Study for efficient production of feline embryos developed from the oocytes of preserved ovaries

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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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.