

The glucocorticoid inhibits neutrophils formed extracellular traps (NETs) and suppresses the inflammation caused by fallopian tube staphylococcal infection

S.-X. LUAN, X.-H. CHEN

Department of Reproductive Medicine, Weifang People's Hospital, Weifang, Shandong, China.

Abstract. – **OBJECTIVE:** Fallopian tube can transport zygote into the uterine cavity, while inflammation may cause certain influences on the fallopian tube's function. Neutrophils formed extracellular traps (NET) can kill pathogenic microorganisms. This study intends to analyze the role of glucocorticoid in the regulation of NETs sterilization during the fallopian tube staphylococcal infection.

MATERIALS AND METHODS: Rat fallopian tube staphylococcal infection model was established. Group A was the control group, group B was the model group, and group C was the dexamethasone intervention group. ELISA was applied to test inflammatory factors, including citrullinated histone H3 (CitH3) and high molecular weight kininogen (HK) content in serum. RT-PCR was performed to test the mRNA expression of glucocorticoid receptor α , β (GR- α , GR- β). Western blot was used to detect the protein levels of GR- α and GR- β .

RESULTS: Microscopically, group A showed clear fallopian tube wall and unobstructed lumen. Group B presented obscured tube wall, blocked lumen, and inflammatory cells infiltration. Group C demonstrated unclear tube wall and a few inflammatory cells infiltration. Serum CitH3 level was increased, while HK was down-regulated in group B compared with group A. CitH3 was declined, whereas HK was enhanced in group C compared with group B ($p < 0.05$). The mRNA expression of GR- β was reduced, while GR- α expression was elevated in group C compared with group A and B. Group B showed upregulated GR- β expression and reduced GR- α mRNA and protein expression compared with group A ($p < 0.05$).

CONCLUSIONS: Rat fallopian tube *Staphylococcus aureus* infection activates NETs, elevates CitH3, and decreases HK. Glucocorticoid can inhibit inflammation through down-regulate GR- β and up-regulate GR- α expression.

Key Words:

Staphylococcus aureus, NETs, Glucocorticoid.

Introduction

Tubal factor accounts for 25-50% of factors that causes women infertility. Fallopian tube inflammatory lesions occur in numerous unfertilized women. Bacterial infection is the main pathogenic factor of chronic salpingitis, including purulent *Staphylococcus*, *Streptococcus*, *E. coli*, and *Pseudomonas aeruginosa*, leading to impurity abortion, incomplete abortion, induced abortion, and even puerperal infection¹. As the main effector cells of innate immune system, neutrophils can resist a variety of pathogenic bacteria invasion in the first line². Studies showed that neutrophils can release a kind of nuclear substance out of the cell, which constitutes the extracellular traps (NETs). It can create synergy together with extracellular chromosome skeleton to form physical barrier against pathogenic microorganisms. Also, it can be used as a scaffold to enhance the effect of antimicrobial drugs^{3,4}. Glucocorticoid (GC) is a commonly used drug that plays an anti-inflammatory effect quickly and strongly. GC can restrain capillary expansion, reduce vascular leakage and edema, and inhibit leukocyte infiltration as well as the phagocytosis of macrophage. Furthermore, it can regulate immunity and reduce cytokines release, thus alleviating inflammation damage to the body and enhancing system function^{5,6}. This study intends to analyze the mechanism of GC in the regulation of NETs sterilization during the fallopian tube staphylococcal infection.

Materials and Methods

Experimental Animals

Healthy female Wistar rats in SPF grade aged 12-14 weeks old and weighted 150-200 g were

provided by Experimental Animal Center of Shandong University.

Rats were used for all experiments, and all procedures were approved by the Animal Ethics Committee of Weifang People's Hospital.

Instruments and Reagents

Staphylococcus aureus was diluted by normal saline to adjust the concentration to be 3×10^9 /ml (provided by microorganism teaching and research section, Liaoning Institute of Traditional Chinese Medicine. Bacterium number: ATCC25923). Na₂EDTA anticoagulant, ELISA kit, CitH3 and HK ELISA kits, RPMI1640 medium, PBS, and GR- β antibody were from NOVUS (St. Louis, MO, USA). GAPDH antibody was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Western blot TMB substrate was from Thermo Fisher Scientific (Waltham, MA, USA). Reverse transcription kit was from Takara (Kusatsu, Shiga, Japan). Trizol was from ABI (Carlsbad, CA, USA). The centrifugal machine was from Shanghai Flying Pigeon (Shanghai, China). The inverted microscope was from Olympus (Tokyo, Japan). Microplate reader was from TECNA (c/o Area Science Park, Padriciano, Trieste, IT). Image analysis system was from HP (Palo Alto, CA, USA).

Rat *Staphylococcus Aureus* Induced Salpingitis Model Establishment

The rats were anesthetized by anhydrous diethyl ether and fixed on supine. After disinfection, a longitudinal incision at 1.5 cm long was made at 0.5 cm above pubic symphysis. The abdominal cavity was opened to expose uterus. Then, the needle was injected at cornua uteri close the fallopian tube. A total of 0.02 ml bacteria suspension was slowly injected into fallopian tube-ovarian direction. After injection, the cornua uteri were clipped by tweezers for 1 min before closing the abdomen^{7,8}.

Grouping

Group A, normal healthy rats received 1 ml normal saline i.v. Qd.

Group B, *Staphylococcus aureus* salpingitis model rats received 1 ml normal saline i.v. Qd.

Group C, *Staphylococcus aureus* salpingitis model rats received 1 ml dexamethasone at 0.1% i.v. Qd.

Sample Collection

The rats were euthanized on day 45 after modeling. Venous blood was centrifuged at 3000 r/min for 20 min and the supernatant was stored. Fallopian tube tissue was fixed with formaldehyde solution for H&E staining. The left tissue was applied for Western blot and RT-PCR.

ELISA

Serum was collected to detect inflammatory factors CitH3 and HK level according to the manual. The diluted standard substance was added into 96-well plate to prepare a standard curve. The sample was added to the well with five replicates. After sampling, washing, developing, and stopping, the plate was read at 450 nm to draw the linear regression equation and calculate standard curve.

RT-PCR

Fallopian tube tissue mRNA was extracted using Trizol. Its concentration was identified by D260 nm/D280 nm. A total of 200 ng RNA was reversely transcribed to cDNA. The primers used were listed in Table I. Reverse transcription system contained 2 μ l RNA and 1 μ l primer. Real-time PCR reaction system contained 3 μ l cDNA, 1 μ l Primer, and 0.2 μ l Taq DNA polymerase. The reaction condition was composed by 94°C for 3 min, followed by 30 cycles of 94°C for 40 s, 56°C for 1 min, and 72°C for 1 min. GAPDH was used as an internal reference. The gel image was analyzed by Quantity One software (Bio-Rad, Hercules, CA, USA).

Western Blot

The tissue was centrifuged at 1200 r/min for 20 min and the supernatant was moved to new tube. Total protein was separated by 8% sodium dodecyl sulphate-polyacrylamide gel electropho-

Table I. Primer sequence.

Gene	5'-3'	
GR- β mRNA	Forward	ACACAGGCTTCAGGTATCTT
	Reverse	CGCCAAGATTGTTGGGATGA
GAPDH	Forward	TGAAGGTCGGTGTGAACGGATTTGG
	Reverse	ACGACATACTCAGCACCAGCATCAC

Table II. ELISA detection of serum CitH3 and HK levels.

Group	Cases	CitH3 ($\mu\text{g/L}$)	HK (ng/L)
Group A	20	118.31	573.92 \pm 149.91
Group B	20	187.25*	243.45 \pm 131.14*
Group C	20	136.81*#	418.02 \pm 131.26*#

* $p < 0.05$, compared with group A; # $p < 0.05$, compared with group B.

resis (SDS-PAGE). Then, it was transferred to NC membrane and blocked with 5% skim milk at room temperature for 1 h. After washed with tris buffered saline-tween (TBST) for 3 times, the membrane was incubated with primary antibody (1:200 dilution) at 4°C overnight. Next, the membrane was incubated with secondary antibody (1:2000 dilution) for 60 min and chemiluminescence reagent was added. The membrane was developed and analyzed by Quantity One software (Bio-Rad, Hercules, CA, USA).

Statistical Analysis

SPSS 17.0 software (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. The data was presented as mean \pm standard deviation (SD) and compared by ANOVA and LSD test. $p < 0.05$ was considered as statistical significance.

Results

Fallopian tube changes

The rats in group A showed balanced and smooth fallopian tube with good elasticity and without adhesion, hydrops, or suppuration. The fallopian tube in-group B became thin, rough, and pale with

adhesion, poor elasticity, hydrops, and suppuration. The fallopian tube in-group C was slightly rough, partly adhesion, and unobvious hydrops. Microscopically, group A showed clear fallopian tube wall and unobstructed lumen. Group B presented obscured tube wall, blocked lumen, fibrous tissue hyperplasia, and inflammatory cells infiltration, such as lymphocytes, plasmocytes, and eosinophils. Group C demonstrated unclear tube wall and a few inflammatory cells infiltration (Figure 1).

ELISA Detection of Serum CitH3 and HK Levels

ELISA was applied to test serum CitH3 and HK levels in rat. The results showed that serum CitH3 level was obviously increased, while HK was down-regulated in-group B compared with group A ($p < 0.05$). CitH3 level was declined, whereas HK enhanced in-group C compared with that in-group B ($p < 0.05$) (Table II).

RT-PCR Detection of GR- α and GR- β mRNA Expressions in Rat Fallopian Tube Tissue

GR- α and GR- β mRNA expressions in rat fallopian tube tissue were tested. GR- β mRNA level was reduced, while GR- α mRNA was elevated in group C compared with that in group A and B. Group B showed upregulated GR- β mRNA level and reduced GR- α mRNA expression compared with group A ($p < 0.05$) (Table III).

Western blot detection of GR- α and GR- β protein levels in rat fallopian tube tissue

GR- α and GR- β protein levels in rat fallopian tube tissue were tested. GR- β protein expression was declined, while GR- α protein level was

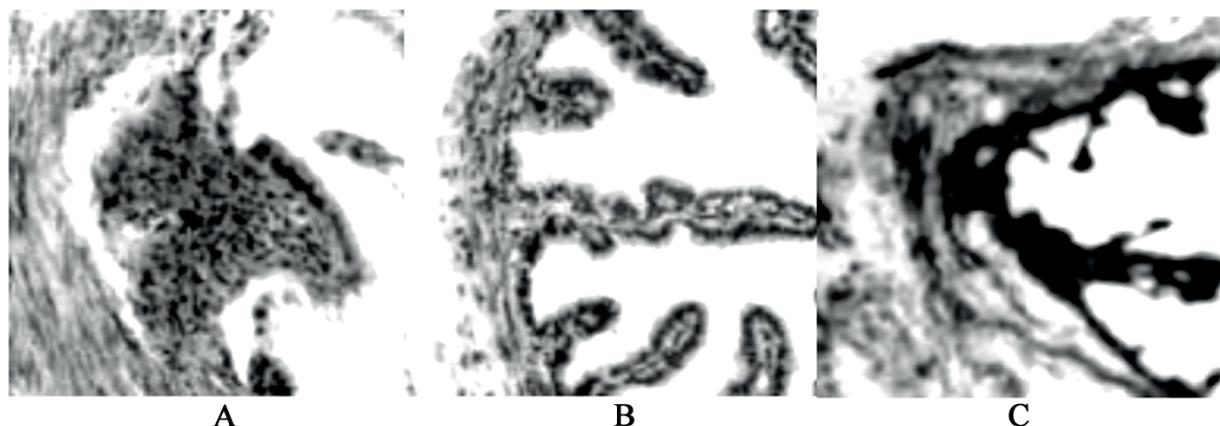


Figure 1. Fallopian tube changes under light microscope ($\times 200$).

enhanced in group C compared with group A and B. Group B demonstrated increased GR- β protein level and reduced GR- α protein level compared with group A ($p < 0.05$) (Figure 2).

Discussion

Chronic salpingitis is a common gynecological disease. Patients may appear abdominal pain, dysmenorrhea, menstruation, and even infertility. Various pathogens infection may cause fallopian tube inflammation. During this process, bacterial infection leads to a large number of leukocytes infiltration, tubal interstitial edema, fallopian tube intima swelling, tubal mucosa epithelium exuviation, fibrous tissue hyperplasia, granulation tissue organization, fimbriated extremity of fallopian adhesion, resulting in fallopian tube obstruction⁹. A previous research showed that pathologically, salpingitis appears exudation, hydrops, empyema, adhesion, and fibrous scar formation. Fallopian tube wall becomes stiffness and thickening that blocks zygote pass through, leading to infertility¹⁰. As an important member of the immune system, neutrophils can reach each part following the circulation. The infection causes a lot of white blood cells and macrophage infiltration and stimulation through the blood vessel wall to reach the lesions¹¹. NETs are released by the nuclear components of neutrophils to the extracellular space, thus forming a network. It is mainly composed of chromosome mesh structure and grain. It is found that NETs appeared in a variety of vertebrates¹².

In this study, we constructed rat *Staphylococcus aureus* infected salpingitis model and treated the rat with dexamethasone. The rats were killed on day 45 after modeling. Group A

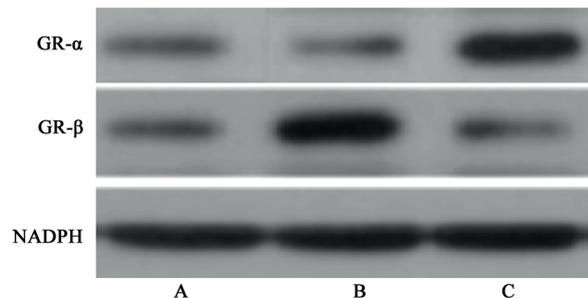


Figure 2. GR- α and GR- β protein levels in rat fallopian tube tissue.

Table III. RT-PCR detection of GR- α and GR- β mRNA expressions in rat fallopian tube tissue.

Group	Cases	GR- α mRNA	GR- β mRNA
Group A	20	2.23±0.53	2.58±0.31
Group B	20	1.32±0.32*	6.25±0.81*
Group C	20	7.61±1.02*#	1.13±0.18*#

* $p < 0.05$, compared with group A; # $p < 0.05$, compared with group B.

showed balanced and smooth fallopian tube with good elasticity and without adhesion, hydrops, or suppuration. The fallopian tube in-group B became thin, rough, and pale with adhesion, poor elasticity, hydrops, and suppuration. The fallopian tube in-group C was slightly rough, partly adhesion, and unobvious hydrops. Microscopically, group A showed clear fallopian tube wall and unobstructed lumen. Group B presented obscured tube wall, blocked lumen, fibrous tissue hyperplasia, and inflammatory cell infiltration, such as lymphocytes, plasmocytes, and eosinophils. Group C demonstrated unclear tube wall and a small amount of inflammatory cells infiltration. It suggested that we successfully established rat *Staphylococcus aureus* infected salpingitis model, and dexamethasone intervention could alleviate inflammation.

Plasma coagulation factor XII (FXII), FXI, prekallikrein, and high molecular weight kininogen (HK) form the body contact system together. The inflammatory reaction is mainly caused by FXII contacting with negative charge, leading to bradykinin activation and contact system factors consumption, and further development of inflammatory symptoms^{13,14}. NETs, released by died neutrophils, are mainly composed of the nucleic acid material and grain with strong killing effect on all kinds of pathogenic microorganisms^{15,16}. A previous study showed that citrullinated histone H3 (CitH3) is an important marker of NETs. It can activate contact system to trigger inflammation¹⁷. In this study, we tested serum CitH3 and HK content in the rats. Serum CitH3 level was increased, while HK decreased in group B compared with that in group A. CitH3 level was declined, whereas HK was enhanced in group C compared with group B. It indicated that *Staphylococcus aureus* caused fallopian tube inflammation and activated NETs, leading to CitH3 elevation. Furthermore, it consumed HK in the contact system and GC can inhibit inflammation and suppress NETs.

GC is a commonly used drug for the treatment of inflammation in clinic. It plays a rapid and strong anti-inflammatory effect through binding to GR- α in the cytoplasm, activating anti-inflammatory genes, and suppressing inflammatory genes. In this process, GR- β , another receptor of GC, can affect inflammation by inhibiting GR- α biological activity though cannot bind to GR- α ¹⁸⁻²⁰. In our research, we used RT-PCR and Western blot to test GR- α and GR- β mRNA and protein expression in rat fallopian tube tissue. The GR- β level was reduced, while GR- α was elevated in group C compared with group A and B. Group B showed up-regulated GR- β and reduced GR- α mRNA and protein expression compared with group A. It suggested that *Staphylococcus aureus* elevated GR- β and declined GR- α level in salpingitis, while GC intervention restrained GR- β expression. Our results indicated that dexamethasone intervention can down-regulate GR- β expression, which facilitates the anti-inflammation effect of GC.

Conclusions

Fallopian tube *Staphylococcus aureus* infection activates NETs, elevates CitH3, and expends HK. GC can inhibit inflammation through down-regulating GR- β expression. However, the exact mechanism by how GC down-regulates GR- β expression remains unclear and requires further investigation.

Acknowledgments

This work was supported by Weifang Health and Family Planning Commission Scientific Research Project (No. 2015066).

Conflict of interest

The authors declare no conflicts of interest.

References

- PEARLMAN MD, MCNEELEY SG, FRANK TS, HOEFT-LOYER C. Antiendotoxin antibody is protective against tubal damage in an Escherichia coli rabbit salpingitis model. *Am J Obstet Gynecol* 1994; 171: 1588-1593.
- MANTOVANI A, CASSATELLA MA, COSTANTINI C, JAILLON S. Neutrophils in the activation and regulation of innate and adaptive immunity. *Nat Rev Immunol* 2011; 11: 519-531.
- CHUAMMITRI P, OSTOJIC J, ANDREASEN CB, REDMOND SB, LAMONT SJ, PALIC D. Chicken heterophil extracellular traps (HETs): novel defense mechanism of chicken heterophils. *Vet Immunol Immunopathol* 2009; 129: 126-131.
- YOUNG RL, MALCOLM KC, KRET JE, CACERES SM, POCH KR, NICHOLS DP, TAYLOR-COUSAR JL, SAAVEDRA MT, RANDELL SH, VASIL ML, BURNS JL, MOSKOWITZ SM, NICK JA. Neutrophil extracellular trap (NET)-mediated killing of *Pseudomonas aeruginosa*: evidence of acquired resistance within the CF airway, independent of CFTR. *PLoS One* 2011; 6: e23637.
- YU FL, LIU TT, ZHU X, YANG WX, ZHANG T, LIN N, LIU Y, LIU CS, JIANG J, GUAN JC. Staphylococcal enterotoxin B and alphatoxin induce the apoptosis of ECV304 cells via similar mechanisms. *Mol Med Rep* 2013; 8: 591-596.
- CORRALES-MEDINA VF, SERPA J, RUEDA AM, GIORDANO TP, BOZKURT B, MADJID M, TWEARDY D, MUSER DM. Acute bacterial pneumonia is associated with the occurrence of acute coronary syndromes. *Medicine (Baltimore)* 2009; 88: 154-159.
- PATTON DL, SWEENEY YT, KUO CC. Demonstration of delayed hypersensitivity in Chlamydia trachomatis salpingitis in monkeys: a pathogenic mechanism of tubal damage. *J Infect Dis* 1994; 169: 680-683.
- FEIN DM, SELLINGER C, FAGAN MJ. Acute salpingitis in a nonsexually active adolescent. *Pediatr Emerg Care* 2015; 31: 853-855.
- TUFFREY M, WOODS C, INMAN C, WARD M. The effect of a single oral dose of azithromycin on chlamydial infertility and oviduct ultrastructure in mice. *J Antimicrob Chemother* 1994; 34: 989-999.
- LIMBERG J, GINSBURG H, LALA S, TOMITA S. Recurrent pelvic infections and salpingitis after perforated appendicitis. *J Pediatr Adolesc Gynecol* 2015; 28: e177-178.
- PILSCZEK FH, SALINA D, POON KK, FAHEY C, YIPP BG, SIBLEY CD, ROBBINS SM, GREEN FH, SURETTE MG, SUGAI M, BOWDEN MG, HUSSAIN M, ZHANG K, KUBES P. A novel mechanism of rapid nuclear neutrophil extracellular trap formation in response to *Staphylococcus aureus*. *J Immunol* 2010; 185: 7413-7425.
- LIN AM, RUBIN CJ, KHANDPUR R, WANG JY, RIBLETT M, YALAVARTHI S, VILLANUEVA EC, SHAH P, KAPLAN MJ, BRUCE AT. Mast cells and neutrophils release IL-17 through extracellular trap formation in psoriasis. *J Immunol* 2011; 187: 490-500.
- OEHMCKE S, HERWALD H. Contact system activation in severe infectious diseases. *J Mol Med (Berl)* 2010; 88: 121-126.
- NEELI I, KHAN SN, RADIC M. Histone deimination as a response to inflammatory stimuli in neutrophils. *J Immunol* 2008; 180: 1895-1902.

- 15) ICHIRO K. Current concept of steroid tachyphylaxis in the skin. *Jpn J Dermatol* 2004; 114: 2088-2092.
- 16) GOLEVA E, LI LB, EVES PT, STRAND MJ, MARTIN RJ, LEUNG DY. Increased glucocorticoid receptor beta alters steroid response in glucocorticoid-insensitive asthma. *Am J Respir Crit Care Med* 2006; 173: 607-616.
- 17) CORRALES-MEDINA VF, FATEMI O, SERPA J, VALAYAM J, BOZKURT B, MADJID M, MUSER DM. The association between *Staphylococcus aureus* bacteremia and acute myocardial infarction. *Scand J Infect Dis* 2009; 41: 511-514.
- 18) LU J. The anti-proliferation mechanism of glucocorticoid mediated by glucocorticoid receptor-regulating gene expression. *Pathophysiology* 2009; 16: 267-272.
- 19) BARNES PJ. Mechanisms and resistance in glucocorticoid control of inflammation. *J Steroid Biochem Mol Biol* 2010; 120: 76-85.
- 20) LEWIS-TUFFIN LJ, JEWELL CM, BIENSTOCK RJ, COLLINS JB, CIDLOWSKI JA. Human glucocorticoid receptor beta binds RU-486 and is transcriptionally active. *Mol Cell Biol* 2007; 27: 2266-2282.