

The protective effect of selenium on the acute injury in *S. aureus*-infected mouse mammary gland

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Abstract

In this study the protective effect of dietary Selenium during pregnancy on the acute mammary injury induced by *S. aureus* were observed. Eighty BALB/c pregnant mice were randomly divided into four groups: Control group (CG; Se-deficient basal diet during pregnancy without *S. aureus* infection; n = 20), Negative control (NG; 0.2 mg/kg Se-supplemented diet during pregnancy without *S. aureus* infection; n = 20), Positive group (PG; Se-deficient basal diet during pregnancy and infected with *S. aureus* four days after pregnancy; n = 20) and Treatment group (TG; 0.2 mg/kg Se-supplemented diet during pregnancy and infected with *S. aureus* four days after pregnancy; n = 20). 24 h after infection, all mice were euthanized and the mammary tissues were sampling. Pathological section and scanning electron microscope were used to observe the pathologic injury in mammary gland, 54146 genes were detected by high throughput sequencing to identify the differentially expressed gene, ELISA was used to determine the expression level of IL-1 β and TNF- α . Results shown that the mammary tissue structure were damaged by *S. aureus*, after selenium treatment the acute injury lightened, meanwhile 24434 were detected differentially expressed after *S. aureus* infection, which mainly regulate T cell activation, keratinization, cytokine production. Whereas only 20467 differentially expressed genes were detected in TG, in which genes code T cell activation and cytokine production were marked downregulated and gene code cell adhesion was upregulated. furthermore, selenium treatment significantly decreased the expression level of IL-1 β and TNF- α induced by *S. aureus*. In conclusion, selenium played a protective effect on the acute injury in *S. aureus*-infected mouse mammary gland.

Introduction

Mastitis is a serious problem in dairy industry worldwide which has a profound impact on animal health [1, 2]. *S. aureus* is a major representative pathogenic bacterium causing bovine mastitis [3, 4]. A number of studies have performed to explore the host/*S. aureus* interactions in the mammary gland. Jolly et al confirmed that *S. aureus* has the ability to survive and persist in host cells [5]. Furthermore, Wang et al recently reported that autophagy is involved in the *S. aureus* survival in cells [6, 7]. Mounting evidence suggests that *S. aureus* uses complex regulatory networks to sense diverse signals that enable it to internalize into host cell and adapt to different environments. As a result, mastitis induced by *S. aureus* is extremely difficult to control by treatment alone. Furthermore, up to 40% of mastitis cases induced by *S. aureus* may be recurrences of previous infections after therapy [8]. Thus, successful control is gained only through prevention of new infections and further study on the underlying mechanisms of *S. aureus* infection will be helpful.

Selenium is a widely used feedstuff additive in dairy industry for a series of biology function [9, 10]. On dairy industry selenium deficiency has proved to be associated with an increased incidence of mastitis [11]. Studies demonstrated that selenium could affect the mammary innate and the adaptive immune responses through cellular activities. For example, Numerous studies have shown selenium deficiency was proved to facilitate inflammation by regulating TLR2, NLRP3, Nod2-related pathways during mastitis [12, 13]. All of these studies suggested that selenium played a protective role in mastitis, however the

underlying mechanisms of selenium in regulating mastitis is complicated. Thus, further research is still needed to study the protective effect of selenium during mastitis.

Materials And Methods

Chemicals

S. aureus (ATCC29213) was purchased from Micro biologics Inc. (St. cloud, MN, US). Mouse IL-1 α , IL-1 β and TNF- α enzyme-linked immunosorbent assay (ELISA) kit was purchased from Cusabio Biotech (Wuhan, Hubei, China). Trizol was purchased from Invitrogen (Carlsbad, CA, US). The reverse transcriptase synthesis kit was purchased from Takara Biotech (Dalian, Liaoning, China). SYBR Premix Ex TaqTM II was purchased from Sheng gong Biotech (Shanghai, China).

Experimental design

Eighty BALB/c female mice and ten male mice (60 days old) were purchased from the Center of Experimental Animals of Hebei Agricultural University and the protocol was approved by the Animal Care and Ethics Committee of the Hebei Agricultural University. The mice were fed separately in an air-conditioned room maintained at a temperature of $24 \pm 1^\circ\text{C}$ for 2 weeks to adapt to the environment. The diet was prepared consulting the standard of AIN-93 purified diets for laboratory rodent in Animal Nutrition and Feed Science Laboratory of Hebei Agricultural University. Pregnancy is determined by vaginal plugs. Pregnant mouse was randomly divided into four groups: Control group and Experimental group. Control group (CG; Se-deficient basal diet during pregnancy without *S. aureus* infection; n=20), Negative control (NG; 0.2 mg/kg Se-supplemented diet during pregnancy without *S. aureus* infection; n=20), Positive group (PG; Se-deficient basal diet during pregnancy and infected with *S. aureus* four days after pregnancy; n=20) and Treatment group (TG; 0.2 mg/kg Se-supplemented diet during pregnancy and infected with *S. aureus* four days after pregnancy; n=20). 24 h after infection, all mice were euthanized and the mammary tissues were sampling.

Histopathologic examination

The tissues were collected and fixed in 10% formaldehyde solution, and then embedded in paraffin wax. The mammary tissue sections were stained with hematoxylin eosin (H&E) staining and the pathological changes were observed under a microscope (Olympus DX45, Japan).

Scanning electron microscope (TEM) examination

Mammary gland was perfusion fixed with 2.5% glutaraldehyde and 2% formaldehyde containing 2 mM CaCl_2 in 0.025 M sodium cacodylate buffer (pH 7.3) at 35°C for 10 minutes. Tissues were then removed and placed in the same fixative for 2–3 hours at 4°C . First, semi-thin slides were made using an ultramicrotome (LKB-2088) and stained with 1% toluidine blue (1% borax) on a 60°C hot plate for 2 min.

Then ultra-thin slices were made and stained with uranyl acetate and lead citrate. The cells microstructures were observed under TEM (JEM-1230, JEOL, Japan).

Enzyme-linked immunosorbent assay (ELISA) analysis

Mammary tissue samples were weighed and grinded with RIPA lysis buffer until homogenized. Then the homogenate was centrifuged at 12000rpm for 15min at 4°C, and the supernatant was collected in -20 °C until measurement. The expression of IL-1 β were measured by ELISA and according to the manufacturer.

Statistical analysis

All data is shown as the mean \pm S.E.M, and experiments were independently repeated at least 3 times. One-way ANOVA and Dunnett's test were used for statistical analyses. Values of $p < 0.05$ and were considered statistically significant.

High throughput sequencing analysis

We first identified all statistically enriched terms (can be GO/KEGG terms, canonical pathways, hall mark gene sets, etc., based on what your choice during the analysis), accumulative hypergeometric p-values and enrichment factors were calculated and used for filtering. Remaining significant terms were then hierarchically clustered into a tree based on Kappa-statistical similarities among their gene memberships (similar to what is used in NCI DAVID site). Then 0.3 kappa score was applied as the threshold to cast the tree into term clusters. The terms within each cluster are exported in the Excel spreadsheet named "Enrichment Analysis".

We then selected a subset of representative terms from this cluster and convert them into a network layout. More specifically, each term is represented by a circle node, where its size is proportional to the number of input genes fall into that term, and its color represent its cluster identity (i.e., nodes of the same color belong to the same cluster). Terms with a similarity score > 0.3 are linked by an edge (the thickness of the edge represents the similarity score). The network is visualized with Cytoscape (v3.1.2) with "force-directed" layout and with edge bundled for clarity. One term from each cluster is selected to have its term description shown as label.

Result

Result of histopathologic examination

Histopathologic examination was used to detect the pathological changes of mammary gland. The results are shown in Fig. 1. There were no obvious pathological changes in CG and NG (Fig. 1A and B); In PG, the acinar contour became unclear (Fig. 1C blue rectangular contour mark) and there was a small amount of inflammatory cell infiltration in the non-lactation acinar (Fig. 1C blue arrow mark); In TG, the

acinar contour was relatively complete, but there was still a small amount of inflammatory cell infiltration in the non-lactation acinar (Fig. 1D blue arrow mark).

Result of TEM examination

TEM examination was used to detect the over-all morphology of the mammary gland. The results are shown in Fig. 2. The profiles of acinar and ducts seen in CG and NG was like those familiar from histological sections. Connective tissue fibers were compacted between acinar and ducts, and capillaries, occasionally still enclosing erythrocytes, may be identified (Fig. 2A and B); In PG the profiles of acinar and ducts was damaged and connective tissue fibers was relaxed between acinar and ducts, furthermore a large number of bacterial particles were observed in the surface of mammary tissue (Fig. 2C); In TG the damage level of cellular structure induced by *S. aureus* and the number of bacterial particles attached to the surface of mammary gland were all reduced (Fig. 2D).

Result of high throughput sequencing examination

54146 genes were detected by high throughput sequencing to identify the differentially expressed gene. The results are shown in Fig. 3. Compared with CG, 24434 differential expressed genes were detected in PG, which mainly code T cell activation, Keratinization, cytokine production and other 17 kinds of biological functions (Fig. 3A) In TG, 20467 differential expressed genes were detected, among them gene code T cell activation and cytokine production were inhibited, meanwhile gene code cell adhesion was up-regulated Fig. 3B.

Result of ELISA examination

ELISA was used to detect the expression level of IL-1 β and TNF- α . The results are shown in Fig. 4. compared with CG and NG group the release of IL-1 β and TNF- α increased significantly in PG group ($P<0.05$) and selenium treatment could significantly decrease the release of these two cytokines in mammary gland ($P<0.05$) (Fig. 4A and B).

Discussion

S. aureus induced mastitis will cause serious economic losses to the bovine industry [14, 15]. in majority of the cases reduced milk production due to mastitis is associated with irreversible mammary tissue damage. Potential mechanism underpinning the characterization of the inflammatory immune responses of mammary tissue damage response to *S. aureus* infection have been reported previously [16, 17]. However, limited studies have been reported on the potential impact of mammary tissue damage due to *S. aureus* infection. In our study, the main aim was to observed the pathology subsequent to infection of the mammary glands with a high dose of *S. aureus* strains. The level of mammary tissue damage was measured based upon histopathologic and TEM examination. Histopathological and TEM examination showed *S. aureus* infection led serious structural damage to acinar, ducts and connective tissue fibers on mammary gland, similar to what has been observed in previous research [18, 19]. In selenium treatment

group (TG), we observed that selenium obviously relieved *S. aureus*-induced mammary tissue damage, indicated that selenium plays a protective effect on the acute injury in *S. aureus*-infected mouse mammary gland.

Mastitis-related immune response is a complex biological process involving multiple signaling pathways and a large number of signaling genes. It is well-known that two PRRs mainly response for *S. aureus* infection: Toll-like receptors family (TLRs) and Nod-like receptors family (NLRs) [20, 21]. It is also known that inflammation related signaling pathways such as NF- κ B and MAPK played an important role during *S. aureus* mastitis [22, 23]. Thus, screening and identifying susceptible genes associated with selenium nutrition will provide novel strategies to reduce the incidence of mastitis and improve the utilization rate of selenium. High-throughput sequencing technique that offers a powerful method for screened and identified immune-regulated gene correlative with mastitis. In this study, A total of 54146 genes were identified, of which 24434 were detected differentially expressed after *S. aureus* infection, which mainly regulate T cell activation, keratinization, cytokine production. Whereas only 20467 differentially expressed genes were detected in selenium treatment, interestingly gene code T cell activation and cytokine production were marked downregulated and gene code cell adhesion was upregulated. The results implicate a role of these genes as important targets for selenium.

High-throughput sequencing have verified that selenium treatment may led to the inhibition of genes that code cytokine production. Then the activation of cytokines such as IL-1 β and TNF- α were test, and the result showed the *S. aureus*-induced IL-1 β and TNF- α secretions were significantly inhibited by selenium. As IL-1 β and TNF- α plays a central role in initiating and regulating the cytokine cascade during an inflammatory response [24, 25], so we think selenium has a greater inhibitory effect on the production of secondary cytokines. The result in the present study indicated that selenium played a protective effect on the acute injury in *S. aureus*-infected mouse mammary gland.

Declarations

Funding Information

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Compliance with Ethical Standards Ethical

Ethical approval: All experimental procedures were conducted with the approval of the Institutional Animal Care and Use Committee of Hebei Agricultural University.

Author contributions: Qin Jianhua and Wang Yueshang contributed to the overall study design and supervised all research. Liu Junjun carried out the experiments, analyzed the data and were also responsible for the final editing of the manuscript. Zhan Guanjian prepared Figures and contributed partly to writing and finally revising the manuscript and data analysis. Zhao Yuping drafted and revised the first version of the manuscript. All the authors reviewed and finally approved the manuscript.

Data availability: All data used during the study appear in the submitted article.

Conflict of Interest

The authors declare that they have no conflict of interest.

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Figures

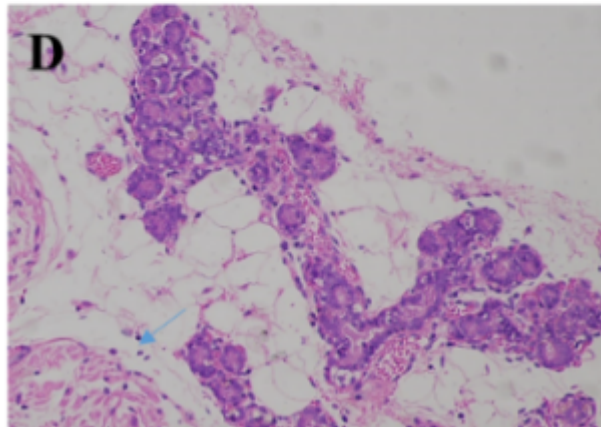
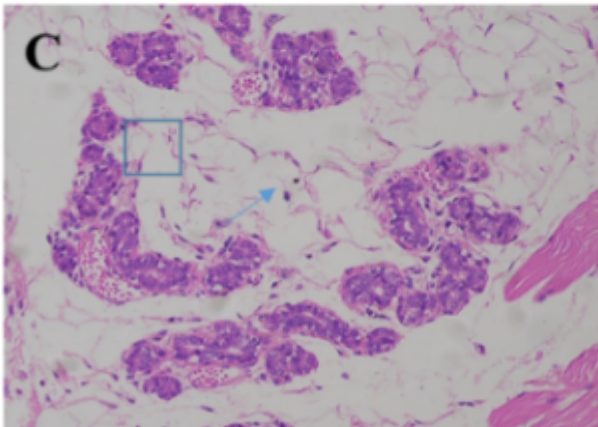
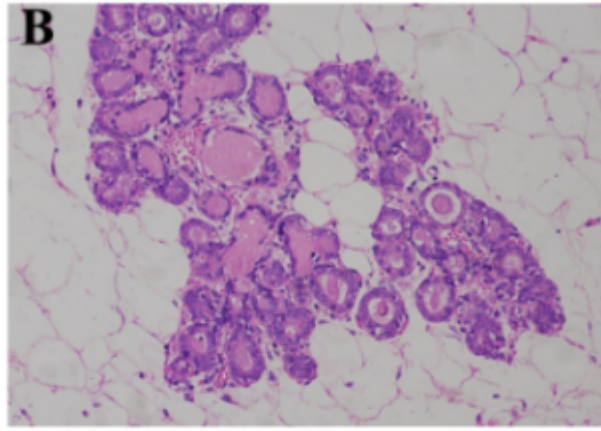
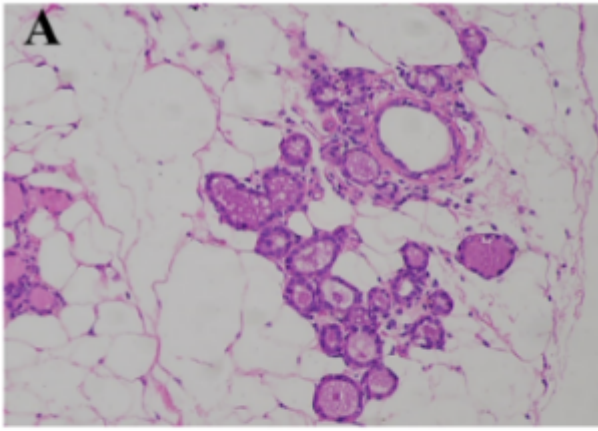


Figure 1

Pathological changes in mammary gland (HE staining, $\times 200$). A: CG; B: NG; C: PG; D: TG.

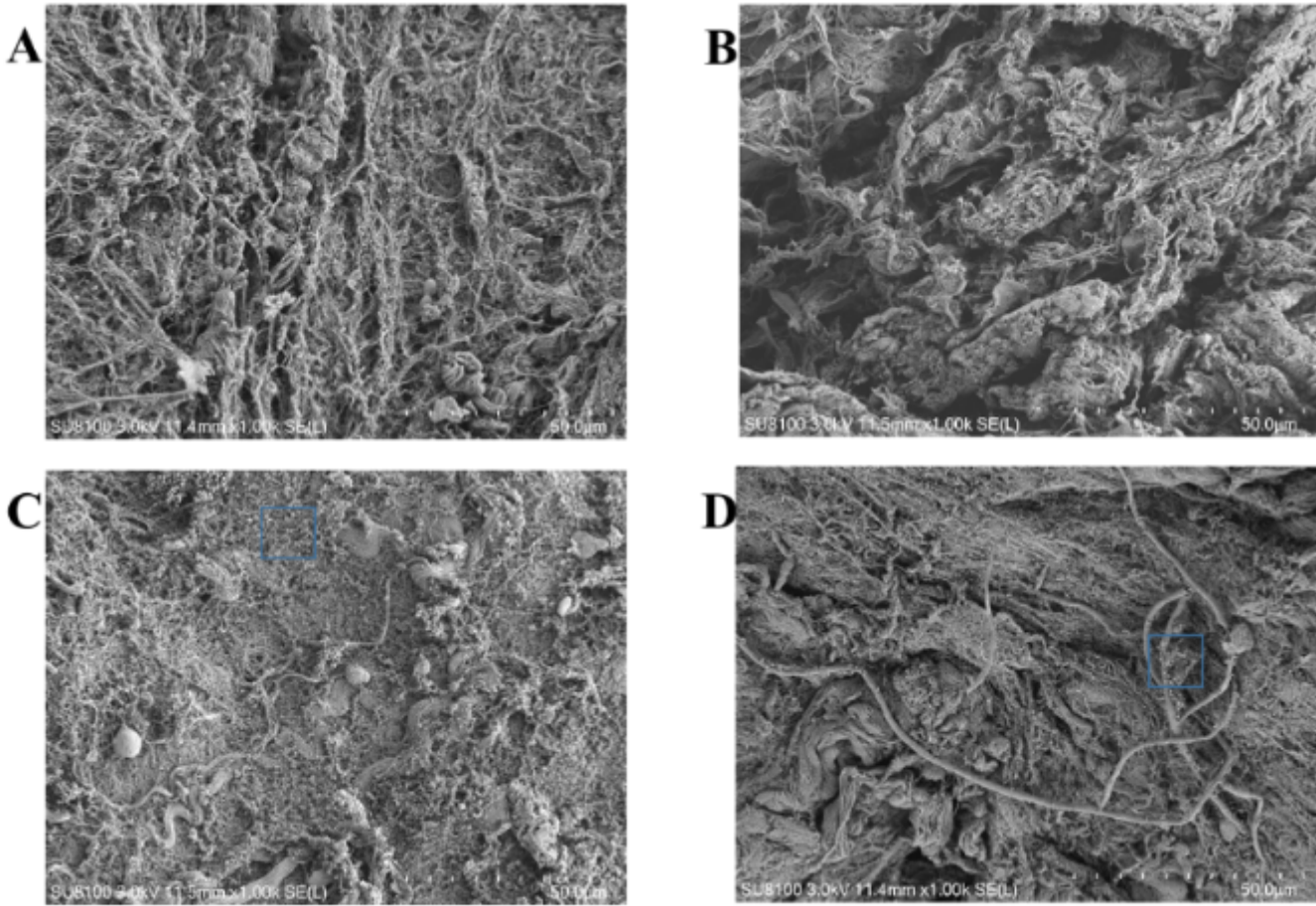


Figure 2

TEM analysis of mammary gland. A: CG; B: NG; C: PG; D: TG.

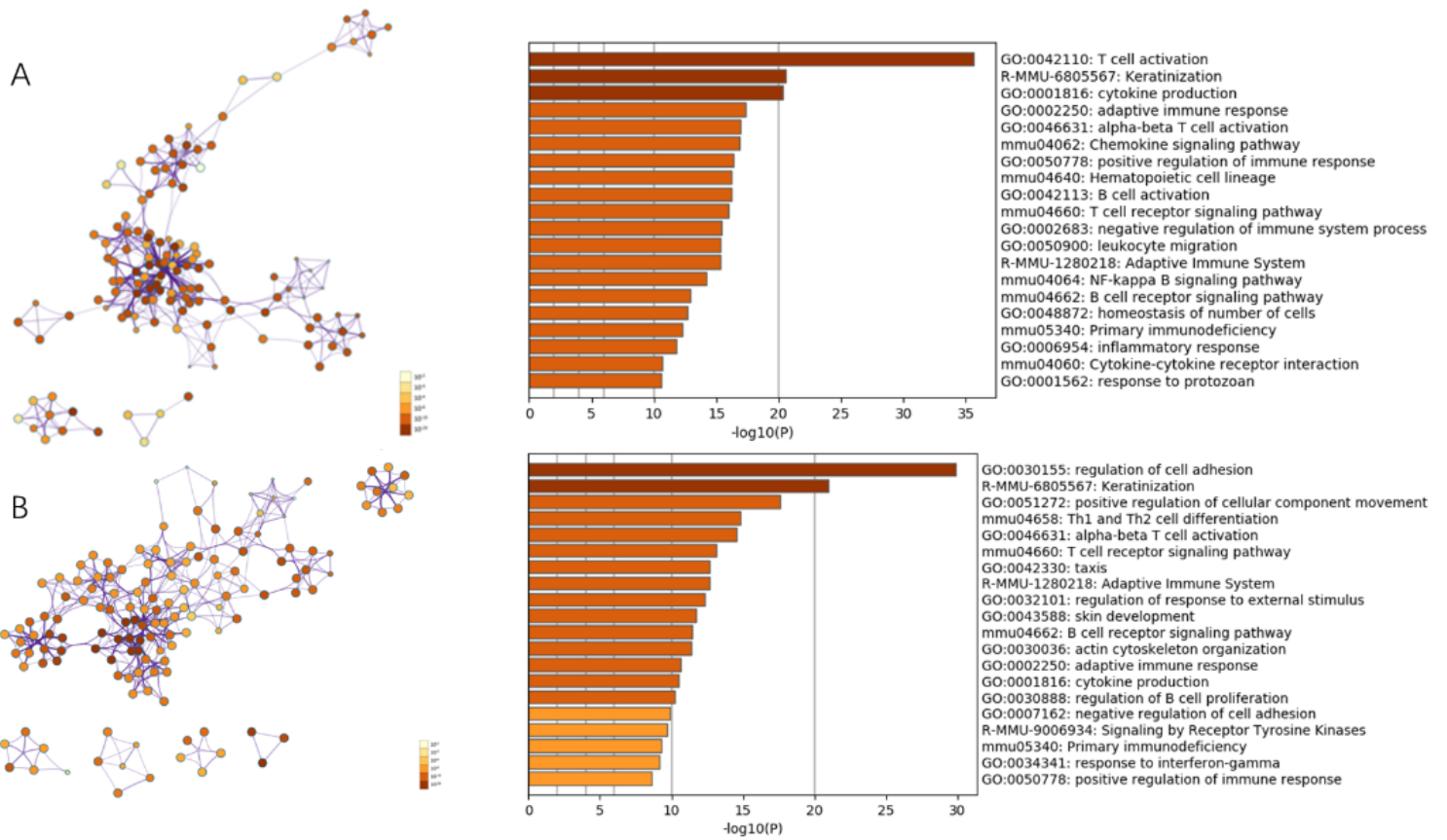


Figure 3

Enriched ontology clusters colored by P-Value. A: PG vs CG; B: TG vs CG

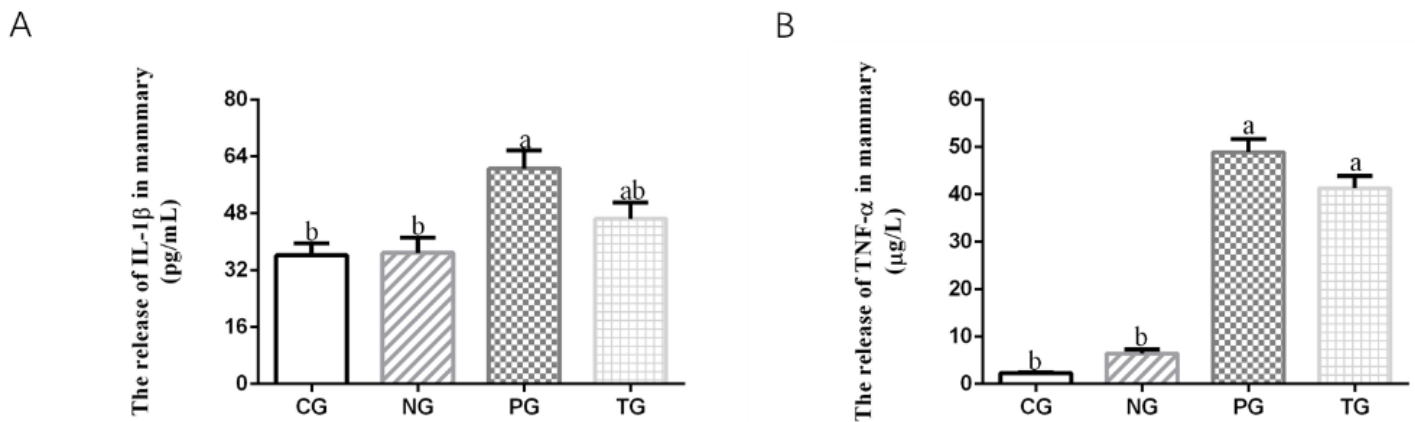


Figure 4

The cytokines release changes in mammary gland. A: the release of IL-1 β in mammary gland; B: the release of TNF- α in mammary gland. Means with different letters (a, b) are significantly different ($P < 0.05$) from each other.