Reproduction Advance Publication first posted on 6 June 2008 as Manuscript REP-08-0175

Changes in the expression of Interleukin-1β and Tumor Necrosis Factor-α in the oviduct of laying hens in response to artificial insemination

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Running title: *Sperm survivability and IL-1β and TNF-α in hen oviduct*

Key words: utero-vaginal junction, sperm survivability, IL-1β, TNF-α, vaginal inflammatory response

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The aim of this study was to determine the physiological significance of two proinflammatory cytokines, interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α), 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 IL-1β and TNF-α, and the immunolocalization of these molecules. Semi-quantitative RT-PCR was used to observe the changes in mRNA expression of these cytokines in the infundibulum, uterus, 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. mRNA expression of IL-1β and TNF-α 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, positive cells for immunoreactive (ir)IL-1β and irTNF-α were localized in the stroma and surface epithelium, respectively. The number of irIL-1β and irTNF-α cells were increased in the vagina but almost unchanged in UVJ after insemination with semen. Significant changes were not observed in the mRNA expression and immunopositive cells for irIL-1β and irTNF-α in the vagina after PBS or seminal plasma insemination. The increase of IL-1β and TNF-α 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.

Introduction

In hens, 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 utero-vaginal junction (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 *et al*. 1994, Bakst 1987). 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 loose 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 vagina (Higaki *et al*. 1995) and UVJ (Das *et al*. 2005a, b) of low fertility hens after AI, suggesting that an immune response to sperm may occur in the avian oviduct. 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-βs (*TGFβs*) and their receptors in UVJ during sperm storage (Das *et al*. 2006). Sperm also expressed *TGFβs* and their receptors by themselves, and their expression is correlated with sperm fertility (Das *et*

al. 2007). Because TGFβs are immunosuppressive cytokines, they may function for the protection of sperm stored in SST by suppressing anti-sperm immunoreactions (Das *et al*. 2006). TGFβ 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, TGFβs may recruit other inflammatory cytokines in the hen oviduct in response to sperm. However, there is no information whether the expression of inflammatory cytokines in the hen oviduct is altered in response to sperm.

Inflammatory responses to semen or sperm have been reported in mammals (Taylor 1982, Claus 1990, Rozeboom *et al*. 1998, 1999, Robertson 2005, 2007), which activates the epithelial cells of the reproductive tract to synthesize a series of inflammatory cytokines (Sanford *et al*. 1992, Robertson *et al*. 1992, Robertson *et al*. 1996). The increase of these cytokines following copulation or AI, however, caused the influx of immunocompetent cells such as macrophages, dendritic cells and granulocytes into the stromal tissue of mammalian uterus (Robertson 2007). There are reports that immunoregulatory TGFβs 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 IL-1β and TNF-α (Peters *et al*. 1986, Rautenschlein *et al*. 1999). If the immunoreactions are enhanced by IL-1β in the oviductal mucosal tissue, it may affect the survivability of sperm. Because TNF-α is known to exhibit cytotoxic activity to cause germ cell death in mammalian testis (Grataroli et al., 2004), it may also cause damage of sperm in the oviduct. The elevated expression of *TGFβs* and their receptors in UVJ after AI (Das *et al*. 2006) may play a role in regulation of the expression of proinflammtory cytokines such as IL-1β and TNF-α in UVJ.

IL-1β and TNF-α are the first-line co-stimulatory cytokines that mostly induce their functions to enhance immunoreactions in biological systems. Therefore, less exposure of these molecules to sperm in the UVJ of the hen oviduct may be significant for sperm survivability. Recently, mRNA expression of IL-1β, lipopolysaccharide-induced TNF-α and TNF-α receptor super family 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, Sundaresan *et al*. 2007a, b, Subedi *et al*. 2007). However, the physiological significance of these molecules in the avian oviduct, particularly in relation to the voidance 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 IL-1β and TNF-α 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 voidance from the vagina; however, if they affect sperm they should not be increased in the tissue of UVJ where sperm are stored. In experiment 1, laying hens were inseminated with fresh semen or PBS or seminal plasma and the mRNA expression of IL-1β (*IL-1β*) and TNF-α related molecule (*TNF-α*) in different oviductal segments were investigated by RT-PCR. In experiment 2, histological observations of oviductal segments were made and immunocytochemistry of immunoreactive IL-1β (irIL-1β) and irTNF-α was performed to confirm their localization in the vagina and UVJ.

Materials and Methods

Birds, treatment and tissue collection

Healthy Single-comb White Leghorn laying hens, approximately 32 weeks of age and laying 5 or more eggs at regular intervals, were maintained in individual cages under a photoperiod of 14L : 10D and provided with free access to feed and water. The hens were intravaginally inseminated with approximately 0.05 ml of fresh semen containing approximately 2 X 10^8 sperm or phosphate-buffered saline (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 *IL-1β* and *TNF-α* at 0, 1, 3, 6, 12 and 24 h after insemination with fresh semen or PBS (n = 4 each). The time of tissue collection for the groups of 0, 1, 3, 6 and 24 h after insemination was 5 h after oviposition, whereas that for the group of 12 h after insemination was just prior to oviposition. The time of oviposition was determined by recording the exact time of oviposition on the day of tissue collection, or estimated based on observation of the oviposition times for several weeks before the day of tissue collection. Because the significant effects of semen insemination on the expression of *IL-1β* and *TNF-α* was observed within 3 h of insemination, the tissues from four oviductal segments were collected at 0, 1 and 3 hrs after insemination with seminal plasma (n = 4 each). When oviductal tissues were collected, the birds were euthanized under anesthesia with Nembutal (Abbott Laboratories, Chicago, USA). Birds were handled in accordance with regulations approved by the Animal Experiment Committee of Hiroshima University, Japan.

Experiment 1. Semi-quantitative RT-PCR analysis for the expression of IL-1β, TNF-α and TNF-α 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 of Sepasol RNA I Super (Nacalai Tesque Inc., Kyoto, Japan). Extraction was performed as described previously by Barua and Yoshimura (2004). Observation was also made for the possible expression of TNF-α receptor mRNA in chicken sperm. Collection of semen samples and RNA isolation were as described in our previous studies (Das *et al*. 2006, 2007). The pellet of RNA was suspended in TE buffer and incubated with DNase 1 (Roche Diagnostics GmbH, Mannheim, Germany) at a concentration of 10 U/µl. RNA concentration was measured with Gene Quant Pro (Amersham Pharmacia Biotech., Uppsala, Sweden) and stored at –80^{o}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^{o}C for 30 min followed by heat inactivation at 99^{o}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 of total RNA, 1×RT buffer, 1mM 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, which was used to confirm the absence of genomic DNA in PCR samples. Primer information for *IL-1β* (Acces. no: NM_204524), *TNF-α* (Hong *et al*. 2006, Acces. no: AY765397), *TNF-α* receptor (Abdalla *et al*. 2004, Acces. no: BAC55966) and chicken *β-actin* (Kost *et al*. 1983, Acces. no: X00182) are shown in Table 1. A mixture of 25 µl

volume consisting of 0.2 μM specific primers, a 0.5 μl aliquot of cDNA, 1×PCR buffer, 0.2 mM dNTP mixture, 0.626 U TaKaRa Taq^{TM} (TaKaRa Bio Inc., Shiga, Japan) and 19 μl of 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^{o}C for 1 min; 35, 30 or 40 cycles of denaturation for *IL-1β*, *TNF-α* and *TNF-α* receptor, respectively at 95^{o}C for 1 min; annealing at 62^{o}C (for *IL-1β* and *TNF-α* receptor) or 68^{o}C (for *TNF-α*) for 1 min; extension at 72^{o}C for 2 min and final extension at 72^{o}C for 10 min in a Programmable Thermal Controller PTC-100 (MJ Research Inc., Waltham, MA, USA). Chicken *β-actin* was used as an internal control. PCR amplification for β-actin cDNA was performed as follows: denaturation at 95^{o}C for 1 min; 30 cycles of denaturation at 95^{o}C for 1 min; annealing at 58^{o}C for 1 min; extension at 72^{o}C for 2 min and final extension at 72^{o}C for 10 min. In preliminary experiments, different numbers of cycles (25, 30, 35 and 40 cycles) for *IL-1β* and *TNF-α* 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 *IL-1β* and *TNF-α* was quantified with reference to that of *β-actin* using UN-SCAN-IT gel$_{TM}$ (ver.6.1, Silk Scientific Corporation, Orem, UT, USA) and the ratios of either *IL-1β* or *TNF-α* to *β-actin* were obtained.

Experiment 2. Histology and immunohistochemistry for irIL-1β and irTNF-α

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. Other sections were used for immunostaining to confirm the localizations of irIL-1β and irTNF-α in tissues of the vagina and UVJ. For irIL-1β immunostaining, sections were incubated with 20 μg/ml

proteinase K (Sigma-Aldrich, St. Louis, MO, USA) for 25 min at 37°C; whereas for that of irTNF-α, sections were autoclaved for 1 min in 2 mM citric acid, pH 6.0 for antigen retrieval. After washing in PBS for 15 min (5 min × 3 times), all sections were incubated with 1.5 μl/ml of normal goat serum (Vector Laboratories, Inc., Burlingame, CA, USA) for 1 h. Sections for irIL-1β immunostaining were then incubated with rabbit polyclonal antibody to chicken IL-1β antibody (Abcam Ltd., Cambridge, UK) diluted to 1:100, and those for irTNF-α immunostaining were incubated with rabbit polyclonal antibody to TNF-α antibody (Abcam Ltd., Cambridge, UK; an antibody raised against recombinant full length protein of human and explained to react with chicken TNF-□ by the supplier) diluted to 1:200 in PBS containing 0.05% bovine serum albumin (Nacalai Tesque, Inc., Kyoto, Japan). All sections were incubated overnight at 4°C. After washing with PBS (5 min × 3 times), sections were incubated with biotinylated anti-rabbit IgG (Vector Laboratories, Inc., Burlingame, CA, USA) for 2 h and with avidin peroxidase complex (Nichirei Corporation, Tokyo, Japan) for 30 min. Sections were washed again in PBS (5 min × 3 times) and immunoprecipitates were visualized by incubating with 0.02% (w/v) 3', 3'-diaminobenzidine tetrahydrochloride (Nacalai Tesque, Inc., Kyoto, Japan) and 0.001% (v/v) H_2O_2 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.

Histological observations

The populations of SST-containing sperm and immunopositive cells for irIL-1β in the stroma and for irTNF-α in the surface epithelium 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 5 different regions (approximately, 7–10×10^4 μm^2 area in each count)

randomly selected from 3–4 slides of each vagina or UVJ section. The number of cells positive for irIL-1β or irTNF-α in a 5×10^4 μm^2 area was then calculated.

Statistical analysis

Differences in *IL-1β* or *TNF-α* expression and in the number of immunopositive cells for irIL-1β or irTNF-α 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 *IL-1β* or *TNF-α* 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 were analyzed using Tukey-Kramer. Differences were considered significant when P value was < 0.05.

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). Degraded sperm were found at 12 h after AI (Fig. 1B), 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 until 6 h after AI showed smooth and long thread-like structures, whereas those of degraded sperm at 12 h after AI showed irregular surface and body structures with small and rounded particles around them. In the UVJ, SST were filled with sperm for 1–24 h after AI at a ratio of approximately 50–60% (data not shown).

Experiment 2. RT-PCR analysis for IL-1β and TNF-α expression in oviductal segments, and TNF-α receptor in sperm

PCR products of *IL-1β and TNF-α* were observed at their predicted size (138 and 229 base pairs, respectively; Fig. 2). The expression of *IL-1β* 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 *IL-1β* 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 *TNF-α* in the oviduct following insemination with semen or PBS. The *TNF-α* expression did not change in the infundibulum and UVJ from 0 to 24 h after insemination with semen or PBS (Fig. 4A, 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 *IL-1β* and *TNF-α* during 0-3 h after insemination with seminal plasma in UVJ and vagina. The expression of these cytokines was not changed in both UVJ (Fig. 5A, C) and vagina (Fig. 5B, D). The mRNA expression of TNF-α receptor in chicken sperm was observed at the predicted product size of 440 base pairs (Fig. 6).

Experiment 3. Immunohistochemistry for irIL-1β and irTNF-α

The irIL-1β cells were observed in the lamina propria of the vagina before and after insemination (Fig. 7A, B) and UVJ (Fig. 7C). Numbers of irIL-1β 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). The irTNF-α immunopositive cells were observed in the surface epithelium of the vagina at before and after insemination (Fig. 7D, E) and UVJ, and also in the SST epithelium (Fig. 7F). Like irIL-1β, the numbers of immunopositive cells for irTNF-α were significantly increased in the surface epithelium of the vagina from 1-3 h when the hens were inseminated with fresh semen but their population was unchanged with PBS insemination (Fig. 8C). Differences between semen and PBS groups were significant at 1 and 3 h. The number of irTNF-α cells did not show any significant alteration when the birds were inseminated with semen or PBS either in the surface epithelium of UVJ or SST epithelium until 24 h (Fig. 8D, E).

Discussion

We report that IL-1β and TNF-α, multifunctional inflammatory cytokines, are expressed in mucosal tissue of the hen oviduct, and their expression changed both in mRNA and immunopositive cells levels in the vagina, but not in UVJ, with artificial insemination. The significant findings of the present study are: (i) among the four oviductal segments (infundibulum, uterus, UVJ and vagina), expression of both *IL-1β* and *TNF-α* was significantly increased only in the vagina in response to semen insemination; (ii) immunopositive cells for irIL-1β were observed in lamina propria, and for irTNF-α were observed in the surface epithelium of the vagina and UVJ, and their populations were significantly increased in the vagina but were almost unchanged in UVJ after semen insemination.

In the vagina, the expression of *IL-1β* and *TNF-α* 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 irIL-1β in the stroma and for irTNF-α in the surface

epithelium 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 or secondary folds until 6 h after insemination but were degraded or 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 one hour 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 IL-1β, IL-6 and leukemia inhibitory factor (LIF) were increased 2–3-folds 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 and the increase of irIL-1β and irTNF-α 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. This is further justified by the fact that mucosal tissue of the vagina failed to show any increased expression in mRNA or immunopositive cell levels for both cytokines when laying hens were inseminated with the same volume of PBS. Because the vaginal tissue did not show significant changes in the expression of *IL-1β* and *TNF-α* 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 *TNF-α* in the uterus was decreased after 12 h of semen insemination. It is assumed that this decline of *TNF-α*

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 as an important factor to select 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 cilliary beat of vaginal epithelial cells to transport them towards the cloaca (Brillard 1993, Bakst & Akuffo 2008). Another one is the response to vaginal cells to release some molecules that may affect the sperm survivability. In addition to the basic functions, i.e. the regulation of inflammatory response and immunity, few subsets of the TNF superfamily, including TNF-α, 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 current study showed an increase of TNF-α in the vagina and confirmed the expression of TNF-α receptor in sperm. The higher level of *TNF-α* in the vagina after insemination with semen observed in the present study and its receptor in sperm may be involved, at least in part, in degrading sperm that were unable to transverse the vaginal part of the hen oviduct.

Histological observation of UVJ revealed that the SST contained sperm for 1–24 h after insemination. The expression of IL-1β and TNF-α in UVJ, both in their mRNA and immunoreactive cell levels, was unchanged except that the populations of irIL-1β 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 *TGFβs* 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 *IL-1β*, *TNF-α* and *TGFβs* in UVJ in the presence of sperm in their SST. TGFβs induce inhibitory functions on macrophages, chemokines and proinflammatory cytokines including that of IL-1β and TNF-α (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 1994, 1995, Ahmad *et al*. 1997). Therefore, the increased level of *TGFβs* in UVJ after AI might suppress the expression of *IL-1β* and *TNF-α* by suppressing macrophage levels. The lower levels of *IL-1β* and *TNF-α* in UVJ after AI suggest lower exposure of sperm to these cytokines, leading to sperm survivability in SST by protecting them from immunoreactions.

In conclusion, we have provided evidence that IL-1β and TNF-α mRNAs and their immunopositive 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 IL-1β and TNF-α 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.

Acknowledgement

We would like to thank Dr. Takashi Bungo, Graduate School of Biosphere Science, Hiroshima University, Japan for help with statistical analyses. This work was supported by a Grant-in-Aid for scientific research from the Japanese Society for the Promotion of Science (JSPS). The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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Figure legends

Fig. 1. Sections of the vagina of hen oviduct after insemination. (A) 1 h after insemination and (B) 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 after insemination (arrows, A). 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, B). E = surface epithelium; LP = lamina propria; L = lumen. Scale bars = 50 μm. Inset of (A) shows magnified view of the vagina with long thread-like structures of intact sperm (arrows) and (B) shows magnified view of the vagina with round particles of sperm fragments (arrowheads). Scale bars = 10 μm.

Fig. 2. RT-PCR analysis of the expression of *IL-1β* and *TNF-α* in tissue of the infundibulum, uterus, uterovaginal junction and vagina. Clear bands are seen for *IL-1β* and *TNF-α* at approximately 138 and 229 base pairs, 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 *IL-1β* and *TNF-α* (right). inf = infundibulum; ut = uterus; uvj = utero-vaginal junction; vag = vagina; M = DNA marker of 100 bp ladder.

Fig. 3. Changes in the expression of *IL-1β* in the oviduct in response to insemination with semen or PBS. (A) infundibulum, (B) uterus, (C) UVJ and (D) vagina. Each value indicates mean ± SE (n = 4). Values were compared by two-way ANOVA (treatment X time) followed by Tukey-Kraner 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 ($F(1,6) = 5.905$, $P< 0.001$). 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).

Fig. 4. Changes in the expression of *TNF-α* in the oviduct in response to insemination with semen or PBS. (A) infundibulum, (B) uterus, (C) UVJ and (D) vagina. Each value indicates mean ± SE (n = 4). Values were compared by two-way ANOVA (treatment X time) followed by Tukey-Kraner 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).

Fig. 5. Effects of seminal plasma insemination on the expression of *IL-1β* and *TNF-α* in the uterovaginal junction and vagina. (A) and (B) *IL-1β* expression in the UVJ and vagina; (C) and (D) *TNF-α* expression in the UVJ and vagina; Values are mean ± SE (n = 4). Data were analyzed by one-way ANOVA, followed by Tukey-Kramer test.

Fig. 6. RT-PCR analysis of the expression *TNF-α* receptor in sperm cells with or without ReverTra Ace. Clear band is seen for *TNF-α* receptor at approximately 440 base pairs 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-α* receptor (right). M = DNA marker of 100 bp ladder.

Fig. 7. Sections of vagina and uterovaginal junction of non-inseminated and inseminated birds immunostained for irIL-1β and irTNF-α. (A) Before insemination and (B) 1 h after insemination of vagina, and (C) before insemination of UVJ immunostained for irIL-1β. (D) Before insemination and (E) 1 h after insemination of

vagina and (F) before insemination of UVJ for immunostained irTNF-α. Arrows indicate positive cells for irIL-1β or irTNF-α in the lamina propria or surface epithelium of vagina and UVJ. E = surface epithelium; LP = lamina propria; L = lumen. Scale bar = 50 μm. Insets of (B) and (E) show magnified view of positive cells for irIL-1β and irTNF-α, respectively. Scale bar = 25 μm.

Fig. 8. Frequency of the populations of positive cells for irIL-1β and irTNF-α in the oviduct inseminated with semen or PBS. (A) irIL-1β in the vaginal stroma, (B) irIL-1β in the UVJ stroma, (C) irTNF-α in the vaginal surface epithelium, (D) irTNF-α in the UVJ surface epithelium, and (E) irTNF-α in the SST epithelium Each value indicates mean ± SE (n = 4). Values were compared by two-way ANOVA (treatment X time) followed by Tukey-Kraner test. In the vagina, significances for irIL-1β 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 ($F(5, 30) = 9.905$, $P <0.001$). The significances for irTNF-α in the vagina were also obtained in the effects of treatment ($F(1, 6) = 6.462$, $P<0.05$), time ($F(5, 30) = 8.480$, $P< 0.001$) and interaction ($F(5, 30) = 6.626$, $P <0.001$). Values with different letters are significantly different within treatment ($P<0.05$). *Significantly different between semen and PBS insemination within an examined time (($P<0.05$).

Table 1: List of primers with references/accession numbers

Primers	Observed base pair	Sequences	References/ Accession number
IL-1β	138	F:: 5′-GGGCATCAAGGGCTACAA-3′ R:5′-CTGTCCAGGCGGTAGAAGAT -3′	Subedi *et al*. (2007) Acces. no: NM_204524
TNF-α	229	F: 5′-TGTGTATGTGCAGCAACCCGTAGT-3′ R: 5′-GGCATTGCAATTTGGACAGAAGT-3′	Hong *et al*. (2006) Acces. no: AY765397
TNF-α receptor	440	F: 5′- CACAGAATGTAAGCCCTGTCC-3′ R: 5′-TGGAGTTCTGCGATCCTGCATT-3′	Abdalla *et al.* (2004) Acces. no: BAC55966
β-actin		F: 5′-TTCCAGCCATCTTTCTTG-3′ R: 5′-TCCTTCTGCATCCTGTCA-3′	Kost *et al*. (1983) Acces. no: X00182

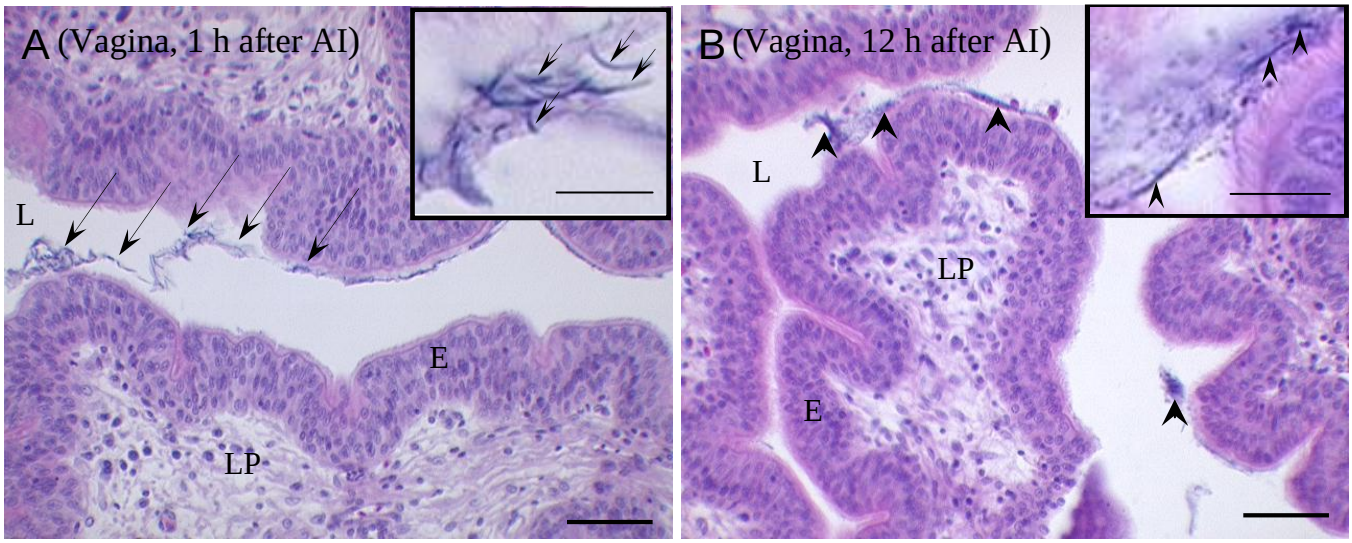

Fig : **1**

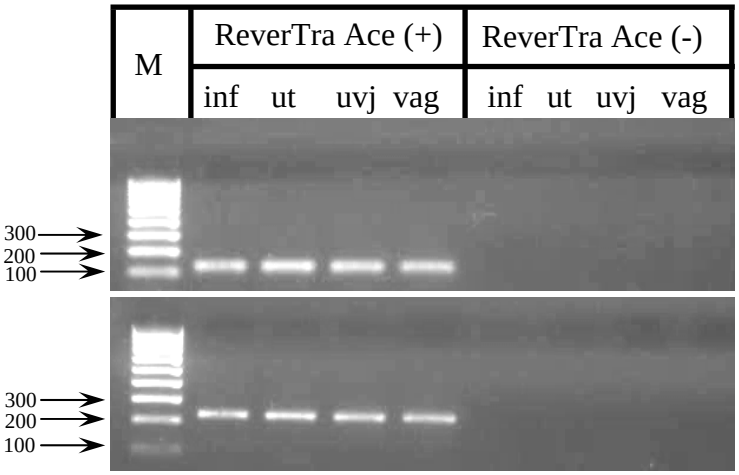
M
ReverTra Ace (+)
ReverTra Ace (-)
inf ut uvj vag
inf ut uvj vag
300
200
100
IL-1β
300
200
100
TNF-α

Fig : 2

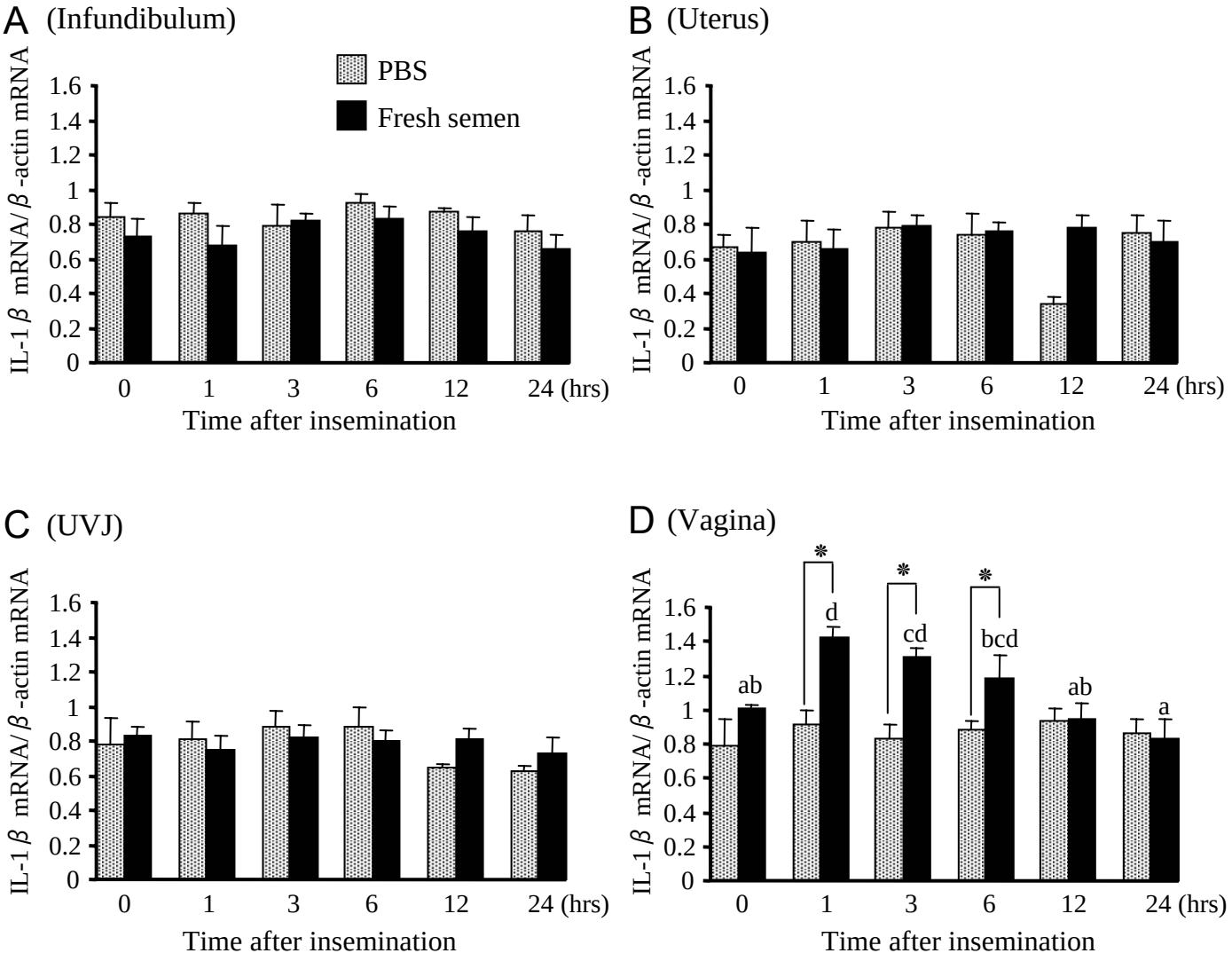
A (Infundibulum)
B (Uterus)
C (UVJ)
D (Vagina)
PBS
Fresh semen
IL-1β mRNA/β-actin mRNA
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
1
3
6
12
24 (hrs)
Time after insemination
ab
d
cd
bcd
ab
a
*

Fig. 3

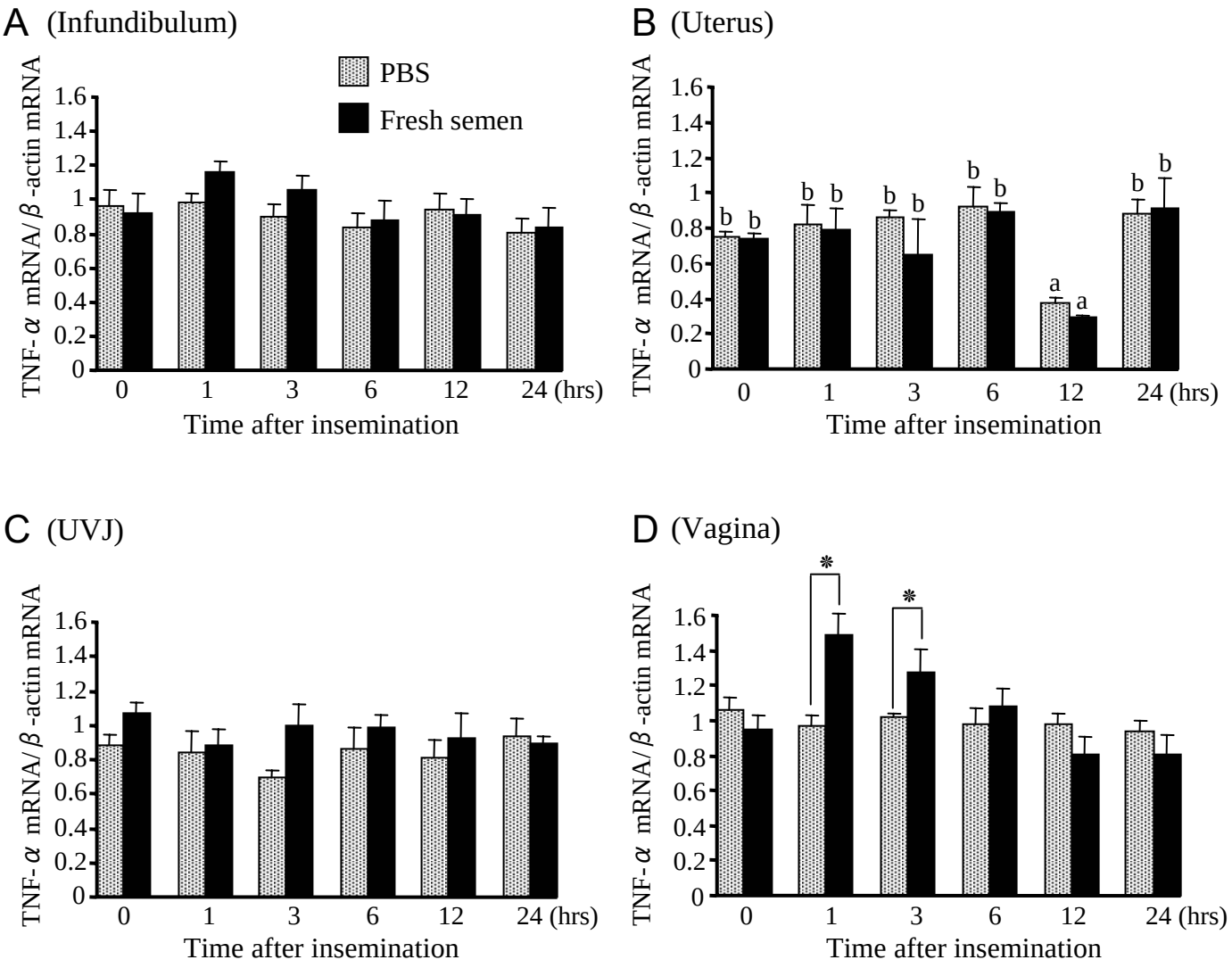
A (Infundibulum)
PBS
Fresh semen
TNF-α mRNA/β-actin mRNA
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
1
3
6
12
24 (hrs)
Time after insemination
B (Uterus)
b b
b b
b b
b b
a a
b b
C (UVJ)
D (Vagina)
*
*

Fig. 4

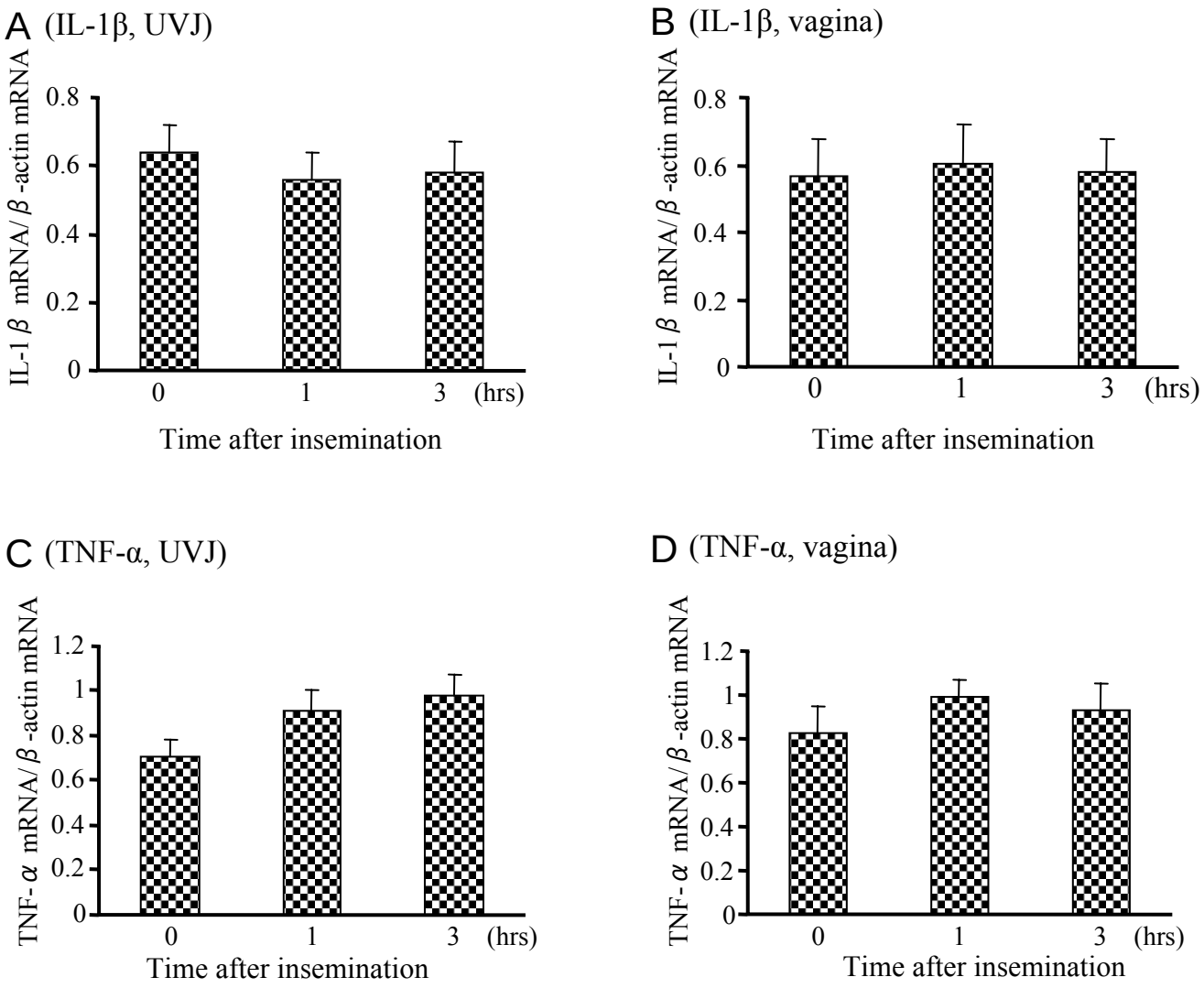
A (IL-1β, UVJ)
IL-1β mRNA/β-actin mRNA
0.8
0.6
0.4
0.2
0
0
1
3
(hrs)
Time after insemination
B (IL-1β, vagina)
IL-1β mRNA/β-actin mRNA
0.8
0.6
0.4
0.2
0
0
1
3
(hrs)
Time after insemination
C (TNF-α, UVJ)
TNF-α mRNA/β-actin mRNA
1.2
1
0.8
0.6
0.4
0.2
0
0
1
3
(hrs)
Time after insemination
D (TNF-α, vagina)
TNF-α mRNA/β-actin mRNA
1.2
1
0.8
0.6
0.4
0.2
0
0
1
3
(hrs)
Time after insemination

Fig. 5

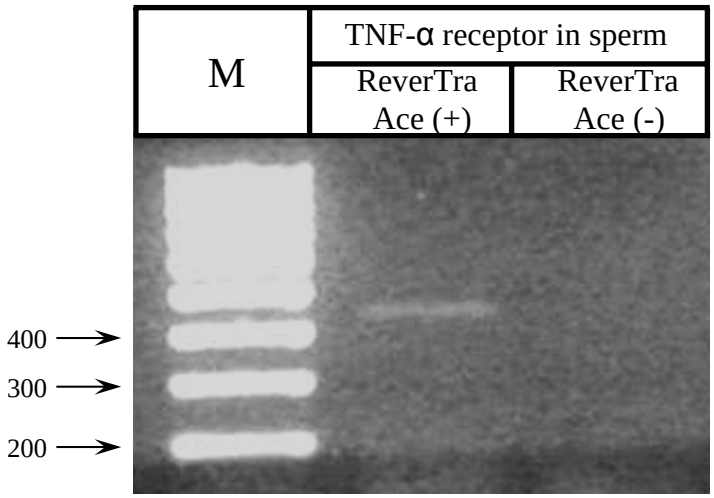
M
TNF-α receptor in sperm
ReverTra Ace (+)
ReverTra Ace (-)
400
300
200

Fig. 6

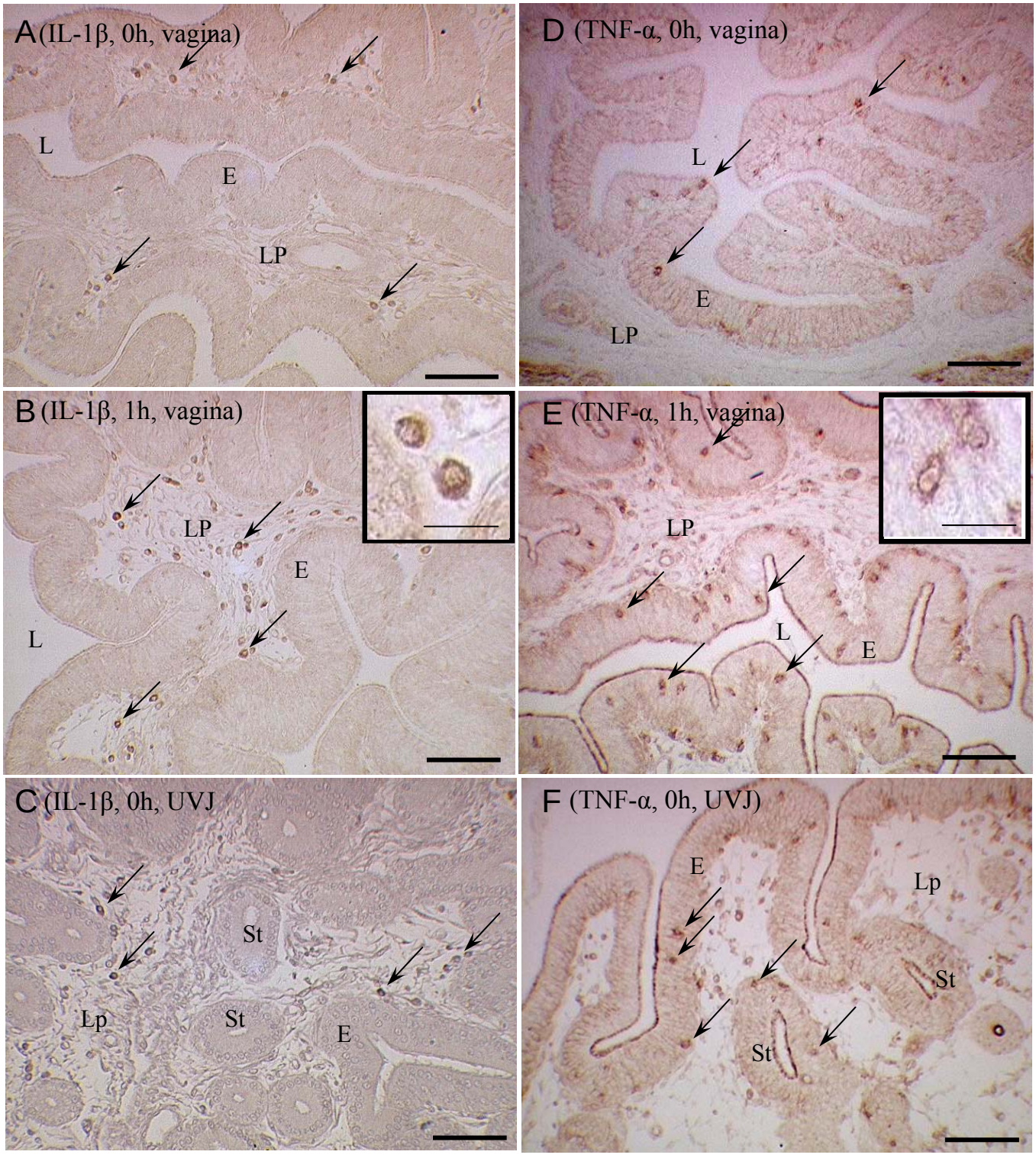
A (IL-1β, 0h, vagina)
L
E
LP
D (TNF-α, 0h, vagina)
L
E
LP
B (IL-1β, 1h, vagina)
LP
E
L
E (TNF-α, 1h, vagina)
LP
L
E
C (IL-1β, 0h, UVJ
St
Lp
St
E
F (TNF-α, 0h, UVJ)
E
Lp
St
St

Fig. 7

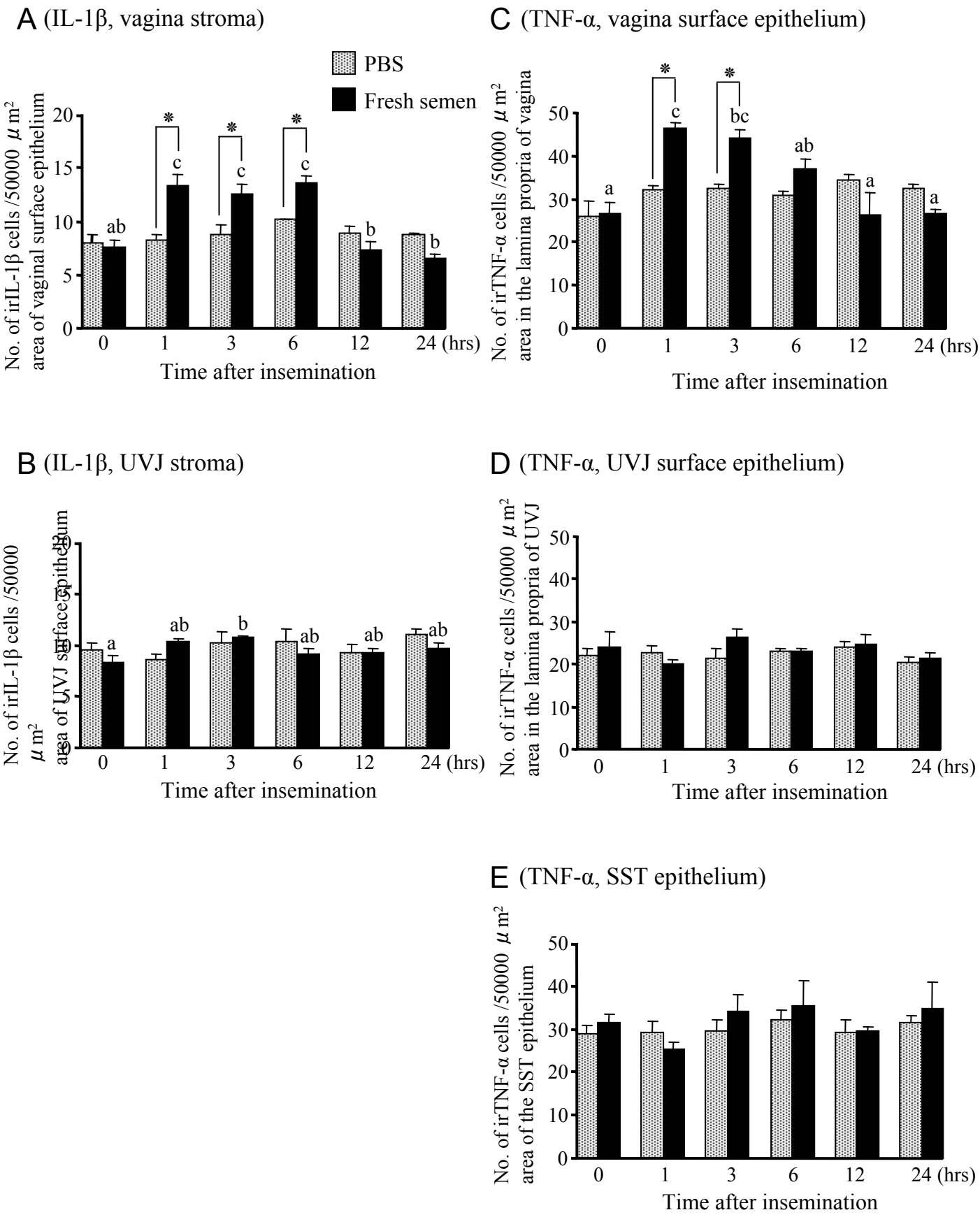
A (IL-1β, vagina stroma)
PBS
Fresh semen
No. of irIL-1β cells /50000 μm² area of vaginal surface epithelium
Time after insemination
0
1
3
6
12
24 (hrs)
C (TNF-α, vagina surface epithelium)
No. of irTNF-α cells /50000 μm² area in the lamina propria of vagina
B (IL-1β, UVJ stroma)
No. of irIL-1β cells /50000 μm² area of UVJ surface epithelium
D (TNF-α, UVJ surface epithelium)
No. of irTNF-α cells /50000 μm² area in the lamina propria of UVJ
E (TNF-α, SST epithelium)
No. of irTNF-α cells /50000 μm² area of the SST epithelium

Fig. 8