Changes in the expression of interleukin-1 β and lipopolysaccharide-induced TNF factor in the oviduct of laying hens in response to artificial insemination

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Abstract

The aim of this study was to determine the physiological significance of interleukin-1 β (IL1B) and lipopolysaccharide-induced TNF factor (LITAF) in the fate of sperm in the oviduct of laying hens after artificial insemination (AI). Laying hens were inseminated with fresh semen, PBS or seminal plasma and tissues from different oviductal segments were collected to observe the general histology, changes in the mRNA expression of *IL1B* and *LITAF* and the localization of positive cells expressing immunoreactive IL1B (irIL1B). Semi-quantitative RT-PCR was used to observe the changes in mRNA expression of these molecules in the infundibulum, uterus, utero-vaginal junction (UVJ), and vagina after insemination. Intact sperm in the lumen and between the primary or secondary folds of the vagina were found until 6 h after insemination but were degraded at 12 h. The mRNA expression of *IL1B* and *LITAF* was significantly increased in the vagina until 6 h after AI but remained unchanged in the other oviductal segments. In the tissue of the vagina and UVJ, irIL1B was localized in the mucosal stroma. The number of irIL1B-positive cells was increased in the vagina but almost unchanged in UVJ after insemination with semen. Significant changes were not observed in the mRNA expression and irIL1B-positive cells in the vagina after PBS or seminal plasma insemination. The increase of IL1B and LITAF in the vagina may lead to sperm degradation and elimination by cilia of surface epithelium, whereas their lower levels in UVJ may permit sperm to survive in sperm storage tubules.

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Introduction

The hen oviduct consists of the infundibulum, magnum, isthmus, uterus and vagina, and the tissue between uterus and vagina which is known as utero-vaginal junction (UVJ; Johnson 2001). Following copulation or artificial insemination (AI), about 1% of the total deposited sperm are transported through the vagina to enter the sperm storage tubules (SST) of the UVJ and infundibulum, and reside there for a prolonged period. In avian species, upon copulation or insemination, the majority of deposited sperm is lost from the vagina (Bakst *et al.* 1994, Steele & Wishart 1996). Such removal of sperm from the vagina is a natural process but may have biological significance in the selection of good-quality sperm, whereas survivability of sperm in the SST for a prolonged period enables female birds to produce one or more clutches of fertile eggs after a single dose of AI. The SST in UVJ is the primary site for sperm residence, and upon release from the SST, they ascend to the infundibulum where fertilization occurs (Bakst 1987, Bakst *et al.* 1994). In a recent study, Froman (2003) explained sperm storage in birds in terms of sperm cell behavior and concluded that the sperm residence and

emergence from the SST may depend on sperm cell motility. Thus, if sperm are degraded to lose motility in the vagina and UVJ, they may not enter and survive in SST. Although a number of studies have focused on and described insights based on mechanical, histological and, to some extent, immunological aspects, a major gap still exists in our understanding of the actual physiological and molecular mechanisms that regulate the sperm removal from vagina and sperm survivability in UVJ in avian species. However, recent reports showed the increase of immunocompetent cells such as antigen presenting cells and T cells in the UVJ of low-fertility hens after AI, suggesting that an immune response to sperm may occur in the avian oviduct (Das *et al.* 2005a, 2005b). Therefore, the suppression of local immunity of UVJ against sperm seems important for sperm survivability in SST.

Our recent study significantly extended relevant findings showing an increased expression of transforming growth factor- β s (TGF β s) and their receptors in UVJ during sperm storage (Das *et al.* 2006). Sperm also expressed TGF β s and their receptors by themselves, and their expression is correlated with sperm fertility (Das *et al.* 2007). Since TGF β s are immunosuppressive

cytokines, they may function for the protection of sperm stored in the SST by suppressing anti-sperm immunoreactions (Das *et al.* 2006). TGF β is a potent regulatory cytokine in the immune system and its regulatory activities are modulated by the presence of varieties of inflammatory cytokines and costimulatory molecules (Li *et al.* 2006). This suggests that, while inducing their function, TGF β s may recruit other inflammatory cytokines in the hen oviduct in response to sperm. However, there is no information as to whether the expression of inflammatory cytokines in the hen oviduct is altered in response to sperm.

In mammals, inflammatory response by immunocompetent cells in response to sperm or semen has been suggested in the reproductive tract (Robertson *et al.* 1996, Rozeboom *et al.* 1998, 1999, Robertson 2005, 2007). There are reports that immunoregulatory TGF β s function mostly as inhibitory molecules on macrophages (Li *et al.* 2006) and also inhibit the production of inflammatory cytokines and chemokines (Fadok *et al.* 1998, McDonald *et al.* 1999). Macrophages, however, are known as major cell types that secrete a series of cytokines, including interleukin-1 β (IL1 β) and tumor necrosis factor- α (TNF; Peters *et al.* 1986, Rautenschlein *et al.* 1999). If the immunoreactions are enhanced by IL1 β in the oviductal mucosal tissue, it may affect the survivability of sperm. In chickens, to date, TNF has not been identified, whereas lipopolysaccharide-induced TNF factor (LITAF) that may induce the expression of TL1A (TNFSF15), a member of TNF ligand superfamily (TNF-related molecule), has been identified (Hong *et al.* 2006). The LITAF may also play roles in the breakdown of protein molecules and other substances because it is located in the lysosomal membrane (Moriwaki *et al.* 2001). Since TNF is known to exhibit cytotoxic activity to cause germ cell death in mammalian testis (Grataroli *et al.* 2004), molecules of the TNF ligand superfamily may also cause damage of sperm in the oviduct. The elevated expression of TGF β s in UVJ after AI (Das *et al.* 2006) may play a role in regulation of the expression of proinflammatory cytokines.

IL1 β and TNF are the first-line co-stimulatory cytokines that mostly induce their functions to enhance immunoreactions in biological systems. Therefore, less exposure of IL1 β and TNF-related molecules to sperm in the UVJ of the hen oviduct may be significant for sperm survivability. Recently, mRNA expression of IL1 β , LITAF, and the TNF receptor superfamily was reported in the ovary and oviduct of laying hens, suggesting the involvement of these cytokines in the process of avian reproductive functions (Birdgham & Johnson 2004, Subedi *et al.* 2007, Sundaresan *et al.* 2007a, 2007b). However, the physiological significance of IL1 β and TNF-related molecules in the avian oviduct, particularly in relation to the avoidance of sperm from the vagina, is not fully understood. It also remains unclear whether these molecules affect the sperm stored in SST in UVJ.

Thus, the goal of our present study was to examine the role of inflammatory cytokines in the fate of sperm in the hen oviduct by observing their changes in expression in different oviductal segments after insemination of the birds with fresh semen, PBS or seminal plasma. If they are enhanced in the vagina following insemination, it may be involved in the process of sperm avoidance from the vagina; however, if they affect sperm they should not be increased in the tissue of UVJ. The mRNA expression of IL1 β and LITAF in different oviductal segments after insemination with fresh semen or PBS or seminal plasma was investigated by RT-PCR. Histological observations of oviductal segments were made and localization of immunoreactive IL1 β (irIL1 β) in the vagina and UVJ was confirmed by immunohistochemistry.

Results

Experiment 1: Histological observation

In the vagina, the mucosal folds were developed and intact sperm were observed on the surface of mucosa for 1–6 h after AI (Fig. 1A and B). Degraded sperm were found at 12 h after AI (Fig. 1C), whereas no sperm and/or sperm fragments were observed in the vagina at 24 h after AI (data not shown). Head regions of the sperm at 1 h after AI showed smooth and long thread-like structures and the feature was kept similar until 6 h (Fig. 1A and B), whereas those of degraded sperm at 12 h after AI showed irregular surface and body structures with small and rounded particles around them (Fig. 1C). In the UVJ, SSTs were filled with sperm for 1–24 h after AI at a ratio of ~50–60% (data not shown). No histological change was observed in the cells of mucosal tissues at all times after insemination examination.

Experiment 2: RT-PCR analysis for IL1 β and LITAF expression in oviductal segments, and TNF receptor in sperm

PCR products of IL1 β and LITAF were observed at their predicted size (138 and 229 bp respectively; Fig. 2). The expression of IL1 β was not changed in the infundibulum, uterus, and UVJ during 0–24 h by insemination with PBS or semen (Fig. 3A–C). In the vagina, insemination with semen resulted in the significant increase in IL1 β expression at 1 and 3 h compared with pretreatment, whereas PBS treated group did not show significant change. Significant differences between semen and PBS treatment groups were found at 1, 3, and 6 h after insemination (Fig. 3D). Figure 4 shows the change in expression of LITAF in the oviduct following insemination with semen or PBS. The expression of LITAF did not change in the infundibulum and UVJ from 0 to 24 h after insemination with semen or PBS (Fig. 4A and C). In the uterus, the expression was decreased at 12 h after treatment with semen or PBS, but there was no difference

in the expression level between semen and PBS insemination groups at all times after insemination (Fig. 4B). In the vagina, a significant difference was not observed during 0–24 h within the semen or PBS insemination groups compared with pretreatment; however, the amount of expression was significantly greater in the semen insemination group than PBS group at 1 and 3 h of treatment (Fig. 4D). Figure 5 shows the

change in expression of *IL1B* and *LITAF* during 0–3 h after insemination with seminal plasma in UVJ and vagina. The expression of them was not changed in both UVJ (Fig. 5A and C) and vagina (Fig. 5B and D). The mRNA expression of TNF receptor in chicken sperm was observed at the predicted product size of 440 bp (Fig. 6).

Experiment 3: Immunohistochemistry for *irIL1B*

The *irIL1B* cells were observed in the lamina propria of the vagina (Fig. 7A and B) and UVJ (Fig. 7C and D) before and after insemination. Numbers of *irIL1B* cells in the vagina were significantly increased from 1 to 6 h by insemination with fresh semen but were unchanged by PBS, and there were significant differences at 1, 3, and 6 h among semen and PBS groups (Fig. 8A). In the UVJ, however, the number of positive cells showed a significant but small increase only at 3 h with fresh semen, whereas in the case of PBS insemination no significant difference was observed (Fig. 8B).

Discussion

We report that *IL1B* and *LITAF* are expressed in mucosal tissue of the hen oviduct, and changed in the vagina, but not in UVJ, with AI. The significant findings of the present study are: (i) among the four oviductal segments (infundibulum, uterus, UVJ, and vagina), expression of both *IL1B* and *LITAF* was significantly increased only in the vagina in response to semen insemination; (ii) immunopositive cells for *irIL1B* were observed in lamina propria and their population was significantly increased in the vagina but were almost unchanged in UVJ after semen insemination, and (iii) sperm expressed TNF receptor mRNA.

In the vagina, the expression of *IL1B* and *LITAF* was significantly increased for 1 to 6 h after insemination, and then returned to the level at 0 h. The numbers of immunopositive cells for *irIL1B* in the stroma of the vagina were also increased during the same period of observation. Histological observation of the vagina confirmed the presence of undegraded sperm in the lumen and between the primary and secondary folds until 6 h after insemination but were degraded or had

Figure 1 Sections of the vagina of hen oviduct after insemination. (A) 1 h after insemination, (B) 6 h after insemination, and (C) 12 h after insemination (AI). Note that intact sperm were observed in the lumen of the vagina and/or in between the primary folds of the vagina at 1 h and 6 h after insemination (arrows, A and B). Degraded parts of sperm showing irregular surface and body structures with round particles are seen in the lumen of the vagina and in between the vaginal folds at 12 h after AI (arrowheads, C). By contrast, the epithelial and stromal cells in the mucosal tissue do not show degenerative features. E, surface epithelium; LP, lamina propria; L, lumen. Scale bars = 50 μ m. Insets of (A) and (B) show magnified view of the vagina with long thread-like structures of intact sperm (arrows) and (C) shows magnified view of the vagina with round particles of sperm fragments (arrowheads). Scale bars = 10 μ m.

Figure 2 RT-PCR analysis of the expression of *IL1B* and *LITAF* in tissue of the infundibulum, uterus, uterovaginal junction, and vagina. Clear bands are seen for *IL1B* and *LITAF* at ~138 and 229 bp respectively, for all oviductal segments when samples are reverse transcribed with ReverTra Ace (left). RNA samples that are subjected to reverse transcription without ReverTra Ace do not show bands either of *IL1B* and *LITAF* (right). Tissue was collected from non-inseminated laying hens at 5 h after oviposition. inf, infundibulum; ut, uterus; uvj, utero-vaginal junction; vag, vagina; M, DNA marker of 100 bp ladder.

disappeared at 12 and 24 h. Previous reports showed that insemination caused a dramatic influx of inflammatory cytokines into the site of semen deposition in different species (Robertson 2007). A surge of proinflammatory cytokines was observed in the stromal tissue of the endometrium within 1 h of insemination in mice (Sanford *et al.* 1992, Robertson *et al.* 1998) as well as in pigs (Rozeboom *et al.* 1998, O'Leary *et al.* 2004). The mRNA expression of *IL1B*, *IL6*, and leukemia inhibitory factor were increased 2–3-fold in the endometrium after stimulation with seminal plasma (Gutsche *et al.* 2003). These cytokines caused influx and accumulation of a variety of inflammatory leukocytes, macrophages, dendritic cells, and granulocytes to the endometrial stroma (Bischof *et al.* 1994, 1995, Engelhardt *et al.* 1997). There are reports suggesting that avian and mammalian

cytokines may perform similar tasks, although their primary structures in most cases are remarkably different (Weining *et al.* 1998, Staeheli *et al.* 2001). The higher mRNA expression of *IL1B* and *LITAF* as well as the increase of irIL1B cells in the vagina of the hen oviduct for 1–6 h after insemination with semen observed in the current study may be the eventual results of the inflammatory response to semen that may affect the fate of sperm. These results were further justified by the fact that the mucosal tissue of the vagina failed to show any increase in expression of those mRNAs or in irIL1B cells when laying hens were inseminated with the same volume of PBS. Since the vaginal tissue did not show significant changes in the expression of *IL1B* and *LITAF* within 3 h of seminal plasma insemination, the increase in their expression with semen might occur in response to sperm rather than seminal plasma. The expression of *LITAF* in the uterus was decreased after 12 h of semen insemination. It is assumed that this decline of *LITAF* expression occurred as the spontaneous changes in its expression during an ovulatory cycle because the same decline was observed in the birds inseminated with PBS.

Sperm cells in the vaginal lumen were degraded at 12 h after AI, suggesting a consequence of sperm elimination from the vagina. A remarkable degree of sperm redundancy in the vagina is considered to be an important factor for the selection of good-quality sperm (Bakst *et al.* 1994). There are two possible factors involved in selection or loss of sperm deposited with vagina. One is the ciliary beat of vaginal epithelial cells to transport them towards the cloaca (Brillard 1993, Bakst & Akuffo 2008). Another is the response of vaginal cells to release some molecules that may affect the sperm survivability. In addition to the basic functions, i.e. the regulation of inflammatory

Figure 3 Changes in the expression of *IL1B* in the oviduct in response to insemination with semen or PBS. (A) infundibulum, (B) uterus, (C) UVJ, and (D) vagina. Each value indicates mean $\pm$ S.E.M. ($n=4$). Values were compared by two-way ANOVA (treatment $\times$ time) followed by Tukey-Kramer test. In the vagina, significances were obtained in the effects of treatment ($F(1, 6)=6.337$, $P<0.05$), time ($F(5, 30)=6.597$, $P<0.001$), and interaction ($F(5, 30)=5.905$, $P<0.001$). Values with different letters are significantly different within treatment ($P<0.05$). *Significantly different between semen and PBS insemination within an examined time ($P<0.05$).

Figure 4 Changes in the expression of *LITAF* in the oviduct in response to insemination with semen or PBS. (A) infundibulum, (B) uterus, (C) UVJ, and (D) vagina. Each value indicates mean $\pm$ S.E.M. ($n=4$). Values were compared by two-way ANOVA (treatment $\times$ time) followed by Tukey-Kramer test. In the vagina, significances were obtained in the effects of time ($F(5, 30)=3.499$, $P<0.05$) and interaction ($F(5, 30)=2.940$, $P<0.05$). *Significantly different between semen and PBS insemination within an examined time ($P<0.05$).

response and immunity, few subsets of the TNF superfamily, including TNF, are also known to exhibit potent cytotoxic activity by inducing apoptosis of varieties of susceptible cell lines (Nagata 1997, Gupta & Gollapudi 2006, Chen *et al.* 2007). Induction of germ cell death during spermatogenesis by TNF-related apoptosis-inducing ligand was also suggested in the adult human testis (Grataroli *et al.* 2004). The present study showed an

increase of *LITAF* in the vagina and confirmed the expression of TNF receptor in sperm. It has been reported that *LITAF* induces the expression of TNF ligand superfamily members like TNFSF15 (TL1A; Hong *et al.* 2006). The increased expression of *LITAF* in the vagina after AI with semen observed in the present study may cause the expression of TNF-related molecules. If such molecules are synthesized, the interaction of them with TNF receptor

Figure 5 Effects of seminal plasma insemination on the expression of *IL1B* and *LITAF* in the uterovaginal junction and vagina. (A) and (B) *IL1B* expression in the UVJ and vagina; (C) and (D) *LITAF* expression in the UVJ and vagina; Values are mean $\pm$ S.E.M. ($n=4$). Data were analyzed by one-way ANOVA, followed by Tukey-Kramer test.

Figure 6 RT-PCR analysis of the expression of TNF receptor mRNA in sperm cells with or without ReverTra Ace. A clear band is seen for TNF receptor at ~440 bp for sperm cells when the RNA sample is reverse transcribed with ReverTra Ace (left). RNA sample subjected to reverse transcription without ReverTra Ace does not show any band for TNF receptor (right). M, DNA marker of 100 bp ladder.

in sperm may cause degradation of sperm that are unable to transverse the vaginal part of oviduct. Recent report suggested that LITAF may be located in the lysosomal membrane and play roles in the breakdown of protein molecules and other substances (Moriwaki *et al.* 2001). However, the significant changes in the lysosomal activity in the vaginal cells may not be induced in response to AI because those cells did not show histological changes.

Histological observation of UVJ revealed that the SST contained sperm for 1–24 h after insemination. The expression of *IL1B* and *LITAF* in UVJ was unchanged, although the populations of irIL1B cells showed only a small and short increase. These results suggest that the inflammatory response to sperm might not be enhanced in the tissue of UVJ even in the presence of abundant sperm in SST. Our previous reports revealed an increased

expression of *TGFBs* and their receptors in UVJ, but not in the vagina after AI (Das *et al.* 2006). These results suggest a possible correlation between expression of *IL1B*, *LITAF*, and *TGFBs* in UVJ in the presence of sperm in their SST. *TGFBs* induce inhibitory functions on macrophages, chemokines, and proinflammatory cytokines (Peters *et al.* 1986, Fadok *et al.* 1998, McDonald *et al.* 1999, Rautenschlein *et al.* 1999, Li *et al.* 2006), and those produced by macrophages can act in a paracrine manner on T cells to suppress immune responses (Reinhold *et al.* 1994, 1995, Ahmad *et al.* 1997). There seems to be an association between the higher expression of *TGFBs* and lower expression of *IL1B* and *LITAF* in the UVJ after AI. The lower levels of *IL1B* and *LITAF* in UVJ after AI suggest lower exposure of sperm to IL1B and TNF-related molecules, leading to sperm survivability in SST by protecting them from immunoreactions.

In conclusion, we have provided evidence that *IL1B* and *LITAF* mRNAs and irIL1B cells are increased in the vagina with AI, probably in response to sperm. Mucosal tissue of UVJ that stored a sufficient number of sperm, however, does not show their increased expression. The increase of IL1B and TNF-related molecules in the vagina may lead to sperm degradation and elimination by cilia of surface epithelium, whereas their lower levels in UVJ may permit sperm to survive in SST.

Materials and Methods

Birds, treatment, and tissue collection

Healthy single-comb White Leghorn laying hens, ~32 weeks of age and laying 5 or more eggs at regular intervals were maintained in individual cages under a photoperiod of 14 h

Figure 7 Sections of vagina and uterovaginal junction of non-inseminated and inseminated birds immunostained for irIL1B. (A) Vagina before insemination, (B) Vagina 1 h after insemination, (C) UVJ before insemination, and (D) UVJ 1 h after insemination. Arrows indicate positive cells for irIL1B in the mucosal tissues of vagina and UVJ. E, surface epithelium; LP, lamina propria; L, lumen, St, sperm storage tubule. Scale bar=50 µm. Inset of (B) shows magnified view of positive cells for irIL1B. Scale bar=25 µm.

Figure 8 Frequency of the populations of positive cells for irIL1B in the oviduct inseminated with semen or PBS. (A) irIL1B in the vaginal stroma, (B) irIL1B in the UVJ stroma. Each value indicates mean $\pm$ S.E.M. ($n=4$). Values were compared by two-way ANOVA (treatment $\times$ time) followed by Tukey-Kramer test. In the vagina, significances for irIL1B were obtained in the effects of treatment ($F(1, 6)=16.986$, $P<0.05$), time ($F(5, 30)=14.981$, $P<0.001$) and interaction ($F(5, 30)=9.905$, $P<0.001$). Values with different letters are significantly different within treatment ($P<0.05$). *Significantly different between semen and PBS insemination within an examined time ($P<0.05$).

light:10 h darkness and provided with free access to food and water. The hens were intravaginally inseminated with ~ 0.05 ml fresh semen containing $\sim 2 \times 10^8$ sperm or PBS (control; Nissui Pharmaceutical Co., Ltd, Tokyo, Japan) from the cloaca using a 1 ml plastic syringe. To examine whether the seminal plasma components were involved in the effects of semen insemination on cytokine expression, seminal plasma (0.05 ml) was also used for insemination. Semen was collected by abdominal massage from White Leghorn roosters ($n=4$) maintained under similar conditions to the laying hens. Seminal plasma was separated from the fresh semen by centrifuging at 1700 g for 10 min.

The mucosal tissues of infundibulum (tubular region), uterus, UVJ, and vagina were collected for observation of the expression of *IL1B* and *LITAF* at 0, 1, 3, 6, 12, and 24 h after insemination with fresh semen or PBS ($n=4$ each). The tissue was collected at 5 h after oviposition (the time of AI was 5, 4, and 2 h after oviposition for the groups of 0, 1, and 3 h after AI,

and was 1 and 19 h before oviposition for the groups of 6 and 24 h after AI), except for the group of 12 h after insemination in which the tissue was collected just prior to oviposition. The time of oviposition was estimated based on the observations of the oviposition times for several weeks and the exact time of oviposition on the day of tissue collection. Since the significant effects of semen insemination on the expression of *IL1B* and *LITAF* was observed within 3 h of insemination, the tissues from four oviductal segments were collected at 0, 1, and 3 h after insemination with seminal plasma ($n=4$ each). When oviductal tissues were collected, the birds were killed under anesthesia with Nembutal (Abbott Laboratories). Birds were handled in accordance with regulations approved by the Animal Experiment Committee of Hiroshima University, Japan.

Experiment 1: Histological observation

Tissues from the vagina and UVJ of all birds were fixed with Bouin's solution followed by embedding in paraffin in the usual manner. Paraffin sections (4 μm in thickness) were air-dried on silane-coated slides. After deparaffinization, sections were stained with hematoxylin and eosin to observe the general structure of the mucosal tissues and sperm distribution in the oviductal lumen.

Experiment 2: RT-PCR analysis for the expression of *IL1B*, *LITAF*, and *TNF receptor*

Extraction of total RNA

For total RNA extraction from oviductal segments, mucosal tissues from the infundibulum (tubular region), uterus, UVJ, and vagina were collected and added to 1 ml Sepasol RNA I Super (Nacalai Tesque Inc., Kyoto, Japan). Extraction was performed as described previously by Barua & Yoshimura (2004). Observation was also made for the possible expression of *TNF receptor* mRNA in chicken sperm. Collection of semen samples and RNA isolation were performed as described in our previous studies (Das *et al.* 2006, 2007). The pellet of RNA was suspended in TE buffer and incubated with DNase I (Roche Diagnostics GmbH) at a concentration of 10 U/ μl . RNA concentration was measured with Gene Quant Pro (Amersham Pharmacia Biotech.) and stored at -80°C until further analysis.

Semi-quantitative RT-PCR

Semi-quantitative RT-PCR was performed as described previously by Das *et al.* (2006). RNA samples were reverse transcribed at 42°C for 30 min followed by heat inactivation at 99°C for 10 min using a Programmable Thermal Controller PTC-100 (MJ Research Inc., Waltham, MA, USA). For transcription, 10 μl reaction mixture was prepared containing 1 μg total RNA, 1 $\times$ RT buffer, 1 mM each dNTP mixture, 20 U RNase inhibitor, 0.5 mM oligo (dT), and 50 U ReverTra Ace (Toyobo Co. Ltd, Osaka, Japan). Some of the samples were treated with the same process but without ReverTra Ace that was used to confirm the absence of genomic DNA in PCR samples. Primer information for *IL1B* (accession no: NM_204524), *LITAF*

Table 1 List of primers with references/accession numbers.

Primers	Observed (bp)	Sequences	References/accession number
<i>IL1B</i>	138	F: 5'-GGGCATCAAGGGCTACAA-3' R: 5'-CTGTCCAGGCGGTAGAAGAT-3'	Subedi <i>et al.</i> (2007) NM_204524
<i>LITAF</i>	229	F: 5'-TGTGTATGTGCAGCAACCCGTAGT-3' R: 5'-GGCATTGCAATTTGGACAGAAAGT-3'	Hong <i>et al.</i> (2006) AY765397
TNF receptor	440	F: 5'-CACAGAATGTAAGCCCTGTCC-3' R: 5'-TGGAGTTCTGCGATCCTGCATT-3'	Abdalla <i>et al.</i> (2004) BAC55966
β -actin		F: 5'-TTCCAGCCATCTTCTTG-3' R: 5'-TCCTTCTGCATCCTGTCA-3'	Kost <i>et al.</i> (1983) X00182

(Hong *et al.* 2006, accession no: AY765397), TNF receptor (Abdalla *et al.* 2004, accession no: BAC55966), and chicken β -actin (Kost *et al.* 1983, accession no: X00182) are shown in Table 1. A mixture of 25 μ l volume consisting of 0.2 μ M specific primers, a 0.5 μ l aliquot of cDNA, 1 $\times$ PCR buffer, 0.2 mM dNTP mixture, 0.626 U TaKaRa Taq (TaKaRa Bio Inc., Shiga, Japan), and 19 μ l DNase-free water was subjected to semi-quantitative PCR to observe mRNA expression. The protocol for the PCR amplification was as follows: denaturation at 95 °C for 1 min; 35, 30 or 40 cycles of denaturation for *IL1B*, *LITAF*, and TNF receptor respectively at 95 °C for 1 min; annealing at 62 °C (for *IL1B* and TNF receptor) or 68 °C (for *LITAF*) for 1 min; extension at 72 °C for 2 min and final extension at 72 °C for 10 min in a programmable thermal controller PTC-100 (MJ Research Inc.). Chicken β -actin was used as an internal control. PCR amplification for β -actin cDNA was performed as follows: denaturation at 95 °C for 1 min; 30 cycles of denaturation at 95 °C for 1 min; annealing at 58 °C for 1 min; extension at 72 °C for 2 min and final extension at 72 °C for 10 min. In preliminary experiments, different numbers of cycles (25, 30, 35, and 40 cycles) for *IL1B* and *LITAF* were tested in various samples from the vagina to optimize amplification. PCR products were electrophoresed in a 2% (w/v) agarose gel with 0.4% ethidium bromide. Band density for *IL1B* and *LITAF* was quantified with reference to that of β -actin using UN-SCAN-IT gel (version 6.1, Silk Scientific Corporation, Orem, UT, USA) and the ratios of either *IL1B* or *LITAF* to β -actin were obtained.

Experiment 3: Immunohistochemistry for *irIL1B*

The sections prepared as described in *Experiment 1* were used for immunostaining to confirm the localizations of *irIL1B* in tissues of the vagina and UVJ. For *irIL1B* immunostaining, sections were incubated with 20 μ g/ml proteinase K (Sigma-Aldrich) for 25 min at 37 °C. After washing in PBS for 15 min (5 min $\times$ 3 times), sections were incubated with 1.5 μ l/ml normal goat serum (Vector Laboratories, Inc., Burlingame, CA, USA) for 1 h. They were then incubated with rabbit polyclonal antibody to chicken *IL1B* antibody (Abcam Ltd, Cambridge, UK) diluted to 1:100 in PBS containing 0.05% BSA (Nacalai Tesque Inc.). All sections were incubated overnight at 4 °C. After washing with PBS (5 min $\times$ 3 times), sections were incubated with biotinylated anti-rabbit IgG (Vector Laboratories, Inc.) for 2 h and with avidin peroxidase complex (Nichirei Corporation, Tokyo, Japan) for 30 min. Sections

were washed again in PBS (5 min $\times$ 3 times) and immuno-precipitates were visualized by incubating with 0.02% (w/v) 3',3'-diaminobenzidine tetrahydrochloride (Nacalai Tesque Inc.) and 0.001% (v/v) H₂O₂ in 0.05 M Tris-HCl buffer (pH 7.6). Slides were counterstained with hematoxylin, dehydrated, and covered. Control slides were prepared as the same manner except that the first antibody was replaced with normal rabbit IgG.

Image analysis

The populations of SST-containing sperm and immunopositive cells for *irIL1B* in the stroma of the vagina and UVJ were observed and analyzed under a light microscope with a computer-assisted image analysis system (Image-Pro Plus; Media Cybernetics, Silver Spring, MD, USA). Immunopositive cells were counted in five different regions ($\sim 7\text{--}10 \times 10^4 \mu\text{m}^2$ area in each count) randomly selected from 3 to 4 slides of each vagina or UVJ section. The number of cells positive for *irIL1B* in a $5 \times 10^4 \mu\text{m}^2$ area was then calculated.

Statistical analysis

Differences in *IL1B* or *LITAF* expression and in the number of immunopositive cells for *irIL1B* among treatment groups (insemination with PBS or fresh semen at 0, 1, 3, 6, 12, and 24 h) were analyzed by repeated measure two-way ANOVA using StatView, version 5, SAS institute, Cary, USA. Data on *IL1B* or *LITAF* expression after seminal plasma insemination were analyzed by one-way ANOVA. Significance of differences between PBS and fresh-semen groups among each time of insemination within each treatment was analyzed using Tukey-Kramer test. Differences were considered significant when $P < 0.05$.

Declaration of interest

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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