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論　文　名	Study for efficient production of feline embryos developed from the oocytes of preserved ovaries （ネコにおける保存卵巣由来卵子からの効率的な胚生産に関する研究）
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論文要旨

Introduction

Studies in the *in vitro* reproductive technologies in the domestic cat would be useful not only for understanding mechanisms of reproduction, but also for conserving endangered feline and the other species and for application to regenerative medicine. The *in vitro* technologies include storage of ovaries, the *in vitro* maturation of oocytes (IVM), the *in vitro* fertilization (IVF) and the *in vitro* culture (IVC) of fertilized oocytes (zygotes), IVC develops zygotes through 2~8 cell-stages and morula stage into blastocysts. Blastocyst can develop to animal body, if transplanted into the uterus of same animal family, even not the same species. Moreover, cells in the inner cell mass of blastocysts can develop to embryonic stem cells used in the regenerative medicine.

Among these technologies, that of IVC has still not been established. Particularly, in feline IVC, it is very difficult to obtain blastocysts because of unknown mechanism named as “morula block”. Therefore, to overcome the morula block, many attempts have been made, and an improvement has been elicited by incubating zygotes with fetal bovine serum (FBS), which

contains many kinds of growth factor, instead of bovine serum albumin (BSA) in the latter period of the IVC. On the other hand, a commercially available serum-free media has recently been developed for human IVC, with two opposing views about the ideal composition. These are the "Single-step medium" that contains all components needed by the embryo during its development and the "Sequential media" that contain a different composition to fulfill the needs of the embryo in early or late development according to environment of the *in vivo* embryonic development. A predefined, commercially available medium is preferable because it would standardize embryo culture among different laboratories. Therefore, it is very interesting to examine development of cat blastocyst using the two kind of human IVC media in the combination of the FBS addition.

The technology of ovary storage significantly effects on the quality of oocytes, which in turn significantly effects on the blastocyst production. In the works for conserving endangered species, it is very difficult to preserve oocytes and fertilized oocyte in frozen because of difficulty to bring liquid nitrogen and specific instrument to the wildlife habitats where are very far from the processing facility. Therefore, long term storage of ovary should be required. However it is reported that significant decrease in the quality of oocytes by using saline for preservation of cat ovaries. Two preservation solutions, Eurocollins (EU) and ET-Kyoto (ETK) have been successfully used in the preservation of different human organs like lung, skin and kidney for transplantation. Therefore, it is worthy to examine preservation efficiency of cat ovary using EC and ETK.

In the present study the author shows in the Chapter 1, the improvement of IVC for the development of cat embryo by the culture methods described above and then in the Chapter 2, shows the improvement of ovary storage for the development of cat embryo using the preservation solutions described above and the improved IVC method obtained by the study in the Chapter 1.

Chapter 1: Improvement of the *in vitro* culture for the production of feline embryos

We first examined the development of feline embryos in the two kind of human IVC medium in combination with FBS supplementation, instead of BSA, in the late period of the culture. Four types of cultures were set for the examination. In the [Single-step BSA/FBS], zygotes were incubated in the single-step medium supplemented with BSA for first 2 days and then incubated with the single-step medium supplemented with FBS for next 5 days. In the [Single-step BSA/BSA], oocytes were incubated in the single-step medium supplemented with BSA for 7 days. In the [Sequential-step BSA/FBS], oocytes were incubated in the Early Culture Medium with BSA for first 2 days, followed by incubating in the Blastocyst Medium with FBS for next 5 days. In the [Sequential-step BSA/BSA], oocytes were incubated in the sequence of media with BSA

for total 7days.

Oocytes collected from ovaries are classified into grades 1, 2 and 3 by quality of cytoplasm and association of surrounded cumulus cells. Oocytes in the grade 1 or 2 (Grade 1, 2 oocytes) have homogenous dark cytoplasm and are surrounded by several layers of compacted cumulus, and are usually possible to develop into blastocysts by the *in vitro* reproductive technologies. In contrast, oocytes in the grade 3 (Grade 3 oocytes) lack uniformity in cytoplasm and less than 2 layers of cumulus cells loosely attached each other, and hardly generate blastocysts even if fertilization is succeeded. In the present study, the blastocyst generation of Grade 3 oocytes was examined as well as that of Grade 1, 2 oocytes in the IVC described above.

The morula plus blastocyst formation of Grade 1, 2 oocytes was not significantly different among the four IVC groups. However, the proportion of blastocyst formation in the [Single-step BSA/FBS] was greatest compared with the other IVC groups, and was significantly higher than those in the [Single-step BSA/BSA] and the [Sequential-step BSA/BSA].

Partially due to significant decrease in fertilization rate, the proportion of morula plus blastocyst of Grade 3 oocyte is significantly small compared with the Grade 1, 2 oocytes. However, Grade 3 oocytes formed blastocysts in the all four IVCs designed. Notably, the [Single-step BSA/FBS] of Grade 3 oocytes showed the highest proportion of blastocyst formation and the proportion was significantly higher than that in the [Single-step BSA/BSA] as well as that of Grade 1, 2 oocytes, although blastocyst cell number was significantly lower in those developed from Grade 3 oocytes.

To clarify the necessity of FBS presenting in the later periods of the IVC, morula and blastocyst formation was compared between in the [Single-step BSA/FBS] and in the [Single-step FBS/FBS]. Using either Grade 1, 2 or Grade 3 oocytes, no difference in the morula plus blastocyst formation was found between two kinds of the IVC. However, a significant reduction of the blastocyst formation was observed in the [Single-step FBS/FBS] compared with the [Single-step BSA/FBS].

In the [Single-step BSA/FBS], the proportion of parthenotes, and there was no oocyte developed to the morula and blastocyst from parthenotes oocytes. Although Grade 3 oocytes showed significantly lesser fertilization rate than Grade 1, 2 oocytes, the two pronuclei (male and female) formation was observed in every fertilized oocyte. These results suggest that only fertilized Grade 3 oocytes developed to embryos.

From these results it is indicated that the [Single-step BSA/FBS] is the best choice for the blastocyst generation of cat oocytes.

Chapter 2: Improvement of the ovary storage for the production of feline embryos

In this study the author examined improvement in the ovary preservation using Eurocollins (EU) and ET-Kyoto (ETK), solutions using in the preservation of human organs. Since it has been reported that in the case of cat ovary storage, the blastocyst development occurred using oocytes from ovaries stored in cold condition, storage was performed at 4°C in this study. In all experiments the storage of ovaries was performed in 24, 48 and 72 hours. After the storage the Grade 1, 2 oocytes were collected and employed to examinations of *in vitro* oocyte maturation (IVM) and embryo development. As a positive control freshly collected Grade 1, 2 oocytes (0 hour storage) were used. As a negative control oocytes stored in saline.

At first, effect of the ovary storage in different preservation solutions on the maturation of oocytes was examined in IVM culture. The proportion of mature (MII) oocytes which have first polar body did not significantly decrease in all kinds of preservation up to 48 hours, compared with the positive control. In the 72 hour-storage the proportion of MII oocytes significantly decreased by the saline and EU storages than positive control. The MII oocytes from the ETK-storage was not statistically lower than that of the positive control.

Then the effect of the preserved solutions on the various stages of embryo development was examined in the [Single-step BSA/FBS], the best IVC methods obtained by the study in the Chapter 1. The rate of cleavage (2 cell stage) significantly decreased, compared with the positive control, by the 72 hour-storage in all solutions. There is no significant decrease by the 24 and 48 hour-storage in all solutions. The development of morula plus blastocyst was not significantly decreased by the 48-hour storage in ETK. But significant decrease was observed by the 72-hour storage of all solutions. The potential for blastocyst formation seemed to keep by the ETK storage up to 48 hours. However, there is no statistical difference by the 24- and 48-hour storage of all solutions for the development of blastocyst, compared with the positive control. Finally there is no significant decrease in the blastocyst cell number, which is a critical indicator for development potential, by the storage of all solutions for any periods.

From these results, storage in ETK elicited better potential both in IVM and IVC.

Conclusion:

1. Significant improvement in the *in vitro* culture of cat embryo was elicited by using a medium of single-step culture for human embryo in the combination with FBS added in the late period.

2. Improvement in the ovarian storage for the oocyte development to embryos was elicited by using ET-Kyoto, a solution for preservation of human organs.

審査結果の要旨

体外生殖は、卵子を受精可能な状態まで成熟させるための *in vitro* maturation（IVM）、体外受精（*in vitro* fertilization: IVF）および受精卵から胚盤胞期胚に至る胚形成のための *in vitro* culture（IVC）の 3 つの過程から成り、これらに関する研究は、有性生殖・胚発達機序の解明や動物生産の向上のみでなく、絶滅危惧種の保存や再生獣医療への応用に必要不可欠である。ネコでは、IVC の適切な方法が確立されておらず、また、morula block と呼ばれる桑実胚での胚発達の停止頻度が高いために最終段階である胚盤胞期胚の形成率が低く、ネコ科動物における絶滅危惧種の保存にとって大きな問題となっている。また、絶滅危惧種の生息域と生殖組織の処理を行う施設が遠く離れており、輸送時に卵子の受精能力や胚生産能力が低下することも報告されている。一方、ヒトの IVC では、近年開発された市販の無血清培地を用いて、胚の発育に必要な成分を全て含んだ培地で培養する方法（Single-step）と胚の各発生段階に適した成分を含む培地で順次培養する方法（Sequential-step）が行われ、良好な成績を得ている。また、ヒト臓器移植において、臓器輸送に用いられる保存液は長時間の冷蔵保存を可能にしている。そこで本研究では、ネコにおける保存卵巣由来卵子からの効率的な胚生産を目的として、ヒト IVC 法を用いた胚盤胞期胚の形成率向上と臓器保存液を用いた保存卵巣の卵子における受精能力および胚生産能力の維持について検討している。

第 1 章では、ヒト IVC 法に牛血清アルブミン（BSA）および様々な栄養と成長因子を含む牛胎子血清（FBS）の添加を組み合わせてネコ受精卵を培養し、胚盤胞期胚の形成を検討している。その結果、Single-step 培地に培養初期 2 日間に BSA を、培養後期 5 日間に FBS を添加した培養法（Single-step BSA/FBS）により、他の培養法に比べて有意に高い胚盤胞期胚の形成率が得られた。また、本培養法では、優良な Grade 1 および 2 卵子の受精卵からの胚盤胞期胚の形成（形成率約 20%）のみでなく、これまでの方法では胚盤胞期胚をほとんど形成しなかった Grade 3 卵子の受精卵からも高い胚盤胞期胚形成率（約 15%）が得られた。しかし、Single-step 培地に培養初期、後期ともに FBS を添加した方法（Single-step FBS/FBS）では、胚盤胞期胚の形成率はかえって低下した。また、Single-step BSA/FBS 法において、未受精卵からの単位生殖は認められず、胚形成は正常受精卵からのみであった。

第 2 章では、臓器保存液である Eurocollins（EU）および ET-KYOTO（ETK）で冷蔵保存した卵巣から回収した卵子の成熟能力および胚形成能力を新鮮摘出卵巣の卵子と比較検討している。その結果、ETK で保存した卵巣の卵子は、成熟率は 72 時間保存後まで、受精後の Single-step BSA/FBS 法における桑実胚および胚盤胞期胚形成率は 48 時間保存後まで、新鮮卵巣の卵子との間で有意差が認められず、EU および生理的食塩水で保存した卵巣の卵子に比べて高い成熟能および胚形成能を示した。

以上、本研究において、臓器保存液である ETK で保存したネコ卵巣の卵子を IVF し、ヒトの Single-step 培地を用いて培養し、培養前期に BSA を、培養後期に FBS を添加する

ことによって、効率的な胚生産を得られることが明らかとなった。これらの成果は、絶滅危惧種の保護に役立つのみでなく、生殖工学研究の新たな展開に資するものである。従って、最終試験の結果と併せて、博士（獣医学）の学位を授与することを適当と認める。