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Transcriptional changes of cytokines in rooster testis and epididymis during sexual maturation stages and *Salmonella* infection

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ABSTRACT

Infection of rooster testis and epididymis by pathogens can lead to impaired fertility, resulting in economic losses in the poultry industry. Antimicrobial protection of rooster reproductive organs is, therefore, an important aspect of reproductive physiology. Salmonellosis is one of the most important zoonotic diseases, caused by *Salmonella* bacteria including *Salmonella Enteritidis* (SE) and is usually the result of infection of the reproductive organs. Thus, knowledge of the endogenous innate immune mechanisms of the rooster testis and epididymis is an emerging aspect of reproductive physiology. Cytokines are key factors for stimulating the immune response and inflammation in chickens to *Salmonella* infection. In the present study the expression profile of 11 pro-inflammatory cytokine genes in the rooster testis and epididymis *in vivo* and transcriptional changes in these organs during sexual maturation and SE infection were investigated. Gene expression analysis data revealed that in both testis and epididymis nine cytokines namely the IL-1β, IL-6, IL-8, IL-10, IL-12, IL-15, IL-16, IL-17 and IL-18 genes were expressed, while no mRNA transcripts were detected in both organs for IL-2 and IL-4. Furthermore, the expression of various cytokine genes during sexual maturation appeared to be developmentally regulated, while SE infection resulted in a significant up-regulation of IL-1β, −6, −12 and −18 genes in the testis and an increase in the mRNA relative abundance of IL-1β, −6, −12, −16 and −18 in the epididymis of SE-infected sexually mature 28-week-old roosters. These results suggest a cytokine-mediated immune response mechanism against *Salmonella* infection in the rooster reproductive tract.

1. Introduction

Infection and inflammation of the male reproductive tract are widely accepted as important etiological factors of subfertility or infertility. Research on antimicrobial protection of male reproductive organs has revealed the importance of the innate immune system in controlling microbial infections of the male genital tract.

In avian species, infection of the reproductive organs can cause orchitis, epididymitis, epididymo-orchitis, induce semen abnormality and, therefore, reduce fertility and spread pathogen contamination via copulation and artificial insemination (Boltz et al., 2006; Jackson et al., 2006; Monleon et al., 2008). Antimicrobial protection of rooster reproductive organs is, therefore, an important aspect of reproductive physiology. Although several microbial

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pathogens are able to invade and colonize the male reproductive tract and semen in livestock, and in particular in avian species, antimicrobial protection and function of the innate immune system in the rooster reproductive tract has not yet been studied extensively. Furthermore, immunological function and the defense to infections, particularly to bacteria, are poorly understood in the rooster testis and epididymis at the molecular level.

The innate immune system has an essential role in host defense against infection. Toll-like receptors (TLRs) have been identified as one of the key components of innate immune recognition in vertebrate species (Akira et al., 2001; Brownlie and Allan 2010) and have been reported to be expressed in the reproductive organs of various vertebrates, including chickens (Fazeli et al., 2005; Andersen et al., 2006; Shimada et al., 2006; Soboll et al., 2006; Herath et al., 2007; Ozoe et al., 2009; Michailidis et al., 2010, 2011; Anastasiadou et al., 2011, 2013). The TLRs recognize a range of microbial molecular patterns and generate intra-cellular signals through nuclear factor-kB (NF-kB) dependent pathways, to induce cellular responses, such as synthesis of antimicrobial peptides and proinflammatory cytokines (Zarember and Godowski 2002; Kogut et al., 2006; Berndt et al., 2007; Cheeseman et al., 2008; Abdel Mageed et al., 2011). Cytokines are important factors for stimulating the immune response and inflammation. They are a group of mediators regulating cellular function that are secreted by specific cells to affect the function of other cells, having a role in the regulation of immune and inflammatory processes (Giansanti et al., 2006). Proinflammatory cytokines, such as interleukins, have an important role in initiating innate and adaptive immune responses and assist in generating a local inflammatory response, with a well-documented role in reproductive physiology (Robertson et al., 1997; Staeheli et al., 2001; Hiscott and Ware 2011).

In chickens, a common downstream result of TLR stimulation is the induction of pro-inflammatory cytokines (St. Paul et al., 2012). It has been reported that several members of the TLRs family are significantly up-regulated in the testis and epididymis of sexually mature roosters infected with *Salmonella Enteritidis* (SE; Anastasiadou et al., 2011, 2013). Furthermore, it has been reported that lipopolysaccharide (LPS), the component of Gram-negative bacteria such as in *Salmonella* species, stimulation of rooster Sertoli cells resulted in an induction of the expression of six TLR genes which further resulted in the induction of the expression of various cytokine genes (Michailidis et al., 2014). Whether stimulation of TLRs can induce pro-inflammatory cytokines, such as interleukins, which may be responsible to initiate innate and adaptive immune responses in the rooster reproductive organs have not been studied extensively. Furthermore, an understanding of molecular mechanisms involved in antimicrobial protection of chicken male reproductive organs through cytokines is relatively limited.

Research on antimicrobial protection of male reproductive organs is becoming a rapidly emerging area of reproductive physiology. Because cytokines are an integral component of the immune response in avian species to *Salmonella* infection and that activation of the innate immune system is characterized by the production of pro-inflammatory cytokines, such as interleukins, the aim of the present study was to (1) investigate the expression profile of eleven cytokine genes in the rooster testis and epididymis *in vivo*, (2) investigate whether sexual maturation affects the mRNA relative abundance as a result of expression of these genes and (3) determine whether expression of the cytokine genes was constitutive or induced in the rooster testis and epididymis *in vivo*, as a response to *Salmonella enteritidis* infection.

2. Materials and methods

2.1. Collection of tissues

The roosters (Rhode Island Reds) used in this study were supplied by a commercial company. Birds were housed in cages under a light regimen of 14 h light: 10 h dark. Feed and water were given *ad libitum*. The management of experimental animals was in concordance with the institutional accepted welfare guidelines. Before the experiment was initiated, faecal samples of all experimental birds were cultured and were confirmed, using *Salmonella*/Shigella (SS) agar (Fluka, St Louis, USA), to be negative for *Salmonella* organisms. Experimental groups consisted of roosters at the beginning of the reproductive period (26-week-old), sexually mature (52-week-old, sexual maturity) and aged (104-week-old) ($n = 6$ at each age). Birds were sacrificed by cervical dislocation. The testes and epididymides from each rooster were removed, snap frozen in liquid nitrogen and stored at −80 °C until analysed.

2.2. Experimental infections

A group of 28-weeks-old male birds ($n = 6$ per group) were orally gavaged with 0.1 ml of inoculum containing approximately 5×10^6 organisms of SE. Age-matched, non infected control birds were housed under similar environmental conditions, and 0.1 ml of phosphate buffered saline (PBS) was introduced by gavage. The *in vivo* bacterial challenge of experimental animals was in concordance with the institutional accepted welfare guidelines. Birds were sacrificed on the fourth day after infection and the testes of each bird was removed, snap-frozen in liquid nitrogen, and stored at −80 °C until analysd.

The presence of *Salmonella* in the testis and epididymis of the infected roosters was confirmed using SS agar. The testes and epididymides of each infected bird were assessed for *Salmonella* by plating different dilutions of homogenised tissue samples, re-suspended in 1X PBS, on SS selective agar plates. Using this medium, growth of the *Salmonella enteritidis* species is uninhibited and appears as a colourless colony with a black centre.

2.3. RNA isolation, cDNA synthesis and quantitative real-time PCR analysis

Total RNA was isolated from rooster testis and epididymis stored at −80 °C. Initially, the tissues were ground to a fine powder and the RNA was extracted using the Total RNA Isolation (TRI) Reagent (Ambion, Texas, USA) according to the instructions provided by the manufacturer. The