Effects of lactoferrin on fetal development

胎仔発生に対するラクトフェリンの影響

Doctoral Thesis

For

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Yamaguchi University

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Contents

I. PREFACE

Modern human society faces the serious problem of infertility. The average age of marriage has increased, which has resulted in some challenges such as infertility. There are many reports about causes of infertility [Abrao et al., 2013, de Kretser, 1997, Ochsendorf, 2008], which can be roughly classified as female-dependent factors (e.g., egg aging, age-related chromosome segregation errors in oocytes, female genital tumors, ovulation disorder, antibody production against sperm) and male-dependent factors (e.g., oligospermia, asthenospermia, teratospermia, sexual dysfunction, genitourinary tract infection). These studies revealed that almost half of the causes of infertility result from male-dependent factors. In addition, there are other reports that bacteria frequently exist in seminal fluid, and many infertile male patients have semen that is contaminated with bacteria such as *Escherichia coli* [Moretti et al., 2009, Philippov et al., 1998, Shukla et al., 2012]. Therefore, seminal bacterial infection remains an important problem.

On the other hand, sperm cells express Toll-like receptor (TLR) on cell surfaces, such as TLR2 and TLR4 which are generally acknowledged to play a key role in immune responses in adaptive and innate immunity [Fujita et al., 2011, Fujita et al., 2011, Shimada et al., 2008]. TLRs are key upstream mediators of inflammation at many tissue sites, and TLR activation results in an inflammatory immune response[Akira and Takeda, 2004]. Approximately 10-15

mammalian TLRs have been identified, with TLR1-10 present in human and TLR1-13 present in mouse [Jiménez-Dalmaroni et al., 2016]. Each TLR recognizes a pathogen-associated molecular pattern (PAMP) as specific components of pathogenic microorganisms, namely, bacteria, fungi, viruses, and parasites[Guan et al., 2010, Yuan and Wilhelmus, 2010]. For example, TLR2 recognizes peptidoglycan (PG), which is derived from Gram-positive bacteria, and TLR4 recognizes lipopolysaccharide (LPS), which is an endotoxin of Gram-negative bacteria [Fu et al., 2013, Poltorak et al., 1998, Qureshi et al., 1999]. In sperm, PAMP recognition by TLRs reduces cell motility and induces apoptosis, in addition to immune response initiation [Fujita et al., 2011, Fujita et al., 2011]. To prevent bacterial contamination of semen, oral administration of antibiotics or addition of antibiotics in semen has been performed [Magri et al., 2009, Okazaki et al., 2010, Vaillancourt et al., 1993]. However, frequent antibiotic use raises other problems such as negative influence on intestinal bacterial flora or antimicrobial-resistant bacteria [Yoon and Yoon, 2018]. Moreover, bacterial components can remain in semen and continue to stimulate sperm TLRs.

On the other hand, I have studied the antibacterial property of lactoferrin (LF). LF is an iron-binding glycoprotein with a molecular weight of 78 kDa, which is contained in mammalian exocrine fluids including milk, tears, and vaginal fluids [García-Montoya et al., 2012]. Moreover, LF is known to have antimicrobial, anti-inflammatory, and antitumor effects [Tung et al., 2013]. Concerning its antimicrobial effect, LF competitively blocks the

binding of LPS to TLR4, which inhibits TLR4-mediated signaling by LPS, and consequently suppresses inflammatory cytokine production [Drago-Serrano et al., 2012]. From there facts, I assumed that LF may also prevent the sperm apoptosis induced by pathogen LPS via sperm TLR4 and may facilitate the pregnancy.

In chapter 1 of this thesis, I investigated the LF potentiality on LPS-induced sperm apoptosis. In chapter 2, I further investigated the effect of LF on subsequent events such as embryo development and pregnancy by using LPS-treated mouse sperm.

II. Chapter 1

Effect of lactoferrin on murine sperm apoptosis induced by intraperitoneal injection of lipopolysaccharide

ABSTRACT

There are many causes of infertility, and genital bacterial infection is one of the most important causes of infertility. However, bacteria frequently exist in seminal fluid and many infertile male patients have bacterial contamination of semen. Moreover, sperm express Toll-like receptors (TLRs) on their cell surfaces and bacterial recognition by TLRs induces sperm apoptosis. I have investigated the antibacterial property of lactoferrin (LF) on murine gametes. In the present study, I examined the LF potentiality on sperm apoptosis induced by bacterial lipopolysaccharide (LPS) by using mouse semen. TUNEL assay indicated that TUNEL-positive sperm cells were scarce in the group treated with LF and LPS (LF/LPS group) compared to the group treated with LPS only (LPS group). In addition, real-time RT-PCR detected lower mRNA expression levels of apoptosis-associated genes in the LF/LPS group compared to the LPS group. These results indicate that LF treatment of semen might decrease LPS-induced apoptosis of sperm.

INTRODUCTION

There are many causes of infertility, and almost half of them result from male-dependent factors [Abrao et al., 2013, de Kretser, 1997, Ochsendorf, 2008]. Bacteria frequently exist in seminal fluid, therefore seminal bacterial infection remains an important problem [Moretti et al., 2009, Philippov et al., 1998, Shukla et al., 2012].

Sperm cells express Toll-like receptor (TLR) on cell surfaces [Fujita et al., 2011, Fujita et al., 2011, Shimada et al., 2008]. TLRs are key upstream mediators of inflammation, and TLR activation results in an inflammatory immune response [Akira and Takeda 2004, Jiménez-Dalmaroni et al., 2016]. Each TLR recognizes a pathogen-associated molecular pattern (PAMP) as specific components of pathogenic microorganisms [Fu et al., 2013, Guan et al., 2010, 2010, Poltorak et Qureshi et al., 1999., 1998, Yuan and Wilhelmus]. In sperm, PAMP recognition by TLRs reduces cell motility and induces apoptosis [Fujita et al., 2011, Fujita et al., 2011]. To prevent bacterial contamination of semen, administration of antibiotics has been performed [Magri et al., 2009, Okazaki et al., 2010, Vaillancourt et al., 1993]. However, frequent antibiotic use raises other problems such as antimicrobial-resistant bacteria [Yoon and Yoon, 2018].

On the other hand, I have studied the antibacterial property of lactoferrin (LF). LF is an iron-binding glycoprotein, which is contained in mammalian exocrine fluids [García-Montoya

et al., 2012]. LF is known to have various functions such as antimicrobial effect [Tung et al., 2013]. For example, LF competitively blocks the binding of LPS to TLR4 and suppresses inflammatory cytokine production [Drago-Serrano et al., 2012]. In this study, I investigated whether LF have the potentiality to decrease LPS-induced apoptosis of sperm.

MATERIALS AND METHODS

Mice:

All mice used in this study were purchased (CLEA Japan, Tokyo, Japan) or bred in our mouse colony. Mice were reared under conventional laboratory housing conditions and allowed free access to water and food (CA-1; CLEA Japan) *ad libitum*. The facility was maintained under a 12 hr light/12 hr dark cycle at 20–25°C. The institutional animal care and use committee approved this study (permission number: h25-T020), and all procedures were conducted according to the guide for the care and use of laboratory animals at Tottori University.

Oral LF administration:

Fifteen male C57BL/6 JJcl mice aged 8–22 weeks were used for oral administration of LF. Mice were equally divided into three groups: saline group (control), LPS group and

LF/LPS group. Then, saline or 3.0 mg/10 g b.w. of bovine LF (NRL Pharma, Tokyo, Japan) diluted with saline was orally administered once a day for 10 days to each group, respectively. The administration dose of LF was determined based on the condition for humans [Otsuki and Imai, 2017]. Simultaneously, 1.0×10^{-3} mg/10 g b.w. of LPS from O111:B4 *Escherichia coli* (Sigma-Aldrich, St. Louis, MO, USA) was also i.p. administered once a day on days 6–10 of saline or LF administration to both groups. The administration dose of LPS was determined according to the previous report for mice [Kajihara et al., 2006]. On day 11, mice were euthanized by cervical dislocation under anesthesia with i.p. administration of a mixed anesthetic agent (hereafter referred to as MMB) comprising 0.75 mg/kg b.w. medetomidine (Nippon Zenyaku Kogyo, Fukushima, Japan), 4.0 mg/kg b.w. midazolam (Astellas Pharma, Tokyo, Japan), and 5.0 mg/kg b.w. butorphanol (Meiji Seika Pharma, Tokyo, Japan). Then, testes and cauda epididymides were removed and used for apoptotic sperm detection and real-time RT-PCR analyses.

Apoptotic sperm detection:

Testes and cauda epididymides were fixed in 4% paraformaldehyde-phosphate buffer (Nacalai Tesque, Tokyo, Japan) for 16 hr at room temperature. Tissue samples were paraffin-embedded and sectioned into 3-μm thickness. Then, sections were placed on MAS-GP type A glass slides (Matsunami Glass, Osaka, Japan) and used to detect apoptotic sperm cells by the

TdT-mediated dUTP-biotin nick end labeling (TUNEL) method. TUNEL assay was performed using the *In situ* Apoptosis Detection Kit (Takara Bio, Kusatsu, Japan) according to the manufacturer's instruction. Thereafter, slides were mounted in Mounting Medium with DAPI (Vector Laboratories, Burlingame, CA, USA) and observed under a fluorescence microscope (BX-53; Olympus, Tokyo, Japan). For an analysis of sperm apoptosis in testes and cauda epididymides, 5 random and non-overlapping fields were examined in control, LPS group and LF/LPS group, respectively. The number of TUNEL-positive cells was counted from 30 or 50 randomly selected DAPI-positive nuclei in testes or cauda epididymides, and relative percentages of TUNEL-positive cells to DAPI-positive total nuclei were indicated.

Real-time RT-PCR:

Cauda epididymides were collected and immersed in RNAiso Plus (Takara Bio) immediately. Thereafter, total RNA was isolated and cDNA was reverse-transcribed by ReverTra Ace® qPCR RT Master Mix with gDNA Remover (Toyobo, Tokyo, Japan) using the Takara PCR thermal cycler dice system (Takara Bio) according to the manufacturer's instruction. The primer sequences used are shown in Table 1. Real-time RT-PCR was performed with the StepOne™ Real-Time PCR system (Applied Biosystems, Foster City, CA, USA), using universal temperature cycles: 10 min of pre-incubation at 95°C, followed by 40

cycles of 2-temperature cycles (15 sec at 95°C and 1 min at 55°C). All PCR reactions were carried out with Power SYBR® Green PCR Master Mix (Applied Biosystems) and relative mRNA levels were calculated after normalization by *GAPDH*.

Statistical analysis:

All values are expressed as the mean ± SEM. For the statistical analysis, results from TUNEL assay were analyzed by one-way ANOVA followed by Tukey's test and the level of significance was set at $P < 0.05$. On the other hand, results from real-time RT-PCR analysis were analyzed by Student's t-test and the level of significance was set at $P < 0.05$.

RESULTS

Apoptotic sperm detection:

From the TUNEL assay of testes, many sperm cells were TUNEL-positive in the control group, LPS group and LF/LPS group, respectively (Fig. 1A). However, TUNEL-positive sperm cells were frequent in LPS and LF/LPS group, compared to that of the control group ($P < 0.05$) (Fig. 1B). Moreover, TUNEL-positive sperm cells of the LF/LPS group were less frequent than that of the LPS group ($P < 0.05$) (Fig. 1B). In the cauda epididymis, sperm cells were TUNEL-positive in the control group, LPS group and LF/LPS group (Fig. 2A). However, TUNEL-positive sperm cells were frequent in LPS and LF/LPS group, compared to that of the control group ($P < 0.05$) (Fig. 2B). Moreover, TUNEL-positive sperm cells were less frequent in the LF/LPS

group than that in the LPS group ($P < 0.05$) (Fig. 2B).

Real-time RT-PCR:

Gene expression of *Caspase3* was measured in cauda epididymides to examine if mRNA levels of these apoptosis-related factors were also inhibited by LF under the stimulation of the apoptotic process by LPS. In cauda epididymides, *Caspase3* mRNA expression seemed to be lower in the LF/LPS group than that in the LPS group, although the difference was not significant (Fig. 3).

DISCUSSION

In this study, I performed TUNEL assay to examine the effect of orally administrated LF against LPS-induced sperm apoptosis. Additionally, I performed real-time RT-PCR analysis to examine if mRNA levels of the apoptosis-related factor was also inhibited by orally administrated LF under the stimulation of the apoptotic process by LPS. As a result, TUNEL-positive sperm cells were scarce in the LF/LPS group compared to the LPS group. Moreover, the mRNA expression of *Caspase3*, the apoptotic biomarkers [Xue et al., 2017], seemed to be slightly lower in the LF/LPS group than in the LPS group. In addition, another apoptosis related gene, *TNFR1 associated death domain protein* [Bender et al., 2005], we have found its expression in the sperm also seems to be lower in LF/LPS group than LPS group (data not shown). Previous research shows that orally ingested LF undergoes limited

proteolysis by trypsin in pancreatic juice into fragments with a molecular weight of 30 kDa [Davidson and Lönnerdal, 1989]; limited proteolysis with a molecular weight of 10 kDa or more is a characteristic of LF. In addition, orally ingested bovine LF is degraded by gastric pepsin and this degradation produces lactoferricin, which expresses higher antibacterial activity [Gifford et al., 2005]. Orally administered LF is then taken up via the LF receptor on the brush border of the small intestinal epithelium and transferred to blood or lymph flow while maintaining physiological activity [Fischer et al., 2007, Takeuchi et al., 2004]. In mouse and human, the LF receptor is expressed in intestinal mucosa cells and the testis or ovary [Suzuki and Lönnerdal, 2004, Suzuki et al., 2001]. These results imply that orally administered LF may be transferred to the testis and decrease LPS-induced apoptosis of sperm. However, bacterial composition in semen varies among mammalian species. For example, Gram-positive bacteria are abundant in human semen compared to Gram-negative bacteria [Fujita et al., 2011]. Since the LF effect on bacteria-induced sperm apoptosis can be expected to be applied to infertility treatment, further studies are required to confirm whether LF can suppress sperm apoptosis induced by Gram-negative and positive bacteria.

Table 1. Primers used for quantitative real-time polymerase chain reaction

Gane	Forward sequence (5'-3')	Reverse sequence (5'-3')
TRADD	CAGACAAGGTGCTTCGGTC	TGCTACAAAAACTGTCGGGA
Caspase3	CCTCAGAGACATTCATGG	GCAGTAGTCGCCTCTGAAGA
GAPDH	TGCCGCCTGGAGAAACCTGC	TGAGAGCAATGCCAGCCCCA

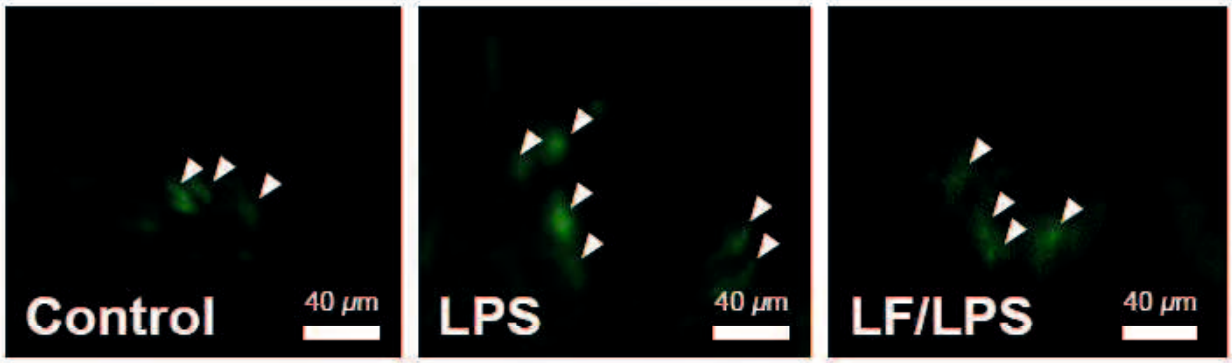

Fig. 1. Apoptotic sperm detection in testes with TUNEL assay. (A) Representative images of TUNEL-positive sperm cells (arrowheads). The number of TUNEL-positive sperm cells is abundant in LPS and LF/LPS compared to the control. Moreover, the number of TUNEL-positive sperm cells is fewer in the LF/LPS than the LPS..

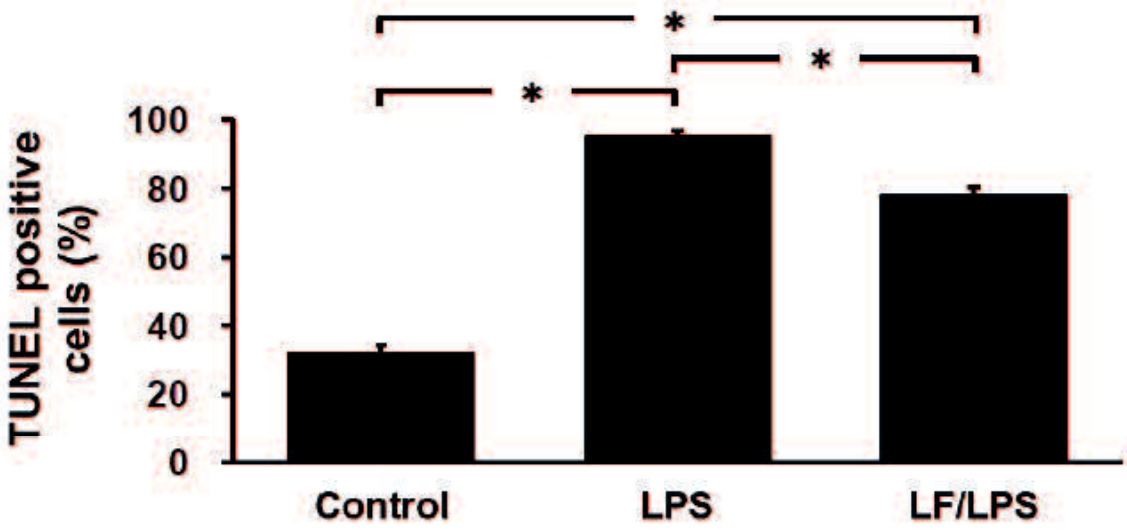

Fig. 1. Apoptotic sperm detection in testes with TUNEL assay. (B) Relative percentages of TUNEL-positive sperm cells to DAPI-positive total nuclei. Each bar represents mean ± SEM. Asterisks indicate significant differences among control, LPS and LF/LPS ($P < 0.05$).

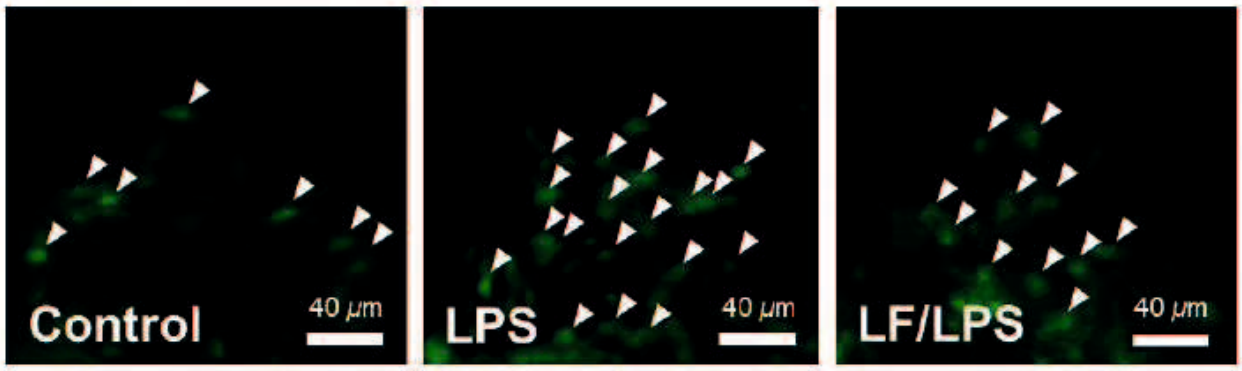

Fig. 2. Apoptotic sperm detection in cauda epididymides with TUNEL assay. (A) Representative images of TUNEL-positive sperm cells (arrowheads). The number of TUNEL-positive sperm cells is abundant in LPS and LF/LPS compared to the control. Moreover, the number of TUNEL-positive sperm cells is fewer in the LF/LPS than the LPS.

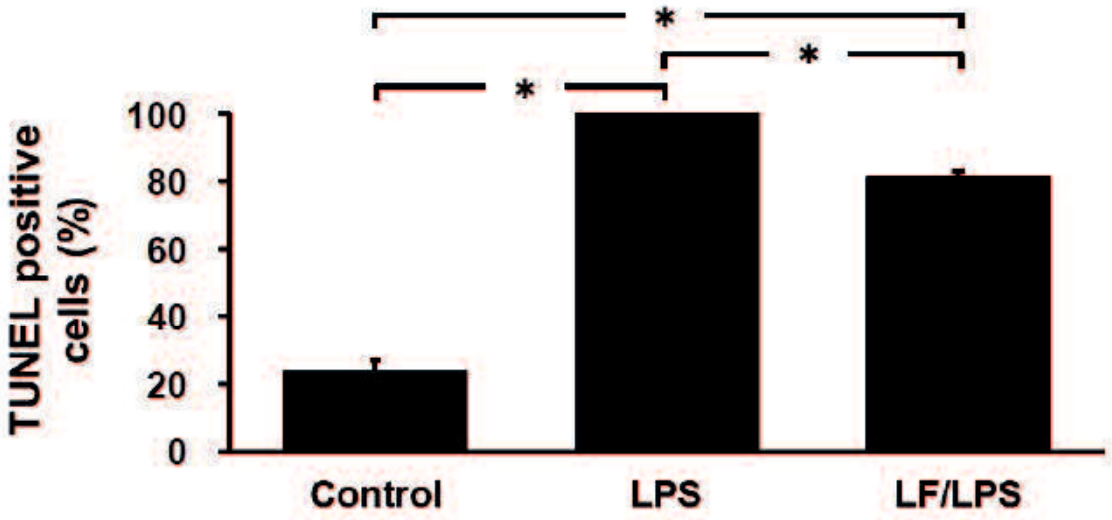

Fig. 2. Apoptotic sperm detection in cauda epididymides with TUNEL assay. (B) Relative percentages of TUNEL-positive sperm cells to DAPI-positive total nuclei. Each bar represents mean ± SEM. Asterisks indicate significant differences among control, LPS and LF/LPS ($P < 0.05$).

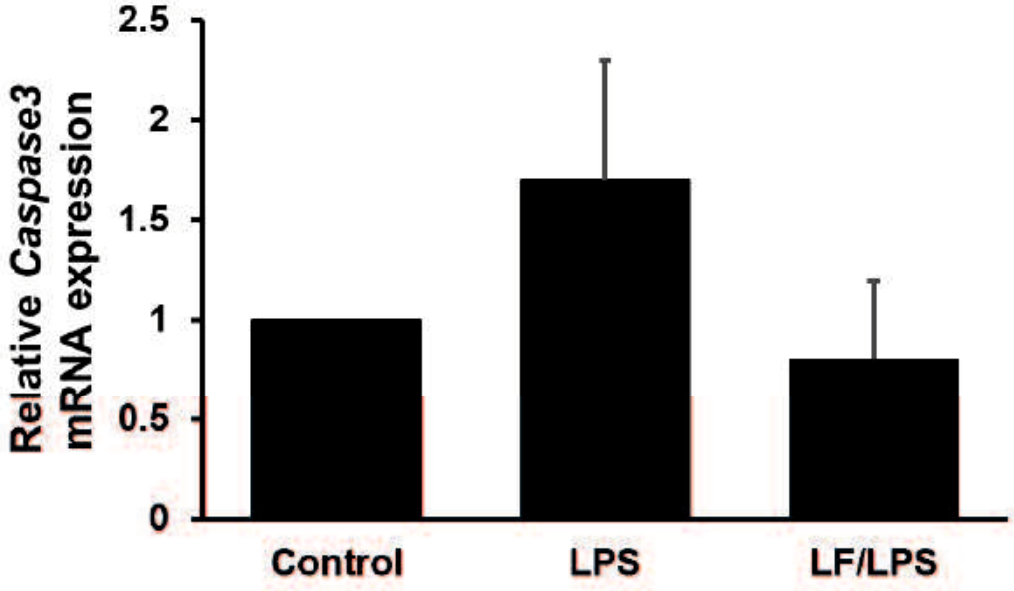

Fig. 3. Relative mRNA expression of the apoptotic biomarker gene (*Caspase3*) in cauda epididymides with a control value as 1 with real-time RT-PCR analysis. *Caspase3* mRNA expression tends to be lower in the LF/LPS group (LF/LPS) than the LPS group (LPS. Each bar represent mean ± SEM.

III. Chapter 2

Effect of lactoferrin on development of murine embryo created from lipopolysaccharide-treated sperm

ABSTRACT

Seminal bacterial infection is one of the most important causes of infertility, while seminal fluid is frequently contaminated by bacteria. Additionally, sperm expresses Toll-like receptor (TLR) 2 and TLR4 on the cell surface, of which bacterial recognition by TLRs induces sperm apoptosis. On the other hand, I have investigated the antibacterial property of lactoferrin (LF) for sperm and demonstrated that LF treatment of semen might prevent LPS-induced apoptosis of sperm. In the present study, I further investigated the effect of LF on murine embryo development and establishment of pregnancy by using mouse sperm that were pre-treated with bovine LF and bacterial lipopolysaccharide (LPS). As a result, the development rate of the 2-cell stage embryo seemed to be higher in the LF/LPS group (sperm treated with bovine LF and LPS) than the LPS group (sperm treated with only LPS). In addition, morphological abnormalities were seen in fetuses or placentas of the LPS group compared with LF and LF/LPS and the control group on 12 days after embryo transfer. These results indicate that LF treatment for sperm might improve infertility caused by bacterial LPS contamination in semen.

INTRODUCTION

Modern human society faces the serious problem of infertility. There are various factors of infertility and about half of them are attributed to male-dependent factors, such as oligospermia, asthenospermia, teratospermia, sexual dysfunction, and genitourinary tract infection [Abrao et al., 2013, de Kretser, 1997, Ochsendorf, 2008, Philippov et al., 1998]. Above all, seminal bacterial infection is believed to be one of the important causes of infertility. However, bacteria are frequently found in semen of both infertile and fertile men [Moretti et al., 2009, Shukla et al, 2012].

Sperm cells express many types of Toll-like receptor (TLR) on their cell surfaces [Fujita et al., 2011, Fujita et al., 2011, Shimada et al., 2011], which are generally acknowledged to play a key role in immune responses in adaptive and innate immunity [Guan et al., 2010, Moretti et al., 2009, Yuan and Wilhelmus, 2010]. Each TLR recognizes a pathogen-associated molecular pattern (PAMP) as a specific ligand, which is a component of the specific invading pathogen. For example, TLR2 recognizes peptidoglycan of Gram-positive bacteria, and TLR4 recognizes lipopolysaccharide (LPS) of Gram-negative bacteria [Fu et al., 2013, Poltorak et al., 1998, Qureshi et al., 1999]. PAMP recognition by sperm via TLR2 and TLR4 induces reduced cellular motility and subsequently causes sperm apoptosis, in addition to initiation of immune responses in sperm [Fujita et al., 2011, Fujita et al., 2011].

To prevent bacterial contamination of semen, antibiotics have been used. However, frequent antibiotic use has resulted in antimicrobial-resistant bacteria [Magri et al., 2009, Okazaki et al., 2010, Vaillancourt et al., 1993, Yoon and Yoon, 2018]. Furthermore, dead bacterial components (peptidoglycan or LPS) remain in semen and continue to stimulate sperm TLRs.

On the other hand, I have been investigating the usefulness of lactoferrin (LF) in bacterially contaminated sperm. LF is an iron-binding glycoprotein that is contained in mammalian exocrine fluids, such as milk, saliva, sweat, and tears [García-Montoya et al., 2012, Suzuki et al., 2001]. Several studies have demonstrated various effects of LF, such as antimicrobial, anti-inflammatory, and antitumor effect [García-Montoya et al., 2012, Tung et al., 2013]. Concerning its antimicrobial effect, competitive binding of LF with LPS for TLR4 inhibits TLR4-mediated signal transduction and suppresses inflammatory cytokine production [Drago-Serrano et al., 2012]. I demonstrated in the previous study that LF treatment of semen might prevent LPS-induced apoptosis of sperm. In this study, I further investigated the effect of LF on subsequent events such as embryo development and pregnancy by using LPS-treated mouse sperm.

MATERIALS AND METHODS

Mice:

All mice used in this study were purchased (CLEA Japan, Tokyo, Japan) or bred in our mouse colony. Mice were reared under conventional laboratory housing conditions and allowed free access to water and food (CA-1; CLEA Japan) *ad libitum*. The facility was maintained under a 12 hr light/12 hr dark cycle at 20–25°C. The institutional animal care and use committee (permission number: h25-T020) approved this study, and all procedures were conducted according to the guide for the care and use of laboratory animals at Tottori University.

Vasectomy:

Ten Male Jcl:ICR mice aged 8–10 weeks underwent vasectomy. Mice were anesthetized with i.p. administration of a mixed anesthetic agent (hereafter referred to as MMB) comprising 0.75 mg/kg b.w. medetomidine (Nippon Zenyaku Kogyo, Fukushima, Japan), 4.0 mg/kg b.w. midazolam (Astellas Pharma, Tokyo, Japan), and 5.0 mg/kg b.w. butorphanol (Meiji Seika Pharma, Tokyo, Japan). After vasectomy, according to the standard method for recovery from deep anesthesia, mice recovered from anesthesia with atipamezole (Nippon Zenyaku Kogyo), which was used as a medetomidine antagonist. Subsequently, mice in good condition were selected and used for preparation of pseudopregnant mice at least 1 week after the operation.

Superovulation:

Forty-seven female BDF1 mice aged 4 weeks were used for superovulation. Each mouse was i.p. administered 0.1 m*l* of CARD HyperOva^{TM} (Kyudo, Saga, Japan) according to the manufacturer's instruction. Forty-eight hours after the first administration, mice were subsequently i.p. administered 7.5 IU of human chorionic gonadotropin (hCG; ASKA Pharmaceutical, Tokyo, Japan). Both administrations were performed at 17:00. Subsequently, ova were collected from these superovulating mice at 9:00 the next morning, 16 hr after hCG administration. Details of ovum collection procedure was described in the section of *In vitro* fertilization (IVF).

Sperm collection and LF treatment:

Thirteen male B6D2F1/Jcl mice aged 8–16 weeks were used for sperm collection. Mice were euthanized by cervical dislocation under anesthesia with i.p. administration of MMB. Cauda epididymides were removed and placed into 35-mm plastic dishes (Corning Life Sciences, Tewksbury, MA, USA) filled with mineral oil suitable for mouse embryo cell culture (Sigma-Aldrich, St. Louis, MO, USA). Then, sperm were collected and equally divided into one of four types of 100 μl medium. Four types of medium were used: TYH medium (LSI Medience, Tokyo, Japan; control group), TYH medium with 1.0 mg/m*l* bovine LF (NRL Pharma, Tokyo, Japan; LF group), TYH medium with 1.0 mg/m*l* bovine LF and

1.0×10^{-3} mg/m*l* LPS from *E. coli* O111:B4 (Sigma-Aldrich; LF/LPS group), and 1.0×10^{-3} mg/m*l* LPS (LPS group). The dose of LF was determined based on the condition for humans [Otsuki et al., 2017]. Additionally, the dose of LPS was determined according to the previous report for mice [Fujita et al, 2011, Wang and Zhuang, 2019]. All media were pre-equilibrated under 5% CO_2 at 37°C for 1 hr. Subsequently, sperm were incubated in these media under 5% CO_2 at 37°C for 3 hr, and then only active sperm, which reached to the periphery of the medium, were used for insemination according to a routine procedure of reproductive engineering techniques.

In vitro fertilization (IVF):

Superovulating mice were euthanized by cervical dislocation under anesthesia of i.p. administration with MMB 16 hr after hCG administration. Oviducts were removed and divided into four groups (13 mice for the control group, 11 mice for the LF group, 10 mice for the LF/LPS group and 13 mice for the LPS group), and they were placed into 35-mm plastic dishes containing 100 μl of mHTF medium (Kyudo) covered with mineral oil. Thereafter, ova were removed from the ampulla of each oviduct and introduced into mHTF medium. Then 6 μl of sperm suspension were collected from the abovementioned four types of media and added to one of four mHTF medium containing ova, respectively. Therefore, these four dishes were also named in the same way (control group, LF group, LF / LPS group and LPS group).

Media were pre-equilibrated under 5% CO_2 at 37°C for 1 hr. After co-incubation of sperm and ova under 5% CO_2 at 37°C for 3 hr, a total of 3,568 fertilized ova were collected (878 from the control group, 767 from the LF group, 895 from the LF/LPS group and 1,028 from the LPS group) and further incubated in M16 medium (Sigma-Aldrich) under 5% CO_2 at 37°C for 16 hr. Subsequently, only 2-cell stage embryos were selected and used for embryo transfer.

Embryo transfer:

Fifteen female ICR mice aged 8–22 weeks were used for recipients of the embryo. Prior to embryo transfer, pseudopregnant mice were prepared by mating female ICR mice in the early estrus stage with male ICR vasectomy mice at 17:00. Then, female mice with vaginal plugs were selected at 9:00 the next morning, 16 hr after mating, and anesthetized by i.p. administration of MMB. Thereafter mice were divided into four groups (4 mice for the control group, 4 mice for the LF group, 3 mice for the LF/LPS group and 4 mice for the LPS group) and 2-cell stage embryos, which were derived from sperm prepared as described above, were separately transferred into each mouse oviduct. For embryo transfer, 16 to 20 randomly selected 2-cell stage embryos were transferred per mouse. After the operation, mice were recovered from anesthesia with atipamezole.

Fetal development:

On day 12 post-embryo transfer, recipient mice were euthanized by cervical dislocation under anesthesia with i.p. administration of MMB. Then, fetuses were removed with placentas.

Statistical analysis:

All values are expressed as the mean ± SEM. Results were analyzed by one-way ANOVA followed by Tukey's test using BellCurve for Excel (Social Survey Research Information, Tokyo, Japan). The level of significance was set at $P < 0.05$.

RESULTS

LF effect on embryo development:

First, development rates of 2-cell stage embryos were measured in each of the four groups after IVF to verify the effect of LF treatment of sperm on embryo development. Average numbers of fertilized ova were 125.4 ± 24.1 in the control group, 127.8 ± 29.8 in the LF group, 162.7 ± 43.0 in the LF/LPS group and 146.9 ± 33.5 in the LPS group, and average numbers of 2 cell-stage embryos were 69.1 ± 15.3 in the control group, 72.7 ± 18.5 in the LF group, 82.9 ± 21.4 in the LF/LPS group and 63.0 ± 16.2 in the LPS group, respectively. The rate of the embryo development into the 2-cell stage were 56.4 ± 3.7 %, 58.7 ± 4.8 %, 53.6

± 4.4 % and 45.9 ± 4.9 % in the control, LF, LF/LPS and LPS groups, respectively (Fig. 4). This result leaded the notion that LF treatment not only rescued the LPS-affected sperm but also facilitated its embryogenesis.

LF effect on fetal development:

Next, morphological observation of the fetus and placenta was performed on day 12 post-embryo transfer with measurement of the pregnancy rate to verify the effect of LF treatment of sperm on establishment of pregnancy. From the morphological observation on day 12 post-embryo transfer, the abnormal structures, that is small placenta-like tissues without fetuses, were frequently found in the uterus transferred the LPS-group embryo, but rarely in the control, LF and LF/LPS groups (Fig. 5). This result suggested that the embryo abnormality occurred in the LPS-affected sperm could be prevented by LF treatment to the sperm.

DISCUSSION

Bacteria are frequently found in semen not only in infertile patient, but also in healthy men, whereas pathogen contamination in semen is considered an important cause of infertility [Moretti et al., 2009, Shukla et al., 2012]. Sperm TLR4 recognize LPS from gram-

negative bacteria, and the stimulation causes sperm apoptosis and reduced fertility [Fujita et al., 2011, Fujita et al., 2011, Shimada et al, 2008]. On the other hand, I demonstrated in the previous study that LF treatment of sperm could suppress the induction of sperm apoptosis by gram- negative bacteria.

In this study, I further clarified the effect of LF on murine embryo development and establishment of pregnancy by using sperm that escaped LPS-induced apoptosis by LF treatment. The contamination of bacterial LPS in the semen is one of the principal factors for the infertility. In this study, it is suggested that LF treatment to the sperm may lead LPS-treated sperm to carry out the embryogenesis normally and facilitate the pregnancy. Unfortunately, the statistical significance could not be observed among 4 examined groups on the development rates of 2-cell stage embryos in this study, maybe because of the low concentration of LPS for our experimental condition. However, considering the similar value of the development rates between the control and the LPS/LF groups, together with the fact that the embryo abnormality found in the LPS group are rarely in the LPS/LF group, this study may represent the novel potency of LF for the treatment of the infertility. The molecular mechanism of the effect of LF not only for the survival of sperm but also for the maintenance of the embryogenesis still remains unclear, and we expect our findings and experiment procedures in this study can contribute to resolve this matter.

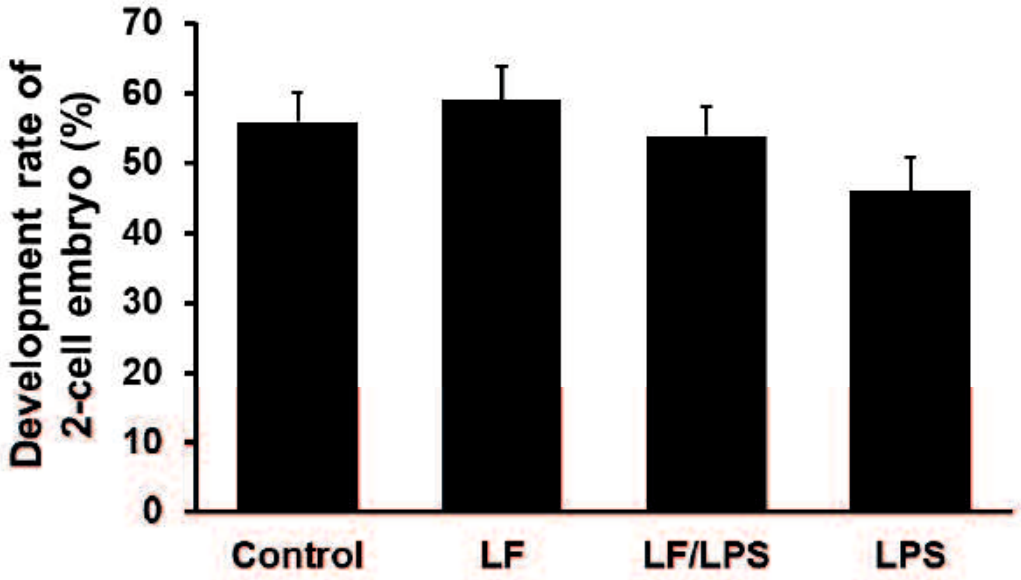

Fig. 4. LF effect on embryo development. Development rates of 2-cell stage embryos are measured in control group (control), LF group (LF), LF/LPS group (LF/LPS) and LPS group (LPS). The development rate seems to be higher in the LF/LPS than the LPS. Each bar represent mean ± SEM.

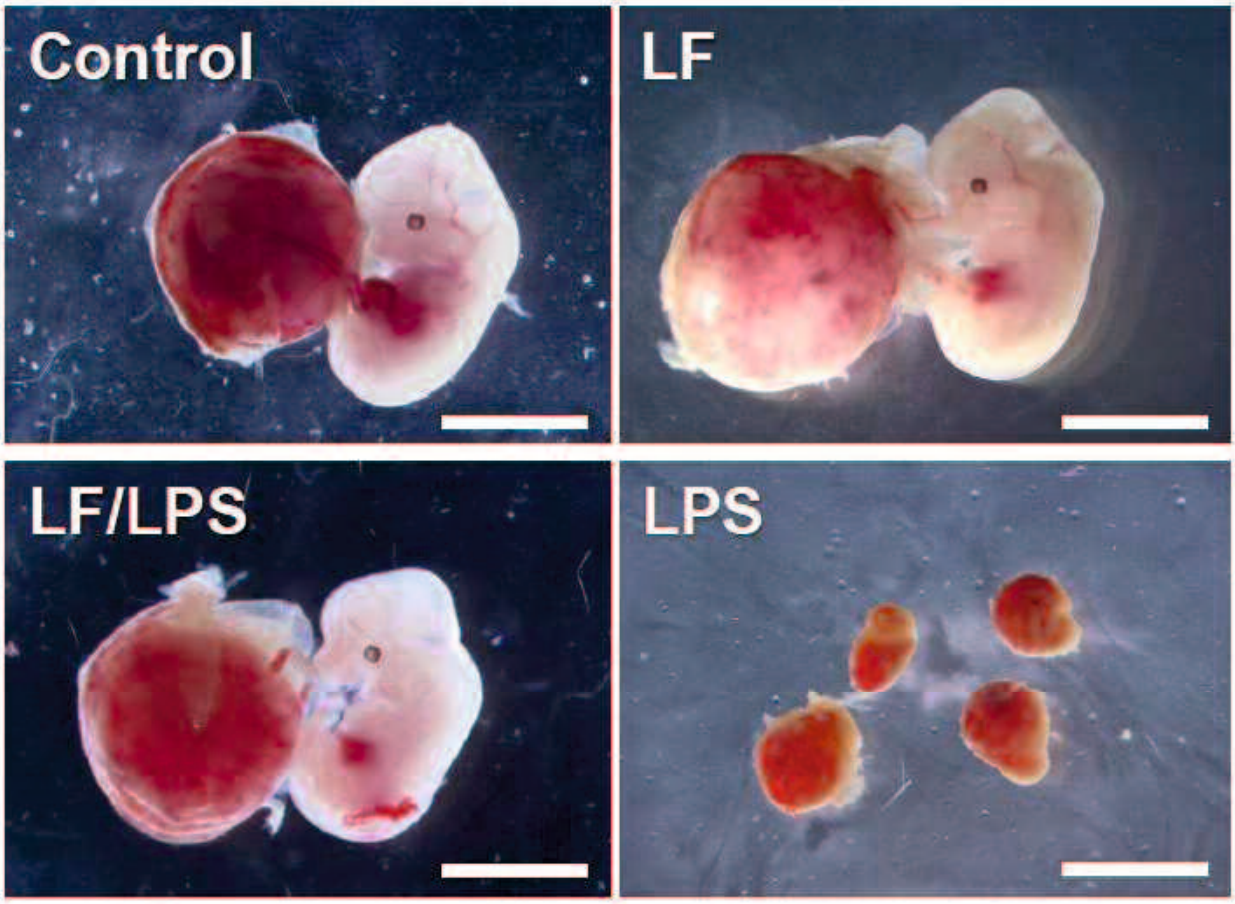

Fig. 5. LF effect on fetal formation. Morphological observation of the fetus and placenta is performed on day 12 post-embryo transfer in control group (control), LF group (LF), LF/LPS group (LF/LPS) and LPS group (LPS). Morphological abnormalities (absence of fetuses and small placenta-like tissues) are seen in fetuses or placentas of the LPS group compared with LF and LF/LPS and the control group. Scale bars: 100 μm.

IV. Summary and Conclusion

Genital bacterial infection is one of the most important causes of infertility, however, bacteria frequently exist in seminal fluid. Moreover, sperm express Toll-like receptors (TLRs) on their cell surfaces and bacterial recognition by TLRs induces sperm apoptosis.

On the other hand, I have investigated the antibacterial property of lactoferrin (LF) on murine gametes. Concerning its antimicrobial effect, LF competitively blocks the binding of LPS to TLR4, which inhibits TLR4-mediated signaling by LPS, and consequently suppresses inflammatory cytokine production. From there facts, I assumed that LF may also prevent the sperm apoptosis induced by pathogen LPS via sperm TLR4 and planned to examine the LF potentiality on sperm.

Firstly, I examined the LF effect on sperm apoptosis induced by bacterial lipopolysaccharide (LPS). From the result of TUNEL assay, TUNEL-positive sperm cells were scarce in the group treated with LF and LPS compared to the group treated with LPS only. In addition, real-time RT-PCR detected lower mRNA expression levels of apoptosis-associated genes in the LF/LPS group compared to the LPS group.

Next, I further investigated the LF effect on embryo development by using LPS-treated mouse sperm. For the development rate of the 2-cell stage embryo, the embryo derived

from LPS- and LF-treated sperm showed similar survival rate to the control embryo. On day 12 after the embryo transfer into the recipient, the frequent abnormality was observed in the embryo derived from LPS-treated sperm, and the abnormality was tended to be inhibited in the embryo derived from LPS- and LF-treated sperm.

These results indicate that the LF treatment on bacteria-contaminated sperm might decrease LPS-induced sperm apoptosis and the LF treatment of sperm may also facilitate the embryo development, which contribute to the improvement of infertility.

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