



Protective effect of Tanshinone IIA on LPS-induced canine endometritis

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ABSTRACT

Canine endometritis is a common disease in dogs. This work intends to establish the model of lipopolysaccharide (LPS) -induced canine endometritis, and investigate the effect of Tanshinone IIA (Tan IIA) on canine endometritis. At first, we tested the rectal temperature and the production of IL-1 β and TNF- α at 6 h, 12 h, 18 h and 24 h after LPS administration. Then 9 beagles were divided into 3 groups on average, all beagles received intraperitoneal injection of saline solution (group 1 and 2) or Tan IIA (group 3) at 6 h before and after LPS challenge. Beagles of group 2 and 3 were performed uterine infusion of LPS, and beagles of group 1 were performed uterine infusion of saline solution. The rectal temperature was measured 6 h, 12 h, 18 h, 24 h post-LPS challenge, all uterus were collected after 24 h post-LPS challenge. The results showed that canine endometritis can be established by LPS at the concentration of 0.5 mg/kg of body weight after 24 h performance. The rectal temperature, the production of IL-1 β and TNF- α increased significantly when the model was established. The results showed that rectal temperature, production of IL-1 β and TNF- α and the expression of IL-6 were significantly reduced after treatment with Tan IIA compared with the group of LPS challenge only. However, the expression of IL-10 increased after Tan IIA treatment. Considering the positive anti-inflammation effect on the LPS-induced canine endometritis, Tan IIA may be used as a therapeutic agent to treat the clinical canine endometritis.

Keywords: Anti-inflammation, Beagles, Endometritis, Tan IIA

Endometritis is a common disease in dogs throughout the world (Hagman and Greko 2005). It also causes severe clinical symptoms, even resulting in dog death (Chu *et al.* 2002). Endometritis primarily infected by a large number of pathogenic microorganisms (Li *et al.* 2015). LPS is the main components in the outer membrane of Gram-negative bacteria, which may induce severe endometritis in mice (Fu *et al.* 2015, Lv *et al.* 2015).

Administration of LPS leads to releases of pro-inflammation cytokines, including IL-1 β and TNF- α . IL-1 β was generated by mononuclear cells, macrophages, epithelial cells and stromal cells (Diwakar *et al.* 2019, Lulijwa *et al.* 2019). IL-1 β is produced in an inactive form, which requires the protease-1 activation (Liu *et al.* 2016). And, mature IL-1 β is required for the activation of body's immune response (Alipour *et al.* 2013). Many studies showed that IL-1 β is a endogenous pyrogen factor, associated with the initial inflammation (Martinon *et al.* 2006). IL-1 β and TNF- α can stimulate the body to produce inflammatory response by improving the phagocytosis of neutrophils and promoting neutrophils adhesion to endothelial cells (Yuan *et al.* 2016). Then activated

neutrophils also induce the release of TNF- α and IL-1 β (Furlaneto and Campa, 2000). This reaction forms a vicious circle that aggravates the inflammatory response. Additionally, many pro- and anti-inflammatory cytokines were involved in the development of inflammation, such as pro-inflammatory cytokines IL-6 and IL-8, anti-inflammatory cytokine IL-10 (Flores-Espinosa *et al.* 2014, Wang *et al.* 2018). They all play an important role in the regulation of inflammation.

Tan IIA is the main active component in the herbal drug Danshen. Previous studies have shown that Tan IIA have anti-apoptotic, anti-oxidative and anti-inflammatory effect (Yao *et al.* 2017). Tan IIA can relief lung injury significantly in LPS induced mice models (Xu *et al.* 2009). We have found that Tan IIA have the effect to inhibit endometrial inflammation induced by LPS in mouse model (Lv *et al.* 2015). However, the anti-inflammatory effects of Tan IIA in the canine endometritis induced by LPS remain unclear. In the present study, we evaluated the anti-inflammatory effects of Tan IIA in the canine endometritis, and found that Tan IIA may be a potential anti-inflammatory drug.

MATERIALS AND METHODS

Dogs: Beagle dogs (20–24 months old) with a body weight at 13 $\pm$ 0.5 kg were purchased from Qingdao Bolong

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Experimental Animal Co. Ltd. (Qingdao, China). All experiments were approved by the Institutional Animal Care and Use Committee at Qingdao Agricultural University and performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Chemical materials: Tan IIA (Cat No: ST8020, Fig. 1) was purchased from Beijing Solarbio Science and technology Co., Ltd. (Beijing, China). LPS (Cat No: L8880) from *Escherichia coli* O55:B5 was purchased from Beijing Solarbio Science and technology Co., Ltd. (Beijing, China). Dog IL-1 β (Cat No: SEA563Ca) and TNF- α (Cat No: SEA133Ca) Enzyme linked immune sorbent assay (ELISA) kits were purchased from Cloud-Clone Corp. (Wuhan, China).

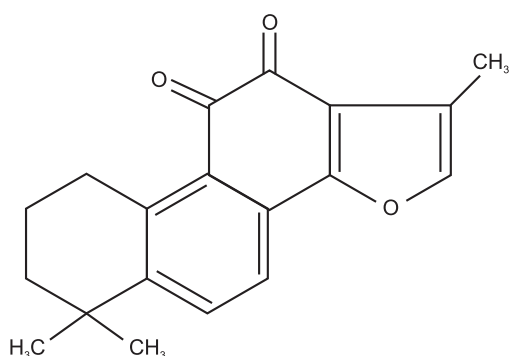


Fig. 1. Chemical structure of Tanshinone IIA.

Canine endometritis model and experimental design: Beagle dogs were anesthetized before LPS administration, then dissolved 0.5 mg/kg LPS into 5 mL saline solution, and infused them into uterus of Beagle dogs. Twenty-four hours later, Beagle dogs were anesthetized, and uterus were collected and stored at -80° until use. Nine Beagle dogs were randomly divided into three groups: (1) saline uterine infusion and saline intraperitoneal injection, (2) LPS uterine infusion and saline intraperitoneal injection, (3) LPS uterine infusion and Tan IIA intraperitoneal injection. Each uterus was infused with 5 mL LPS, which was made up of 0.5 mg/kg LPS and appropriate volume saline solution. Tan IIA or saline solution was administered at 6 h before and 6 h after the LPS infusion.

ELISA: Uteri samples were collected 24 h after LPS administration for ELISA analysis. The endometrium of each group was taken from the same site and weighed, homogenized in RIPA buffer (1:9, w:v), and centrifuged at 20,000 g for 15 min at 4°C . The supernatants were extracted to assay the levels of IL-1 β and TNF- α by ELISA kits according to the manufacturer's instructions.

RNA extraction and RT-qPCR: The total RNA from each uterus was isolated by using EASYsPin (Aidlab, Beijing, China) according to the manufacturer's instructions. RNA concentration and purity were determined and reverse-transcribed to cDNA with PrimeScript RT reagent kit (TaKaRa, Tokyo, Japan), as described in the manufacturer's protocol. Then the expression levels of mRNA were measured by using SYBR II (Vazyme, Nanjing, China) on

Table 1. Primers for qPCR analysis

Gene	Sequence
IL-6-F	GGCCACACGTAGAGGAAAGG
IL-6-R	CAGGATCGGATGTGCTTCCT
IL-10-F	CAAAGCAAAGGAAGCGGACC
IL-10-R	AGGCTGCGCTGATGAAACAT
β -actin-F	TGTGTTATGTGGCCCTGGAC
β -actin-R	TTCCATGCCCAGGAAGGAAG

the real-time PCR analysis instrument (ABI, MA, USA). The primers for RT-qPCR were designed by Primer 3 software and listed in Table 1.

Statistical analysis: All values are expressed as the means $\pm$ SEM. Difference between mean values of normally distributed data were analyzed using two-tailed Student's T-Test. Statistical significance was accepted at $P < 0.05$ or $P < 0.01$.

RESULTS AND DISCUSSION

LPS induced the model of canine endometritis: To determine the best conditions of making canine endometritis model, different concentration of LPS was infused into the uterus. Then we tested the rectal temperature at 6 h, 12 h, 18 h and 24 h after LPS administration. As shown in Table 2, the temperature was significantly increased at 12 h after LPS administration with 0.5 mg/kg and 1 mg/kg compared to the saline solution treatment. To test the inflammation intensity, we measured the levels of IL-1 β and TNF- α with ELISA kits at 24 h after LPS administration. As shown in Fig. 2A and B, the levels of IL-1 β and TNF- α were significantly increased after LPS administration with 0.5 mg/kg. Based on the rectal temperature and the levels of IL-1 β and TNF- α , we determined that the model of canine endometritis can be constructed after 24 h using LPS administration with 0.5 mg/kg.

There is no canine endometritis model which can be used for the drug development. In this study, we have recorded the rectal temperature and the expression of pro-inflammatory cytokines after LPS administration with different doses or different times. At last, we determined the optimal condition to construct the canine endometritis is

Table 2. Change of rectal temperature ($^{\circ}\text{C}$) after LPS administration with different doses

	0	6 h	12 h	18 h	24 h
Saline solution		38.40 $\pm$ 0.10	38.50 $\pm$ 0.00	38.70 $\pm$ 0.10	38.65 $\pm$ 0.15
0.25 mg/kg LPS		38.60 $\pm$ 0.10	38.80 $\pm$ 0.10	39.05 $\pm$ 0.05	39.10 $\pm$ 0.10*
0.5 mg/kg LPS		38.40 $\pm$ 0.10	39.10 $\pm$ 0.00*	39.55 $\pm$ 0.05*	40.40 $\pm$ 0.30*
1 mg/kg LPS		38.50 $\pm$ 0.10	39.55 $\pm$ 0.05**	39.95 $\pm$ 0.05**	40.70 $\pm$ 0.20**

* $P < 0.05$ and ** $P < 0.01$ significantly different from saline solution group.

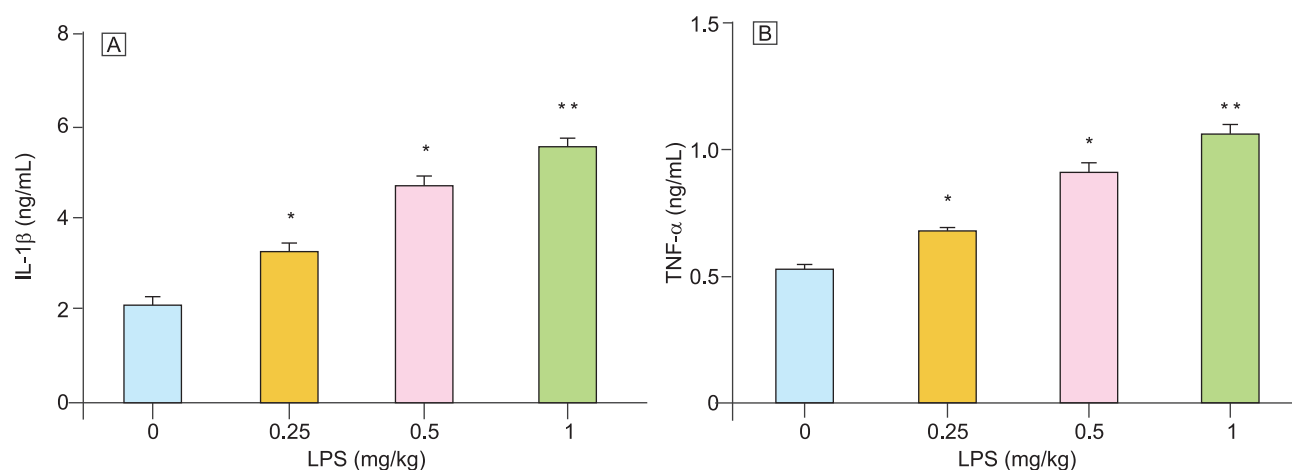


Fig. 2. LPS induced the production of IL-1 β and TNF- α in a dose-dependent manner. A. Production of IL-1 β was induced by LPS with different dose; B. Production of TNF- α was induced by LPS with different dose. * P <0.05 and ** P <0.01 significantly different from Saline solution group.

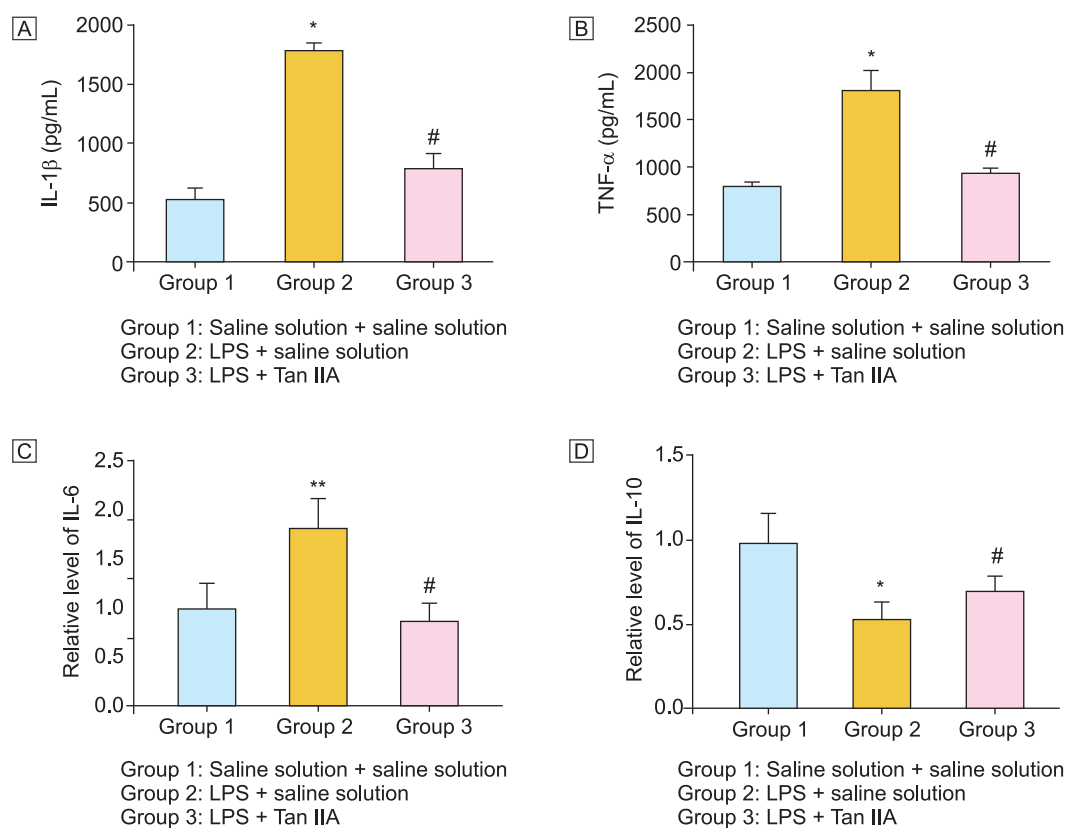


Fig. 3. Tan IIA suppressed the production of pro-inflammatory cytokines induced by LPS. A. The production of IL-1 β induced by LPS was suppressed by Tan IIA; B. The production of TNF- α induced by LPS was suppressed by Tan IIA; C. Tan IIA inhibited the mRNA expression of IL-6 induced by LPS; D. Tan IIA increased the mRNA expression of IL-10 induced by LPS.

* P <0.05 significantly different from Saline solution group; # P <0.05 significantly different from LPS treatment group.

giving LPS at the concentration of 0.5 mg/kg of body weight by uterine infusion. After 24 h administration, we found that the rectal temperatures, the levels of pro-inflammatory cytokines were significantly different from saline solution group. This canine endometritis model can be used to develop canine endometritis associated drugs.

Tan IIA improved the rectal temperature during LPS administration: LPS administration raised the rectal temperature significantly with the passage of time. The rectal temperature increased close to 40°C after 24 h using LPS administration. In Tan IIA treatment group, the rectal temperature was controlled, and decreased significantly

compared to the model group. Dogs in the Tan IIA treatment group returned to normal temperature basically.

Tan IIA decreased the levels of IL-1 β and TNF- α induced by LPS: IL-1 β and TNF- α are the most important pro-inflammatory factors in the inflammation. As results showed that the production of IL-1 β and TNF- α were significantly increased after LPS administration compared to control group (Fig. 3A and B). In contrast, these increased cytokines induced by LPS were declined by Tan IIA.

Tan IIA coordinated the expression of inflammatory cytokines: In order to make clear that pro-inflammatory cytokines were decreased through control the gene expression or neutralization by Tan IIA. We tested the mRNA level of IL-6 using qPCR. As shown in Fig. 3C, Tan IIA decreased significantly the expression of IL-6 compared to the model group. To make sure that anti-inflammatory cytokines were decreased by Tan IIA, we tested the mRNA level of IL-10 using qPCR. As shown in Fig. 3D, Tan IIA increased significantly the expression of IL-10 compared to the model group.

IL-1 β and TNF- α are the main pro-inflammatory cytokines, which induced lots of neutrophils to come to the infection site and lead to serious inflammatory response (Chen *et al.* 2017). We found the levels of IL-1 β and TNF- α were significantly increased after LPS administration. To investigate whether Tan IIA improve the canine endometritis, we examined the levels of IL-1 β and TNF- α . The results showed that Tan IIA inhibit the production of IL-1 β and TNF- α . According, the anti-inflammatory effects of Tan IIA may be exerted by limiting the production of pro-inflammatory cytokines.

Tan IIA is derived from the root or rhizome of *Salvia miltiorrhiza bunge*. Previous studies have showed that Tan IIA exerts anti-inflammatory effect on vulnerable atherosclerotic plaque (VAP) via the NF-KB pathway, and inhibits glucose metabolism leading to apoptosis of cervical cancer cells (Munagala *et al.* 2015, Zhao *et al.* 2016). In our previous studies, we have certified that Tan IIA exerts anti-inflammatory effect in the mouse endometritis model through inhibiting the activity of NF-KB pathway (Lv *et al.* 2015). However, Tan IIA exerted the effect of anti-inflammation via coordinating the signaling pathway or neutralizing the pro-inflammatory cytokines. We tested the mRNA level of IL-6 and IL-10, and found that Tan IIA promoted the expression of IL-10 while inhibited IL-6.

In summary, Tan IIA plays important role in the canine endometritis. Tan IIA can inhibit the inflammatory response via coordinating the expression of pro- and anti-inflammatory cytokines. These results indicated that Tan IIA might be a potential therapeutic agent against the canine endometritis.

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