The Journal of Veterinary Medical Science

NOTE

Anatomy

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

Takahiro AYA[1)], Yukiko TOMIOKA[1)] and Takashi TAKEUCHI[1)]*

[1)]Department of Laboratory Animal Science, Faculty of Agriculture, Tottori University, Tottori 680-8553, Japan

J. Vet. Med. Sci.
83(8): 1173–1177, 2021
doi: 10.1292/jvms.21-0269

Received: 10 May 2021
Accepted: 24 May 2021
Advanced Epub:
11 June 2021

ABSTRACT. Genital bacterial infection is one of the most important causes of infertility, however, bacteria frequently exist in seminal fluid. Sperm express Toll-like receptors (TLRs) on their cell surfaces and bacterial recognition by TLRs induces sperm apoptosis. In this study, we examined the lactoferrin (LF) potentiality on sperm apoptosis induced by bacterial lipopolysaccharide (LPS). The TdT-mediated dUTP-biotin nick end labeling (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.

KEY WORDS: apoptosis, bacteria, lactoferrin, sperm, Toll-like receptor

The average age of marriage has increased in modern society, which has resulted in some challenges such as infertility. There are many reports about causes of infertility [1, 5, 18], 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* [17, 21, 25]. 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 [8, 9, 24]. TLRs are key upstream mediators of inflammation at many tissue sites, and TLR activation results in an inflammatory immune response [2, 14]. Approximately 10–15 mammalian TLRs have been identified, with TLR1–10 present in human and TLR1–13 present in mouse [13]. Each TLR recognizes a pathogen-associated molecular pattern (PAMP) as specific components of pathogenic microorganisms, namely, bacteria, fungi, viruses, and parasites [13, 33]. 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 [8, 22, 23]. In sperm, PAMP recognition by TLRs reduces cell motility and induces apoptosis, in addition to immune response initiation [9, 10]. To prevent bacterial contamination of semen, oral administration of antibiotics or addition of antibiotics in semen has been performed [16, 19, 30]. However, frequent antibiotic use raises other problems such as negative influence on intestinal bacterial flora or antimicrobial-resistant bacteria [32]. Moreover, bacterial components can remain in semen and continue to stimulate sperm TLRs. We 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 [11]. Moreover, LF is known to have antimicrobial, anti-inflammatory, and antitumor effects [11, 29]. 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 [6]. In this study, we investigated whether LF have the potentiality to decrease LPS-induced apoptosis of sperm.

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 (CA-1; CLEA Japan) *ad libitum*. The facility was maintained under a 12 hr light/12 hr dark cycle at 20–25°C.

Fifteen male C57BL/6JJcl 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 body weight (b.w.) of bovine LF

*Correspondence to: Takeuchi, T.: ttakeuch@tottori-u.ac.jp

(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 [20]. 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 [15]. 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.

For an analysis of sperm apoptosis, 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 4',6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Burlingame, CA, USA) and observed under a fluorescence microscope (BX-53; Olympus, Tokyo, Japan). For examination, 5 random and non-overlapping fields were used 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.

For real-time RT-PCR analysis, 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*. 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 $P<0.05$.

In 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 was 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). In relation to the inhibition of the sperm apoptosis, the expression of *Caspase3* mRNA in cauda epididymides also seemed to be lower in LF/LPS group than LPS group (Fig. 3).

In this study, we performed TUNEL assay to examine the effect of orally administrated LF against LPS-induced sperm apoptosis. Additionally, we 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 [31], 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* [3], 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 [4]; 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 [12]. 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 [7, 28]. In mouse and human, the LF receptor is expressed in intestinal mucosa cells and the testis or ovary [26, 27]. 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 [9]. 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′)
Caspase3	CCTCAGAGACATTCATGG	GCAGTAGTCGCCTCTGAAGA
GAPDH	TGCCGCCTGGAGAAACCTGC	TGAGAGCAATGCCAGCCCCA

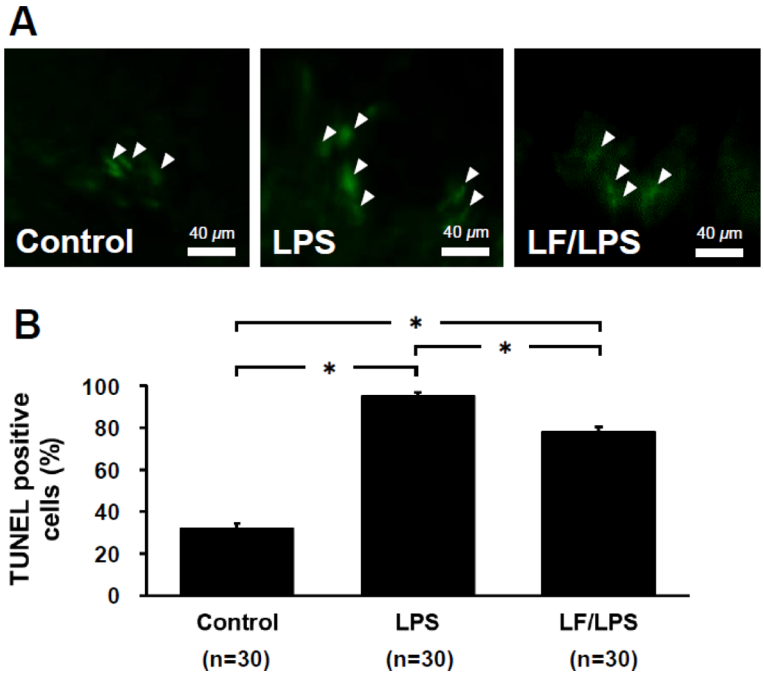

Fig. 1. Apoptotic sperm detection in testes with the TdT-mediated dUTP-biotin nick end labeling (TUNEL) assay. (A) Representative images of TUNEL-positive sperm cells (arrowheads) in saline group (Control), lipopolysaccharide group (LPS) and lactoferrin (LF)/LPS group (LF/LPS). (B) Relative percentages of TUNEL-positive sperm cells to 4',6-diamidino-2-phenylindole (DAPI)-positive total nuclei in Control, LPS and LF/ LPS. Each bar represents mean ± standard error of the mean (SEM). Asterisks indicate significant differences among control, LPS and LF/LPS (P<0.05).

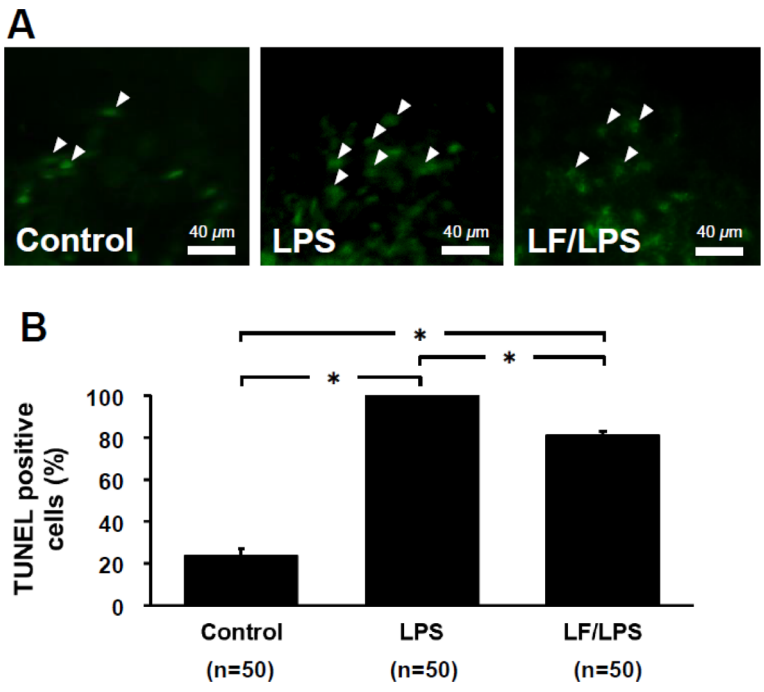

Fig. 2. Apoptotic sperm detection in cauda epididymides with the TdT-mediated dUTP-biotin nick end labeling (TUNEL) assay. (A) Representative images of TUNEL-positive sperm cells (arrowheads) in saline group (Control), lipopolysaccharide group (LPS) and lactoferrin (LF)/LPS group (LF/LPS). (B) Relative percentages of TUNEL-positive cells to 4',6-diamidino-2-phenylindole (DAPI)-positive total nuclei in Control, LPS and LF/ LPS. Each bar represents mean ± standard error of the mean (SEM). Asterisks indicate significant differences among control, LPS and LF/LPS (P<0.05).

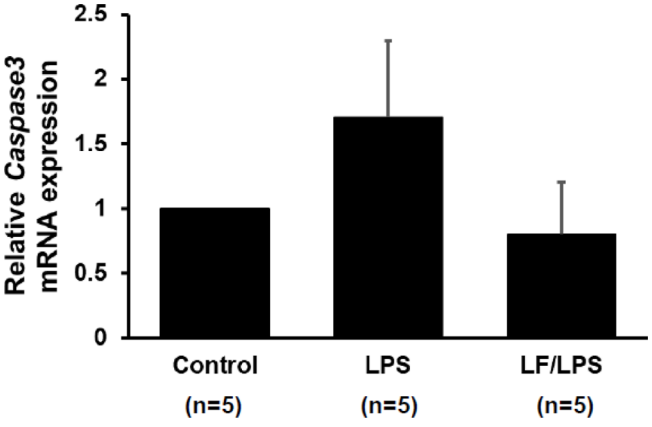

Fig. 3. Relative mRNA expression of the apoptotic biomarker gene (*Caspase3*) in cauda epididymides in saline group (Control), lipopolysaccharide group (LPS) and lactoferrin (LF)/LPS group (LF/LPS). With a control value as 1 with real-time RT-PCR analysis. Each bar represent mean ± standard error of the mean (SEM).

POTENTIAL CONFLICTS OF INTEREST. The authors have nothing to disclose.

ACKNOWLEDGMENT. This work was supported in part by JSPS KAKENHI Grant Number JP26450456.

REFERENCES

1. Abrao, M. S., Muzii, L. and Marana, R. 2013. Anatomical causes of female infertility and their management. *Int. J. Gynaecol. Obstet.* **123** Suppl 2: S18–S24. [Medline] [CrossRef]
2. Akira, S. and Takeda, K. 2004. Toll-like receptor signalling. *Nat. Rev. Immunol.* **4**: 499–511. [Medline] [CrossRef]
3. Bender, L. M., Morgan, M. J., Thomas, L. R., Liu, Z. G. and Thorburn, A. 2005. The adaptor protein TRADD activates distinct mechanisms of apoptosis from the nucleus and the cytoplasm. *Cell Death Differ.* **12**: 473–481. [Medline] [CrossRef]
4. Davidson, L. A. and Lönnerdal, B. 1989. Fe-saturation and proteolysis of human lactoferrin: effect on brush-border receptor-mediated uptake of Fe and Mn. *Am. J. Physiol.* **257**: G930–G934. [Medline]
5. de Kretser, D. M. 1997. Male infertility. *Lancet* **349**: 787–790. [Medline] [CrossRef]
6. Drago-Serrano, M. E., de la Garza-Amaya, M., Luna, J. S. and Campos-Rodríguez, R. 2012. Lactoferrin-lipopolysaccharide (LPS) binding as key to antibacterial and antiendotoxic effects. *Int. Immunopharmacol.* **12**: 1–9. [Medline] [CrossRef]
7. Fischer, R., Debbabi, H., Blais, A., Dubarry, M., Rautureau, M., Boyaka, P. N. and Tome, D. 2007. Uptake of ingested bovine lactoferrin and its accumulation in adult mouse tissues. *Int. Immunopharmacol.* **7**: 1387–1393. [Medline] [CrossRef]
8. Fu, Y., Liu, B., Feng, X., Liu, Z., Liang, D., Li, F., Li, D., Cao, Y., Feng, S., Zhang, X., Zhang, N. and Yang, Z. 2013. Lipopolysaccharide increases Toll-like receptor 4 and downstream Toll-like receptor signaling molecules expression in bovine endometrial epithelial cells. *Vet. Immunol. Immunopathol.* **151**: 20–27. [Medline] [CrossRef]
9. Fujita, Y., Negishi, H. and Shimada, M. 2011. Innate immune functions of sperm -Toll like receptor family expressed on sperm recognize bacteria infection to decrease fertilization ability. *J. Reprod. Eng.* **14**: 13–18.
10. Fujita, Y., Mihara, T., Okazaki, T., Shitanaka, M., Kushino, R., Ikeda, C., Negishi, H., Liu, Z., Richards, J. S. and Shimada, M. 2011. Toll-like receptors (TLR) 2 and 4 on human sperm recognize bacterial endotoxins and mediate apoptosis. *Hum. Reprod.* **26**: 2799–2806. [Medline] [CrossRef]
11. García-Montoya, I. A., Cendón, T. S., Arévalo-Gallegos, S. and Rascón-Cruz, Q. 2012. Lactoferrin a multiple bioactive protein: an overview. *Biochim. Biophys. Acta* **1820**: 226–236. [Medline] [CrossRef]
12. Gifford, J. L., Hunter, H. N. and Vogel, H. J. 2005. Lactoferricin: a lactoferrin-derived peptide with antimicrobial, antiviral, antitumor and immunological properties. *Cell. Mol. Life Sci.* **62**: 2588–2598. [Medline] [CrossRef]
13. Guan, Y., Ranoa, D. R. E., Jiang, S., Mutha, S. K., Li, X., Baudry, J. and Tapping, R. I. 2010. Human TLRs 10 and 1 share common mechanisms of innate immune sensing but not signaling. *J. Immunol.* **184**: 5094–5103. [Medline] [CrossRef]
14. Jiménez-Dalmaroni, M. J., Gerswhin, M. E. and Adamopoulos, I. E. 2016. The critical role of toll-like receptors--From microbial recognition to autoimmunity: a comprehensive review. *Autoimmun. Rev.* **15**: 1–8. [Medline] [CrossRef]
15. Kajihara, T., Okagaki, R. and Ishihara, O. 2006. LPS-induced transient testicular dysfunction accompanied by apoptosis of testicular germ cells in mice. *Med. Mol. Morphol.* **39**: 203–208. [Medline] [CrossRef]
16. Magri, V., Montanari, E., Škerk, V., Markotić, A., Marras, E., Restelli, A., Naber, K. G. and Perletti, G. 2011. Fluoroquinolone-macrolide combination therapy for chronic bacterial prostatitis: retrospective analysis of pathogen eradication rates, inflammatory findings and sexual dysfunction. *Asian J. Androl.* **13**: 819–827. [Medline] [CrossRef]
17. Moretti, E., Capitani, S., Figura, N., Pammolli, A., Federico, M. G., Giannerini, V. and Collodel, G. 2009. The presence of bacteria species in semen and sperm quality. *J. Assist. Reprod. Genet.* **26**: 47–56. [Medline] [CrossRef]
18. Ochsendorf, F. R. 2008. Sexually transmitted infections: impact on male fertility. *Andrologia* **40**: 72–75. [Medline] [CrossRef]
19 Okazaki, T., Mihara, T., Fujita, Y., Yoshida, S., Teshima, H. and Shimada, M. 2010. Polymyxin B neutralizes bacteria-released endotoxin and improves the quality of boar sperm during liquid storage and cryopreservation. *Theriogenology* **74**: 1691–1700. [Medline] [CrossRef]
20. Otsuki, K. and Imai, N. 2017. Effects of lactoferrin in 6 patients with refractory bacterial vaginosis. *Biochem. Cell Biol.* **95**: 31–33. [Medline] [CrossRef]
21. Philippov, O. S., Radionchenko, A. A., Bolotova, V. P., Voronovskaya, N. I. and Potemkina, T. V. 1998. Estimation of the prevalence and causes of infertility in western Siberia. *Bull. World Health Organ.* **76**: 183–187. [Medline]
22. Poltorak, A., He, X., Smirnova, I., Liu, M. Y., Van Huffel, C., Du, X., Birdwell, D., Alejos, E., Silva, M., Galanos, C., Freudenberg, M., Ricciardi-Castagnoli, P., Layton, B. and Beutler, B. 1998. Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. *Science* **282**: 2085–2088. [Medline] [CrossRef]
23. Qureshi, S. T., Larivière, L., Leveque, G., Clermont, S., Moore, K. J., Gros, P. and Malo, D. 1999. Endotoxin-tolerant mice have mutations in Toll-like receptor 4 (Tlr4). *J. Exp. Med.* **189**: 615–625. [Medline] [CrossRef]
24. Shimada, M., Yanai, Y., Okazaki, T., Noma, N., Kawashima, I., Mori, T. and Richards, J. S. 2008. Hyaluronan fragments generated by sperm-secreted hyaluronidase stimulate cytokine/chemokine production via the TLR2 and TLR4 pathway in cumulus cells of ovulated COCs, which may enhance fertilization. *Development* **135**: 2001–2011. [Medline] [CrossRef]
25. Shukla, K. K., Mahdi, A. A. and Rajender, S. 2012. Apoptosis, spermatogenesis and male infertility. *Front. Biosci. (Elite Ed.)* **4**: 746–754. [Medline] [CrossRef]
26. Suzuki, Y. A. and Lönnerdal, B. 2004. Baculovirus expression of mouse lactoferrin receptor and tissue distribution in the mouse. *Biometals* **17**: 301–309. [Medline] [CrossRef]
27. Suzuki, Y. A., Shin, K. and Lönnerdal, B. 2001. Molecular cloning and functional expression of a human intestinal lactoferrin receptor. *Biochemistry* **40**: 15771–15779. [Medline] [CrossRef]
28. Takeuchi, T., Kitagawa, H. and Harada, E. 2004. Evidence of lactoferrin transportation into blood circulation from intestine via lymphatic pathway in adult rats. *Exp. Physiol.* **89**: 263–270. [Medline] [CrossRef]
29. Tung, Y. T., Chen, H. L., Yen, C. C., Lee, P. Y., Tsai, H. C., Lin, M. F. and Chen, C. M. 2013. Bovine lactoferrin inhibits lung cancer growth through suppression of both inflammation and expression of vascular endothelial growth factor. *J. Dairy Sci.* **96**: 2095–2106. [Medline] [CrossRef]

30. Vaillancourt, D., Guay, P. and Higgins, R. 1993. The effectiveness of gentamicin or polymyxin B for the control of bacterial growth in equine semen stored at 20 degrees C or 5 degrees C for up to forty-eight hours. *Can. J. Vet. Res.* **57**: 277–280. [Medline]
31. Xue, L., Borné, Y., Mattisson, I. Y., Wigren, M., Melander, O., Ohro-Melander, M., Bengtsson, E., Fredrikson, G. N., Nilsson, J. and Engström, G. 2017. FADD, Caspase-3, and Caspase-8 and incidence of coronary events. *Arterioscler. Thromb. Vasc. Biol.* **37**: 983–989. [Medline] [CrossRef]
32. Yoon, M. Y. and Yoon, S. S. 2018. Disruption of the gut ecosystem by antibiotics. *Yonsei Med. J.* **59**: 4–12. [Medline] [CrossRef]
33. Yuan, X. and Wilhelmus, K. R. 2010. Toll-like receptors involved in the pathogenesis of experimental Candida albicans keratitis. *Invest. Ophthalmol. Vis. Sci.* **51**: 2094–2100. [Medline] [CrossRef]