Antimicrobial responses in the male reproductive tract: insights into the regulatory mechanisms and the potential to limit infections

DOCTOR OF PHILOSOPHY

By

Barnali Biswas

Department of Animal Sciences

School of Life Sciences

University of Hyderabad

Hyderabad-500 046, India

March 2013

Antimicrobial responses in the male reproductive tract: insights into the regulatory mechanisms and the potential to limit infections

A thesis submitted to University of Hyderabad
for the award of Ph.D. degree in Animal Sciences

By

Barnali Biswas

Enrolment No: 07LAPH07

Department of Animal Sciences
School of Life Sciences
University of Hyderabad
Hyderabad-500 046, India

March 2013

University of Hyderabad

(A Central University established in 1974 by an Act of Parliament)

Department of Animal Sciences
School of Life Sciences
Hyderabad-500 046, INDIA

DECLARATION

I, **Barnali Biswas**, hereby declare that this thesis entitled "Antimicrobial responses in the male reproductive tract: insights into the regulatory mechanisms and the potential to limit infections" submitted by me under the guidance and supervision of Dr. Suresh Yenugu is an original and independent research work. I also declare that it has not been submitted previously in part or in full to this University or any other University or Institution for the award of any degree or diploma.

Name : Barnali Biswas

Regd. No : 07LAPH07

Signature :

Date :

University of Hyderabad

(A Central University established in 1974 by an Act of Parliament)

Department of Animal Sciences
School of Life Sciences
Hyderabad-500 046, INDIA

CERTIFICATE

This is to certify that this thesis entitled "Antimicrobial responses in the male reproductive tract: insights into the regulatory mechanisms and the potential to limit infections" is a record of bonafide work done by **Miss. Barnali Biswas,** a research scholar for Ph.D. programme in the Department of Animal Sciences, University of Hyderabad under my guidance and supervision. The thesis has not been submitted previously in part or full to this or any other University or Institution for the award of any degree or diploma.

Dr. Suresh Yenugu
Supervisor

Head
Department of Animal Sciences

Dean
School of Life Sciences

Acknowledgements

One of the joys of completion is to look over the journey over the past and remember all the people who have supported me along this long but fulfilling road. At the outset I would like to express my heartfelt appreciation to Dr. Suresh Yenugu; to work with you has been a real pleasure to me. You have been a steady influence throughout my Ph.D. career; you have oriented and supported me with promptness and care, and have always been patient and encouraging in times of new ideas and difficulties; I admire your ability to balance research interests and personal pursuits. I am extremely lucky to have you as my supervisor as well as being the 1st student.

Furthermore, I am very grateful to members of the doctoral committee, Prof. Aparna Dutta Gupta for insightful comments in my work and for many motivating discussions. I thank Prof. B. Senthilkumaran for his constructive feedback.

My thanks to the Head, Department of Animal Sciences Prof. B. Senthilkumaran, and former Heads Prof. Manjula Sritharan and Prof. S. Dayananda for providing us with good equipment and facilities to carry out the work.

I thank the Dean Prof. R. P. Sharma, and former Dean, Prof. M. Ramanadham and Prof A. S. Raghavendra for allowing me to use the school facilities.

I would like to express my sincere thanks to Prof. Andreas Meinhardt (Justus Liebig University), Germany for the collaboration in developing epididymo-orchitis in rat. I would like to thank Dr. Sanjeev Khosla CDFD for providing guidance in DNA methylation work. I am grateful to Dr. Niyaz Ahmed and Dr. Arun Kumar for allowing me to use the real time machine.

I thank UGC, CSIR for providing financial assistance through Junior and Senior research fellowship. DST-FIST, UGC-SAP, UPE, DBT-CREBB and University of Hyderabad are accredited for providing necessary facilities in school. Financial assistance to my supervisor's laboratory by DST, DBT, CSIR, ICMR and UGC are gratefully acknowledged.

I thank DST-PURSE, UPE grant for sponsoring my trip for attending the international conferences Aegean conference 2011(Greece) and EMBO meeting on innate immunity 2012 (Germany).

A special thanks to my wonderful colleagues Rajesh, Narmadha, Madhu, Reejuana for their help cooperation and enjoyable working hours.

I would like to thank Dr. Suraj for his invaluable assistance in experiments with rat.

Completing this work would have been all the more difficult were it not for the support and friendship provided by the members of the school of life science (Arif, Nabamita, Oindrilla, Samrah, Sarita, Jyoti, Maruti, Ravi J, Venkat, Sunil P, Vincent, Malavika, Dr. Anand, Dr. Sridevi, Dr. Chaitanya) and others (Moushumi, Sanjeev, Tridib, Anindita, Suchira, Pavan, Kiran). I thank Surendra and Kishore in helping me with the thesis compilation.

To my friends (Abdul and Lakshmi) thank you for your continued friendship, support, and cups of tea along the way. I am truly lucky to have you as my friends.

This is a great opportunity to express my gratitude to my best friend Sapan Borah, thank you for all the support and encouragement you give me, and the patience and unwavering faith you have in me. For that I am eternally grateful.

Mr. Narsimha is gratefully appreciated for maintaining laboratory. I thank Mr. Ankinedu, Mr. Gopi, Mr. Jagan, Mrs. Jyothi & Mr.Baburao from department office who helped me in different endeavors during my work here.

I would like to thank Mrs. Leena Bashyam, Mrs. Monika and Miss. Kalpana for their technical help in using genomics and proteomics facility.

I take this opportunity to express the unconditional support from my deep heart to my beloved sisters (Er. Sonali Biswas and Dr. Moushumi Biswas), uncle and aunt (Mr. Maniklal Biswas and Mrs. Manju Biswas), Grandmother (Mrs. Bishnupriya Bose), jiju (Dr. Debajit Das), niece (pakhi) for their everlasting support and love. Many thanks for always being there for inspiring me with lots of love.

Lastly, and most importantly, I am deeply indebted to my parents, I dedicate this thesis to

Baba (Mr. ChittaRanjan Biswas) & Ma (Mrs. Malaya Biswas)……

Barnali Biswas

CONTENTS

ABBREVIATIONS

5'-Azacytidine	Aza
Androgen Receptor	AR
Antimicrobial peptide	AMP
Defb	Defensin
Degree Celsius	^{0}C
Deoxynucleotide triphosphate	dNTP
Deoxyribonucleic acid	DNA
Dimethyl Sulfoxide	DMSO
Dithiothreitol	DTT
DNA methyltransferase	DNMT
E-twenty six	ETS
Histone Deacetylase	HDAC
Interleukin -6	IL-6
K	Lysine
Kilobases	kb
Kilodalton	kd
Lipopolysacharride	LPS
Male reproductive tract	MRT
Methylation	Me
Nuclear Factor Kappa Beta	NF-kB
Nuclear factor-1	NF-1
Pathogen associated molecular pattern	PAMP
Phosphorylation	P
Polyacrylamide gel electrophoresis	PAGE
Pyrollidine dithiocarbamate	PDTC
Reverse transcription- Polymerase chain reaction	RT-PCR
Sodium dodecyl sulphate	SDS
Toll Like Receptor	TLR
Transcription Factor	TF
Transcription Start site	TSS
Trichostatin A	TSA
Tumor necrosis factor - alpha	TNFα
Urinary tract infection	UTI
Uropathogenic E.coli	UPEC

Innate immune machinery including the Toll-like receptors (TLRs) confers the first line of defense mechanisms to counter pathogenic microorganisms that enter the body. Although the majority of Toll-like receptors (TLRs) are reported in many species, some of them are not yet described in the rat. Further, factors that govern *Tlr* expression in the male reproductive tract have received little attention. We attempt to identify and characterize *Tlrs* in the rat and determine the expression profile under conditions that affect male reproductive tract gene expression. Although 40 defensins have been identified in the male reproductive tract (epididymis and testis), little is known about their role in immune function. Using epididymal and testicular tissues cultured *in vitro*, we demonstrate that lipopolysaccharide (LPS) induces the mRNA expression of β-defensins and the defensin like *Spag11* genes. Further, in an *in vivo* model system we demonstrate that the expression of *Spag11* and defensin genes are induced in the male reproductive tract when challenged with lipopolysaccharide (LPS), with a concomitant increase in protein expression. An increase in the mRNA levels of proinflammatory cytokines was observed parallel to the induction of *Spag11* and defensin gene expression.

The molecular mechanisms that operate to induce antimicrobial gene expression in male reproductive tract are not well studied. In epididymal and testicular tissue culture *in vitro*, LPS-induced antimicrobial gene expression involved NF-kB activation and decreased levels of histone deacetylase 1 (HDAC1) and DNA methyltransferase (DNMT), all of which possibly allow antimicrobial gene transcription. LPS induced defensin and *Spag11* expression was abrogated when NF-kB and IkB phosphorylation was inhibited, thus providing evidence that NF-kB activation is crucial. Inhibition of endogenous HDAC1 and DNMT1 resulted in higher

antimicrobial gene expression when compared to the LPS alone treated conditions. Increased trimethylation of histone 3, its binding to the upstream region of *Spag11e* gene, and demethylation of this region were observed during endotoxin challenge.

In silico analysis revealed that the upstream region contained consensus binding sites for the transcription factors androgen receptor(AR), nuclear factor-kappa beta (NF-kB), nuclear factor-1 (NF-1), E-twenty six(ETS) and activator protein 2 (AP2). Reporter gene assays for promoter activity revealed that the mutations in either AR or NF-kB binding sites caused a significant reduction in luciferase activity of the responsiveness to LPS. These results suggest that the AR and NF-kB binding sites are essential for the LPS induced *Spag11e* gene expression.

The testis and epididymis are immune privilege organs where even a minor infection can threaten organ integrity and function. Uropathogenic *Escherichia coli* (UPEC) are the leading agents for the etiology of reproductive tract infections. Infection of the urinary tract occurs in an ascending manner, with colonization of the bladder leading to possible kidney infection and also epididymo-orchitis**.** Bacteria eliciting epididymo-orchitis are either sexually transmitted or originate from common urinary tract infections. UPEC are among the most frequently isolated microbial agents in epididymo-orchitis and in semen. In this study, we report that antimicrobial genes, including defensins and *Spag11*s are inducible in UPEC infected condition. UPEC induced antimicrobial gene expression may be mediated by NF-kB signalling pathway. Histone modification (HDAC1, H3k36me3) and DNA methylation are involved in the regulation of UPEC induced antimicrobial gene expression. Recombinant Defensin 21 when injected to UPEC infected rats was able to reduce the bacterial load in the epididymis and testis. Its ability to limit infections suggests

that this protein can be used as a potential alternative to antibiotics to treat UPEC infections.

In summary, we report that antimicrobial genes of male reproductive tract respond to endotoxin or bacterial challenge; and they seem to be regulated by NF-kB pathway and epigenetic factors. The potential of one of the antimicrobial protein (Defensin 21) that responded to endotoxin or bacterial challenge to limit UPEC infection provides impetus to explore the possibility of using recombinant antimicrobial proteins to treat reproductive tract infections.

Due to growing antibiotic resistance by pathogens, there is an urgent need to identify alternate anti-infective agents that can be used in place of conventional antibiotics. Since innate immune system produces antimicrobial proteins and peptides in response to an infection, it will be of potential therapeutic significance to exploit these antimicrobial proteins as alternatives for antibiotics. In this study, the overall objective is to identify the antimicrobial proteins in male reproductive tract that respond to endotoxin challenge or bacterial infection and then use the recombinant proteins to treat reproductive tract infections. To accomplish this, the study is carried out with the following aims.

Objectives of the study:

I) To investigate the *in vitro* and *in vivo* antimicrobial responses in the male reproductive tract during endotoxin (lipopolysaccharide) challenge.

II) To study the molecular mechanisms involved during LPS mediated antimicrobial gene expression.

III) To determine the potential of antimicrobial proteins to limit or prevent male reproductive tract infections.

MOTIVATION

Evolutionarily, the ability of an organism to protect itself from pathogenic microbes has been a key factor for survival. At the same time a large number of pathogenic organisms have evolved ways to adapt to host environment and also develop resistance to antibiotics. The widespread resistance of pathogens to conventional antibiotics has generated interest to develop alternative anti-infectives. Infection due to antibiotic resistant microorganisms presents a major challenge for both the medical community and the society in general. The emergence of antibiotic resistant pathogens also underscores the need for proper use of antibiotics. It also demands that novel antibiotics with a mode of action different from currently used antibiotics are urgently needed. The development of alternate antimicrobial agents has mainly focused on ways to eliminate the pathogen, either by a direct killing or inhibiting growth or strengthening the host's immune system to clear the invaders. Obviously, the development and spread of multi-drug resistant microorganisms nullifies these modes of elimination and development of new drugs that rely on this strategy has lagged behind because of a lack of new microbial targets to aim at. Thus, in the last decennium research has shifted toward exploring the possibility for an alternative way of coping with infections, a way that has proven its merit in human evolution: employment of naturally occurring human antimicrobial peptides/proteins as possible alternative for current antibiotics. Besides the direct antimicrobial activity of antimicrobial peptides, attention has shifted towards the immune modulatory properties of some antimicrobial peptides. It is hypothesized that elimination of a pathogen by enhancement of the hosts' immune response will less likely result in resistance of pathogens against these peptides.

INTRODUCTION

The Immune System

The Immune System has evolved as a means to protect the host from harmful pathogens, tissue injury, toxic components and harmful derailment of body cells. If recognised as harmful, the immune system becomes activated and begins to neutralise or actively fight and eliminate the causative agent. Disorder of the immune system can result in autoimmune disease, inflammation and cancer. The complex immune system is comprised of innate and adaptive immunity.

Innate Immunity

The innate immune system is robust and forms the first line of defense. The innate immune system includes epithelial cells and phagocytes which produce microbicidal molecules. Neutrophilic granulocyte, a type of phagocyte engulfs invading microbes and utilises antimicrobial molecules to kill the microbes. Some of the antimicrobial peptides secreted by immune cells (Ganz 1987) have the capacity to attract other immune cells and thus enhance the immune response (Chertov, Michiel et al. 1996). The innate immune system is not as specific as the adaptive immunity and no clonal expansion of cells occurs. Instead an innate immune response is triggered by recognition of common structural patterns of microbes (pathogen-associated molecular patterns/PAMPs) (Medzhitov and Janeway 2000). Cells of innate immunity express pattern recognition receptors (PRRs), which recognise these microbial structures. These include family of Toll like receptors (TLRs), Nod like receptors (NLRs) and Rig like receptors (RLRs) (Schutze, Machleidt et al. 1999; Takeuchi, Hoshino et al. 1999; Akira 2003).

Adaptive Immunity

Adaptive immunity is acquired and involves lymphocytes with receptors of unique specifities to foreign antigens. An immune response during adaptive immunity can be either humoral or cellular. The humoral immunity is directed towards extracellular antigens, where B-cells with their surface immunoglobulin receptors (antibodies) are crucial. The cellular immunity is directed towards intracellular antigens eg: antigens from virus infected cells or tumour cells. T- Cells are mediators of cellular immunity and utilise the T-cell receptor (TCR) to recognise foreign peptide fragments presented on the surface of cells by the major histocompatibility complex (MHC). The adaptive immunity is also capable of generating a memory of encountered pathogens and at reinfection a rapid response is elicited, hindering disease progression (Janeway, Chervonsky et al. 1997).

Antimicrobial peptides

Antimicrobial peptides are cytolytic peptides that serve in the vertebrates and invertebrates for both offensive and defensive purposes. They can rapidly kill a broad range of microbes including Gram-positive and Gram-negative bacteria, fungi, parasites, enveloped viruses, and tumor cells (Hancock and Scott 2000; Zasloff 2002). In mammals, they are mainly expressed in the epithelia and in blood cells. The activity spectrum is unique for every peptide and single amino acid substitutions can affect the activity. Hundreds of antimicrobial peptides have been isolated so far and irrespective of their origin, spectrum of activity, and structure, most of these peptides share several common properties. The antimicrobial peptide interacts with membranes and alters the organization of the bilayer and makes it permeable to ions, causing membrane depolarization (Matsuzaki, Sugishita et al.

1997; Kourie and Shorthouse 2000). This has led to the general conclusion that the interaction of most antimicrobial peptides with bacterial membranes, involving electrostatic and hydrophobic interactions, is a necessary precursor to cause cell death (Hancock and Rozek 2002).

Several models explaining their mechanism of action have been proposed, of which barrel-stave pore, thoroidal pore, and carpet models are well established (Brogden 2005) (Figure 1). In the barrel-stave pore model, the peptides transverse the membrane with the hydrophobic surfaces directed towards the lipid membrane and the hydrophilic surfaces inwards forming a pore which allows water and electrolyte leakage (Oren and Shai 1998). The toroidal pore model describes the killing mechanism in a similar manner as the barrel stave pore model. However, here the peptides are suggested to aggregate and induce the lipid monolayers to bend continuously through the pore, causing the lipid head groups to be directed towards the water core (Brogden 2005). In the carpet model, the bacterial membrane surface is covered with the peptides after an electrostatic interaction, and the membrane is disrupted in a detergent-like manner (Oren and Shai 1998).

Beside this, antimicrobial peptides also act by binding to intracellular targets or by influencing host defense mechanisms (Hancock and Rozek 2002; Ganz 2003; Jenssen, Hamill et al. 2006). Further, certain peptides have been found to interact directly with host cells to stimulate host gene products, including specific chemokines, chemokine receptors, integrins, transcriptional factors etc (Hancock and Scott 2000). It has been suggested that the cationic antimicrobial peptides have many potential roles in inflammatory responses, which represent an orchestration of the mechanisms of innate immunity (Hancock and Diamond 2000).

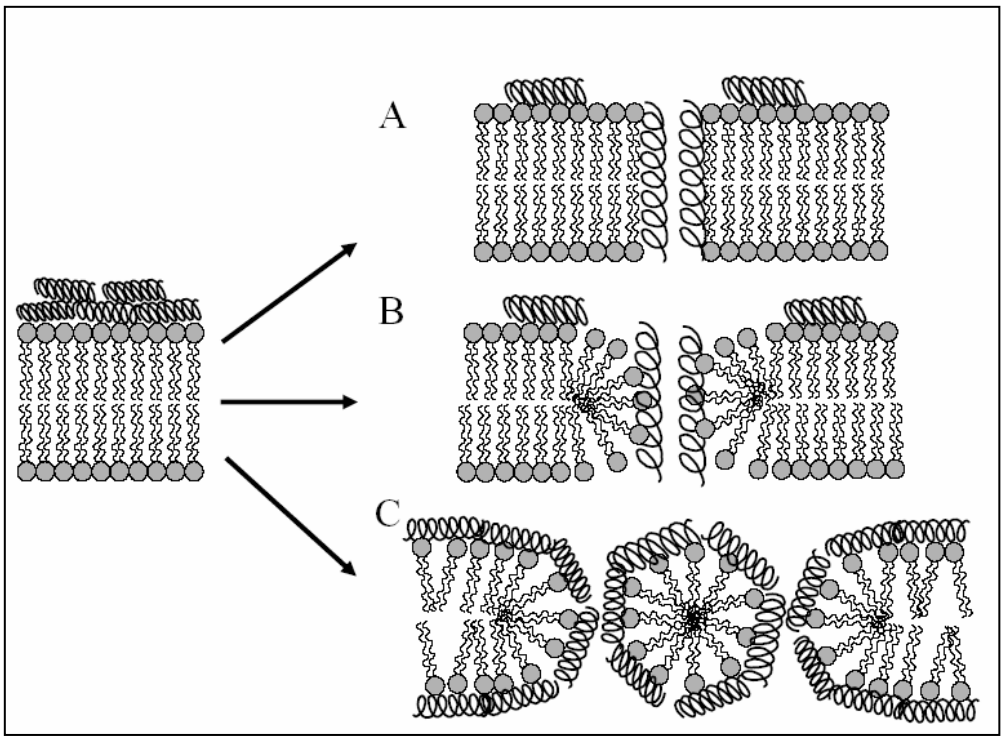

Figure 1: Mechanism of action of antimicrobial peptides. A. Barrel stave pore model, B. Toroidal pore model and C. Carpet model. *Adapted from (Papo and Shai 2003).*

Functions of Antimicrobial Peptides

Apart from being able to directly kill or inhibit growth of microorganisms, antimicrobial peptides also have other functions in immunity. They have been shown to chemoattract cells of both the innate and adaptive immunity, i.e. polymorphonuclear leukocytes, monocytes (Territo, Ganz et al. 1989), and T-cells (Chertov, Michiel et al. 1996). The peptides thereby form a link between innate and adaptive immunity. Furthermore, upregulation of the expression of chemokines in epithelial cells and macrophages has been reported (Tjabringa, Aarbiou et al. 2003), adding additional immunomodulatory functions. These spectra of immunomodulatory activities of antimicrobial peptides have lead some researchers to question whether the primary function of antimicrobial peptides really is to kill pathogens (Bowdish, Davidson et al. 2005). Many other activities also have been

attributed to these peptides, such as neutralising LPS (Larrick, Hirata et al. 1995), increasing phagocytosis (Ichinose, Asai et al. 1996), induction of mast cell degranulation (Prohaszka, Nemet et al. 1997; Befus, Mowat et al. 1999), and participation in the regulation of the complement system (Prohaszka, Nemet et al. 1997). In addition, stimulation of angiogenesis (Frantz, Vincent et al. 2005) and re-epithelialisation in wounds, have also been shown indicating that these peptides may be multifunctional *in vivo*.

A wide variety of antimicrobial peptides present in mammals belong to different class such as cathelicidins and defensins. Basing on the arrangement of disulfide bridges they are subdivided into α (alpha), β (Beta) and Θ (theta) defensins (Figure 2). They share a similar amphipathic β-sheet-rich structure (defensin fold) (Ganz 2003), but differ in primary structure and disulphide patterns. α- defensins are mainly expressed by neutrophils and Paneth cells of the small intestine. β-defensins are primarily expressed in epithelia and are produced as preproteins, hence the mature peptides of 35-45 residues are only preceded by a signal peptide. The θ-defensins are circular and are formed by ligation of two short peptides. The first discovered θ-defensin, was isolated from leukocytes of the rhesus macaque and characterized as an 18-residue circular peptide active towards both bacteria and fungi. It was found that its circular property was important for its activity since an open chain analogue was much less active (Tang, Yuan et al. 1999).

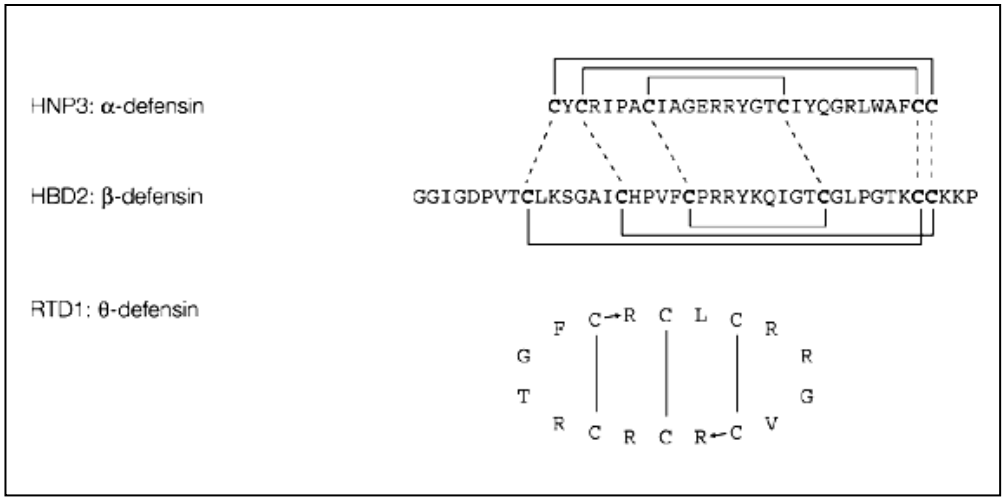

Figure 2: Sequences and disulfide pairing of cysteines in α, β and Θ defensins. *Adapted from (Ganz 2003).*

β- Defensins

β-defensins are antimicrobial peptides involved in the resistance of epithelial surfaces to microbial colonization. They are 2-6 kDa, cationic, microbicidal peptides active against many Gram-negative and Gram-positive bacteria, fungi, and enveloped viruses. They are generally composed of < 60 amino acid residues (mostly common L-amino acids), with amphipathic and cationic nature. Similar to the α and Θ defensins, they also contain the characteristic 6 cysteine motif that is responsible for the formation of three disulfide bonds. Except for the 6-cysteine domain, rich in positively charged amino acids, defensins differ considerably in their amino acid sequences and target pathogen specificity. β-defensin are generally produced by epithelial cells lining various organs such as the epidermis, bronchial trachea and genitourinary tract. β-defensins differ in their amino acid sequence and target pathogenicity, but they have similarity in the 6 cysteine signature domain.

Figure 3 shows the localisation of β-defensins in the human genome. They are mainly clustered on chromosomes 8 and 20. About 50 defensins are identified till date. Similar to humans, a large number of β-defensins have been identified in rodents (Froy, Hananel et al. 2005).

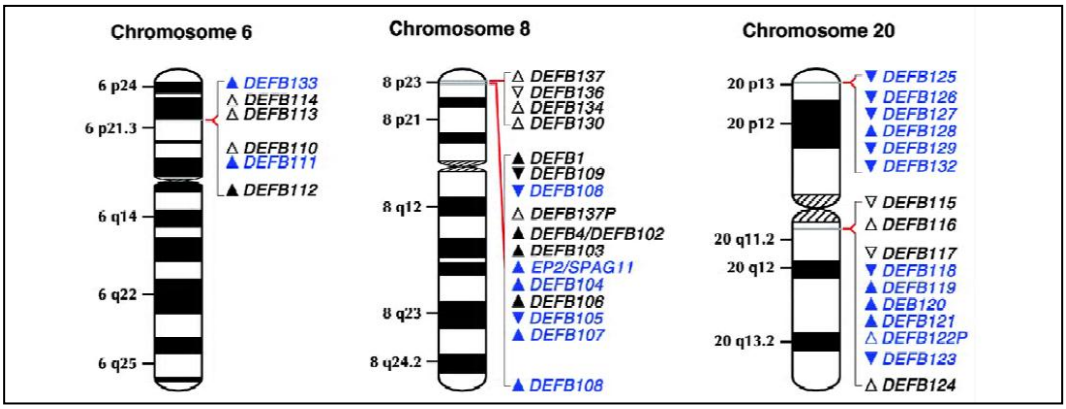

Figure 3: Major human defensin gene clusters. Gene names in black indicate widespread expression. Gene names in blue indicate expression predominantly in the male reproductive tract. Triangles point in the direction of transcription. The filled triangles indicate active genes, whereas no transcripts are known for the open triangle genes. Adapted from (Hall, Yenugu et al. 2007).

The Sperm Associated Antigen 11(*Spag11*) gene

The *Spag11* gene is found within the cluster of β-defensin genes on chromosome 8 (Figure 3). It is also referred to as human epididymis 2 (HE2) or epididymal protein 2 (EP2) gene. The human SPAG11 gene is a fusion of two independent β-defensin genes (Figure 4). Because of 2 promoters and due to alternative splicing, many transcripts are formed. It has been reported that species specific exons are formed for human, monkey and bovine *Spag11*. Translation of these alternatively spliced RNAs produces a complex protein family. Some of the

proteins encoded by SPAG11E gene contain the characteristic feature of β-defensins such as the presence of 6 cysteine array and the N terminal pro-peptide.

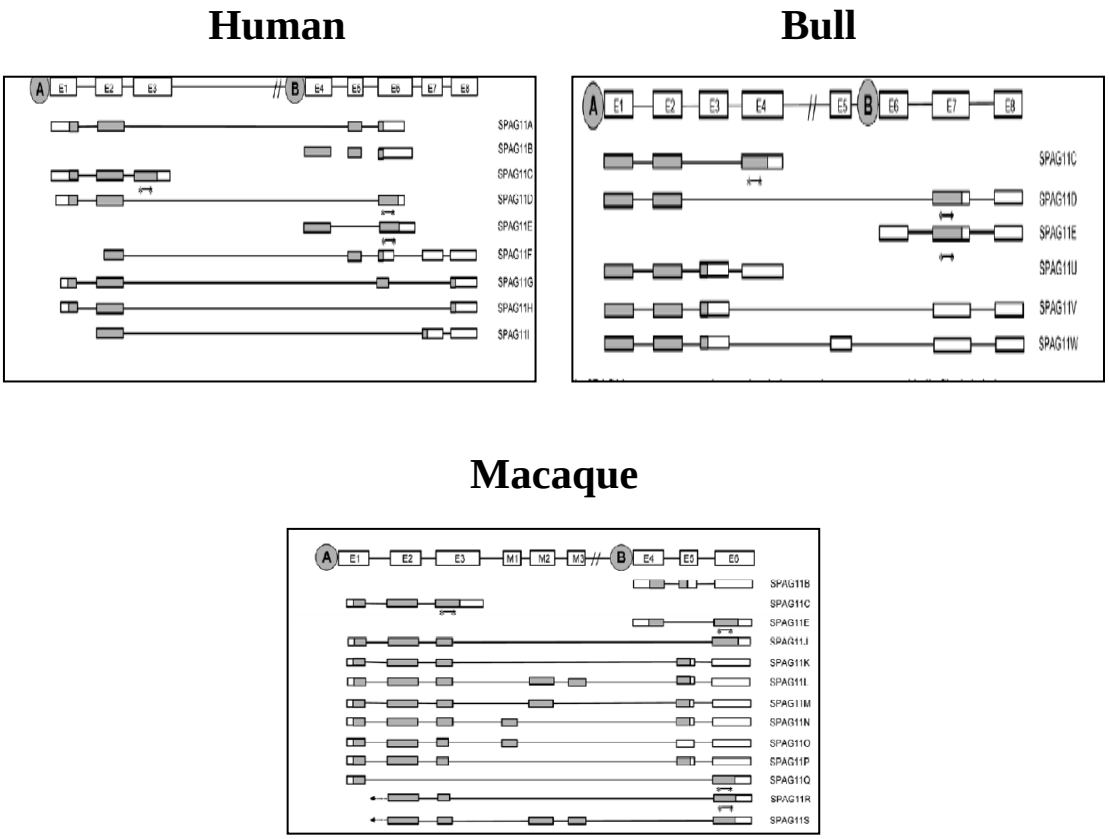

Figure 4: *SPAG11* gene structure and transcripts in human, rhesus macaque and bull. Shaded circles represent promoters with adjacent exons represented by rectangles. Splice variant mRNAs are aligned with their exons of origin. Shaded portions of the transcript rectangles indicate regions encoding amino acid sequences. *–* indicates exons encoding the β-defensin signature. *Adapted from (Hall, Yenugu et al. 2007).*

In the rat, two *Spag11* genes viz *Spag11c/t* and *Spag11e* were identified (Figure 5). The former codes for two different alternate transcripts resulting in the generation of SPAG11C and SPAG11T protein isoforms. Amino acid analysis of rat SPAG11C (RSPC) and SPAG11E (RSPE) reveal that they conserve the six cysteine motif similar to β-defensins (Figure 6).

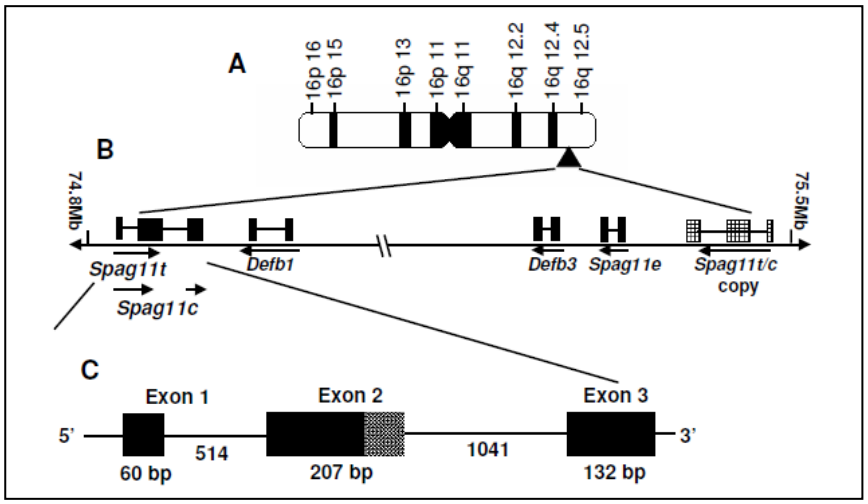

Figure 5: Rat *Spag11t/Spag11c* localization on Chromosome 16.

A. Ideogram of rat Chromosome 16.

B. Arrangement of genes from 75.5 megabases to 74.8 megabases (not to scale). Arrows indicate direction of transcription. Positions were taken from the MapView (build 3.1) at the National Center for Biotechnology Information (NCBI) website.

C. Intron-Exon structure of rat *Spag11t/Spag11c* gene. Black boxes indicate translated regions of the exons. The region of exon 2 not included in *Spag11c* after the intrinsic splice site is shown as a hashed box. *Adapted from (Yenugu, Hamil et al. 2006)*.

```
>HBD2---CLKSGAICHPVFCPRRYKQIGTCGLP-GTKCCKKP-----
>RBD1---CVSSGGQCLYSACPIFTKIQGTC-YRGKAKCCK-------
>RBD2---CLREKGFCLNAVCPRSTLFVGTC-FPYKFYCCKFKR----
>RSPC-VNCKKNEGFCQ-KYCNFMETQVGYC-SKKKEACCLHPF----
>RSPE---CFMQRGHCRLFMCRSGERKGDIC-SDPWNRCCVSSSIKNR
```

Figure 6: Alignment of human β-defensin signature motifs with similar arrays in rat β defensins and Spag11. The defensin and defensin like 6-cysteine motifs are highlighted in grey.

Male reproductive tract defensins are known only in mammals. These may be as large as 18 kDa (human DEFB129) and have long N-terminal or C-terminal extensions, generally of unknown function. Several defensins, including SPAG11

(Osterhoff, Kirchhoff et al. 1994; Hamil, Sivashanmugam et al. 2000) , DEFB118 (Liu, Hamil et al. 2001) and DEFB126 (Tollner, Yudin et al. 2004) which are located in the sperm surfaces are known to have reproductive functions. The rat *Spag11e* (*Bin1b*) is associated with the motility of immature spermatozoa (Yudin, Tollner et al. 2003; Zhou, Zhang et al. 2004). The highly anionic C-terminus of DEFB118 is thought to have a role in antibacterial action (Yudin, Treece et al. 2005) which typically depends on cationic amino acids. The male reproductive tract DEFB123 has a novel function to protect against endotoxemia (Motzkus, Schulz-Maronde et al. 2006).

The Male Reproductive System

The male reproductive system encompasses the anatomical structures and physiological functions that produce mature sperm. The processes of sex determination and embryonic development produce a male child, setting the stage for the virilization and onset of fertility that begin with puberty. The study of the physiology of reproduction began as early as 300 B. C. by gonadal extirpation technique of Aristotle and the first microscopic examination of germ cells was done in 17th century by Van Leeuwenhoek. The discovery that the spermatozoa develop from cells residing in testis was followed by the description of the microscopic characteristics of the interstitial cells by Leydig in 1857 and Sertoli cells by Sertoli in 1865. The male reproductive system (Figure 7) includes:

- ***The testicles.***
- The duct system, which is made up of the ***epididymis*** and the ***vas deferens.***
- The accessory glands, which include the ***seminal vesicles*** and ***prostate gland.***

The male reproductive system functions to:

- Produce, maintain and transport spermatozoa (the male reproductive cells) and protective fluid (semen).
- Discharge spermatozoa within the female reproductive tract.
- Produce and secrete male sex hormones.

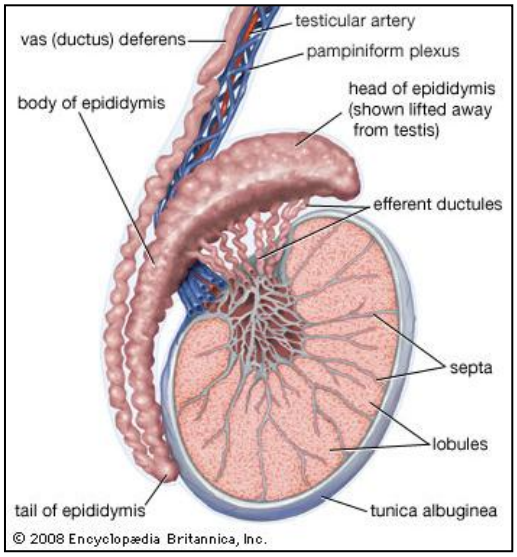

Figure 7: The male reproductive system. *Adapted from (Encyclopaedia Britanica).*

Previous studies have demonstrated a correlation between bacterial infection and male infertility (Cunningham and Beagley 2008; De Francesco, Negrini et al. 2011; Sarkar, Bahrainwala et al. 2011). Orchitis is an inflammatory condition of the testes secondary to infections whereas epididymitis is the inflammation of the epididymis. It results in leukocytes infiltration outside the seminiferous tubules, which can be observed inside the tubules in more severe cases as well (Yule, Mahi-Brown et al. 1990). With the exception of specific viral infections, orchitis is usually in combination with acute epididymitis due to retrograde infection, known as epididymo-orchitis. Among the sexually active men between 14-35 year of age,

sexually transmitted pathogens including *Chlamydia trachomatis* and *Neisseria gonorrhoeae* are among the most common pathogens related to epididymo-orchitis (Berger, Alexander et al. 1979; Ludwig 2008; Ochsendorf 2008). Uropathogenic *E. coli* (UPEC) account for the vast majority of urinary tract infections and thus are assumed to be the likely substrain responsible for the *E. coli* elicited epididymo-orchitis (Pellati, Mylonakis et al. 2008).

Pattern recognition Receptors

The innate immune system responds to invading pathogens by the activation of pro-inflammatory cascades aiming at eradication of the infectious agents. Pattern recognition receptors (PRRs) are crucial parts of this innate immune reaction (Mogensen 2009). A variety of intracellular and extra-cellular PRRs are known till date, which includes Toll-like receptors (TLRs), Nod-like receptors (NLRs), and RIG-like receptors (RLRs) (Janeway and Medzhitov 2002; Yoneyama, Kikuchi et al. 2004; Kanneganti, Lamkanfi et al. 2007). Out of this intricate system of innate immune receptors, TLRs are the best characterized. To date, thirteen different TLRs have been discovered in humans and their specific ligands are identified. They all have the same basic structure of a type 1 transmembrane glycoprotein receptor. However, some TLRs are localized at the cell membrane (TLRs 1, 2, 4, 5, 6, 10), whereas others are anchored in the endosome (TLRs 3, 7, 8, 9) and therefore lie intracellularly. The N-terminal end of all TLRs contains a leucine-rich repeat (LRR) motif and mediates ligand binding. The cytoplasmic domain is highly conserved and termed Toll-IL-1R (TIR) domain according to the strong similarity between the *Drosophila* Toll and the mammalian IL-1R1 protein. The TIR domain functions as binding site for downstream adaptor molecules. Each TLR specifically recognizes conserved

fragments of pathogens; so-called pathogen associated molecular patterns (PAMPs), which can be found on Gram-positive and Gram-negative bacteria, DNA and RNA viruses, fungi and protozoa (Figure 8). Interaction between TLR and PAMP causes a signaling cascade (Figure 9) that leads to the production of pro-inflammatory cytokines, type-1 interferons and chemokines, and promotes direct killing of the pathogen, activates phagocytosis and influences adaptive immune responses (Newton and Dixit 2012). Activation of TLRs is not restricted to the initiation of innate and adaptive immune reactions. It is understood that after binding of a ligand, TLRs homodimerize or heterodimerize and then the signal is mediated via adaptors to downstream signaling proteins(Takeda and Akira 2005). In one pathway, the main adaptor protein is MyD88, binds directly to the cytosolic domain of TLR5, 7, 8, and 9 (Figure 9). TLR2/TLR4 interacts with MyD88 via Mal (MyD88 adaptor-like) also called TIRAP (TIR-domain-containing adaptor protein). Depending on the downstream signaling molecules, activation of the MyD88 pathway can lead to the induction of gene expression via the transcription factors NF-kB, AP (activating protein)-1 or IRF (interferon-response factor) 1, 5 and 7. In the other pathway, recruitment of TRIF (Toll-receptor-associated activator of interferon) transmits the signal downstream, which mainly leads to activation of IRF3, but can also induce activation of NF-kB (Figure 9). It has been proposed that TLR4-ligand-binding leads to activation of the MyD88 pathway at the plasma membrane (Fitzgerald, Rowe et al. 2003). Whereas dendritic cells (DCs), neutrophils or macrophages express an almost complete panel of the different TLRs, other cells only express a restricted repertoire.

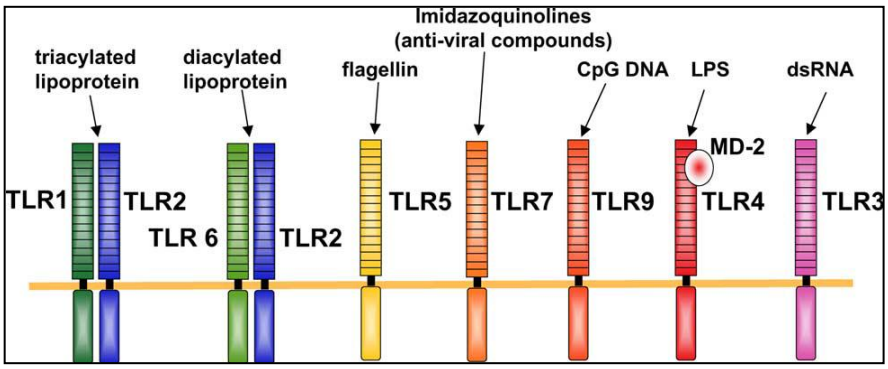

Figure 8: TLRs and their ligands. *Adapted from (Takeda and Akira 2004).*

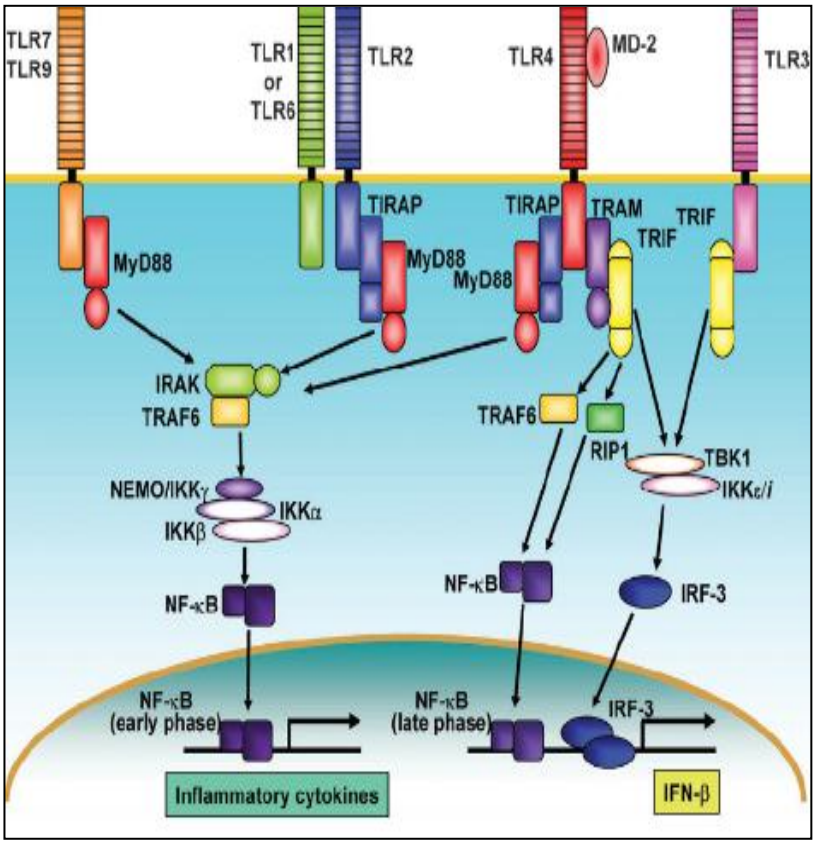

Figure 9: TLRs and their signalling Pathways. *Adapted from (Takeishi and Kubota 2009)*

Epigenetic Regulation

Histone modification

Heritable changes in gene expression that are not based upon alterations in the DNA sequence are defined as epigenetics. Historically the word epigenetics has been used to describe events that cannot be explained by genetic principles. Early observations have demonstrated a remarkable correlation between chromatin structure and DNA methylation (Ng and Bird 1999). Inactive chromatin is associated with hypermethylated DNA, whereas active chromatin is associated with demethylated DNA. Active genes are organized in chromatin, which is characterized by specific histone modifications as well as DNA demethylation (Li 2002; Bartova, Krejci et al. 2008). However the mechanism linking these two levels of epigenetic modifications was unknown. In addition, since epigenetic deregulation is postulated to be involved in stable gene expression in diverse human pathologies (Feng, Fouse et al. 2007; Sadikovic, Al-Romaih et al. 2008; Paschos and Allday 2010), studying these molecular mechanisms will have an impact on understanding the molecular pathology of male reproductive tract infections.

The basic unit of the chromatin is the nucleosome, which is a dimer of a two identical complexes each consisting of four histone proteins: H2A, H2B, H3 and H4. Histone H3 and H4 are highly charged and are tightly associated with DNA. Figure 10 summarizes these modifications and their sites on the different histones tails. These modifications are believed to form a ¨histone code¨ that regulates chromatin function by affecting the structural dynamics of the nucleosome, ultimately dictating gene expression patterns. Several histone residues can be modified on histone tails. Three major outcomes of histone modification states are regulation of DNA replication,

repair and transcription. There are two different types of chromatin environments in the genome, silent heterochromatin where chromatin is "inaccessible" for transcription; and active euchromatin, where chromatin is "accessible" for transcription. Active euchromatin is associated with high levels of acetylation, trimethylation on H3K4, H3K36 and H3K79.

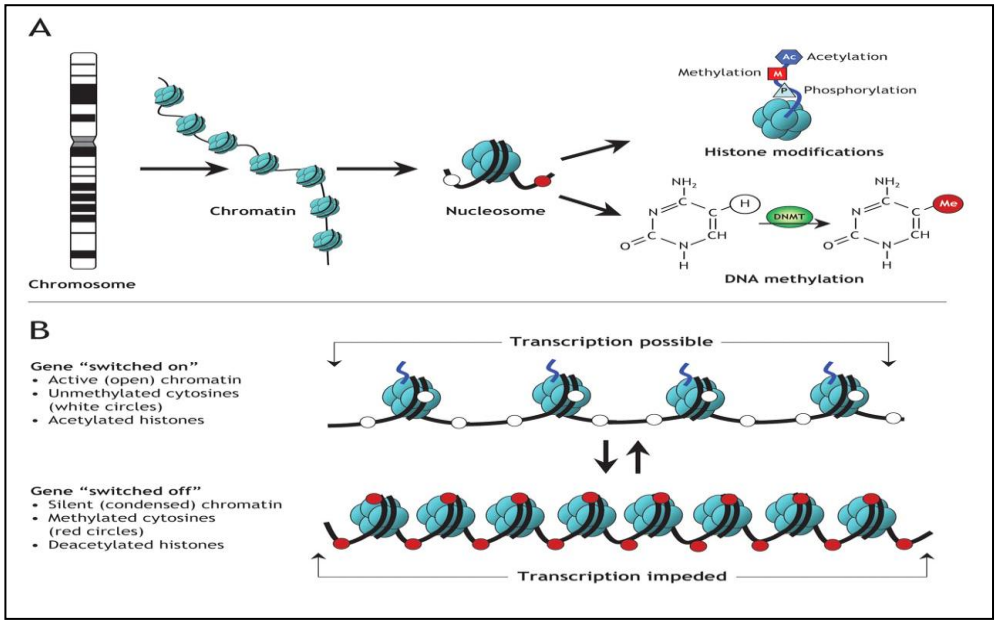

Figure 10: Histones and their sites of modification. The nucleosome is the composed of an octamer of four core histones (H2A, H2B, H3 and H4). N and C terminal histone tails can be modified in several residues. Posttranslational histone modifications have an important role in the regulation of chromatin dynamics. *Adapted from (Margueron, Trojer et al. 2005)*.

Acetylation is believed to serve as a predominant signal for active chromatin by enhancing the accessibility of the transcription machinery to regulatory regions of genes. Histone acetyltransferases (HATs) function enzymatically by transferring an acetyl group from acetyl-coenzyme A (acetyl-CoA) to the ε-amino group of lysine side chains within a histone's N-terminal tail. Histone acetylation signal is removed by the action of histone deacetylases (HDACs), leading to chromatin condensation and repression. As mentioned above, both HATs and HDACs are able to acetylate/deacetylate non-histone substrates as well. There is a considerable

division of labour between the different HDAC subtypes which often have unique functions. This increases their potential as targets for therapeutic intervention (Wagner, Hackanson et al. 2010). Several inhibitors of HATs and HDACs have been identified, including both endogenous proteins as well as pharmacological agents. A large number of structurally diverse HDAC inhibitors (HDACi) have been purified from natural sources or were synthetically developed. In particular, trichostatin A (TSA), a fungistatic antibiotic is more specific inhibitors that inhibit HDACs at the nanomolar range (Yoshida, Kijima et al. 1990).

DNA methylation

The other component of the epigenome, DNA methylation, in contrast to chromatin, is not just associated with DNA but it is part of the DNA chemistry itself. DNA methylation is present throughout diverse biological kingdoms. In higher eukaryotes DNA methylation occurs at cytosines residing at CpG dinucleotides. The majority of CpG islands are unmethylated and correspond to housekeeping genes. In contrast, methylation occurs on promoters of certain tissue specific genes in non-expressing tissues and not in the tissues that express the gene (Razin and Szyf 1984). In humans, approximately 70% of the human genes are associated to CpG areas. The remaining 30% tend to be depleted of CpGs. In mammals, DNA methylation has been proposed to regulate gene expression. An established consequence of CpG methylation is transcriptional silencing (Razin and Riggs 1980; Kass, Pruss et al. 1997). The process of DNA methylation takes place after DNA replication (Araujo, Knox et al. 1998) and is catalyzed by DNA methyltransferases (DNMTs), which transfer the methyl group from S-adenosylmethionine to the 5^{th} position on the cytosine ring (Huang, Kuo et al. 2011). It is believed that DNA methylation patterns are laid down during development by *de novo* DNMTs (DNMT3a and DNMT3b)

(Okano, Bell et al. 1999). These patterns are then copied in somatic cells by a faithful semi-conservative maintenance DNA methyltransferase (DNMT1) that replicates the methylation pattern from a methylated parental template strand to an unmethylated daughter strand (Razin and Cedar 1984).

When compared to direct genetic mutation, epigenetic alterations have begun to show a high range of therapeutic potentials for a rapidly increasing catalogue of associated diseases. To date, there are a few methods of epigenetic therapy that are most popular, including DNMT inhibitors and HDAC inhibitors. The FDA approval of three cancer-related, epigenetically targeting drugs has also provided hope in a promising future of epigenetic therapy. Although the inhibition of DNMTs may be expected to decrease the overall DNA methylation levels and result in the reactivation of many genes, the use of specific chemical agents has shown to specifically target aberrantly methylated tumorigenic cells. The demethylating agent 5'-azacytidine was first synthesized as a possible chemotherapy agent, and has since been FDA-approved under the name Vidaza (Kaminskas, Farrell et al. 2005). This works by becoming incorporated into the nucleic acids of cells in the S-phase of the cell cycle due to their analogous properties to nucleosides. The DNMTs become covalently linked to the nucleoside analogues, creating enzyme-DNA adducts, with a consequent cellular depletion of DNMTs (Juttermann, Li et al. 1994). These demethylating agents have been shown to work by inducing apoptosis or differentiation on various forms of cancer cells. Azacitidine has been shown to induce apoptosis in Myelodysplastic syndrome (MDS) derived cells, and is also currently being studied in the treatment of other types of cancer (Issa 2005; Gore 2011).

REFERENCES

Akira, S. (2003). "Mammalian Toll-like receptors." Curr Opin Immunol 15(1): 5-11.

Araujo, F. D., J. D. Knox, et al. (1998). "Concurrent replication and methylation at mammalian origins of replication." Mol Cell Biol 18(6): 3475-3482.

Bartova, E., J. Krejci, et al. (2008). "Histone modifications and nuclear architecture: a review." J Histochem Cytochem 56(8): 711-721.

Befus, A. D., C. Mowat, et al. (1999). "Neutrophil defensins induce histamine secretion from mast cells: mechanisms of action." J Immunol 163(2): 947-953.

Berger, R. E., E. R. Alexander, et al. (1979). "Etiology, manifestations and therapy of acute epididymitis: prospective study of 50 cases." J Urol 121(6): 750-754.

Bowdish, D. M., D. J. Davidson, et al. (2005). "A re-evaluation of the role of host defence peptides in mammalian immunity." Curr Protein Pept Sci 6(1): 35-51.

Brogden, K. A. (2005). "Antimicrobial peptides: pore formers or metabolic inhibitors in bacteria?" Nat Rev Microbiol 3(3): 238-250.

Chertov, O., D. F. Michiel, et al. (1996). "Identification of defensin-1, defensin-2, and CAP37/azurocidin as T-cell chemoattractant proteins released from interleukin-8-stimulated neutrophils." J Biol Chem 271(6): 2935-2940.

Cunningham, K. A. and K. W. Beagley (2008). "Male genital tract chlamydial infection: implications for pathology and infertility." Biol Reprod 79(2): 180-189.

De Francesco, M. A., R. Negrini, et al. (2011). "Bacterial species present in the lower male genital tract: a five-year retrospective study." Eur J Contracept Reprod Health Care 16(1): 47-53.

Feng, J., S. Fouse, et al. (2007). "Epigenetic regulation of neural gene expression and neuronal function." Pediatr Res 61(5 Pt 2): 58R-63R.

Fitzgerald, K. A., D. C. Rowe, et al. (2003). "LPS-TLR4 signaling to IRF-3/7 and NF-kappaB involves the toll adapters TRAM and TRIF." J Exp Med 198(7): 1043-1055.

Frantz, S., K. A. Vincent, et al. (2005). "Innate immunity and angiogenesis." Circ Res 96(1): 15-26.

Froy, O., A. Hananel, et al. (2005). "Differential expression of rat beta-defensins." IUBMB Life 57(1): 41-43.

Ganz, T. (1987). "Extracellular release of antimicrobial defensins by human polymorphonuclear leukocytes." Infect Immun 55(3): 568-571.

Ganz, T. (2003). "Defensins: antimicrobial peptides of innate immunity." Nat Rev Immunol 3(9): 710-720.

Ganz, T. (2003). "The role of antimicrobial peptides in innate immunity." Integr Comp Biol 43(2): 300-304.

Gore, S. D. (2011). "New ways to use DNA methyltransferase inhibitors for the treatment of myelodysplastic syndrome." Hematology Am Soc Hematol Educ Program 2011: 550-555.

Hall, S. H., S. Yenugu, et al. (2007). "Characterization and functions of beta defensins in the epididymis." Asian J Androl 9(4): 453-462.

Hamil, K. G., P. Sivashanmugam, et al. (2000). "HE2beta and HE2gamma, new members of an epididymis-specific family of androgen-regulated proteins in the human." Endocrinology 141(3): 1245-1253.

Hancock, R. E. and G. Diamond (2000). "The role of cationic antimicrobial peptides in innate host defences." Trends Microbiol 8(9): 402-410.

Hancock, R. E. and A. Rozek (2002). "Role of membranes in the activities of antimicrobial cationic peptides." FEMS Microbiol Lett 206(2): 143-149.

Hancock, R. E. and M. G. Scott (2000). "The role of antimicrobial peptides in animal defenses." Proc Natl Acad Sci U S A 97(16): 8856-8861.

Huang, Y. W., C. T. Kuo, et al. (2011). "An overview of epigenetics and chemoprevention." FEBS Lett 585(13): 2129-2136.

Ichinose, M., M. Asai, et al. (1996). "Enhancement of phagocytosis by corticostatin I (CSI) in cultured mouse peritoneal macrophages." Immunopharmacology 35(2): 103-109.

Issa, J. P. (2005). "Optimizing therapy with methylation inhibitors in myelodysplastic syndromes: dose, duration, and patient selection." Nat Clin Pract Oncol 2 Suppl 1: S24-29.

Janeway, C. A., A. V. Chervonsky, et al. (1997). "T-cell receptors: is the repertoire inherently MHC-specific?" Curr Biol 7(5): R299-300.

Janeway, C. A., Jr. and R. Medzhitov (2002). "Innate immune recognition." Annu Rev Immunol 20: 197-216.

Jenssen, H., P. Hamill, et al. (2006). "Peptide antimicrobial agents." Clin Microbiol Rev 19(3): 491-511.

Juttermann, R., E. Li, et al. (1994). "Toxicity of 5-aza-2'-deoxycytidine to mammalian cells is mediated primarily by covalent trapping of DNA methyltransferase rather than DNA demethylation." Proc Natl Acad Sci U S A 91(25): 11797-11801.

Kaminskas, E., A. T. Farrell, et al. (2005). "FDA drug approval summary: azacitidine (5-azacytidine, Vidaza) for injectable suspension." Oncologist 10(3): 176-182.

Kanneganti, T. D., M. Lamkanfi, et al. (2007). "Intracellular NOD-like receptors in host defense and disease." Immunity 27(4): 549-559.

Kass, S. U., D. Pruss, et al. (1997). "How does DNA methylation repress transcription?" Trends Genet 13(11): 444-449.

Kourie, J. I. and A. A. Shorthouse (2000). "Properties of cytotoxic peptide-formed ion channels." Am J Physiol Cell Physiol 278(6): C1063-1087.

Larrick, J. W., M. Hirata, et al. (1995). "Human CAP18: a novel antimicrobial lipopolysaccharide-binding protein." Infect Immun 63(4): 1291-1297.

Li, E. (2002). "Chromatin modification and epigenetic reprogramming in mammalian development." Nat Rev Genet 3(9): 662-673.

Liu, Q., K. G. Hamil, et al. (2001). "Primate epididymis-specific proteins: characterization of ESC42, a novel protein containing a trefoil-like motif in monkey and human." Endocrinology 142(10): 4529-4539.

Ludwig, M. (2008). "Diagnosis and therapy of acute prostatitis, epididymitis and orchitis." Andrologia 40(2): 76-80.

Margueron, R., P. Trojer, et al. (2005). "The key to development: interpreting the histone code?" Curr Opin Genet Dev 15(2): 163-176.

Matsuzaki, K., K. Sugishita, et al. (1997). "Interactions of an antimicrobial peptide, magainin 2, with outer and inner membranes of Gram-negative bacteria." Biochim Biophys Acta 1327(1): 119-130.

Medzhitov, R. and C. Janeway, Jr. (2000). "Innate immune recognition: mechanisms and pathways." Immunol Rev 173: 89-97.

Mogensen, T. H. (2009). "Pathogen recognition and inflammatory signaling in innate immune defenses." Clin Microbiol Rev 22(2): 240-273, Table of Contents.

Motzkus, D., S. Schulz-Maronde, et al. (2006). "The novel beta-defensin DEFB123 prevents lipopolysaccharide-mediated effects in vitro and in vivo." FASEB J 20(10): 1701-1702.

Newton, K. and V. M. Dixit (2012). "Signaling in innate immunity and inflammation." Cold Spring Harb Perspect Biol 4(3).

Ng, H. H. and A. Bird (1999). "DNA methylation and chromatin modification." Curr Opin Genet Dev 9(2): 158-163.

Ochsendorf, F. R. (2008). "Sexually transmitted infections: impact on male fertility." Andrologia 40(2): 72-75.

Okano, M., D. W. Bell, et al. (1999). "DNA methyltransferases Dnmt3a and Dnmt3b are essential for de novo methylation and mammalian development." Cell 99(3): 247-257.

Oren, Z. and Y. Shai (1998). "Mode of action of linear amphipathic alpha-helical antimicrobial peptides." Biopolymers 47(6): 451-463.

Osterhoff, C., C. Kirchhoff, et al. (1994). "Molecular cloning and characterization of a novel human sperm antigen (HE2) specifically expressed in the proximal epididymis." Biol Reprod 50(3): 516-525.

Papo, N. and Y. Shai (2003). "Can we predict biological activity of antimicrobial peptides from their interactions with model phospholipid membranes?" Peptides 24(11): 1693-1703.

Paschos, K. and M. J. Allday (2010). "Epigenetic reprogramming of host genes in viral and microbial pathogenesis." Trends Microbiol 18(10): 439-447.

Pellati, D., I. Mylonakis, et al. (2008). "Genital tract infections and infertility." Eur J Obstet Gynecol Reprod Biol 140(1): 3-11.

Prohaszka, Z., K. Nemet, et al. (1997). "Defensins purified from human granulocytes bind C1q and activate the classical complement pathway like the transmembrane glycoprotein gp41 of HIV-1." Mol Immunol 34(11): 809-816.

Razin, A. and H. Cedar (1984). "DNA methylation in eukaryotic cells." Int Rev Cytol 92: 159-185.

Razin, A. and A. D. Riggs (1980). "DNA methylation and gene function." Science 210(4470): 604-610.

Razin, A. and M. Szyf (1984). "DNA methylation patterns. Formation and function." Biochim Biophys Acta 782(4): 331-342.

Sadikovic, B., K. Al-Romaih, et al. (2008). "Cause and consequences of genetic and epigenetic alterations in human cancer." Curr Genomics 9(6): 394-408.

Sarkar, O., J. Bahrainwala, et al. (2011). "Impact of inflammation on male fertility." Front Biosci (Elite Ed) 3: 89-95.

Schutze, S., T. Machleidt, et al. (1999). "Inhibition of receptor internalization by monodansylcadaverine selectively blocks p55 tumor necrosis factor receptor death domain signaling." J Biol Chem 274(15): 10203-10212.

Takeda, K. and S. Akira (2004). "TLR signaling pathways." Semin Immunol 16(1): 3-9.

Takeda, K. and S. Akira (2005). "Toll-like receptors in innate immunity." Int Immunol 17(1): 1-14.

Takeishi, Y. and I. Kubota (2009). "Role of Toll-like receptor mediated signaling pathway in ischemic heart." Front Biosci 14: 2553-2558.

Takeuchi, O., K. Hoshino, et al. (1999). "Differential roles of TLR2 and TLR4 in recognition of gram-negative and gram-positive bacterial cell wall components." Immunity 11(4): 443-451.

Tang, Y. Q., J. Yuan, et al. (1999). "A cyclic antimicrobial peptide produced in primate leukocytes by the ligation of two truncated alpha-defensins." Science 286(5439): 498-502.

Territo, M. C., T. Ganz, et al. (1989). "Monocyte-chemotactic activity of defensins from human neutrophils." J Clin Invest 84(6): 2017-2020.

Tjabringa, G. S., J. Aarbiou, et al. (2003). "The antimicrobial peptide LL-37 activates innate immunity at the airway epithelial surface by transactivation of the epidermal growth factor receptor." J Immunol 171(12): 6690-6696.

Tollner, T. L., A. I. Yudin, et al. (2004). "Macaque sperm release ESP13.2 and PSP94 during capacitation: the absence of ESP13.2 is linked to sperm-zona recognition and binding." Mol Reprod Dev 69(3): 325-337.

Wagner, J. M., B. Hackanson, et al. (2010). "Histone deacetylase (HDAC) inhibitors in recent clinical trials for cancer therapy." Clin Epigenetics 1(3-4): 117-136.

Yenugu, S., K. G. Hamil, et al. (2006). "Identification, cloning and functional characterization of novel sperm associated antigen 11 (SPAG11) isoforms in the rat." Reprod Biol Endocrinol 4: 23.

Yoneyama, M., M. Kikuchi, et al. (2004). "The RNA helicase RIG-I has an essential function in double-stranded RNA-induced innate antiviral responses." Nat Immunol 5(7): 730-737.

Yoshida, M., M. Kijima, et al. (1990). "Potent and specific inhibition of mammalian histone deacetylase both in vivo and in vitro by trichostatin A." J Biol Chem 265(28): 17174-17179.

Yudin, A. I., T. L. Tollner, et al. (2003). "ESP13.2, a member of the beta-defensin family, is a macaque sperm surface-coating protein involved in the capacitation process." Biology of Reproduction 69(4): 1118-1128.

Yudin, A. I., C. A. Treece, et al. (2005). "The carbohydrate structure of DEFB126, the major component of the cynomolgus Macaque sperm plasma membrane glycocalyx." J Membr Biol 207(3): 119-129.

Yule, T. D., C. A. Mahi-Brown, et al. (1990). "Role of testicular autoantigens and influence of lymphokines in testicular autoimmune disease." J Reprod Immunol 18(1): 89-103.

Zasloff, M. (2002). "Antimicrobial peptides of multicellular organisms." Nature 415(6870): 389-395.

Zhou, C. X., Y. L. Zhang, et al. (2004). "An epididymis-specific beta-defensin is important for the initiation of sperm maturation." Nat Cell Biol 6(5): 458-464.

INTRODUCTION

The molecular components of the innate immune system play a key role in host defence. Dysregulation of innate immunity in human disease generated a lot of interest to study the key signalling mechanisms, especially the ones mediated by Toll-like receptors (TLRs), Nod-like receptors (NLRs), and RIG-like receptors (RLRs) (Emma M Creigh 2006). Immune receptors specifically recognise conserved fragments of pathogens i.e pathogen associated molecular patterns (PAMPs), found on Gram-positive and Gram-negative bacteria, DNA and RNA viruses, fungi and protozoa. These recognitions initiate a signalling cascade that lead to the production of pro-inflammatory cytokines, direct killing of the pathogens and initiation of adaptive immune responses. The major site of primary immune responses is generally the epithelial surfaces (Kawai and Akira 2009).

The epithelial surfaces of many organs such as the respiratory, reproductive, and digestive systems are exposed to the external environment and are constantly under threat from invading pathogenic microorganisms. Innate immune mechanisms that exist in the body counter these microbial infections. In most organ systems, the presence of complex infection-driven signalling pathways is very well established. The ability of innate systems to recognize and respond to these attacks is largely mediated by a family of type I transmembrane receptors called Toll-like receptors (TLRs). They are primarily expressed on many cell types and have the ability to discriminate and recognize distinct microbial components. Recognition of microbial products by TLRs triggers a variety of signal transduction pathways that differ in nature magnitude and duration of the inflammatory response (Dinarello 2000). The TLR family consists of 13 members (TLR1–13) that are widely expressed

in most of the vertebrate species. Among them TLR1– 10 have been identified in humans and the remaining three are confined to rodents (Roach, Glusman *et al*. 2005). Each TLR has been shown to recognize specific components of pathogens. For example, TLR4 recognizes lipopolysaccharide, whereas TLR2 is specific for microbial lipopeptides such as peptidoglycan, lipoteichoic acid, and porins (Pandey and Agarwal 2006).

In the recent past, mechanisms involved in the innate immune responses of the male reproductive tract have become an active area of investigation. For example, the expression of antimicrobial proteins and peptides including defensins has been extensively characterized in the male reproductive tract (Yenugu, Chintalgattu *et al*. 2006; Yenugu, Hamil *et al*. 2006). The expression of lipopolysaccharide-binding protein, an acute phase protein known to play a central role in defense against Gram-Negative bacteria, was demonstrated in the human epididymis (Malm, Nordahl et al. 2005). A recent study reported the presence of *Tlr1*-*Tlr11* and some of their adaptors in the male reproductive tract of rats (Palladino, Savarese et al. 2008). Although, majority of TLRs are reported in the rat, TLR5, TLR10, and TLR11 are not. Further, TLR12 and TLR13 have not been characterized in the male reproductive tract of this species. Hence, *in silico* and *in vivo*, analyses were carried out in this study to identify and characterize *Tlr5*, *Tlr10*, and *Tlr11*. Further, the male reproductive tract specific expression profiles of all previously reported *TLRs* besides *Tlr12* and *Tlr13* in the rat were also studied. Gene expression in the male reproductive tract is dependent on tissue levels of androgens (Rodriguez, Kirby *et al*. 2001) that are known to change during development. As very little is known about the factors (such as androgens) that may affect *Tlr* expression

in the male reproductive tract, attempts were made to characterize the expression profile of all known *Tlrs* under conditions of androgen variation.

During sperm maturation in the epididymis, a variety of secreted epididymal proteins belonging to different classes such as the sperm associated antigen 11 (SPAG11), cathelicidins, defensins, protease inhibitors like cystatins were identified to be added on to the sperm surface and are thought to play an important role in sperm maturation (Hamil, Sivashanmugam *et al*. 2000; Hamil, Liu *et al*. 2002; Hamil, Liu *et al*. 2003; Hsia and Cornwall 2003). However, recent evidence indicates that epididymal proteins exhibit potent antimicrobial activity, suggesting their important role in male reproductive tract immunity (Li, Chan et al. 2001; Hall, Hamil et al. 2002; Hamil, Liu et al. 2002; Com, Bourgeon et al. 2003; Bourgeon, Evrard et al. 2004; Liao, Ruddock et al. 2005). One such class of proteins is the SPAG11 family, also referred to as human epididymis 2 (HE2) or epididymal protein 2. The SPAG11 gene in humans is located on chromosome 8p.23 whereas in rats it is located on chromosome 16q (Yenugu, Hamil *et al*. 2006). It was also identified in other species including mouse, macaque and bull. Some of the SPAG11 C-terminal peptides encoded by combinations of different exons contain the 6- cysteine array characteristic of β-defensins, the predominant antimicrobial peptides in the body.

Previous studies characterized the nature of SPAG11 proteins and peptides and demonstrated their antimicrobial activity (Yenugu, Hamil *et al*. 2003; Avellar, Honda *et al*. 2004; Yenugu, Hamil *et al*. 2004; Yenugu, Hamil *et al*. 2006). Their antimicrobial activity is attributed to their potent bacterial membrane permeabilizing ability. Further, the SPAG11 alpha (HE2 alpha) peptide was shown to exhibit potent antimicrobial activity against *Neisseria gonorrhoea* and

Staphylococcus aureus (Liao, Ruddock *et al*. 2005). Though *Spag11* transcripts are extensively characterized in humans and monkeys, only a single *Spag11* variant, *Spag11e* (also referred to as Bin1b) was reported in rodents (Li, Chan *et al*. 2001). We previously identified and characterized novel SPAG11 isoforms namely, SPAG11T and SPAG11C in the rat and their antimicrobial activity demonstrated (Yenugu, Chintalgattu *et al*. 2006). Further, we also identified and characterized novel rat β-defensin genes (*Defb21*, *Defb24*, *Defb27, Defb30* and *Defb36*), that are expressed in an androgen-dependent manner and the proteins exhibit potent antibacterial activity (Yenugu, Hamil *et al*. 2006).

Recognition of specific cell wall components by TLRs triggers a cascade of events involving a variety of adaptor proteins and protein kinases, finally resulting in the activation of immune response genes (Takeda and Akira 2007; Randhawa and Hawn 2008). The role of endotoxins in eliciting cytokine response in the male reproductive tract was demonstrated. For example, increased levels of IL-18 and its receptor, IL18R and interleukin-1 beta converting enzyme were observed in the testes and Leydig cells 3 and 24 hr after intraperitoneal injection of lipopolysaccharide (LPS) to male mice (Abu Elhija, Lunenfeld *et al*. 2008). Similarly the involvement of LPS in the production of IL-1 and IL-6-like molecules by sperm cells was demonstrated (Huleihel, Lunenfeld *et al*. 2000). However, the involvement of SPAG11 isoforms and defensins during an immune response has not received much attention. Administration of endotoxin (LPS) generates inflammatory conditions in the male reproductive tract similar to that observed during infections (Hales, Diemer *et al*. 2000; O'Bryan, Schlatt *et al*. 2000; Gow, O'Bryan *et al*. 2001). Hence in this study using LPS induced inflammatory model, the role of male reproductive tract antimicrobial genes and proteins was studied.

APPROACHES

Genomics: Using mouse *Tlr5*, *Tlr10*, and *Tlr11* gene sequence, the rat genome (build RGSC v3.4) was searched using the BLAST program at the NCBI website to identify the rat orthologs. Five to six sets of intron spanning primers (Table 1.1) were designed for each *Tlr* mRNA so that the entire sequence can be amplified in parts. RT PCR was performed using rat epididymis mRNA as the template. The specific products were sequenced, aligned and deposited in GenBank. The corresponding exon / intron boundaries were determined by aligning the cDNA with the genomic sequence. The sequences were translated using the tools available at ExPASy proteomics server (http://ca.expasy.org/tools/dna.html). In silico, domain analyses were carried out using the InterProScan, a signature recognition search against the integrated resource of protein domains and functional sites (http://www.ebi.ac.uk/Tools/InterProScan). The physical and predicted features of the deduced amino acid sequences were analyzed using tools available at Expasy Proteomics Server.

Tissue Specimens and RT-PCR: Wistar rats (aged 60–90 days; n = 3) tissues were obtained from NCLAS, National Institute of Nutrition. The animals were sacrificed and caput, corpus, cauda, testis and seminal vesicles were obtained. The tissues were immediately stored at -70^{o}C. Total RNA was extracted using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) from the following tissues: caput, corpus, cauda, and testis. Total RNA (2 µg) was reverse transcribed using 50 U Stratascript (Stratagene, La Jolla, CA, USA) and 0.5 µg of oligodT (Invitrogen) according to the manufacturer's instructions. 2uL of the resultant cDNA was amplified by PCR using gene specific primers (Table 1.1) for *Tlrs* as well as for *defensins*. The number of

cycles to amplify each cDNA in the linear range was determined by preliminary PCR under the following conditions: 94^0C for 1 min followed by 25–35 cycles at 94^0C for 30 sec, 58^0C for 30 sec and 72^0C for 30 sec, and with a final round of extension at 72^0C for 10 min. *Tlrs* were amplified using gene specific primers (Table 1.1) for 28–32 cycles and glyceraldehyde phosphate dehydrogenase (*Gapdh*) for 28 cycles. PCR amplified gene products were analyzed by electrophoresis on 2% agarose gels. *Gapdh* expression was used as the internal control. To study the androgen regulation of *Tlr* transcripts, epididymides were obtained from sham operated, castrated and testosterone supplemented Wistar rats (n = 5 in each group). Testosterone supplementation was by a 20 mg dihydrotestosterone pellet implanted subcutaneously immediately after castration. All the animals were killed 14 days after castration. All procedures involving animals were performed in accordance with the Guiding Principles in the Care and Use of Animals established by the institute for animal ethical committee (IAEC). For studies on the developmental regulation of *Tlrs*, epididymides were obtained from 10- to 60-day-old Wistar rats.

***In vitro* LPS treatment:** *In vitro* cultures were maintained as described earlier (Abu Elhija, Lunenfeld *et al*. 2008; Rodrigues, Queiroz *et al*. 2008). Briefly, Caput, Cauda and Testis from 90 day old rats were dissected and cut into two longitudinal halves. One half of the tissue was used as control, and the other for LPS treatments. Tissues were transferred to 2 ml nutritive media (136.89 mM NaCl, 5.63 mM KCl, 1.80 mM $CaCl_2$, 0.36 mM NaH_2PO_4, 14.88 mM $NaHCO_3$ and 5.55 mM glucose pH 7.6-7.8) and cultured at 30^0 C with aeration. After 15 min of incubation, tissues were transferred to nutritive solution with or without LPS (1µg/ml) and incubated for different time points (3-9 h for RNA studies) and (15-180 min for protein studies).

The LPS dose and treatment periods were chosen based on previous studies (Rodrigues, Queiroz *et al*. 2008). During these incubations nutritive solution with or without LPS was renewed every 30 min. The tissues were collected, rinsed with PBS, frozen in liquid nitrogen and stored in -80 C until use.

***In vivo* LPS treatment:** Adult male Wistar rats (90 days old), maintained on a 12L: 12D lighting schedule, at 22–25^{0}C, with food and water ad libitum, were injected intraperitoneally with LPS (1 mg/ kg body weight; from *E. coli* 0111:B4; Sigma, St Louis, MO, USA) or saline (control). LPS dose and site of injection was chosen based on previous reports. Rats were sacrificed at 3, 6, 9, 15 and 24 hr after LPS treatment. Testis, caput and cauda epididymis and seminal vesicle were identified, stripped of connective tissues, frozen in liquid nitrogen, and kept at -70^{0}C until use. Experiments were conducted using the guidelines for the care and use of laboratory animals, approved by the Institutional Animal Ethics Committee of University of Hyderabad.

Real-time PCR: Total RNA was extracted using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) from the caput, cauda and testis of LPS treated rats. Total RNA (2µg) was reverse transcribed using 50 U Stratascript (Invitrogen) and 0.5 µg of oligodT (Invitrogen) according to the manufacturer's instructions. One microliters of the resultant cDNA was used for real time RT-PCR analysis using Power SYBR Green PCR master mix kit (Applied Biosystems, Warrington, UK) in a real time thermal cycler (Applied Biosystems). *Tlr4*, proinflammatory cytokines (*Tnf, Il6*), *Spag11* and defensin gene expression was studied using the gene specific primers (Table 1.1). Beta-actin expression was used as the internal control. A typical real time PCR reaction was carried out with the following conditions: initial denaturation at 94^{0}C

for 10 min; 40 cycles with 94^0C for 15s and 60^0C for 1 min. After 40 cycles, melting curve analyses was performed to confirm the absence of non-specific amplification. Fluorescence data was obtained after the end of second step (60^0C for 1 min) in each cycle. Negative controls (no template control and minus RT control (only with RNA) were included in the assays to detect non specific amplification. The amplicons were sequenced to confirm their identity. Results shown are representative of two separate experiments. Further, data points (3–24 hr) were compared only with the 0 hr control.

Immunostaining : Antibodies to detect SPAG11E were a kind gift from Dr. Susan Hall, Laboratories for Reproductive Biology, University of North Carolina, USA. Epididymides and testes obtained from control and LPS treated rats were fixed in 4% paraformaldehyde and embedded in paraffin. Five micron thick sections were taken and treated with xylene and graded alcohol (70– 100%). The sections were then treated with 1% Triton- X 100 to facilitate permeabilisation followed by treatment with 3% H_2O_2. SPAG11E was detected by incubating the sections using polyclonal antibodies (1:200 dilution) raised in rabbit followed by biotin conjugated secondary antibody (1:500 dilution) against rabbit IgG raised in goat. Immunostaining was detected using a Vectastain Elite ABC kit (avidin- biotin-complex horse radish peroxidase) (Vector Laboratories Inc., Burlingame, CA, USA). Diaminobenzidine, the chromogen, produced a brown reaction product. Sections were counter-stained with hematoxylin. For the control staining, antibodies were preincubated with antigen peptide. Photographs were taken using a color digital imaging system attached to a Leica Photomicroscope.

Statistical Analysis: Statistical analyses were performed using ANOVA and Student's t-test available in Sigma Plot software (SPSS Inc., Chicago, IL, USA). Values shown are mean ± S.D. To maintain simplicity and avoid confusion in the figures, for every gene analyzed, data points that show a significance level of $P < 0.01$ and 0.001 were assigned the same symbol.

RESULTS

Identification and characterisation of toll like receptors in the male reproductive tract of rats.

Identification of *Tlr* transcripts in the rat

Tlr5, *Tlr10*, and *Tlr11* transcripts were discovered on the rat chromosome 13, 14, and 15. The rat *Tlr5*, *Tlr10*, and *Tlr11* cDNA sequences were submitted to GenBank and were assigned the accession numbers FJ750588, FJ755908, and FJ539013 respectively. Expression sequence analysis revealed that *Tlr5* was localized at 13q26 region, whereas *Tlr10* was localized at 14p11 and *Tlr11* is present at 15p14 (Figure 1.1). *In silico*, protein translation analyses revealed that single exon encodes the full length protein. Further analyses reveal that the predicted amino acid sequences of the three TLRs contain the leucine rich repeat (LRR) and Toll / interleukin-1 receptor (TIR) domains; characteristic of the known TLRs (Figure 1.2). TLR5 in the rat is predicted to have five LRRs whereas *Tlr10* and *Tlr11* contain three LRRs each. Similar to the known TLRs, there is a single TIR domain, a signal peptide and a transmembrane domain in the rat TLR5, TLR10, and TLR11. Further, the cysteine-rich flanking region (CRFR) was found to be present on the C-terminal side of rat TLR5 and TLR10, whereas the same is absent in the rat TLR11 (Figure 1.2).

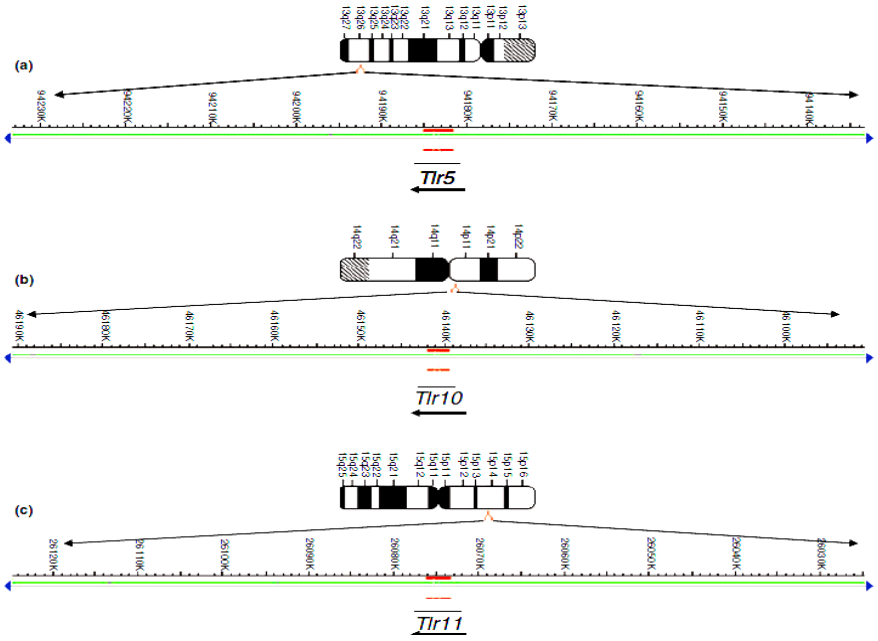

Figure 1.1: Rat *Tlr* localization on chromosomes (a) *Tlr5* on chromosome 13; (b) *Tlr10* on chromosome 14; (c) *Tlr11* on chromosome 15. Arrows indicate direction of transcription. Positions were taken from the MapView (build 3.4) at the National Center for Biotechnology information (NCBI) website.

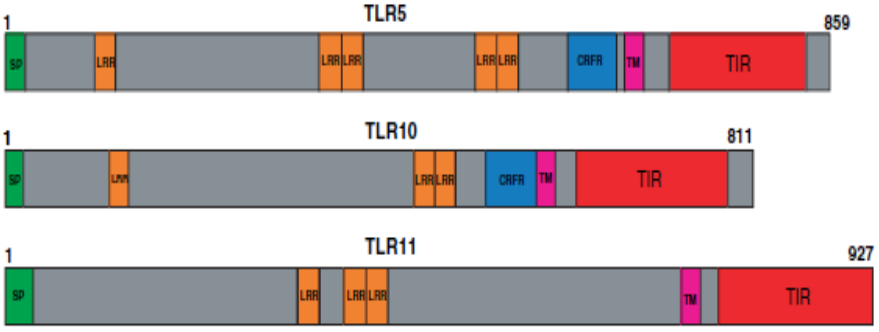

Figure1.2: Rat TLR5, TLR10, and TLR11 predicted domain structure. The predicted amino acid sequences were analyzed using InterProScan sequence search. Numbers indicate the length of the protein. Different domains are indicated in colors – signal peptide (green); LRR (orange); CRFR (blue); transmembrane domain (pink); TIR domain (red).

Tlr expression in the rat

The male reproductive tract is generally a sterile environment and is not routinely exposed to pathogens as are the respiratory and gastrointestinal tracts. However, an exposure to pathogens would be expected to mount an immediate immune response to prevent any damage to the male reproductive tract and to fertility. To determine whether the male tract constitutively expresses the toll-like receptors of the innate immune machinery, the expression of *Tlr1–13* mRNAs was analysed. Majority of the *Tlr* mRNA transcripts (*Tlr2*, *Tlr4*, *Tlr5*, *Tlr6*, *Tlr7*, *Tlr8*, *Tlr10*, and *Tlr11*) were found to be abundantly expressed in all tissues of the male reproductive tract (Figure 1.3). However, *Tlr1* and *Tlr12* expression was only weakly detected in the male tract. *Tlr3* expression was mostly restricted to the caput with minimal expression in corpus, cauda, and testis (Figure 1.3). To determine whether *Tlr5*, *Tlr10*, and *Tlr11* are ubiquitously expressed, RT-PCR was performed in a variety of tissues obtained from male and female rats. *Tlr5*, *Tlr10*, and *Tlr11* were found to be expressed in the non-reproductive as well as the female reproductive tract tissues in the rat (Figure 1.4).

Gene regulation in the male reproductive tract is androgen dependent, which in turn varies with the developmental stage of the animal. To determine whether the innate immune machinery is regulated during the course of development, *Tlr* expression was analyzed in the epididymis, testis, and seminal vesicle of 10 to 60 day old rats. In the epididymis, as seen in the adult rat, *Tlr1* seems to be not expressed abundantly during the course of the development (Figure 1.5). The presence of *Tlr 2*, *4*, *5*, *6*, *7*, *8*, *10*, *11*, and *12* mRNA was observed at all the stages during development (Figure 1.5). The expression of *Tlr3*, *9*, and *13* was detected only in the epididymides obtained from 20, 50, and 60 day old rats. An interesting feature is the presence of all the *Tlr* mRNA transcripts in the

epididymides of 20 and 50 day old rats (Figure 1.5), suggesting a possible role for TLRs at 20 and 50 day time points during development, besides their role in innate immunity. In the testis, *Tlr5*, *7*, *8*, *10*, *11*, and *12* were found to be abundantly present in all the states during development (Figure 1.6), whereas *Tlr1*, *2*, *4*, *6*, *9*, and *13* were weakly expressed. *Tlr3*, although weakly expressed in the adult rat testes, was not detected in the testes of developing rats (Figure 1.6).

To develop our understanding of the relation between androgen levels and immune receptor expression, *Tlr* mRNA levels were analyzed in the epididymides of rats that were either castrated or castrated and supplemented with testosterone (Figure 1.7). Interestingly, *Tlr1* and *13* mRNA expression was detected abundantly in the castrated rats, whereas the same was weak in the sham operated and testosterone supplemented groups. Abundant expression of *Tlr2*, *5*, *6*, *7*, *8*, *9*, *10*, *11*, and *12* and weak expression of *Tlr3* and *Tlr4* was detected in all the three groups suggesting an androgen independent expression (Figure 1.7).

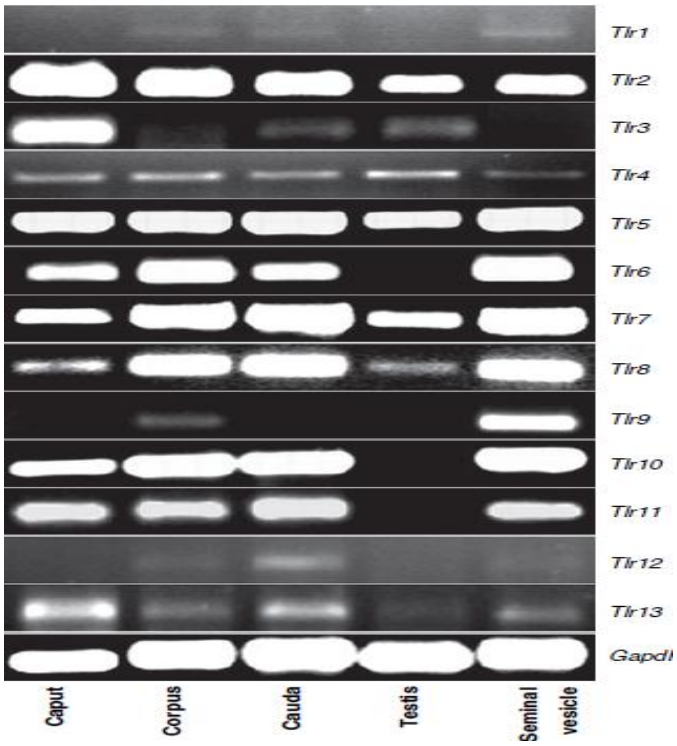

Figure 1.3: *Tlr* expression in the rat male reproductive tract. Total RNA was isolated from caput, corpus, cauda, testis, and seminal vesicle were reverse transcribed and PCR amplified. *Gapdh* was used as the internal control.

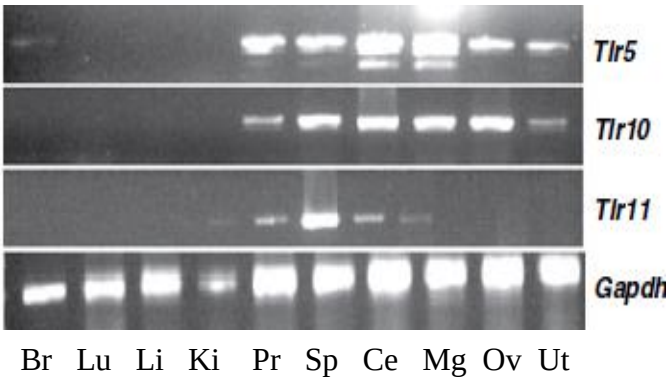

Figure 1.4: Tissue distribution of *Tlr5*, *Tlr10*, and *Tlr11* in the rat. RT-PCR analysis was performed using total RNA isolated from **Br**ain**, Lu**ng, **Li**ver, **Ki**dney, **Pr**ostate, **Sp**leen, **Ce**rvix, **Ma**mmary gland, **Ov**ary, **Ut**erus. *Gapdh* was used as the internal control.

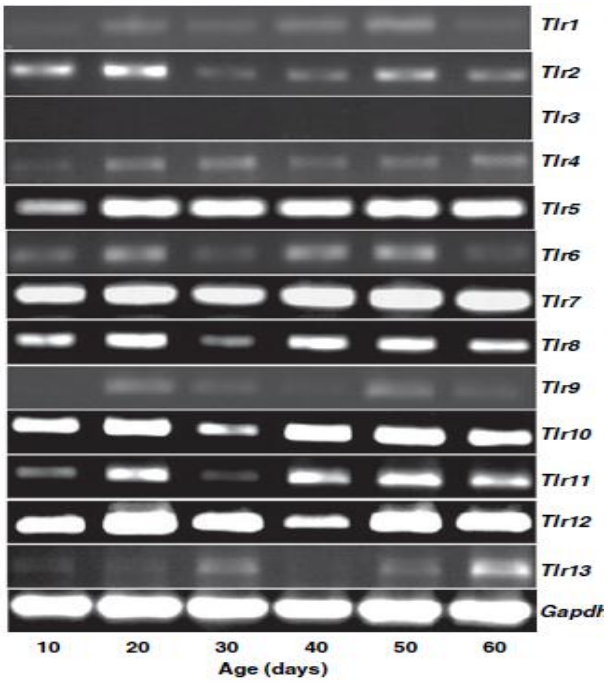

Figure1.5: Developmental regulation of *Tlr1–13* expression in the epididymides of 10- to 60-day old rats. RT-PCR for *Tlr1–13* in RNA isolated from epididymides of rats aged 10–60 days.

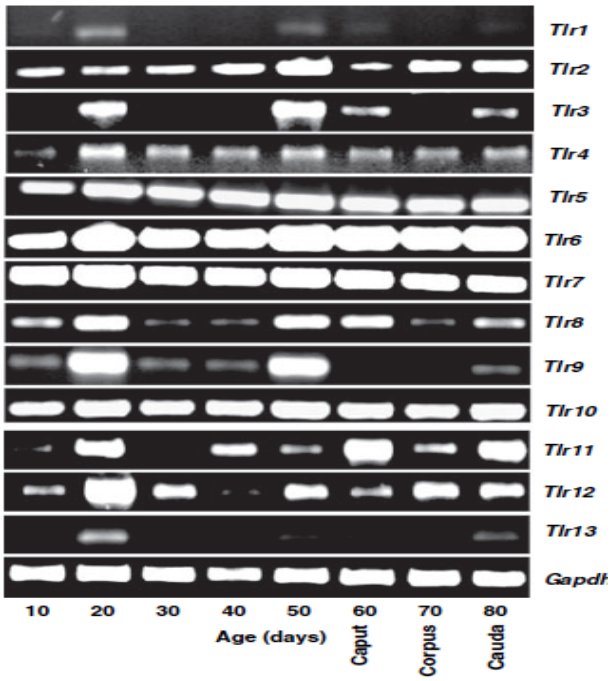

Figure 1.6: Age-dependent regulation of *Tlr1–13* in the testes of 10- to 60-day rats. RNA from 10- to 60-day-old rat testes were isolated and reverse transcribed followed by PCR. *Gapdh* expression served as the internal control.

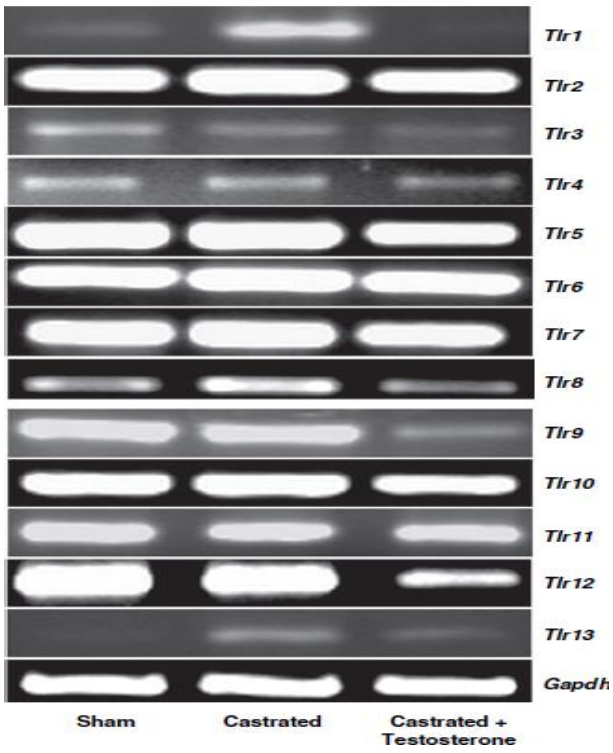

Figure 1.7: Effect of androgen ablation on *Tlr1–13* expression in the epididymis. Rats (n = 5 for each group) were sham operated, castrated, or castrated and testosterone replaced immediately after castration. Epididymides were removed 14 days after castration. Gene expression was analyzed using RT-PCR with *Gapdh* as the internal control.

Antimicrobial gene expression during endotoxin (LPS) challenge- an in vitro analysis

In vitro studies using testicular and epididymal cell lines were initiated. We used the testicular cell line LC540 and the epididymal cell line PC1 to study antimicrobial gene expression induced by endotoxin *in vitro*. Our attempts to determine basal expression of defensins and *Spag11* genes in these cells lines yielded disappointing results. None of the defensins and *Spag11* transcript were expressed in both these cell lines. Hence, further studies could not be conducted using these cell lines.

However, earlier reports suggested that epididymal and testicular primary culture systems using specific medium can be used to conduct studies on the functionality of male reproductive tract under various conditions (Rodrigues, Queiroz et al. 2008). Hence, we adopted this system to determine the impact of endotoxins on antimicrobial gene expression. Before attempting to determine the effect of endotoxins on the antimicrobial and *Tlr* gene expression, the basal expression of these genes in the male reproductive tract tissue of rat was analysed. As shown in the table 1.2, majority of the defensins are abundantly expressed in the male reproductive tract.

Table 1.1: Gene specific primers used to amplify *Tlrs* and Defensin

Primer	Sequence 5'-3'
Tlr1 F	TCTTACCCTGAACAACGTGGACAC
Tlr1 R	AAGCATGTGGACCATGCGTGTTC
Tlr2 F	CGCTTCCTGAACTTGTCCAGTACAG
Tlr2 R	GGTTGTCACCTGCTTCCAGAGTCTC
Tlr3 F	TGCACGAACCTGACAGAGCTCCAT
Tlr3 R	GCTTCTCTGTGAGGTTGAGGTTCAG
Tlr4 F	GAGACCAGGAAGCTTGAATCCCTGC
Tlr4 R	TGTCTCCACAGCCACCAGATTCTC
Tlr5 F	GCCAGAGCCAGATTGAAGTCTTGA
Tlr5 R	GAGAGGCTGGAGTTCATCTTCACA
Tlr6 F	GGTGTGTTCTTGCTCAATCGGGAC
Tlr6 R	CACCAAGTCCAGAAGAACAACACAGCTC
Tlr7 F	CTCTCGATAGCACAAACACCCATG
Tlr7 R	AGAGTCCACTAAAGCTTCCAGGTC
Tlr8 F	CCAGAGTCTTCCAAACTTGGCAAC
Tlr8 R	CAAGGCCTTGCCATAAGCAGTACA
Tlr9 F	CATGCTGGAAGAGCTGAACCTGAG
Tlr9 R	TATAGGACAGCAGGAGGTACTCCA
Tlr10 F	GAACTCTATCTGGCCCACCACAAT
Tlr10 R	AACCTTGGCAATCCGCGTTAGGCTT
Tlr11 F	CTCTGATGTCACCTTTACTTGCCAG
TLR11 R	AGAGGTGATGAAGCCAGGACCATT
Tlr12 F	GCCCATGTATCTGACCAGCTTAGAG
TLR12 R	ACTGAAGTTTGGGGAGCTGCCAGA
Tlr13 F	GGATTCAGGTTGTTCCGAGCAACT
TlR13 R	AGCTGGAGAACATGTCAGGAACCA
Gapdh F	AGACAGCCGCATCTTCTTGT
Gapdh R	CTTGCCGTGGGTAGAGTCAT
Defb1 F	GACCCTGACTTCACCGACAT
Defb1 R	CCTGCAACAGTTGGGCTTAT
Defb2 F	ATTTCTCCTGGTGCTGCTGT
Defb2 R	TCCACAAGTGCCAATCTGTC
Defb21 F	ATACCTGGATCTACTGTCCTACCT
Defb21 R	TTATGTGTCCATCCGTGAAGTC
Defb24 F	GTCATCACCTTCACCCCGGGA
Defb24 R	CAGCTTCTCTGGAAGTCTGTGCAT
Defb27 F	CACGAGGAACACCCTGGATTTCC
Defb27 R	TGCCTAGGTCCACCTTCGTTTCTG
Defb30 F	GAGTGACTTTCCTTTCCTCAG
Defb30 R	TCAGAATTCCCAGAGGAACCCTGGA

Primer	Sequence 5'-3'
Spag11t F	CTGCAGTCCCCTCCACAGCC
Spag11t R	CATCCACGCTGTCACCTCCC
Spag11c F	GTGTGCAGGTCACTTCAACTTC
Spag11c R	CATCCACGCTGTCACCTCCC
Spag11e F	CACATCTGCTTTCCTGCACAG
Spag11e R	GCACCCCACATCTCAGATCTTC
Il6 F	ACAAAGCCAGAGTCATTCAGAGCAA
Il6 R	AATGTCCACAAACTGATATGCTTAGGC
Tnf F	CGTCGTAGCAAACCACCAAGC
Tnf R	ATGGCGGAGAGGAGGCTGACT
Actin F	AACACGGCATTGTCACCAACTG
*A*ctin R	ACTCTCAGCTGTGGTGGTGAA

Table 1.2: ***Tissue distribution of β- defensins in the male reproductive tract of rat.***

Gene Name	Caput	Corpus	Cauda	Testis	Seminal Vesicle
Defb 1	+	+	+	++	+
Defb 2	+	+	+	++	+
Defb 3	-	-	-	-	-
Defb 4	-	-	-	-	-
Defb 5	-	-	-	-	-
Defb 9	-	-	-	-	-
Defb 10	-	-	-	-	-
Defb 11	-	-	-	-	-
Defb 12/35	+	+	+	+	+
Defb 13	++	-	-	+	+
Defb 14	-	-	+	+	+
Defb 15	+	-	-	-	-
Defb 17	+	-	-	-	-
Defb 18	+	-	+	+	+
Defb 21	+	+	+	+	-
Defb 22	-	-	+	-	-
Defb 24	+	+	+	++	+
Defb 25	+	-	+	+	+
Defb27	+	+	+	-	-
Defb 29	-	-	-	-	-
Defb 30	+	+	+	-	-
Defb 33	-	-	-	+	-
Defb 36	-	+	+	+	-
Defb 37	+	-	+	+	+
Defb 38	+	-	+	+	-
Defb 43	++	-	-	-	-
Defb 44	-	-	-	-	-
Defb 49	+	-	+	+	+
Defb 51	+	-	+	+	+
Defb 52	+	-	+	+	+
Spag11t/Spag11c	+	-	-	-	-
Spag11e	+	+	+	+	-

- indicates no expression, + indicates faint expression, ++ indicates abundant expression.

Antimicrobial gene expression

In the caput, the expression pattern of all the defensins analyzed was increased in response to LPS treatment (Figure 1.8). Significant increase in the mRNA expression of *Defb1*, *Defb2* and *Defb21* was observed at the 3 h time point, whereas *Defb24* and *Defb27* mRNA levels were increased 6 h after LPS treatment. Except for *Defb27*, the mRNA levels of other defensins reverted to control levels at the subsequent time points (Figure 1.8A). Similar expression pattern was observed in the cauda wherein 3 h after LPS treatment the mRNA levels of *Defb1*, *Defb2*, *Defb21* and *Defb27* were increased (Figure 1.8B). *Defb24* expression on the other hand was found to be increased 6 h after LPS challenge. Further, the expression of defensins decreased to control levels at the later time points. In the testis, increased levels of *Defb2*, *Defb21* and *Defb27* were observed 3 h after LPS challenge (Figure 1.8C). *Defb1* and *Defb27* mRNA levels were increased 6 and 9 h after LPS treatment respectively. Except for *Defb2*, the expression of all other defensins reverted to control values at the later time points.

The expression pattern of *Spag11* genes was also analyzed in response to LPS challenge. In the caput, significant increase in the levels of *Spag11t* and *Spag11c* were observed 6 h after LPS challenge, whereas the *Spag11e* levels were significantly increased at the 9 h time point (Figure 1.9A). *Spag11 c, t* and *e* gene expression in response to LPS challenge was significantly increased in the cauda 3 h after LPS challenge and decreased to control levels at the later time points (Figure 1.9B). In the testis, *Spag11c* and *t* expression was significantly increased 3 h after LPS exposure (Figure 1.9C), with a decrease to control levels at the later time points.

Tlr4 and pro-inflammatory cytokine expression

LPS is known to act via the Toll-like receptor 4 and induces inflammatory responses such as the production of cytokines and acute phase proteins. *Tlr4* expression was not significantly altered in the caput, cauda and testis after LPS challenge at all the time points tested (Figure 1.10A, B and C). In the caput and cauda, the mRNA expression of pro-inflammatory cytokines, *Tnf-α* and *Il-6* were increased 3 h after LPS treatment followed by a decrease in the later time points (Figure 1.10A and B). On the other hand, there seems to be a biphasic increase in *TNF-α* and *IL-6* gene expression in the testicular tissues treated with LPS (Figure 1.10C). Increased expression was observed at the 3 and 9 h time points following LPS addition, suggesting differential expression pattern of pro-inflammatory genes in different tissues.

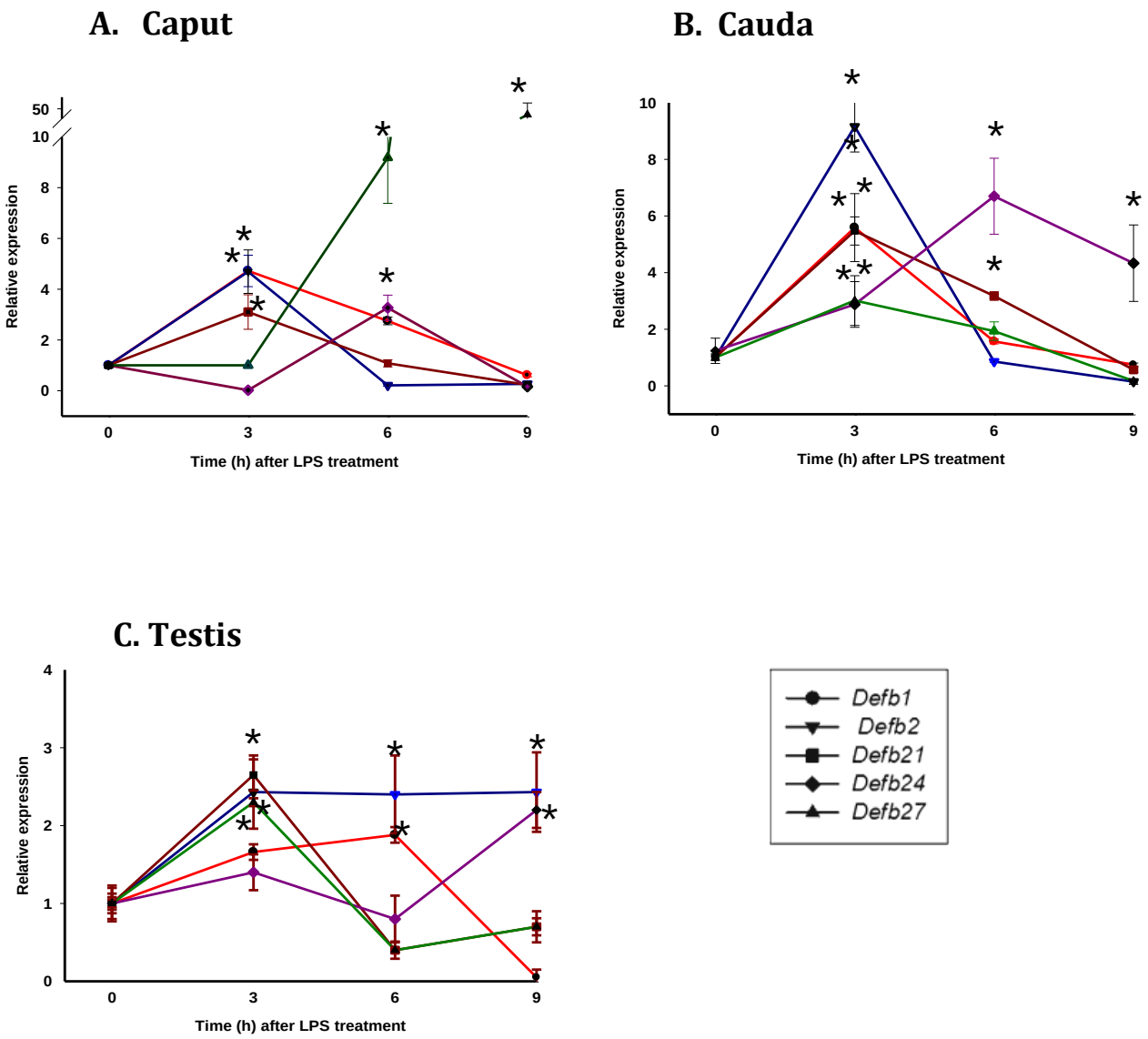

Figure 1.8: Defensin gene expression in response to LPS. Male reproductive tract tissues were cultured in nutritive medium and challenged with 1µg/ml LPS. RNA was isolated from the tissues collected after 0, 3, 6 and 9 h after incubation and defensin expression analysed by real time PCR. **A)** Caput, **B)** Cauda and **C)** Testis. (●) *Defb1*; (▼) *Defb2*; (■) *Defb21*; (♦) *Defb24*; (▲) *Defb27*. Values shown are Mean ± S.E. * indicates $p<0.05$.

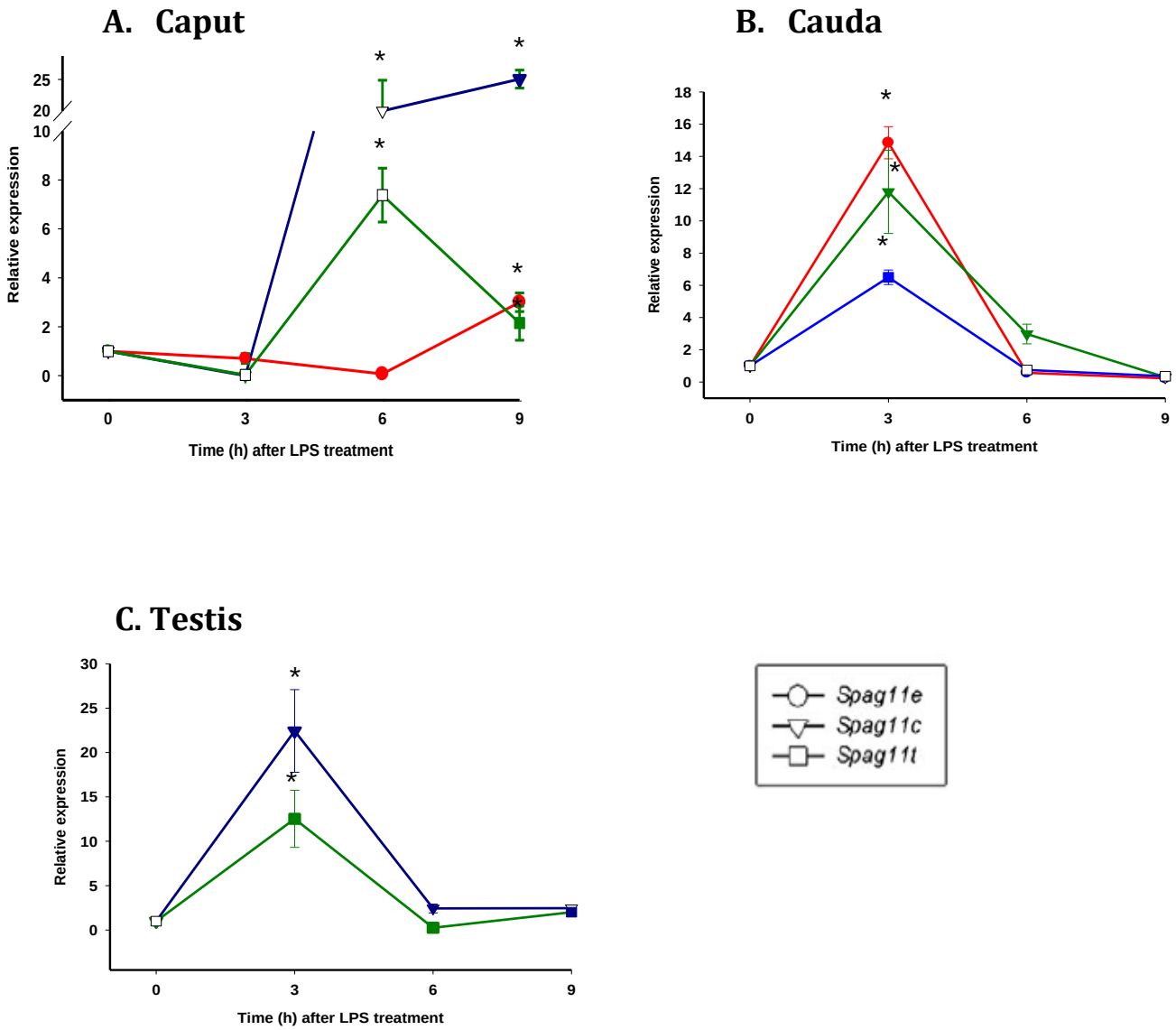

Figure 1.9: *Spag11* gene expression in response to LPS. Real time PCR was performed using RNA isolated from the male reproductive tract tissues challenged with 1 µg/ml LPS for 0-9 h. **A)** Caput, **B)** Cauda and **C)**Testis. (●) *Spag11e*; (▼) *Spag11c*; (■) *Spag11t*. Values shown are Mean ± S.E. * indicates $p<0.05$.

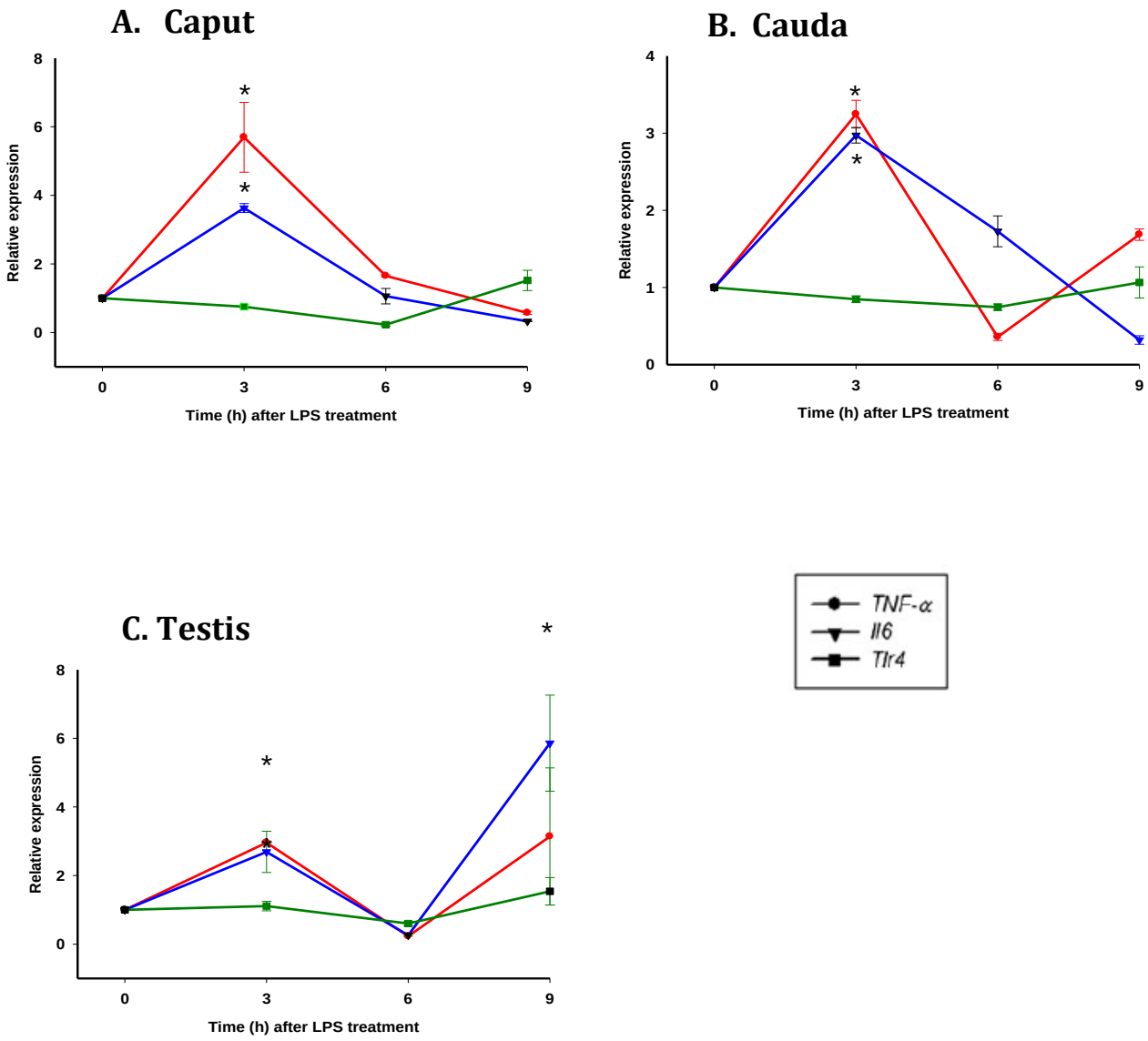

Figure 1.10: *Tlr* and pro-inflammatory gene expression. Caput, cauda and testis cultured in vitro were treated with 1 µg/ml LPS for 0-9 h. Real time PCR analyses was carried out using RNA isolated from these tissues. (●) *Tnf- α*; (▼) *Il-6*; (■) *Tlr4*. **A)** Caput, **B)** Cauda and **C)** Testis. Values shown are Mean ± S.E. * indicates $p<0.05$.

Immunohistochemistry

Immunohistochemical analyses for *SPAG11E* in the epididymis of LPS treated rats were performed. Intense staining was observed after LPS treatment (Figure 1.11) indicating that the *Spag11e* mRNA induced in response to LPS is indeed translated into protein.

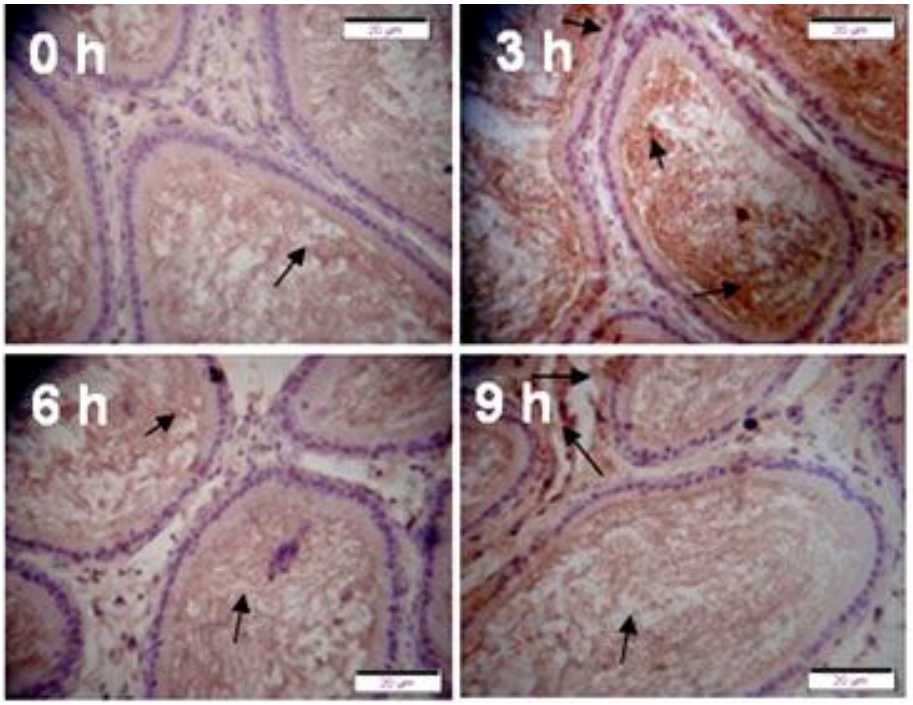

Figure 1.11: Immunostaining to detect SPAG11E. Five micron sections obtained from caput treated with LPS for 0–9 h were immunodetected for SPAG11E using polyclonal antibodies against the rat SPAG11E. Hematoxylin was used for counterstaining. All the photographs were taken at ×40 magnification.

Impact of a single dose of bacterial endotoxin on Spag11 and defensin expression in the male reproductive tract of rat- an in vivo analysis

Tlr4 expression and plasma testosterone

In the caput, increased *Tlr4* gene expression was observed 3 hr after LPS administration, whereas in the cauda, the increase was time dependent and sustained for a longer duration (up to 9 hr) (Figure 1.12). In the testis, a time dependent increase in *Tlr4* expression was observed up to 9 hr after LPS treatment followed by a sharp decline at 15 hr. Seminal vesicle *Tlr4* expression was biphasic with a significant increase at 3 and 15 hr after LPS administration (Figure 1.12). To determine the relation between androgen levels and antimicrobial gene (*Spag11* and Defensin) expression after LPS treatment, testosterone was measured in the plasma. LPS treatment resulted in a significant time-dependent reduction of plasma testosterone levels within 6 hr post-injection (Figure 1.13).

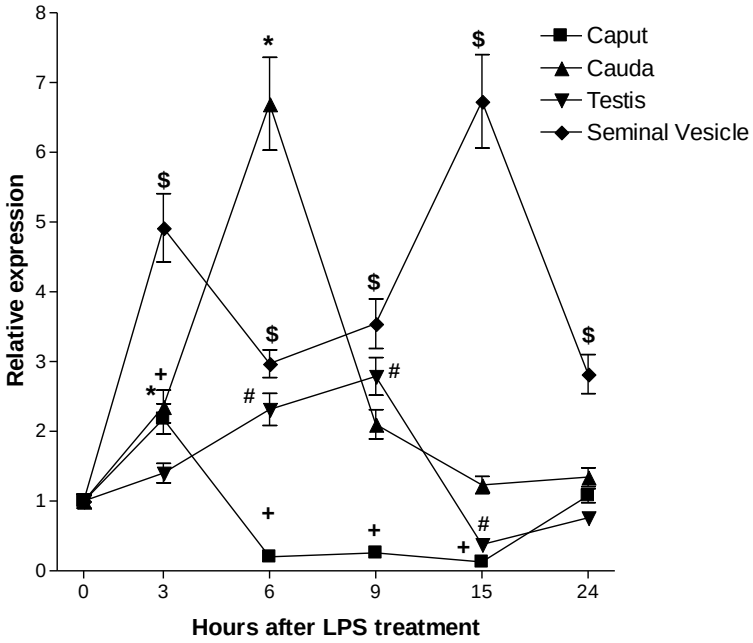

Figure 1.12: *Tlr4* mRNA expression was analyzed by real time RT-PCR. RNA isolated from reproductive tract tissues obtained from rats treated with LPS. Values shown are mean ± S.E. *, # and $ indicates $p < 0.01$ compared to 0 hr control.

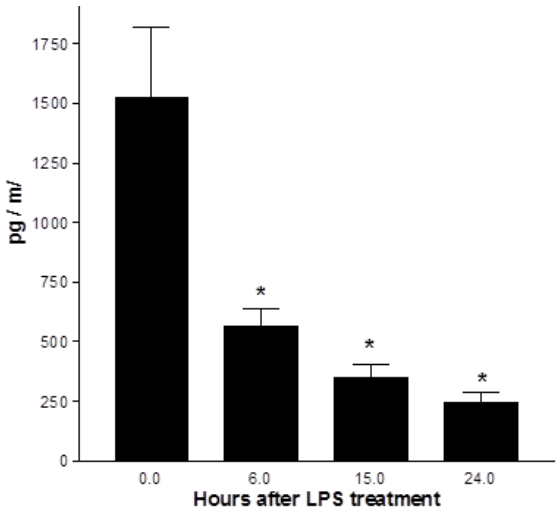

Figure 1.13: Plasma testosterone levels in LPS treated rats. LPS was injected (1 mg / kg body weight) intraperitoneally to adult rats and testosterone determined in plasma by ELISA based kit. Values shown are mean ± S.E,* indicates $p < 0.05$ compared to 0 hr control.

Spag11 and defensin expression in the caput

In the caput, *Spag11t* expression increased significantly up to 6 hr followed by a sharp decline reaching minimal levels by 9 hr. *Spag11c* expression also increased significantly within 3 hr and continued to remain at elevated levels up to 24 hr after LPS administration. *Spag11e* expression was similar to that of *Spag11t* (Figure 1.14A).

Defb1, *Defb24* and *Defb36* expression increased significantly after LPS administration reaching peak levels at 6 hr, followed by a decrease at the latter time points. However, the increase was much earlier for *Defb24* (3 hr) (Figure 1.14B).

An interesting observation is the sharp decline in the expression of *Defb2*, *Defb21* and *Defb27* immediately after LPS treatment (Figure 1.14C). *Spag11* and defensin expression observed in this study are contrary to the loss of expression previously reported under conditions of androgen withdrawal (Yenugu, Chintalgattu et al. 2006; Yenugu, Hamil et al. 2006). LPS treatment in these animals may be affecting levels of other regulatory molecules that overcome the effects of suppression of testosterone.

Concentrations of the pro- and anti-inflammatory cytokines play key roles in the downstream responses to LPS challenge (Thirunavukkarasu, Watkins et al. 2006). To determine if there may be correlative induction of pro-inflammatory cytokines and antimicrobial gene expression during LPS challenge, real time RT-PCR analyses were carried out to study *Il-6* and *Tnf-a* expression. In the caput, *Il-6* expression increased significantly up to 24 hr after LPS exposure. *Tnf-a* expression was increased significantly at 3 and 6 hr followed by a transient decline up to 15 hr and an increase at 24 hr after LPS treatment (Figure 1.14D). Increase in *Tnf-a* and

Il-6 seems to correlate with *Spag11* and defensin expression pattern at the early time points.

Antimicrobial gene expression in the cauda

Since gene expression varies greatly along the length of the epididymis, *Spag11* and defensin expression in the cauda of LPS treated rats was studied. *Spag11t, c* and *e* expression was significantly increased within 3 hr after LPS administration with differences in the duration of continued response over the experimental period (Figure 1.15A). In the case of *Spag11c*, the increased response sustained until 24 hr after LPS administration, whereas a biphasic response was observed for *Spag11t* and *Spag11e* (Figure 1.15B).

Defb24 expression increased significantly immediately after LPS injection and its levels remained elevated throughout the experimental period. A delayed response was observed for *Defb27* and *Defb36* wherein their mRNA levels increased 9 and 15 hr after treatment respectively (Figure 1.15C).

Defb2 levels increased significantly in a time dependent manner up to 6 hr followed by a steady decrease up to 24 hr. *Defb1* and *Defb21* mRNA levels were decreased reaching levels below the untreated control after LPS administration, though an increase was observed for *Defb1* at the initial time point (3 hr) (Figure 1.15C).

The expression of *Tnf-α* increased significantly within 3 hr, whereas the response was at the 9 hr time point for *Il-6*, an increase that correlates with the expression of *Spag11* and defensins (Figure 1.15D).

Antimicrobial gene expression in the testis

In the testis, *Spag11e* expression increased within 3 hr after LPS exposure and continued to remain elevated throughout the experimental period. An increase in *Spag11c*, *Defb27* and *Defb36* expression was also observed within 3 hr, though not as robust as *Spag11e* followed by a time dependent decrease (Figure 1.16A). In the case of *Defb21*, the response was delayed reaching significant levels 9 hr after LPS exposure (Figure 1.16B). A decrease in the mRNA levels of *Defb1* and *Defb24* were observed (Figure 1.16C).

The expression of pro-inflammatory cytokines, *Il-6* and *Tnf-α* in the testis increased 3–15 hr after LPS treatment with a decline at the 24 hr time point. The increase at the 3–15 hr time points corresponds to the elevated levels of *Spag11* and defensin transcripts (Figure 1.16D).

Antimicrobial gene expression in the seminal vesicle

In the seminal vesicle, *Defb24* mRNA levels were increased significantly within 3 hr of LPS administration followed by a decline below the untreated control levels in a time dependent manner (Figure 1.17A). Similar expression pattern was observed for *Defb1*, though the increased response occurred 6 hr after LPS injection. *Defb27* levels remained unaltered. Further, a significant increase in *Il-6* and *Tnf- α* levels at the 3–15 hr time points correlated with those of *Defb24* and *Defb1*(Figure 1.17B).

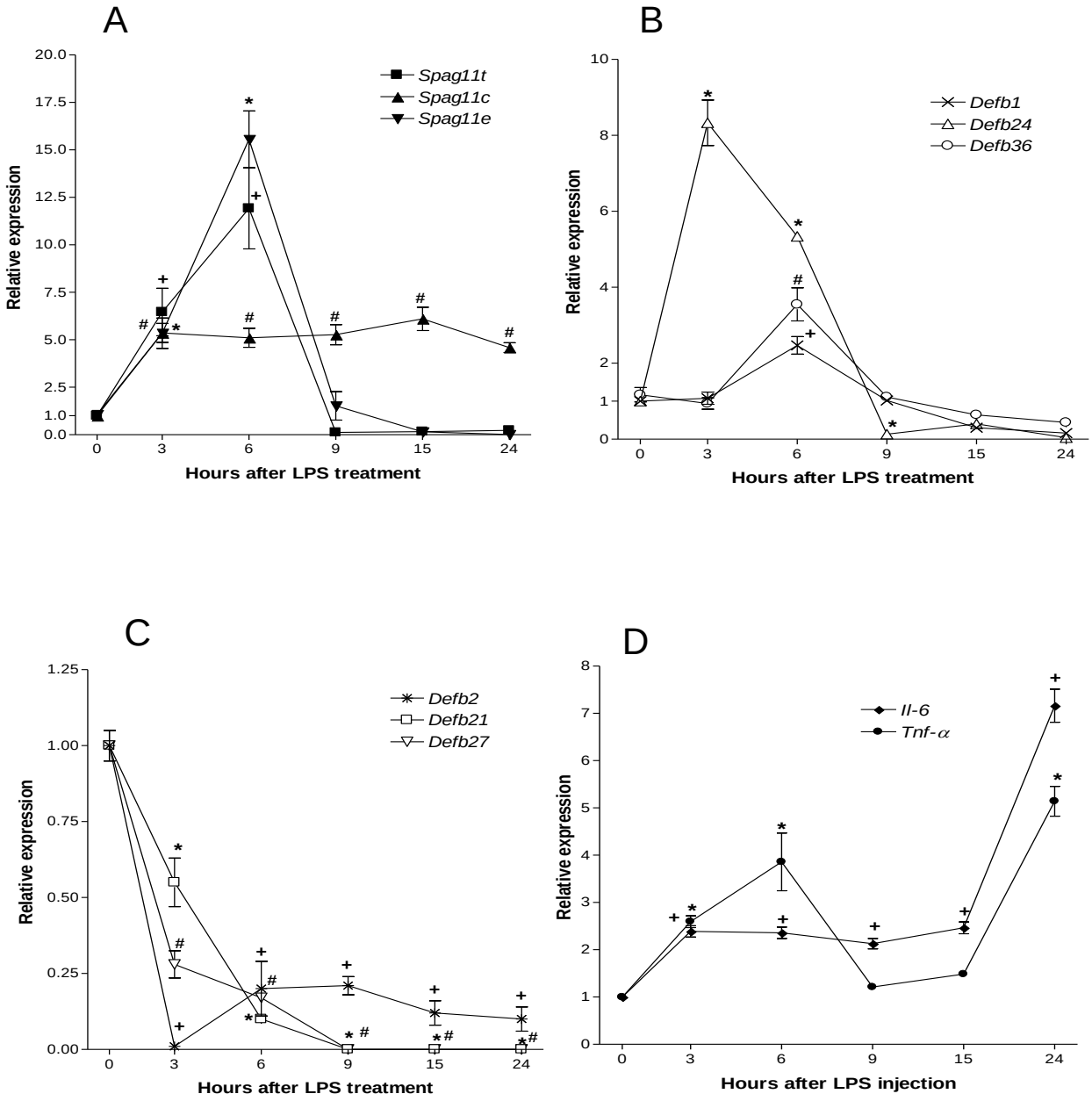

Figure 1.14: *Spag11*, *Defb* and pro-inflammatory gene expression in the caput of LPS challenged rats. RNA isolated from caput collected at different time points after treating the rats with 1 mg / kg body weight LPS was used to study gene expression by real time RT-PCR. Values shown are mean ±S.E. *, + and # indicate $p < 0.05$ compared to 0 hr control.

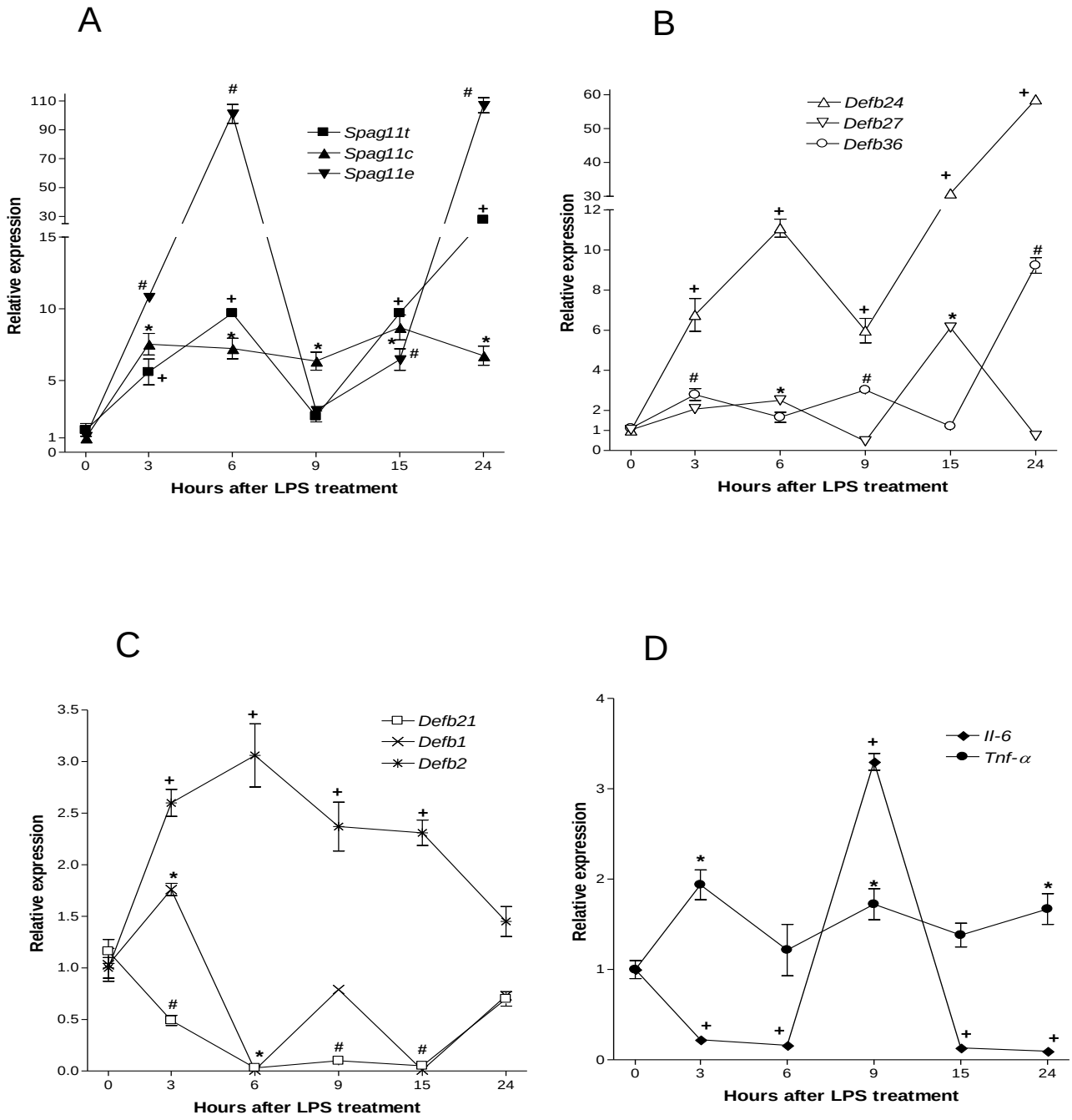

Figure 1.15: Time course of antimicrobial and proinflammatory gene expression in the cauda of LPS treated rats. Real time RT-PCR analysis was carried out using RNA isolated from the cauda of LPS treated (1 mg / kg body weight) rats. Values shown are mean ± S.E. *, + and # indicate $p < 0.05$ compared to 0 hr control.

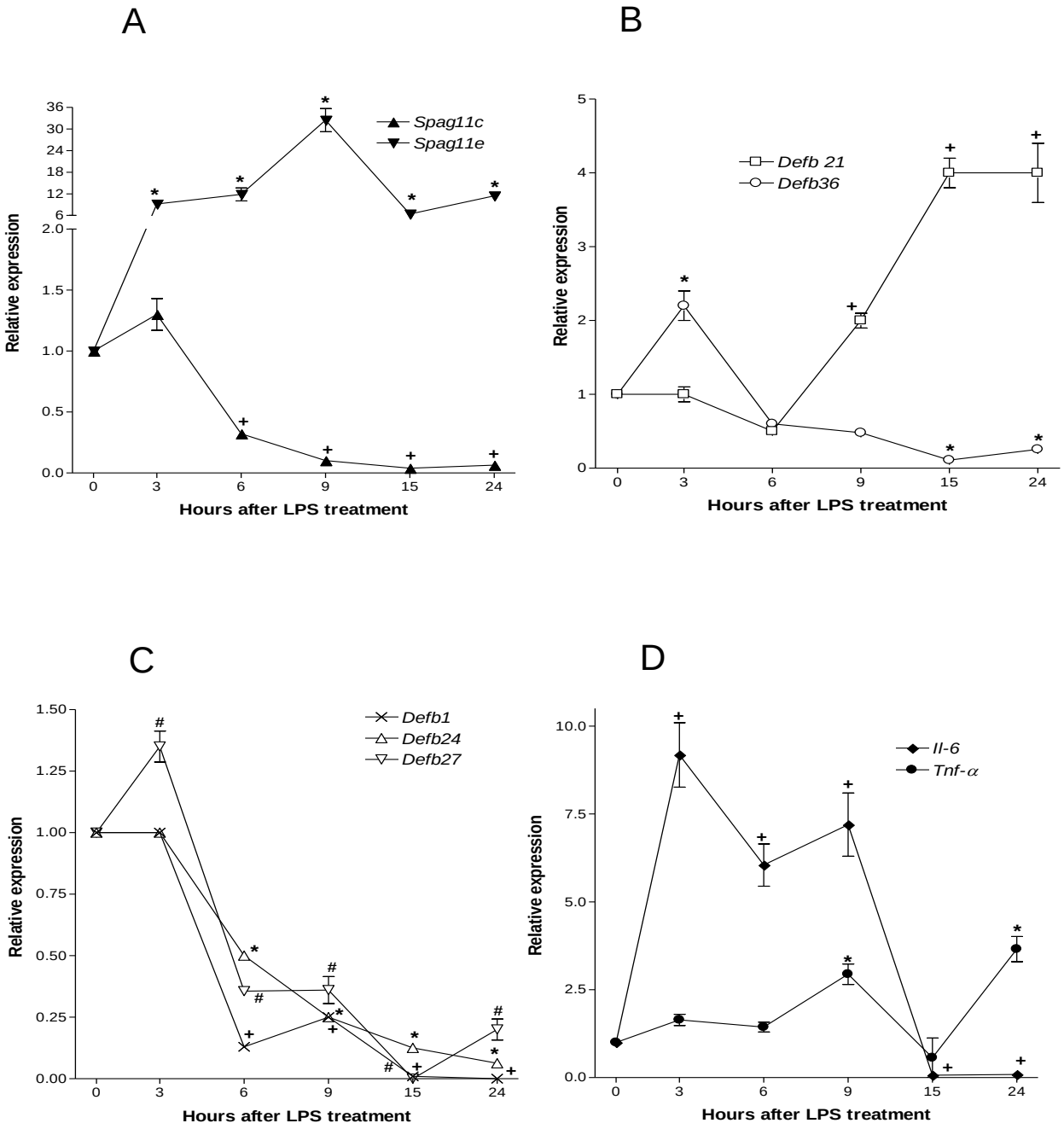

Figure 1.16: Expression profile of *Spag11* mRNA variants, defensins, *Il-6* and *Tnf-a* in the testis of LPS treated rats. RNA isolated from the testes of LPS treated rats was analyzed by real-time RT-PCR using gene specific primers. Values shown are mean ±S.E. *, + and # indicate $p < 0.05$ compared to 0 hr control.

.

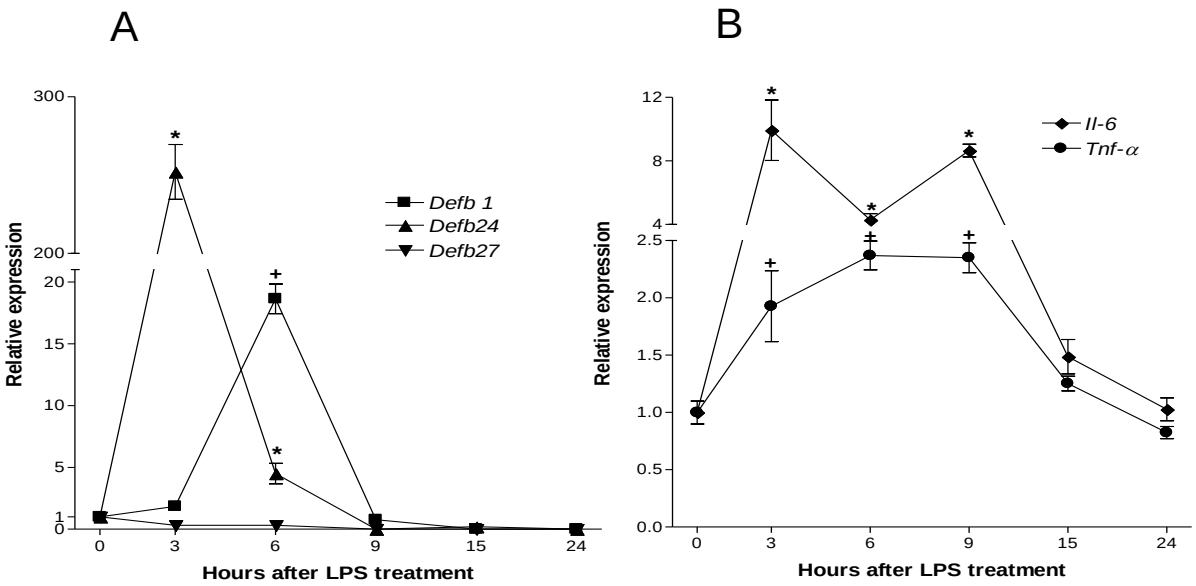

Figure 1.17: Pro-inflammatory cytokine and defensin expression in the seminal vesicle of LPS treated rats. RNA isolated from the seminal vesicle of LPS treated rats was analyzed by real-time RT-PCR using gene specific primers. Values shown are mean ±S.E. *, + and # indicate $p < 0.05$ compared to 0 hr control.

Immunostaining

In the epididymis, intense staining was observed for SPAG11E at 6 h after LPS administration in the epithelial cells as well on the sperm surface, whereas in the untreated control minimal staining was observed only on the sperm surface. At the 9 and 15 h time points, the staining gradually decreased and was mainly confined to the sperm surface similar to that observed in the untreated control (Figure 1.18). Similar protein expression pattern was also observed in the testes. At the 6 h time point, the intensity of staining was higher in the epithelium and on the sperm surface than the untreated control. *SPAG11E* localization was primarily restricted to the sperm surface at the 9 h time point whereas minimal staining was observed at 24 h (Figure 1.18). The increase in *SPAG11E* protein expression seems to correlate with its increased mRNA expression.

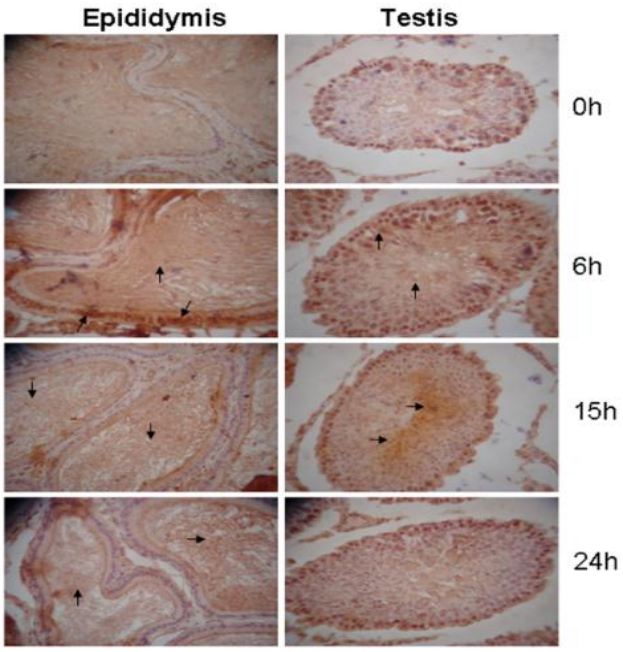

Figure 1.18: SPAG11E protein expression in the epididymides and testes of LPS treated rats. Testis and epididymis sections obtained from LPS treated rats were immuno detected for SPAG11E using polyclonal antibodies against the rat SPAG11E. Hematoxylin was used for counterstaining. All the photographs were taken at 40Xmagnification.

DISCUSSION

We report the identification of rat *Tlr5*, *Tlr10*, and *Tlr11*. The presence of characteristic LRR and TIR domains in the predicted amino acid sequences of these TLRs suggests that they primarily function to detect pathogens thus sharing the functional features with the known TLRs. Toll-like receptors which form an important component of innate immunity have been implicated in many roles in the male and female reproductive tracts. In the male reproductive tract, TLRs appear to play a role in the control of testicular steroidogenesis and spermatogenesis both in normal and disease conditions. A recent study demonstrates the abundance of TLRs in the male reproductive tract of rats (Girling and Hedger 2007). However evidence is lacking in describing the regulation of TLRs in general in the male reproductive tract. Such evidence can provide insights into the role of these innate immune molecules during an infection.

This study demonstrates the identification of three *Tlrs* namely *Tlr5*, *Tlr10*, and *Tlr11* in the rat. Further, the expression profile under conditions of androgen variation was also demonstrated, which was not reported in a previous study, wherein the presence of *Tlrs* in the male reproductive tract of rats was analyzed. In this study, the abundant expression of the majority of TLRs suggests the preparedness of the male reproductive tract to respond to an infection. The presence of other innate molecules such as CD14 and MYD88 in the male tract along with the *TLR2* and *4* strongly points to the fact that invasion by a pathogen might result in the activation of immune effector pathways that can trigger the production of cytokines and antimicrobial peptides. Abundant *Tlr3* expression was reported in contrast to the weak expression of *Tlr11* in the male reproductive tract (Palladino, Johnson *et al*.

2007). However, by contrast we observed an abundant *Tlr11* and weak *Tlr13* expression. This discrepancy could be because of age and strains of the animals used. Further, male reproductive tract tissues analyzed in this study contain different cell types including the immune cells. It is possible that the expression of *Tlrs* in male reproductive tract tissues could partly be contributed by the immune cells.

Developmental regulation of a wide variety of genes in the male reproductive system has been studied extensively (Rodriguez, Kirby *et al*. 2001) because of the fluctuations of androgens at various stages. For example, rat epididymis tissue androgen decreases from birth until 20 days, but remains at a substantial level of approximately 10 ng /g tissue (35 nm) until approximately 40 days when it begins to increase to adult levels of between 15 and 20 ng /g (Charest, Petrusz *et al*. 1989) Serum testosterone levels in the rat remain low and do not begin to increase to adult levels until 35–40 days (Nayfeh, Barefoot *et al*. 1966). The expression pattern of majority of *Tlrs* in the epididymis did not seem to correlate with the minimal androgen levels from day 20 to day 40 or increased androgen in the adult, suggesting that *Tlr* expression is not androgen dependent during development. However, androgen concentration differs in the testis compared with the epididymis. A steady increase in testosterone levels was reported in the rete testis of 30 to 130 day old rats, (Harris and Bartke 1974; Harris and Bartke 1981). In this study, abundant expression of *Tlrs* was detected in the testes of 10 to 60 day old rats suggesting their constitutive nature of expression, thereby keeping the testes ready for any microbial challenge. Lack of *Tlr3* expression in the testes of developing rats (10–60 days old) is in contrast to its presence in the testes obtained from adult rats (60–90 days old). Conversely, *Tlr10*, *11*, and *12* mRNA were detected in the testes of

developing rats (10–60 days old), whereas their expression was not detected in testes obtained from adult rats (60–90 days old). These discrepancies could be because of the age differences and actual levels of testicular testosterone at the time of tissue collection. Further analyses are required to determine whether *Tlr3*, *10*, *11*, and *12* expressions could be drastically affected within a short window of time. Earlier studies indicate that androgens are important to the development and physiology of the prostate and seminal vesicle (Kinghorn, Bate et al. 1987; Martikainen, Harkonen et al. 1987).

We observed that *in vitro* LPS challenge induced the expression of all the defensin and *Spag11* genes analysed in the male reproductive tract tissues of rat. LPS induced defensin and *Spag11* gene expression observed in this study is in agreement with intraperitoneal injection of LPS which also induces antimicrobial gene expression in the epididymis and testis of rats. The increased expression of defensin and *Spag11* mRNA in response to LPS demonstrates the robust innate immune mechanisms operating in the male reproductive tract. Signalling mechanisms that operate during LPS exposure is primarily mediated by TLR4 (Peri, Piazza *et al*.). In an *invitro* LPS study we observe no significant changes in the expression pattern of *Tlr4* in the epididymides and testis during LPS challenge, suggesting that *Tlr4* expression seems to be constitutive and abundant levels are present to combat endotoxin challenge. *Tlr4* mRNA expression increased in the epididymides and testis challenged with LPS obtained from rats treated intraperitoneally with LPS. Increase in pro-inflammatory cytokine production leading to the transcriptional activation of many response genes is one of the hall marks of endotoxin induced cellular responses (McIntyre, Modur *et al*. 1997). In the mouse testes, the importance of

proinflammatory cytokines during enodotoxin challenge is reported (Abu Elhija, Lunenfeld *et al*. 2008). Increased levels of *Il-6* and *Tnf-a* observed in an *in vitro* and intraperitoneal LPS study could influence the expression of a variety of genes including the defensin and *Spag11* genes analysed in this study. Administration of LPS resulted in the up regulation of *Tlr4* mRNA in a time dependent manner in the caput, cauda, and testis. Such increased expression in *Tlr4* mRNA was also reported in other organ systems such as liver (Kitazawa, Tsujimoto *et al*. 2008) articular cartilage (Haglund, Bernier *et al*. 2008) and mesenchymal cells (Shi, Wang *et al*. 2007). Increased levels of *Tlr4* expression after LPS challenge suggests its possible role in recognition of LPS and initiate an immune response to combat the invading pathogen. However, the molecular machinery that is involved in initiating such a response by *TLR4* during LPS challenge or infection in the male reproductive tract remains unanswered.

In this study, *Spag11* and defensin gene expression in response to LPS varied in the caput, cauda, testis and seminal vesicle. In view of the key importance of testosterone in maintaining male reproductive tract function in general and *Spag11* and defensin gene expression specifically, (Yenugu, Chintalgattu et al. 2006; Yenugu, Hamil et al. 2006) testosterone was measured in the plasma of LPS treated rats. Reduction in plasma testosterone after LPS treatment as we here report and as previously reported (O'Bryan, Schlatt *et al*. 2000; Reddy, Mahipal *et al*. 2006) might be expected to result in reduced expression of these antimicrobial genes. However our findings suggest that some of the *Spag11* and defensin expression levels are instead increased in caput and cauda during the window of low serum testosterone suggest that stimulatory signaling intermediates, such as the cytokines (*Il-6* and *Tnf-*

α) may overcome the effects of suppressed testosterone. Evidence for the possible involvement of cytokines in modulating antimicrobial gene expression was demonstrated for *Il-6*, wherein it induces the expression of hepcidin, an antimicrobial protein (Lee, Peng *et al*. 2005). This inverse regulation confirms the role of factors other than androgens, which may collaborate to maintain antimicrobial gene expression to prevent damage to the reproductive tract by pathogens. These observations suggest that androgen dependent genes of the male reproductive tract may become androgen independent under conditions that threaten the integrity of the system. Further, there was a fluctuation (biphasic) in response to LPS treatment. It is possible that these fluctuations could be due to immediate and delayed immune responses by these organ systems in response to LPS. However, further studies are required to demonstrate the relation between these fluctuations and the immediate and delayed immune responses (Abrahamson, Ritonja *et al*. 1987).

Spag11e has been implicated to have definite role in sperm maturation (Zhou, Zhang *et al*. 2004). Similarly, the defensin-like protein ESP13.2 is implicated in sperm capacitation (Yudin, Tollner et al. 2003). Decreased expression of some of the *Spag11* variants and defensins immediately upon LPS exposure may be a mechanism to prevent sperm maturation, thereby avoiding fertilization with defective sperm that may have arisen due to DNA damage by free radicals generated upon LPS administration. In order to determine whether pro-inflammatory responses occur in the reproductive tract during LPS challenge, mRNA levels of *Il-6* and *Tnf-a* were monitored. In all the tissues analyzed the mRNA levels of *Il-6* and *Tnf-a* transiently increased in parallel with an increase in the antimicrobial gene

expression. It is possible that increase in cytokine levels may trigger protective mechanisms indirectly resulting in the induction of antimicrobial genes. The increased expression of *Il-6* and *Tnf-α* may augment germ cell production under stress conditions induced by LPS thereby providing conditions to create uncompromised fertility. In this study it is not clear whether antimicrobial gene induction observed is due to protective mechanisms or the direct effect of pro-inflammatory cytokines. Further, SPAG11E protein expression was found to be induced in the epididymis and testis of LPS treated rats. These findings are in agreement with the increased *Spag11e* gene expression observed in this study. A time dependent increase in *Tlr4* expression in the reproductive tissues obtained from rats treated with LPS suggests that increased *Tlr4* expression may effectively initiate and amplify the signaling pathways via NF-kB required for downstream processing leading to induction of antimicrobial gene and protein responses. We report that the male reproductive tract exhibits potent antimicrobial protection against LPS by inducing the expression of *Spag11* and defensins. Induction of some of the antimicrobial gene expression seems to be androgen independent under conditions that threaten the reproductive system; and may involve complex pathway(s) that mediate the innate immune responses. Consistent with the gene expression, *SPAG11E* protein expression also increased during LPS challenge. Identification of antimicrobial genes predominant in the male reproductive tract and their functions in innate immune responses will contribute to the design of novel peptide antibiotics to treat sexually transmitted diseases. Increased understanding of the regulation of these genes can facilitate the development of strategies to increase their synthesis in the treatment of microbial infections.

REFERENCES

Abrahamson, M., A. Ritonja, et al. (1987). "Identification of the probable inhibitory reactive sites of the cysteine proteinase inhibitors human cystatin C and chicken cystatin." J Biol Chem 262(20): 9688-9694.

Abu Elhija, M., E. Lunenfeld, et al. (2008). "LPS Increases the Expression Levels of IL-18, ICE and IL-18 R in Mouse Testes." American Journal of Reproductive Immunology 60(4): 361-371.

Avellar, M. C., L. Honda, et al. (2004). "Differential expression and antibacterial activity of epididymis protein 2 isoforms in the male reproductive tract of human and rhesus monkey (Macaca mulatta)." Biol Reprod 71(5): 1453-1460.

Bourgeon, F., B. Evrard, et al. (2004). "Involvement of semenogelin-derived peptides in the antibacterial activity of human seminal plasma." Biol Reprod 70(3): 768-774.

Charest, N. J., P. Petrusz, et al. (1989). "Developmental expression of an androgen-regulated epididymal protein." Endocrinology 125(2): 942-947.

Com, E., F. Bourgeon, et al. (2003). "Expression of antimicrobial defensins in the male reproductive tract of rats, mice, and humans." Biol Reprod 68(1): 95-104.

Dinarello, C. A. (2000). "Proinflammatory cytokines." Chest 118(2): 503-508.

Emma M Creigh, L. A. J. O. n. (2006). "TLRs, NLRs and RLRs: a trinity of pathogen sensors that co-operate in innate immunity." trends in immunology

Girling, J. E. and M. P. Hedger (2007). "Toll-like receptors in the gonads and reproductive tract: emerging roles in reproductive physiology and pathology." Immunol Cell Biol 85(6): 481-489.

Gow, R. M., M. K. O'Bryan, et al. (2001). "Differential effects of dexamethasone treatment on lipopolysaccharide-induced testicular inflammation and reproductive hormone inhibition in adult rats." J Endocrinol 168(1): 193-201.

Haglund, L., S. M. Bernier, et al. (2008). "Proteomic analysis of the LPS-induced stress response in rat chondrocytes reveals induction of innate immune response components in articular cartilage." Matrix Biol 27(2): 107-118.

Hales, K. H., T. Diemer, et al. (2000). "Diametric effects of bacterial endotoxin lipopolysaccharide on adrenal and Leydig cell steroidogenic acute regulatory protein." Endocrinology 141(11): 4000-4012.

Hall, S. H., K. G. Hamil, et al. (2002). "Host defense proteins of the male reproductive tract." J Androl 23(5): 585-597.

Hamil, K. G., Q. Liu, et al. (2003). "LCN6, a novel human epididymal lipocalin." Reprod Biol Endocrinol 1(1): 112.

Hamil, K. G., Q. Liu, et al. (2002). "Cystatin 11: a new member of the cystatin type 2 family." Endocrinology 143(7): 2787-2796.

Hamil, K. G., P. Sivashanmugam, et al. (2000). "HE2beta and HE2gamma, new members of an epididymis-specific family of androgen-regulated proteins in the human." Endocrinology 141(3): 1245-1253.

Harris, M. E. and A. Bartke (1974). "Concentration of testosterone in testis fluid of the rat." Endocrinology 95(3): 701-706.

Harris, M. E. and A. Bartke (1981). "Androgen levels in the rete testis fluid during sexual development." Experientia 37(4): 426-427.

Hsia, N. and G. A. Cornwall (2003). "Cres2 and Cres3: new members of the cystatin-related epididymal spermatogenic subgroup of family 2 cystatins." Endocrinology 144(3): 909-915.

Huleihel, M., E. Lunenfeld, et al. (2000). "Involvement of serum and lipopolysaccharide in the production of interleukin-1- and interleukin-6-like molecules by human sperm cells." Am J Reprod Immunol 43(1): 41-46.

Kawai, T. and S. Akira (2009). "The roles of TLRs, RLRs and NLRs in pathogen recognition." Int Immunol 21(4): 317-337.

Kinghorn, E. M., A. S. Bate, et al. (1987). "Growth of rat seminal vesicle epithelial cells in culture: neurotransmitters are required for androgen-regulated synthesis of tissue-specific secretory proteins." Endocrinology 121(5): 1678-1688.

Kitazawa, T., T. Tsujimoto, et al. (2008). "Expression of Toll-like receptor 4 in various organs in rats with D-galactosamine-induced acute hepatic failure." J Gastroenterol Hepatol 23(8 Pt 2): e494-498.

Lee, P., H. Peng, et al. (2005). "Regulation of hepcidin transcription by interleukin-1 and interleukin-6." Proc Natl Acad Sci U S A 102(6): 1906-1910.

Li, P., H. C. Chan, et al. (2001). "An antimicrobial peptide gene found in the male reproductive system of rats." Science 291(5509): 1783-1785.

Liao, M., P. S. Ruddock, et al. (2005). "Cationic peptide of the male reproductive tract, HE2{alpha}, displays antimicrobial activity against *Neisseria gonorrhoeae*, *Staphylococcus aureus* and *Enterococcus faecalis*." J Antimicrob Chemother 56(5): 957-961.

Malm, J., E. A. Nordahl, et al. (2005). "Lipopolysaccharide-binding protein is produced in the epididymis and associated with spermatozoa and prostasomes." J Reprod Immunol 66(1): 33-43.

Martikainen, P., P. Harkonen, et al. (1987). "Multihormonal control of synthesis and secretion of prostatein in cultured rat ventral prostate." Endocrinology 121(2): 604-611.

McIntyre, T. M., V. Modur, et al. (1997). "Molecular mechanisms of early inflammation." Thrombosis and Haemostasis 78(1): 302-305.

Nayfeh, S. N., S. W. Barefoot, Jr., et al. (1966). "Metabolism of progesterone by rat testicular homogenates. II. Changes with age." Endocrinology 78(5): 1041-1048.

O'Bryan, M. K., S. Schlatt, et al. (2000). "Bacterial lipopolysaccharide-induced inflammation compromises testicular function at multiple levels in vivo." Endocrinology 141(1): 238-246.

Palladino, M. A., T. A. Johnson, et al. (2007). "Members of the Toll-like receptor family of innate immunity pattern-recognition receptors are abundant in the male rat reproductive tract." Biol Reprod 76(6): 958-964.

Palladino, M. A., M. A. Savarese, et al. (2008). "Localization of Toll-like receptors on epididymal epithelial cells and spermatozoa." Am J Reprod Immunol 60(6): 541-555.

Pandey, S. and D. Agarwal (2006). "Immunology of toll-like receptors: emerging trends." Immunol Cell Biol 84: 333-341.

Peri, F., M. Piazza, et al. "Exploring the LPS/TLR4 signal pathway with small molecules." Biochemical Society Transactions 38(5): 1390-1395.

Randhawa, A. K. and T. R. Hawn (2008). "Toll-like receptors: their roles in bacterial recognition and respiratory infections." Expert Rev Anti Infect Ther 6(4): 479-495.

Reddy, M. M., S. V. Mahipal, et al. (2006). "Bacterial lipopolysaccharide-induced oxidative stress in the impairment of steroidogenesis and spermatogenesis in rats." Reprod Toxicol 22(3): 493-500.

Roach, J. C., G. Glusman, et al. (2005). "The evolution of vertebrate Toll-like receptors." Proc Natl Acad Sci U S A 102(27): 9577-9582.

Rodrigues, A., D. B. Queiroz, et al. (2008). "Activation of toll-like receptor 4 (TLR4) by in vivo and in vitro exposure of rat epididymis to lipopolysaccharide from Escherichia Coli." Biology of Reproduction 79(6): 1135-1147.

Rodriguez, C. M., J. L. Kirby, et al. (2001). "Regulation of gene transcription in the epididymis." Reproduction 122(1): 41-48.

Shi, L., J. S. Wang, et al. (2007). "Upregulated functional expression of Toll like receptor 4 in mesenchymal stem cells induced by lipopolysaccharide." Chin Med J (Engl) 120(19): 1685-1688.

Takeda, K. and S. Akira (2007). "Toll-like receptors." Curr Protoc Immunol Chapter 14: Unit 14 12.

Thirunavukkarasu, C., S. C. Watkins, et al. (2006). "Mechanisms of endotoxin-induced NO, IL-6, and TNF-alpha production in activated rat hepatic stellate cells: role of p38 MAPK." Hepatology 44(2): 389-398.

Yenugu, S., V. Chintalgattu, et al. (2006). "Identification, cloning and functional characterization of novel beta-defensins in the rat (Rattus norvegicus)." Reprod Biol Endocrinol 4(1): 1-7.

Yenugu, S., K. G. Hamil, et al. (2003). "Antibacterial properties of the sperm-binding proteins and peptides of human epididymis 2 (HE2) family; salt sensitivity, structural dependence and their interaction with outer and cytoplasmic membranes of *Escherichia coli*." Biochem J 372(Pt 2): 473-483.

Yenugu, S., K. G. Hamil, et al. (2006). "Antimicrobial actions of human and macaque sperm associated antigen (SPAG)11 isoforms: Influence of the N-terminal peptide." Molecular and Cellular Biochemistry 284(1-2): 25-35.

Yenugu, S., K. G. Hamil, et al. (2006). "Identification, cloning and functional characterization of novel Spag11 isoforms in the Rat." Reprod Biol Endocrinol 4(23): 1-14.

Yenugu, S., K. G. Hamil, et al. (2006). "Identification, cloning and functional characterization of novel sperm associated antigen 11 (SPAG11) isoforms in the rat." Reprod Biol Endocrinol 4: 23.

Yenugu, S., K. G. Hamil, et al. (2004). "The androgen-regulated epididymal sperm-binding protein, human beta-defensin 118 (DEFB118) (formerly ESC42), is an antimicrobial beta-defensin." Endocrinology 145(7): 3165-3173.

Yudin, A. I., T. L. Tollner, et al. (2003). "ESP13.2, a member of the beta-defensin family, is a macaque sperm surface-coating protein involved in the capacitation process." Biology of Reproduction 69(4): 1118-1128.

Zhou, C. X., Y. L. Zhang, et al. (2004). "An epididymis-specific beta-defensin is important for the initiation of sperm maturation." Nat Cell Biol 6(5): 458-464.

INTRODUCTION

The male reproductive tract, a dynamic organ system consisting of extensive duct system with mucosal surfaces, is responsible for the maintenance of fertility and other physiological functions as well. Immature spermatids produced in the testis pass through the epididymis, during which a number of proteins secreted by the mucosal surfaces are added and these proteins contribute to maturation, motility, and fertilizing ability of spermatozoa. Cathelicidins, defensins, protease inhibitors such as cystatins, and members of the Sperm-Associated Antigen 11 (SPAG11) family are some of the proteins that were identified to be added on to the sperm surface (Hamil, Sivashanmugam et al. 2000; Hamil, Liu et al. 2002; Cornwall, Cameron et al. 2003; Hamil, Liu et al. 2003). Besides their role in sperm maturation, the antimicrobial activities of sperm-bound proteins have been demonstrated (Malm, Sorensen et al. 2000; Li, Chan et al. 2001; Hamil, Liu et al. 2002; Bourgeon, Evrard et al. 2004; Liao, Ruddock et al. 2005). For example, SPAG11 proteins and peptides of humans, macaque, bovine and mouse were found to exhibit potent broad spectrum antimicrobial activity and the mechanism of action involves bacterial membrane permeabilization (Yenugu, Hamil et al. 2003; Yenugu, Hamil et al. 2004; Yenugu, Hamil et al. 2006). Further, identification and characterization of novel SPAG11 isoforms and β-defensins in the rat was done (Yenugu, Hamil et al. 2006) and demonstrated their antimicrobial activity. Infections of the male reproductive tract caused by *Escherichia coli*, *Chlamydia trachomatis* and *Neisseria gonorrhea* cause epididymitis, a clinical condition characterized by damage to the epididymal epithelial lining, leading to loss of fertility (Trojian, Lishnak et al. 2009). The male reproductive tract being a sterile organ system has developed (Girling and Hedger 2007) robust defence mechanisms to combat infections. During infection, damage to

spermatozoa by endotoxins occurs primarily because of the generation of free radicals (Fraczek and Kurpisz 2007).

In most of the organ systems, the immune responses that operate to combat infections involve Toll-like receptors (TLRs) and their adaptor proteins (Takeda and Akira 2004). In humans, 11 TLRs (TLR1-11) are identified, whereas in rodents, 13 are reported (Roach, Glusman et al. 2005; Biswas, Narmadha et al. 2009). Among them, TLR4 is shown to be very crucial for the immune responses elicited by Lipopolysaccharide (LPS). TLR4 functions in association with other accessory proteins such as lipopolysaccharide binding protein (LBP), CD14, and MD2 to activate NF-kB and IRF-3 via the Myd88-dependent and Myd88-independent pathways, respectively (Akira and Takeda 2004; Roach, Glusman et al. 2005). The signalling pathways initiated by TLRs induce the transcription of genes such as tumour necrosis factor-alpha (*Tnf-α*), Interleukin-6 (*Il-6)*, *IL-12p40*, interferon-beta (*IFN-β*), chemokines, adhesion molecules, acute phase proteins, antimicrobial peptides, inducible nitric oxide synthase (iNos), and cyclooxygenase 2 (Cox-2) (Akira 2001). Further, the signalling pathways utilized by the pathogens and endotoxins are quite different in inducing the immune responses of epithelial cells (Chung and Dale 2004). TLR4 is constitutively expressed in the male reproductive organs of humans and rodents (Quintar, Roth et al. 2006; Girling and Hedger 2007; Palladino, Johnson et al. 2007) and other accessory gland tissues (Gatti, Rivero et al. 2006; Gatti, Quintar et al. 2009). Further, the TLR-associated molecules MYD88 and CD14 were reported in the prostate (Mackern-Oberti, Maccioni et al. 2006). Activation of TLR4 and NF-kB in the male reproductive tract of rat during LPS challenge has been demonstrated (Rodrigues, Queiroz et al. 2008). However, studies on the role of defensins and

defensin like proteins (SPAG11 isoforms) in protecting the male reproductive tract during endotoxin challenge or infection are limited. Earlier we demonstrated that the expression of defensins and members of the *Spag11* family is elevated during endotoxin challenge in rats (Biswas and Yenugu 2011).

It was also reported that LPS disrupts epididymal β-defensin expression in the male reproductive tract of rats (Cao, Li et al. 2010). However, signalling pathways that govern the regulation of antimicrobial gene expression in the male reproductive tract during endotoxin challenge or infectious conditions have not been previously investigated. Further, it is possible that antimicrobial gene expression in the male reproductive tract could be epigenetically regulated. Epigenetic modifications in general include histone acetylation/ deacetylation, histone methylation/ demethylation, and DNA methylation, which are tightly, controlled by enzymes such as histone deacetylases (HDACs), histone demethylases and DNA methyl transferases (DNMTs). Acetylation of histones including histone 3 and demethylation of DNA in general promote transcriptional activation (Kouzarides 2007). Previous studies have demonstrated the epigenetic regulation of β-defensin 2 expression in gingival epithelial cells in response to oral bacteria (Yin and Chung 2011). Hence, in this study, we attempt to determine whether epigenetic changes would contribute to the innate immune responses of the male reproductive tract. In an *in vitro* culture system, we demonstrate that LPS induces *Spag11* and β-defensin mRNA expression and involves NF-kB activation.

Upon recognition of microorganisms or their products by epithelial cells and macrophages of many organ systems, immune defences include the production of antimicrobial proteins and peptides. Epithelial cells and macrophages of the testis

and epididymis also produce various immune effectors such as cytokines, chemokines, and antimicrobial peptides in response to inflammatory stimuli, and regulate the activation and recruitment of professional phagocytes (neutrophils and macrophages) and immune cells (T cells and dendritic cells). Among the antimicrobial peptides, β defensins play a unique role linking innate and acquired immunities and regulate the activation and recruitment of professional phagocytes (neutrophils and macrophages) and immune cells (T cells and dendritic cells). Recent studies including ours have demonstrated that defensin gene is upregulated during endotoxin challenge. Taken together, it is important to elucidate the molecular mechanisms regulating their expression, especially the role of transcription factors.

A wide variety of transcription factors such as Nuclear factor kappa beta (NF-kB), Activator protein-1 (AP-1), E-twenty six (ETS) etc are known to regulate immune response related gene expression. Recently, the *Spag11e* promoter has been cloned, and found to contain several consensus transcription factor binding sites. The *Spag11e* upstream region between -780 and +27, contains binding sites for androgen receptor (AR), nuclear factor-kB (NF-kB), nuclear factor -1 (NF-1), E-twenty six (ETS) and activator protein 2 (Ap2) binding sites. However, studies that describe the involvement of transcription in regulating β-defensins in the male reproductive tract are lacking. Hence, we analysed the presence of transcription factor binding elements in the *Spag11e* gene upstream sequence and whether the cognate transcription factors do bind to these elements during endotoxin challenge.

APPROACHES

***In vitro* culture and treatments:** Briefly, caput, cauda, and testis from 90-day-old rats were dissected and cut into two longitudinal halves. One half of the tissue was used as control, and the other for LPS (Sigma Aldrich, St. Louis, USA) treatments. Tissues were transferred to 2 ml of freshly prepared nutritive medium containing 136.89 mM NaCl, 5.63 mM KCl, 1.80 mM $CaCl_2$, 0.36 mM NaH_2PO_4, 14.88 mM $NaHCO_3$, and 5.55 mM glucose, pH 7.6-7.8 and cultured at 30°C with aeration. After 15 min of incubation, tissues were transferred to fresh nutritive medium and treated with 1 µg/ml LPS and incubated for different time points (0–9 h for RNA studies) and (0–180 min for protein studies). The LPS dose and treatment periods were chosen based on previous studies (Rodrigues *et al*. 2008). During these incubation periods, nutritive solution without or with LPS was renewed every 30 min. The tissues were collected, rinsed with PBS, frozen in liquid nitrogen, and stored in −80 °C until use. To study the effect of NF-kB inhibitors (Sigma Aldrich) on LPS-mediated antimicrobial gene expression, caput, cauda, and testis tissues were pretreated with either 100 µM PDTC (Pyrollidine dithiocarbamate) or 100 µM Bay11 for 1 h. The tissues were then transferred to fresh wells containing nutritive medium without or with LPS (1 µg/ml) and incubated for 0–9 h. Similarly, caput and testis tissues were pretreated with 100 µM TSA (a histone deacetylase 1 (HDAC1) inhibitor; Sigma Aldrich) or 50 µM 5'-azacytidine (a DNA methyl transferase1 (DNMT1) inhibitor; Sigma Aldrich) for 1 h and challenged with LPS (1 µg/ml) for 6 h to determine the role of HDAC1 and DNMT1 in LPS induced antimicrobial responses. All the inhibitors were dissolved in DMSO, and control tissues were treated with an equivalent amount of DMSO that is present in the volume of the inhibitor added.

Experiments were conducted using the Guidelines for the Care and Use of Laboratory Animals, approved by the Institutional Animal Ethics Committee of the University of Hyderabad.

Real-time RT-PCR: Total RNA (2µg) extracted using the TRIzol reagent (Invitrogen, Carlsbad, USA) was reverse-transcribed using 50 U Stratascript (Invitrogen, USA) and 0.5 µg of oligodT (Invitrogen). Two microliters of the resultant cDNA was used for real-time RT-PCR analysis using Power SYBR Green PCR master mix kit (Applied Biosystems, Warrington, UK) in a real-time thermal cycler (Applied Biosystems). A typical real-time PCR reaction was carried out with the following conditions: initial denaturation at 94 °C for 10 min, 40 cycles with 94 °C for 15 s, and 60 °C for 1 min. After 40 cycles, melting curve analyses was performed to confirm the absence of non-specific amplification. Fluorescence data were obtained at the end of the second step (60 °C for 1 min) in each cycle. Negative controls (no template control and minus RT control (only with RNA)) were included in the assays to detect nonspecific amplification. The amplicons were sequenced to confirm their identity. Results shown are representative of three separate experiments. Statistical analyses were performed using ANOVA and Student's t test available in Sigma Plot software (SPSS Inc., Chicago, IL, USA). Values shown are mean ± SD.

Immunoblotting: Total protein from LPS-treated (1 µg/ml) tissues was harvested in freshly prepared radioimmunoprecipitation assay buffer followed by homogenization and centrifugation at 10,000 rpm. The supernatant was collected and the concentration of the total protein was determined by the Lowry method using bovine serum albumin as a standard. One hundred micrograms of each protein sample was separated on a 10 % SDS-PAGE. After transfer to a nitrocellulose

membrane and blocking with 5 % skimmed milk in TBS-T, the membrane was incubated with specific antibodies (Cell Signalling Technology, Danvers, USA) directed against rat phosphorylated P65, phosphorylated IkBα, histone H3 (trimethyl K36), and HDAC1 for 3 h at room temperature. The membrane was then washed and incubated with secondary antibody against rabbit IgG conjugated with alkaline phosphatase or horse radish peroxidase (Cell Signaling Technology).

Chromatin immunoprecipitation: Caput tissue treated with LPS (1 μg/ml) was washed and homogenized in 1 ml PBS, followed by incubation with formaldehyde (1 % final concentration) at room temperature for 10 min. The fixation reaction was stopped by adding 10 ml glycine and centrifuged for 3 min at 3,000 rpm. Samples were lysed for 30 min in lysis buffer on ice, and the chromatin was sheared by sonicating 20 times for 40 s each at maximum power with an ultrasonic processor. Cross-linked and released chromatin fractions were immunoprecipitated with agarose beads coupled to histone H3 (trimethyl K36) antibodies on a rolling shaker overnight at 4°C. Cross-linking of the immunoprecipitates containing fragmented DNA was chemically reversed, and polymerase chain reaction (PCR) was performed using primers (Table 2.1) specific to the upstream region of *Spag11e*. The PCR amplicons were analyzed on a 2 % agarose gel. For quantification, real-time PCR analyses were performed. The identity of the PCR product was confirmed by sequencing. Input control was performed by amplifying the target sequence from the total pool of DNA before immunoprecipitation. A negative control (antibody against rat IgG; Cell Signaling Technology) was also included in the immunoprecipitation. Data are representative of three independent experiments.

DNA methylation analyses: Using the standard bioinformatics tools, Emboss 6.3.1, MethPrimer (http://www.urogene.org/methprimer/), the CG rich regions and methylation primers in the *Spag11e* upstream sequence were identified. The methylation status of the region corresponding to 350 base pairs on either side of the transcription start site of *Spag11e* gene was analyzed using standard protocol described earlier (Rosenfeld *et al*. 2009). Following LPS treatment (1 µg/ml), genomic DNA was isolated from the caput tissue and digested with XhoI and HindIII (Fermentas Life Sciences, Opelstrasse, Germany) and boiled for 10 min to allow denaturation. The DNA sample was then mixed with two volumes of low melting point agarose (Sigma Aldrich) and incubated at 50°C. Ten microliters of DNA-agarose was mixed with bisulfite modification solution (1.9 g sodium bisulfite dissolved in 2.5 ml water and 750 µl of 2 M NaOH) and hydroquinone solution (0.55 g dissolved in 500 µl water). The mixture was overlaid with mineral oil and incubated for 3.5 h at 50 °C in the dark. The beads were then washed with 1×TE (pH8.0), 0.2 M NaOH, and water (four times each at room temperature) and air-dried. Using methylation-specific primers (Table 2.1), the region corresponding to 350 base pairs on either side of the transcription start site of *Spag11e* gene was PCR-amplified and the amplicons were purified, cloned into T-overhang vector and sequenced. To determine whether the methylation pattern analyzed in the caput is comparable to another organ, as a control, the methylation pattern was also analyzed in the testis.

***In silico* analysis:** The *Spag11e* gene upstream sequence (1 kb) was obtained from rat genome and the putative transcription factor binding sites were predicted using the Transcription Element Search System database (TESS), MatInspector and TRANSFAC Version 2.2 and by inspection for DNA sequences reported in other defensin genes.

Site-Directed Mutagenesis: *Spag11e* gene upstream sequence (1 kb) from the transcriptional start site was cloned in pGL3 basic firefly luciferase vector between the restriction sites *XhoI* and *HindIII* sites such that the expression of luciferase is under the control of the cloned sequence. Point mutations were generated into one of the transcription factor binding sites of the *Spag11e* promoter using QuickChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA) according to the manufacturer's instructions. Briefly, two synthetic oligonucleotide primers (Table 2.1), each complementary to opposite strands of the vector, were designed for the desired mutation. Incorporation of the primers into the deletant was performed by PCR using PfuTurbo DNA polymerase (Stratagene) according to the following conditions: denaturing at 95^0C for 30 s, and 18 cycles of denaturing at 95^0C for 30 s, annealing at 60^0C for 1 min, and elongation at 68^0C for 8 min. After the temperature cycling, the product was treated with DpnI, which digested the parental DNA template and selected for the synthesized DNA containing mutations. The plasmid DNA of each mutant was prepared using the Plasmid Midi Kit (Qiagen) and used for transfections.

Cell culture, transfection, and reporter assay: Human embryonic kidney 293 (HEK 293) were obtained from National Centre for Cell Sciences (Pune, India). They were grown in Dulbeco modified Eagle medium (DMEM) supplemented with 10% fetal bovine, 2 mM L-glutamine, 20 U/ml pencillin, 20 µg/ml streptomycin and 50 ng/ml amphothericin B in 5% CO2 at 37°C. The cells were transfected in 24-well plates using lipofectamine reagent (Invitrogen) with the following plasmids: 800 ng of deletion construct / mutant of *Spag11e* upstream sequence cloned into pGL3-basic (firefly luciferase) vector, and 40 ng/well pRL-TK (Renilla luciferase) vector (Promega) was employed as an internal control. The confluency of the cell lines was maintained at 90% at the time of transfection. After 5 hrs of transfection, cells were treated with 1µg/ml LPS for 24hrs. Cells were washed in PBS 24 h after LPS treatment and lysed in 100 µl luciferase lysis buffer. Luciferase reporter activity was determined with the dual-luciferase reporter assay system according to the manufacturer's instructions. Light intensity was measured using Turner Design 20/20 luminometer (Promega).

Electrophoretic gel-mobility shift assay: Preparation of nuclear extract from epididymal tissue and gel-mobility shift assay was performed as described previously (Watanabe *et al*., 1999; Yoshiura *et al*., 2003; Wang *et al*., 2007) with few modifications. Briefly, 10-50 µg of nuclear extract was incubated with 15 f mol of ATP, [γ-^{32}P] end labelled double stranded wild or mutated androgen receptor binding site and NF-kB binding site oligonucleotides (Table 2.1) in the presence of 0.5 µg of poly dI:dC in binding buffer (20 mM HEPES, pH 7.9, 0.5 mM EDTA, 0.4 mM DTT and 5% glycerol) for 30 min at 37 °C. The DNA-protein complex formed was separated from free oligonucleotide on a 6% native polyacrylamide gel.

RESULTS

LPS-mediated TLR signalling

In order to determine whether the antimicrobial gene induction by LPS in the male reproductive tract tissues is mediated by the NF-kB signaling pathway, we analyzed the phosphorylation of p65 and IkBα. We observed significantly increased levels of phosphorylated p65 and IkBα within 15 min after the addition of LPS both in the epididymis and testis (Figure 2.1), suggesting that NF-kB translocation into the nucleus could be the signalling mechanism that operates during LPS-induced antimicrobial gene expression in the male reproductive tract.

To lend further support for the results explained above, antimicrobial gene expression was analyzed in the male reproductive tract tissues that were pretreated with pyrrolidine dithiocarbamate (PDTC), an inhibitor of NF-kB activation, and Bay-11, an inhibitor of IkBα phosphorylation. We observed that LPS-induced β-defensin gene expression is inhibited in the caput (Figure 2.2A), pretreated with PDTC. In the cauda pretreated with PDTC, there was no significant increase in LPS-induced β-defensin gene expression till the experimental period for all the genes analysed (Figure 2.2B). Defensin expression in the testis pretreated with PDTC was also analyzed. No significant increase in LPS-induced expression of *Defb1*, *Defb21*, *Defb24*, and *Defb27* was observed. *Defb2* expression in the testis seems to be unaffected by NF-kB inhibitor treatment (Figure 2.2C).

LPS-induced *Spag11* expression seems to be generally repressed in the male reproductive tract tissues pretreated with PDTC. In the caput, *Spag11e* was suppressed at all time points tested, whereas *Spag11c* and *Spag11t* expression was

suppressed at 6 and 9 h after LPS stimulation (Figure 2.3A). In the cauda, pretreatment with PDTC suppressed LPS induced *Spag11* gene expression at all the time points tested (Figure 2.3B). In the testis, *Spag11c* expression was inhibited only at 6 and 9 h time points, whereas *Spag11t* was suppressed at all the time points (Figure 2.3C).

To confirm whether phosphorylation of IkBα is a key event controlling LPS-induced antimicrobial gene expression in the male reproductive tract, tissues were pretreated with Bay11 for 1 h and challenged with LPS. The effect of Bay11 on LPS-induced defensin expression was similar to that observed with PDTC. In the caput, cauda, and testis pretreated with Bay11, suppressed defensin gene expression in response to LPS was observed (Figure 2.4).

Spag11c and *Spag11e* gene expression in the caput pretreated with Bay11 was found to be suppressed at all the time points tested after LPS challenge, whereas expression of *Spag11t* was suppressed at 6 and 9 h time points (Figure 2.5A). In the cauda, *Spag11e* and *Spag11t* expression was suppressed at all the time points tested during LPS challenge, whereas *Spag11c* expression was found to be suppressed only at 3 and 6 h time points (Figure 2.5B). LPS-induced *Spag11* gene expression in the testis pretreated with Bay11 was suppressed up to 6 h, after which there was a significant increase (Figure 2.5C).

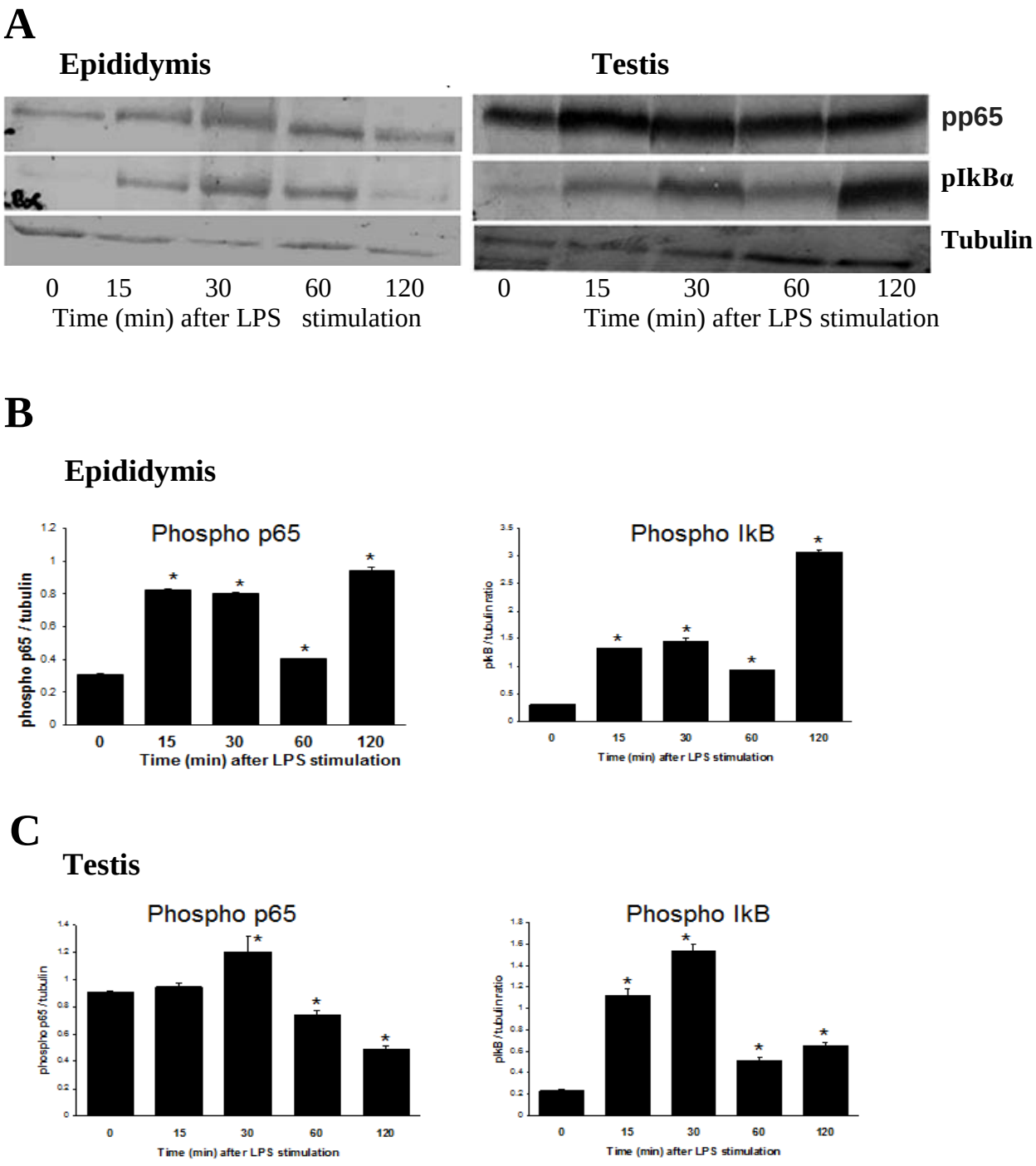

Figure 2.1: NF-kB activation in the male reproductive tract during LPS challenge. [A] Caput and testis obtained from adult Wistar rats were cultured in nutritive medium and challenged with 1 µg/ml LPS for 0–9 h. Tissues were then homogenized and the lysate was separated on SDS-PAGE and Western blotting performed using monoclonal antibodies specific to phosphoP65 and phosphoIkBα. β-Tubulin was used as the internal control.**[B, C]** Densitometric analyses for the Western blots shown to determine phospho P65 and phospho IkBα protein expression in the epididymis **[B]** and testis **[C]** after LPS stimulation. Values shown are mean ± S.D. *$p<0.05$ compared to 0 h control.

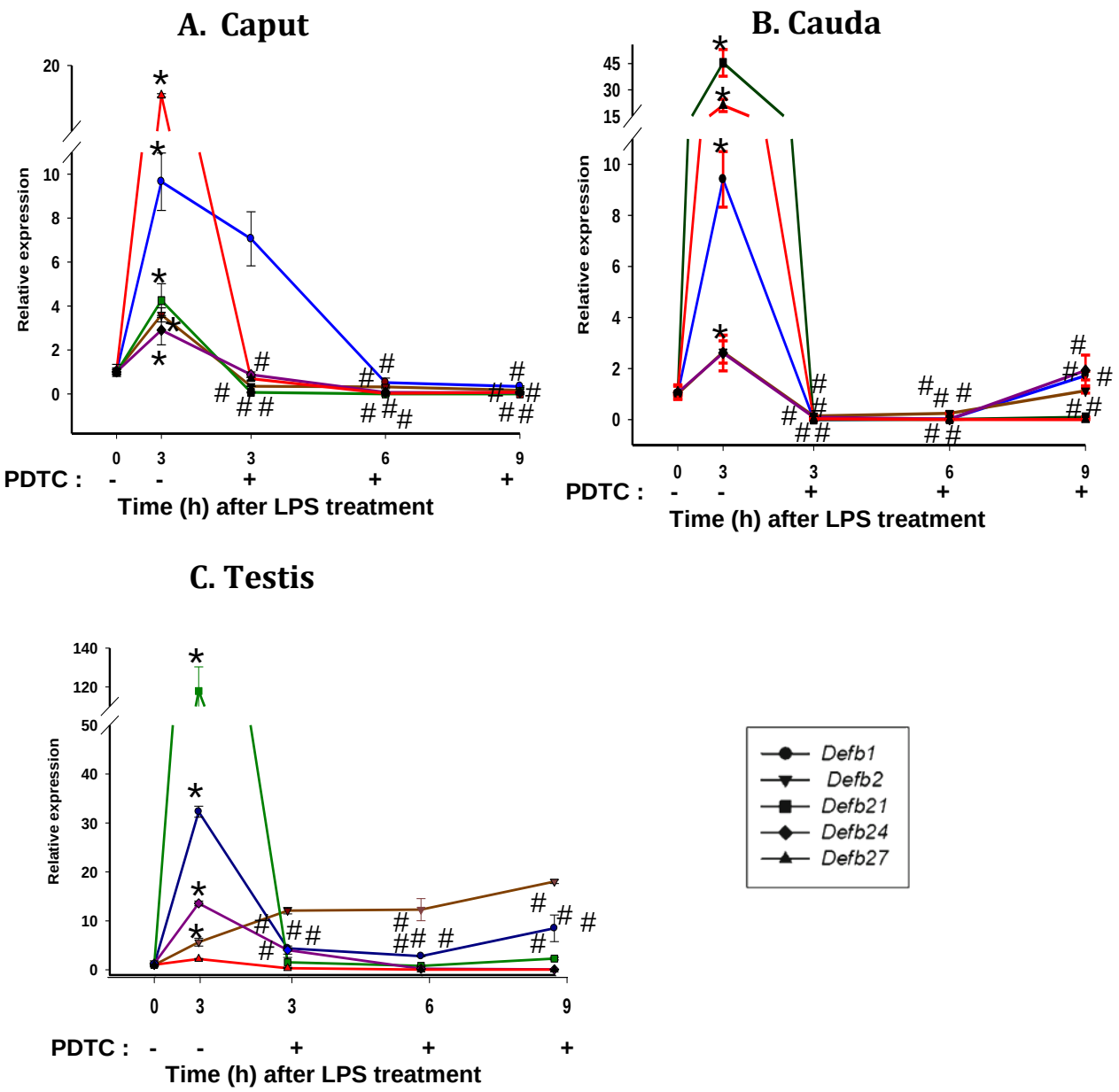

Figure 2.2: Effect of PDTC on LPS-induced defensin gene expression. Caput, cauda, and testis obtained from adult Wistar rats were pretreated with 100 µM PDTC for 1 h. The tissues were then challenged with 1 µg/ml LPS for 0–9 h and defensin expression analyzed using realtime PCR. Values shown are mean ± SD. *p<0.05 compared to 0 h (untreated control). #p<0.05 compared to LPS alone treated.

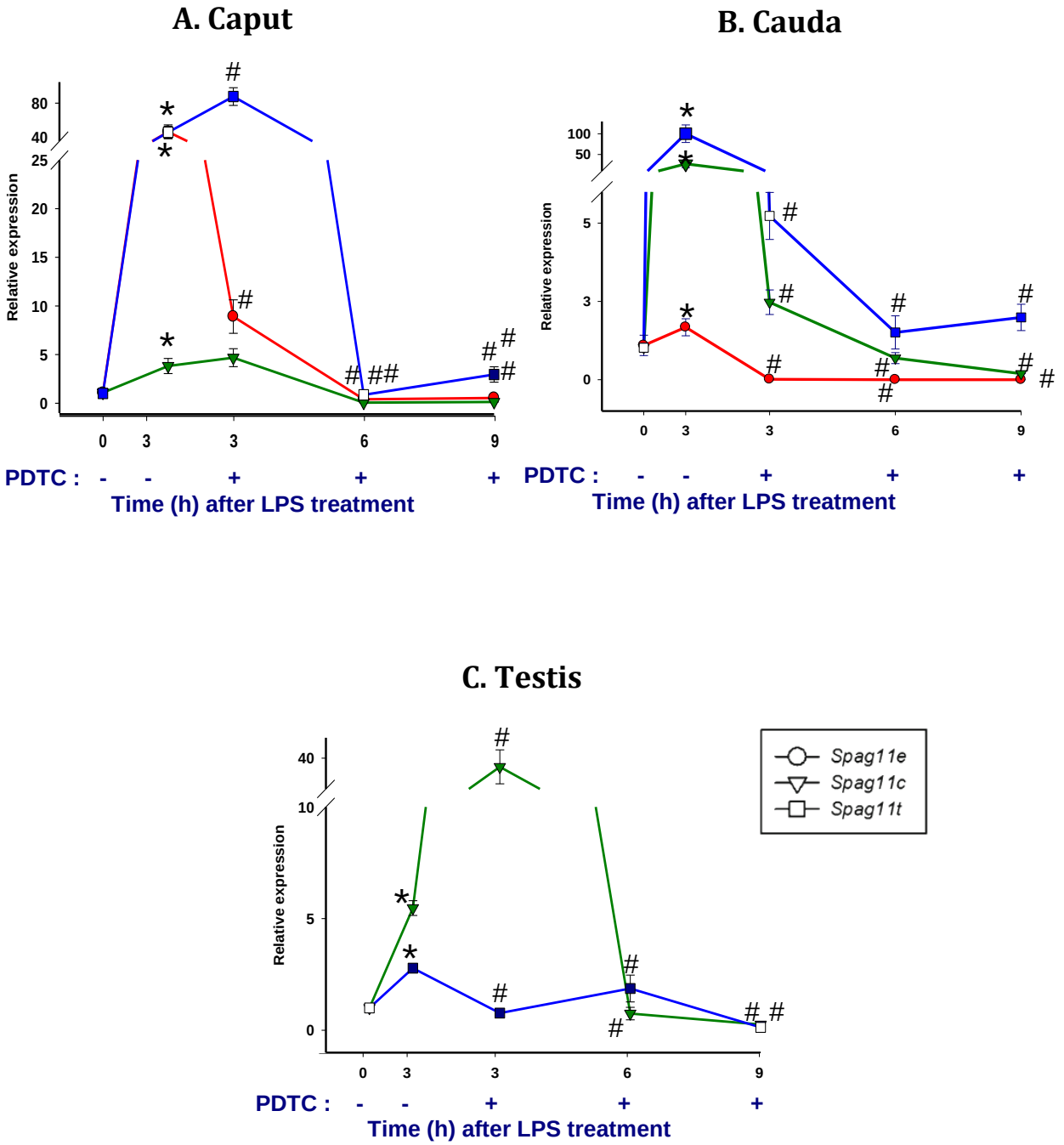

Figure 2.3: Effect of NF-kB inhibitor on LPS induced *Spag11* gene expression. Caput, cauda, and testis obtained from adult Wistar rats were pretreated with 100 µM PDTC for 1 h. The tissues were then challenged with 1 µg/ml LPS for 0–9 h and *Spag11* expression analyzed using real-time PCR. Values shown are mean ± SE. *p<0.05 compared to 0 h (untreated control). #p<0.05 compared to LPS alone treated.

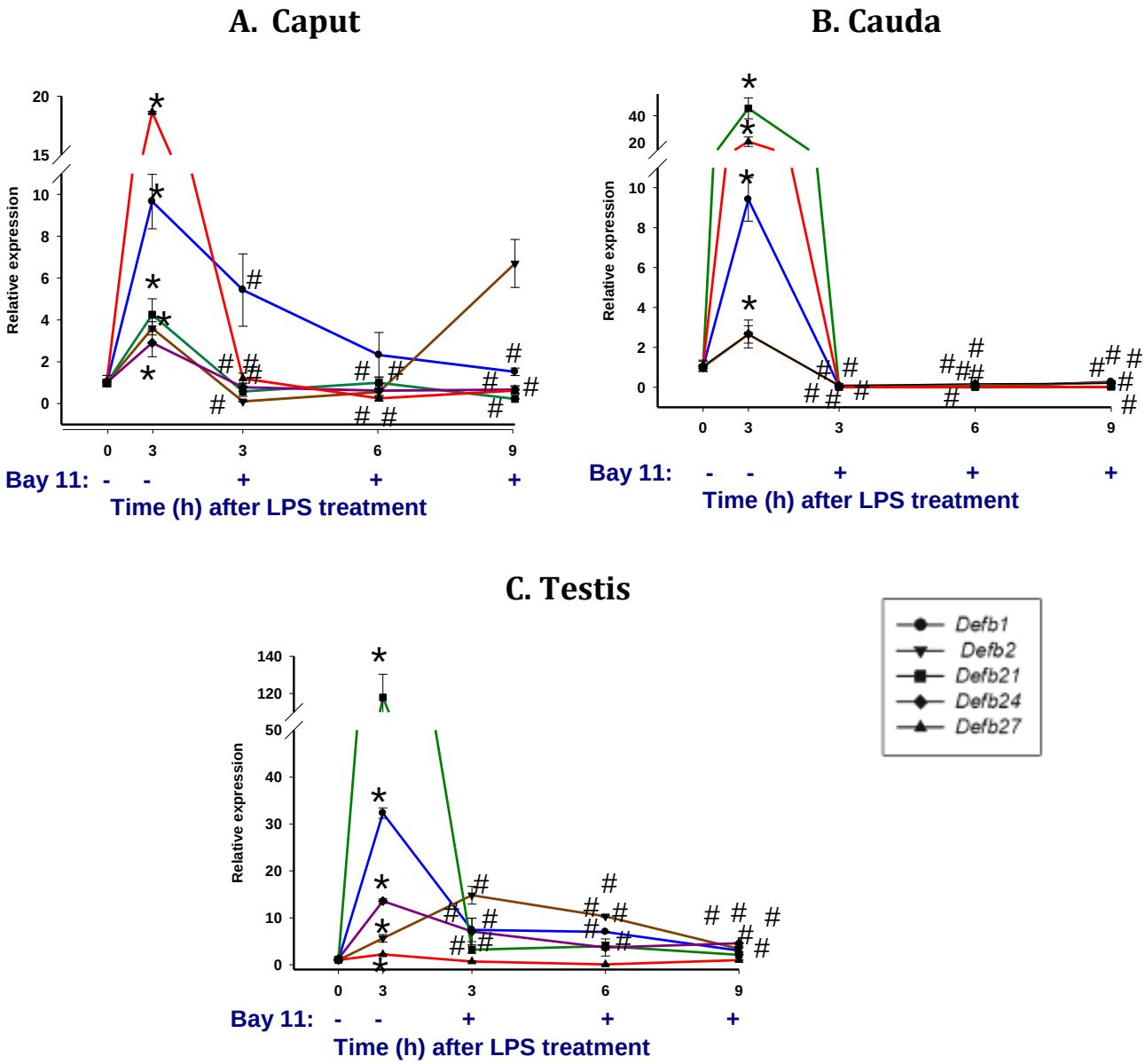

Figure 2.4: Defensin gene expression in Bay11-pretreated male reproductive tissues and challenged with LPS. Real-time PCR analyses for defensins were performed using RNA isolated from caput, cauda, and testis that were pretreated with 100 μM Bay11 for 1 h and followed by 1 μg/ml LPS for 0–9 h. Values shown are mean ± SD. *p<0.05 compared to 0 h (untreated control). #p<0.05 compared to LPS alone treated.

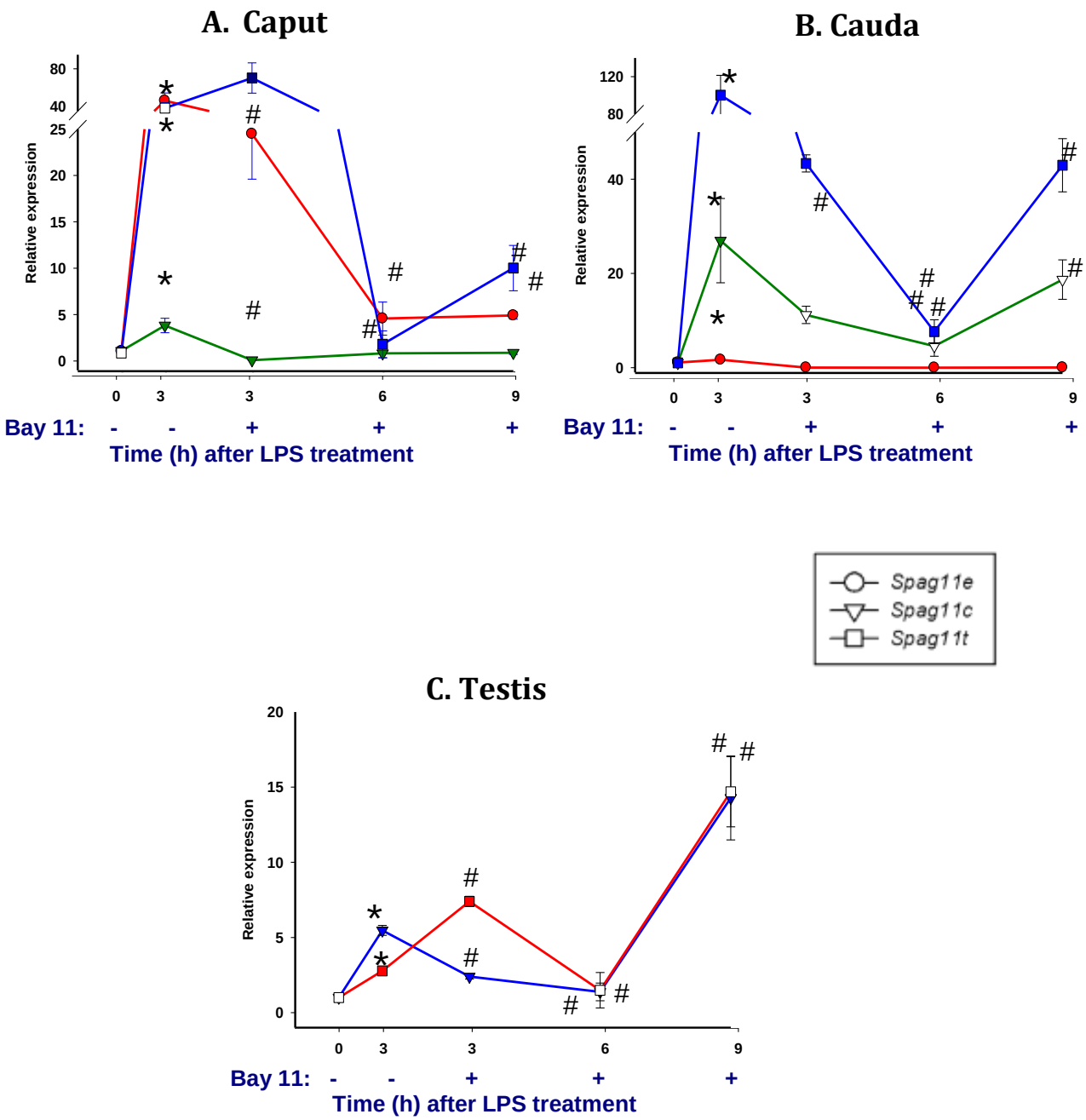

Figure 2.5: *Spag11* gene expression in Bay11-pretreated male reproductive tissues and challenged with LPS. Real-time PCR analyses for defensins were performed using RNA isolated from caput, cauda, and testis that were pretreated with 100 µM Bay11 for 1 h and followed by 1 µg/ml LPS for 0–9 h. Values shown are mean ± SD. *p<0.05 compared to 0 h (untreated control). #p<0.05 compared to LPS alone treated.

LPS-mediated Epigenetic Regulation

Histone Deacetylase:

Gene expression in normal and stress conditions is influenced by a host of epigenetic changes, such as histone modification (acetylation/deacetylation and methylation) and DNA methylation. The possibility that histone modifications could be one of epigenetic changes occurring during LPS-induced defensin and Spag11 expression is unknown. In the epididymis (caput) and testis challenged with LPS *in vitro*, histone deacetylase 1 protein expression decreased significantly (Figure 2.6), suggesting that reduced levels of histone deacetylases could allow histone acetylation thereby allowing increased defensin and *Spag11* gene expression. To further confirm the role of histone deacetylases, defensin and *Spag11* gene expression was analyzed in the epididymis (caput) and testis pretreated with trichostatin A (TSA), a HDAC1 inhibitor. β-Defensin and *Spag11e* gene expression was increased significantly in the epididymis and testis pretreated with TSA when compared to the LPS-treated tissues (Figure 2.7). These results suggest that inhibition of HDAC1 allows transcriptional activation during LPS challenge in the male reproductive tract.

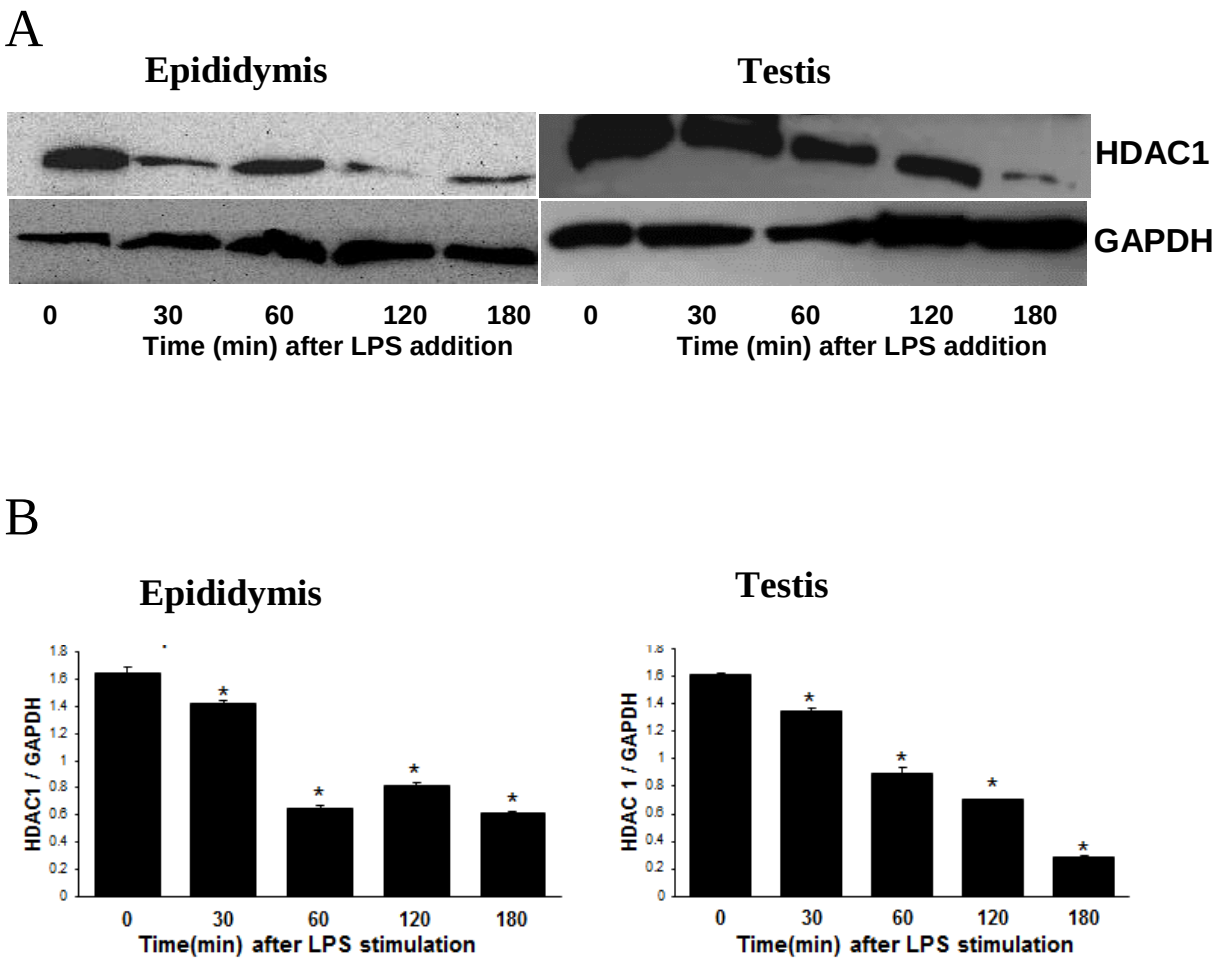

Figure 2.6: Involvement of histone deacetylase 1 (HDAC1) during LPS induced antimicrobial gene expression in the male reproductive tract. [A] Caput and testis obtained from adult Wistar rats were cultured in nutritive medium and challenged with 1 µg/ml LPS for 0–180 min. Tissues were then homogenized and the lysate was separated on SDSPAGE and Western blotting performed using monoclonal antibodies specific to HDAC1. GAPDH was used as the internal control. **[B]** Densitometric analyses for the Western blots shown to determine HDAC1 levels in the epididymis and testis after LPS stimulation. Values shown are mean ± S.D.

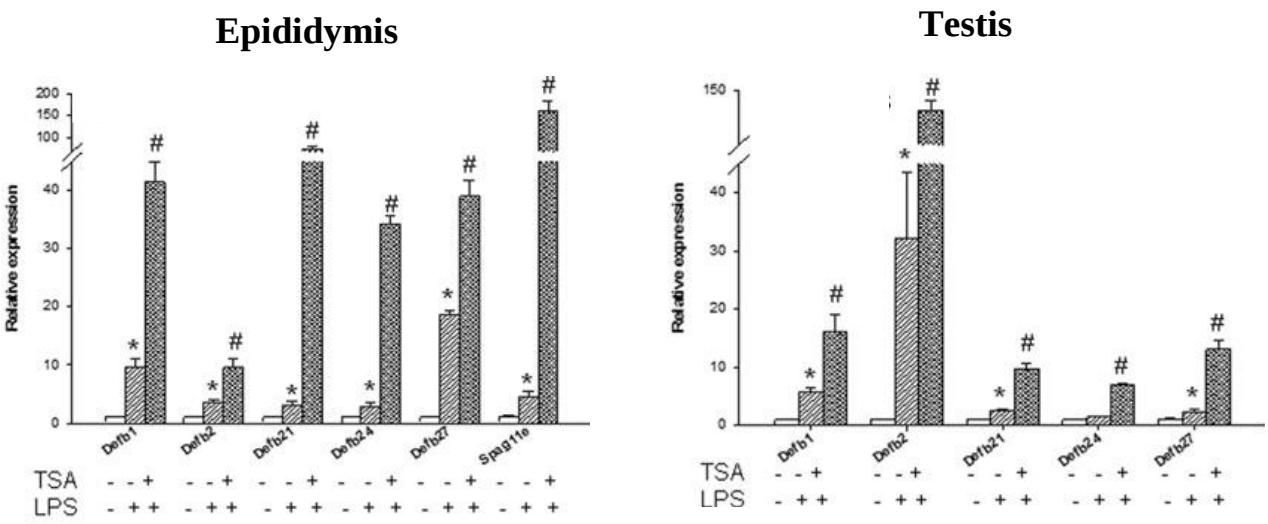

Figure 2.7: Defensin and *Spag11* gene expression after HDAC inhibition. Caput and testis tissues were pretreated with 50 µM trichostatin A followed by 1 µg/ml LPS challenge for 6 h. Real time PCR carried out to analyse defensin and *Spag11* expression. Values shown are mean ± SD. *p<0.05 compared to 0 h (untreated control). # p<0.05 compared to LPS alone treated.

Histone Methylation:

Methylation of histone 3 at lysine 36 (H3K36) is known to correlate with transcriptional activation. To study whether antimicrobial gene induction by LPS in the epididymis and testis involves the methylation of histones, we determined the levels of methylated H3K36 using specific antibodies. Methylated H3K36 protein levels were significantly increased up to 60 and 120 min in the epididymis and testis treated with LPS (Figure 2.8 A). However, a decrease at later time points was observed. To provide further evidence that H3K36 methylation occurs in the upstream region of the antimicrobial genes induced by LPS, we employed chromatin immunoprecipitation to determine methylated H3K36 in the promoter region of antimicrobial gene (*Spag11e*). This gene was analyzed, since it is one of the best characterized among the rat *Spag11* isoforms. Further, we used caput samples only since *Spag11e* is specific to this tissue. Increased methylated H3K36 protein was observed in the LPS-treated caput (Figure 2.8C). The same was also quantified by real-time PCR analyses (Figure 2.8D). These results suggest that LPS-induced antimicrobial gene expression in the male reproductive tract tissues involves epigenetic changes.

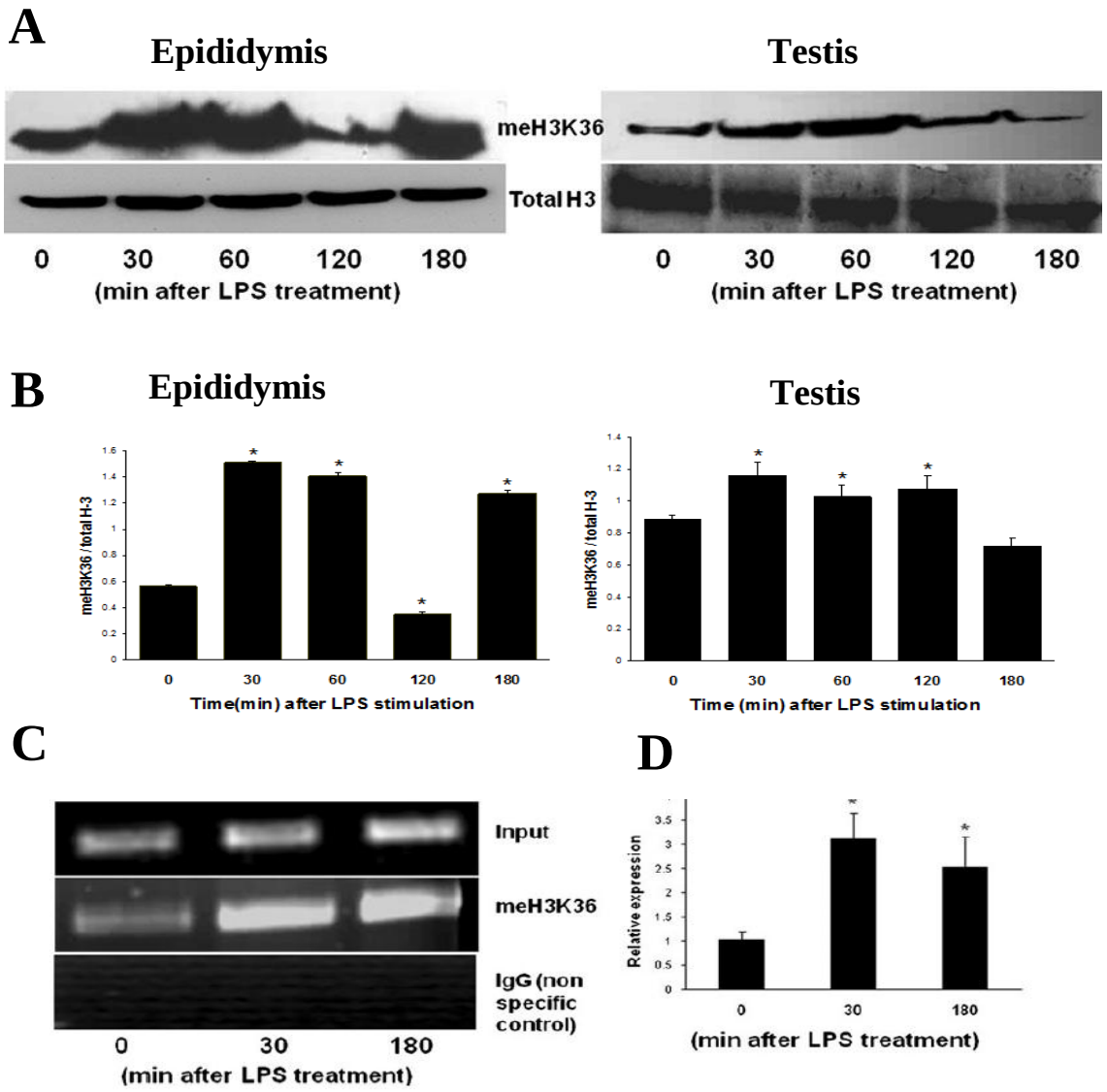

Figure 2.8: Histone methylation in the male reproductive tract tissues during LPS challenge. [A] Caput and testis cultured *in vitro* were treated with 1 µg/ml LPS for 0–180 min, followed by homogenization and separation on SDS-PAGE. The levels of trimethylated histone 3(me3H3K36) were analyzed using monoclonal antibodies by Western blotting. **[B]** Densitometric analyses for the Western blots shown to determine trimethylate histone 3 levels in the epididymis and testis after LPS stimulation. Values shown are mean ± S.D. *p<0.05 compared to 0 min control(untreated control). **[C]** Chromatin immunoprecipitation analyses to determine me3H3K36 levels during LPS challenge in the epididymis. Caput tissue was challenged with 1 µg/ml LPS for 0–180 min, followed by immunoprecipitation using antibodies against rat me3H3K36. Immunoprecipitated DNA was amplified using *Spag11e* upstream region primers and the amplicons run on a 1.5 % agarose gel. **[D]** Real-time PCR analyses to quantify me3H3K36. Using *Spag11e* upstream region primers, DNAwas amplified in a realtime thermal cycler. Values shown are mean ± SD. *p<0.05 compared to 0 min control (untreated control).

DNA methylation:

It is possible that the epigenetic changes observed could be influenced by the activation of NF-kB by LPS or vice versa. Besides histone modifications, cytosine 5′methylation of CpG dinucleotides in the promoter regions or within a gene is an important epigenetic factor in regulating gene expression. It is possible that LPS-induced antimicrobial gene expression in the male reproductive tract tissues may involve CpG modifications. Using bioinformatic tools, we identified two CpG dinucleotides in each of the DNA regions that are 350-bp upstream and downstream of rat *Spag11e* gene transcription start site (Figure 2.9A). Sequencing analyses confirmed the presence of the four predicted CpGs in the DNA isolated from the caput. To determine whether endotoxin challenge could have an effect on *Spag11e* gene, methylation status, CpGs were analyzed in the DNA isolated from the caput tissue challenged with LPS for 0–9 h. We demonstrate that the number of methylated CpG dinucleotides decreased in a time-dependent manner (Figure 2.9B), suggesting demethylation of DNA of *Spag11e* gene and thereby allowing transcriptional activation. During LPS challenge, there was a time-dependent reduction in the expression of DNA methyl transferase 1 (*Dnmt1*) in the caput (Figure 2.9C). Inhibition of *Dnmt1* expression could be one of the innate immune defense mechanisms occurring in the male reproductive tract during endotoxin challenge. The role of *Dnmt1* in endotoxin-induced antimicrobial expression was further confirmed by analyzing *Spag11e* expression in caput tissues pretreated with DNMT1 inhibitor, 5'-azacytidine followed by LPS challenge. *Spag11e* expression was found to be significantly higher in the caput subjected to DNMT1 inhibition when compared to the LPS alone treated tissues (Figure 2.9D), lending further evidence

that repression of *Dnmt1* is one of the innate mechanisms to allow antimicrobial gene expression during endotoxin challenge.

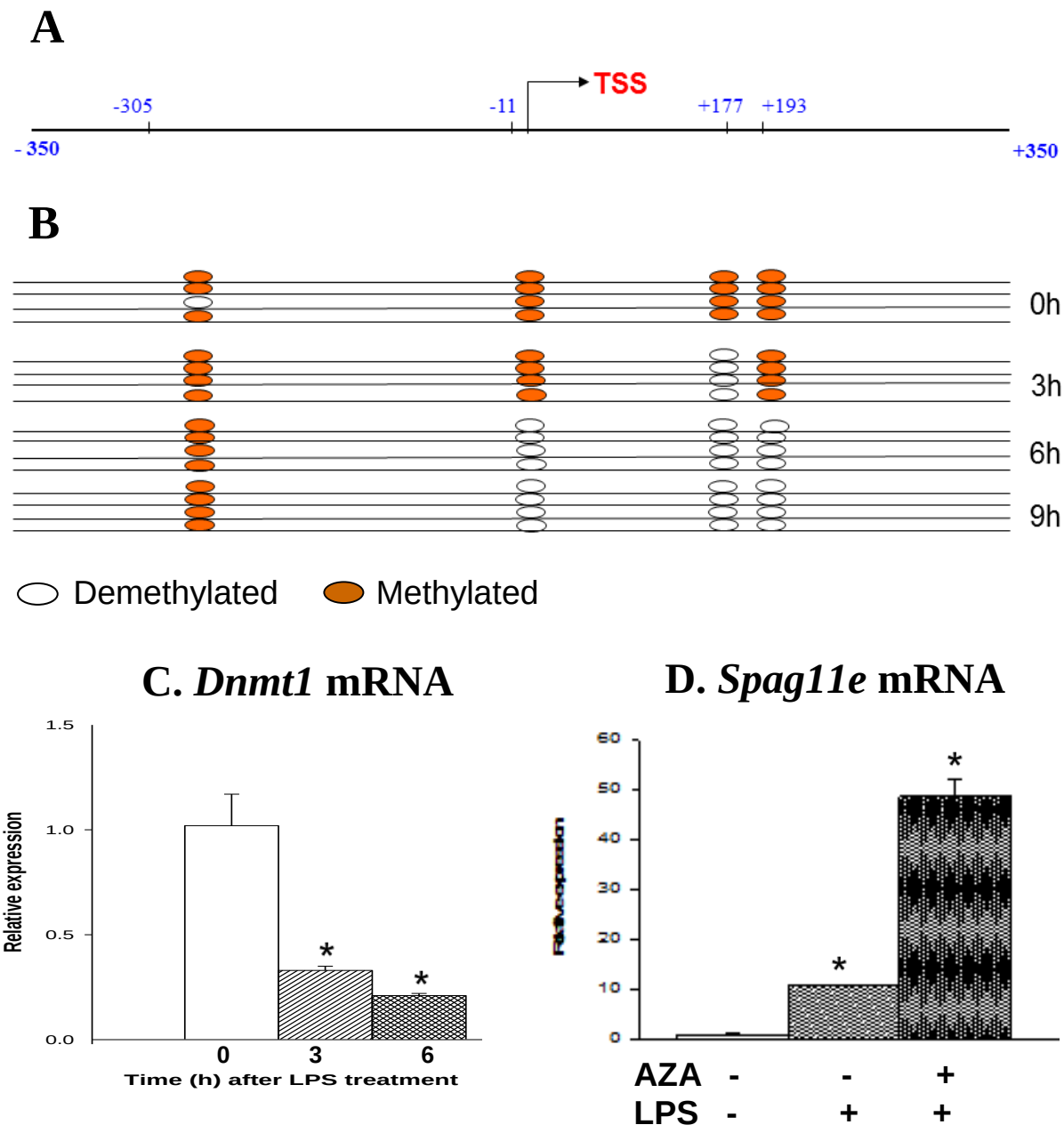

Figure 2.9: Involvement of DNA methylation and DNMT1 during LPS induced antimicrobial gene expression. **[A]** Location of CpG dinucleotides in the *Spag11e* gene. **[B]** Methylation status of the CpG dinucleotides of *Spag11e* gene. Filled oblong indicates methylated and empty oblong indicates unmethylated. Four representative sequencing results are shown. **[C]** Real-time PCR for *Dnmt1* using RNA isolated from the caput tissue treated with 1 µg/ml LPS for 0– 6 h. **[D]** Prior to LPS challenge (3h), rat caput tissues were pretreated with 50 µM 5'-azacytidine and the expression of *Spag11e* was analyzed by real-time PCR. Values shown are mean ± SD. *p<0.05 compared to 0 h (untreated control).

LPS-mediated transcriptional regulation

Spag11e upstream region contains binding sites for transcription factors:

Using specific primers, approximately 1kb of genomic sequence present upstream of *Spag11e* transcription start site was amplified and cloned. Sequence analysis revealed the presence of consensus binding sites for androgen receptor (AR), nuclear factor kappa beta (NF-kB), nuclear factor1 (NF-1), E- twenty six (ETS) and activator protein2 (AP2) and a TATA box (Figure 2.10).

LPS induced gene expression is dependent on AR and NF-kB binding:

Gene expression in the male reproductive tract is androgen dependent. Besides this we have demonstrated that NF-kB phosphorylation and translocation occurs during LPS induced antimicrobial gene expression. To determine whether androgen receptor and Nuclear factor-kappa B are important for antimicrobial gene expression, plasmids were generated that contained mutated binding sites for these two transcription factors. LPS induced *Spag11e* upstream region mediated luciferase activity, whereas the same was not observed in cells transfected with plasmids in which either androgen receptor binding site or NF-kB binding site was mutated (Figure 2.11). These results clearly demonstrate that androgen receptor and NF-kB may play a crucial role in LPS induced *Spag11e* gene expression.

In order to confirm the binding of AR and NF-kB to their binding sites in the upstream region of *Spag11e* during LPS challenge, EMSA were performed. Gel-shift assays were also performed using normal probes designed according to the sequences for AR and NF-kB respectively. AR and NF-kB proteins present in the nuclear extract bound to the radiolabelled oligonucleotides, resulting in the formation of specific band of protein/DNA complex (Figure 2.12).

Figure 2.10: Nucleotide sequence of the 5' flanking region of the rat *Spag11e* gene. The putative transcription start site is depicted by an arrow and the nucleotide sequence is numbered on the left. The potential transcription binding sites are underlined and labeled

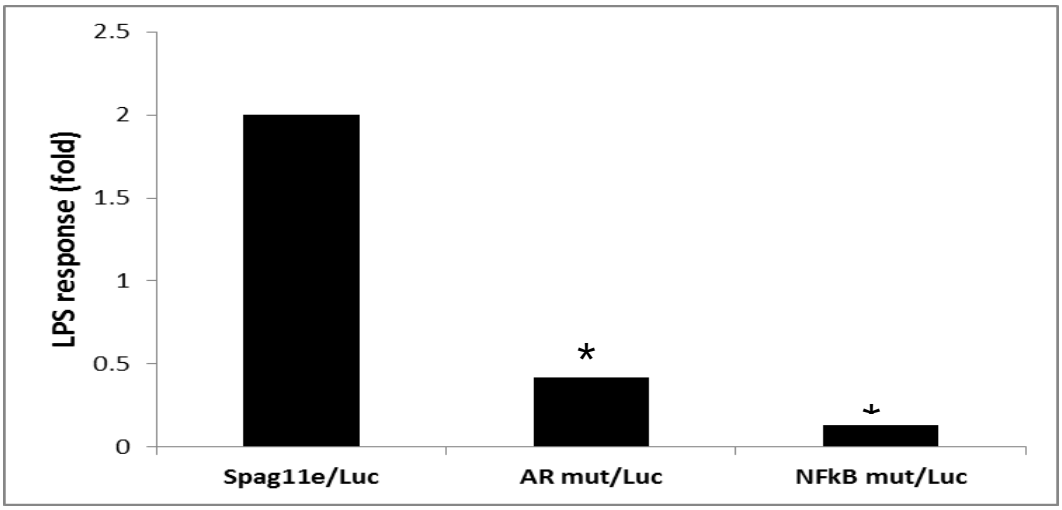

Figure 2.11: Effect of mutagenesis of AR and NF-kB binding sites on *Spag11e* promoter activity in HEK- 293 cells. The wild type or mutant construct was transfected into HEK293 ($5X10^4$), and the cells were incubated for 24 h with or without LPS. The relative luciferase value is indicated as a fold increase in the luciferase activity for each construct relative to that without LPS for the *Spag11e* construct containing point mutations for AR and NF-kB.

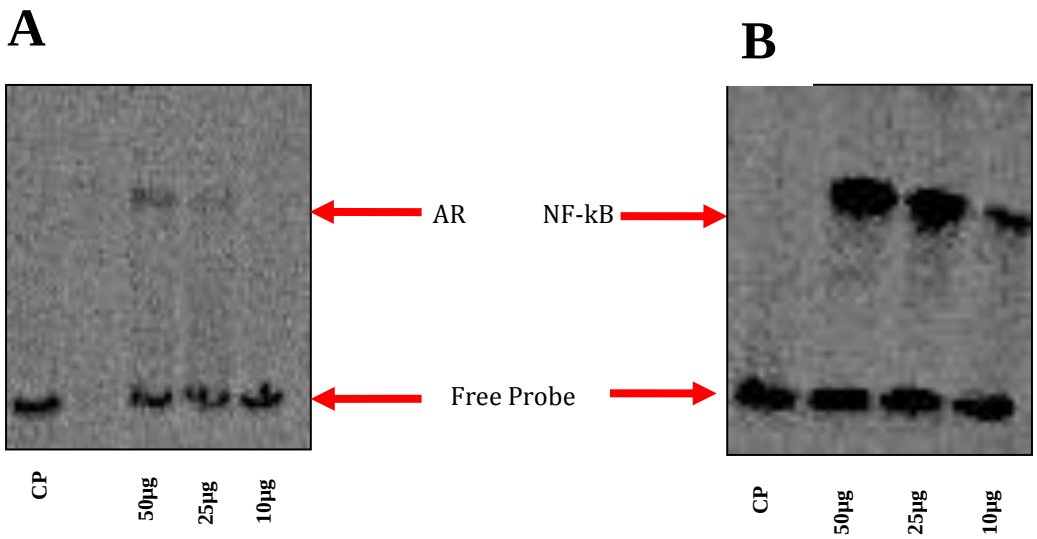

Figure 2.12: Electrophoretic mobility gel shift assay. Gel shift assays showing the binding of **[A] AR, [B] NF-kB** (10- 50 µg of nuclear extract) to the respective oligomers (probe) synthesized from the upstream nucleotide sequence of *Spag11e*. CP-cold probe.

Table 2.1: List of primers used for the study

Primer	Sequence (5'-3')	Purpose
Spag11e F *Spag11e* R	TAGCTCGAGTCTGGGGAATGCCACAATG GCTAAGCTTCCTTCATGTTTTATGGCGCTC	ChIP
Spag11e F *Spag11e* R	GTGTGGTTTTTGGTTATATTTTTTGTGGTG CTATACAAAAAAACAAATATAACTAATAAC	Methylation
AR mut F *AR mut* R	CGTCACGCTCTCCTG**c**T**a**TCGTCGCTGTGTGGG CCCACACAGCGACGATAGCAGGAGAGCGTGACG	Site Directed Mutagenesis
NF-kb mut F *NF-kb mut* R	CAGGTTCACACG**c**GACT**c**TGTTTCTTTGGGCAGG CCTGCCCAAAGAAACAGAGTCGCGTGTGAACCTG	Site Directed Mutagenesis
NF-1 mut F *NF-1 mut* R	CTTTGAA**g**GCC**g**AGGACACATGTTTTATAGAGTTGGG CCCAACTCTATAAAACATGTGTCCTCGGCCTTCAAAG	Site Directed Mutagenesis
AR F *AR* R	CTT CGTCACGCTCTCC**TGTTCTC**GTCGCTGTG CACAGCGACGAGAACAGGAGAGCGTGACGAAG	EMSA
NF-kB F *NF-kB* R	GTTCACAC**GGGACTTT**GTTTCTTTGGGCAGGTT AACCTGCCCAAAGAAACAAAGTCCCGTGTGAAC	EMSA

DISCUSSION

Infections of the male reproductive tract lead to temporary or permanent infertility and are considered as one of the major health problems throughout the world. The general causative agents are *Escherichia coli*, *Neisseria gonorrhea*, and *Staphylococcus aureus*. Epididymitis represents one of the common types of infection. Current treatment regimens for reproductive tract infections include prescription of antibiotics, and over the years, antibiotic resistance has evolved. Strategies to develop alternate candidates to treat reproductive tract infections in place of conventional antibiotics have gained importance. In this study, we attempt to determine the antimicrobial responses in the male reproductive tract tissues challenged with LPS with a long-term goal of identifying the antimicrobial proteins that respond to an infection and develop them as potential candidates to treat reproductive tract infections in place of conventional antibiotics.

We observed that *in vitro* LPS challenge induced the expression of all the β-defensins and *Spag11* genes analyzed in the male reproductive tract tissues of rat. Studies that report the role of β-defensins and *Spag11* genes during endotoxin challenge are limited. LPS-induced β-defensin and *Spag11* gene expression observed in this study is in agreement with our previous study (Biswas and Yenugu 2011) wherein intraperitoneal injection of LPS induces antimicrobial gene expression in the epididymis and testis of rats. The increased LPS demonstrates the robust innate immune mechanisms operating in the male reproductive tract. However, it is also reported that in a rat model of epididymitis induced by LPS, the expression of *Spag11e* and β-defensins in the epididymis is repressed (Cao, Li et al.). This discrepancy could be due to difference in the treatment protocols and the model

systems used. Earlier report suggest the temporal expression pattern of defensin and *Spag11* genes in the male reproductive tract of rat, and they are thought to have a role in sperm maturation and innate immunity (Yenugu, Chintalgattu et al. 2006; Yenugu, Hamil et al. 2006). In this study, though all the defensins and *Spag11* genes analyzed were upregulated in response to LPS, their expression pattern among the tissues seems to vary both in terms of time and abundance. It was demonstrated that LPS disrupts steroidogenesis in the male reproductive tract of rats (Tulassay, Viczian et al. 1970; Wallgren, Kindahl et al. 1993), resulting in altered levels of circulating testosterone (Biswas and Yenugu 2011). Further, gene expression of the epididymides and testis is largely androgen regulated. The variations in defensin and *Spag11* gene observed along the epididymis and testis during endotoxin challenge could be due to alterations in the testosterone levels in the microenvironment.

Signalling mechanisms that operate during LPS exposure is primarily mediated by TLR4 (Peri, Piazza et al.). The presence of TLR4, CD14, and LBP, the major components of LPS recognition on the cell surface, is reported in the male reproductive tract of rat (Palladino, Johnson et al. 2007; Palladino, Savarese et al. 2008). In this study, we observed no significant changes in the expression pattern of *Tlr4* in the epididymides and testis during LPS challenge, suggesting that *Tlr4* expression seems to be constitutive and abundant levels are present to combat endotoxin challenge. *Tlr4* mRNA and protein expression remained unchanged in the epididymides either challenged with LPS *in vitro* or obtained from rats treated intraperitoneally with LPS (Rodrigues, Queiroz et al. 2008).

Increase in pro-inflammatory cytokine production leading to the transcriptional activation of many response genes is one of the hallmarks of

endotoxin-induced cellular responses (McIntyre, Modur et al. 1997). In the mouse testes, the importance of pro-inflammatory cytokines during endotoxin challenge is reported (Abu Elhija, Lunenfeld et al. 2008). Keratinocyte-derived cytokine production in the primary cultures of prostate, seminal vesicle, and epididymis was found to be increased during C. trachomatis infection (Mackern-Oberti, Maccioni et al. 2011). We observed an increase in *Il-6* and *Tnf-α* mRNA expression in the epididymis and testis during LPS challenge, indicating an inflammatory response that could affect epididymal and testicular function. Increased levels of *Il-6* and *Tnf-α* observed in this study could influence the expression of a variety of genes including the defensin and *Spag11* genes analyzed in this study. However, further studies are required to demonstrate whether pro-inflammatory cytokines can modulate the expression of defensin and *Spag11*genes in the male reproductive tract.

Recognition of LPS by TLR4 initiates signaling pathways that involves the activation and translocation of the transcription factor NF-kB. Binding of NF-kB to DNA mediates changes in gene expression following LPS binding to TLRs (Han, Ko et al. 2002). In this study, an increase in antimicrobial gene expression in response to LPS accompanied the phosphorylation of p65 and IkBα both in the epididymis and testis, suggesting that the transcriptional activation could be mediated by NF-kB. Activation of NFkB in the epididymides during LPS challenge in vitro was observed and the same was abrogated in the presence of PDTC (Rodrigues, Queiroz et al. 2008). Though the involvement of NF-kB in LPS-induced effects is reported in the male reproductive tract (Rodrigues, Queiroz et al. 2008), its role in regulating antimicrobial gene expression is not yet studied. We provide further evidence that pretreatment with inhibitors of NF-kB activation (PDTC) and IkBα phosphorylation

(Bay11) resulted in the abrogation of antimicrobial gene expression in response to LPS (Figure 2.2- 2.5). Inhibition of LPS induced gene expression in many organ systems is reported. For example, in the kidney cells, the increased expression of MCP-1 via the NF-kB pathway by LPS was inhibited by PDTC (Zhai, Zhang et al. 2012). Similarly, LPS-induced expression of COX-2 is inhibited by PDTC in macrophage cell line (Zhang, Lei et al. 2010). Sulfasalazine, also an NF-kB inhibitor, suppressed the expression of a variety of cytokines induced by LPS in the gestational membranes (Keelan, Khan et al. 2009).

Epigenetic modifications play a pivotal role in the regulation of gene expression, and until recently, majority of the studies focused on the role of these modifications in developmental biology and cancer progression. Since innate immune mechanisms also involve the regulation of a variety of genes, there has been a growing interest in the recent past on the role of epigenetic modifications in controlling gene expression during immunological responses in different organ systems. In host responses to bacterial infections, selective gene induction by inflammatory signals that activate NF-kB involved histone methylation and deacetylation (Ito 2007). However, studies on the role of epigenetic factors to control immune response-related gene expression in the male reproductive tract are lacking. We report that LPS induced antimicrobial gene expression in the epididymis and testis is epigenetically regulated. The decrease of HDAC1 in the epididymis and testis exposed to LPS suggests an intrinsic mechanism to slow down histone deacetylation thereby allowing gene transcription. Similar observations were made in the gingival cells exposed to oral bacteria (Yin and Chung 2011). In this study, β-defensin and *Spag11* gene expression was significantly higher in epididymis and

testis pretreated with HDAC1 inhibitor (TSA) when compared to LPS alone treated tissues. This increase could be due to the inhibition of the remaining endogenous HDAC1, suggesting an additive effect. However, from this study, it is not clear whether suppression of HDAC1 expression during LPS challenge is directly regulated by the endotoxin itself or whether it involves any other signaling components. Further, the actual acetylation status of histones (H3 and H4) that influence defensin transcription during LPS challenge needs further investigation. Since TSA is a general inhibitor of all HDACs, it is possible that the increased defensin expression during LPS challenge could also be due to inhibition of HDACs other than HDAC1. The role of other HDACs besides HDAC1 in LPS-induced defensin expression is yet to be investigated. Recently, it was demonstrated that sodium butyrate, a HDAC1 inhibitor, stimulates endogenous antimicrobials in the gut (Halili, Andrews et al. 2009). Similarly, treatment with TSA amplified the expression of proatherogenic factors in mouse macrophages in vitro (Halili, Andrews et al. 2010). In this study, since HDAC1 inhibition by TSA resulted in an additive effect on antimicrobial gene expression during LPS challenge, the results reported here may suggest that HDAC1 inhibitors may be used as therapeutic agents to treat reproductive tract infections.

Methylation of histone H3 at K36 (H3K36) is an important event for the activation of transcription. A time dependent increase in the levels of trimethylated histone 3 was observed in the epididymis and testis challenged with LPS, suggesting multiple levels of epigenetic regulation. Such epigenetic regulation was observed for β-defensin 2 in gingival epithelial cells exposed to *Fusobacterium nucleatum*. Further, it is demonstrated that LPS-induced IL-8 and p19 production in human intestinal epithelial cells and mouse bone marrow cells, respectively, is accompanied

by the methylation of histone 3 (Angrisano, Pero et al. ; El Mezayen, El Gazzar et al. 2009). The binding of methylated histones to the promoter regions is an important event in the regulation of target genes. For example, in the murine macrophage cell line, it was shown that LPS induces the binding of H3K4 to the promoter region of TNF-α, an LPS-inducible gene (Ara, Xia et al. 2008). The results of the ChIP analyses suggest that the methylation of histone 3 and its binding to *Spag11e* is an epigenetic regulatory mechanism during the immune response to LPS in the male reproductive tract.

One of the well-known mechanisms that govern gene transcription is the methylation of DNA (Bird and Wolffe 1999). Hypermethylation within or in sequences that flank a gene generally results in gene repression and vice versa. In this study, using *in silico* tools, CpG dinucleotides were identified in the upstream and downstream regions of the transcription site of *Spag11e* gene. Presence of CpG dinucleotides gives a clue that this gene could be epigenetically regulated. We demonstrate demethylation of four CpG dinucleotides in LPS-treated epididymides in a time dependent manner. Loss of DNA methylation favors gene transcription and increase in *Spag11e* expression during LPS challenge could be due to the demethylation observed. This seems to be an innate mechanism to allow enhanced expression of antimicrobial gene expression under infectious conditions. The observed demethylation could be due to inhibition of endogenous DNA methyl transferases (DNMTs). Reduced *Dnmt1* expression during LPS challenge observed in this study could contribute to the demethylated status of *Spag11e* promoter. Increased *Spag11e* expression under conditions where DNMT1 is inhibited with 5'-azacytidine provides ample evidence that inhibition of DNMT1 or reduction in the

transcription of *Dnmt1* could be one of the innate mechanisms that operate in the reproductive tract to allow increased transcription of antimicrobial genes during infections.

In conclusion, we report that LPS induces the expression of β-defensin and *Spag11* gene expression in the epididymal and testicular tissues *in vitro* and involves the activation of NF-kB signaling pathway. Further, induction of antimicrobial gene expression is accompanied by a decrease in HDAC1 and *Dnmt1* expression, increased methylation of histone 3, and its binding to the upstream region of *Spag11e* gene and demethylation of DNA, suggesting an epigenetic regulation in the male reproductive tract tissues under conditions that mimic an infection. The results of this study provide clues to develop novel strategies, such as the use of HDAC1 and DNMT1 inhibitors to induce antimicrobial gene responses and thereby treat reproductive tract infections.

REFERENCES

Abu Elhija, M., E. Lunenfeld, et al. (2008). "LPS Increases the Expression Levels of IL-18, ICE and IL-18 R in Mouse Testes." American Journal of Reproductive Immunology 60(4): 361-371.

Akira, S. (2001). "Toll-like receptors and innate immunity." Adv Immunol 78: 1-56.

Akira, S. and K. Takeda (2004). "Toll-like receptor signalling." Nature Reviews. Immunology 4(7): 499-511.

Angrisano, T., R. Pero, et al. "LPS-induced IL-8 activation in human intestinal epithelial cells is accompanied by specific histone H3 acetylation and methylation changes." BMC Microbiology 10: 172.

Ara, A. I., M. Xia, et al. (2008). "S-adenosylmethionine inhibits lipopolysaccharide-induced gene expression via modulation of histone methylation." Hepatology 47(5): 1655-1666.

Bird, A. P. and A. P. Wolffe (1999). "Methylation-induced repression--belts, braces, and chromatin." Cell 99(5): 451-454.

Biswas, B., G. Narmadha, et al. (2009). "Identification of Toll-like receptors in the rat (Rattus norvegicus): messenger RNA expression in the male reproductive tract under conditions of androgen variation." American Journal of Reproductive Immunology 62(4): 243-252.

Biswas, B. and S. Yenugu (2011). "Antimicrobial responses in the male reproductive tract of lipopolysaccharide challenged rats." American Journal of Reproductive Immunology 65(6): 557-568.

Bourgeon, F., B. Evrard, et al. (2004). "Involvement of semenogelin-derived peptides in the antibacterial activity of human seminal plasma." Biol Reprod 70(3): 768-774.

Cao, D., Y. Li, et al. "Lipopolysaccharide-induced epididymitis disrupts epididymal beta-defensin expression and inhibits sperm motility in rats." Biology of Reproduction 83(6): 1064-1070.

Cao, D., Y. Li, et al. (2010). "Lipopolysaccharide-induced epididymitis disrupts epididymal beta-defensin expression and inhibits sperm motility in rats." Biol Reprod 83(6): 1064-1070.

Chung, W. O. and B. A. Dale (2004). "Innate immune response of oral and foreskin keratinocytes: utilization of different signaling pathways by various bacterial species." Infection and Immunity 72(1): 352-358.

Cornwall, G. A., A. Cameron, et al. (2003). "The cystatin-related epididymal spermatogenic protein inhibits the serine protease prohormone convertase 2." Endocrinology 144(3): 901-908.

El Mezayen, R., M. El Gazzar, et al. (2009). "Aging-dependent upregulation of IL-23p19 gene expression in dendritic cells is associated with differential transcription factor binding and histone modifications." Aging Cell 8(5): 553-565.

Fraczek, M. and M. Kurpisz (2007). "Inflammatory mediators exert toxic effects of oxidative stress on human spermatozoa." Journal of Andrology 28(2): 325-333.

Gatti, G., A. A. Quintar, et al. (2009). "Expression of Toll-like receptor 4 in the prostate gland and its association with the severity of prostate cancer." Prostate 69(13): 1387-1397.

Gatti, G., V. Rivero, et al. (2006). "Prostate epithelial cells can act as early sensors of infection by up-regulating TLR4 expression and proinflammatory mediators upon LPS stimulation." Journal of Leukocyte Biology 79(5): 989-998.

Girling, J. E. and M. P. Hedger (2007). "Toll-like receptors in the gonads and reproductive tract: emerging roles in reproductive physiology and pathology." Immunol Cell Biol 85(6): 481-489.

Halili, M. A., M. R. Andrews, et al. (2010). "Differential effects of selective HDAC inhibitors on macrophage inflammatory responses to the Toll-like receptor 4 agonist LPS." J Leukoc Biol 87(6): 1103-1114.

Halili, M. A., M. R. Andrews, et al. (2009). "Histone deacetylase inhibitors in inflammatory disease." Current Topics in Medicinal Chemistry 9(3): 309-319.

Hamil, K. G., Q. Liu, et al. (2003). "LCN6, a novel human epididymal lipocalin." Reprod Biol Endocrinol 1(1): 112.

Hamil, K. G., Q. Liu, et al. (2002). "Cystatin 11: a new member of the cystatin type 2 family." Endocrinology 143(7): 2787-2796.

Hamil, K. G., P. Sivashanmugam, et al. (2000). "HE2beta and HE2gamma, new members of an epididymis-specific family of androgen-regulated proteins in the human." Endocrinology 141(3): 1245-1253.

Han, S. J., H. M. Ko, et al. (2002). "Molecular mechanisms for lipopolysaccharide-induced biphasic activation of nuclear factor-kappa B (NF-kappa B)." J Biol Chem 277(47): 44715-44721.

Ito, K. (2007). "Impact of post-translational modifications of proteins on the inflammatory process." Biochemical Society Transactions 35(Pt 2): 281-283.

Keelan, J. A., S. Khan, et al. (2009). "Prevention of inflammatory activation of human gestational membranes in an ex vivo model using a pharmacological NF-kappaB inhibitor." Journal of Immunology 183(8): 5270-5278.

Kouzarides, T. (2007). "Chromatin modifications and their function." Cell 128(4): 693-705.

Li, P., H. C. Chan, et al. (2001). "An antimicrobial peptide gene found in the male reproductive system of rats." Science 291(5509): 1783-1785.

Liao, M., P. S. Ruddock, et al. (2005). "Cationic peptide of the male reproductive tract, HE2{alpha}, displays antimicrobial activity against *Neisseria gonorrhoeae*, *Staphylococcus aureus* and *Enterococcus faecalis*." J Antimicrob Chemother 56(5): 957-961.

Mackern-Oberti, J. P., M. Maccioni, et al. (2011). "Innate immunity in the male genital tract: Chlamydia trachomatis induces keratinocyte-derived chemokine production in prostate, seminal vesicle and epididymis/vas deferens primary cultures." J Med Microbiol 60(Pt 3): 307-316.

Mackern-Oberti, J. P., M. Maccioni, et al. (2006). "Susceptibility of prostate epithelial cells to Chlamydia muridarum infection and their role in innate immunity by recruitment of intracellular Toll-like receptors 4 and 2 and MyD88 to the inclusion." Infection and Immunity 74(12): 6973-6981.

Malm, J., O. Sorensen, et al. (2000). "The human cationic antimicrobial protein (hCAP-18) is expressed in the epithelium of human epididymis, is present in seminal plasma at high concentrations, and is attached to spermatozoa." Infect Immun 68(7): 4297-4302.

McIntyre, T. M., V. Modur, et al. (1997). "Molecular mechanisms of early inflammation." Thrombosis and Haemostasis 78(1): 302-305.

Palladino, M. A., T. A. Johnson, et al. (2007). "Members of the Toll-like receptor family of innate immunity pattern-recognition receptors are abundant in the male rat reproductive tract." Biol Reprod 76(6): 958-964.

Palladino, M. A., M. A. Savarese, et al. (2008). "Localization of Toll-like receptors on epididymal epithelial cells and spermatozoa." Am J Reprod Immunol 60(6): 541-555.

Peri, F., M. Piazza, et al. "Exploring the LPS/TLR4 signal pathway with small molecules." Biochemical Society Transactions 38(5): 1390-1395.

Quintar, A. A., F. D. Roth, et al. (2006). "Toll-like receptor 4 in rat prostate: modulation by testosterone and acute bacterial infection in epithelial and stromal cells." Biol Reprod 75(5): 664-672.

Roach, J. C., G. Glusman, et al. (2005). "The evolution of vertebrate Toll-like receptors." Proc Natl Acad Sci U S A 102(27): 9577-9582.

Rodrigues, A., D. B. Queiroz, et al. (2008). "Activation of toll-like receptor 4 (TLR4) by in vivo and in vitro exposure of rat epididymis to lipopolysaccharide from Escherichia Coli." Biology of Reproduction 79(6): 1135-1147.

Takeda, K. and S. Akira (2004). "TLR signaling pathways." Semin Immunol 16(1): 3-9.

Trojian, T. H., T. S. Lishnak, et al. (2009). "Epididymitis and orchitis: an overview." Am Fam Physician 79(7): 583-587.

Tulassay, Z., M. Viczian, et al. (1970). "Quantitative histological studies on the injury of spermatogenesis induced by endotoxin in rats." Journal of Reproduction and Fertility 22(1): 161-164.

Wallgren, M., H. Kindahl, et al. (1993). "Alterations in testicular function after endotoxin injection in the boar." International Journal of Andrology 16(3): 235-243.

Yenugu, S., V. Chintalgattu, et al. (2006). "Identification, cloning and functional characterization of novel beta-defensins in the rat (Rattus norvegicus)." Reprod Biol Endocrinol 4(1): 1-7.

Yenugu, S., K. G. Hamil, et al. (2003). "Antibacterial properties of the sperm-binding proteins and peptides of human epididymis 2 (HE2) family; salt sensitivity, structural dependence and their interaction with outer and cytoplasmic membranes of *Escherichia coli*." Biochem J 372(Pt 2): 473-483.

Yenugu, S., K. G. Hamil, et al. (2004). "Antimicrobial actions of the human epididymis 2 (HE2) protein isoforms, HE2alpha, HE2beta1 and HE2beta2." Reprod Biol Endocrinol 2(1): 61.

Yenugu, S., K. G. Hamil, et al. (2006). "Antimicrobial actions of human and macaque sperm associated antigen (SPAG)11 isoforms: Influence of the N-terminal peptide." Molecular and Cellular Biochemistry 284(1-2): 25-35.

Yenugu, S., K. G. Hamil, et al. (2006). "Identification, cloning and functional characterization of novel sperm associated antigen 11 (SPAG11) isoforms in the rat." Reprod Biol Endocrinol 4: 23.

Yin, L. and W. O. Chung (2011). "Epigenetic regulation of human beta-defensin 2 and CC chemokine ligand 20 expression in gingival epithelial cells in response to oral bacteria." Mucosal Immunol 4(4): 409-419.

Zhai, J. X., Z. X. Zhang, et al. (2012). "PDTC attenuate LPS-induced kidney injury in systemic lupus erythematosus-prone MRL/lpr mice." Mol Biol Rep 39(6): 6763-6771.

Zhang, J., T. Lei, et al. (2010). "Resistin up-regulates COX-2 expression via TAK1-IKK-NF-kappaB signaling pathway." Inflammation 33(1): 25-33.

INTRODUCTION

The male reproductive tract, a dynamic organ system consisting of extensive duct system with mucosal surfaces, is responsible for the maintenance of fertility and other physiological functions as well. Immature spermatids produced in the testis pass through the epididymis, during which a number of proteins secreted by the mucosal surfaces are added and these proteins contribute to maturation, motility, and fertilizing ability of spermatozoa. Cathelicidins, defensins, protease inhibitors such as cystatins, and members of the Sperm-Associated Antigen 11 (SPAG11) family are some of the proteins that were identified to be added on to the sperm surface (Hamil, Sivashanmugam et al. 2000; Hamil, Liu et al. 2002; Cornwall, Cameron et al. 2003; Hamil, Liu et al. 2003). Besides their role in sperm maturation, the antimicrobial activities of sperm-bound proteins have been demonstrated (Malm, Sorensen et al. 2000; Li, Chan et al. 2001; Hamil, Liu et al. 2002; Bourgeon, Evrard et al. 2004; Liao, Ruddock et al. 2005). For example, SPAG11 proteins and peptides of humans, macaque, bovine and mouse were found to exhibit potent broad spectrum antimicrobial activity and the mechanism of action involves bacterial membrane permeabilization (Yenugu, Hamil et al. 2003; Yenugu, Hamil et al. 2004; Yenugu, Hamil et al. 2006). Further, identification and characterization of novel SPAG11 isoforms and β-defensins in the rat was done (Yenugu, Hamil et al. 2006) and demonstrated their antimicrobial activity. Infections of the male reproductive tract caused by *Escherichia coli*, *Chlamydia trachomatis* and *Neisseria gonorrhea* cause epididymitis, a clinical condition characterized by damage to the epididymal epithelial lining, leading to loss of fertility (Trojian, Lishnak et al. 2009). The male reproductive tract being a sterile organ system has developed (Girling and Hedger 2007) robust defence mechanisms to combat infections. During infection, damage to

spermatozoa by endotoxins occurs primarily because of the generation of free radicals (Fraczek and Kurpisz 2007).

In most of the organ systems, the immune responses that operate to combat infections involve Toll-like receptors (TLRs) and their adaptor proteins (Takeda and Akira 2004). In humans, 11 TLRs (TLR1-11) are identified, whereas in rodents, 13 are reported (Roach, Glusman et al. 2005; Biswas, Narmadha et al. 2009). Among them, TLR4 is shown to be very crucial for the immune responses elicited by Lipopolysaccharide (LPS). TLR4 functions in association with other accessory proteins such as lipopolysaccharide binding protein (LBP), CD14, and MD2 to activate NF-kB and IRF-3 via the Myd88-dependent and Myd88-independent pathways, respectively (Akira and Takeda 2004; Roach, Glusman et al. 2005). The signalling pathways initiated by TLRs induce the transcription of genes such as tumour necrosis factor-alpha (*Tnf-α*), Interleukin-6 (*Il-6)*, *IL-12p40*, interferon-beta (*IFN-β*), chemokines, adhesion molecules, acute phase proteins, antimicrobial peptides, inducible nitric oxide synthase (iNos), and cyclooxygenase 2 (Cox-2) (Akira 2001). Further, the signalling pathways utilized by the pathogens and endotoxins are quite different in inducing the immune responses of epithelial cells (Chung and Dale 2004). TLR4 is constitutively expressed in the male reproductive organs of humans and rodents (Quintar, Roth et al. 2006; Girling and Hedger 2007; Palladino, Johnson et al. 2007) and other accessory gland tissues (Gatti, Rivero et al. 2006; Gatti, Quintar et al. 2009). Further, the TLR-associated molecules MYD88 and CD14 were reported in the prostate (Mackern-Oberti, Maccioni et al. 2006). Activation of TLR4 and NF-kB in the male reproductive tract of rat during LPS challenge has been demonstrated (Rodrigues, Queiroz et al. 2008). However, studies on the role of defensins and

defensin like proteins (SPAG11 isoforms) in protecting the male reproductive tract during endotoxin challenge or infection are limited. Earlier we demonstrated that the expression of defensins and members of the *Spag11* family is elevated during endotoxin challenge in rats (Biswas and Yenugu 2011).

It was also reported that LPS disrupts epididymal β-defensin expression in the male reproductive tract of rats (Cao, Li et al. 2010). However, signalling pathways that govern the regulation of antimicrobial gene expression in the male reproductive tract during endotoxin challenge or infectious conditions have not been previously investigated. Further, it is possible that antimicrobial gene expression in the male reproductive tract could be epigenetically regulated. Epigenetic modifications in general include histone acetylation/ deacetylation, histone methylation/ demethylation, and DNA methylation, which are tightly, controlled by enzymes such as histone deacetylases (HDACs), histone demethylases and DNA methyl transferases (DNMTs). Acetylation of histones including histone 3 and demethylation of DNA in general promote transcriptional activation (Kouzarides 2007). Previous studies have demonstrated the epigenetic regulation of β-defensin 2 expression in gingival epithelial cells in response to oral bacteria (Yin and Chung 2011). Hence, in this study, we attempt to determine whether epigenetic changes would contribute to the innate immune responses of the male reproductive tract. In an *in vitro* culture system, we demonstrate that LPS induces *Spag11* and β-defensin mRNA expression and involves NF-kB activation.

Upon recognition of microorganisms or their products by epithelial cells and macrophages of many organ systems, immune defences include the production of antimicrobial proteins and peptides. Epithelial cells and macrophages of the testis

and epididymis also produce various immune effectors such as cytokines, chemokines, and antimicrobial peptides in response to inflammatory stimuli, and regulate the activation and recruitment of professional phagocytes (neutrophils and macrophages) and immune cells (T cells and dendritic cells). Among the antimicrobial peptides, β defensins play a unique role linking innate and acquired immunities and regulate the activation and recruitment of professional phagocytes (neutrophils and macrophages) and immune cells (T cells and dendritic cells). Recent studies including ours have demonstrated that defensin gene is upregulated during endotoxin challenge. Taken together, it is important to elucidate the molecular mechanisms regulating their expression, especially the role of transcription factors.

A wide variety of transcription factors such as Nuclear factor kappa beta (NF-kB), Activator protein-1 (AP-1), E-twenty six (ETS) etc are known to regulate immune response related gene expression. Recently, the *Spag11e* promoter has been cloned, and found to contain several consensus transcription factor binding sites. The *Spag11e* upstream region between -780 and +27, contains binding sites for androgen receptor (AR), nuclear factor-kB (NF-kB), nuclear factor -1 (NF-1), E-twenty six (ETS) and activator protein 2 (Ap2) binding sites. However, studies that describe the involvement of transcription in regulating β-defensins in the male reproductive tract are lacking. Hence, we analysed the presence of transcription factor binding elements in the *Spag11e* gene upstream sequence and whether the cognate transcription factors do bind to these elements during endotoxin challenge.

APPROACHES

***In vitro* culture and treatments:** Briefly, caput, cauda, and testis from 90-day-old rats were dissected and cut into two longitudinal halves. One half of the tissue was used as control, and the other for LPS (Sigma Aldrich, St. Louis, USA) treatments. Tissues were transferred to 2 ml of freshly prepared nutritive medium containing 136.89 mM NaCl, 5.63 mM KCl, 1.80 mM $CaCl_2$, 0.36 mM NaH_2PO_4, 14.88 mM $NaHCO_3$, and 5.55 mM glucose, pH 7.6-7.8 and cultured at 30°C with aeration. After 15 min of incubation, tissues were transferred to fresh nutritive medium and treated with 1 µg/ml LPS and incubated for different time points (0–9 h for RNA studies) and (0–180 min for protein studies). The LPS dose and treatment periods were chosen based on previous studies (Rodrigues *et al*. 2008). During these incubation periods, nutritive solution without or with LPS was renewed every 30 min. The tissues were collected, rinsed with PBS, frozen in liquid nitrogen, and stored in −80 °C until use. To study the effect of NF-kB inhibitors (Sigma Aldrich) on LPS-mediated antimicrobial gene expression, caput, cauda, and testis tissues were pretreated with either 100 µM PDTC (Pyrollidine dithiocarbamate) or 100 µM Bay11 for 1 h. The tissues were then transferred to fresh wells containing nutritive medium without or with LPS (1 µg/ml) and incubated for 0–9 h. Similarly, caput and testis tissues were pretreated with 100 µM TSA (a histone deacetylase 1 (HDAC1) inhibitor; Sigma Aldrich) or 50 µM 5'-azacytidine (a DNA methyl transferase1 (DNMT1) inhibitor; Sigma Aldrich) for 1 h and challenged with LPS (1 µg/ml) for 6 h to determine the role of HDAC1 and DNMT1 in LPS induced antimicrobial responses. All the inhibitors were dissolved in DMSO, and control tissues were treated with an equivalent amount of DMSO that is present in the volume of the inhibitor added.

Experiments were conducted using the Guidelines for the Care and Use of Laboratory Animals, approved by the Institutional Animal Ethics Committee of the University of Hyderabad.

Real-time RT-PCR: Total RNA (2µg) extracted using the TRIzol reagent (Invitrogen, Carlsbad, USA) was reverse-transcribed using 50 U Stratascript (Invitrogen, USA) and 0.5 µg of oligodT (Invitrogen). Two microliters of the resultant cDNA was used for real-time RT-PCR analysis using Power SYBR Green PCR master mix kit (Applied Biosystems, Warrington, UK) in a real-time thermal cycler (Applied Biosystems). A typical real-time PCR reaction was carried out with the following conditions: initial denaturation at 94 °C for 10 min, 40 cycles with 94 °C for 15 s, and 60 °C for 1 min. After 40 cycles, melting curve analyses was performed to confirm the absence of non-specific amplification. Fluorescence data were obtained at the end of the second step (60 °C for 1 min) in each cycle. Negative controls (no template control and minus RT control (only with RNA)) were included in the assays to detect nonspecific amplification. The amplicons were sequenced to confirm their identity. Results shown are representative of three separate experiments. Statistical analyses were performed using ANOVA and Student's t test available in Sigma Plot software (SPSS Inc., Chicago, IL, USA). Values shown are mean ± SD.

Immunoblotting: Total protein from LPS-treated (1 µg/ml) tissues was harvested in freshly prepared radioimmunoprecipitation assay buffer followed by homogenization and centrifugation at 10,000 rpm. The supernatant was collected and the concentration of the total protein was determined by the Lowry method using bovine serum albumin as a standard. One hundred micrograms of each protein sample was separated on a 10 % SDS-PAGE. After transfer to a nitrocellulose

membrane and blocking with 5 % skimmed milk in TBS-T, the membrane was incubated with specific antibodies (Cell Signalling Technology, Danvers, USA) directed against rat phosphorylated P65, phosphorylated IkBα, histone H3 (trimethyl K36), and HDAC1 for 3 h at room temperature. The membrane was then washed and incubated with secondary antibody against rabbit IgG conjugated with alkaline phosphatase or horse radish peroxidase (Cell Signaling Technology).

Chromatin immunoprecipitation: Caput tissue treated with LPS (1 μg/ml) was washed and homogenized in 1 ml PBS, followed by incubation with formaldehyde (1 % final concentration) at room temperature for 10 min. The fixation reaction was stopped by adding 10 ml glycine and centrifuged for 3 min at 3,000 rpm. Samples were lysed for 30 min in lysis buffer on ice, and the chromatin was sheared by sonicating 20 times for 40 s each at maximum power with an ultrasonic processor. Cross-linked and released chromatin fractions were immunoprecipitated with agarose beads coupled to histone H3 (trimethyl K36) antibodies on a rolling shaker overnight at 4°C. Cross-linking of the immunoprecipitates containing fragmented DNA was chemically reversed, and polymerase chain reaction (PCR) was performed using primers (Table 2.1) specific to the upstream region of *Spag11e*. The PCR amplicons were analyzed on a 2 % agarose gel. For quantification, real-time PCR analyses were performed. The identity of the PCR product was confirmed by sequencing. Input control was performed by amplifying the target sequence from the total pool of DNA before immunoprecipitation. A negative control (antibody against rat IgG; Cell Signaling Technology) was also included in the immunoprecipitation. Data are representative of three independent experiments.

DNA methylation analyses: Using the standard bioinformatics tools, Emboss 6.3.1, MethPrimer (http://www.urogene.org/methprimer/), the CG rich regions and methylation primers in the *Spag11e* upstream sequence were identified. The methylation status of the region corresponding to 350 base pairs on either side of the transcription start site of *Spag11e* gene was analyzed using standard protocol described earlier (Rosenfeld *et al*. 2009). Following LPS treatment (1 µg/ml), genomic DNA was isolated from the caput tissue and digested with XhoI and HindIII (Fermentas Life Sciences, Opelstrasse, Germany) and boiled for 10 min to allow denaturation. The DNA sample was then mixed with two volumes of low melting point agarose (Sigma Aldrich) and incubated at 50°C. Ten microliters of DNA-agarose was mixed with bisulfite modification solution (1.9 g sodium bisulfite dissolved in 2.5 ml water and 750 µl of 2 M NaOH) and hydroquinone solution (0.55 g dissolved in 500 µl water). The mixture was overlaid with mineral oil and incubated for 3.5 h at 50 °C in the dark. The beads were then washed with 1×TE (pH8.0), 0.2 M NaOH, and water (four times each at room temperature) and air-dried. Using methylation-specific primers (Table 2.1), the region corresponding to 350 base pairs on either side of the transcription start site of *Spag11e* gene was PCR-amplified and the amplicons were purified, cloned into T-overhang vector and sequenced. To determine whether the methylation pattern analyzed in the caput is comparable to another organ, as a control, the methylation pattern was also analyzed in the testis.

***In silico* analysis:** The *Spag11e* gene upstream sequence (1 kb) was obtained from rat genome and the putative transcription factor binding sites were predicted using the Transcription Element Search System database (TESS), MatInspector and TRANSFAC Version 2.2 and by inspection for DNA sequences reported in other defensin genes.

Site-Directed Mutagenesis: *Spag11e* gene upstream sequence (1 kb) from the transcriptional start site was cloned in pGL3 basic firefly luciferase vector between the restriction sites *XhoI* and *HindIII* sites such that the expression of luciferase is under the control of the cloned sequence. Point mutations were generated into one of the transcription factor binding sites of the *Spag11e* promoter using QuickChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA) according to the manufacturer's instructions. Briefly, two synthetic oligonucleotide primers (Table 2.1), each complementary to opposite strands of the vector, were designed for the desired mutation. Incorporation of the primers into the deletant was performed by PCR using PfuTurbo DNA polymerase (Stratagene) according to the following conditions: denaturing at 95^0C for 30 s, and 18 cycles of denaturing at 95^0C for 30 s, annealing at 60^0C for 1 min, and elongation at 68^0C for 8 min. After the temperature cycling, the product was treated with DpnI, which digested the parental DNA template and selected for the synthesized DNA containing mutations. The plasmid DNA of each mutant was prepared using the Plasmid Midi Kit (Qiagen) and used for transfections.

Cell culture, transfection, and reporter assay: Human embryonic kidney 293 (HEK 293) were obtained from National Centre for Cell Sciences (Pune, India). They were grown in Dulbeco modified Eagle medium (DMEM) supplemented with 10% fetal bovine, 2 mM L-glutamine, 20 U/ml pencillin, 20 µg/ml streptomycin and 50 ng/ml amphothericin B in 5% CO2 at 37°C. The cells were transfected in 24-well plates using lipofectamine reagent (Invitrogen) with the following plasmids: 800 ng of deletion construct / mutant of *Spag11e* upstream sequence cloned into pGL3-basic (firefly luciferase) vector, and 40 ng/well pRL-TK (Renilla luciferase) vector (Promega) was employed as an internal control. The confluency of the cell lines was maintained at 90% at the time of transfection. After 5 hrs of transfection, cells were treated with 1µg/ml LPS for 24hrs. Cells were washed in PBS 24 h after LPS treatment and lysed in 100 µl luciferase lysis buffer. Luciferase reporter activity was determined with the dual-luciferase reporter assay system according to the manufacturer's instructions. Light intensity was measured using Turner Design 20/20 luminometer (Promega).

Electrophoretic gel-mobility shift assay: Preparation of nuclear extract from epididymal tissue and gel-mobility shift assay was performed as described previously (Watanabe *et al*., 1999; Yoshiura *et al*., 2003; Wang *et al*., 2007) with few modifications. Briefly, 10-50 µg of nuclear extract was incubated with 15 f mol of ATP, [γ-^{32}P] end labelled double stranded wild or mutated androgen receptor binding site and NF-kB binding site oligonucleotides (Table 2.1) in the presence of 0.5 µg of poly dI:dC in binding buffer (20 mM HEPES, pH 7.9, 0.5 mM EDTA, 0.4 mM DTT and 5% glycerol) for 30 min at 37 °C. The DNA-protein complex formed was separated from free oligonucleotide on a 6% native polyacrylamide gel.

RESULTS

LPS-mediated TLR signalling

In order to determine whether the antimicrobial gene induction by LPS in the male reproductive tract tissues is mediated by the NF-kB signaling pathway, we analyzed the phosphorylation of p65 and IkBα. We observed significantly increased levels of phosphorylated p65 and IkBα within 15 min after the addition of LPS both in the epididymis and testis (Figure 2.1), suggesting that NF-kB translocation into the nucleus could be the signalling mechanism that operates during LPS-induced antimicrobial gene expression in the male reproductive tract.

To lend further support for the results explained above, antimicrobial gene expression was analyzed in the male reproductive tract tissues that were pretreated with pyrrolidine dithiocarbamate (PDTC), an inhibitor of NF-kB activation, and Bay-11, an inhibitor of IkBα phosphorylation. We observed that LPS-induced β-defensin gene expression is inhibited in the caput (Figure 2.2A), pretreated with PDTC. In the cauda pretreated with PDTC, there was no significant increase in LPS-induced β-defensin gene expression till the experimental period for all the genes analysed (Figure 2.2B). Defensin expression in the testis pretreated with PDTC was also analyzed. No significant increase in LPS-induced expression of *Defb1*, *Defb21*, *Defb24*, and *Defb27* was observed. *Defb2* expression in the testis seems to be unaffected by NF-kB inhibitor treatment (Figure 2.2C).

LPS-induced *Spag11* expression seems to be generally repressed in the male reproductive tract tissues pretreated with PDTC. In the caput, *Spag11e* was suppressed at all time points tested, whereas *Spag11c* and *Spag11t* expression was

suppressed at 6 and 9 h after LPS stimulation (Figure 2.3A). In the cauda, pretreatment with PDTC suppressed LPS induced *Spag11* gene expression at all the time points tested (Figure 2.3B). In the testis, *Spag11c* expression was inhibited only at 6 and 9 h time points, whereas *Spag11t* was suppressed at all the time points (Figure 2.3C).

To confirm whether phosphorylation of IkBα is a key event controlling LPS-induced antimicrobial gene expression in the male reproductive tract, tissues were pretreated with Bay11 for 1 h and challenged with LPS. The effect of Bay11 on LPS-induced defensin expression was similar to that observed with PDTC. In the caput, cauda, and testis pretreated with Bay11, suppressed defensin gene expression in response to LPS was observed (Figure 2.4).

Spag11c and *Spag11e* gene expression in the caput pretreated with Bay11 was found to be suppressed at all the time points tested after LPS challenge, whereas expression of *Spag11t* was suppressed at 6 and 9 h time points (Figure 2.5A). In the cauda, *Spag11e* and *Spag11t* expression was suppressed at all the time points tested during LPS challenge, whereas *Spag11c* expression was found to be suppressed only at 3 and 6 h time points (Figure 2.5B). LPS-induced *Spag11* gene expression in the testis pretreated with Bay11 was suppressed up to 6 h, after which there was a significant increase (Figure 2.5C).

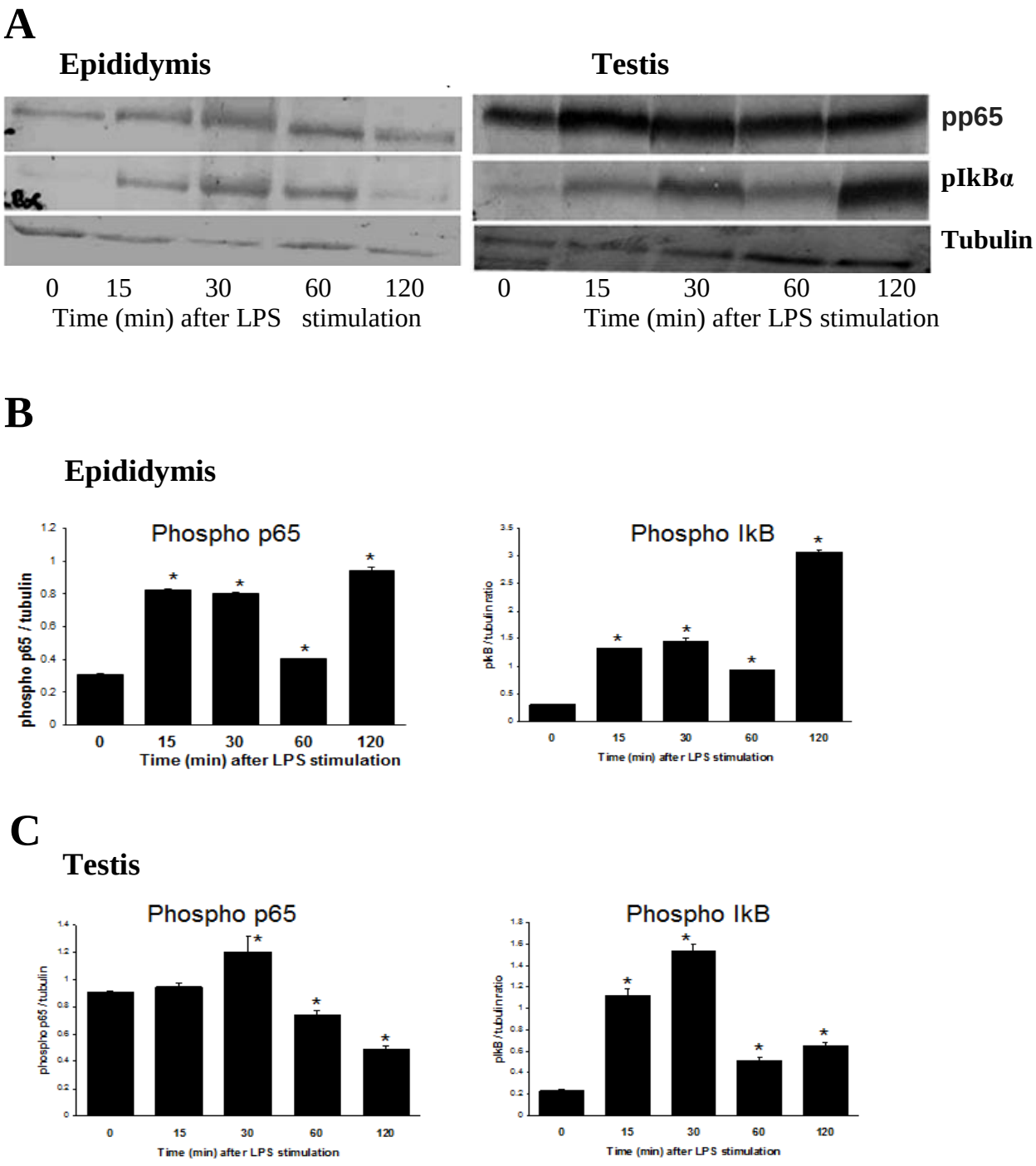

Figure 2.1: NF-kB activation in the male reproductive tract during LPS challenge. [A] Caput and testis obtained from adult Wistar rats were cultured in nutritive medium and challenged with 1 µg/ml LPS for 0–9 h. Tissues were then homogenized and the lysate was separated on SDS-PAGE and Western blotting performed using monoclonal antibodies specific to phosphoP65 and phosphoIkBα. β-Tubulin was used as the internal control.**[B, C]** Densitometric analyses for the Western blots shown to determine phospho P65 and phospho IkBα protein expression in the epididymis **[B]** and testis **[C]** after LPS stimulation. Values shown are mean ± S.D. *$p<0.05$ compared to 0 h control.

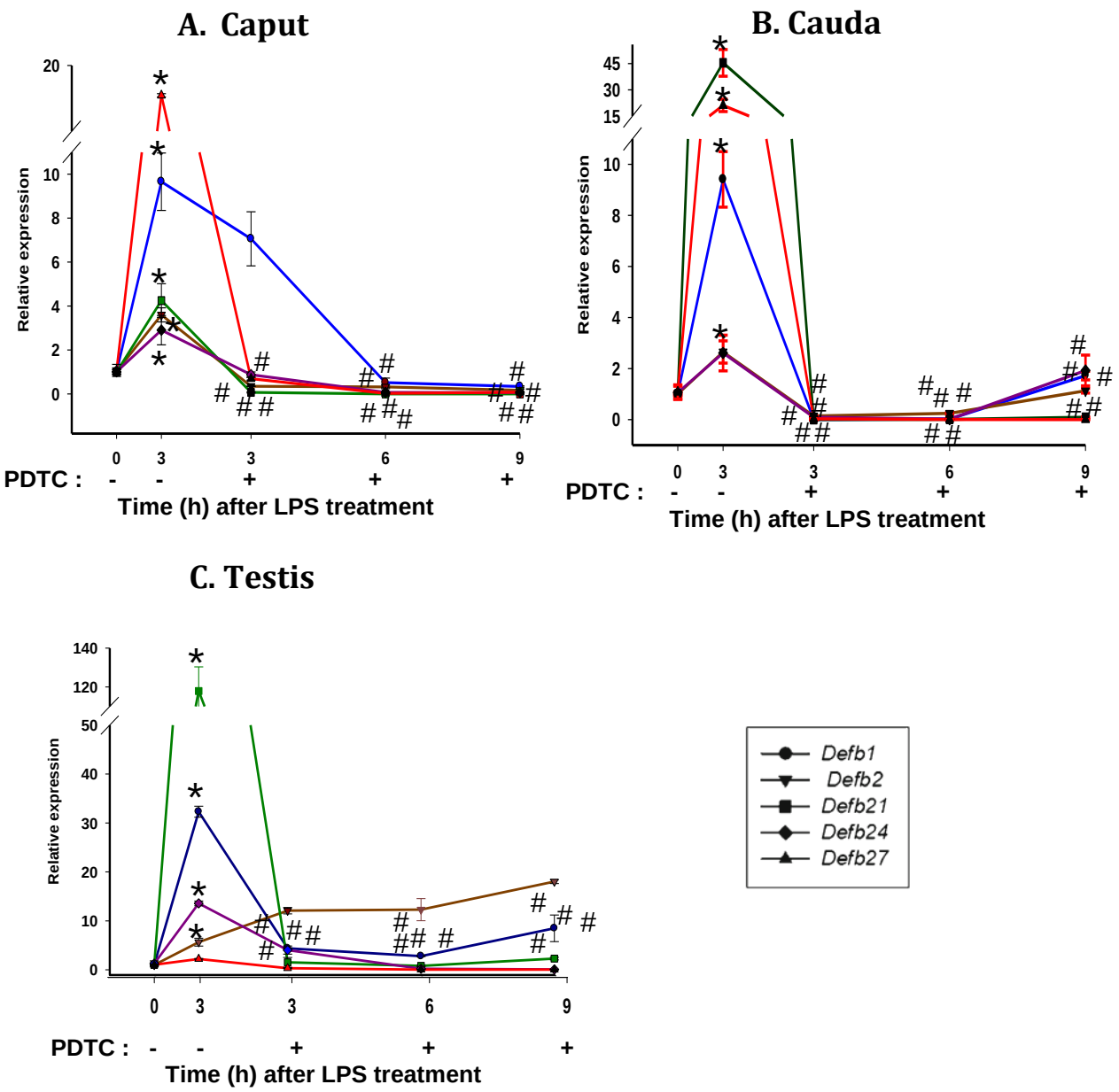

Figure 2.2: Effect of PDTC on LPS-induced defensin gene expression. Caput, cauda, and testis obtained from adult Wistar rats were pretreated with 100 µM PDTC for 1 h. The tissues were then challenged with 1 µg/ml LPS for 0–9 h and defensin expression analyzed using realtime PCR. Values shown are mean ± SD. *p<0.05 compared to 0 h (untreated control). #p<0.05 compared to LPS alone treated.

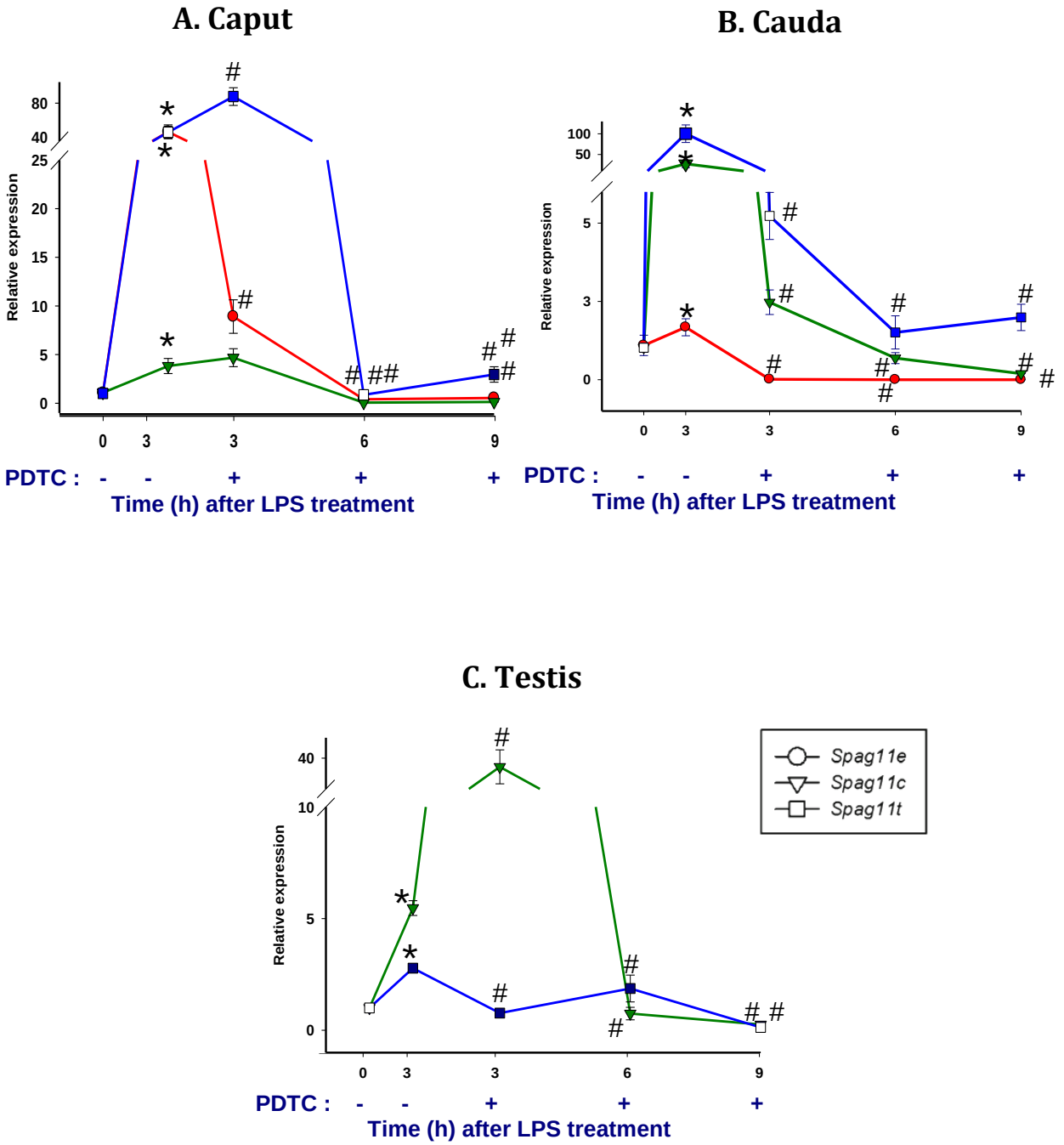

Figure 2.3: Effect of NF-kB inhibitor on LPS induced *Spag11* gene expression. Caput, cauda, and testis obtained from adult Wistar rats were pretreated with 100 µM PDTC for 1 h. The tissues were then challenged with 1 µg/ml LPS for 0–9 h and *Spag11* expression analyzed using real-time PCR. Values shown are mean ± SE. *p<0.05 compared to 0 h (untreated control). #p<0.05 compared to LPS alone treated.

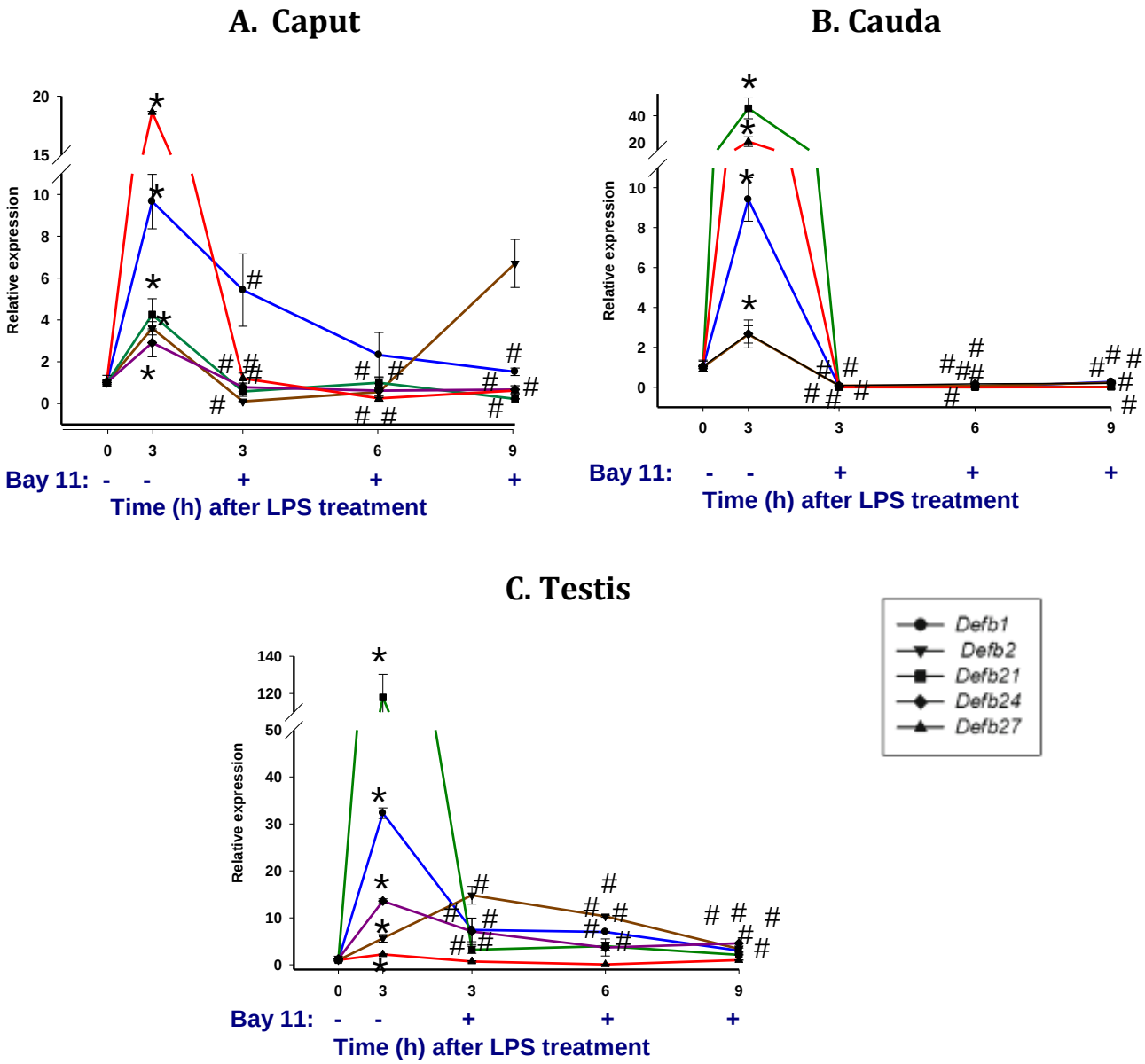

Figure 2.4: Defensin gene expression in Bay11-pretreated male reproductive tissues and challenged with LPS. Real-time PCR analyses for defensins were performed using RNA isolated from caput, cauda, and testis that were pretreated with 100 μM Bay11 for 1 h and followed by 1 μg/ml LPS for 0–9 h. Values shown are mean ± SD. *p<0.05 compared to 0 h (untreated control). #p<0.05 compared to LPS alone treated.

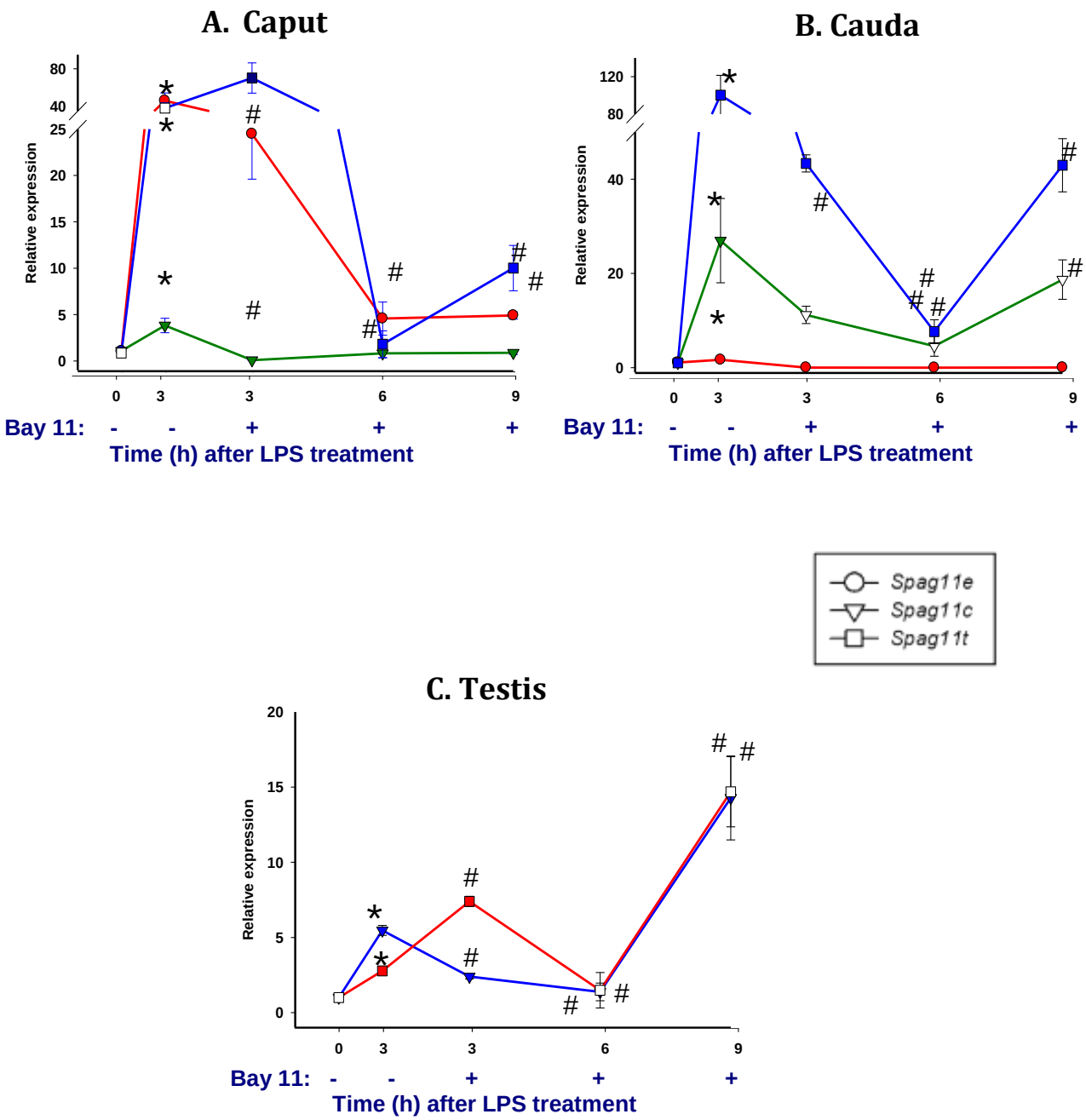

Figure 2.5: *Spag11* gene expression in Bay11-pretreated male reproductive tissues and challenged with LPS. Real-time PCR analyses for defensins were performed using RNA isolated from caput, cauda, and testis that were pretreated with 100 µM Bay11 for 1 h and followed by 1 µg/ml LPS for 0–9 h. Values shown are mean ± SD. *p<0.05 compared to 0 h (untreated control). #p<0.05 compared to LPS alone treated.

LPS-mediated Epigenetic Regulation

Histone Deacetylase:

Gene expression in normal and stress conditions is influenced by a host of epigenetic changes, such as histone modification (acetylation/deacetylation and methylation) and DNA methylation. The possibility that histone modifications could be one of epigenetic changes occurring during LPS-induced defensin and Spag11 expression is unknown. In the epididymis (caput) and testis challenged with LPS *in vitro*, histone deacetylase 1 protein expression decreased significantly (Figure 2.6), suggesting that reduced levels of histone deacetylases could allow histone acetylation thereby allowing increased defensin and *Spag11* gene expression. To further confirm the role of histone deacetylases, defensin and *Spag11* gene expression was analyzed in the epididymis (caput) and testis pretreated with trichostatin A (TSA), a HDAC1 inhibitor. β-Defensin and *Spag11e* gene expression was increased significantly in the epididymis and testis pretreated with TSA when compared to the LPS-treated tissues (Figure 2.7). These results suggest that inhibition of HDAC1 allows transcriptional activation during LPS challenge in the male reproductive tract.

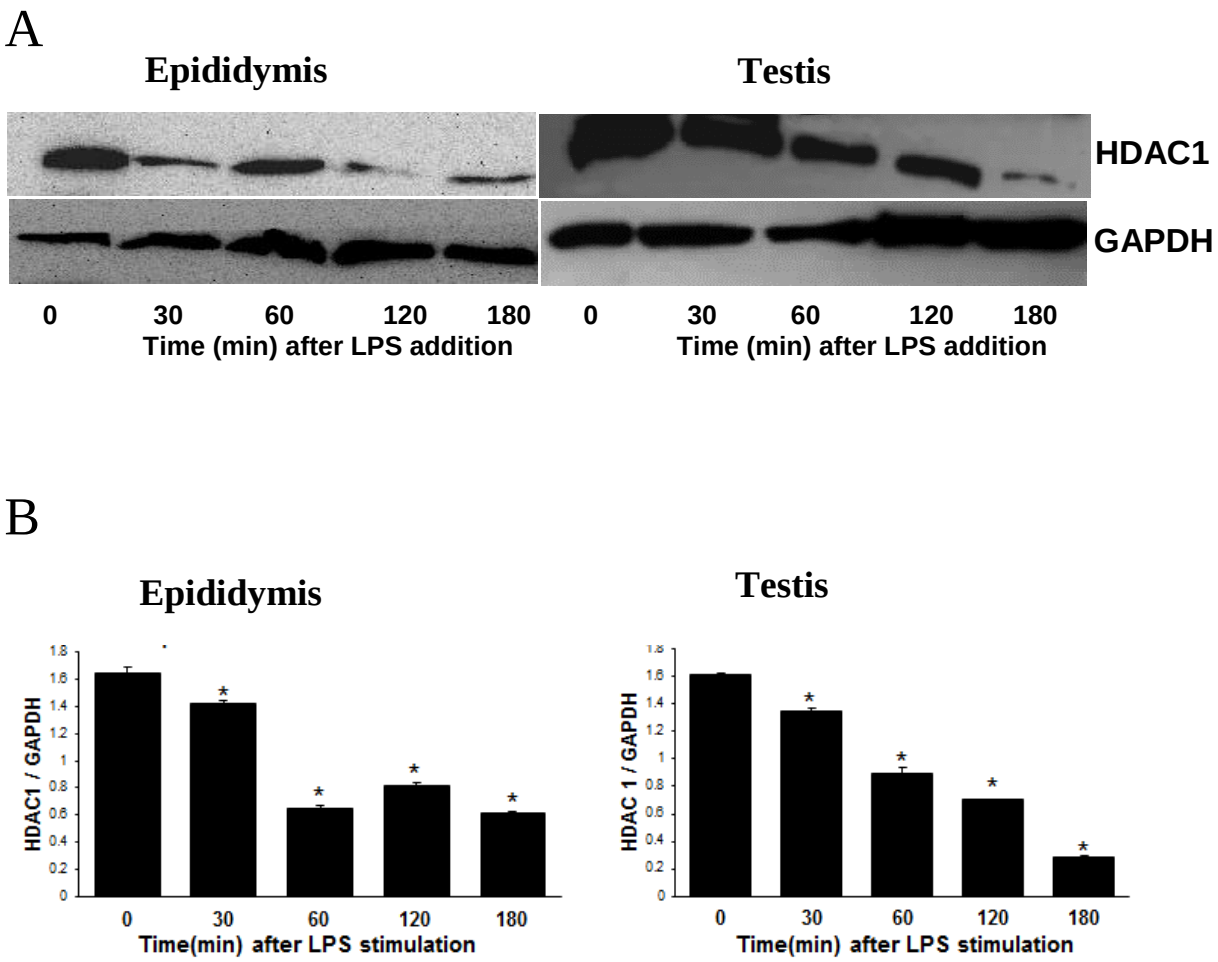

Figure 2.6: Involvement of histone deacetylase 1 (HDAC1) during LPS induced antimicrobial gene expression in the male reproductive tract. [A] Caput and testis obtained from adult Wistar rats were cultured in nutritive medium and challenged with 1 µg/ml LPS for 0–180 min. Tissues were then homogenized and the lysate was separated on SDSPAGE and Western blotting performed using monoclonal antibodies specific to HDAC1. GAPDH was used as the internal control. **[B]** Densitometric analyses for the Western blots shown to determine HDAC1 levels in the epididymis and testis after LPS stimulation. Values shown are mean ± S.D.

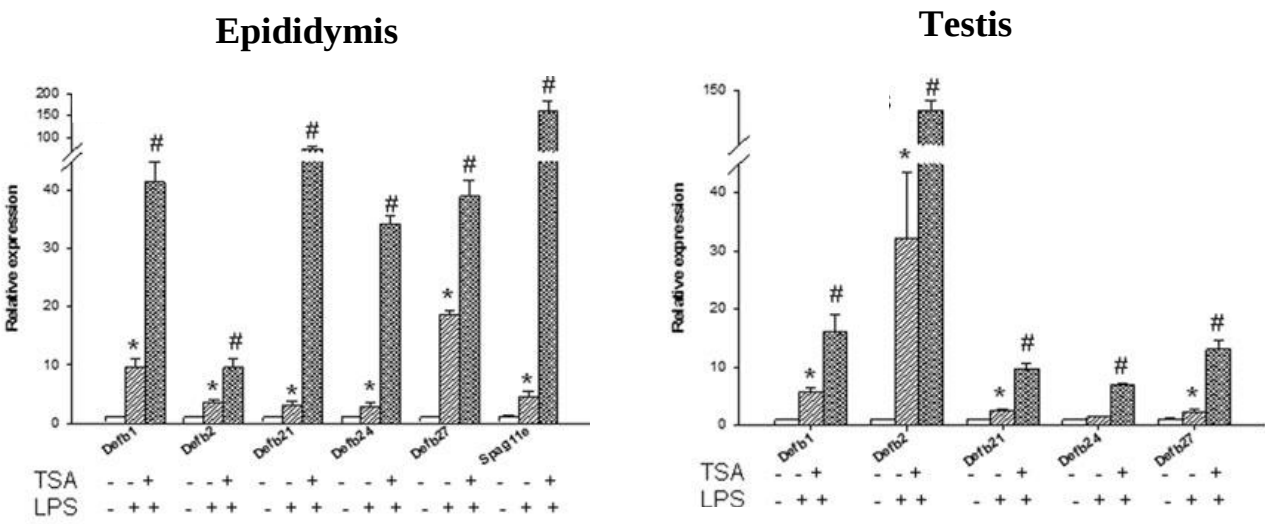

Figure 2.7: Defensin and *Spag11* gene expression after HDAC inhibition. Caput and testis tissues were pretreated with 50 µM trichostatin A followed by 1 µg/ml LPS challenge for 6 h. Real time PCR carried out to analyse defensin and *Spag11* expression. Values shown are mean ± SD. *p<0.05 compared to 0 h (untreated control). # p<0.05 compared to LPS alone treated.

Histone Methylation:

Methylation of histone 3 at lysine 36 (H3K36) is known to correlate with transcriptional activation. To study whether antimicrobial gene induction by LPS in the epididymis and testis involves the methylation of histones, we determined the levels of methylated H3K36 using specific antibodies. Methylated H3K36 protein levels were significantly increased up to 60 and 120 min in the epididymis and testis treated with LPS (Figure 2.8 A). However, a decrease at later time points was observed. To provide further evidence that H3K36 methylation occurs in the upstream region of the antimicrobial genes induced by LPS, we employed chromatin immunoprecipitation to determine methylated H3K36 in the promoter region of antimicrobial gene (*Spag11e*). This gene was analyzed, since it is one of the best characterized among the rat *Spag11* isoforms. Further, we used caput samples only since *Spag11e* is specific to this tissue. Increased methylated H3K36 protein was observed in the LPS-treated caput (Figure 2.8C). The same was also quantified by real-time PCR analyses (Figure 2.8D). These results suggest that LPS-induced antimicrobial gene expression in the male reproductive tract tissues involves epigenetic changes.

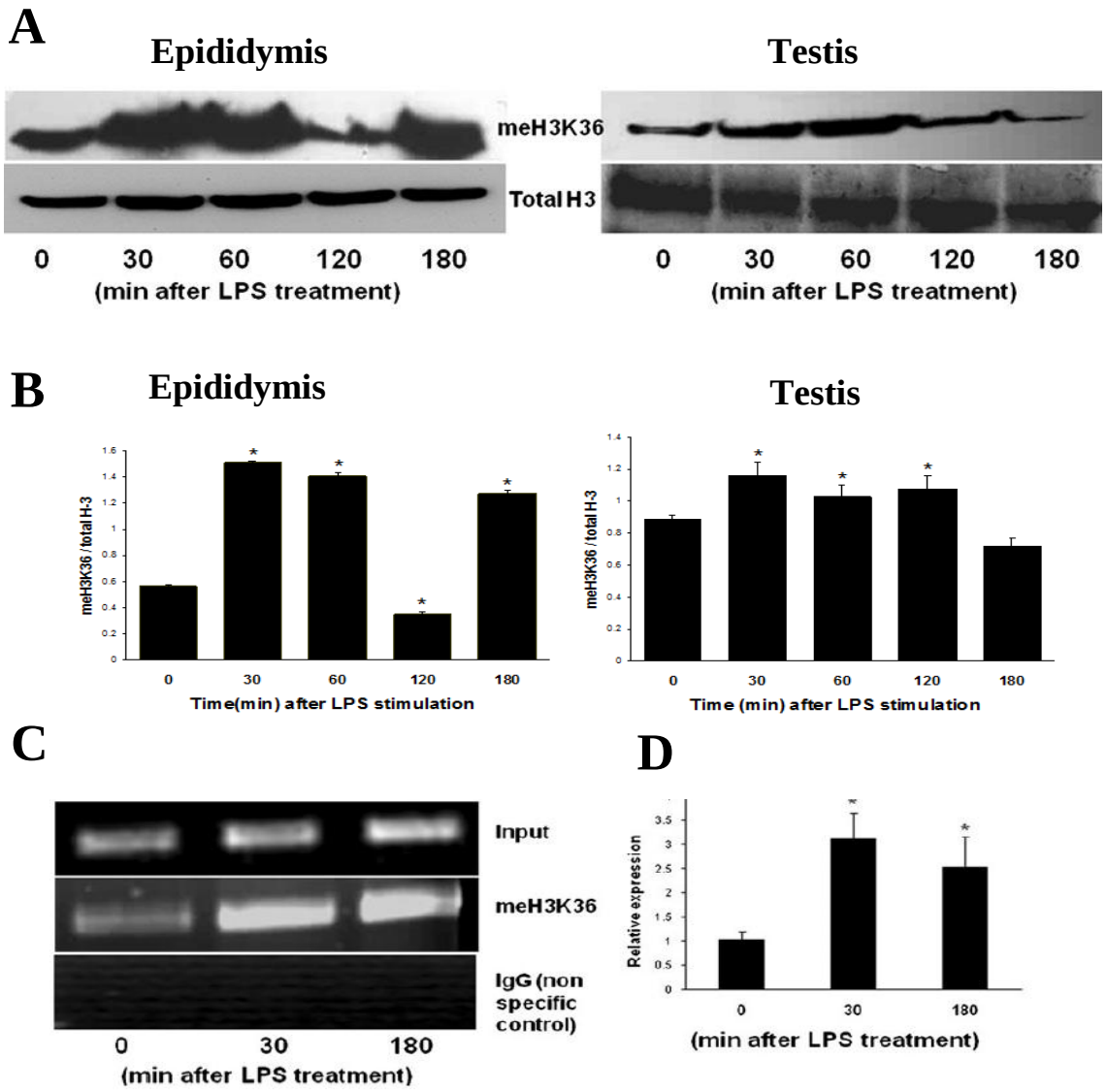

Figure 2.8: Histone methylation in the male reproductive tract tissues during LPS challenge. [A] Caput and testis cultured *in vitro* were treated with 1 µg/ml LPS for 0–180 min, followed by homogenization and separation on SDS-PAGE. The levels of trimethylated histone 3(me3H3K36) were analyzed using monoclonal antibodies by Western blotting. **[B]** Densitometric analyses for the Western blots shown to determine trimethylate histone 3 levels in the epididymis and testis after LPS stimulation. Values shown are mean ± S.D. *p<0.05 compared to 0 min control(untreated control). **[C]** Chromatin immunoprecipitation analyses to determine me3H3K36 levels during LPS challenge in the epididymis. Caput tissue was challenged with 1 µg/ml LPS for 0–180 min, followed by immunoprecipitation using antibodies against rat me3H3K36. Immunoprecipitated DNA was amplified using *Spag11e* upstream region primers and the amplicons run on a 1.5 % agarose gel. **[D]** Real-time PCR analyses to quantify me3H3K36. Using *Spag11e* upstream region primers, DNAwas amplified in a realtime thermal cycler. Values shown are mean ± SD. *p<0.05 compared to 0 min control (untreated control).

DNA methylation:

It is possible that the epigenetic changes observed could be influenced by the activation of NF-kB by LPS or vice versa. Besides histone modifications, cytosine 5′methylation of CpG dinucleotides in the promoter regions or within a gene is an important epigenetic factor in regulating gene expression. It is possible that LPS-induced antimicrobial gene expression in the male reproductive tract tissues may involve CpG modifications. Using bioinformatic tools, we identified two CpG dinucleotides in each of the DNA regions that are 350-bp upstream and downstream of rat *Spag11e* gene transcription start site (Figure 2.9A). Sequencing analyses confirmed the presence of the four predicted CpGs in the DNA isolated from the caput. To determine whether endotoxin challenge could have an effect on *Spag11e* gene, methylation status, CpGs were analyzed in the DNA isolated from the caput tissue challenged with LPS for 0–9 h. We demonstrate that the number of methylated CpG dinucleotides decreased in a time-dependent manner (Figure 2.9B), suggesting demethylation of DNA of *Spag11e* gene and thereby allowing transcriptional activation. During LPS challenge, there was a time-dependent reduction in the expression of DNA methyl transferase 1 (*Dnmt1*) in the caput (Figure 2.9C). Inhibition of *Dnmt1* expression could be one of the innate immune defense mechanisms occurring in the male reproductive tract during endotoxin challenge. The role of *Dnmt1* in endotoxin-induced antimicrobial expression was further confirmed by analyzing *Spag11e* expression in caput tissues pretreated with DNMT1 inhibitor, 5'-azacytidine followed by LPS challenge. *Spag11e* expression was found to be significantly higher in the caput subjected to DNMT1 inhibition when compared to the LPS alone treated tissues (Figure 2.9D), lending further evidence

that repression of *Dnmt1* is one of the innate mechanisms to allow antimicrobial gene expression during endotoxin challenge.

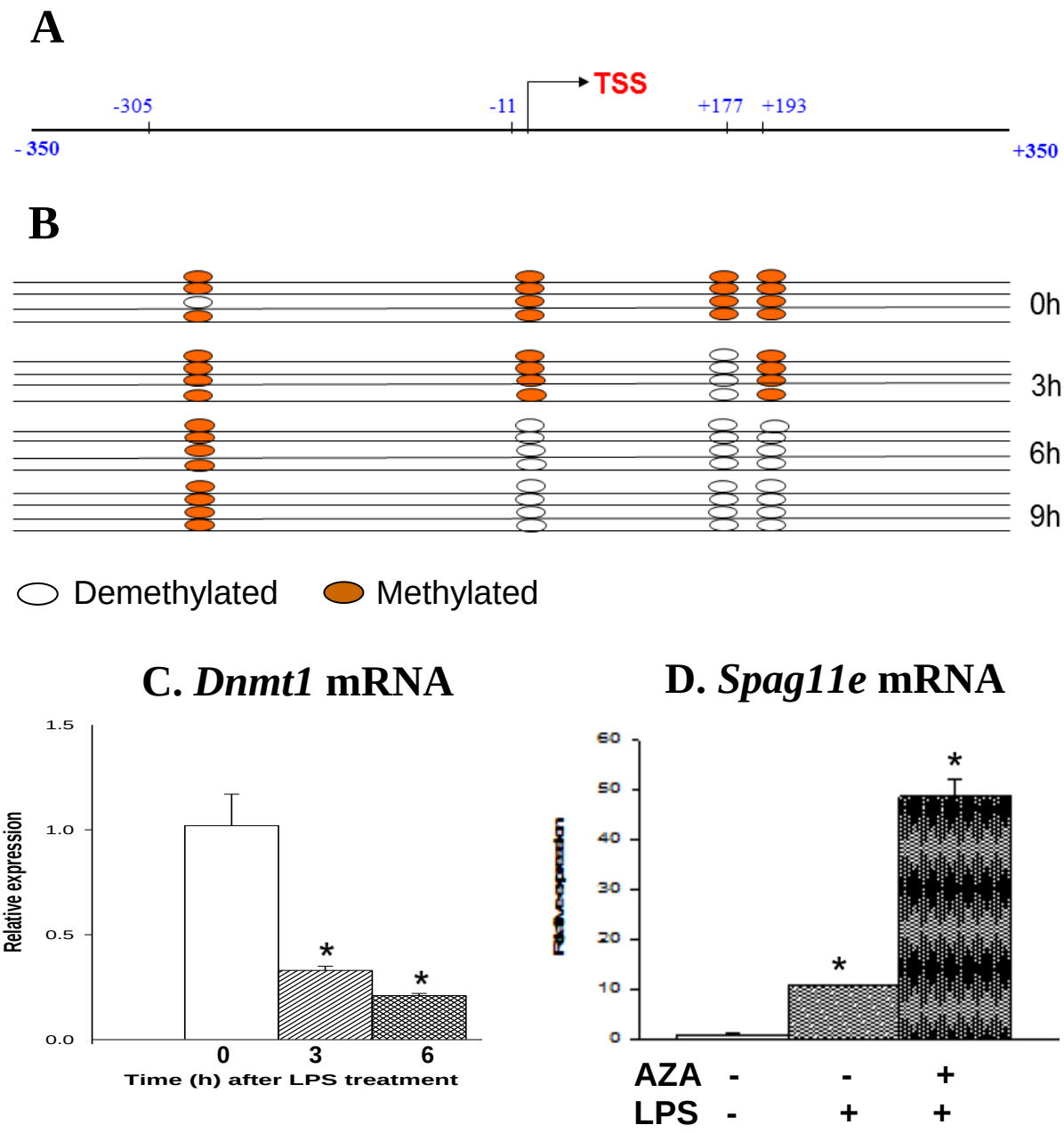

Figure 2.9: Involvement of DNA methylation and DNMT1 during LPS induced antimicrobial gene expression. **[A]** Location of CpG dinucleotides in the *Spag11e* gene. **[B]** Methylation status of the CpG dinucleotides of *Spag11e* gene. Filled oblong indicates methylated and empty oblong indicates unmethylated. Four representative sequencing results are shown. **[C]** Real-time PCR for *Dnmt1* using RNA isolated from the caput tissue treated with 1 µg/ml LPS for 0– 6 h. **[D]** Prior to LPS challenge (3h), rat caput tissues were pretreated with 50 µM 5'-azacytidine and the expression of *Spag11e* was analyzed by real-time PCR. Values shown are mean ± SD. *p<0.05 compared to 0 h (untreated control).

LPS-mediated transcriptional regulation

Spag11e upstream region contains binding sites for transcription factors:

Using specific primers, approximately 1kb of genomic sequence present upstream of *Spag11e* transcription start site was amplified and cloned. Sequence analysis revealed the presence of consensus binding sites for androgen receptor (AR), nuclear factor kappa beta (NF-kB), nuclear factor1 (NF-1), E- twenty six (ETS) and activator protein2 (AP2) and a TATA box (Figure 2.10).

LPS induced gene expression is dependent on AR and NF-kB binding:

Gene expression in the male reproductive tract is androgen dependent. Besides this we have demonstrated that NF-kB phosphorylation and translocation occurs during LPS induced antimicrobial gene expression. To determine whether androgen receptor and Nuclear factor-kappa B are important for antimicrobial gene expression, plasmids were generated that contained mutated binding sites for these two transcription factors. LPS induced *Spag11e* upstream region mediated luciferase activity, whereas the same was not observed in cells transfected with plasmids in which either androgen receptor binding site or NF-kB binding site was mutated (Figure 2.11). These results clearly demonstrate that androgen receptor and NF-kB may play a crucial role in LPS induced *Spag11e* gene expression.

In order to confirm the binding of AR and NF-kB to their binding sites in the upstream region of *Spag11e* during LPS challenge, EMSA were performed. Gel-shift assays were also performed using normal probes designed according to the sequences for AR and NF-kB respectively. AR and NF-kB proteins present in the nuclear extract bound to the radiolabelled oligonucleotides, resulting in the formation of specific band of protein/DNA complex (Figure 2.12).

Figure 2.10: Nucleotide sequence of the 5' flanking region of the rat *Spag11e* gene. The putative transcription start site is depicted by an arrow and the nucleotide sequence is numbered on the left. The potential transcription binding sites are underlined and labeled

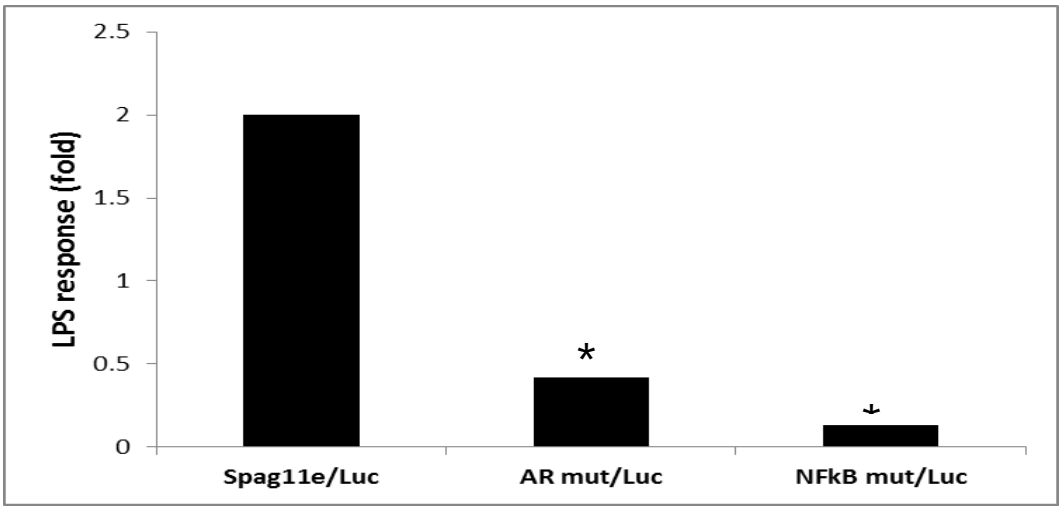

Figure 2.11: Effect of mutagenesis of AR and NF-kB binding sites on *Spag11e* promoter activity in HEK- 293 cells. The wild type or mutant construct was transfected into HEK293 ($5X10^4$), and the cells were incubated for 24 h with or without LPS. The relative luciferase value is indicated as a fold increase in the luciferase activity for each construct relative to that without LPS for the *Spag11e* construct containing point mutations for AR and NF-kB.

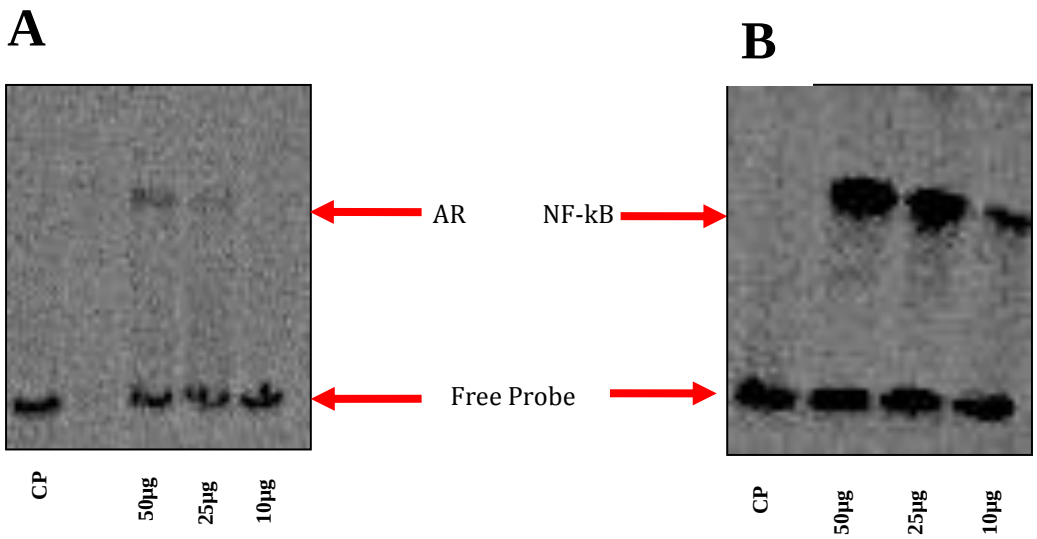

Figure 2.12: Electrophoretic mobility gel shift assay. Gel shift assays showing the binding of **[A] AR, [B] NF-kB** (10- 50 µg of nuclear extract) to the respective oligomers (probe) synthesized from the upstream nucleotide sequence of *Spag11e*. CP-cold probe.

Table 2.1: List of primers used for the study

Primer	Sequence (5'-3')	Purpose
Spag11e F *Spag11e* R	TAGCTCGAGTCTGGGGAATGCCACAATG GCTAAGCTTCCTTCATGTTTTATGGCGCTC	ChIP
Spag11e F *Spag11e* R	GTGTGGTTTTTGGTTATATTTTTTGTGGTG CTATACAAAAAAACAAATATAACTAATAAC	Methylation
AR mut F *AR mut* R	CGTCACGCTCTCCTG**c**T**a**TCGTCGCTGTGTGGG CCCACACAGCGACGATAGCAGGAGAGCGTGACG	Site Directed Mutagenesis
NF-kb mut F *NF-kb mut* R	CAGGTTCACACG**c**GACT**c**TGTTTCTTTGGGCAGG CCTGCCCAAAGAAACAGAGTCGCGTGTGAACCTG	Site Directed Mutagenesis
NF-1 mut F *NF-1 mut* R	CTTTGAA**g**GCC**g**AGGACACATGTTTTATAGAGTTGGG CCCAACTCTATAAAACATGTGTCCTCGGCCTTCAAAG	Site Directed Mutagenesis
AR F *AR* R	CTT CGTCACGCTCTCC**TGTTCTC**GTCGCTGTG CACAGCGACGAGAACAGGAGAGCGTGACGAAG	EMSA
NF-kB F *NF-kB* R	GTTCACAC**GGGACTTT**GTTTCTTTGGGCAGGTT AACCTGCCCAAAGAAACAAAGTCCCGTGTGAAC	EMSA

DISCUSSION

Infections of the male reproductive tract lead to temporary or permanent infertility and are considered as one of the major health problems throughout the world. The general causative agents are *Escherichia coli*, *Neisseria gonorrhea*, and *Staphylococcus aureus*. Epididymitis represents one of the common types of infection. Current treatment regimens for reproductive tract infections include prescription of antibiotics, and over the years, antibiotic resistance has evolved. Strategies to develop alternate candidates to treat reproductive tract infections in place of conventional antibiotics have gained importance. In this study, we attempt to determine the antimicrobial responses in the male reproductive tract tissues challenged with LPS with a long-term goal of identifying the antimicrobial proteins that respond to an infection and develop them as potential candidates to treat reproductive tract infections in place of conventional antibiotics.

We observed that *in vitro* LPS challenge induced the expression of all the β-defensins and *Spag11* genes analyzed in the male reproductive tract tissues of rat. Studies that report the role of β-defensins and *Spag11* genes during endotoxin challenge are limited. LPS-induced β-defensin and *Spag11* gene expression observed in this study is in agreement with our previous study (Biswas and Yenugu 2011) wherein intraperitoneal injection of LPS induces antimicrobial gene expression in the epididymis and testis of rats. The increased LPS demonstrates the robust innate immune mechanisms operating in the male reproductive tract. However, it is also reported that in a rat model of epididymitis induced by LPS, the expression of *Spag11e* and β-defensins in the epididymis is repressed (Cao, Li et al.). This discrepancy could be due to difference in the treatment protocols and the model

systems used. Earlier report suggest the temporal expression pattern of defensin and *Spag11* genes in the male reproductive tract of rat, and they are thought to have a role in sperm maturation and innate immunity (Yenugu, Chintalgattu et al. 2006; Yenugu, Hamil et al. 2006). In this study, though all the defensins and *Spag11* genes analyzed were upregulated in response to LPS, their expression pattern among the tissues seems to vary both in terms of time and abundance. It was demonstrated that LPS disrupts steroidogenesis in the male reproductive tract of rats (Tulassay, Viczian et al. 1970; Wallgren, Kindahl et al. 1993), resulting in altered levels of circulating testosterone (Biswas and Yenugu 2011). Further, gene expression of the epididymides and testis is largely androgen regulated. The variations in defensin and *Spag11* gene observed along the epididymis and testis during endotoxin challenge could be due to alterations in the testosterone levels in the microenvironment.

Signalling mechanisms that operate during LPS exposure is primarily mediated by TLR4 (Peri, Piazza et al.). The presence of TLR4, CD14, and LBP, the major components of LPS recognition on the cell surface, is reported in the male reproductive tract of rat (Palladino, Johnson et al. 2007; Palladino, Savarese et al. 2008). In this study, we observed no significant changes in the expression pattern of *Tlr4* in the epididymides and testis during LPS challenge, suggesting that *Tlr4* expression seems to be constitutive and abundant levels are present to combat endotoxin challenge. *Tlr4* mRNA and protein expression remained unchanged in the epididymides either challenged with LPS *in vitro* or obtained from rats treated intraperitoneally with LPS (Rodrigues, Queiroz et al. 2008).

Increase in pro-inflammatory cytokine production leading to the transcriptional activation of many response genes is one of the hallmarks of

endotoxin-induced cellular responses (McIntyre, Modur et al. 1997). In the mouse testes, the importance of pro-inflammatory cytokines during endotoxin challenge is reported (Abu Elhija, Lunenfeld et al. 2008). Keratinocyte-derived cytokine production in the primary cultures of prostate, seminal vesicle, and epididymis was found to be increased during C. trachomatis infection (Mackern-Oberti, Maccioni et al. 2011). We observed an increase in *Il-6* and *Tnf-α* mRNA expression in the epididymis and testis during LPS challenge, indicating an inflammatory response that could affect epididymal and testicular function. Increased levels of *Il-6* and *Tnf-α* observed in this study could influence the expression of a variety of genes including the defensin and *Spag11* genes analyzed in this study. However, further studies are required to demonstrate whether pro-inflammatory cytokines can modulate the expression of defensin and *Spag11*genes in the male reproductive tract.

Recognition of LPS by TLR4 initiates signaling pathways that involves the activation and translocation of the transcription factor NF-kB. Binding of NF-kB to DNA mediates changes in gene expression following LPS binding to TLRs (Han, Ko et al. 2002). In this study, an increase in antimicrobial gene expression in response to LPS accompanied the phosphorylation of p65 and IkBα both in the epididymis and testis, suggesting that the transcriptional activation could be mediated by NF-kB. Activation of NFkB in the epididymides during LPS challenge in vitro was observed and the same was abrogated in the presence of PDTC (Rodrigues, Queiroz et al. 2008). Though the involvement of NF-kB in LPS-induced effects is reported in the male reproductive tract (Rodrigues, Queiroz et al. 2008), its role in regulating antimicrobial gene expression is not yet studied. We provide further evidence that pretreatment with inhibitors of NF-kB activation (PDTC) and IkBα phosphorylation

(Bay11) resulted in the abrogation of antimicrobial gene expression in response to LPS (Figure 2.2- 2.5). Inhibition of LPS induced gene expression in many organ systems is reported. For example, in the kidney cells, the increased expression of MCP-1 via the NF-kB pathway by LPS was inhibited by PDTC (Zhai, Zhang et al. 2012). Similarly, LPS-induced expression of COX-2 is inhibited by PDTC in macrophage cell line (Zhang, Lei et al. 2010). Sulfasalazine, also an NF-kB inhibitor, suppressed the expression of a variety of cytokines induced by LPS in the gestational membranes (Keelan, Khan et al. 2009).

Epigenetic modifications play a pivotal role in the regulation of gene expression, and until recently, majority of the studies focused on the role of these modifications in developmental biology and cancer progression. Since innate immune mechanisms also involve the regulation of a variety of genes, there has been a growing interest in the recent past on the role of epigenetic modifications in controlling gene expression during immunological responses in different organ systems. In host responses to bacterial infections, selective gene induction by inflammatory signals that activate NF-kB involved histone methylation and deacetylation (Ito 2007). However, studies on the role of epigenetic factors to control immune response-related gene expression in the male reproductive tract are lacking. We report that LPS induced antimicrobial gene expression in the epididymis and testis is epigenetically regulated. The decrease of HDAC1 in the epididymis and testis exposed to LPS suggests an intrinsic mechanism to slow down histone deacetylation thereby allowing gene transcription. Similar observations were made in the gingival cells exposed to oral bacteria (Yin and Chung 2011). In this study, β-defensin and *Spag11* gene expression was significantly higher in epididymis and

testis pretreated with HDAC1 inhibitor (TSA) when compared to LPS alone treated tissues. This increase could be due to the inhibition of the remaining endogenous HDAC1, suggesting an additive effect. However, from this study, it is not clear whether suppression of HDAC1 expression during LPS challenge is directly regulated by the endotoxin itself or whether it involves any other signaling components. Further, the actual acetylation status of histones (H3 and H4) that influence defensin transcription during LPS challenge needs further investigation. Since TSA is a general inhibitor of all HDACs, it is possible that the increased defensin expression during LPS challenge could also be due to inhibition of HDACs other than HDAC1. The role of other HDACs besides HDAC1 in LPS-induced defensin expression is yet to be investigated. Recently, it was demonstrated that sodium butyrate, a HDAC1 inhibitor, stimulates endogenous antimicrobials in the gut (Halili, Andrews et al. 2009). Similarly, treatment with TSA amplified the expression of proatherogenic factors in mouse macrophages in vitro (Halili, Andrews et al. 2010). In this study, since HDAC1 inhibition by TSA resulted in an additive effect on antimicrobial gene expression during LPS challenge, the results reported here may suggest that HDAC1 inhibitors may be used as therapeutic agents to treat reproductive tract infections.

Methylation of histone H3 at K36 (H3K36) is an important event for the activation of transcription. A time dependent increase in the levels of trimethylated histone 3 was observed in the epididymis and testis challenged with LPS, suggesting multiple levels of epigenetic regulation. Such epigenetic regulation was observed for β-defensin 2 in gingival epithelial cells exposed to *Fusobacterium nucleatum*. Further, it is demonstrated that LPS-induced IL-8 and p19 production in human intestinal epithelial cells and mouse bone marrow cells, respectively, is accompanied

by the methylation of histone 3 (Angrisano, Pero et al. ; El Mezayen, El Gazzar et al. 2009). The binding of methylated histones to the promoter regions is an important event in the regulation of target genes. For example, in the murine macrophage cell line, it was shown that LPS induces the binding of H3K4 to the promoter region of TNF-α, an LPS-inducible gene (Ara, Xia et al. 2008). The results of the ChIP analyses suggest that the methylation of histone 3 and its binding to *Spag11e* is an epigenetic regulatory mechanism during the immune response to LPS in the male reproductive tract.

One of the well-known mechanisms that govern gene transcription is the methylation of DNA (Bird and Wolffe 1999). Hypermethylation within or in sequences that flank a gene generally results in gene repression and vice versa. In this study, using *in silico* tools, CpG dinucleotides were identified in the upstream and downstream regions of the transcription site of *Spag11e* gene. Presence of CpG dinucleotides gives a clue that this gene could be epigenetically regulated. We demonstrate demethylation of four CpG dinucleotides in LPS-treated epididymides in a time dependent manner. Loss of DNA methylation favors gene transcription and increase in *Spag11e* expression during LPS challenge could be due to the demethylation observed. This seems to be an innate mechanism to allow enhanced expression of antimicrobial gene expression under infectious conditions. The observed demethylation could be due to inhibition of endogenous DNA methyl transferases (DNMTs). Reduced *Dnmt1* expression during LPS challenge observed in this study could contribute to the demethylated status of *Spag11e* promoter. Increased *Spag11e* expression under conditions where DNMT1 is inhibited with 5'-azacytidine provides ample evidence that inhibition of DNMT1 or reduction in the

transcription of *Dnmt1* could be one of the innate mechanisms that operate in the reproductive tract to allow increased transcription of antimicrobial genes during infections.

In conclusion, we report that LPS induces the expression of β-defensin and *Spag11* gene expression in the epididymal and testicular tissues *in vitro* and involves the activation of NF-kB signaling pathway. Further, induction of antimicrobial gene expression is accompanied by a decrease in HDAC1 and *Dnmt1* expression, increased methylation of histone 3, and its binding to the upstream region of *Spag11e* gene and demethylation of DNA, suggesting an epigenetic regulation in the male reproductive tract tissues under conditions that mimic an infection. The results of this study provide clues to develop novel strategies, such as the use of HDAC1 and DNMT1 inhibitors to induce antimicrobial gene responses and thereby treat reproductive tract infections.

REFERENCES

Abu Elhija, M., E. Lunenfeld, et al. (2008). "LPS Increases the Expression Levels of IL-18, ICE and IL-18 R in Mouse Testes." American Journal of Reproductive Immunology 60(4): 361-371.

Akira, S. (2001). "Toll-like receptors and innate immunity." Adv Immunol 78: 1-56.

Akira, S. and K. Takeda (2004). "Toll-like receptor signalling." Nature Reviews. Immunology 4(7): 499-511.

Angrisano, T., R. Pero, et al. "LPS-induced IL-8 activation in human intestinal epithelial cells is accompanied by specific histone H3 acetylation and methylation changes." BMC Microbiology 10: 172.

Ara, A. I., M. Xia, et al. (2008). "S-adenosylmethionine inhibits lipopolysaccharide-induced gene expression via modulation of histone methylation." Hepatology 47(5): 1655-1666.

Bird, A. P. and A. P. Wolffe (1999). "Methylation-induced repression--belts, braces, and chromatin." Cell 99(5): 451-454.

Biswas, B., G. Narmadha, et al. (2009). "Identification of Toll-like receptors in the rat (Rattus norvegicus): messenger RNA expression in the male reproductive tract under conditions of androgen variation." American Journal of Reproductive Immunology 62(4): 243-252.

Biswas, B. and S. Yenugu (2011). "Antimicrobial responses in the male reproductive tract of lipopolysaccharide challenged rats." American Journal of Reproductive Immunology 65(6): 557-568.

Bourgeon, F., B. Evrard, et al. (2004). "Involvement of semenogelin-derived peptides in the antibacterial activity of human seminal plasma." Biol Reprod 70(3): 768-774.

Cao, D., Y. Li, et al. "Lipopolysaccharide-induced epididymitis disrupts epididymal beta-defensin expression and inhibits sperm motility in rats." Biology of Reproduction 83(6): 1064-1070.

Cao, D., Y. Li, et al. (2010). "Lipopolysaccharide-induced epididymitis disrupts epididymal beta-defensin expression and inhibits sperm motility in rats." Biol Reprod 83(6): 1064-1070.

Chung, W. O. and B. A. Dale (2004). "Innate immune response of oral and foreskin keratinocytes: utilization of different signaling pathways by various bacterial species." Infection and Immunity 72(1): 352-358.

Cornwall, G. A., A. Cameron, et al. (2003). "The cystatin-related epididymal spermatogenic protein inhibits the serine protease prohormone convertase 2." Endocrinology 144(3): 901-908.

El Mezayen, R., M. El Gazzar, et al. (2009). "Aging-dependent upregulation of IL-23p19 gene expression in dendritic cells is associated with differential transcription factor binding and histone modifications." Aging Cell 8(5): 553-565.

Fraczek, M. and M. Kurpisz (2007). "Inflammatory mediators exert toxic effects of oxidative stress on human spermatozoa." Journal of Andrology 28(2): 325-333.

Gatti, G., A. A. Quintar, et al. (2009). "Expression of Toll-like receptor 4 in the prostate gland and its association with the severity of prostate cancer." Prostate 69(13): 1387-1397.

Gatti, G., V. Rivero, et al. (2006). "Prostate epithelial cells can act as early sensors of infection by up-regulating TLR4 expression and proinflammatory mediators upon LPS stimulation." Journal of Leukocyte Biology 79(5): 989-998.

Girling, J. E. and M. P. Hedger (2007). "Toll-like receptors in the gonads and reproductive tract: emerging roles in reproductive physiology and pathology." Immunol Cell Biol 85(6): 481-489.

Halili, M. A., M. R. Andrews, et al. (2010). "Differential effects of selective HDAC inhibitors on macrophage inflammatory responses to the Toll-like receptor 4 agonist LPS." J Leukoc Biol 87(6): 1103-1114.

Halili, M. A., M. R. Andrews, et al. (2009). "Histone deacetylase inhibitors in inflammatory disease." Current Topics in Medicinal Chemistry 9(3): 309-319.

Hamil, K. G., Q. Liu, et al. (2003). "LCN6, a novel human epididymal lipocalin." Reprod Biol Endocrinol 1(1): 112.

Hamil, K. G., Q. Liu, et al. (2002). "Cystatin 11: a new member of the cystatin type 2 family." Endocrinology 143(7): 2787-2796.

Hamil, K. G., P. Sivashanmugam, et al. (2000). "HE2beta and HE2gamma, new members of an epididymis-specific family of androgen-regulated proteins in the human." Endocrinology 141(3): 1245-1253.

Han, S. J., H. M. Ko, et al. (2002). "Molecular mechanisms for lipopolysaccharide-induced biphasic activation of nuclear factor-kappa B (NF-kappa B)." J Biol Chem 277(47): 44715-44721.

Ito, K. (2007). "Impact of post-translational modifications of proteins on the inflammatory process." Biochemical Society Transactions 35(Pt 2): 281-283.

Keelan, J. A., S. Khan, et al. (2009). "Prevention of inflammatory activation of human gestational membranes in an ex vivo model using a pharmacological NF-kappaB inhibitor." Journal of Immunology 183(8): 5270-5278.

Kouzarides, T. (2007). "Chromatin modifications and their function." Cell 128(4): 693-705.

Li, P., H. C. Chan, et al. (2001). "An antimicrobial peptide gene found in the male reproductive system of rats." Science 291(5509): 1783-1785.

Liao, M., P. S. Ruddock, et al. (2005). "Cationic peptide of the male reproductive tract, HE2{alpha}, displays antimicrobial activity against *Neisseria gonorrhoeae*, *Staphylococcus aureus* and *Enterococcus faecalis*." J Antimicrob Chemother 56(5): 957-961.

Mackern-Oberti, J. P., M. Maccioni, et al. (2011). "Innate immunity in the male genital tract: Chlamydia trachomatis induces keratinocyte-derived chemokine production in prostate, seminal vesicle and epididymis/vas deferens primary cultures." J Med Microbiol 60(Pt 3): 307-316.

Mackern-Oberti, J. P., M. Maccioni, et al. (2006). "Susceptibility of prostate epithelial cells to Chlamydia muridarum infection and their role in innate immunity by recruitment of intracellular Toll-like receptors 4 and 2 and MyD88 to the inclusion." Infection and Immunity 74(12): 6973-6981.

Malm, J., O. Sorensen, et al. (2000). "The human cationic antimicrobial protein (hCAP-18) is expressed in the epithelium of human epididymis, is present in seminal plasma at high concentrations, and is attached to spermatozoa." Infect Immun 68(7): 4297-4302.

McIntyre, T. M., V. Modur, et al. (1997). "Molecular mechanisms of early inflammation." Thrombosis and Haemostasis 78(1): 302-305.

Palladino, M. A., T. A. Johnson, et al. (2007). "Members of the Toll-like receptor family of innate immunity pattern-recognition receptors are abundant in the male rat reproductive tract." Biol Reprod 76(6): 958-964.

Palladino, M. A., M. A. Savarese, et al. (2008). "Localization of Toll-like receptors on epididymal epithelial cells and spermatozoa." Am J Reprod Immunol 60(6): 541-555.

Peri, F., M. Piazza, et al. "Exploring the LPS/TLR4 signal pathway with small molecules." Biochemical Society Transactions 38(5): 1390-1395.

Quintar, A. A., F. D. Roth, et al. (2006). "Toll-like receptor 4 in rat prostate: modulation by testosterone and acute bacterial infection in epithelial and stromal cells." Biol Reprod 75(5): 664-672.

Roach, J. C., G. Glusman, et al. (2005). "The evolution of vertebrate Toll-like receptors." Proc Natl Acad Sci U S A 102(27): 9577-9582.

Rodrigues, A., D. B. Queiroz, et al. (2008). "Activation of toll-like receptor 4 (TLR4) by in vivo and in vitro exposure of rat epididymis to lipopolysaccharide from Escherichia Coli." Biology of Reproduction 79(6): 1135-1147.

Takeda, K. and S. Akira (2004). "TLR signaling pathways." Semin Immunol 16(1): 3-9.

Trojian, T. H., T. S. Lishnak, et al. (2009). "Epididymitis and orchitis: an overview." Am Fam Physician 79(7): 583-587.

Tulassay, Z., M. Viczian, et al. (1970). "Quantitative histological studies on the injury of spermatogenesis induced by endotoxin in rats." Journal of Reproduction and Fertility 22(1): 161-164.

Wallgren, M., H. Kindahl, et al. (1993). "Alterations in testicular function after endotoxin injection in the boar." International Journal of Andrology 16(3): 235-243.

Yenugu, S., V. Chintalgattu, et al. (2006). "Identification, cloning and functional characterization of novel beta-defensins in the rat (Rattus norvegicus)." Reprod Biol Endocrinol 4(1): 1-7.

Yenugu, S., K. G. Hamil, et al. (2003). "Antibacterial properties of the sperm-binding proteins and peptides of human epididymis 2 (HE2) family; salt sensitivity, structural dependence and their interaction with outer and cytoplasmic membranes of *Escherichia coli*." Biochem J 372(Pt 2): 473-483.

Yenugu, S., K. G. Hamil, et al. (2004). "Antimicrobial actions of the human epididymis 2 (HE2) protein isoforms, HE2alpha, HE2beta1 and HE2beta2." Reprod Biol Endocrinol 2(1): 61.

Yenugu, S., K. G. Hamil, et al. (2006). "Antimicrobial actions of human and macaque sperm associated antigen (SPAG)11 isoforms: Influence of the N-terminal peptide." Molecular and Cellular Biochemistry 284(1-2): 25-35.

Yenugu, S., K. G. Hamil, et al. (2006). "Identification, cloning and functional characterization of novel sperm associated antigen 11 (SPAG11) isoforms in the rat." Reprod Biol Endocrinol 4: 23.

Yin, L. and W. O. Chung (2011). "Epigenetic regulation of human beta-defensin 2 and CC chemokine ligand 20 expression in gingival epithelial cells in response to oral bacteria." Mucosal Immunol 4(4): 409-419.

Zhai, J. X., Z. X. Zhang, et al. (2012). "PDTC attenuate LPS-induced kidney injury in systemic lupus erythematosus-prone MRL/lpr mice." Mol Biol Rep 39(6): 6763-6771.

Zhang, J., T. Lei, et al. (2010). "Resistin up-regulates COX-2 expression via TAK1-IKK-NF-kappaB signaling pathway." Inflammation 33(1): 25-33.

INTRODUCTION

Infections of the male reproductive system are the major causes of infertility. Because of the anatomical architecture of this system, wherein the testis and epididymis are connected to urethra, the chances of microbial invasion are very high. Epididymitis, urethritis and prostatitis, which are generally sexually transmitted, are the common forms of male reproductive tract infections resulting in male infertility (WHO 1987; Yule, Mahi-Brown *et al*. 1990; Hawes and Kiviat 2002). Besides *Chlamydia trachomatis* and *Neisseria gonorrhea* (Trojian, Lishnak *et al*. 2009), *Uropathogenic E. coli* (UPEC) is responsible for the etiology of reproductive tract infections (Gupta, Hooton *et al*. 1999; Gupta, Scholes *et al*. 1999) and is also known to be predominant in the isolates obtained from epididymo-orchitis (Eggert-Kruse, Rohr *et al*. 1995; Merino, Carranza-Lira *et al*. 1995; Willen, Holst *et al*. 1996) subjects. Following infection, UPEC adheres to the epithelial cells of the urethra and invades other organs of the reproductive system by endocytic and invagination mechanisms (Song, Bishop *et al*. 2007; Song, Bishop *et al*. 2009), leading to loss of fertility (Trojian, Lishnak *et al*. 2009). Virulent factors of UPEC that are responsible for infection include fimbriae with adhesion tips, protectins and endotoxins such as haemolysin, colony necrotizing factor and lipopolysaccharide (LPS).

Innate immune defense mechanisms in the male reproductive tract in response to endotoxins such as LPS are widely reported. The presence of toll-like receptors (TLRs) and the components of the TLR signaling pathway that lead to initiation of immune response is reported in this organ system (Palladino, Johnson *et al*. 2007). In general, TLR4 activation by endotoxins results in initiation of a cascade of events mediated by adaptor proteins and protein kinases to activate NF-kB

(Nuclear factor-kappa beta) and IRF-3 (Interferon regulatory factor-3) via the Myd88 dependent and Myd88 independent pathways respectively (Akira and Takeda 2004; Roach, Glusman *et al*. 2005); to influence the expression of a variety of cytokines, acute phase proteins, adhesion molecules and antimicrobial proteins and peptides (O'Neill and Dinarello 2000; Akira, Takeda *et al*. 2001). Besides the classical immune response pathways, the regulation of antimicrobial gene expression is also influenced by epigenetic mechanisms that involve histone modifications (deacetylation and methylation). Antimicrobial gene expression was found to be epigenetically regulated in the male reproductive tract tissues challenged with LPS *in vitro* (Biswas and Yenugu 2012). The mechanisms of antimicrobial gene regulation in a male reproductive tract infection model such as the epididymo-orchitis is not yet reported.

Spermatozoa produced in the testis transit through the epididymis to undergo maturation, during which a wide variety of proteins are added on to their surface. Secreted testicular and epididymal proteins that are thought to play an important role in spermatogenesis and sperm maturation (Hamil, Sivashanmugam *et al*. 2000; Hamil, Liu *et al*. 2002; Hamil, Liu *et al*. 2003; Hsia and Cornwall 2003) were found to be antimicrobial in nature, suggesting their role in male reproductive tract immunity as well (Malm, Sorensen *et al*. 2000; Li, Chan *et al*. 2001; Hall, Hamil *et al*. 2002; Hamil, Liu *et al*. 2002; Com, Bourgeon *et al*. 2003; Bourgeon, Evrard *et al*. 2004; Liao, Ruddock *et al*. 2005). For example, the Sperm Associated Antigen 11 (SPAG11) family members (also referred to as HE2 (Human Epididymis 2) or EP2 (Epididymal Protein 2)) of the male reproductive tract are antimicrobial in nature and their mechanism of action involved membrane permeabilization (Yenugu, Hamil

et al. 2003; Avellar, Honda *et al*. 2004; Yenugu, Hamil *et al*. 2004; Yenugu, Hamil *et al*. 2006). In the rat, for SPAG11 isoforms namely, SPAG11T and SPAG11C, their antimicrobial activity was demonstrated (Yenugu, Hamil *et al*. 2006), besides the previously reported SPAG11E (also referred to as BIN1B) (Li, Chan *et al*. 2001). In addition to these, we also demonstrated the antibacterial activity of rat β-defensin genes (*Defb21, Defb24, Defb27, Defb30* and *Defb36*) (Yenugu, Chintalgattu *et al*. 2006). SPAG11 isoforms and defensins respond to *in vitro* and *in vivo* endotoxin challenge and that the expression is epigenetically regulated (Biswas and Yenugu 2011; Biswas and Yenugu 2012). Further, SPAG11E transgenic mice were found to be resistant to bacterial infection (Fei, Hu *et al*. 2012). However, the role of *Spag11* and defensin genes in diseased conditions such as epididymitis is not yet known.

Emergence of antibiotic resistance due to their widespread use has generated the need to develop newer antimicrobial agents to combat infections (Shea 2003; Verhoef 2003). Among a variety of anti-infectives that are being developed, antimicrobial peptides (AMPs) have received the most attention. The therapeutic applications of AMPs can be exploited by using them alone or in combination with conventional antibiotics. They can also be used as immunostimulatory agents or endotoxin neutralizing agents (Gordon, Romanowski *et al*. 2005). A number of studies including ours have demonstrated the synergistic effects of AMPs when used in combination with antibiotics. The synergistic effects of AMPs when used in combination with antibiotics was demonstrated earlier (Yenugu and Narmadha 2010). Further, the efficacy of AMPs to limit infections in different animal disease models is also reported. β–defensin3 administration showed beneficial effects in mouse influenza A model (Jiang, Yang *et al*. 2012). Similarly, humanized theta-

defensins protected mice from experimental anthrax infections (Welkos, Cote *et al*. 2011). In the female reproductive tract of mouse, lactoferricin was found to inhibit HSV2 infection (Shestakov, Jenssen *et al*. 2012). However, the efficacy of antimicrobial proteins in male reproductive tract infections is not yet reported.

In this part of the study, we report the induction of defensin and *Spag11* gene expression in a rat model of epididymo-orchitis. Antimicrobial gene expression seems to be mediated by NF-kB activation and epigenetic regulation. UPEC infection was found to be decreased in rats treated with recombinant defensin 21, suggesting the potential of antimicrobial proteins to treat reproductive tract infections in place of conventional antibiotics.

APPROACHES

Animals and bacterial strain: Adult male Wistar rats were housed in standard conditions (12 h light/dark cycle, 20-22°C), with free access to water and food. All experiments were carried out in strict accordance with the recommendations by Institutional Biosafety committee (IBSC) and the Institutional Animal Ethics Committee (IAEC) at University of Hyderabad. All surgeries were performed under Ketamine and Xylazine anaesthesia, and efforts were made to minimize suffering.

UPEC strain MTCC 729 obtained from MTCC, Chandigarh, India, was used to induce epididymo-orchitis in rat. UPEC was propagated overnight in LB agar without antibiotics. The overnight culture was inoculated into fresh LB medium and grown to exponential phase (A600= 0.5 - 1) at 37^{o}C in a shaking incubator. The concentration of viable bacteria was calculated using standard growth curves. Bacteria were

centrifuged at 5000 rpm for 10 min at room temperature and the pellet was washed once with PBS and resuspended in sterile saline to achieve 2 x 10^5 cells.

UPEC induced epididymo-orchitis: Adult male Wistar rats (250-300 g) were anaesthetized with 50 mg/ml Ketamine and 2% xylazine. After general anesthesia, a scrotal incision was made to expose the testis, epididymis and vas deferens. The organs were not taken out to minimize contamination. The vas deferens was ligated at the sight of injection to prevent the bacteria from moving in the upward direction and as well as to prevent spreading of infection. 50µl of UPEC suspension containing 1 x 10^5 was injected in each vasa deferentia using an insulin syringe. The scrotum was then ligated and the animals were kept under standard conditions. Rats were sacrificed after day 1, 3, 5 and 7 post infections. The sham operated rats that received 50 µl of PBS and sacrificed on day 7 after injection served as uninfected control. The testes and epididymides were removed aseptically, for further analysis. One of the pairs of the testis and epididymides were fixed in Bouin's fluid (75 ml picric acid solution + 25 ml 37% formaldehyde solution + 5 ml acetic acid) and 4% PFA, and the other of the pair were preserved at -80°C. Before placement of each testicle into fixative, the tunica albuginea was punctured with a 24-gauge needle to facilitate the penetration of fixative.

UPEC detection

Colony forming unit assay: The number of UPEC present in the caput, cauda and testis after infection was analysed by colony forming unit (CFU) assay. Approximately 1g of epididymidis and testes from saline and UPEC injected rats were homogenized in 10 ml sterile PBS. The homogenate was then serially diluted

and 100 µl was streaked on LB agar plates and incubated overnight at 37°C. Bacterial colonies were then hand counted.

Polymerase chain reaction: The presence of UPEC in reproductive tissue following UPEC injection was also confirmed by PCR using primers specific for UPEC *P pili* gene (Table 3.1). Briefly, homogenates of epididymides and testes obtained from sham operated control and UPEC infected rats were diluted and subjected to PCR. The resulting amplicons were analyzed on a 1% agarose gel.

Immunofluorescence: Fluorescent microscopy was used to detect the presence of UPEC following bacterial injection. Epididymides and testes obtained from control and UPEC treated rats were fixed in 4% paraformaldehyde and embedded in paraffin. Five micron thick sections were taken and treated with xylene and graded alcohol (70–100%). The sections were then treated with 1% Triton-X 100 to facilitate permeabilisation followed by treatment with 3% H_2O_2. After three times washing with PBS, tissue sections were incubated with blocking agent (5% BSA + 5% normal goat serum) for an hour at room temperature, followed by incubation with rabbit anti *E.coli* (Abcam, Cambridge, UK ab68451). Sections were then rinsed with PBS and incubated with anti rabbit FITC conjugated secondary antibody (dilution 1:1000) in dark. The sections were washed thrice with PBS for 10 min each, and then mounted with Vectashield mounting medium containing DAPI. Photographs were taken using a fluorescence microscope (Leica).

Real Time PCR: RNA was isolated from the caput, cauda and testis obtained from sham operated and UPEC infected rats. 2 µg of RNA was reverse transcribed and the resulting cDNA was used for real time RT-PCR analysis using Power SYBR Green PCR

master mix kit (Applied Biosystems, Warrington, UK) in a real time thermal cycler (Applied Biosystems). *Spag11c, Spag11e, Spag11t*, *Defb1, Defb2*, *Defb21, Defb24* and *Defb27* was studied using the gene specific primers (Table 3.1). *β-actin* expression was used as the internal control. A typical real time PCR reaction was carried out with the following conditions: initial denaturation at 94^0C for 10 min; 40 cycles with 94^0C for 15s and 60^0C for 1 min. After 40 cycles, melting curve analyses was performed to confirm the absence of non-specific amplification. Fluorescence data was obtained after the end of second step (60^0C for 1 min) in each cycle. Negative controls (no template control) and minus RT control (only with RNA) were included in the assays to detect non specific amplification. The amplicons were sequenced to confirm their identity.

Western Blotting: Tissues obtained from control and UPEC infected rats were lysed using RIPA lysis buffer (25 mM Tris-HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% Sodium deoxycholate and 0.1% SDS) supplemented with proteinase cocktail inhibitor. The concentration of protein in lysates was determined by the Bradford's method. 100 µg protein of each sample was resolved on SDS-polyacrylamide gel and protein was electrophoretically transferred onto a 0.2 µm pore size nitrocellulose membrane (Millipore). The nonspecific binding sites were blocked by incubating the membranes in 5% non-fat milk in Tris-buffered saline (TBS) with 0.1% Tween (TBS-T) for 1 h at room temperature. The membranes were probed with primary antibodies specific to phospho P65, P65, phospho IkBα, IkBα, Histone Deaceylase 1 (HDAC1) and methylated histone 3 at lysine 36 (me3H3K36) in blocking solution overnight at 4°C and then rinsed three times with TBS-T. Membranes were then incubated with anti-mouse or anti-rabbit secondary antibody conjugated with HRP

for 1 h at room temperature and then rinsed three times with TBS-T for 10 min. Immunoreactive proteins were detected using an ECL prime Western blot detection kit (GE Healthcare Life Sciences, MA, USA), according to the manufacturer's instructions.

Recombinant Protein Production: Rat defensin 21 cDNA sequence without the signal peptide was cloned directionally between EcoRI and SalII sites of the pQE30 expression vector and the recombinant protein expressed in *E.coli* BL21. Briefly, *E. coli* transformed with the vector were grown to exponential phase in nutrient-rich liquid medium and the recombinant protein expression was induced by addition of 50 mM isopropyl beta-D-1-thiogalactopyranoside for 3h. Bacterial cells collected by centrifugation were lysed in 6 M guanidine-HCl. Defensin 21 fused to 6X His tag was purified by affinity chromatography on nickel-NTA columns**.**

Immunohistochemistry: Immunostaining for SPAG11E was performed in the caput of sham operated and UPEC infected animals using standard protocol described earlier (Chapter 1).

Statistical analysis: Statistical analyses were performed using ANOVA and Student's t-test available in Sigma Plot software (SPSS Inc., Chicago, IL, USA). Values shown are mean ± S.D. To maintain simplicity and avoid confusion in the figures, for every gene analyzed, data points that show any significance level were assigned the same symbol. Further, data points (1, 3, 5 and 7 days) were compared only with the uninfected control.

RESULTS

Epididymo-orchitis

Epididymo-orchitis was successfully induced by injecting UPEC bilaterally in to the vasa deferentia. Morphologically, swelling of the scrotal sac was observed five to seven days after UPEC infection (Figure 3.1). Swollen epididymides, atrophic and inflamed testes with increased vascularization and vasocongestion were observed in UPEC injected rats. Reddening and oedema formation was observed in the testis indicating Orchitis (Figure 3. 2).

To verify whether UPEC is able to reach the testis by ascending canalicular infection originating at the vas deferens, the presence of bacteria in the epididymis and testis was analysed by colony forming unit assay, PCR and fluorescence microscopy. PCR amplicon (328 bp) obtained using specific primers to UPEC *P pili* was detected in the epididymis and testes at all the time points analysed after infection (Figure 3.3). CFU assay revealed the presence of UPEC starting from one day after infection and the number of colonies formed increased in a time dependent manner until the seventh day after infection (Figure 3.4). Increase in the number of immunofluorescent foci that represent UPEC was observed in a time dependent manner in the caput, cauda and testis obtained from rats infected with UPEC (Figure 3.5). Taken together, these results suggest that UPEC injected in to the vas deferens migrates upward to infect the epididymis and testis, thereby causing epididymo-orchitis.

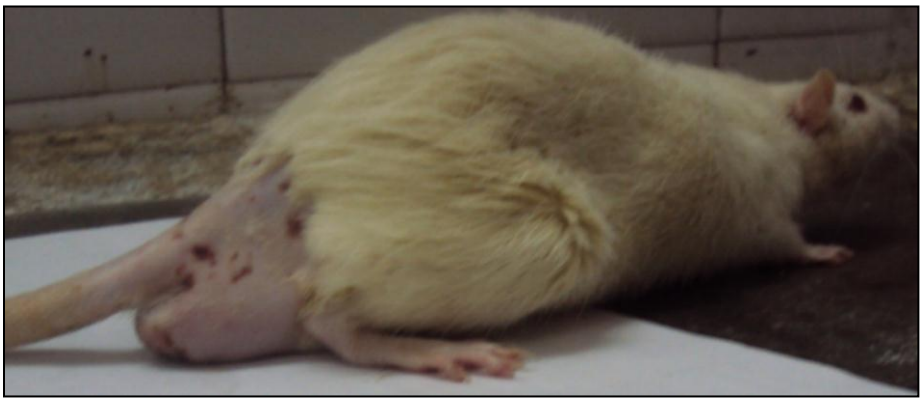

Figure 3.1: Development of epididymo-orchitis in rat. Adult male Wistar rats were injected 50µl of UPEC ($1X10^5$) suspension in the vas deferens and the development of epididymo- orchitis was monitored.

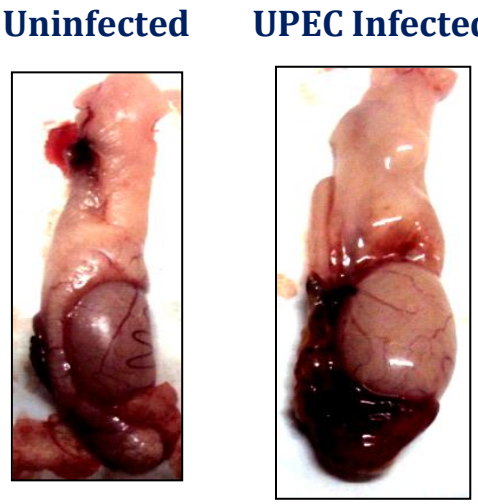

Figure 3.2: Morphological features of the epididymis and testis due to epididymo-orchitis.

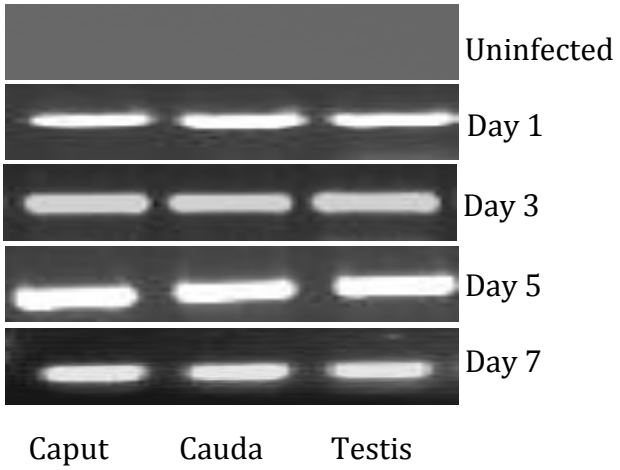

Figure 3.3: PCR detection of UPEC. Homogenates of epididymides and testes obtained from uninfected and UPEC infected rats were diluted and amplified by PCR. Amplification was performed for UPEC P pili Primers.

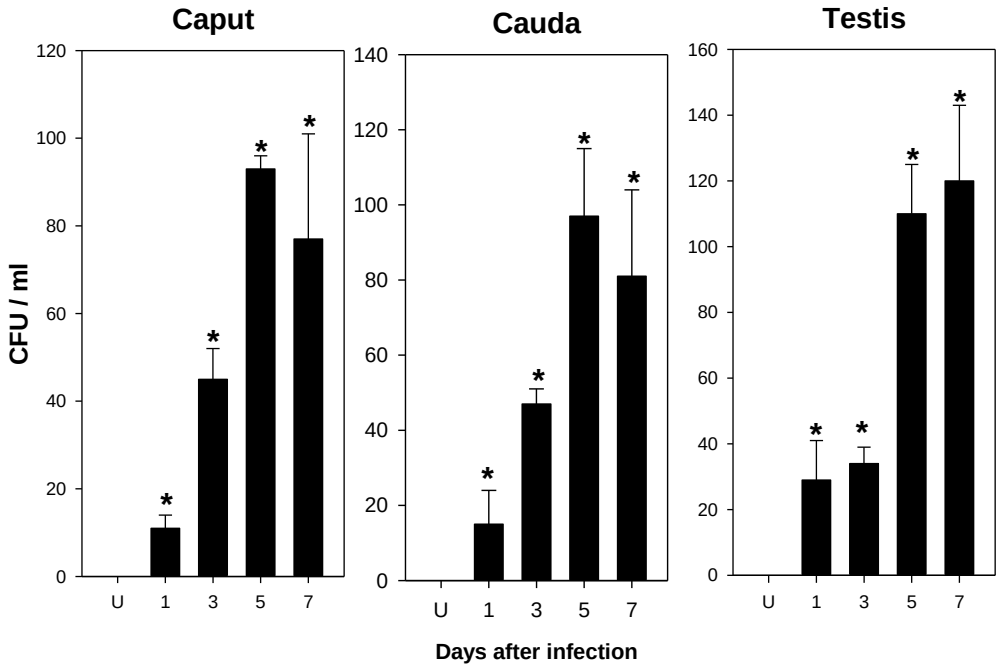

Figure 3.4: Colony forming unit assay for UPEC. Caput, Cauda and Testes from uninfected and UPEC infected rats were homogenized in PBS, and serially diluted. 100µl of the diluted homogenates were plated on agar plates without antibiotics and kept at 37^0C. After overnight incubation, colonies were hand counted.

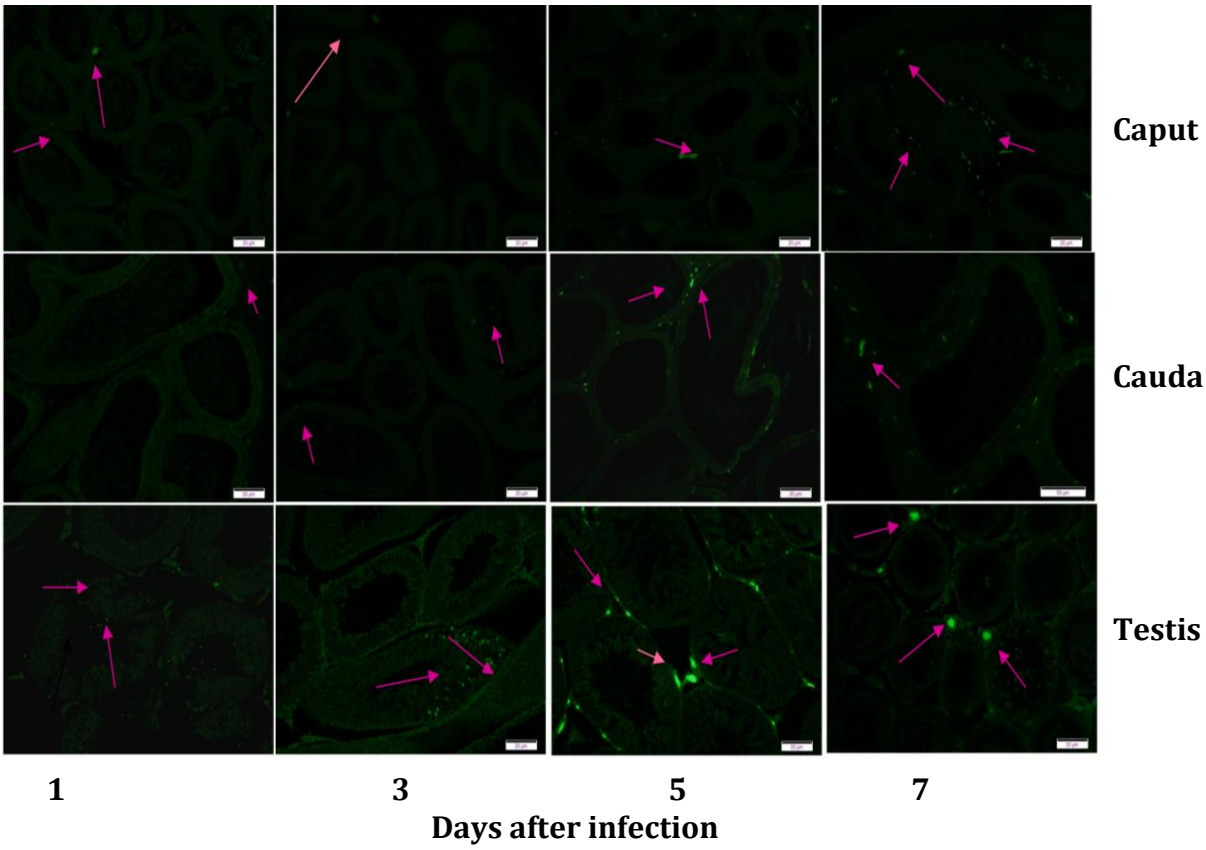

Figure 3.5: Localisation of UPEC by fluorescence microscopy. Sections of caput, cauda and testes from UPEC infected rats were incubated with anti -*E. coli* antibody and anti-rabbit IgG antibody conjugated to FITC.

Antimicrobial gene expression

To determine the antimicrobial responses in a rat epididymo-orchitis model, mRNA expression of defensins and *Spag11* genes were analysed using real time PCR. In the epididymis *Defb1*, *Defb2* and *Defb21* expression was induced in a biphasic manner, significant increases at day 1 and day 5 post infection (Figure 3. 7). *Defb24* expression was significantly increased at 1 and 3 days after infection. No significant changes in *Defb27* expression was observed at all the time points (Figure 3.7). *Spag11e* and *Spag11c* expression was significantly increased within one day after infection with a decline at the later time points. *Spag11t* expression was induced significantly in a biphasic manner with steep increase at day 1 and day 5 post infection Figure 3. 8).

In the testis, the expression of the majority of defensins analysed was upregulated within 1 day after UPEC infection. *Defb1*, *Defb2* and *Defb21* expression was significantly induced within 1 day and declined at the later time points with the exception of *Defb2* (Figure 3.9). *Spag11t* and *Spag11c* expression was induced 1 day after infection (Figure 3.10). Taken together, these data suggest that defensin and *Spag11* mRNA expression is induced, which could be one of the immune responses by the male reproductive tract in response to UPEC infection.

Table 3.1: List of primers used for this study

Primer	Sequence (5'-3')	Purpose
UPEC Pilli F	GACGGCTGTACTGCAGGGTGTGGCG	PCR
UPEC Pilli R	ATATCCTTTCTGCAGGGATGCAATA	
Spag11e F	TTCAAGTGGGGAGAACCTTGA	DNA Methylation
Spag11e R	CTTCTGGCCACACTCTTT	
Dnmt1 F	ACTATTGGAAGGTGAGCATCGACG	Real Time PCR
Dnmt1 R	CAGAACCAGTGTGCATGGAACATC	
Defb1 F	GACCCTGACTTCACCGACAT	Real Time PCR
Defb1 R	CCTGCAACAGTTGGGCTTAT	
Defb2 F	ATTTCTCCTGGTGCTGCTGT	Real Time PCR
Defb2 R	TCCACAAGTGCCAATCTGTC	
Defb21 F	ATACCTGGATCTACTGTCCTACCT	Real Time PCR
Defb21 R	TTATGTGTCCATCCGTGAAGTC	
Defb24 F	GTCATCACCTTCACCCCGGGA	Real Time PCR
Defb24 R	CAGCTTCTCTGGAAGTCTGTGCAT	
Defb27 F	CACGAGGAACACCCTGGATTTCC	Real Time PCR
Defb27 R	TGCCTAGGTCCACCTTCGTTTCTG	
Defb30 F	GAGTGACTTTCCTTTCCTCAG	Real Time PCR
Defb30 R	TCAGAATTCCCAGAGGAACCCTGGA	
Spag11t F	CTGCAGTCCCCTCCACAGCC	Real Time PCR
Spag11t R	CATCCACGCTGTCACCTCCC	
Spag11c F	GTGTGCAGGTCACTTCAACTTC	Real Time PCR
Spag11c R	CATCCACGCTGTCACCTCCC	
Spag11e F	CACATCTGCTTTCCTGCACAG	Real Time PCR
Spag11e R	GCACCCCACATCTCAGATCTTC	

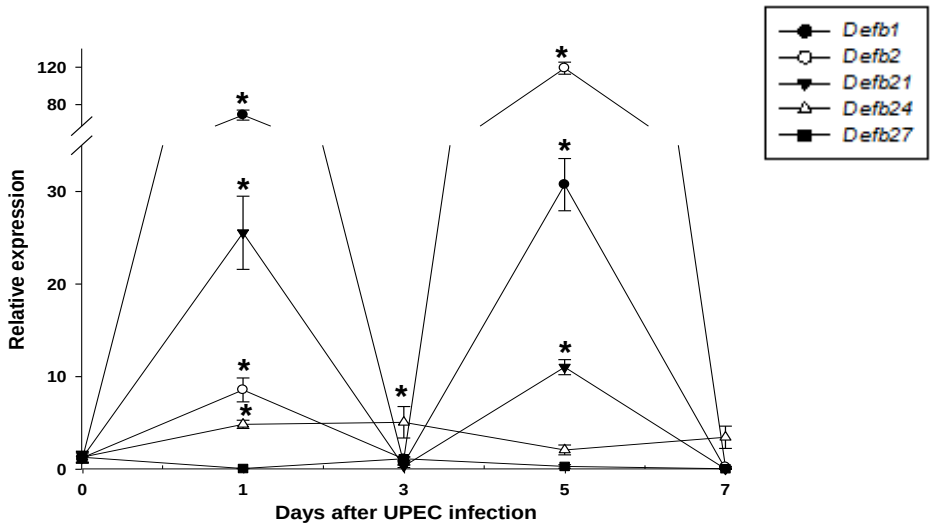

Figure 3.7: Defensin gene expression in response to UPEC infection in epididymis. Caput epididymis was collected from uninfected and UPEC infected rats (1-7 days). RNA was isolated from the tissues collected at 0, 1, 3, 5 and 7day after infection and defensin mRNA expression analysed by real time PCR. Values shown are mean±SD. *p<0.05 compared to untreated control.

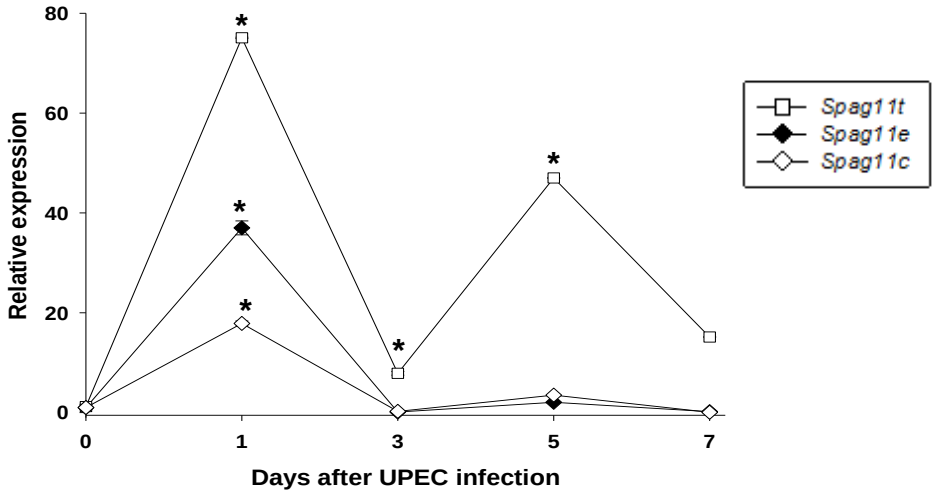

Figure 3.8: *Spag11* gene expression in response to UPEC in epididymis. Caput was obtained from control and UPEC infected rats. RNA was isolated from the tissues collected at 0, 1, 3, 5 and 7day after infection and *Spag11* mRNA expression analysed by real time PCR. Values shown are mean ±SD. *p<0.05 compared to untreated control.

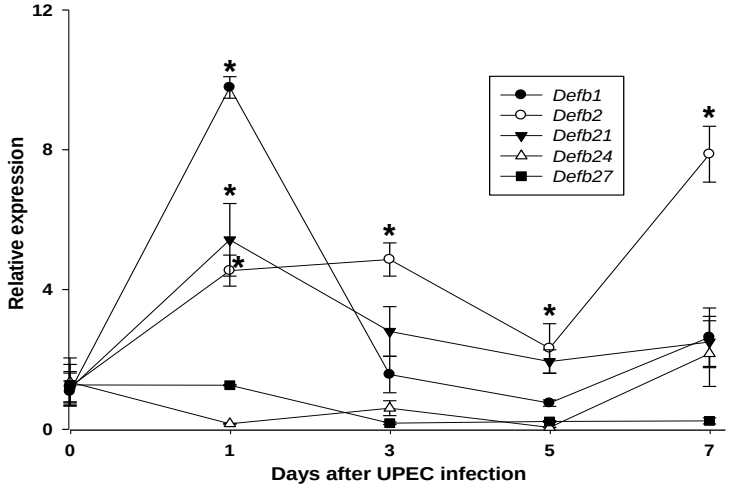

Figure 3.9: Defensin gene expression in the testis. Testes were collected from uninfected and UPEC infected rats. RNA was isolated from the tissues collected at 0, 1, 3, 5 and 7day after infection and defensin expression analysed by real time PCR. Values shown are mean ±SD. * p<0.05 compared to untreated control.

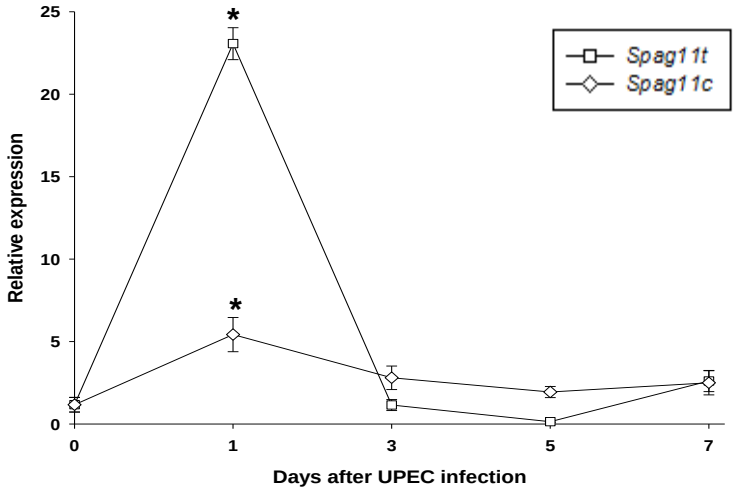

Figure 3.10: *Spag11 g*ene expression in response to UPEC in testis. Testes were collected from uninfected and UPEC infected rats (1-7 days). RNA was isolated from the tissues collected after 0, 1, 3, 5 and 7day after infection and defensin expression analysed by real time PCR. Values shown are mean ±SD. *p<0.05 compared to untreated control.

Immunohistochemistry

To determine whether antimicrobial protein expression is also induced consistent with the mRNA induction observed after UPEC infection, SPAG11E was detected using immunohistochemical staining. Intense staining was detected for SPAG11E within one day after UPEC infection, when compared to the uninfected control (Figure 3.11). The intensity of staining decreased at the later time points.

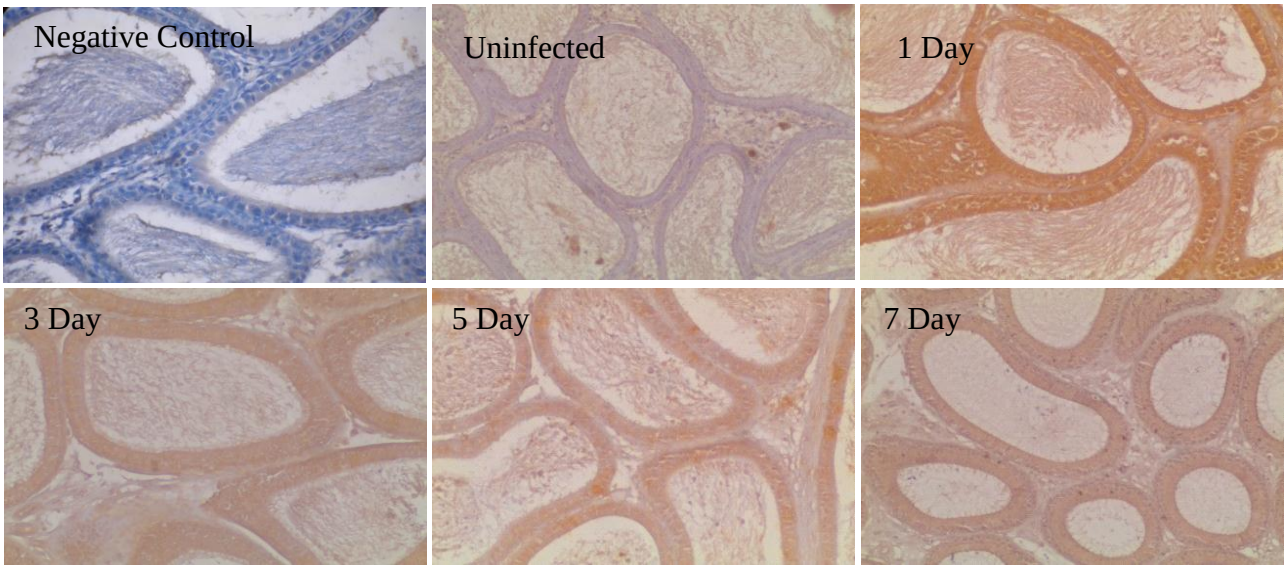

Figure 3.11: Immunhistochemical analysis to detect SPAG11E in the epididymis. Caput epididymides were serially sectioned and the expression of SPAG11E was detected by probing with specific antibodies followed by development with Diaminobenzamidine (DAB).

NF-κB signalling

The NF-κB signalling pathway plays a pivotal role in innate immune responses. To determine whether antimicrobial responses induced during UPEC infection may involve activation of NF-kB, the levels of phospho p65 was analysed by immunoblotting. In both epididymis and testis, the levels of phosphorylated p65 were significantly increased within one day after infection (Figure 3.12). p65 translocation into the nucleus requires IkB phosphorylation, which is effected by the phosphorylated form of IkB-alpha. We also observed significant increase in the levels of phosphorylated IkB-α within one day after UPEC infection followed by a decline (Figure 3.12). These results indicate that defensin and *Spag11e* gene regulation during UPEC infection could be mediated by NF-kB signaling pathway.

Epigenetic regulation

Gene expression is under the tight control of epigenetic modification, such as histone deacetylation and methylation. In order to determine whether defensin expression during UPEC infection are influenced by epigenetic factors, we analysed the levels of HDAC1, the enzyme that deacetylates histones to inhibit gene transcription, UPEC infection resulted in significantly decreased levels of HDAC1 levels in a time dependent manner in both epididymis and testis. (Figure 3.13). Methylation of histone 3 at lysine 36 is generally associated with transcriptionally active chromatin to favour gene transcription. Increased levels of me3H3K36 were observed in the epididymis and testis after UPEC infection (Figure 3.13). These results indicate that defensin and *Spag11e* gene induction may also be regulated by epigenetic modifications. Besides histone modifications, alteration in DNA methylation status influences gene expression. To determine whether antimicrobial

gene expression induced in the male reproductive tract involves changes in DNA methylation status, *Spag11e* upstream gene was sequenced in the DNA obtained from caput of control and infected rats. A time dependent increase in the demethylation of *Spag11e* upstream region was observed (Figure 3.14). Increased demethylation was consistent with the decreased levels of *Dnmt1* levels, suggesting that during infection in the male reproductive tract, antimicrobial gene expression could be influenced by DNA modifications.

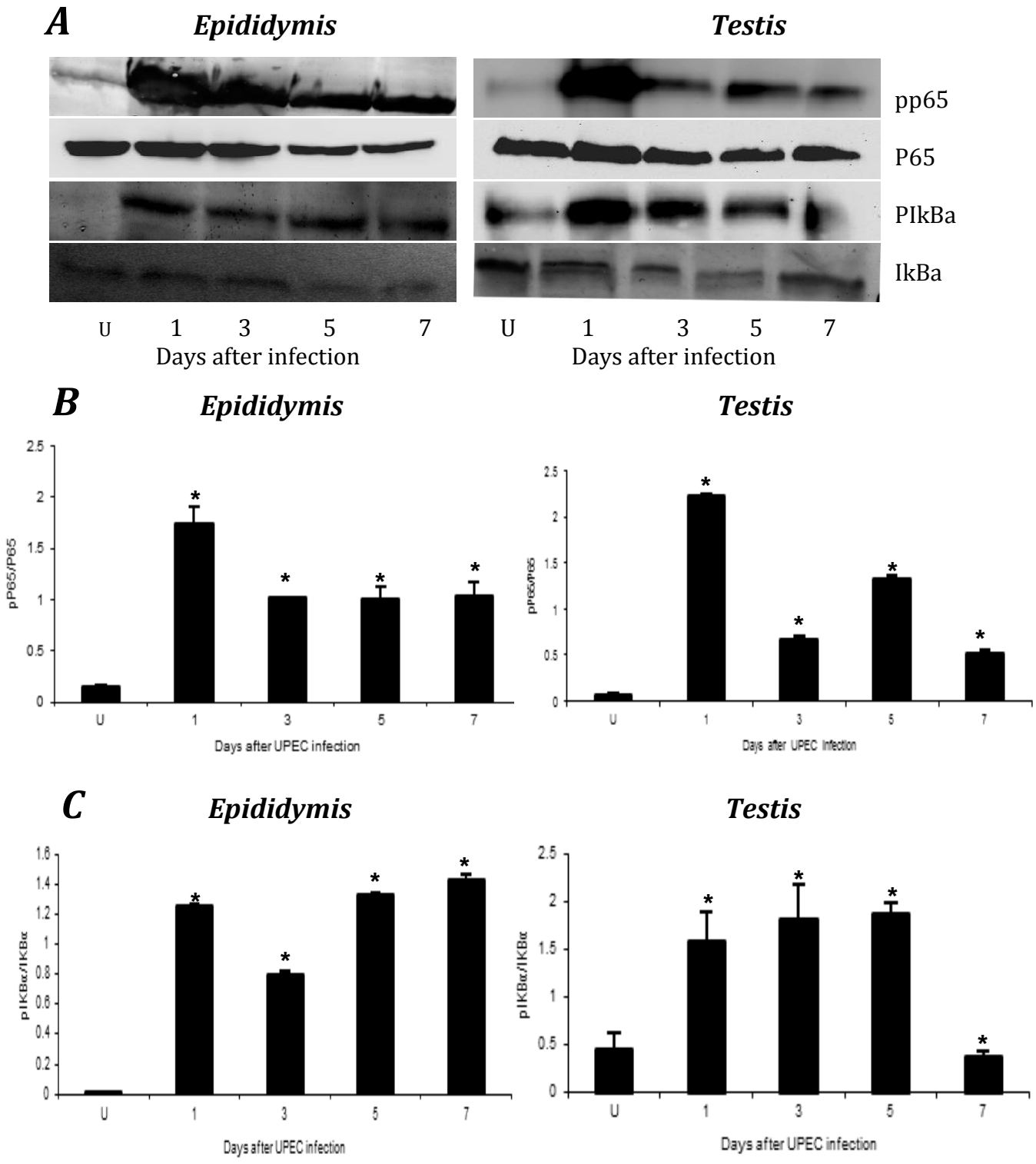

Figure 3.12: NF-kB activation in the male reproductive tract during UPEC infection.

[A] Caput and testis were obtained at different time intervals from adult Wistar rats injected with UPEC or PBS. Tissues were then homogenized and the lysate was separated on SDS-PAGE and Western blotting performed using monoclonal antibodies specific to phosphoP65 and phosphoIkBα, p65, IkBa. Densitometric analysis for the Western blots shown to determine phospho p65 **[B]** and Phospho IKBα **[C]** protein expression in the epididymis and testis after UPEC infection. Values shown are mean ±S.D. *p<0.05 compared to untreated control.

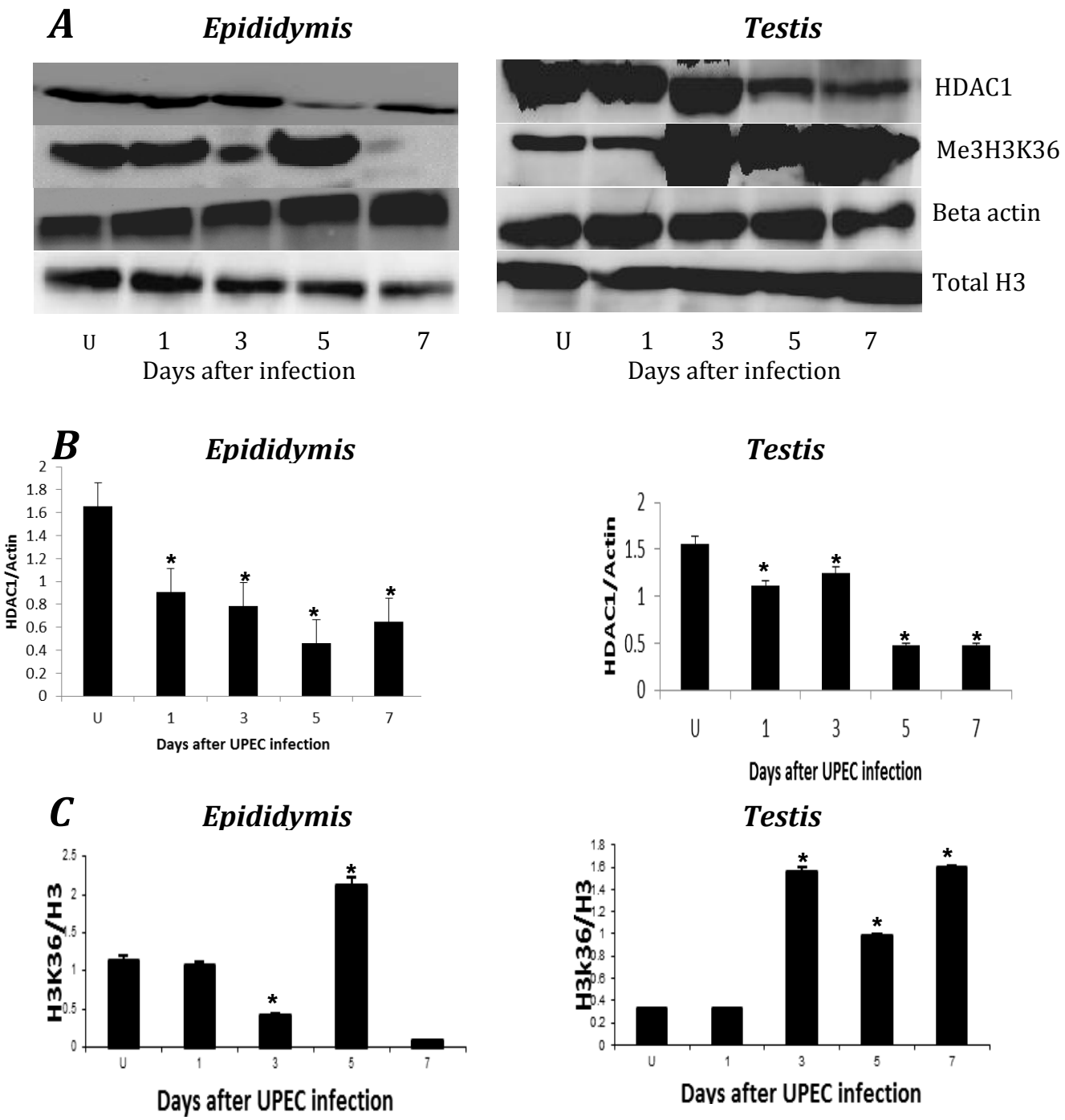

Figure 3.13: Histone deacetylation and Histone methylation in the male reproductive tract tissues during UPEC infection. [A] Caput and testis were obtained at different time points from adult Wistar rats injected with UPEC or PBS. Tissues were then homogenized and the lysate was separated on SDS-PAGE and Western blotting performed using monoclonal antibodies specific to HDAC1, Me3H3K36, total H3 and beta actin. Densitometric analysis for the Western blots shown to determine HDAC1 **[B]** and Phospho IKBα **[C]** protein expression in the epididymis and testis after UPEC infection. Values shown are mean ±S.D. *p<0.05 compared to untreated control.

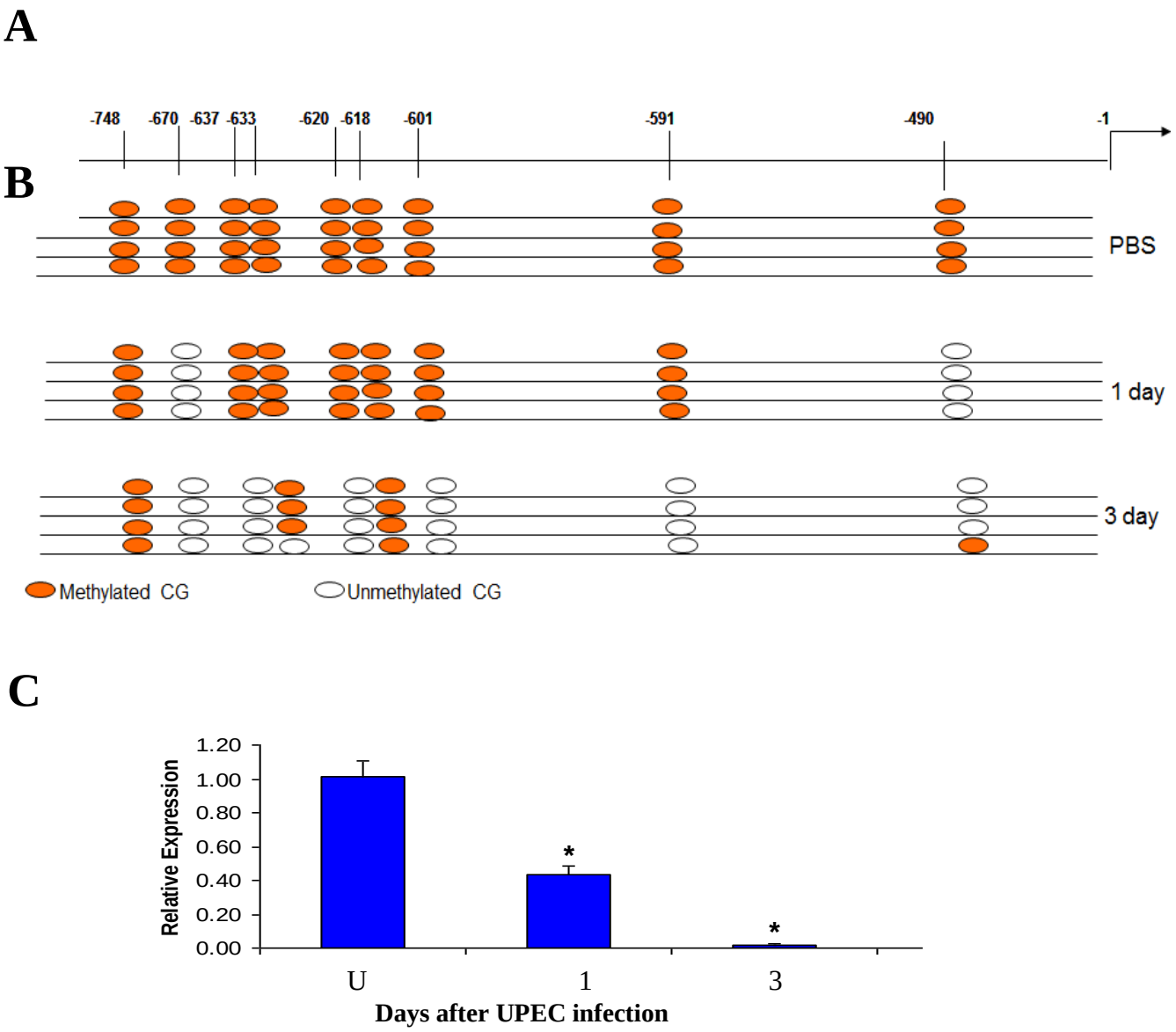

Figure 3.14: Involvement of DNA methylation and DNMT1 during UPEC induced antimicrobial gene expression. [A] Location of CpG dinucleotide in the *Spag11e* upstream region. **[B]** CG methylation status in the *Spag11e* upstream sequence. DNA isolated from the caput of PBS control and UPEC infected rats was sequenced to determine the methylation status of CGs . ⬭ indicates methylated and ⬭ indicates unmethylated. **[C]** Real time PCR expression for *Dnmt1* using RNA isolated from the caput tissue of UPEC infected animals. *p<0.05 compared to untreated control.

Efficacy of DEFB21 to contain UPEC infection

The potential of DEFB21 to contain or eliminate UPEC infection in the male reproductive tract was analyzed by administering the protein intravenously to infected rats. As a first step, the *in vivo* toxicity of DEFB21 was determined by injecting 5, 10, 20 and 40 mg/kg body weight of DEFB21 recombinant protein. No mortality was observed when DEFB21 was administered up to 20 mg/kg, whereas 40 mg/kg resulted in 80% mortality within 3 days after injection. Hence further experiments were conducted with 20 mg/kg dose. As a positive control, a separate group of rats infected with UPEC were treated with 5 mg/kg body weight gentamycin.

CFU assay revealed that the number of UPEC in the caput, cauda and testis of infected rats treated with DEFB21 were significantly decreased when compared with the control (Figure 3.15). Similar results were observed in reproductive tissues obtained from UPEC infected rats treated with gentamycin. Interestingly, the number of CFU in the DEFB21 group was lesser than the gentamycin treated group. Immunofluorescence analyses revealed localization of UPEC in the control group. In the DEFB21 and gentamycin groups, there was complete absence of localization of UPEC. These results taken together suggest that DEFB21 has the potential to limit or contain reproductive tract infections caused by UPEC.

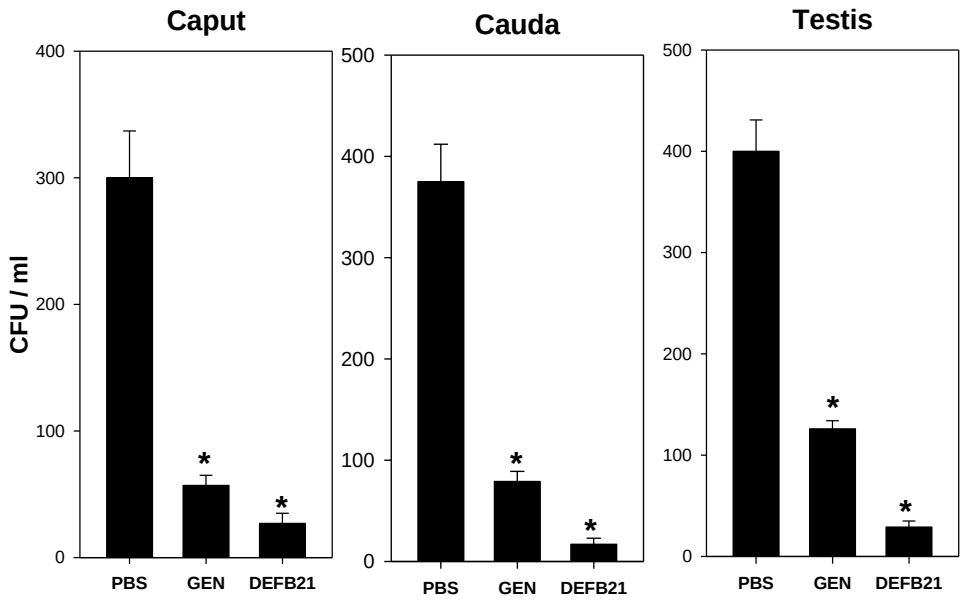

Figure 3.15: Colony forming unit assay in UPEC infected rats treated with Defensin21. Caput, cauda and testis obtained from UPEC infected rats treated with gentamycin or Defensin21 were homogenized in PBS, and serially diluted. 100µl of the diluted homogenates were plated on agar plates without antibiotics and kept at 37^0C. After overnight incubation, colonies were hand counted. Values shown are mean ±S.D. *$p<0.05$ compared to untreated control.

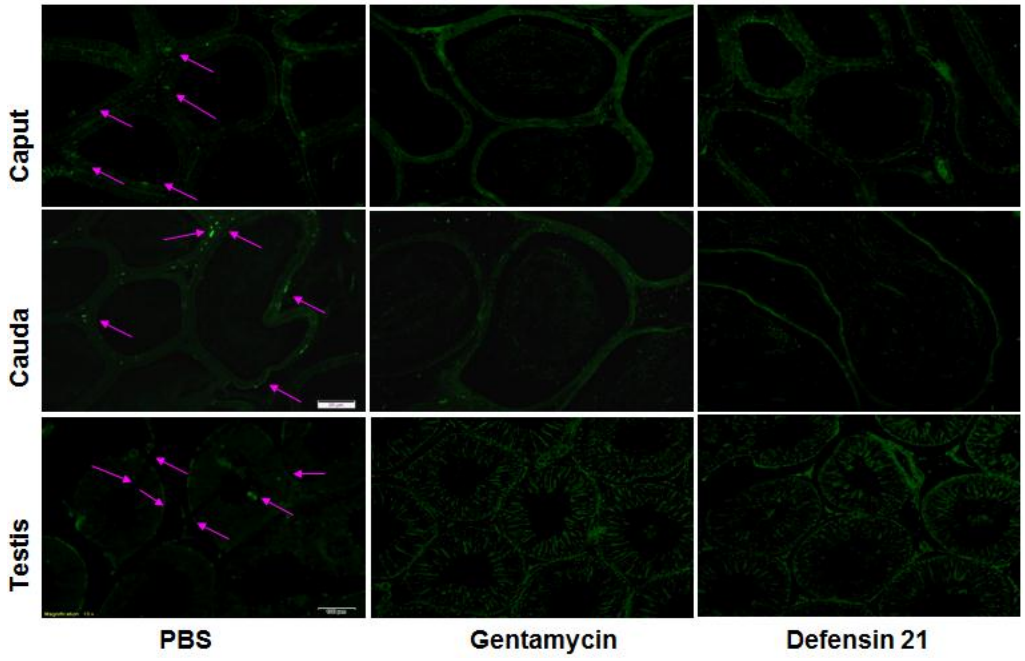

Figure 3.16: Localisation of UPEC in infected rats treated with Gentamycin or Defensin 21. Sections of caput, cauda and testes from UPEC infected rats treated with Gentamycin or Defensin21 were incubated with anti -*E. coli* antibody and anti-rabbit IgG antibody conjugated to FITC. The nuclei were counterstained with DAPI (Blue).

DISCUSSION

The rationale of our study was based on our previous reports that defensins and SPAG11 proteins of the male reproductive tract possess potent antimicrobial activity and that lipopolysaccharide induces their expression regulated by NF-kB signaling and epigenetic factors (Biswas and Yenugu 2012). However, the role of defensins and SPAG11 proteins in an infection model, especially that of the male reproductive tract is not yet known. We successfully generated a rat model of epididymo-orchitis by injecting UPEC into the vas deferentia. The presence of UPEC in the epididymis and testis indicates that the bacteria were able to cause retrograde infection, a condition similar to epididymitis patients (Nickel 2003; Curtis and Darren 2007). Uropathogenic *E. coli* induced epididymo-orchitis is reported earlier (Ludwig, Johanness et al. 2002). The mode of retrograde infection observed in our study is in agreement with the earlier reports (Ludwig, Jantos *et al*. 1997) suggesting that the epididymo-orchitis model we have established is a suitable model system to study antimicrobial responses during reproductive tract infections.

The male reproductive system being a sterile organ system is expected to initiate an array of immune reponses during endotoxin or pathogen challenge. Due to the development of development of antibiotic resistance to conventionally used antibiotics, attempts to identify host antimicrobial proteins or peptides that respond to infections have gained importance. In this study, we demonstrate that UPEC challenge induces defensin and *Spag11* gene expression indicating that epididymal and testicular antimicrobial genes have a role in innate immune responses in this organ system. Endotoxins influence defensins and *Spag11* expression in the male reproductive tract. LPS induced epididymitis down regulates Spag11e expression

with a concomitant decrease in sperm function (Cao, Li *et al*. 2010). Further, defensin 1 and 2 expression was altered in the testis of rats challenged with endotoxin (Palladino, Mallonga *et al*. 2003). Our previous studies also indicated induction of defensins and *Spag11e* expression both *in vitro* and *in vivo* (Biswas and Yenugu 2011; Biswas and Yenugu 2013). In this study, it is interesting to note that a biphasic induction of defensins genes was observed in the epididymis. Gene expression in the male reproductive tract is largely androgen dependent (Rodriguez, Kirby *et al*. 2001). Further, altered levels of serum and tissue testosterone were observed in rats challenged with endotoxins (Reddy, Mahipal et al. 2006; Biswas and Yenugu 2012). The biphasic expression could be due to changes in androgen status post infection. On the other hand, *Spag11* expression in the epididymis and antimicrobial gene expression in the testis after UPEC infection did not display a biphasic pattern, with majority of them being induced within 1 day after infection. Though androgen status influences gene expression in the male reproductive tract and it is expected that there should be a correlation with testosterone levels, it was earlier reported that under conditions of endotoxin challenge, antimicrobial expression seems to become androgen independent (Biswas and Yenugu 2011). The variations observed in the epididymis and testis could be due to differential responsiveness. Consistent with *Spag11e* mRNA induction, increased intensity was detected for SPAG11E protein in the caput of UPEC infected rats, suggesting that antimicrobial responses also occur at the protein level.

Gene expression under a variety of stress conditions is mediated by NF-kB. Its binding to DNA has been shown to regulate transcription during endotoxin challenge (Han SJ 2002). In this study, increased phosphorylation of p65 and IkB-α after

UPEC infection in both epididymis and testis indicates the involvement of NF-kB pathway. Phosphorylation and translocation of p65 in the male reproductive tissues during LPS challenge was reported (Rodrigues, Queiroz *et al*. 2008; Biswas and Yenugu 2013). However, this study, for the first time demonstrates that antimicrobial gene expression in the male reproductive tract during UPEC infection seems to be mediated by NF-kB signalling. Besides immune signalling pathways, epigenetic mechanisms play a significant role in modulating genes involved in innate immunity (Ito 2007). We observed decreased levels of HDAC1 expression in the epididymides and testes obtained from UPEC infected rats. These results indicate that decreased levels of HDAC1 may allow histone acetylation, which in turn may favour defensin and *Spag11* expression. The role of HDACs in modulating gene expression in response to pathogens is reported (Mombelli, Lugrin *et al*. 2011). Since histone deacetylation is regulated by many HDACs, the possibility of other HADCs in regulating defensins and Spag11 gene expression cannot be ruled out and further studies are required. Increased demethylation in *Spag11e* upstream region after infection could be one of the innate immune mechanisms in the male reproductive tract to allow increased antimicrobial gene expression to successfully eliminate the pathogens.

Due to emergence of antibiotic resistance, novel approaches to treat infections including use of antimicrobial proteins and peptides have gained importance. Lactoferrin, hCAP18, CAP37, LL37, protegrins and SLPI have been analyzed for their ability to limit infections in different model systems (Brackett, Lerner *et al*. 1997; Giacometti, Cirioni *et al*. 2003; Ohgami, Ilieva *et al*. 2003; Takakura, Wakabayashi *et al*. 2003; Cirioni, Giacometti *et al*. 2006; Williams, Brown

et al. 2006). Further, a number of antimicrobial proteins and peptides have entered clinical trials with some benefits (Levy 2004). To the best of our knowledge, the potential of male reproductive tract defensins to limit infections is not reported. We tested the ability of rat DEFB21 recombinant protein to limit infection in a rat epididymo-orchitis model. The advantage of using an antimicrobial protein belonging to the same species may not induce antibodies thereby allowing more bioavailability. Preliminary results showed that DEFB21 significantly decreased bacterial load in the epididymis and testis. Antimicrobial peptides are known to be immunostimulatory or endotoxin neutralizing agents (Gordon, Romanowski *et al*. 2005). Further, male reproductive tract defensins exhibit their antibacterial activity by membrane permeabilization was demonstrated earlier (Yenugu, Hamil et al. 2004). In this study, it is possible that DEFB 21 could induce responses that may strengthen the immune system to clear UPEC or may affect direct killing of the pathogen. However, the mechanism by which DEFB21 reduced bacterial load and its bioavailability in different tissues to eliminate the pathogen needs further investigation. This study provides a base to initiate experiments to test whether antimicrobial peptides of the male reproductive tract can be used in combination with conventional antibiotics or agents that strengthen the innate immune system for better management of reproductive tract infections.

In conclusion, we report that UPEC induced defensin and Spag11 gene expression in the male reproductive tract involves NF-kB activation and epigenetic regulation. Recombinant DEFB21 displayed potent ability to limit bacterial load in a rat model of epididymo-orchitis. Results of this study provide a clue to design novel peptide antibiotics to effectively treat reproductive tract infections.

REFERENCES

Akira, S. and K. Takeda (2004). "Toll-like receptor signalling." Nature Reviews. Immunology 4(7): 499-511.

Akira, S., K. Takeda, et al. (2001). "Toll-like receptors: critical proteins linking innate and acquired immunity." Nat Immunol 2(8): 675-680.

Avellar, M. C., L. Honda, et al. (2004). "Differential expression and antibacterial activity of epididymis protein 2 isoforms in the male reproductive tract of human and rhesus monkey (Macaca mulatta)." Biol Reprod 71(5): 1453-1460.

Biswas, B. and S. Yenugu (2011). "Antimicrobial responses in the male reproductive tract of lipopolysaccharide challenged rats." American Journal of Reproductive Immunology 65(6): 557-568.

Biswas, B. and S. Yenugu (2012). "Lipopolysaccharide induces epididymal and testicular antimicrobial gene expression in vitro: insights into the epigenetic regulation of sperm-associated antigen 11e gene." Immunogenetics.

Biswas, B. and S. Yenugu (2013). "Lipopolysaccharide Induces Epididymal and Testicular Antimicrobial Gene Expression In Vitro: Insights Into The Epigenetic Regulation of Sperm Associated Antigen 11e Gene." Immunogenetics in press.

Bourgeon, F., B. Evrard, et al. (2004). "Involvement of semenogelin-derived peptides in the antibacterial activity of human seminal plasma." Biol Reprod 70(3): 768-774.

Brackett, D. J., M. R. Lerner, et al. (1997). "A synthetic lipopolysaccharide-binding peptide based on the neutrophil-derived protein CAP37 prevents endotoxin-induced responses in conscious rats." Infection and Immunity 65(7): 2803-2811.

Cao, D., Y. Li, et al. (2010). "Lipopolysaccharide-induced epididymitis disrupts epididymal beta-defensin expression and inhibits sperm motility in rats." Biology of Reproduction 83(6): 1064-1070.

Cirioni, O., A. Giacometti, et al. (2006). "LL-37 protects rats against lethal sepsis caused by gram-negative bacteria." Antimicrobial Agents and Chemotherapy 50(5): 1672-1679.

Com, E., F. Bourgeon, et al. (2003). "Expression of antimicrobial defensins in the male reproductive tract of rats, mice, and humans." Biol Reprod 68(1): 95-104.

Curtis, N. J. and B. T. Darren (2007). Prostatitis, orchitis, and epididymitis., Lippincott Williams & Wilkins.

Eggert-Kruse, W., G. Rohr, et al. (1995). "Anaerobes in ejaculates of subfertile men." Human Reproduction Update 1(5): 462-478.

Fei, Z., S. Hu, et al. (2012). "mBin1b transgenic mice show enhanced resistance to epididymal infection by bacteria challenge." Genes Immun 13(6): 445-451.

Giacometti, A., O. Cirioni, et al. (2003). "Administration of protegrin peptide IB-367 to prevent endotoxin induced mortality in bile duct ligated rats." Gut 52(6): 874-878.

Gordon, Y. J., E. G. Romanowski, et al. (2005). "A review of antimicrobial peptides and their therapeutic potential as anti-infective drugs." Current Eye Research 30(7): 505-515.

Gupta, K., T. M. Hooton, et al. (1999). "The prevalence of antimicrobial resistance among uropathogens causing acute uncomplicated cystitis in young women." International Journal of Antimicrobial Agents 11(3-4): 305-308.

Gupta, K., D. Scholes, et al. (1999). "Increasing prevalence of antimicrobial resistance among uropathogens causing acute uncomplicated cystitis in women." JAMA 281(8): 736-738.

Hall, S. H., K. G. Hamil, et al. (2002). "Host defense proteins of the male reproductive tract." J Androl 23(5): 585-597.

Hamil, K. G., Q. Liu, et al. (2003). "LCN6, a novel human epididymal lipocalin." Reprod Biol Endocrinol 1(1): 112.

Hamil, K. G., Q. Liu, et al. (2002). "Cystatin 11: a new member of the cystatin type 2 family." Endocrinology 143(7): 2787-2796.

Hamil, K. G., P. Sivashanmugam, et al. (2000). "HE2beta and HE2gamma, new members of an epididymis-specific family of androgen-regulated proteins in the human." Endocrinology 141(3): 1245-1253.

Han SJ, K. H., Choi JH, Seo KH, Lee HS, Choi EK, Choi IW, Lee HK, Im SY. (2002). "Molecular mechanisms for lipopolysaccharide-induced biphasic activation of nuclear factor-kappa B (NFkappaB)." J Biol Chem 277(44715-44721).

Hawes, S. E. and N. B. Kiviat (2002). "Are genital infections and inflammation cofactors in the pathogenesis of invasive cervical cancer?" Journal of the National Cancer Institute 94(21): 1592-1593.

Hsia, N. and G. A. Cornwall (2003). "Cres2 and Cres3: new members of the cystatin-related epididymal spermatogenic subgroup of family 2 cystatins." Endocrinology 144(3): 909-915.

Ito, K. (2007). "Impact of post-translational modifications of proteins on the inflammatory process." Biochemical Society Transactions 35(Pt 2): 281-283.

Jiang, Y., D. Yang, et al. (2012). "Antiviral activity of recombinant mouse beta-defensin 3 against influenza A virus in vitro and in vivo." Antiviral Chemistry and Chemotherapy 22(6): 255-262.

Levy, O. (2004). "Antimicrobial proteins and peptides: anti-infective molecules of mammalian leukocytes." Journal of Leukocyte Biology 76(5): 909-925.

Li, P., H. C. Chan, et al. (2001). "An antimicrobial peptide gene found in the male reproductive system of rats." Science 291(5509): 1783-1785.

Liao, M., P. S. Ruddock, et al. (2005). "Cationic peptide of the male reproductive tract, HE2{alpha}, displays antimicrobial activity against *Neisseria gonorrhoeae*, *Staphylococcus aureus* and *Enterococcus faecalis*." J Antimicrob Chemother 56(5): 957-961.

Ludwig, M., C. A. Jantos, et al. (1997). "Tissue penetration of sparfloxacin in a rat model of experimental Escherichia coli epididymitis." Infection 25(3): 178-184.

Ludwig, M., S. Johanness, et al. (2002). "Experimentia Escherichia coli epididymitis in rats: a mode to assess the outcome of antibiotic treatment." BJU International 90: 933-938.

Malm, J., O. Sorensen, et al. (2000). "The human cationic antimicrobial protein (hCAP-18) is expressed in the epithelium of human epididymis, is present in seminal plasma at high concentrations, and is attached to spermatozoa." Infect Immun 68(7): 4297-4302.

Merino, G., S. Carranza-Lira, et al. (1995). "Bacterial infection and semen characteristics in infertile men." Archives of Andrology 35(1): 43-47.

Mombelli, M., J. Lugrin, et al. (2011). "Histone deacetylase inhibitors impair antibacterial defenses of macrophages." Journal of Infectious Diseases 204(9): 1367-1374.

Nickel, J. C. (2003). "Chronic epididymitis: a practical approach to understanding and managing a difficult urologic enigma." Rev Urol 5(4): 209-215.

O'Neill, L. A. and C. A. Dinarello (2000). "The IL-1 receptor/toll-like receptor superfamily: crucial receptors for inflammation and host defense." Immunol Today 21(5): 206-209.

Ohgami, K., I. B. Ilieva, et al. (2003). "Effect of human cationic antimicrobial protein 18 Peptide on endotoxin-induced uveitis in rats." Investigative Ophthalmology and Visual Science 44(10): 4412-4418.

Palladino, M. A., T. A. Johnson, et al. (2007). "Members of the Toll-like receptor family of innate immunity pattern-recognition receptors are abundant in the male rat reproductive tract." Biol Reprod 76(6): 958-964.

Palladino, M. A., T. A. Mallonga, et al. (2003). "Messenger RNA (mRNA) expression for the antimicrobial peptides beta-defensin-1 and beta-defensin-2 in the male rat reproductive tract: beta-defensin-1 mRNA in initial segment and caput epididymidis is regulated by androgens and not bacterial lipopolysaccharides." Biol Reprod 68(2): 509-515.

Reddy, M. M., S. V. Mahipal, et al. (2006). "Bacterial lipopolysaccharide-induced oxidative stress in the impairment of steroidogenesis and spermatogenesis in rats." Reprod Toxicol 22(3): 493-500.

Roach, J. C., G. Glusman, et al. (2005). "The evolution of vertebrate Toll-like receptors." Proc Natl Acad Sci U S A 102(27): 9577-9582.

Rodrigues, A., D. B. Queiroz, et al. (2008). "Activation of toll-like receptor 4 (TLR4) by in vivo and in vitro exposure of rat epididymis to lipopolysaccharide from Escherichia Coli." Biology of Reproduction 79(6): 1135-1147.

Rodriguez, C. M., J. L. Kirby, et al. (2001). "Regulation of gene transcription in the epididymis." Reproduction 122(1): 41-48.

Shea, K. M. (2003). "Antibiotic resistance: what is the impact of agricultural uses of antibiotics on children's health?" Pediatrics 112(1 Pt 2): 253-258.

Shestakov, A., H. Jenssen, et al. (2012). "Lactoferricin but not lactoferrin inhibit herpes simplex virus type 2 infection in mice." Antiviral Research 93(3): 340-345.

Song, J., B. L. Bishop, et al. (2007). "TLR4-initiated and cAMP-mediated abrogation of bacterial invasion of the bladder." Cell Host Microbe 1(4): 287-298.

Song, J., B. L. Bishop, et al. (2009). "TLR4-mediated expulsion of bacteria from infected bladder epithelial cells." Proceedings of the National Academy of Sciences of the United States of America 106(35): 14966-14971.

Takakura, N., H. Wakabayashi, et al. (2003). "Oral lactoferrin treatment of experimental oral candidiasis in mice." Antimicrobial Agents and Chemotherapy 47(8): 2619-2623.

Trojian, T. H., T. S. Lishnak, et al. (2009). "Epididymitis and orchitis: an overview." Am Fam Physician 79(7): 583-587.

Verhoef, J. (2003). "Antibiotic resistance: the pandemic." Advances in Experimental Medicine and Biology 531: 301-313.

Welkos, S., C. K. Cote, et al. (2011). "Humanized theta-defensins (retrocyclins) enhance macrophage performance and protect mice from experimental anthrax infections." Antimicrobial Agents and Chemotherapy 55(9): 4238-4250.

WHO (1987). "Towards more objectivity in diagnosis and management of male infertility. Int J Androl 1987; 7(Suppl):1." International Journal of Andrology 7(1).

Willen, M., E. Holst, et al. (1996). "The bacterial flora of the genitourinary tract in healthy fertile men." Scandinavian Journal of Urology and Nephrology 30(5): 387-393.

Williams, S. E., T. I. Brown, et al. (2006). "SLPI and elafin: one glove, many fingers." Clin Sci (Lond) 110(1): 21-35.

Yenugu, S., V. Chintalgattu, et al. (2006). "Identification, cloning and functional characterization of novel beta-defensins in the rat (Rattus norvegicus)." Reprod Biol Endocrinol 4(1): 1-7.

Yenugu, S., K. G. Hamil, et al. (2003). "Antibacterial properties of the sperm-binding proteins and peptides of human epididymis 2 (HE2) family; salt sensitivity, structural dependence and their interaction with outer and cytoplasmic membranes of *Escherichia coli*." Biochem J 372(Pt 2): 473-483.

Yenugu, S., K. G. Hamil, et al. (2004). "Antimicrobial actions of the human epididymis 2 (HE2) protein isoforms, HE2alpha, HE2beta1 and HE2beta2." Reprod Biol Endocrinol 2(1): 61.

Yenugu, S., K. G. Hamil, et al. (2006). "Antimicrobial actions of human and macaque sperm associated antigen (SPAG) 11 isoforms: influence of the N-terminal peptide." Molecular and Cellular Biochemistry: 1-13.

Yenugu, S., K. G. Hamil, et al. (2006). "Identification, cloning and functional characterization of novel Spag11 isoforms in the Rat." Reprod Biol Endocrinol 4(23): 1-14.

Yenugu, S., K. G. Hamil, et al. (2004). "The androgen-regulated epididymal sperm-binding protein, human beta-defensin 118 (DEFB118) (formerly ESC42), is an antimicrobial beta-defensin." Endocrinology 145(7): 3165-3173.

Yenugu, S. and G. Narmadha (2010). "The human male reproductive tract antimicrobial peptides of the HE2 family exhibit potent synergy with standard antibiotics." J Pept Sci 16(7): 337-341.

Yule, T. D., C. A. Mahi-Brown, et al. (1990). "Role of testicular autoantigens and influence of lymphokines in testicular autoimmune disease." Journal of Reproductive Immunology 18(1): 89-103.

Reproductive tract infections (RTIs) are recognised as a major public health problem in our country. The current treatment strategies include the use of powerful antibiotics. Prolonged use of antibiotics has resulted in the emergence of multidrug resistant strains of a variety of microorganisms. Thus, the urgent need is to explore alternative ways of coping with infections. Employment of naturally occurring human antimicrobial peptides/proteins as possible alternative for current antibiotics is gaining importance in the last decade. Their use to prevent a variety of infections has reached clinical stage and researchers continue to identify more antimicrobial peptides/proteins. In this study, we attempt to identify antimicrobial proteins that respond to infection and use them to treat reproductive tract infection in a rat model system.

Chapter I: Investigate the in vitro and in vivo antimicrobial gene expression in the male reproductive tract of rats during endotoxin (lipopolysacharride) challenge.

We report the identification of rat *Tlr5*, *Tlr10*, and *Tlr11* transcripts. The presence of characteristic LRR and TIR domains in the predicted amino acid sequences of these TLRs suggests that they primarily function to detect pathogens thus sharing the functional features with the known TLRs. The mRNA expression of *Tlrs* is abundant in the male reproductive tract of adult and developing rats. Their expression was also detected in the male reproductive tract of androgen ablated rats. This provides evidence for regulation of TLRs in general in the male reproductive tract. Such evidence can provide insights into the role of these innate immune molecules during an infection. We attempt to demonstrate the increase in

antimicrobial responses in the male reproductive tract tissues challenged with LPS (*in vitro* and *in vivo).* Results of this study help to understand male reproductive tract innate immune defense mechanisms and to design novel peptide antibiotics to prevent sexually transmitted diseases. We provide the first line of evidence that the male reproductive tract induces the expression of Sperm Associated Antigen 11 (*Spag11*) mRNA variants and defensins when challenged with lipopolysaccharide (LPS) with a concomitant increase in protein expression. An increase in the mRNA levels of proinflammatory cytokines was observed parallel to the induction of *Spag11* variants and majority of defensin expression in the male reproductive tract.

Chapter2: Study the Molecular mechanisms during LPS mediated antimicrobial gene.

Infections of the male reproductive tract lead to infertility, and the molecular mechanisms that operate under these conditions are not well studied. Using epididymal and testicular tissues cultured *in vitro*, LPS-induced antimicrobial gene expression involved NF-kB activation and decreased levels of histone deacetylase 1 (HDAC1) and DNA methyltransferase (DNMT), all of which possibly allow antimicrobial gene transcription. Inhibition of endogenous HDAC1 and DNMT1 resulted in higher antimicrobial gene expression when compared to the LPS alone treated conditions. Increased trimethylation of histone 3, its binding to the upstream region of *Spag11e* gene, and demethylation of this region were observed during endotoxin challenge. We demonstrate that antimicrobial gene expression in the male reproductive tract tissues during endotoxin challenge involves NF-kB activation and epigenetic changes. Promoter analysis revealed the presence of binding sites for AR, NF-kB and NF-1 in the upstream region of *Spag11e* gene. Reporter gene assays for

promoter activity revealed that mutations in AR and NF-kB binding sites caused a significant reduction of the responsiveness to LPS. Electrophoretic mobility shift assays demonstrated that LPS induced the binding of AR and NF-kB to their respective binding sites. We report that the transcription of *Spag11e* gene induced by LPS is dependent on the binding of AR and NF-kB to its upstream sequence.

Chapter 3: Determine the potential of antimicrobial proteins to limit or prevent male reproductive tract infection

Resistance to antibiotics is of great social and economic importance and is regarded as a threat to the national security of any country and the global community as a whole. Hence, identification of antimicrobuial peptides to treat antibiotic resistant pathogen is essential. We attempt to characterise the ability of Defensin21, a male reproductive tract defensin to prevent epididymitis in a rat model. The ability of *Uropathogenic E. coli* to induce epididymitis and testicular Orchitis was successfully established by injecting UPEC strain (MTCC 729) bilaterally in vasa deferentia. Antimicrobial (Defensins and *Spag11*) gene expression was inducible during UPEC infection. UPEC induced antimicrobial gene expression may be mediated by NF-kB signalling pathway. Further, epigenetic changes such as histone modification and DNA methylation seem to be involved in the regulation of antimicrobial gene expression. Recombinant defensin21, when injected into rats infected with UPEC, caused a dramatic decrease in the colony count in the male reproductive tract tissues. The potency of defensin 21 was better than gentamycin, a conventional antibiotic. These results provide strong evidence that antimicrobial

peptides can be possible treatment alternatives for male reproductive tract infections.

In conclusion, results of the present study demonstrate that antimicrobial gene expression is inducible in the male reproductive tract of rats during endotoxin challenge or bacterial infection. The induction seems to be TLR signalling mediated and epigenetically controlled. The ability of defensin 21 to bring down infectivity gives an impetus to carry out further studies to develop reproductive tract antimicrobial proteins as alternative agents to treat infections.