Histological and Metabolomic Analysis of Testis Tissues in Japanese Macaques (*Macaca fuscata*) Aiming the Establishment of *In Vitro* Spermatogenesis Technique in Primates

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Recent studies demonstrated successful *in vitro* spermatogenesis from spermatogonia using organ culture methods of mouse and rat testicular tissues. Application of this technique to other mammals including endangered species and humans would be helpful to preserve the fertility of immature males and to obtain gametes from individuals with spermatogenic failure. However, the mechanisms of spermatogenesis and the physiological environment in the testis seem to vary widely among species. In this study, the testis organ culture of Japanese macaques (*Macaca fuscata*) was carried out based on the previous studies of mice. In addition, we performed a histological and metabolomic analysis to collect useful information for the establishment of *in vitro* spermatogenesis techniques for primates. Testes were obtained from Japanese macaques aged 5-14 years in Tama Zoological Park (Tokyo, Japan) from November to January by skilled veterinarians for the management of population. Then tissues were transported to the laboratory on ice and used for the following experiments. Testicular fragments (1-2 mm^3) were cultured for 8 weeks on agarose gels half-soaked with culture medium containing 10% knockout serum replacement (n=5). Cultured tissues were collected at 1,2,4,6,8 weeks of culture followed by a 4% PFA fixation. The states and functions of germ cells and Sertoli cells (SC) were evaluated by immunohistochemistry for DDX4 (germ cell marker), Ki67 (proliferating marker), acrosin (acrosome protein), SOX9 (SC nucleus marker), vimentin (SC marker), androgen receptor (AR) and aldehyde dehydrogenase (ALDH)1/2 which oxidizes retinal to retinoic acid. Non-target metabolome analysis in testis tissues of macaques (n=7) and C57BL/6 mice (26 weeks old, n=4) was conducted using gas chromatography-mass spectrometry to identify the difference in metabolomics profile between the two species. MetaboAnalyst (https://www.metaboanalyst.ca) was used for the statistical analysis. Each type of germ cell (spermatogonia, spermatocytes, and spermatids) and proliferating cells were observed in all testis tissues before culture. However, the number of germ cells and proliferating cells decreased immediately after the beginning of the culture ($p<0.05$). The acrosin immunosignal

was not detected at 6-8 weeks of culture although elongated nuclei of spermatids were observed throughout the culture period. These results indicate spermatogenesis and cell proliferation in monkey testes did not progress successfully in culture condition that works in mice. SC markers SOX9 and vimentins were detected before and after culture suggesting SCs remained on the basal membrane of seminiferous tubules. Clear immunosignals of AR and ALDH1/2 were observed before culture in nuclei and cytoplasm of SCs, respectively, but attenuated within two weeks. Since both AR and ALDH are crucial for spermatogenesis, optimal conditions to maintain the SC function need to be identified. Finally, the metabolomic analysis revealed that peak intensities of 37 metabolites such as L-cystine and L-glutamic acid were higher, and 21 metabolites such as cytidine and xanthine were lower in testes of Japanese macaques compared with mice ($p<0.05$). It suggests that spermatogenesis in each animal proceeds in much different nutrient environments. These results provided useful information to improve the culture methods for successful *in vitro* spermatogenesis in primates. In the further study, culture medium and its supplements will be examined based on the metabolic profiles. This study was conducted by JSPS KAKENHI 23H02232.