Animal Nutrition 2 (2016) 119–126

Contents lists available at ScienceDirect

Animal Nutrition

journal homepage: http://www.keaipublishing.com/en/journals/aninu/

Original research article

Reproductive stage associated changes in plasma fatty acid profile and proinflammatory cytokine expression in rat mammary glands

Sen Lin [1], Jia Hou [1], Fang Xiang [1], Xiaoling Zhang, Lianqiang Che, Yan Lin, Shengyu Xu, De Wu, Zhengfeng Fang*

Key Laboratory for Animal Disease Resistance Nutrition of the Ministry of Education, Animal Nutrition Institute, Sichuan Agricultural University, Chengdu 611130, China

ARTICLE INFO

Article history:
Received 4 December 2015
Received in revised form
29 February 2016
Accepted 8 March 2016
Available online 16 April 2016

Keywords:
Mastitis
Reproductive stage
Proinflammatory cytokine
Fatty acid
Rat

ABSTRACT

Mastitis is a common disease for mammals all around the world. Figuring out why mastitis mainly occurs around parturition may be helpful for dealing with the disease. Lipolytic activity and oxidative stress take place around parturition, which may leads to alteration in fatty acids profile and proinflammatory cytokine expression. Thus, the aim of the present study was to further our understanding about the high incidence of mastitis around parturition by comparison of plasma fatty acid profile and mammary inflammation indicators at different reproductive stages. A total of 47 female rats were included in the present study. After mating, all the pregnant and non-pregnant rats began to receive the same experimental diet. Blood samples were collected at day 1 and 14 of gestation as well as day 3 postpartum. Mammary samples were collected at day 14 of gestation and day 3 postpartum from pregnant and non-pregnant rats. The results showed that rats at d 3 postpartum had greater *($P < 0.05$)* plasma concentrations of non-esterified fatty acids (NEFA), arachidonic acid (ARA) and docosahexaenoic acid (DHA) as well as ARA: eicosapentaenoic acid (EPA) ratio than those at d 14 of gestation. The mRNA abundances of interleukin-1β (*IL-1β*), tumor necrosis factor-α (*TNF-α*), *IL-8* and xanthine oxidoreductase (*XOR*) in mammary of the pregnant rats were greater *($P < 0.05$)* than those in age-matched non-pregnant rats. Rats at d 3 postpartum had higher *($P < 0.05$)* protein expression levels of IL-1β and TNF-α as well as meloperoxidase (MPO) activity and polymorphonuclear neutrophils (PMN) prevalence than those at d 1 of gestation. The rats at d 3 postpartum also had greater *($P < 0.05$)* IL-1β and MPO activity than those at d 14 of gestation. The results indicated that elevated mammary expression of proinflammatory cytokines and *XOR* as well as altered fatty acid profile around parturition might facilitate the recruitment of neutrophils into mammary glands.

© 2016, Chinese Association of Animal Science and Veterinary Medicine. Production and hosting by Elsevier B.V. on behalf of KeAi Communications Co., Ltd. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

1. Introduction

Mastitis is a common disease for mammals all over the world that occurs frequently around parturition (Compton et al., 2007). Previous studies showed the average prevalence of mastitis in sows was about 13% (Gerjets and Kemper, 2009) while it was about 25.5% in lactating heifers in Dutch (Santman-Berends et al., 2012). Piglet mortality around one week in the litters of coliform mastitis-affected sows varies from 5.0% to 38.6% (Gerjets and Kemper, 2009). Cows often have mastitis without obvious clinical symptoms named subclinical mastitis (Hansen et al., 2004). Subclinical mastitis results in leakage of plasma constituents into milk and causes gut damage of infants. Antibiotics are often used to deal with mastitis while overuse of antibiotics may lower the quality and safety of animal products and thus threaten the health of humans (Hortet and Seegers, 1998; Seegers et al., 2003).

Up to date, we know little about how to effectively prevent mastitis, and there is little knowledge about the underlying

* Corresponding author.
E-mail address: fangzhengfeng@hotmail.com (Z. Fang).
[1] These authors contributed equally to this work.
Peer review under responsibility of Chinese Association of Animal Science and Veterinary Medicine.

Production and Hosting by Elsevier on behalf of KeAi

http://dx.doi.org/10.1016/j.aninu.2016.03.008
2405-6545/© 2016, Chinese Association of Animal Science and Veterinary Medicine. Production and hosting by Elsevier B.V. on behalf of KeAi Communications Co., Ltd. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

mechanism. Although *Escherichia coli* has been proposed to play critical roles in triggering mastitis through the toll-like receptor (TLR) pathway (Porcherie et al., 2012) and infusion of lipopolysaccharide (LPS) has been proved to potentially stimulate expression of proinflammatory cytokines (Frank et al., 2003), why mammary glands are more susceptible around parturition (Burvenich et al., 2007) remains to be elucidated. In humans, increased concentration of non-esterified fatty acid (NEFA) can stimulate systemic immune response and is linked to inflammatory-based diseases (Wood et al., 2009). Notably, increased lipolysis takes place around parturition and thus results in large increases in NEFA concentration (Drackley et al., 2001). In addition, enhanced lipolytic activity around parturition also results in break down of fat depots and rapid changes of plasma fatty acid composition (Amusquivar et al., 2010). It was reported that saturated fatty acids (SFA) concentration in plasma of women at parturition was greater than that at week 24 of gestation (Stark et al., 2005). Saturated fatty acids were convinced to be capable of stimulating TLR-mediated proinflammatory signaling pathways (Huang et al., 2012). Moreover, our previous study indicated that consumption of fish oil could attenuate mammary inflammation which might be linked to reduced plasma concentration of SFA (Lin et al., 2013). These results suggested that increased fatty acids metabolism around parturition may play roles in regulation of inflammatory responses in mammary glands. Therefore, detecting the variation in fatty acid composition of mammals across the reproductive cycle may be helpful for understanding why mammary inflammation occurs frequently around parturition.

On the other hand, it is well documented in cows (Castillo et al., 2005) and sows (Berchieri-Ronchi et al., 2011; Xie et al., 2015) that oxidative stress occurs at late gestation and early lactation due to severe catabolic status. And oxidative stress was known to be strongly linked to production of proinflammatory cytokines (Escobar et al., 2009; Yin et al., 2013; 2014; 2015), which has been known to be key factors in inducing mastitis (Oviedo-Boyso et al., 2007). In consequence, enhanced oxidative stress around parturition may affect the expression of proinflammatory cytokines in mammary gland. However, the variation of the proinflammatory cytokines across the reproductive cycle in mammary gland of mammals is poorly understood.

In the present study, the pregnant and age-matched non-pregnant rats were used as models to evaluate changes in plasma fatty acids profile and mammary proinflammatory cytokines expression in different time point of a reproductive cycle, which may further our understanding about the high incidence of mammary inflammation around parturition.

2. Materials and methods

2.1. Animals and facilities

All procedures outlined in this study were approved by the Animal Care and Use Committee of the Animal Nutrition Institute, Sichuan Agricultural University. All experiments involved animals were conducted in conformity with the Public Health Service Policy on Humane Care and Use of Laboratory Animals. The experimental rats (Virgin female Sprague-Dawley rats) were purchased from Sichuan Academy of Medical Sciences-Sichuan Provincial People's Hospital Experimental Animal Research Institute (Sichuan, China), and housed in galvanized-steel cages with bedding and maintained at a controlled temperature (22 ± 2°C) and relative humidity (60 ± 10%) with a 12-h light/dark cycle.

Table 1
Ingredients and composition of the experimental diet (air-dry basis).

Ingredient	Content, %	Composition	Content, %
Corn starch	39.75	Crude protein	16.23
Casein	20	ME, Mcal/kg	3.81
Gelatinization starch	13.2	Lysine	1.53
Sucrose	10	Methionine	0.57
Fat[1]	7	Calcium	0.50
Fiber	5	Available phosphorus	0.16
Mineral premix[2]	3.5		
Vitamin premix[3]	1		
L-cysteine	0.3		
Choline Chloride	0.25		
Total	100		

[1] The 7 kg fat was composed of 5 kg lard and 2 kg soybean oil in the lard diet.
[2] Provided per kg of diet: calcium 5000 mg, phosphorus 1561 mg, potassium 3600 mg, sodium 1019 mg, chlorine 1517 mg, magnesium 510 mg, iron 35 mg, zinc 30 mg, manganese 10 mg, copper 6 mg, selenium 0.15 mg, iodine 0.2 mg.
[3] Provided per kg of diet: vitamin A 4000 IU, vitamin D_3 1000 IU, vitamin K_3 0.75 mg, vitamin B_1 6.0 mg, vitamin B_2 7.0 mg, vitamin B_6 6.0 mg, vitamin B_{12} 0.02 mg, nicotinic acid 30.0 mg, D-calcium pantothenate 15.3 mg, folic acid 2.0 mg, biotin 0.2 mg.

2.2. Diets and treatments

The experimental diet (Table 1) was formulated to meet or exceed the nutrient requirements of gestating and lactating rats as recommended by AIN-93G. To set a proposed level of dietary SFA and n-3 polyunsaturated fatty acid (PUFA), the 7 kg fat included in the experimental diet was composed of 5 kg lard and 2 kg soybean oil. The fatty acid (FA) composition of the diet is showed in Table 2. The diet was stored at −20°C to avoid PUFA oxidation during the experimental period. A total of 47 rats were included in the experiment. When rats grew to sexual maturity, 1 female rat (231 ± 5 g) per cage was housed together with 1 male rat (weighing 300 to 330 g) in the same cage to complete mating. The females

Table 2
Fatty acid composition of the lard (g/100 g) and the diet (g/kg) (as fed basis).

Fatty acid[1]	Lard	Lard diet
C14:0	1.25	0.42
C16:0	26.14	9.91
C18:0	19.89	7.20
C20:0	0.34	0.17
C16:1	1.48	0.52
C18:1	37.62	15.70
C20:1	0.91	0.40
C22:1	ND	0.17
C18:2n6	10.20	10.05
C18:3n3	0.98	0.96
C20:5n3	ND	0.087
C22:6n3	ND	ND
Other	1.19	0.41
∑FA	100	46
∑SFA	47.62	17.70
∑MUFA	40.01	16.80
∑PUFA	11.18	11.09
∑SFA/∑FA	47.62	38.48
∑MUFA/∑FA	40.01	36.52
∑PUFA/∑FA	11.18	24.11
∑n-3	0.98	1.04
∑n-6	10.2	10.05
∑n-6/∑n-3	10.41	9.62

ND = not detected; FA = fatty acids; SFA = saturated fatty acids; MUFA = monounsaturated fatty acid; PUFA = polyunsaturated fatty acid.
[1] ∑FA means the sum of content of all fatty acids evaluated; ∑SFA means the sum of C14:0, C16:0, C18:0 and C20:0 content; ∑MUFA means the sum of C16:1, C18:1, C20:1 and C22:1 content; ∑PUFA means the sum of C18:2n6, C18:3n3, C20:5n3 and C22:6n3 content; ∑n-3 means the sum of C18:3n3, C20:5n3 and C22:6n3 content; ∑n-6 means the content of C18:2n6.

were examined each morning for the presence of a seminal plug in the vagina. The day detecting a plug was designated embryonic day 1 of gestation and plug-positive females were considered 'pregnant'. Immediately after the first observation of a plug, the female rats were removed and housed individually, and began to receive the experimental diet. At the same time, age- and body weight-matched virgin rats also began to receive the experimental diet. All rats had free access to water at all times. Mammary tissue samples were obtained at days 1 and 14 of gestation as well as day 3 postpartum, respectively. All blood samples were obtained (about 3 mL) through intra-orbital bleeding (for rats to be slaughtered thereafter) or by tail-cutting (for virgin rats that continued to be reared) and collected in duplicate in heparinized tubes. The samples were then centrifuged at 1500 × *g* for 15 min at 4°C. All blood samples were obtained after a 12-h fast and stored at −20°C until analysis. Immediately after anesthesia, the mammary tissue samples for detection of mRNA abundance were collected, snap frozen in liquid nitrogen and stored at −80°C. The mammary tissue samples for histopathologic examination and immunohistochemistry detection were fixed in 4% paraformaldehyde and stored at 4°C until analysis.

2.3. ELISA analysis

Non-esterified fatty acids concentration was determined in duplicate by commercial ELISA kits (GBD, San Diego, CA, USA) as described by the manufacturer's protocol. About 100 μL of standard, blank, and sample was added to the wells of the assay plate, respectively, and the assay plate was covered with the adhesive strip. After incubation for 2 h at 37°C, the liquid of each well was removed without washing. Then, 100 μL of Biotin-antibody working solution was added to each well. After incubation for 1 h at 37°C, liquid in the wells was aspirated, and 200 mL of wash buffer was used to wash the wells, guaranteeing that liquid was removed completely in each step. Next, 100 μL of HRP-avidin working solution was added to each well, and a new adhesive strip was used to cover the plate, allowing incubation for 1 h at 37°C. Afterward, the wells were washed as described above. This was followed by adding 90 μL of Trimethyl boron substrate to each well with incubation for 30 min at 37°C. Keep the plate away from drafts and other temperature fluctuations in the dark. At last, 50 μL of stop solution was added to each well and the reaction was thus halted. The optical density of each well was determined within 30 min using a microplate reader set at 450 nm. A standard curve was created and the corresponding NEFA concentrations were determined according to the standard curve.

The plasma meloperoxidase (MPO) activity was determined using a spectrophotometric method. The MPO activity was calculated with the absorbance (at 460 nm) changes causing by reducing H_2O_2 with the presence of o-dianisidine. All reagents were included in a commercial MPO kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

2.4. FA composition analysis

Plasma FA composition were analyzed according to previous research (Fernandez-Real et al., 2003) with modification. Briefly, 30 to 50 mg plasma sample was weighed in glass tubes. About 4 mL acetyl chloride and methanol solution (1:10, volume) was added slowly. After transesterification, the pooled solvent extracts were dried under a gentle stream of nitrogen at room temperature. Residues were dissolved in 5 mL hexane with internal standard. After water bath at 80°C for 2 h, 5 mL 7% potassium carbonate was added, vibrating until uniform, standing for stratification and collecting supernatant. Analysis was performed on a Hewlett-Packard 6890 gas chromatograph equipped with a flame ionization detector. The column temperature was held at 180°C for 10 min and in a stepwise fashion reached a plateau of 230°C. The detector temperature was 270°C. Helium was used as carrier gas.

2.5. RNA analysis

RNA analysis was performed as we described previously (Lin et al., 2013). Briefly, total RNA was extracted using a TRIzol reagent kit (Invitrogen, Carlsbad, CA, USA). The cDNA was prepared using a reverse transcription (RT) kit (TAKARA, Dalian, China) following the manufacture's instruction. Primers were synthesized by Chengdu Tiantai Biological Company (Chengdu, China). β-actin was used as an internal control according to the work of Gu et al. (2010). The nucleotide primer sequences are listed in Table 3. Quantitative real-time RT-PCR analysis was performed using a 7900 real-time PCR system (Applied Biosystems, Foster City, CA, USA) and SYBR Green assays (Master Mix SYBR Green TAKARA, Dalian, China). The specificity of PCR products were examined with melting curve analysis. Results (fold changes) were expressed as $2^{-\Delta\Delta Ct}$ with $\Delta\Delta Ct = (Ct\ ij - Ct\ \beta\text{-actin}\ j) - (Ct\ i1 - Ct\ \beta\text{-actin1})$, where Ct *ij* and Ct β-actin *j* are the Ct for gene *i* and for β-actin in a sample (named *j*), and where Ct *i*1 and Ct β-actin1 are the Ct in sample 1, expressed as the standard.

2.6. Histopathologic examination

Tissue specimens were fixed in 4% paraformaldehyde for 24 h. Standard dehydration and paraffin-wax embedding procedures were used to produce tissue blocks. Hematoxylin and eosin stained slides were prepared using standard methods. The prevalence of the polymorphonuclear neutrophils (PMN) in alveoli was estimated by light microscopic (Olympus BH2, Tokyo, Japan) analyses at a magnification of 400× as previously described (Miao et al., 2007). Briefly, 4 sections of the rat mammary tissue samples were quantified for each animal. Ten fields were selected randomly per sample. Results are presented as average PMN infiltration scores for each time point.

2.7. Immunohistochemistry

Polyclonal antibodies combined with the avidin-biotinper oxidase complex technique were used for the immunohistochemical detection of interleukin-1β (IL-1β) (Abnova, Walnut, CA, USA) and tumor necrosis factor-α (TNF-α) (Novus, SaintCharles, MO, USA). All samples from 1 animal were analyzed within the same assay run, and within each assay run treatment animals to be compared were included. The quantification of IL-1β and TNF-α protein expression level in mammary tissue samples was performed as previously described (Zhu et al., 2007). For each sample, a relative value of the amount of cytokine produced was expressed as the average percentage of the positively stained areas in 10 (for IL-1β) or 5 (for TNF-α) randomly selected view fields.

2.8. Statistical analysis

Data were analyzed using the General Linear Model procedures of SAS statistical package (V8.1, SAS Institute Inc., Cary, NC). Least-squares means comparison was used to evaluate differences among treatments. *P*-value ≤0.05 were considered statistical significance. Values were presented as means ± SE.

Table 3
Primer sequences used to amplify rat cytokines.

Gene	Primer sequences (5′ to 3′)		Product size, bp	GenBank accession No.
IL-1β	Forward	TGACCTGTTCTTTGAGGCTGAC	113	M98820.1
	Reverse	CGAGATGCTGCTGTGAGATTTG		
TNF-α	Forward	CCACTCTGACCCCTTTACTCTGA	154	NM_013693.2
	Reverse	CTGTCCCAGCATCTTGTGTTTC		
IL-8	Forward	CCAGCAGGAAACCAGAAGAAAG	123	NM_001173399.2
	Reverse	CAACTTTGTCACGACCATACCC		
XOR	Forward	GATTCTCACACACCTCCTGACG	156	NM_011723.2
	Reverse	CCCCACACACACACACACACTAT		
β-actin	Forward	CTGTGTGGATTGGTGGCTCTATC	133	NM_031144.2
	Reverse	GCTCAGTAACAGTCCGCCTAGAA		

IL-1β = interleukin-1β; *TNF-α* = tumor necrosis factor-α; *IL-8* = interleukin-8; *XOR* = xanthine oxidoreductase.

3. Results

3.1. Plasma NEFA concentration and FA profile at different reproductive stages

As shown in Fig. 1, plasma NEFA concentration in non-pregnant rats was not changed ($P > 0.05$) with the advance of time. It was also not different ($P > 0.05$) between the value of the pregnant rats at day 1 and those at day 14 of gestation. However, a significant ($P < 0.05$) increase in plasma NEFA concentration was observed at day 3 postpartum compared with that at day 14 of gestation. The concentration of several FA greatly changed, especially the PUFA (Table 4). At day 3 postpartum, plasma arachidonic acid (ARA), docosahexaenoic acid (DHA) and total n-3 PUFA concentrations were significantly ($P < 0.05$) greater than those at day 14 of gestation. Notably, ARA:eicosapentaenoic acid (EPA) ratio at day 3 postpartum was higher than that at day 1 and day 14 of gestation.

3.2. Mammary inflammation mediators at different reproductive stages

Real time-PCR results indicated that the mRNA abundance of *IL-1β*, *TNF-α*, *IL-8* and *XOR* in mammary of the pregnant rats at day 14 of gestation was greater ($P < 0.05$) than that of age-matched non-pregnant rats (Fig. 2). Immunohistochemistry analysis revealed that the protein levels of IL-1β and TNF-α were greater at day 3 postpartum than those at day 1 of gestation. In addition, the protein level of IL-1β at day 3 postpartum was higher than that at day 14 of gestation (Fig. 3). Moreover, MPO activity (Fig. 4) and PMN prevalence (Fig. 5) were greater ($P < 0.05$) at day 3 postpartum than those at day 1 of gestation. Meloperoxidase activity at day 3 postpartum was also greater ($P < 0.05$) than that at day 14 of gestation.

Table 4
Plasma fatty acid concentration (μg/mL) of the rats at different reproductive stages.

Fatty acid	Day 1 of gestation		Day 14 of gestation		Day 3 postpartum	
	Mean	SE	Mean	SE	Mean	SE
C18:3n3	10.32	1.16	12.42	1.59	9.03	1.10
C20:5n3	6.28	0.93	6.16	0.59	4.97	0.72
C22:6n3	65.09^{ab}	6.19	45.71^{b}	1.95	91.36^{a}	15.86
C20:4n6	309.89^{ab}	50.52	248.16^{b}	13.24	406.70^{a}	52.01
ARA:EPA	50.61^{b}	7.80	42.06^{b}	2.31	84.31^{a}	7.22
n-3 PUFA	81.69^{ab}	6.11	65.42^{b}	3.87	106.47^{a}	16.37
SFA	1542.50	150.96	1230.73	178.83	1431.02	145.23
Total FA	2577.71	192.64	2209.09	183.43	2632.85	286.82

ARA = arachidonic acid; EPA = eicosapntemacnioc acid; PUFA = polyunsaturated fatty acids; SFA = saturated fatty acids; FA = fatty acids; SE = standard error.
^{a,b} Mean values within a row without a common superscript differ ($P < 0.05$).

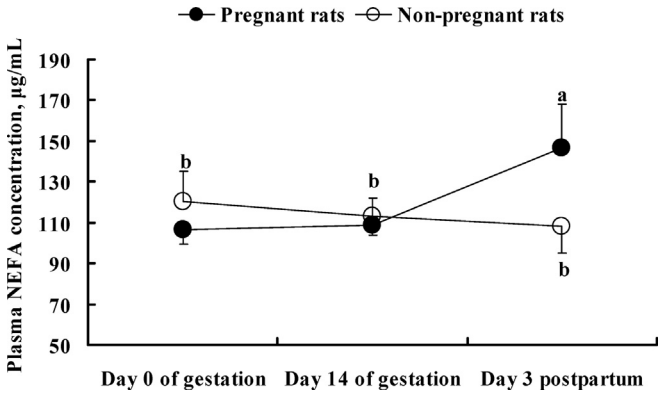

Fig. 1. Plasma non-esterified fatty acids (NEFA) concentration (μg/mL) of the rats at different reproductive stages. Blood samples were collected at day 1 of gestation from non-pregnant rats ($n = 5$) and pregnant rats ($n = 13$), day 14 of gestation from non-pregnant rats ($n = 6$) and pregnant rats ($n = 12$) and day 3 postpartum from non-pregnant rats ($n = 6$) and pregnant rats ($n = 5$) and determined using ELISA kits. Values are presented as means ± SE. Different letters signify statistical difference ($P < 0.05$).

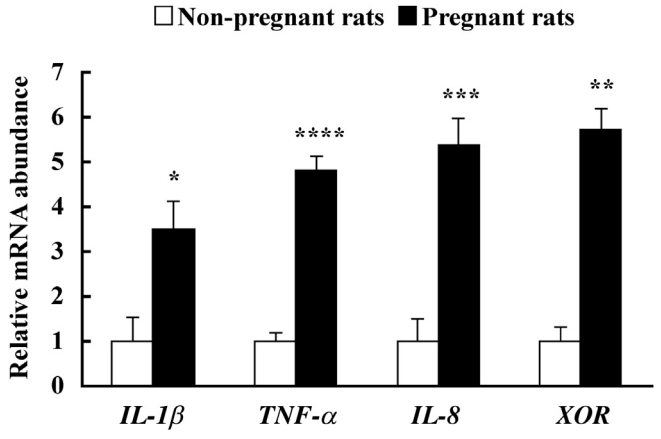

Fig. 2. The mRNA abundance of interleukin-1β *(IL-1β)*, tumor necrosis factor-α *(TNF-α)*, *IL-8* and xanthine oxidoreductase (*XOR*) in udder of rats. The mRNA abundances of *IL-1β*, *TNF-α*, *IL-8* and *XOR* were determined by RT-PCR with mammary tissues obtained after saline infusion at day 14 of gestation from pregnant rats ($n = 7$) and age-matched non-pregnant rats ($n = 11$). Values are presented as means ± SE. Differences between pregnant and non-pregnant rats were indicated by asterisks ($^{****}P < 0.0001$, $^{***}P < 0.001$, $^{**}P < 0.01$ and $^{*}P < 0.05$).

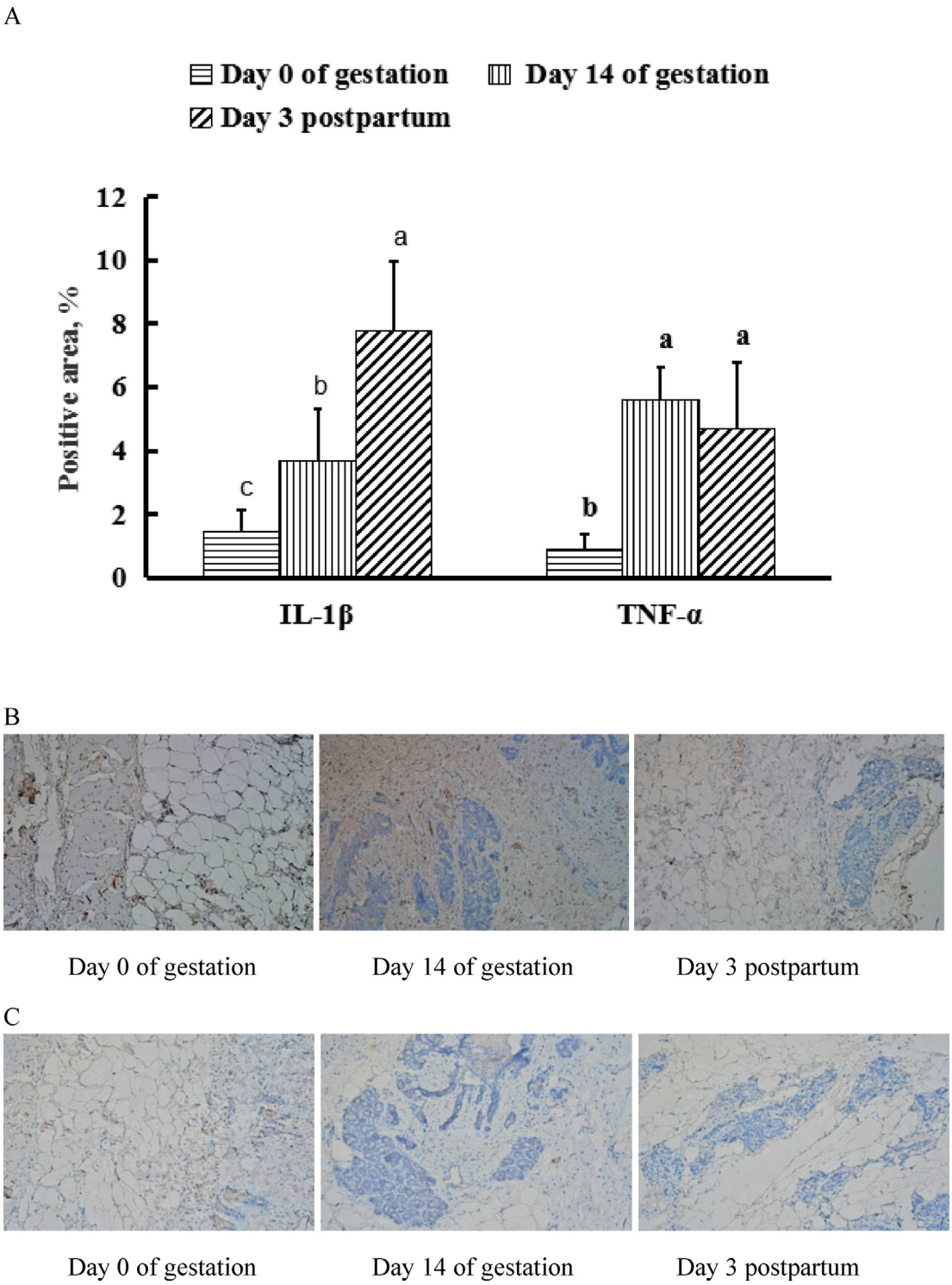

Fig. 3. Immunohistochemical localization of interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) in mammary glands of the rats at different reproductive stages. Mammary glands were collected after saline infusion from non-pregnant rats that designated as rats at day 1 of gestation ($n = 6$), pregnant rats that was infused by saline at day 14 of gestation ($n = 5$) and day 3 postpartum ($n = 5$). (A) Interleukin-1β and TNF-α production is presented as the average percentage of the positively stained areas. The microphotograph from one rat with the positive primary IL-1β or TNF-α antibody was visualized with diaminobenzidine reaction. The area positive for (B) IL-1β and (C) TNF-α in mammary tissue of rats at different reproductive stages was quantified by Easy Image 3000 software at a magnification of 400×. Values are presented as means ± SE. Different letters signify statistical difference ($P < 0.05$) within the same protein.

4. Discussion

In the present study, the plasma FA profile and mammary inflammation indicators at different reproductive stages were determined to explain the high incidence of mastitis around parturition. Across the reproductive cycle, great changes took place in plasma FA profiles. From day 14 of gestation to day 3 postpartum, the increased plasma concentration of DHA resulted

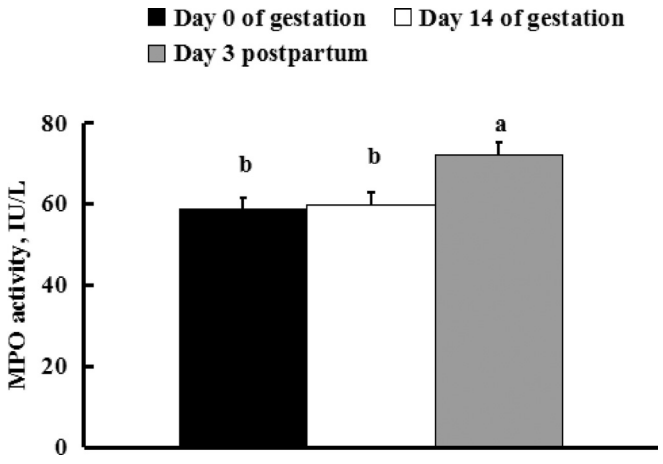

Fig. 4. Plasma meloperoxidase (MPO) activity (IU/L) at different reproductive stages. Meloperoxidase activity was determined using a spectrophotometric method with blood samples collected from the rats infused by saline at day 1 of gestation ($n = 6$), day 14 of gestation ($n = 6$) and day 3 postpartum ($n = 6$). Values are presented as means ± SE. Different letters signify statistical difference ($P < 0.05$).

in elevation of n-3 PUFA. The elevated DHA here could be ascribed to increased NEFA concentration in plasma (Spector, 2001). At day 3 postpartum, NEFA concentration increased significantly compared with that at days 1 and 14 of gestation which was in line with a previous study (Contreras et al., 2010). Increased plasma concentration of NEFA might indicate the reduction in the body content of adipose tissue which could lead to impaired immune cell function (Mora and Pessin, 2002) and increased susceptibility to exogenous pathogens. Moreover, the predominant FA in plasma NEFA was shown to be palmitic and stearic acids, both of which were known to be capable of enhancing the production of inflammatory cytokines. For example, palmitic acid can enhance the production of cytokines TNF-α, IL-1β and IL-8 in monocytes through its activated molecule palmitoyl-CoA (Contreras et al., 2010). Moreover, stearate and palmitate can induce the expression of cyclooxygenase 2, an inflammatory eicosanoid enzyme (Lee et al., 2001). Notably, ARA:EPA ratio at day 3 postpartum increased to twice that at day 14 of gestation and was significantly higher than that at day 1 of gestation. Plasma concentration of ARA at day 3 postpartum was also higher than that at day 14 of gestation. It was demonstrated that increased production of ARA could cause inflammation by triggering proinflammatory eicosanoid generation (Harizi et al., 2008), which was attributable to oxygenation of ARA (Davies et al., 1984). As mentioned above, parturition is accompanied by extensive oxidative stress and may thus facilitate oxygenation of ARA. Previous studies also showed that the decreased production of IL-1 and TNF was accompanied by a decreased ARA:EPA ratio in the membrane phospholipids of mononuclear cells, indicating the significant role of ARA in proinflammatory responses (Endres et al., 1989). In turn, the increased ARA:EPA ratio with the advance of gestation might be accompanied by higher production of IL-1 and TNF.

Consequently, proinflammatory cytokines in mammary glands across the reproductive cycle were determined. An important finding was that with the advance of pregnancy, there was up-regulated mRNA expression of proinflammatory cytokines in mammary glands including *IL-1β*, *TNF-α* and *IL-8*, the mRNA abundance of which was significantly greater in pregnant rats at day 14 of gestation than that in the match-up non-pregnant rats. Moreover, immunohistochemistry analysis revealed that the protein expression level of TNF-α was greater at day 14 of gestation and day 3 postpartum than that at day 1 of gestation and the protein expression level of IL-1β was the greatest at day 3 postpartum among the three time points evaluated. Previous study showed that there was a significant increase in IL-1β, TNF-α and IL-8 at both mRNA levels (Zhu et al., 2008) and protein levels (Zhu et al., 2007) in the inoculated mammary glands of sows that developed clinical signs of mastitis. It was therefore inferred that proinflammatory cytokines in mammary glands, linked closely with mastitis, tended to increase with the advance of gestation and lactation. Those inflammatory cytokines were known to play critical roles in neutrophils recruitment into the mammary glands (Oviedo-Boyso et al., 2007). In the present study, plasma MPO activity and PMN prevalence in mammary glands were observed to reach a peak at day 3 postpartum. Meloperoxidase activity was known as a marker reflecting the function of neutrophil (Roth and Kaeberle, 1981). Polymorphonuclear neutrophils prevalence has been proved to be a principal marker of mastitis after intramammary infection with *E. coli* (Shuster et al., 1997). The aggravated PMN prevalence in the mammary glands might be induced by the over expression of *IL-1β* and *TNF-α*, both of which were documented as key mediators that participate in neutrophil recruitment to the mammary glands. Moreover, neutrophils recruited to the site of infection would phagocytize bacteria and produce reactive oxygen species, low molecular weight antibacterial peptides, and defensins, which eliminate a wide variety of pathogens that cause mastitis (Oviedo-Boyso et al., 2007).

Another important finding was that the mRNA abundance of *XOR* was also greater in pregnant rats at day 14 of gestation than that in the match-up non-pregnant rats. Previous studies have shown that increased *XOR* expression contributed to increased synthesis of numerous ROS (Vorbach et al., 2003), which may facilitate activation of TLR4 signaling pathway (Enos et al., 2013). Toll-like receptor 4 is a member of pattern-recognition receptors that play critical roles in the innate immune system in response to microbial pathogens. The high activity of TLR4 may enhance the innate immune responses of the organisms. Therefore, with the advance of gestation, TLR4 in mammary gland cells can be activated more easily in response to exogenous pathogens owing to enhanced expression of XOR. Activation of TLR4 may further promote the production of proinflammatory cytokines and recruitment of neutrophils to mammary glands.

5. Conclusion

In conclusion, our results indicate that expression of proinflammatory cytokines and *XOR* as well as plasma NEFA and ARA:EPA ratio were all increased with the advance of gestation. Increased plasma NEFA and ARA:EPA ratio as well as the mRNA expression level of *XOR* could enhance the production of proinflammatory cytokines, which would facilitate the neutrophil recruitment to mammary glands. Therefore, in late gestation and early lactation, the mammary glands are in a vulnerable status. Once invaded by pathogens, inflammatory responses may be

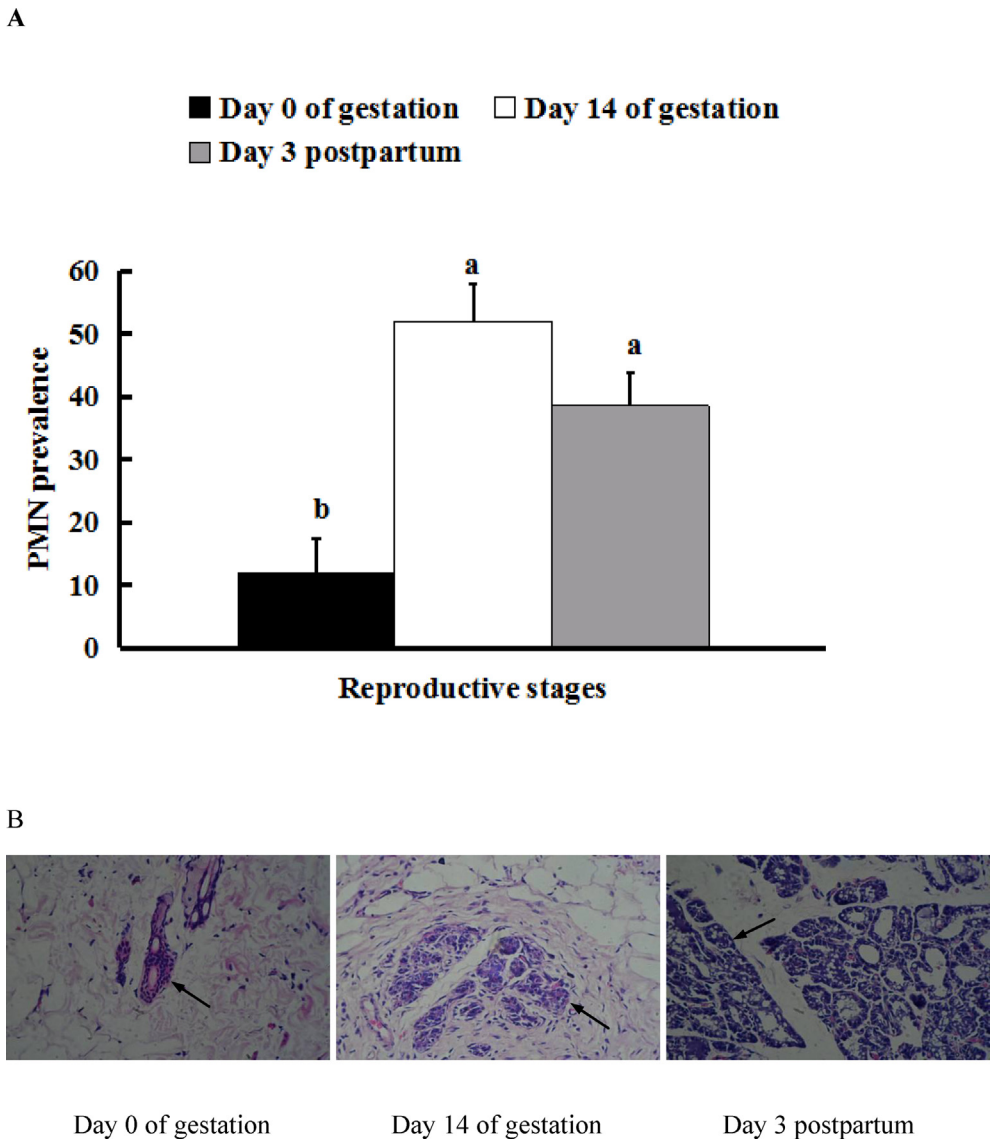

Fig. 5. Histopathology of mammary glands of rats at different reproductive stages. (A) Hematoxylin and eosin stained slides were made with mammary tissues collected from non-pregnant rats that designated as rats at day 1 of gestation ($n = 6$), pregnant rats at day 14 of gestation ($n = 5$) and day 3 postpartum ($n = 5$). (B) Polymorphonuclear neutrophils prevalence in alveoli was estimated by using light microscopic (Olympus BH2, Toyko, Japan) analysis at a magnification of 400×. Values are presented as means ± SE. Different letters signify statistical difference ($P < 0.05$).

stimulated. These results may be helpful for understanding the high incidence of mastitis around parturition.

Acknowledgments

The work was supported by the National Natural Science Foundation of China (30901042), Sichuan Province Science Foundation for Fostering Youths Talents (2011JQ0015), Key Program Fundation of the Education Department of Sichuan Province, and Novus Research Fellowship (NRF).

References

Amusquivar E, Laws J, Clarke L, Herrera E. Fatty acid composition of the maternal diet during the first or the second half of gestation influences the fatty acid composition of sows' milk and plasma, and plasma of their piglets. Lipids 2010;45(5):409–18.

Berchieri-Ronchi CB, Kim SW, Zhao Y, Correa CR, Yeum KJ, Ferreira AL. Oxidative stress status of highly prolific sows during gestation and lactation. Animal 2011;5(11):1774–9.

Burvenich C, Bannerman DD, Lippolis JD, Peelman L, Nonnecke BJ, Kehrli ME, et al. Cumulative physiological events influence the inflammatory response of the bovine udder to *Escherichia coli* infections during the transition period. J Dairy Sci 2007;90:E39–54.

Castillo C, Hernandez J, Bravo A, Lopez-Alonso M, Pereira V, Benedito JL. Oxidative status during late pregnancy and early lactation in dairy cows. Vet J 2005;169(2):286–92.

Compton CWR, Heuer C, Parker K, McDougall S. Risk factors for peripartum mastitis in pasture-grazed dairy heifers. J Dairy Sci, 2007;90(9):4171–80.

Contreras GA, O'Boyle NJ, Herdt TH, Sordillo LM. Lipomobilization in periparturient dairy cows influences the composition of plasma nonesterified fatty acids and leukocyte phospholipid fatty acids. J Dairy Sci 2010;93(6):2508–16.

Davies P, Bailey PJ, Goldenberg MM, Ford-Hutchinson AW. The role of arachidonic acid oxygenation products in pain and inflammation. Annu Rev Immunol 1984;2(1):335–57.

Drackley JK, Overton TR, Douglas GN. Adaptations of glucose and long-chain fatty acid metabolism in liver of dairy cows during the periparturient period. J Dairy Sci 2001;84:E100–12.

Endres S, Ghorbani R, Kelley VE, Georgilis K, Lonnemann G, Vandermeer JWM, et al. The effect of dietary supplementation with n-3 poly-unsaturated fatty-acids on the synthesis of interleukin-1 and tumor necrosis factor by mononuclear-cells. New Engl J Med 1989;320(5):265–71.

Enos RT, Davis JM, Velazquez KT, McClellan JL, Day SD, Carnevale KA, et al. Influence of dietary saturated fat content on adiposity, macrophage behavior,

inflammation, and metabolism: composition matters. J Lipid Res 2013;54(1):152–63.
Escobar J, Pereda J, Arduini A, Sandoval J, Sabater L, Aparisi L, et al. Cross-talk between oxidative stress and proinflammatory cytokines in acute pancreatitis: a key role for protein phosphatases. Curr Pharm Des 2009;15(26):3027–42.
Fernandez-Real JM, Broch M, Vendrell J, Ricart W. Insulin resistance, inflammation, and serum fatty acid composition. Diabetes Care 2003;26(5):1362–8.
Frank JW, Carroll JA, Allee GL, Zannelli ME. The effects of thermal environment and spray-dried plasma on the acute-phase response of pigs challenged with lipopolysaccharide. J Anim Sci 2003;81(5):1166–76.
Gerjets I, Kemper N. Coliform mastitis in sows: a review. J Swine Health Prod 2009;17(2):97–105.
Gu B, Miao J, Fa Y, Lu J, Zou S. Retinoic acid attenuates lipopolysaccharide-induced inflammatory responses by suppressing TLR4/NF-κB expression in rat mammary tissue. Int Immunopharmacol 2010;10(7):799–805.
Hansen PJ, Soto P, Natzke RP. Mastitis and fertility in cattle – possible involvement of inflammation or immune activation in embryonic mortality. Am J Reprod Immunol 2004;51(4):294–301.
Harizi H, Corcuff J-B, Gualde N. Arachidonic-acid-derived eicosanoids: roles in biology and immunopathology. Trends Mol Med 2008;14(10):461–9.
Hortet P, Seegers H. Loss in milk yield and related composition changes resulting from clinical mastitis in dairy cows. Prev Vet Med 1998;37(1):1–20.
Huang S, Rutkowsky JM, Snodgrass RG, Ono-Moore KD, Schneider DA, Newman JW, et al. Saturated fatty acids activate TLR-mediated proinflammatory signaling pathways. J Lipid Res 2012;53(9):2002–13.
Lee JY, Sohn KH, Rhee SH, Hwang D. Saturated fatty acids, but not unsaturated fatty acids, induce the expression of cyclooxygenase-2 mediated through Toll-like receptor 4. J Biol Chem 2001;276(20):16683–9.
Lin S, Hou J, Xiang F, Zhang X, Che L, Lin Y, et al. Mammary inflammation around parturition appeared to be attenuated by consumption of fish oil rich in n-3 polyunsaturated fatty acids. Lipids Health Dis 2013;12:190.
Miao JF, Zhu YM, Gu BB, Wang XB, Zou SX, Deng YE. Evaluation of the changes of immune cells during lipopolysaccharide-induced mastitis in rats. Cytokine 2007;40(2):135–43.
Mora S, Pessin JE. An adipocentric view of signaling and intracellular trafficking. Diabetes Metab Res Rev 2002;18(5):345–56.
Oviedo-Boyso J, Valdez-Alarcón JJ, Cajero-Juárez M, Ochoa-Zarzosa A, López-Meza JE, Bravo-Patino A, et al. Innate immune response of bovine mammary gland to pathogenic bacteria responsible for mastitis. J Infect 2007;54(4):399–409.
Porcherie A, Cunha P, Trotereau A, Roussel P, Gilbert FB, Rainard P, et al. Repertoire of *Escherichia coli* agonists sensed by innate immunity receptors of the bovine udder and mammary epithelial cells. Vet Res 2012;43(1):14.
Roth JA, Kaeberle M. Evaluation of bovine polymorphonuclear leukocyte function. Vet Immunol Immunopathol 1981;2(2):157–74.
Santman-Berends IM, Olde Riekerink RG, Sampimon OC, van Schaik G, Lam TJ. Incidence of subclinical mastitis in Dutch dairy heifers in the first 100 days in lactation and associated risk factors. J Dairy Sci 2012;95(5):2476–84.
Seegers H, Fourichon C, Beaudeau F. Production effects related to mastitis and mastitis economics in dairy cattle herds. Vet Res 2003;34(5):475–91.
Shuster DE, Kehrli M, Rainard P, Paape M. Complement fragment C5a and inflammatory cytokines in neutrophil recruitment during intramammary infection with *Escherichia coli*. Infect Immun 1997;65(8):3286–92.
Spector AA. Plasma free fatty acid and lipoproteins as sources of polyunsaturated fatty acid for the brain. J Mol Neurosci 2001;16(2–3):159–65.
Stark KD, Beblo S, Murthy M, Buda-Abela M, Janisse J, Rockett H, et al. Comparison of bloodstream fatty acid composition from African–American women at gestation, delivery, and postpartum. J Lipid Res 2005;46(3):516–25.
Vorbach C, Harrison R, Capecchi MR. Xanthine oxidoreductase is central to the evolution and function of the innate immune system. Trends Immunol 2003;24(9):512–7.
Wood LG, Scott HA, Garg ML, Gibson PG. Innate immune mechanisms linking non-esterified fatty acids and respiratory disease. Prog Lipid Res 2009;48(1):27–43.
Xie C, Guo X, Long C, Fan Z, Xiao D, Ruan Z, et al . Supplementation of the sow diet with chitosanoligosaccharide during late gestation and lactation affects hepatic gluconeogenesis of suckling piglets. Anim Reprod Sci 2015;159:109–17.
Yin J, Ren W, Liu G, Duan J, Yang G, Wu L, et al. Birth oxidative stress and the development of an antioxidant system in newborn piglets. Free Radic Res 2013;47:1027–35.
Yin J, Wu M, Xiao H, Ren W, Duan J, Yang G, et al. Development of an antioxidant system after early weaning in piglets. J Anim Sci 2014;92:612–9.
Yin J, Duan J, Cui Z, Ren W, Li T, Yin Y. Hydrogen peroxide-induced oxidative stress activates NF-kB and Nrf2/Keap1 signals and triggers autophagy in piglets. RSC Adv 2015;5:15479–86.
Zhu Y, Fossum C, Berg M, Magnusson U. Morphometric analysis of proinflammatory cytokines in mammary glands of sows suggests an association between clinical mastitis and local production of IL-1beta, IL-6 and TNF-alpha. Vet Res 2007;38(6):871–82.
Zhu Y, Magnusson U, Fossum C, Berg M. Escherichia coli inoculation of porcine mammary glands affects local mRNA expression of Toll-like receptors and regulatory cytokines. Vet Immunol Immunopathol 2008;125(1):182–9.