, and they were highly significantly lower ($P < 0.01$) at 42 days of age (Table 4).

Table 3

Changes of catalase (CAT) activities (U/g protein) in the duodenum, jejunum and ileum of broiler chickens

Intestine	Groups	14 days	28 days	42 days
Duodenum	Control	411.32 ± 15.54	404.67 ± 12.34	399.56 ± 9.75
	Met-deficient	405.44 ± 21.12	384.83 ± 13.41*	379.77 ± 11.75*
Jejunum	Control	402.55 ± 15.13	398.37 ± 13.76	398.56 ± 12.87
	Met-deficient	385.46 ± 14.43*	383.78 ± 14.65*	372.53 ± 13.31**
Ileum	Control	407.35 ± 16.31	411.36 ± 12.66	414.92 ± 9.97
	Met-deficient	399.756 ± 20.12	389.63 ± 10.55*	378.31 ± 8.917**

Data are presented as means ± standard deviation (n = 6); Met = methionine; $^{*}P < 0.05$ compared to the control group; $^{**}P < 0.01$ compared to the control group

Table 4

Changes of glutathione peroxidase (GSH-Px) activities (U/g protein) in the duodenum, jejunum and ileum of broiler chickens

Intestine	Groups	14 days	28 days	42 days
Duodenum	Control	121.44 ± 4.43	119.43 ± 6.72	123.86 ± 6.65
	Met-deficient	116.75 ± 7.73	98.65 ± 5.66*	89.87 ± 4.53**
Jejunum	Control	109.26 ± 7.50	113.41 ± 3.78	118.11 ± 3.21
	Met-deficient	105.47 ± 3.33	92.61 ± 3.58**	88.94 ± 3.88**
Ileum	Control	110.17 ± 5.21	104.34 ± 4.14	109.59 ± 3.87
	Met-deficient	105.23 ± 3.50	90.44 ± 2.56*	86.75 ± 4.66**

Data are presented as means ± standard deviation (n = 6); Met = methionine; $^{*}P < 0.05$ compared to the control group; $^{**}P < 0.01$ compared to the control group

Change in the ability to inhibit hydroxyl radicals. The results in Table 5 show that the ability to inhibit hydroxyl radicals was lower ($P < 0.05$) only in the jejunum of the Met-deficient group than in the control at 14 days of age. However, at 28 and 42 days of age, the ability to inhibit hydroxyl radicals in the duodenum, jejunum and ileum showed a varying but significant decrease ($P < 0.05$ or $P < 0.01$) in the Met-deficient group as compared to the control.

Changes in GSH content. At 14 days of age, only the GSH content of the duodenum was reduced ($P < 0.05$) in the Met-deficient group as compared to the control. However, at 28 and 42 days of age, the GSH contents of all of the three intestinal segments were significantly decreased ($P < 0.05$ or $P < 0.01$) in the Met-deficient group (Table 6).

Table 5

Change of the ability to inhibit hydroxyl radicals (U/mg protein) in the duodenum, jejunum and ileum of broiler chickens

Intestine	Groups	14 days	28 days	42 days
Duodenum	Control	171.66 ± 6.66	165.56 ± 4.78	180.21 ± 6.71
	Met-deficient	170.48 ± 4.98	156.10 ± 3.94*	163.30 ± 5.33*
Jejunum	Control	167.32 ± 5.57	169.42 ± 5.11	171.60 ± 4.56
	Met-deficient	148.41 ± 5.24*	151.30 ± 5.22**	153.61 ± 4.30**
Ileum	Control	151.34 ± 5.37	154.36 ± 4.22	155.33 ± 5.19
	Met-deficient	148.77 ± 4.21	143.14 ± 3.97*	136.03 ± 4.51**

Data are presented as means ± standard deviation (n = 6); Met = methionine; $^{*}P < 0.05$ compared to the control group; $^{**}P < 0.01$ compared to the control group

Table 6

Changes of glutathione (GSH) content (mg /g protein) in the duodenum, jejunum and ileum of broiler chickens

Intestine	Groups	14 days	28 days	42 days
Duodenum	Control	4.65 ± 0.33	4.38 ± 0.31	3.46 ± 0.41
	Met-deficient	4.07 ± 0.19*	3.46 ± 0.41*	2.53 ± 0.36*
Jejunum	Control	3.92 ± 0.32	3.73 ± 0.149	2.97 ± 0.27
	Met-deficient	3.86 ± 0.28	2.35 ± 0.22**	1.47 ± 0.33**
Ileum	Control	3.49 ± 0.29	3.38 ± 0.38	2.76 ± 0.32
	Met-deficient	3.27 ± 0.19	2.66 ± 0.44*	1.43 ± 0.38**

Data are presented as means ± standard deviation (n = 6); Met = methionine; $^{*}P < 0.05$ compared to the control group; $^{**}P < 0.01$ compared to the control group

Changes in MDA content. The results in Table 7 show that the MDA content of the ileum was increased ($P < 0.05$) in the Met-deficient group at 14 days of age when compared to that of the control. The MDA contents in the duodenum, jejunum and ileum of the Met-deficient group were all significantly increased ($P < 0.05$) at 28 days of age, and significantly or highly significantly increased ($P < 0.05$ or $P < 0.01$) at 42 days of age.

Changes in small intestinal apoptosis

Changes in the number of apoptotic cells determined by the TUNEL assay. Apoptotic cells had brown-stained nuclei with condensed and irregular morphologic changes. Apoptotic cells were mainly distributed in the lamina propria and the epithelial layer (Fig. 1). As shown in Histogram 1, there were no significant

changes in the number of apoptotic cells in the Met-deficient group in the duodenum, jejunum and ileum at 14 days of age, only a tendency of increase was demonstrated; however, significantly higher ($P < 0.05$ or $P < 0.01$) numbers of apoptotic cells were detected in the Met-deficient group in the duodenum and jejunum at 28 days of age, and the number of apoptotic cells in the ileum was significantly higher ($P < 0.01$) at 42 days of age as compared to control.

Table 7

Changes of malondialdehyde (MDA) content (nmol/mg protein) in the duodenum, jejunum and ileum of broiler chickens

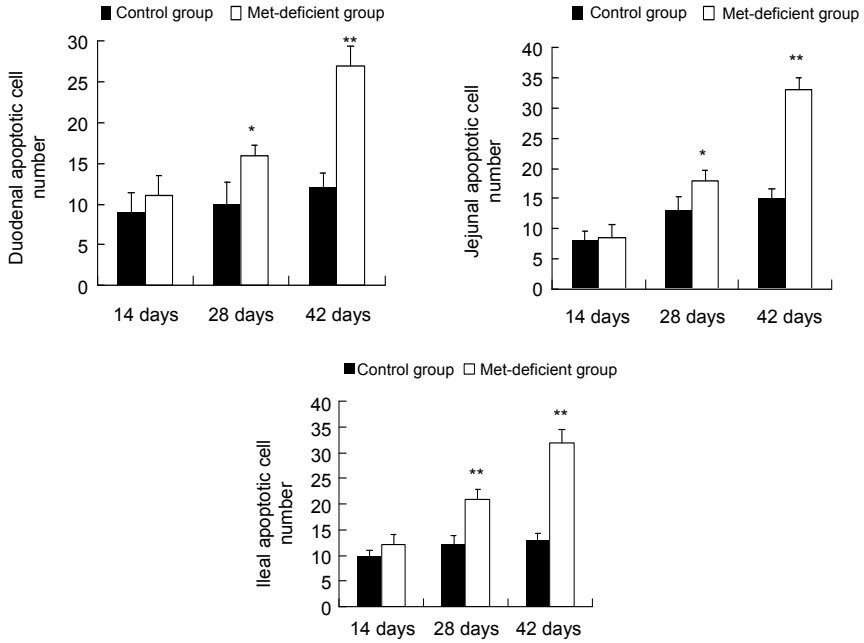
Intestine	Groups	14 days	28 days	42 days
Duodenum	Control	1.47 ± 0.18	1.50 ± 0.31	1.89 ± 0.22
	Met-deficient	1.76 ± 0.31	2.34 ± 0.35*	2.45 ± 0.23*
Jejunum	Control	2.06 ± 0.13	1.99 ± 0.28	2.26 ± 0.37
	Met-deficient	2.30 ± 0.33	2.81 ± 0.32*	3.43 ± 0.23**
Ileum	Control	1.46 ± 0.16	1.50 ± 0.31	1.62 ± 0.20
	Met-deficient	1.95 ± 0.34*	2.27 ± 0.35*	2.49 ± 0.23**

Data are presented as means ± standard deviation (n = 6); Met = methionine; *$P < 0.05$ compared to the control group; **$P < 0.01$ compared to the control group

Percentage of apoptotic cells determined by flow cytometry. Flow cytometry was used to quantitatively determine the percentage of cells within a population that were actively undergoing apoptosis (Fig. 2). As shown in Histogram 2, at 14 days of age, this percentage tended to increase in the duodenum and ileum, but the differences were not statistically significant. The percentages of apoptotic cells in the duodenum and ileum were significantly elevated ($P < 0.05$ or $P < 0.01$) in the Met-deficient group compared to the control at 28 and 42 days of age. The percentages of apoptotic cells in the jejunum were significantly elevated ($P < 0.05$ or $P < 0.01$) in the Met-deficient group at 14, 28 and 42 days of age.

Discussion

The intestinal mucosa is vulnerable to oxidative damage due to its constant exposure to reactive oxygen species (ROS) generated by the luminal content (Deitch, 1990). Met acts as a key antioxidant factor pivotal to the protection of the intestinal mucosa. It has been reported that a variety of ROS readily react with Met residues in proteins to form methionine sulphoxide, thus scavenging the reactive oxygen species (Luo and Levine, 2009). In this study we found that Met deficiency led to intestinal oxidative damage in broilers.



Histogram 1. Number of apoptotic cells measured by TUNEL in the duodenum (a), jejunum (b) and ileum (c) of broiler chickens

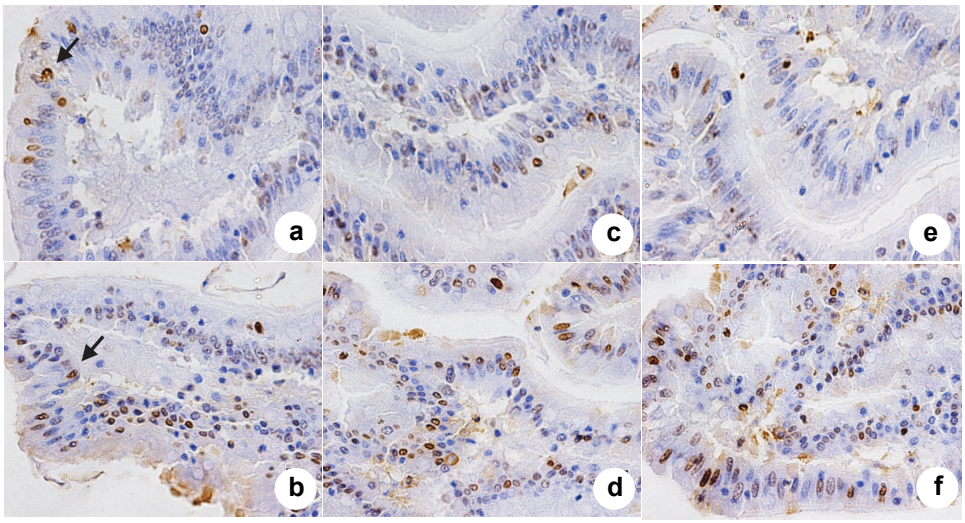
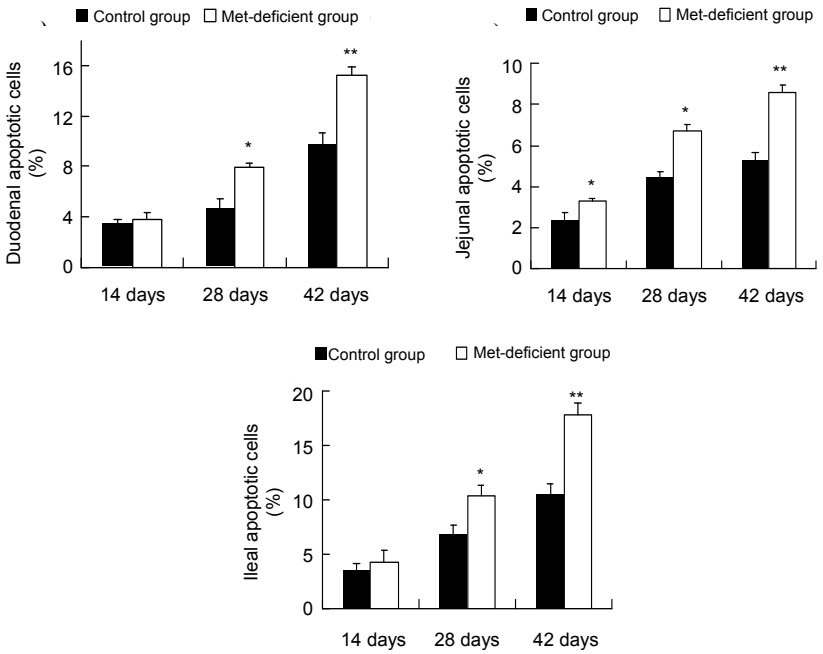


Fig. 1. Apoptotic cells in the duodenum (a and b), jejunum (c and d) and ileum (e and f) stained by TUNEL at 42 days of age. The numbers of apoptotic cells are significantly increased in the Met-deficient group in the duodenum, jejunum and ileum (b, d and f) as compared to those of the control groups (a, c and e). Apoptotic cells have brown-staining nuclei (arrow). Bar = 20 μm



Histogram 2. The percentage of apoptotic cells determined by flow cytometry (FCM) in the duodenum (a), jejunum (b) and ileum (c)

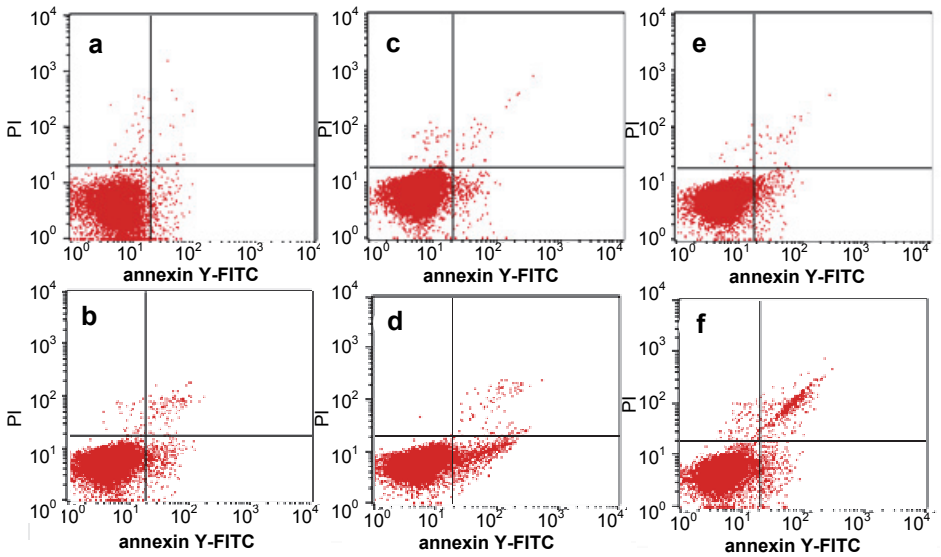


Fig. 2. Cells staining positive for annexin V-FITC and negative for propidium iodide (PI) are undergoing apoptosis. Quadrant diagram shows that a minor percentage of cells in the duodenum (a), jejunum (c) and ileum (e) of the control group are undergoing apoptosis, and an increased percentage of cells is undergoing apoptosis in the duodenum (b), jejunum (d) and ileum (f) of the Met-deficient group

Enhanced ROS generation can overwhelm intrinsic antioxidant cellular defences and result in a condition known as 'oxidative stress' (Halliwell et al., 2000). There are some antioxidants (including antioxidant enzymes and non-enzymatic antioxidants) that play a role in preventing oxidative stress (Adedara et al., 2010). In this study, the activities of SOD, GSH-Px and CAT, which antioxidant enzymes considered to be the first line of cellular defence against oxidative damage (Ferreccio et al., 1998), decreased in the Met-deficient group. SOD activity is in close connection with other antioxidant enzymes, such as GSH-Px and CAT, which decompose hydrogen peroxide (Naziroglu, 2009). Among the non-enzymatic antioxidants, GSH plays a primary role and is regarded as an early biological marker of oxidative stress (Gagliano et al., 2006). In our study, the GSH contents were significantly reduced; as a cellular antioxidant, GSH serves as a co-substrate for GSH-Px to maintain oxidative balance (Aw, 2005).

The decreased activities of the above enzymes can lead to an excessive availability of superoxide and hydrogen peroxide (Naziroglu, 2012). In the present study, it was found that the ability to inhibit hydroxyl radicals in the intestine was decreased in the Met-deficient group, implying that antioxidant function was impaired and caused oxidative stress. Additionally, an increased level of MDA was also found; MDA is one of the several low-molecular-weight end products of lipid peroxidation, which alters membrane fluidity and increases its fragility (Chen and Yu, 1994; Naziroglu, 2012). Moreover, high levels of MDA indicate the enhancement of lipid peroxidation and an accumulation of lipid peroxides and, thus, an impairment of antioxidative and other functions in the intestine. Most cells contain Met sulphoxide reductases and, consequently, Met residues may act as catalytic antioxidants, protecting both the protein where they are located and other macromolecules (Luo and Levine, 2009). Therefore, we assume that Met deficiency may negatively influence antioxidant capacity in the intestine. This could be a consequence of Met residue oxidation of the enzyme proteins, impairment of post-translation modification/activation of these enzymes or perhaps other cause(s). The higher rate of apoptosis suggests that activation of the Nrf2-ARE signal mechanism is impaired and another signal transmission pathway, e.g. NFkB, is activated, resulting in apoptosis. Further studies would be needed to clarify the exact pathways.

In this study, the TUNEL assay and flow cytometric assay revealed an increased percentage of cells undergoing apoptosis in the Met-deficient group, suggesting that dietary Met deficiency could induce apoptosis. Previous studies have clarified that oxidative stress could result in apoptosis (Kannan and Jain, 2000), and our results suggest that oxidative stress in the intestine was induced by Met deficiency, subsequently enhancing apoptosis. The present results may be helpful in explaining the pathogenesis of cellular apoptosis induced by Met deficiency.

It is concluded that dietary Met deficiency results in a decrease of antioxidant enzyme activities, lower activity to inhibit hydroxyl radicals, and enhancement of lipid peroxidation, which consequently induce oxidative damage in the intestinal mucosa and result in cellular apoptosis in the intestine of broilers. Intestinal functions such as absorption and mucosal immunity are eventually impaired due to oxidative damage of the intestinal mucosa and cellular apoptosis. Oxidative damage-induced apoptosis may be the main mechanism underlying the effects of Met deficiency on intestinal health.

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