



## RESEARCH ARTICLE

### Protective Effects of Salidroside and Dexamethasone against *E.coli*-Induced Inflammatory Response on Endometrial Epithelium Cells in Yaks

Hailong Dong<sup>1</sup>, Muhammad Ijaz<sup>2</sup>, Khalid Mehmood<sup>3</sup>, Muhammad Muddassir Ali<sup>4</sup>, Fayi Tian<sup>1</sup>, Jiakui Li<sup>1</sup>, Shakeel Ahmed<sup>5</sup>, Aftab Shaukat<sup>6</sup>, Zhenyu Chang<sup>1\*</sup> and Qingxia Wu<sup>1\*</sup>

<sup>1</sup>Animal Science College, Tibet Agriculture & Animal Husbandry University, Linzhi 860000, Tibet, People's Republic of China; <sup>2</sup>Department of Clinical Medicine & Surgery, University Veterinary and Animal Sciences Lahore-54000, Punjab, Pakistan; <sup>3</sup>University College of Veterinary & Animal Sciences, the Islamia University of Bahawalpur, 63100, Pakistan; <sup>4</sup>Institute of Biochemistry and Biotechnology, University of Veterinary and Animal Sciences, Lahore, 54600, Pakistan; <sup>5</sup>Lab of Drug Discovery and Molecular Engineering, Department of Medicinal Plants, College of Plant Science and Technology, Huazhong Agricultural University, Wuhan 430070, P.R. China; <sup>6</sup>Department of Clinical Veterinary Medicine, College of Veterinary Medicine, Huazhong Agricultural University, Wuhan 430070, P.R. China  
\*Corresponding author: zychang2014@sina.com; wuqx2014@sina.com

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#### ABSTRACT

Endometritis is an inflammation of the uterus, which can reduce the reproductive performance in yaks. Salidroside is a natural monomer of *Rhodiolarosea*, it has anti-inflammatory, antioxidative and immune regulation properties. We conduct this study to investigate the effect of salidroside and dexamethasone on *E. coli*-induced inflammatory response on endometrial epithelium cells in yaks. For this purpose, the endometrial epithelial cells were cultured and dived into four groups: control group, *E.coli* group, salidroside group and dexamethasone group. The antioxidant response, cytokine assays and immunofluorescence staining analysis were performed. The results showed that the intracellular activities of SOD were decreased, while MDA contents were increased significantly in *E.coli*-induced inflammation group. Whereas, the MDA contents decreased and SOD increased in salidroside and dexamethasone supplement groups. Moreover, *E. coli* results in significantly increase in cytokine production. The TNF- $\alpha$ , IL-1 $\alpha$ , IL-6 and IL-10 levels in the cells treated with salidroside and dexamethasone were decreased significantly compared to those of the *E.coli* group. *E.coli*-induced inflammatory cells showed an increase in the level of TNF- $\alpha$  protein compared to control group. The salidroside administration reduced the TNF- $\alpha$  expression significantly just like dexamethasone. In conclusion, salidroside and dexamethasone play important role in regulating *E.coli*-induced inflammatory response in endometrial epithelium cells. Furthermore, salidroside treatment is encouraged for the control of endometritis, which may target to reduce the incidence of endometritis.

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#### INTRODUCTION

The yak (*Bos grunniens*) is relatively an ancient and indigenous breed in China with the characteristic of cold and oxygen deficit tolerance lying at altitude of more than 3000m above the sea level in Qinghai-Tibet plateau, China (Wu *et al.*, 2018a, 2018b). As the main animal species of highland grassland, yak plays an irreplaceable role in the production of animal husbandry in Tibet (Han *et al.*, 2013). However, due to the unique nature of

plateau, cultural beliefs of herdsmen, lack of knowledge for feeding and aquaculture model, endometritis occurs frequently in yak after delivery. It seriously affects the reproductive performance of yaks with the main cause of infertility in yaks. It is mainly caused by a variety of opportunistic pathogens in the uterus, especially in the first three weeks after parturition, which is the main reason that cause the endometritis (Williams *et al.*, 2005; Wang *et al.*, 2013). The mainly clinical symptoms of endometritis are fever, abdominal pain, and infertility.

Some studies have shown that metritis and clinical endometritis in cows are related to non-specific mixed infections involving environmental bacteria (Williams *et al.*, 2005; Westermann *et al.*, 2010; Wang *et al.*, 2013). Among these opportunistic pathogen species causing endometrial diseases, *Staphylococcus aureus* and *Escherichia coli* are considered as the most prevalent (Williams *et al.*, 2005; Wang *et al.*, 2013). In uterus infection, pathogenic microorganisms invade and accumulate in the endometrial epithelium cells, releasing toxins such as lipopolysaccharides (Piras *et al.*, 2017).

The inflammatory reaction is a complex process that is triggered by cell infection or tissue damage and can result in a series of chain reaction (Abuelsaad *et al.*, 2014). Inflammation plays an important role in most chronic diseases, so anti-inflammatory drugs are needed to prevent it. Although, several different steroids and non steroidal anti-inflammatory drugs such as semicoxi, aspirin, ibuprofen, and butethasone can be used to treat inflammation, but most of them have side effects (Ancelin *et al.*, 2012).

Salidroside (Sal), the 8-O-b-d-glucoside of tyrosol, is the main natural monomer extracted from *Rhodiolarosea* (Li *et al.*, 2013). The evidence suggests that salidroside has anti-hypoxia, anti-cancer, anti-fatigue, antioxidative, anti-injury, anti-inflammatory and immune regulation properties (Guan *et al.*, 2011; Li *et al.*, 2013).

The salidroside has wide prospect in treating inflammation in animals. However, no study has been performed to evaluate its beneficial effects on *E.coli*-induced endometrial epithelial cells in yaks. Thus, the purpose of this study was to investigate the anti-inflammatory and antioxidant effects of salidroside on *E.coli*-induced proinflammatory cytokine production in endometrial epithelial cells.

## MATERIALS AND METHODS

**Chemicals and Reagents:** The TNF- $\alpha$  antibody (Abclonal, A11534), DMEM-F 12 media (Cat No. SH30023.01B), Dimethylsulfoxide (DMSO, 67685), 4% paraformaldehyde, Ascorbic acid (Catalog# 0764 Amresco) and trypsin (No. 25300054) were purchased from the commercial company. Fetal bovine serum was purchased from Gibco (Lot: 1414426) by life Technologies Corporation, while SOD and MDA detection kits were purchased from Jiancheng Bio-Engineering Research Institute (Nanjing, China; No. A001-3). The TNF- $\alpha$ , IL-1 $\alpha$ , IL-6 and IL-10 detection kit (ELISA, No. 20170624) was purchased from the commercial company.

**Cell culture and identification:** Primary cultures of yak endometrial epithelial cells were performed in DMEM-F12 media according to Wu *et al.* (2018b). Briefly, the neonatal uterus tissues were quickly and aseptically excised from the healthy 2-month old yak, cut into pieces, digested with trypsin for 2h, then centrifuged at 2000 $\times$ g for 10min, washed two times with PBS and then collected the cells. The DMEM-F12 media was supplemented with 10% FBS, 50 U/mL penicillin, 50  $\mu$ g/mL streptomycin and 50  $\mu$ g/mL ascorbic acid. Immunohistochemistry was performed by using specific antibody against cytokeratin

18 (CK18) to identify the cells according to Wu *et al.* (2018b).

***E. coli*-induced inflammatory models and salidroside treatment protocol:** The endometrial epithelial cells were cultured on 6-well plates at 37°C for 24 h. After that, the cells were distributed into four groups: (1) Control groups (give normal media), (2) *E. coli* group (incubated with 10<sup>3</sup>CFU/ml of *E. coli* O<sub>111</sub> with normal media and cultured for 12h), (3) Salidroside (Sal) group (*E. coli* group + 40 ng/ml Sal), and (4) Dexamethasone (Dex) group (*E. coli* group + 10<sup>-7</sup> M/ml Dex). All the groups were incubated with 5% CO<sub>2</sub> at 37°C for 12h.

**Antioxidant response assay:** The concentration in wells after 24 h of the drug intervention were set up and assayed for SOD activity and MDA contents. Briefly, the cells were immediately homogenized in ice-cold saline. Then it was centrifugation @ 3000 rpm for 10 min at 4°C, while the supernatant was collected to conduct further analysis. The SOD level and MDA contents in the supernatant was deliberated by commercial kits as per manufacturer's instructions according to previous studies (Mehmood *et al.*, 2018; Zhang *et al.*, 2018).

**Cytokine assays in cells:** The endometrial epithelial cells were cultured on 24-well plates for 12h and incubated in the presence of *E. coli*, salidroside and dexamethasone treatments for 12 h at 37°C with 5% CO<sub>2</sub>. The concentrations of cytokine TNF- $\alpha$ , IL-1 $\alpha$ , IL-6 and IL-10 in the supernatants of endometrial epithelial cells were measured by using ELISA kit (ELISA, No. 20170624) as per manufacturer's instructions.

**Immunofluorescence staining:** The cells were stained with immunofluorescence staining when the cells were grown to almost 70% confluence. Briefly, the cells were fixed in 4% paraformaldehyde for the 30 min, and then washed for three times with PBS solution (0.1 M). After that cells were incubated for 15 min with Triton (0.1%) x-100 and blocked with bovine serum albumin (5%) for 30 min at the room temperature. Then cells were incubated with special antibody for TNF- $\alpha$  (1:200) at 4°C overnight and then again incubated with secondary antibody (1:200) for 1h at room temperature. Finally, the 4,6-diamidino-2-phenylindole was added for 2h at 25°C and washed again. Images were recorded with the fluorescence microscopy (Olympus, Japan).

**Statistical analysis:** The data was analyzed by statistical software (SPSS Statistics 19) and presented as mean  $\pm$  SD. The comparison between the groups was performed through student's *t*-test. P<0.05 is used for significance.

## RESULTS AND DISCUSSION

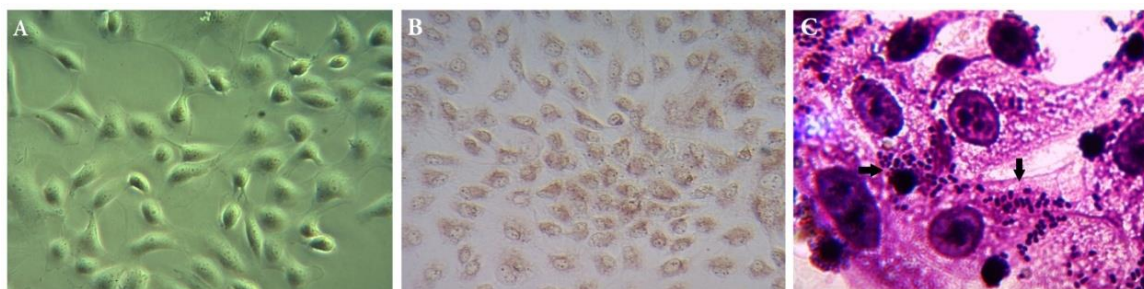
Endometritis mostly happen due to air, semen, urine and microbes like *Streptococcus zooepidemicus* and *Escherichia coli* into uterus. Endometritis is the most common after the parturition due to manipulation during parturition, damage to the epithelium of uterus or birth canal that causes the inflammation (Williams *et al.*, 2005; Wang *et al.*, 2013). Previous studies have shown that the

incidence of endometritis has increased significantly in cases of dystocia, miscarriage, stillbirth and placenta depletion (Williams *et al.*, 2005; Wang *et al.*, 2013). The treatment of endometrial epithelial cells with *E. coli* result in significant increase of cytokine production compared with control group. In order to construct the inflammatory model of endometritis in yaks, the endometrial epithelial cells were cultured. Our results showed that endometrial epithelial cells were observed in a distinct area of the culture dish after 24h of primary culture (Fig. 1A), and their proliferative capacity increased for 48h after isolation and exhibited a typical discontinuous cobblestone appearance with tightly packed cells (Fig. 1A). When the cells grew to 60% confluence after 48h, the immunohistochemical identification of CK18 was done in endometrial epithelium cells for the identification of cells. The results showed that CK18 antibodies were localized and positively stained in the cells (Fig. 1B). After that, the inflammatory model was constructed by using *E. coli*. The results showed that the *E. coli* can adhere to the cells easily and the cells can find typical inflammation damage (Fig. 1C).

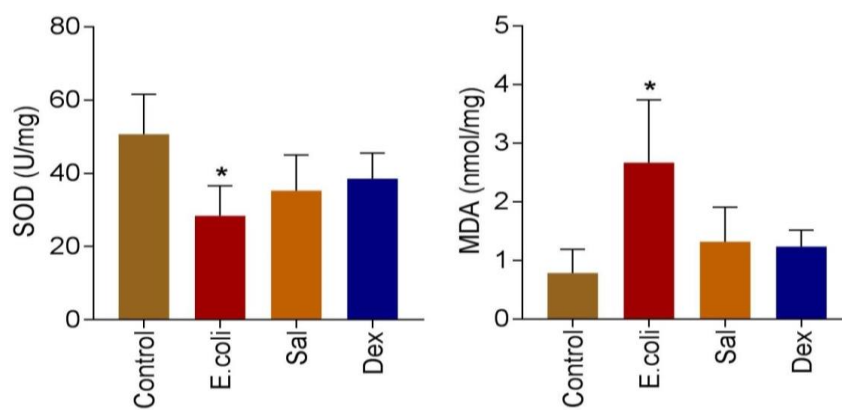
In order to evaluate the antioxidant response capacity of salidroside and dexamethasone, we measured the SOD activity along with MDA levels. Our results showed that the intracellular activity of SOD was decreased significantly in *E. coli*-induced inflammation group as compared with the control group. Differently, pre-treating endometrial epithelial cells with salidroside and dexamethasone improved cells SOD activity (Fig. 2). Additionally, the MDA contents increased significantly in *E. coli* group as compared with control group, and the salidroside and dexamethasone supplements decreased the level of MDA (Fig. 2). It indicates the protective effect as

well as antioxidant properties of salidroside. Our results are in line with Ju *et al.* (2017) and Lan *et al.* (2017), who reported that salidroside is a natural antioxidant. Salidroside enhanced SOD and decreased MDA prominently in mice (Zhu *et al.*, 2015a; Ju *et al.*, 2017). Salidroside significantly reduced the level of MDA in acute lung injury in mice (Lan *et al.*, 2017).

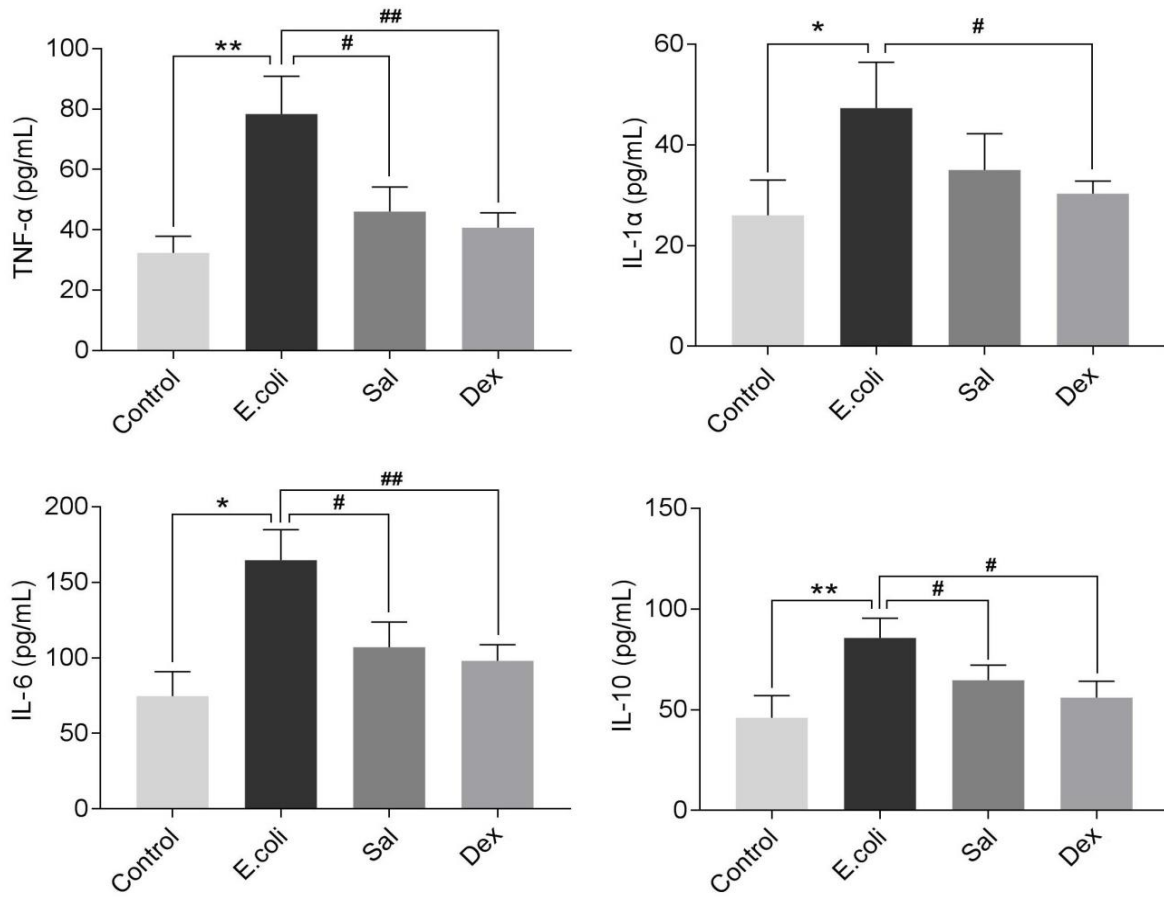
TNF- $\alpha$ , IL-1 $\alpha$ , IL-6 and IL-10 are involved in cell damage (Hervé *et al.*, 2011). TNF- $\alpha$  mainly produced by the activation of mononuclear macrophages, not only can cause cell death but also can activate mononuclear macrophage, promote the release of inflammatory mediators, and play a synergy with other cytokines. It can accelerate progress of IL-6, which is a multifunctional cytokine and plays an important role in immune and inflammatory response. Except mononuclear-macrophage activation of endothelial cells can also produce a large number of IL-6. The IL-6 can affect many kinds of cell growth, differentiation and gene expression. Excessive inflammation factor TNF- $\alpha$ , IL-6, and IL-1 $\alpha$  secretion can lead to inflammation damage (Hervé *et al.*, 2011; Lee *et al.*, 2012). Our results indicated that TNF- $\alpha$ , IL-1 $\alpha$ , IL-6 and IL-10 concentrations in the culture supernatant of endometrial epithelial cells was increased significantly in *E. coli* treated endometrial epithelial cells as compared with control group. However, their levels in cell-free supernatant treated with salidroside and dexamethasone was decreased significantly compared to *E. coli* group (Fig. 3). The results of present study are congruent with Wu *et al.* (2018) that salidroside decrease the concentration of TNF- $\alpha$  and IL-6. Previous studies stated that salidroside attenuated the TNF- $\alpha$  and IL-6 in LPS induced myocardial injury in rats (Zhu *et al.*, 2015a; Zhu *et al.*, 2015b; Chen *et al.*, 2017).



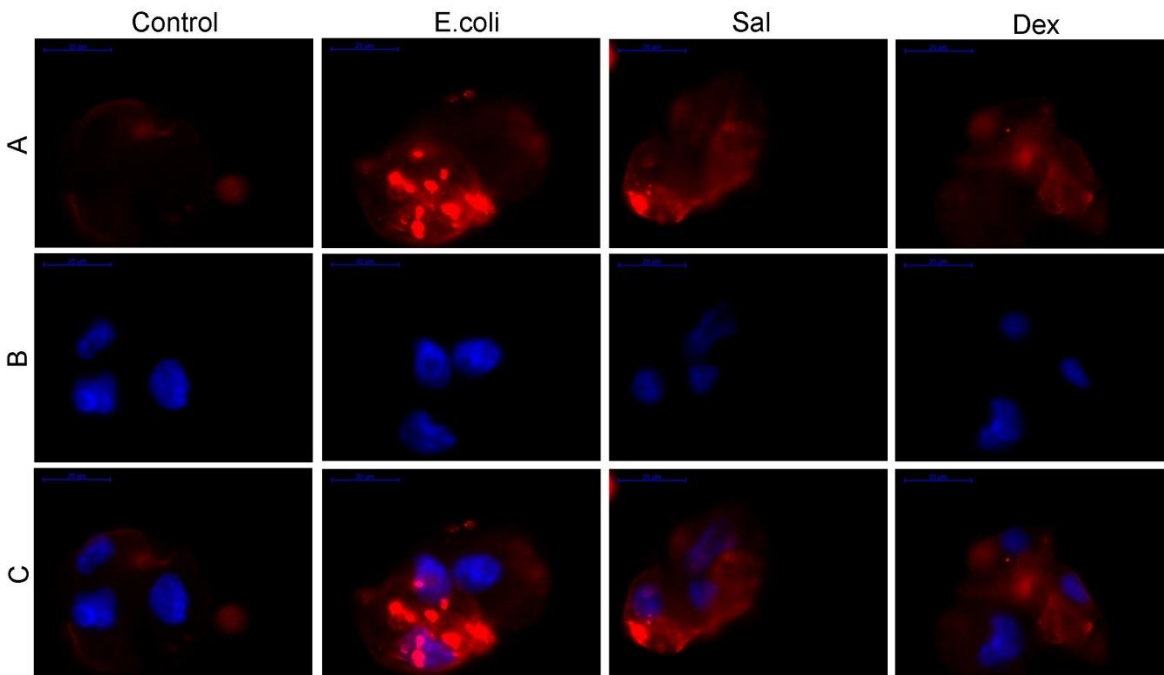
**Fig. 1:** The culture of endometrial epithelial cells in yaks (A: Primary cells culture; B: The CK18 immunohistochemical identification; C: Inflammatory model construction using *E. coli*).



**Fig. 2:** Superoxide dismutase (SOD) activity and malonaldehyde (MDA) levels of endometrial epithelial cells with *E. coli*, salidroside and dexamethasone for 24 h were measured by spectrophotometer.



**Fig. 3:** Effect of salidroside and dexamethasone on cell-free supernatants of TNF- $\alpha$ , IL-1 $\alpha$ , IL-6 and IL-10 by *E. coli*-induced endometrial epithelial cells inflammation. The values are the mean  $\pm$  SD of five independent experiments. \*P<0.05 or \*\*P<0.01 vs control group; #P<0.05 or ###P<0.01 vs *E.coli* group.



**Fig. 4:** Effects of *E. coli*, salidroside and dexamethasone treatments on TNF- $\alpha$  in immunofluorescence sections in endometrial epithelial cells. A, TNF- $\alpha$ ; B, DAPI; C, Merge.

The expression of TNF- $\alpha$  among four groups were evaluated using immunofluorescence analysis. As shown in Fig. 4, the *E. coli*-induced inflammation cells showed an increased level of TNF- $\alpha$  protein as compared with

control group. The results of present study showed that the salidroside and dexamethasone administration reduced the TNF- $\alpha$  expression significantly. Previous study found that salidroside could attenuate pro-inflammatory

cytokines and lymphocyte migration (Guan *et al.*, 2011; Li *et al.*, 2013). The TNF- $\alpha$ , IL-1 $\alpha$ , IL-6 and IL-10 levels in the cell-free supernatant treated with salidroside and dexamethasone was significantly decreased compared to *E. coli* treatment group. Our results showed that the effects of salidroside on endometrial epithelial cells in inhibition of TNF- $\alpha$ , IL-1 $\alpha$ , IL-6 and IL-10 levels were similar to dexamethasone.

**Conclusions:** Salidroside play an important role in weakening *E. coli*-induced inflammatory response in endometrial epithelium cells via regulating the gene expression of pro-inflammatory cytokines including TNF- $\alpha$ , IL-1 $\alpha$ , IL-6 and IL-10 levels, which may target to reduce the incidence of endometritis in yaks.

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**Authors contribution:** HD, QW and JL provided the research idea. FT and ZC contributed reagents, materials, and analysis tools. KM, MMA and MI wrote the manuscript. KM, SA and AS handled the revision.

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