学 位 論 文 の 要 旨

論文題目　Studies on the Sperm Storage Mechanism in the Hen Oviduct
（ニワトリ卵管における精子貯蔵機構に関する研究）

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Improvement of the fertility of hens is one of the important issue of poultry production, which is very crucial for successful production of fertile eggs and hatching enough day-old-chicks for the growing poultry production chain. It is well known that the unique function of sperm storage tubules (SST) in the utero-vaginal junction (UVJ) to store sperm for a prolonged period enables hens to lay a series of fertilized eggs, after single dose of artificial insemination (AI) or natural mating. The function of SST is thereby considered as one of the main intrinsic factors that affect the hen fertility. It is assumed that regulation of pH in SST lumen, lipid supply to sperm in SST, and factors to deliver the substances from SST to resident sperm are important for sperm survival. The aim of this study was to determine the role of above factors in sperm survivability in SST of hen oviduct and their significance in hen fertility.

1. Protein and gene expression of carbonic anhydrase 2 (CA2) in the UVJ of oviduct: comparison between before and after AI, and their correlation with aging and fertility

It was reported that the activity of CAs present in the epithelium of UVJ and SST. The carbonic anhydrase 2 (CA2) is one of the isozymes of CAs that play a major role in the uterus to catalyzes the hydration of CO_2. Therefore, the aim of the study in Chapter 2 is to determine the protein and gene expression of CA2 in UVJ and SST of hen oviduct. The localization of CA2 protein was examined by immunochemistry, and the density of CA2 protein was determined by western blot. The expression of *CA2* gene was determined semi-quantitate PCR and real-time in the mucosal tissues of UVJ and SST cells in the hens at different age and fertility with or without AI. The results showed CA2 was localized in the UVJ mucosal epithelium and the SST cells. The RT-PCR products of *CA2* were identified in SST cells. No significant differences were found in the localization, or the expression level of CA2 protein and gene between control and AI groups, or among different age groups. No correlation was found between CA2 expression level and hen fertility. These results suggest that CA2 may participate in the pH regulation by UVJ mucosal epithelium and SST cells, whereas it remains unknown whether CA2 play a role in sperm survivability in SST.

2. Expression of lipases and lipid receptors in sperm storage tubules and the possible role of fatty acids in sperm survivability in the hen oviduct

The cells in the peripheral tissues accumulate lipids through receptors and store them in lipid droplets. It is generally accepted that lipid droplets are hydrolyzed by lipases and released as free fatty acids. Lipid droplets can be found in the SST cells. They may be an intercellular lipid source for usage by resident sperm. Therefore, the aim of the study in Chapter 3 was to determine the role of lipids in sperm survivability in the hen oviduct. In Experiment 1, the localization of lipids in UVJ mucosal epithelium and SST from hens with or without AI was determined by Sudan Black B staining. The lipid droplets were scattered in the mucosal epithelium of UVJ, and

the SSTs in the UVJ contained dense lipid droplets. In Experiment 2, the gene expression of lipid receptors and lipases were determined in the mucosal tissues of UVJ and SST cells isolated by laser microdissection using RT-PCR analysis. The PCR products of lipid receptors including *FAT/CD36*, *VLDLR* and *LDLR* were identified in the mucosal tissues of UVJ and SST cells. Lipases genes including *HTGL*, *EL*, *LIPH*, *ATGL*, *LPL* and *CEL* were identified in the mucosal tissues of UVJ. Among those lipases, only ATGL expression was identified in the SST cells. The difference in expression level of *ATGL* was further analyzed in SST cells from hens with or without AI by real-time PCR. The relative expression levels of *ATGL* were significantly higher in AI hens than non-inseminated hens. In Experiment 3, to identify the predominant fatty acids composition of UVJ mucosa, lipids were extracted and the fatty acid were analyzed using gas chromatography. Saturated fatty acids including myristic acid (C14), palmitic acid (C16) and stearic acid (C18), and unsaturated fatty acids including oleic acid (C18:1n9) and linoleic acid (C18:2n6) were contained in the UVJ as predominant fatty acids. In Experiment 4, to examine the effect of fatty acids identified in the UVJ mucosa on sperm viability, sperm were cultured for 24 h at different concentrations of fatty acids, and their viability was examined using LIVE/DEAD® Sperm Viability Kit. The viability of control group sperm (cultured without fatty acids) were approximately 50%. The viability of sperm cultured with myristic acid, palmitic acid, stearic acid was not different from the control sperm. However, the viability of sperm cultured with 1 mM oleic acid or linoleic acid was significantly higher (approximately 90%) than control sperm. In Experiment 5, the effect of oleic acid on hen fertility was determined *in vivo*. The hens fed with diet (commercial feed) added with or without different concentration of oleic acid reagent or olive pomace were subjected to AI. The egg production, fertility rate and fertility duration of each group was analyzed in 21 days after AI. The supplementation with oleic acid in diet did not affect the egg production. However, the hens fed diet supplemented with 2.5% or 5% oleic acid showed a higher fertility rate and a longer duration of fertile egg laying than hens without the supplementation. The concentration of oleic acid was approximately 35 mg/g in olive pomace, while it was 15 mg/g in basal diet. No significant difference was found in the egg production, fertility rate, fertility duration and egg yolk fatty acid between the hens fed with and without olive supplementation. These results suggest the lipid droplets in SST cells may be the source of fatty acids to be supplied to resident sperm. The lipid receptors and lipase(s) may participate in the lipid metabolism in SST cells. The unsaturated fatty acid in the UVJ and SST may participate in the sperm survival in SST. The oleic acid supplementation may improve the fertility of hens, although the olive pomace may not be an enough resource for that expected effects.

3. Changes in the localization and density of CD63-positive exosome-like substances in the hen oviduct with artificial insemination and their effect on sperm viability

Exosomes are small membrane vesicles that play a role in intercellular transportation of nucleic acid, protein and fatty acid. The CD63 is known as one of the exosome markers. SST epithelial cell microvilli release exosome-like blebs that fuse with the plasma membrane of sperm within the SST lumen. The aim of this study in Chapter 4 was to determine the role of exosomes in sperm survival in the hen oviduct. The change in the exosomes localization with AI in oviduct were examined by CD63 immunohistochemistry. The expression level of CD63 protein in mucosal tissues of UVJ with or without AI was examined by western blot. The UVJ- and vagina-exosomes were isolated by ultracentrifugation of the medium of UVJ and vagina cell cultures. The protein composition and the expression of CD63 protein in UVJ- or vagina-exosomes were examined by SDS-PAGE and western blot. Sperm were incubated for 36 h with or without different concentrations of those exosomes and their viability was analyzed using LIVE/DEAD® Sperm Viability Kit. The motility of incubated sperm was also examined. The results showed the CD63 was mostly present in the mucosal epithelium and lamina propria cells in UVJ, and abundant CD63 protein was present in the SST cells. The CD63 was also detected in the mucosal epithelium of the vagina, while it was negligible in the other oviductal segments. The localization of CD63 was decreased in SST cells surrounding the sperm and it tended to be transfered into the SST lumen. The density of CD63 protein in the hens inseminated with semen was significantly higher than non-inseminated hens. However, no significance differences were found between hens inseminated with semen

and seminal plasma. Four different molecular weight protein bands, and specific CD63 band were identified in UVJ- and vagina-exosomes. The viability of sperm incubated with vagina-exosomes was significantly lower compared with the viability of sperm incubated with same does of UVJ-exosomes or without exosomes. No significant differences were found in the motility of sperm incubated with or without UVJ- or vagina-exosomes. These results suggest that the exosomes may be synthesized by SST cells and secreted into the SST lumen in response to resident sperm. The production of exosomes may be upregulated by AI. The vagina-exosomes may participate in sperm selection by delivering damaging substances to sperm. However, specific effects of exosomes derived from SST on sperm viability remains unknown.

Conclusion

The CA2 may participate in the pH regulation in SST, although it is unknown whether it is related to SST function for sperm survival. SST cells may accumulate lipids through lipid receptors, and those lipids may be hydrolyzed by ATGL when sperm are stored in SST. Then, unsaturated fatty acids such as oleic acid may be released from SST cells and be utilized by resident sperm for their survivability in SST. The supplementation of oleic acid in chicken diet may facilitate SST function and ultimately improve hen fertility. The exosomes may be one of the factors that contribute to sperm storage function by delivering sperm key substances. These knowledges may help us to establish a feeding strategy which aims to increase the SST function and hen fertility not only in poultry but also in endangered avian species.