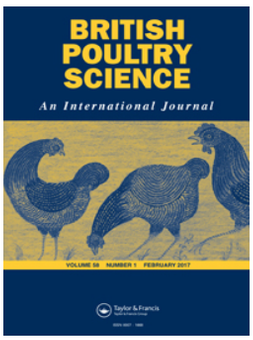

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Lipopolysaccharide reduces food passage rate from the crop by a prostaglandin-independent mechanism in chickens

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ABSTRACT

1. We examined the effect of lipopolysaccharide (LPS), a component of Gram-negative bacteria, on food passage in the digestive tract of chickens (*Gallus gallus*) in order to clarify whether bacterial infection affects food passage in birds.
2. Food passage in the crop was significantly reduced by intraperitoneal (IP) injection of LPS while it did not affect the number of defecations, suggesting that LPS may affect food passage only in the upper digestive tract.
3. Similar to LPS, prostaglandin E2 (PGE2), one of the mediators of LPS, also reduced crop-emptying rate in chickens while it had no effect on the number of defecations.
4. Pretreatment with indomethacin, which is an inhibitor of cyclooxygenase (COX), a prostaglandin synthase, had no effect on LPS-induced inhibition of crop emptying.
5. IP injection of LPS did not affect the mRNA expression of COX2 in the upper digestive tract of chickens.
6. It is therefore likely that LPS and PGE2 reduced food passage rate in the crop by a prostaglandin-independent pathway in chickens.

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Chick; crop-emptying rate; faeces; lipopolysaccharide; prostaglandin E2

Introduction

Bacterial infections are associated with non-specific symptoms including fever, somnolence, anorexia and adipsia in animals (Kent *et al*., 1992). These symptoms are triggered by bacterial components such as lipopolysaccharide (LPS), a component of the outer membrane of Gram-negative bacteria. LPS has been demonstrated to affect the function of the digestive tract. For example, LPS suppresses gastric emptying and/or motility in rats (van Miert and De la Parra, 1970; van Miert and van Duin, 1980; Wirthlin *et al*., 1996; Collares, 1997; Okumura *et al*., 1998), mice (Inada, *et al*., 2006), sheep (Leek and van Miert, 1971), goats (van Miert, 1971), dogs (Cullen *et al*., 1995) and horses (King and Gerring, 1991). Furthermore, intraperitoneal (IP) injection of LPS inhibits gastrointestinal transit and increases diarrhoea in mice (Liang *et al*., 2005). However, intravenous injection of LPS accelerates small intestinal transit of liquid diets in rats (Wirthlin *et al*., 1996) and dogs (Cullen *et al*., 1997). Moreover, colonic transit is also changed by intravenous injection of LPS in dogs (Cullen *et al*., 1995; Spates *et al*., 1998). These results demonstrate that LPS affects food passage and motility in the digestive tract in mammals although the effect is location-dependent within the digestive tract.

The effect of LPS on food passage in the digestive tract of rodents is thought to be mediated by several bioactive molecules such as tumour-necrosis factor-alpha, nitric oxide and epinephrine/norepinephrine (Wirthlin *et al*., 1996; Takakura *et al*., 1997; Inada, *et al*., 2006; Hamano *et al*., 2007). Prostaglandins (PGs) are also candidates that are proposed to mediate the effect of LPS on food passage in the digestive tract. Prostaglandin E2 (PGE2) is thought to be related to the inhibition of bowel motility by LPS in horses (King and Gerring, 1991). In addition, in rats, carbachol-induced contraction is inhibited by LPS *in vitro* and the effect is restored by indomethacin (IND), an inhibitor of cyclooxygenase (COX), a PG synthase (Hori *et al*., 2001). IND also restores LPS-induced inhibition of gastric emptying in rats (Calatayud *et al*., 2002). Moreover, LPS-induced diarrhoea and suppression of gastrointestinal transit is restored by IND in mice (Liang *et al*., 2005). There are two isoforms of COX: COX1 is thought to be the constitutively expressed isoform while COX2 is thought to be induced during inflammation. Indeed, IP injection of LPS upregulates mRNA expression of COX2 but not COX1 in the stomach of neonate and adult rats (Martinez *et al*., 2001). Another study showed that injection of LPS induces COX2 mRNA expression in the stomach of rats (Ferraz *et al*., 1997). These results suggest that PG, which can be synthesised by the action of COX2, is related to LPS-induced change in the function of the digestive tract in mammals.

In chickens (*Gallus gallus*), IP injections of LPS have been demonstrated to induce hyperthermia and anorexia (Johnson *et al*., 1993*a*, 1993*b*). In addition, intravenous injection of LPS has been reported to increase plasma PGE2 concentrations in chickens (De Boever *et al*., 2010), and PG has been demonstrated to be involved in LPS-induced hyperthermia and anorexia in chickens (Johnson *et al*., 1993*b*). However, little is known about bacterial infection or the effect of LPS on food passage in the digestive tract in chickens. Furthermore, the effect of PG on food

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passage in the digestive tract has not been clarified. Thus, the purpose of the present study was to examine whether IP injection of LPS and PGE2 affects food passage in chickens. For this purpose, effects on crop emptying and the number of defecations were investigated. Finally, the relationship between LPS and PG was examined.

Materials and methods

Animals

One-day-old male layer chickens (*G gallus*, Julia, Nihon Layer, Gifu, Japan) were raised in a room kept at 30°C with continuous lighting. A commercial diet (crude protein: 24%, metabolisable energy: 12.8 MJ/kg, Toyohashi Feed Mills Co. Ltd, Aichi, Japan) and water were available *ad libitum* to the chickens. Chickens were transferred to their individual cages 1 d before each experiment. They were weighed and distributed into experimental groups so that the average body weight was as uniform as possible between treatment groups. The chickens were maintained in accordance with the recommendations of the National Research Council (1996). This study was approved by the Committee of Animal Care and Use in Ehime University (no. 08-o3-10).

Drugs and injection

All injections were performed between 06:00 and 08:00. LPS (*Escherichia coli*, O-127:H6, Wako Pure Chemical Industries, Osaka, Japan), PGE2 (Wako Pure Chemical Industries, Osaka, Japan) and IND (Tokyo Chemical Industries, Tokyo Japan) were used for the experiments. LPS was dissolved in phosphate buffered saline (pH 7.4), PGE2 was dissolved in a normal saline solution and IND was dissolved in dimethyl sulfoxide and then diluted 10-fold with sodium carbonate. Each vehicle alone was used for the respective control treatment. These solutions were injected via the IP route at a volume of 0.2 ml per chick except as noted below.

Measurement of crop emptying

Crop-emptying rate was measured based on a method previously reported (Tachibana *et al*., 2010). Chickens were food-deprived for 15 h to empty residual ingesta within the crop. Feeders were removed from individual cages, while water was available to chickens. Chickens were gavaged into the crop with a feed slurry at a mass of approximately 4% body weight. The feed slurry was made by mixing 40% powdered diet with 60% distilled water on a weight basis. No chickens vomited post gavage in the present study. After gavage, chickens were returned to individual cages and water cups were withheld. One or two hours after the gavage, chickens were deeply anesthetised by inhalation of diethyl ether, after which their crops were exposed, the upper and lower oesophagus clamped and the crop excised. Total content of the crop was recovered and dried at 55°C for 48 h and further air-dried for 24 h. The air-dried slurry was weighed using a digital balance with a precision of 1 mg. Based on the dry weight, the wet slurry weight was calculated. The weight of the slurry emptied from the crop through the lower oesophagus was calculated by subtracting the weight of slurry within the crop from the weight of administered slurry. Crop-emptying rate was expressed as the percentage of slurry emptied from the crop to the amount gavaged.

Effect of IP injection of LPS on crop emptying

Seven-day-old chickens, which were food-deprived for 15 h, were IP injected with vehicle (control), 2 or 4 mg/kg LPS. The dose of LPS was based on previous studies of chickens (Johnson *et al*., 1993*a*, 1993*b*) and rodents (Okumura *et al*., 1998; Martinez *et al*., 2001). Immediately following injection, chickens were gavaged with the feed slurry. Chickens were then returned to individual cages. Two hours after the gavage, the crop content was obtained, dried and weighed, and then crop-emptying rate was calculated as aforementioned.

Time-course change in crop emptying after IP injection of LPS

In the first experiment, 7-d-old chickens, which were food-deprived for 15 h, were IP injected with vehicle (control) or 2 mg/kg LPS. Immediately following injection, chickens were gavaged with the feed slurry. After gavage, chickens were returned to individual cages. One or two hours after the gavage, the crop content was obtained, dried and weighed, and then crop-emptying rate was calculated as aforementioned.

In the second experiment, 7-d-old chickens, which were food-deprived for 12 h, were IP injected with the vehicle (control) or 2 mg/kg LPS and thereafter food deprivation was continued for 3 h. Then chickens were gavaged with the feed slurry, crops were removed and crop-emptying rate was calculated as described above.

Effect of IP injection of LPS on the number of defecations

Seven-day-old chickens were IP injected with the vehicle (control) or 100 μg LPS under *ad libitum* feeding conditions. The food and water cups were removed from their cages. At 30 and 60 min post injection, the number of defecations was counted on a white sheet under each cage.

Effect of IP injection of PGE2 on crop emptying

Seven-day-old chickens were food-deprived for 15 h and then IP injected with vehicle (control), 40 or 80 μg/kg PGE2. Immediately after the injection, chickens were gavaged with the feed slurry as described for the other experiments. Chickens were deeply anesthetised with diethyl ether at 2 h after the gavage, and thereafter the crop content was obtained, dried and weighed for the calculation of the crop-emptying rate.

Effect of IP injection of PGE2 on the number of defecations

Eight-day-old chickens were IP injected with the vehicle (control) or 80 μg/kg PGE2 under *ad libitum* feeding conditions. Food and water cups were removed from cages and

the number of defecations counted as for the previous experiment.

Effect of IND on IPS-induced inhibition of crop emptying

This experiment was performed to clarify the involvement of PG on LPS-induced inhibition of crop emptying in chickens using IND, an inhibitor of COX.

Seven-day-old chickens, which were food-deprived for 14.5 h, were divided into 4 groups; vehicle plus vehicle, vehicle plus 2 mg/kg LPS, 10 mg/kg IND plus vehicle and 10 mg/kg μg IND plus 2 mg/kg LPS. Chickens were IP injected with vehicle or 10 mg/kg IND at a volume of 0.1 ml. Thirty minutes after the injection, chickens were IP injected with vehicle or 2 mg/kg LPS at a volume of 0.1 ml and then gavaged with the feed slurry. After gavage, chickens were returned to individual cages. Two hours after the gavage, the crop content for each chick was obtained, dried and weighed, and crop-emptying rate was calculated. The second experiment was performed using 20 mg/kg IND and identical procedures.

Effects of LPS on the mRNA expression of COX2 in the digestive tract

Gene expression of COX2 was determined in the digestive tract in order to investigate whether LPS affects the synthesis of PG.

Eight-day-old *ad libitum*-fed chickens were IP injected with vehicle (control) or 2 mg/kg LPS. At 3 h post injection, crop, lower oesophagus (Es), proventriculus (Pv), gizzard (Gz) and duodenum (Du) were collected, weighed and then frozen with liquid nitrogen and stored at −80°C until analysis.

The expression of COX2 mRNA expression was measured in these tissues. Tissues were homogenised with Sepasol-RNA I Super G (Nacalai Tesque, Kyoto, Japan), and then total RNA was isolated as per the manufacturer's instructions. First-strand cDNA was synthesised from DNase I (Ambion, Austin, TX, USA)-treated total RNA using the reverse transcription kit ReverTra Ace qPCR RT Master Mix with gDNA Remover (Toyobo, Osaka, Japan) with random primers. The cDNA of COX2 was amplified and quantified by quantitative real-time PCR using specific primers for COX2 (product size, 131 bp; forward primer, 5′-ctgctccctcccatgtcaga-3′; reverse primer, 5′-cacgtgaagaattccggtgtt-3′). As an internal standard, polymerase (RNA) II (DNA directed) polypeptide B (RPII) was also amplified (product size, 172 bp; forward primer, 5′-cagaatttgccgacctcttc-3′; reverse primer, 5′-ggccagcatcacagtctctt-3′). PCR parameters for assays were as follows: 95°C for 10 min then 45 cycles at 95°C for 10 s, 60°C for 10 s and 72°C for 15 s. The accuracy of these primers was verified by sequencing each PCR product. The *Ct* value was calculated using the $2^{-\Delta\Delta Ct}$ method.

Statistical analysis

Data for the effects of LPS and PGE2 on the weight of slurry in the crop and crop emptying were analysed with one-way analysis of variance (ANOVA) and the Tukey–Kramer test *post hoc*. Time-course changes in the effect of LPS on the weight of slurry in the crop and crop emptying were analysed with two-way ANOVA with respect to LPS and time, with *t*-tests performed at each time. The number of defecations was analysed with two-way repeated-measures ANOVA. Real-time PCR data were analysed with *t*-tests. Data are expressed as means ± SEM, and statistical significance was set at $P < 0.05$. The numbers of chickens are stated in the figures.

Results

Effect of IP injection of LPS on crop emptying

Chickens were orally administered the slurry at 2.226 ± 0.006 g. IP injection of 2 and 4 mg/kg LPS significantly increased the amount of slurry remaining in the crop at 2 h after the injection and thereby significantly decreased the crop-emptying rate (Figure 1). The effect of LPS was not different among doses.

Time-course change in crop emptying after IP injection of LPS

Chickens were orally administered the slurry at 2.230 ± 0.002 g. Although the effect of LPS was not significant in terms of the amount of slurry remaining in the crop [$F(1,26) = 2.531$, $P = 0.124$] and crop-emptying rate [$F(1,26) = 2.504$, $P = 0.126$], there was a significant interaction between LPS and time for the amount of slurry remaining in the crop [$F(1,26) = 6.489$, $P < 0.05$] and crop-emptying rate [$F(1,26) = 6.535$, $P < 0.05$]. The IP injection of 2 mg/kg LPS did not affect the amount of slurry remaining in the crop and hence the crop-emptying rate at 1 h (Figure 2(a)). However, at 2 h LPS significantly increased the amount of slurry and significantly decreased the crop-emptying rate (Figure 2(a)).

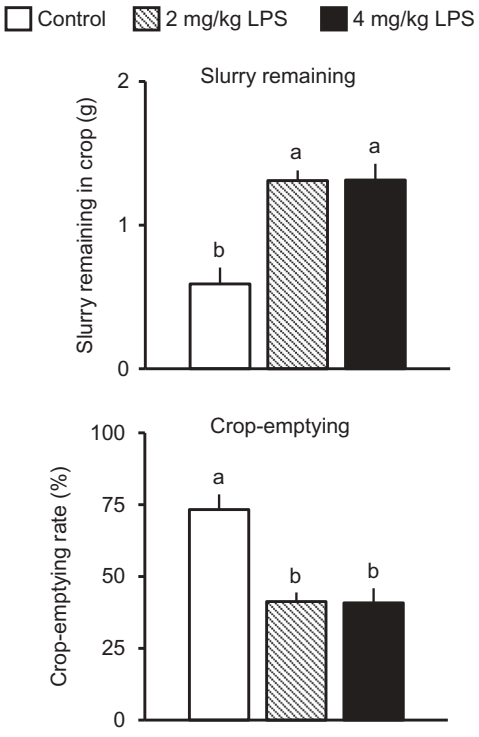

Figure 1. Effect of intraperitoneal injection of lipopolysaccharide (LPS) on slurry remaining in the crop and crop emptying in chickens. Data are expressed as mean ± SEM ($n = 7$ in each group). Groups with different letters within a time period are significantly different ($P < 0.05$).

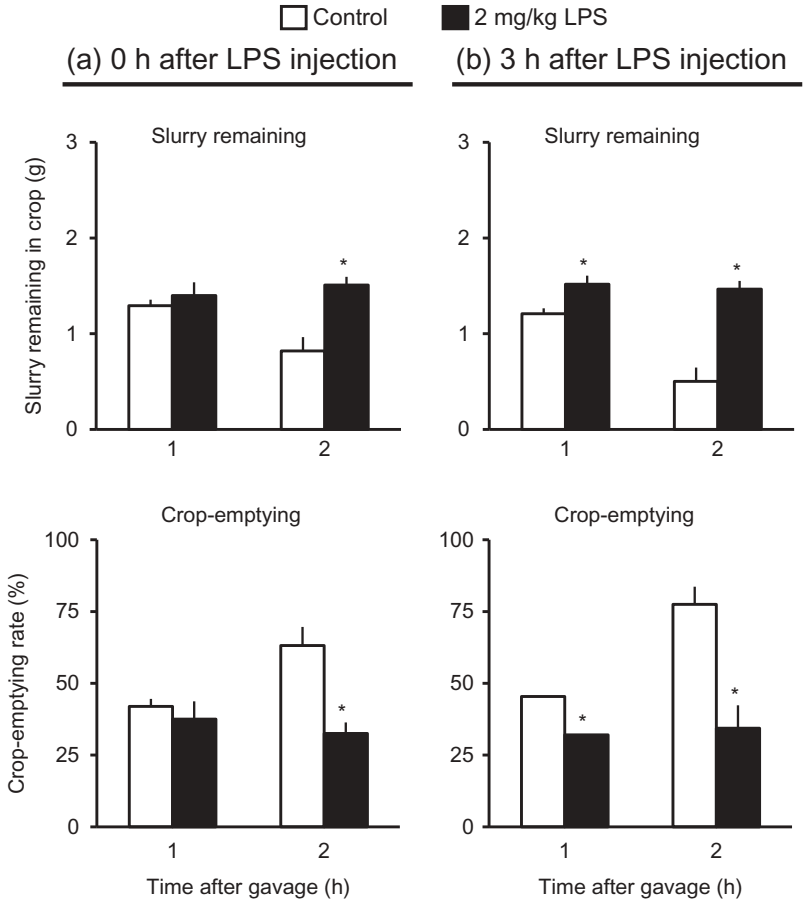

Figure 2. Slurry remaining in the crop and crop emptying after intraperitoneal (IP) injection of lipopolysaccharide (LPS) in chickens. (a) The IP injection was performed immediately before the feed gavage. Data are expressed as mean ± SEM (n = 7 in each group for 1 h and n = 8 in each group for 2 h). (b) The IP injection was performed 3 h before the feed gavage. Data are expressed as mean ± SEM (n = 8–9 for 1 h and n = 8 in each group for 2 h). Asterisks indicate a significant difference from the control group at each time (P < 0.05).

When LPS was injected at 3 h before the gavage, a significant effect of LPS was observed for the amount of slurry remaining the crop [$F(1,29)$ = 9.526, P < 0.01] and crop-emptying rate [$F(1,29)$ = 9.789, P < 0.01]. The interaction between LPS and time was also significant for the amount of slurry remaining the crop [$F(1,29)$ = 7.123, P < 0.05] and crop-emptying rate [$F(1,29)$ = 7.338, P < 0.05]. There was a significant increase in the amount of slurry remaining the crop and decrease in the crop emptying rate from 1 h that continued to 2 h (Figure 2(b)).

Effect of IP injection of LPS on the number of defecations

The number of defecations of chickens in the control group was 1.3 ± 0.3 and 2.2 ± 0.4 at 30 and 60 min after the injection, respectively. On the other hand, the number of defecations in the LPS group was 1.7 ± 0.4 and 2.8 ± 0.4 at 30 and 60 min, respectively. The effect of LPS on the number of defecations was not significant [$F(1,23)$ = 1.223, P = 0.280]. The interaction between LPS and time was also not significant [$F(1,23)$ = 0.266, P = 0.611].

Effect of IP injection of PGE2 on crop emptying

Chickens were orally administered the slurry at 2.234 ± 0.004 g. IP injection of 40 and 80 μg/kg PGE2 significantly increased the amount of slurry remaining in the crop and thereby decreased the crop-emptying rate at 2 h (Figure 3).

Effect of IP injection of PGE2 on the number of defecations

The number of defecation of chickens in the control group was 1.4 ± 0.3 and 2.2 ± 0.4 at 30 and 60 min after the injection, respectively. On the other hand, the number of defecations in the LPS group was 1.8 ± 0.4 and 2.9 ± 0.4 at 30 and 60 min, respectively. IP injection of 80 μg/kg PGE2 did not affect the number of defecations [$F(1,18)$ = 1.231, P = 0.282]. The interaction between PGE2 and time was not significant [$F(1,18)$ = 0.491, P = 0.493].

Effect of IND on IPS-induced inhibition of crop emptying

Chickens were orally administered slurry at 2.227 ± 0.004 g. IP injection of 2 mg/kg LPS alone significantly increased the amount of slurry and decreased crop emptying as noted before, and 10 mg/kg IND treatment had no effect on crop emptying (Figure 4). Pretreatment with 10 mg/kg IND did not affect the amount of LPS-induced slurry in the crop or LPS-induced decreases in crop emptying (Figure 4).

Similarly, LPS-induced increases in the amount of feed slurry and decreased crop emptying were not affected by pretreatment with 20 mg/kg IND (Figure 4).

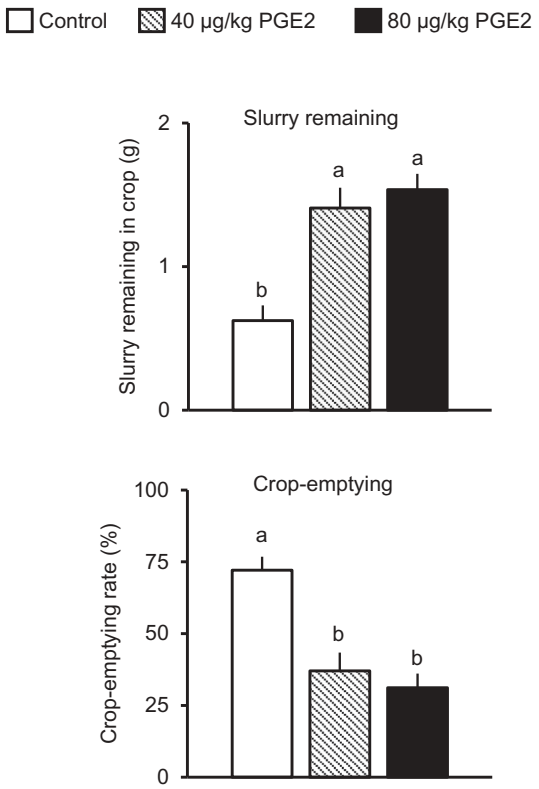

Figure 3. Slurry remaining in the crop and crop emptying after intraperitoneal injection of prostaglandin E2 (PGE2) in chickens. Data are expressed as mean ± SEM (n = 7 in each group). Groups with different letters within a time period are significantly different (P < 0.05).

Effects of LPS on the mRNA expression of COX2 in the digestive tract

The IP injection of 100 μg LPS did not affect the mRNA expression of COX2 in any portion of the digestive tract studied (Figure 5).

Discussion

The present study demonstrated that IP injection of 2 and 4 mg/kg LPS significantly reduced crop-emptying rate in chickens. Because the crop is located in the middle oesophagus and stores ingesta (Denbow, 2000), Gram-negative bacteria may suppress food passage in the upper digestive tract in chickens, a result similar to mammals. In mammals, many studies have revealed that injection of LPS inhibits gastric emptying and/or motility (van Miert and De la Parra, 1970; Leek and van Miert, 1971; van Miert, 1971; van Miert and van Duin, 1980; King and Gerring, 1991; Cullen *et al.*, 1995; Wirthlin *et al.*, 1996; Collares, 1997; Okumura *et al.*, 1998; Inada, *et al.*, 2006). For example, IP injection of 4.5 mg/kg LPS significantly decreased gastric emptying in rats (Okumura *et al.*, 1998). Furthermore, IP injection of 2 mg/kg LPS significantly suppressed gastric emptying in mice (Inada, *et al.*, 2006). These results imply that LPS suppresses food passage from the Pv to Gz and thus crop emptying might be reduced in chickens. Indeed, it is likely that whether the diet remains in the crop or is passed on to the Pv is dependent on the contraction of the Gz, while evacuation of the diet from the crop itself is mediated by contraction of the crop wall (Denbow, 2000). Further studies which investigate the effect of LPS on Pv and Gz will clarify whether LPS directly affects crop emptying. On the other hand, IP injection of 2 mg/kg LPS had no effect on the number of defecations in chickens. This result is

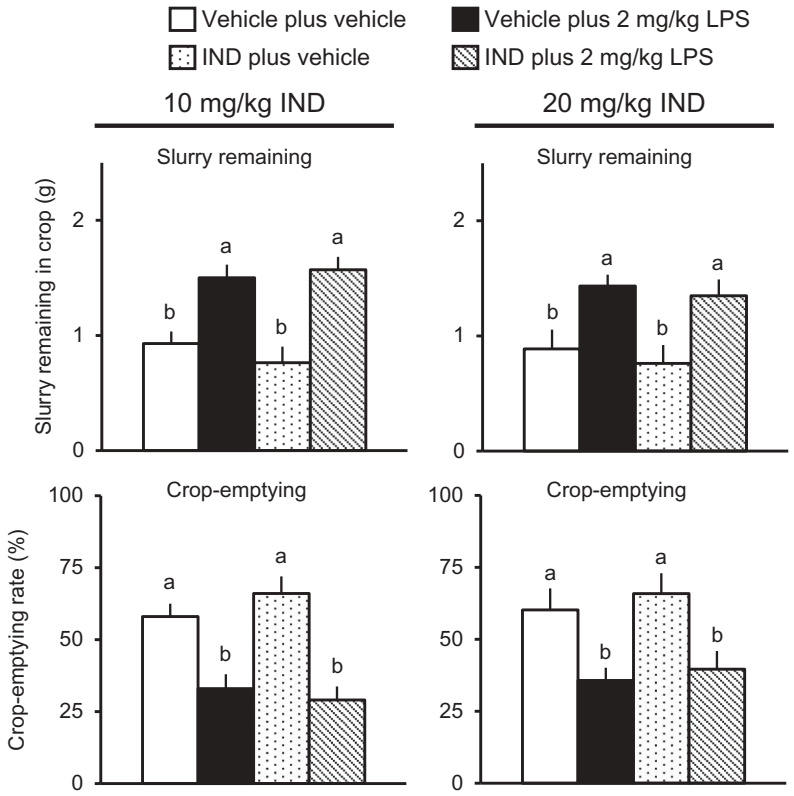

Figure 4. Effect of intraperitoneal (IP) injection of indomethacin (INDO) on lipopolysaccharide (LPS)-induced increase in slurry remaining in the crop and the inhibition of crop emptying. (a) 10 mg/kg INDO was injected at 30 min prior to IP injection of 2 mg/kg LPS. Data are expressed as means ± SEM (n = 6–7 in each group). (a) 20 mg/kg INDO was injected at 30 min prior to IP injection of 2 mg/kg LPS. Data are expressed as mean ± SEM (n = 6–7 in each group). Groups with different letters within a time period are significantly different (P < 0.05).

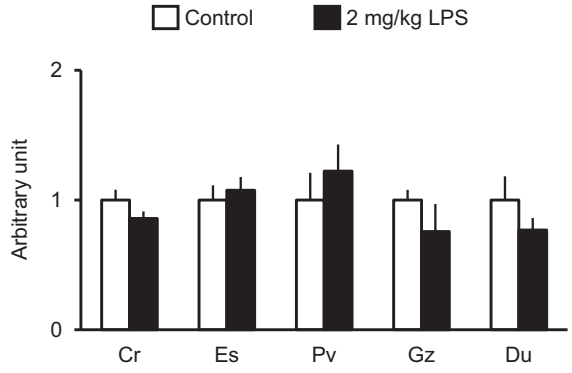

Figure 5. Effect of IP injection of 2 mg/kg LPS on mRNA expression of COX2 in the upper digestive tract. Cr, crop; Es, lower oesophagus; Pv, proventriculus; Gz, gizzard; Du, duodenum. Data are expressed as mean ± SEM (n = 7–8 in each group).

inconsistent with mammalian studies because LPS affects colonic transit in dogs (Cullen *et al.*, 1995; Spates *et al.*, 1998) and colonic contraction in horses (King and Gerring, 1991). It is thus likely that the effect of LPS on the lower digestive tract is different between chickens and mammals. However, Spates *et al.* (1998) showed that intravenous injection of 200 μg/kg LPS facilitated colonic transit in dogs. Thus, it remains a possibility that the difference in the experimental conditions affects the present result.

Although IP injection of LPS significantly decreased crop emptying in chickens, the effect is not fast-acting because LPS did not affect the crop-emptying rate at 1 h after the injection. This late onset suggests that LPS might affect crop emptying indirectly and that some mediator might be related to the effect of LPS. PG is one of the candidates thought to mediate LPS-induced changes in motility of and transit in the gastrointestinal tract of mammals (King and Gerring, 1991; Hori *et al.*, 2001; Calatayud *et al.*, 2002; Liang *et al.*, 2005).

Among the PG, PGE2 is associated with food passage in the gastrointestinal tract in mammals. For example, it is reported that PGE2 contributes to suppression of gastric emptying induced by intragastric injection of a hyperosmolar NaCl solution in rats (Nishiyama *et al.*, 1992; Mizuguchi *et al.*, 2010). Intragastric injection of enprostil, a PGE2 derivative, also reduces gastric emptying of barium grains in rats (Sakurai *et al.*, 1996). On the other hand, enprostil does not reduce gastric emptying in humans while it increases mouth-to-caecum transit time (Nicholl *et al.*, 1989). In contrast to these results, Berstad *et al.* (1988) reported that enprostil accelerates gastric emptying in humans. Nompleggi *et al.* (1980) showed that 15(*S*)-15-methyl PGE2, which is a PGE2 analogue, facilitates gastric emptying in rhesus monkeys. Rowart and Rush (1984) demonstrated that PGE2 and its analogue 16, 16-dimethyl PGE2 affect gastric emptying and small intestinal transit in rats but that their effects are dependent on dosage and injection route. Thus, the effect of PGE2 on food passage in the digestive tract is complex in mammals. In the present study, on the other hand, IP injection of 40 and 80 μg/kg PGE2 clearly inhibited crop emptying in chickens while it had no effect on the number of defecations. It is therefore likely that PGE2 modifies food passage in the upper but not lower digestive tract in chickens as observed in the LPS study.

However, pretreatment with IND, an inhibitor of PG synthase, did not attenuate the effect of LPS, suggesting that PG might not be associated with LPS-induced inhibition of crop emptying in chickens. In addition, IP injection of LPS had no effect on the mRNA expression of COX2 in the crop, Es, Pv, Gz or Du. Thus, it is likely that PG, including PGE2, does not mediate LPS-induced changes in the upper digestive tract in chickens. In contrast to chickens, IND restores LPS-induced inhibition of gastric emptying in rats (Calatayud *et al.*, 2002) and IP injection of LPS upregulates mRNA expression of COX2 but not COX1 in the stomach of neonate and adult rats, whereas expression was not altered in the small and large intestines (Martinez *et al.*, 2001). Thus, the responsiveness of PG synthesis to LPS treatment seems to be different between chickens and rodents. On the other hand, it has been reported that LPS-induced COX2 mRNA expression is dependent on the sources of LPS: COX2 mRNA expression in gastric epithelial cells is increased by *E. coli* LPS but not *Helicobacter pylori* LPS *in vitro* (Smith *et al.*, 2003). Although *E. coli* LPS did not affect COX2 mRNA expression in the upper digestive tract in the present study, LPS from other sources might affect COX2 transcription.

The factors that are involved in LPS-induced inhibition of crop emptying in chickens are still unknown. Since interleukin 1, nitric oxide, tumour-necrosis factor alpha and the alpha2-adrenergic system are thought to mediate LPS-induced inhibition of gastric emptying in mammals (Takakura *et al.*, 1997; Endo and Kumagai, 1998; De Winter *et al.*, 2002; Liang *et al.*, 2005; Inada, *et al.*, 2006; Hamano *et al.*, 2007), some of them might be related to the inhibition of crop emptying induced by LPS in chickens as well. Another candidate is corticosterone because IP injection of LPS increases plasma corticosterone (Johnson *et al.*, 1993*a*) and subcutaneous injection of corticosterone retards crop emptying in chickens (Ogino *et al.*, 2016).

In sum, we showed that LPS injection suppresses food passage in the upper digestive tract in chickens. Further study will clarify the actual mechanism underlying the inhibition of food passage in chickens.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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