NOTE Anatomy

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

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Running head: LACTOFERRIN EFFECT ON PREGNANCY

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ABSTRACT

The effect of lactoferrin (LF) on embryo development was investigated by using lipopolysaccharide (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 imply that LF treatment on sperm contaminated with bacteria may facilitate the embryo development, which contribute to the improvement of infertility.

KEY WORDS: bacteria, infertility, lactoferrin, sperm, Toll-like receptor
There are various factors of infertility and about half of them are attributed to male-dependent factors [1, 2, 10, 13]. Above all, seminal bacterial infection is one of the important causes of infertility. However, bacteria are frequently found in semen of both infertile and fertile men [9, 15]. Sperm cells express the Toll-like receptor (TLR) on their cell surfaces [4, 5, 14], which are generally acknowledged to play a key role in immune responses in innate immunity [7, 9, 20], but pathogen recognition by sperm via TLR2 and TLR4 induces reduced cellular motility and subsequently causes sperm apoptosis [4, 5]. Antibiotics have been used to prevent bacterial contamination of semen, however, frequent antibiotic use has resulted in antimicrobial-resistant bacteria [8, 11, 18, 19]. On the other hand, we have investigated the effect of lactoferrin (LF) on the reproductive system. LF is contained in mammalian exocrine fluids [6, 16] and express various effects, such as the antimicrobial activity [6, 12, 17]. For example, competitive binding of LF with lipopolysaccharide (LPS) for TLR4 inhibits TLR4-mediated signal transduction and suppresses inflammatory cytokine production [3]. In this study, we investigated the effect of LF on embryo development and pregnancy by using mouse sperm which were pre-treated with LPS.

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. 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 ad libitum. The facility
was maintained under a 12 hr light/12 hr dark cycle at 20–25°C.

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 a mixed anesthetic agent (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, sperm were collected from cauda epididymides and equally divided into one of four types of 100 µl medium: TYH medium (LSI Medience, Tokyo, Japan; control group), TYH medium with 1.0 mg/ml bovine LF (NRL Pharma, Tokyo, Japan; LF group), TYH medium with 1.0 mg/ml bovine LF and 1.0×10⁻³ mg/ml LPS from E. coli O111:B4 (Sigma-Aldrich, St. Louis, MO, USA; LF/LPS group), and 1.0×10⁻³ mg/ml LPS (LPS group). After incubation under 5% CO₂ at 37°C for 3 hr, sperm were used for insemination.

Forty-seven female BDF1 mice aged 4 weeks were used for in vitro fertilization, following the superovulation with CARD HyperOva™ (Kyudo, Saga, Japan) according to the manufacture’s instruction. Mice were euthanized by cervical dislocation under anesthesia of i.p. administration with MMB. Then, ova were collected and divided into four groups (control group, LF group, LF/LPS group and LPS group), and co-incubated with 6 µl of sperm suspension from the abovementioned four types of media in mHTF medium (Kyudo) under 5% CO₂ at 37°C for 3 hr, respectively. Thereafter fertilized ova were incubated in M16 medium (Sigma-Aldrich) under 5% CO₂ at 37°C for 16 hr, and only 2-cell stage embryos were used for embryo transfer.

Fifteen female Jcl:ICR mice aged 8–22 weeks were used for recipients of the embryo. Prior to embryo transfer, pseudopregnant mice were prepared by
mating female Jcl:ICR mice with male Jcl:ICR vasectomy mice. Mice were divided into four groups (control group, LF group, LF/LPS group and LPS group) and 2-cell stage embryos, which were derived from sperm prepared as described above, were separately transferred into each mouse oviduct under anesthesia with i.p. administration of MMB. On day 12 post-embryo transfer, recipient mice were euthanized by cervical dislocation under anesthesia with i.p. administration of MMB, and fetuses were removed with placentas.

As a result, 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. 1). This result leaded the notion that LF treatment not only rescued the LPS-affected sperm but also facilitated its embryogenesis. 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. 2). This result suggested that the embryo abnormality occurred in the LPS-affected sperm could be prevented by LF treatment to the sperm.

We examined the effect of LF treatment to the sperm for the embryogenesis of LSP-treated sperm. The contamination of bacterial LPS in the semen is one of the principal factors for the infertility [5]. 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.

POTENTIAL CONFLICTS OF INTEREST
The authors have nothing to disclose.

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FIGURE LEGENDS

Fig. 1. Lactoferrin (LF) effect on embryo development. Development rates of 2-cell stage embryos are measured in control group (control), LF group (LF), LF/ lipopolysaccharide (LPS) group (LF/LPS) and LPS group (LPS). Each bar represent mean ± standard error of the mean (SEM).

Fig. 2. 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.
Fig. 1

Development rate of 2-cell embryo (%)

- Control
- LF
- LF/LPS
- LPS
Fig. 2