The effect of dietary supplementation of nitric oxide donor and inhibitor on nNOS expression in and motility of the small intestine of broilers

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Abstract

We investigated the effect of dietary supplementation of sodium nitroprusside (SNP), a nitric oxide (NO) donor, and N-nitro-L-arginine methyl ester (L-NAME), a NO inhibitor, on neuronal nitric oxide synthase (nNOS) expression in and motility of small intestine in broilers. A total of 560, one-day-old Ross 308 hybrid mixed sex broiler chicks were divided randomly into one control and seven treatment groups for a 42 day feeding trial including starter phase (0–21 days) and grower phase (22–42 days). The control group was fed a basal diet and the experimental groups were fed basal diet supplemented with 25, 50, 100 and 200 mg/kg SNP and 25, 50 or 100 mg/kg L-NAME. Ten chickens from each group were sacrificed to collect samples on days 21 and 42. The expression patterns of nNOS immunoreactivity in nerve fibers were determined by immunohistochemistry. In the contractility studies, longitudinal isolated strips of duodenum, jejunum and ileum were treated with 10⁻⁵ M L-arginine and 10⁻⁴ M SNP. Immunohistochemistry revealed that nNOS expression was not detectable in the duodenum or ileum of either the control or experimental groups. On the other hand, nNOS immunoreactivity in the jejunum control group showed a strong reaction on day 21, but the reaction was weak on day 42. nNOS expression clearly was suppressed on day 21 by the diet supplemented with L-NAME, while the diet supplemented with SNP stimulated nNOS expression on day 21. Contractility experiments revealed that spontaneous contractility of isolated strips of duodenum, jejunum and ileum showed no significant difference among groups. Spontaneous contractions of all strips were inhibited by L-arginine and SNP in all groups. The percentage inhibition rate of spontaneous contractions of jejunum application on days 21 and 42 after L-arginine decreased in the group supplemented with 100 mg/kg L-NAME. The percentage inhibition rate on day 21 after SNP application decreased in both groups that received 50 and 100 mg/kg L-NAME. We demonstrated the expression pattern of nNOS in nerve fibers in jejunum of broiler chickens. Contractility studies revealed that the NOS-NO pathway may play a role in smooth muscle contraction of small intestine of chickens. Feeding strategies that supplement NO donor and NO inhibitor can be of physiological importance to small intestine motility owing to alteration of nNOS expression in the jejunum.

Key words: broiler, jejunum, motility, nNOS

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1996), vasoactive intestinal peptide (VIP) (Goyal et al. 1980) and ATP (Fernandez et al. 1998) are among the neurotransmitters of the NANC neuronal plexus.

NO is a short half-life, highly reactive free radical (Xie et al. 1992) that after release from the endothelium can pass through membranes by diffusion and cause relaxation of smooth muscle cells (Moncada and Higgs 1993). NO is synthesized by the NO synthase (NOS) enzyme group, which uses the semi-essential amino acid, L-arginine, as a substrate (Palmer et al. 1987, Hiramatsu et al. 1999, Hiramatsu and Ohshima 2005). An exogenous NO effect can be derived from nitrate-containing compounds, such as sodium nitroprusside (SNP) (Yallampalli et al. 1993). NOS is an enzyme group that includes inducible (iNOS) and constitutive (cNOS); the latter includes neuronal NOS (nNOS) and endothelial NOS (eNOS) isoforms (Försterman et al. 1991, 1998). cNOS activity requires a Ca$^{2+}$/calmodulin complex (Försterman et al. 1991, Moncada and Higgs 1993, Weiner et al. 1994), while iNOS does not require Ca$^{2+}$ (Ann Word and Cornwell 1998).

It has been reported that nNOS is present in nerves of the mesenteric plexus associated with the autonomic nervous system in small intestines (Blottner et al. 1995). NO produced by nNOS that is present in NANC nerves plays a key role in motility of the intestinal system and in controlling passage time through digestive system (Shah et al. 2001). The relaxation of smooth muscle of the intestinal system is mediated by NO released from NANC nerves by NOS following electrical field stimulation of intestine (Toda and Herman 2005). NO may dilate the stomach to accommodate the internal pressure of this organ. Moreover, NO regulates the physiological functions of digestive system organs by relaxation of sphincters (Schleifer and Raul 1997, Lecointe Besancon et al. 1999, Toda and Okamura 2003).

There are some reports related to distribution of nNOS in small intestine (Föstermann et al. 1998, Qu et al. 1999) and evaluation of the effects and mechanisms of dietary supplementation using NO donors and inhibitors on motility of the small and large intestines of mammals in vitro (Mule et al. 1999). We found no reports concerning dietary supplementation of NO donors or inhibitors in broiler chickens, however. We investigated the effect of dietary supplementation of a NO donor and a NO inhibitor on nNOS expression and motility in vitro of the small intestine in broiler chickens.

### Material and methods

#### Animals

Our experiments were conducted at the poultry research farm in the Animal Husbandry Research Center, Afyon Kocatepe University and were approved by Ethical Committee for Animal Experiments. A total of 560 one-day-old Ross 308 hybrid mixed sex broiler chicks were divided randomly into one control and seven treatment groups for a 42 day feeding trial including starter phase (0–21 days) and grower phase (22–42 days).

The chicks were housed on saw dust bedding at a density of 12 animals/m$^2$. House temperature was maintained at approximately 32°C from 1 to 7 days of age, 29°C from 8 to 14 days of age, 26°C from 15 to 21 days of age, and 21°C thereafter. Broiler chicks were reared under continuous incandescent lighting for 23 h/day. Feed and water were provided ad libitum.

Throughout the experiments, all broilers were provided a common basal diet designed according to NRC (1994) recommendations (Table 1). The nutrient composition of the basal diet, including dry matter, crude protein, crude fat, crude fiber and crude ash contents was determined according to the AOAC (2000). Metabolizable energy (ME) including calcium, phosphorus, arginine, lysine, methionine, and cysteine content, was determined as described by Jurgens (1996). The control group was fed the basal diet only, while experimental groups were fed the basal diet supplemented with 25, 50, 100 and 200 mg/kg of the NO donor, SNP (S0501; Sigma-Aldrich Chemical Co., St. Louis, MO), and 25, 50 and 100 mg/kg of the NO inhibitor, L-NAME (S5501; Sigma).

#### Sample collection

Ten chickens from each group were sacrificed and the abdominal regions of the animals were opened to collect samples of duodenum, jejunum and ileum at the end of day 21. The same procedure was performed for the chickens from each group at the end of day 42. Therefore, intestinal tissue samples were evaluated for each feeding period.

Samples were collected from the middle portions of the duodenum, jejunum and ileum to determine the nNOS expression and motility of the small intestinum.

#### Immunohistochemistry

Samples of duodenum, jejunum and ileum were excised from each animal and fixed by immersion
Table 1. Ingredients and chemical composition of the basal diet (g/kg)

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Starter (days 0–21)</th>
<th>Grower (days 22–42)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corn</td>
<td>497.54</td>
<td>493.59</td>
</tr>
<tr>
<td>Wheat</td>
<td>100.00</td>
<td>150.00</td>
</tr>
<tr>
<td>Corn gluten</td>
<td>120.00</td>
<td>107.33</td>
</tr>
<tr>
<td>Soybean meal</td>
<td>147.13</td>
<td>95.06</td>
</tr>
<tr>
<td>Sunflower meal</td>
<td>26.83</td>
<td>53.64</td>
</tr>
<tr>
<td>Full fat soy</td>
<td>21.02</td>
<td>22.30</td>
</tr>
<tr>
<td>Blood meal</td>
<td>30.00</td>
<td>20.00</td>
</tr>
<tr>
<td>Vegetable oil</td>
<td>30.00</td>
<td>30.00</td>
</tr>
<tr>
<td>Limestone</td>
<td>6.15</td>
<td>9.58</td>
</tr>
<tr>
<td>Dicalcium phosphate</td>
<td>9.97</td>
<td>6.92</td>
</tr>
<tr>
<td>Salt</td>
<td>2.70</td>
<td>2.82</td>
</tr>
<tr>
<td>DL-Methionine</td>
<td>0.20</td>
<td>8.34</td>
</tr>
<tr>
<td>L-Lysine hydrochloride</td>
<td>2.71</td>
<td>3.01</td>
</tr>
<tr>
<td>Sodium bicarbonate</td>
<td>2.25</td>
<td>2.25</td>
</tr>
<tr>
<td>Vitamin premix a</td>
<td>2.50</td>
<td>2.50</td>
</tr>
<tr>
<td>Mineral premix b</td>
<td>1.00</td>
<td>1.00</td>
</tr>
</tbody>
</table>

Analysis results

- Dry matter: 918.60 917.50
- Crude protein: 220.70 198.60
- Crude fat: 67.70 66.40
- Crude fiber: 28.30 31.30
- Crude ash: 54.00 50.20

Calculation results:

- Calcium: 10.00 9.00
- Available phosphorus: 4.50 3.50
- Arginine: 12.50 11.00
- Lysine: 11.00 10.00
- Methionine: 5.00 4.50
- Methionine + cystein: 9.15 8.34
- Metabolic energy, kcal/kg: 3200 3200

a Provides per kg diet: Trans-retinol, 12000 IU; cholecalciferol, 1500 IU; α-tocopherol acetate, 75 mg; thiamin, 3 mg; riboflavin, 6 mg; pyridoxine, 5 mg; cobalamin, 0.03 mg; nicotinamide, 40 mg; panthotenic acid, 10 mg; folic acid, 0.75 mg; choline, 375 mg; biotin, 0.075 mg.

b Provides per kg diet: Mn, 80 mg; Fe, 40 mg; Zn, 60 mg; Cu, 5 mg; I, 0.5 mg; Co, 0.2 mg; Se, 0.15 mg.

c Calculated using values in the table (Jurgens 1996).
the solution was refreshed at 15 min intervals. The appropriate resting tension for the muscle strips was determined by preliminary experiments. The strips were placed under progressive increments of tension. Optimal tension relationships for the strips were achieved with resting tensions of 1 g to stimulate maintenance of the physiological contractile activity of the tissue. Therefore, a resting tension of 1 g was applied to the tissues. After the 30 min baseline period, contractions of longitudinal strips for each portion of small intestine for each animal were recorded to determine normal spontaneous contractions. The muscle strips then were treated with 10^{-5} M L-arginine (A8094; Sigma) to determine endogenous NO activity and 10^{-4} M SNP (S0501; Sigma) to evaluate the exogenous NO pathway. All treatments were performed on the same samples. The mean tension of spontaneous contractions calculated for each strip for a 10-min period was set as 100% (control period). Changes in intestinal contractions caused by the test substances were recorded and compared to the control period (Bulbul et al. 2007a, Yilmaz et al. 2012).

**Statistical analysis**

All values are presented as mean ± S.E. One-way ANOVA was used for statistical evaluation of the data. The Tukey test was performed to compare individual means of treatment groups. Differences were considered significant at *p* < 0.05.

**Results**

**Immunohistochemistry**

No layers of the duodenum and ileum samples showed nNOS immunoreactivity on day 21 or day 42. nNOS expression was observed, however, in nerve fibers of jejunum on both days. Strong nNOS expression was detected in the jejunum of the control at all doses of SNP day 21 (*p* < 0.001). We observed that nNOS expression was suppressed by L-NAME on day 21 (*p* < 0.001) (Table 2; Fig. 1).

Weak nNOS immunoreactivity was detected in nerve fibers of the control group on the day 42, whereas nNOS expression was increased in groups supplemented with 50, 100 and 200 mg/kg SNP (*p* < 0.001). Moreover, nNOS expression in the group supplemented with 100 mg/kg L-NAME was lower in the controls and all SNP groups (*p* < 0.001) (Table 2; Fig. 1).

**Contractility**

The spontaneous contractility of isolated strips of duodenum, ileum and jejunum showed no significant differences in any groups (Table 3).

L-arginine (10^{-5} M) and SNP (10^{-4} M) did not alter the contractile tension of duodenum and ileum in any group on day 21, while 10^{-5} M L-arginine decreased the contractile tension in the group supplemented with 100 mg/kg L-NAME (*p* < 0.01). Similarly, 10^{-4} M SNP decreased the contractile tension of jejunum in the groups supplemented with 50 and 100 mg/kg L-NAME (*p* < 0.05) (Table 4).

Isolated strips of duodenum and ileum following administration of 10^{-5} M L-arginine and 10^{-4} M SNP at the end of the 42^{nd} day, like that at the end of 21^{st} day, showed no significant differences compared to the control group; however, 10^{-5} M L-arginine, decreased the contractile tension of jejunum in the group supplemented with 100 mg/kg L-NAME (*p* < 0.05).

**Discussion**

No reports appear in the literature concerning the effect of dietary supplementation of SNP and L-NAME on nNOS expression in and small intestinal motility of broiler chickens. Therefore, we used the quantities of SNP and L-NAME that were used in previous studies of other birds (Choi et al. 1996, Khan et al. 2007) or mammals (Morley and

**Table 2. nNOS immunoreactivity in nerve fibers of jejunum sampled on days 21 and 42**

<table>
<thead>
<tr>
<th></th>
<th>SNP</th>
<th>L-NAME</th>
<th>SEM</th>
<th><em>p</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>25</td>
<td>50</td>
<td>100</td>
</tr>
<tr>
<td>Day 21</td>
<td>3.00^a</td>
<td>2.66^a</td>
<td>2.83</td>
<td>2.83</td>
</tr>
<tr>
<td>Day 42</td>
<td>1.33^b</td>
<td>1.50^b</td>
<td>2.66</td>
<td>2.50</td>
</tr>
</tbody>
</table>

SNP, sodium nitroprusside; L-NAME, *N*-nitro-L-arginine methyl ester.
Scoring: 0, no reaction; 1, weak reaction; 2, moderate reaction; 3, strong reaction; 4, very strong reaction.

^a,b,c Superscripts in the same row indicate significant differences among the groups.
Our study provides new evidence that NO may play an important role in small intestinal motility in broiler chickens. It has been shown that nNOS and NO in nerve fibers of the proventriculus of chickens may have similar functions in birds as in mammals (Martinez et al. 2000). NADPH diaphorase activity has been reported in nerve fibers of the jejunum in chickens (Hiramatsu et al. 1999), whereas there is no evidence of nNOS expression in the small intestine of other birds. Observations in cattle have indicated that nNOS and NADPH diaphorase activities in nitrergic neurons throughout digestive system from esophagus to rectum might be co-localized (Vittoria et al. 2000). We demonstrated by immunohistochemistry the presence of nNOS expression in nerve fibers of the jejunum and the absence of nNOS expression in nerve fibers of the duodenum and ileum on days 21 and 42 (Table 2; Fig. 1).

We found that nNOS immunoreactivity in nerve fibers of jejunum of all groups supplemented with all doses of SNP did not change on day 21 compared to the control group. We observed, however, that the nNOS expression of nerve fibers of the jejunum on day 21 was inhibited in groups supplemented with 25, 50 and 100 mg/kg L-NAME (Table 2). The effect of oral administration of nitric oxide donor and/or inhibitor on nNOS expression

![Representative image of neuronal nitric oxide synthase immunoreactivity in nerve fibers of jejunum on the day 21 and 42. Different reactions (arrows) were detected in samples obtained from day 21; strong reaction in control group (A), moderate reaction in the group supplemented 200 mg/kg SNP (B), weak reaction in the group supplemented 100 mg/kg L-NAME (C) and day 42; weak reaction in control group (D), strong reaction in the group supplemented 200 mg/kg SNP (E), weak reaction in the group supplemented 100 mg/kg L-NAME (F).](image)

### Table 3. The amplitude (g) of spontaneous contractility of duodenum, jejunum and ileum on days 21 and 42

<table>
<thead>
<tr>
<th></th>
<th>SNP</th>
<th>L-NAME</th>
<th>SEM</th>
<th>p &lt;</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Day 21</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Duodenum</td>
<td>0.60</td>
<td>0.67</td>
<td>0.61</td>
<td>0.51</td>
<td>0.53</td>
</tr>
<tr>
<td>Jejunum</td>
<td>0.72</td>
<td>0.77</td>
<td>0.71</td>
<td>0.70</td>
<td>0.61</td>
</tr>
<tr>
<td>Ileum</td>
<td>0.50</td>
<td>0.49</td>
<td>0.42</td>
<td>0.40</td>
<td>0.42</td>
</tr>
<tr>
<td><strong>Day 42</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Duodenum</td>
<td>0.97</td>
<td>0.86</td>
<td>0.98</td>
<td>0.98</td>
<td>0.80</td>
</tr>
<tr>
<td>Jejunum</td>
<td>0.85</td>
<td>0.87</td>
<td>0.85</td>
<td>0.85</td>
<td>0.79</td>
</tr>
<tr>
<td>Ileum</td>
<td>0.61</td>
<td>0.65</td>
<td>0.62</td>
<td>0.51</td>
<td>0.49</td>
</tr>
</tbody>
</table>

SNP, sodium nitroprusside; L-NAME, Nω-nitro-L-arginine methyl ester; NS, not significant.
nNOS expression and motility in broiler small intestine

In our contractility experiments, the diet supplemented with NO donor and inhibitor did not alter the spontaneous contractile tension of duodenum, ileum or jejunum on day 21 or day 42 (Table 3). Furthermore, both 10⁻⁵ M L-arginine and 10⁻⁴ M SNP decreased the spontaneous contractile tension of the duodenum, ileum and jejunum on day 21 (Table 4) and 42 (Table 5). NO has a short half-life (Ignorra 1990, Xie et al., 1992, Kiechle and Malinski 1993), which suggests that the similarity of spontaneous contractile tension of duodenum, ileum and jejunum may be related to the absence of NO in these tissues owing to the short half-life of NO.

It has been reported that L-arginine inhibits completely smooth muscle contractility in vitro at doses of 0.01–1 mM (Izzo et al. 1998). Bulbul et al. (2007a) demonstrated that the most effective dose of L-arginine was 10⁻⁵ M in rat intestine. Moreover, Word and Cornwell (1998) asserted that the treatment doses of SNP should be 10⁻⁸ – 10⁻³ M in vitro and that doses greater than 10⁻³ M cause irreversible inhibition owing to toxicity. It is known also that 10⁻³ M SNP treatment has a relaxant effect on smooth muscle of intestine (Bulbul et al. 2007b). We observed that SNP at dose of 10⁻³ M showed irreversible inhibition owing to toxicity (data not shown). Therefore, we used 10⁻⁴ M SNP for the present study.

We determined that the effect of L-arginine in jejunum decreased in the groups supplemented with 100 mg/kg L-NAME during both starter (Table 4) and grower phases (Table 5). Intestinal motility changes under different physiologic conditions in intestine of other birds and humans has not been reported, but there are some reports of increased NO levels in blood and other tissues after oral administration of NO donors (Chen et al. 1999, Rytlewski et al. 2005).

It has been reported that arginine, a NO donor, either stimulates the immune system or inhibits pulmonary hypertension by increasing NO levels (Hampl and Herget 2000). It is known that NO inhibitors decrease the efficiency of NO in vitro (Vapaatalo et al. 2000, Bulbul et al. 2007b). Our results were consistent with reports that SNP increased nNOS expression in nerve fibers of the jejunum, whereas nNOS expression was inhibited by L-NAME on day 42. We found that strong nNOS expression was evident in the control group on day 21. Moreover, we observed that nNOS expression among all groups supplemented with all doses of SNP showed intensity similar to the control group. Therefore, we suggest that SNP did not alter the nNOS expression and nNOS did not exist throughout the nerve fibers of the small intestine and that the intensity of nNOS expression changed during growth of the chickens.

The absorption activity of the small intestine and the contraction/relaxation of smooth muscle in the small intestine are the major factors in promoting and regulating the transport and absorption of nutrients (Rawson et al. 1990, Thompson and Applegate 2006). Therefore, we focused on the effect of NO on contractile activity of the small intestine of chickens during different feeding periods. It has been reported that NO is both an inhibitor in NANC and a neurotransmitter in isolated canine ileocolonic junction (Bult et al. 1990), longitudinal smooth muscle of the duodenum (Toda et al. 1990), and rat gastric fundus (Lie and Rand 1990).

In our contractility experiments, the diet supplemented with NO donor and inhibitor did not alter the spontaneous contractile tension of duodenum, ileum or jejunum on day 21 or day 42 (Table 3). Furthermore, both 10⁻⁵ M L-arginine and 10⁻⁴ M SNP decreased the spontaneous contractile tension of the duodenum, ileum and jejunum on day 21 (Table 4) and 42 (Table 5). NO has a short half-life (Ignorra 1990, Xie et al., 1992, Kiechle and Malinski 1993), which suggests that the similarity of spontaneous contractile tension of duodenum, ileum and jejunum may be related to the absence of NO in these tissues owing to the short half-life of NO.

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We determined that the effect of L-arginine in jejunum decreased in the groups supplemented with 100 mg/kg L-NAME during both starter (Table 4) and grower phases (Table 5). Intestinal motility changes under different physiologic...
motility owing to alteration of nNOS expression in the jejunum of broilers. It appears that the nNOS immunoreactivity in nerve fibers of the jejunum of broiler chickens. Feeding strategies including supplementation with NO donors and inhibitors can be of physiological importance to small intestinal muscle contractile activity of the small intestine of broiler chickens. It has been reported that SNP inhibits the contractility of smooth muscles by activating guanylate cyclase (GC), which diffuses into smooth muscle cells (Buhimschi et al. 1995). Activated GC increases cGMP-dependent or -independent pathways and smooth muscle consequently is relaxed (Anggard 1992, Bulbul et al. 2007b). The cGMP-dependent pathway is associated with NOS, whereas K+ channels are activated by the cGMP-independent pathway. We demonstrated that groups supplemented 25, 50 and 100 mg/kg L-NAME showed no alteration of the effect of SNP by day 42. Therefore, we suggest that SNP relaxes the smooth muscle of the jejunum by the cGMP-independent pathway mediated by L-arginine in these groups. It has been reported that SNP inhibits the contractility of smooth muscles by activating guanylate cyclase (GC), which diffuses into smooth muscle cells (Buhimschi et al. 1995). Activated GC increases cGMP-dependent or -independent pathways and smooth muscle consequently is relaxed (Anggard 1992, Bulbul et al. 2007b). The cGMP-dependent pathway is associated with NOS, whereas K+ channels are activated by the cGMP-independent pathway. We demonstrated that groups supplemented 25, 50 and 100 mg/kg L-NAME showed no alteration of the effect of SNP by day 42. Therefore, we suggest that SNP relaxes the smooth muscle of the jejunum by the cGMP-independent pathway during the grower phase in broilers.

Our results showed the expression pattern of nNOS immunoreactivity in nerve fibers of the jejunum of broiler chickens. It appears that the NOS-NO pathway may play a role in smooth muscle contractile activity of the small intestine of broiler chickens. Feeding strategies including supplementation with NO donors and inhibitors can be of physiological importance to small intestinal motility owing to alteration of nNOS expression in the jejunum of broilers.

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