F-3.2.3 Developing animal model system for reproduction technologies in endangered wildlife.

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Abstract

Electro -ejaculated deer (*Cervus nippon*) semen and epididymal spermatozoa collected from hunted Japanese serow (*Capriconis crispus*) were diluted with the first diluent (NF3-A) and the second diluent (NF3-B) before filling into 0.5 ml straws. The straws were then exposed to liquid nitrogen vapor for 10 min. This freezing techniques resulted in approximately 30-40% motile spermatozoa after thawing.

To examine a function of embryonic diapause of Tammar Wallaby bovine embryos were co-cultured with endometrium cell monolayer, which was collected from Tammar Wallaby at embryonic diapause state. Under the conditions of present study, no embryos changed to diapause state.

Ovaries and testis were removed from Japanese serow *(Capriconis crispus)* shot by gun for an authorized habitat control in Nagano Prefecture. Epididymal sperm were collected and frozen in NF3 and used for in vitro insemination. Nine to 41 oocytes were collected from three female serow. The oocytes were cultured in TCM 199 at 38.5C for maturation. But the maturation rate was low. Several oocytes were penetrated by sperm but no cleaved. The IVM/IVF system of cattle was adapted for serow with a limited success.

The effects of concentration of glycerol and sugars added in Lake solution were researched for the cryo-preservation of fowl spermatozoa. Seventy % of motile spermatozoa were observed in the composition of 2.5% glycerol, raffinose and torehalose. This result indicates that freezing of fowl spermatozoa above solution will be possible to direct insemination of hen without removing glycerol. It was also demonstrated that the semen of the Japanese copper pheasant was well frozen with the modified Lake solution.

Key Words : Cryopreservation Sperm Oocytes Pheasant Deer Wallaby

(1)Freezing preservation of spermatozoa of deers(*Cervus nippon*) and serow(*Capricornis crispus*)

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1. Introduction

The loss of genetic diversity caused by reducing of population in a certain wildlife species may induce low fertility and in the extreme case accelerate the extermination of a species. For preventing from the extermination of a certain wildlife species, it is a necessary means to enhance propagation and to sustain current level of bio- and genetic diversity.

Certain bio-techniques like artificial insemination, gamete and embryo cryo-preservation has been developed in domestic animals, especially in cattle. It is possible to inseminate several hundred cows with a single ejaculate of a bull by A.I. and to preserve its potential fertility for a long period. These techniques could be instrumental in assisting in the conservation and management of wild life species.

2. Research Objective

This experiment was carried out to clarify the semen collection method for non-domesticate animals and the possibility of the cryo-preservation of sperm collected from dead animals by means of the biotechniques in domestic animals.

3. Research Method

The semen was collected from deer(*Cervus nippon*) raised in a zoo and epididymal sperm were collected from hunted wild serow (*Capricornis crispus*) and a dead koala (*Phascolarctos cinereus adustus*) of illness at other zoo.

a. Deer(*Cervus nippon*)

Semen was collected by rectal-probe electro-ejaculation from 5 males anesthetized with xylazine in the breeding season. The teflon rectal probe was 280 mm long with a diameter of 28 mm containing three ringed stainless-steel electrodes spaced 19 mm apart. Ejaculation was achieved by stimuli of 5-10 volts with a range of 0.05-0.10A, 5-10min intervals. The motility of collected spermatozoa was observed immediately under the microscope at 38℃.

The semen was extended with the first solution of NF-3 diluent developed for bull semen (table 1) and then cooled to 5℃ for 2-3hrs. After cooling, the second diluent containing 14% glycerol was added. The second diluted semen was loaded into 0.5 ml plastic straws and then the straws were immediately placed horizontary in a rack positioned 30-40 mm above the liquid nitrogen surface in a freezing box. After 10 min, all straws were plunged into liquid nitrogen and then transferred to a liquid nitrogen storage and stored until evaluation of sperm motility.

Straws of semen were removed from liquid nitrogen and thawed immediately in a 40 ℃ water bath. Thawed semen was incubated at 37℃ for 180 min and the motility and survival rate of spermatozoa were evaluated microscopically in progress of incubation.

b. Serow(*Capricornis crispus*)

Epididymides were collected from 6 males after several hours of death by hunt and then transferred to the laboratory at 4 - 5C. Sperm were collected from epididymides after 12-15 hrs of the death. Collected sperm suspension was added to the first solution of NF-3 and then stored at 5C for 16-18 hrs. After the second dilution, semen was frozen in 0.5ml straws in the liquid nitrogen vapor.

c. Koala(*Phascolarcts*)

The epididymides were collected from a dead koala of illness at Kanazawa zoological garden, Yokohama city and stored at 5C for about 15 hrs and then transferred to our laboratory. Sperm were collected from the epididymides under a stereomicroscope. Three kinds of diluents, i.e., GLR for freezing of boar semen, NF-3 for freezing of bull semen and Lake solution for freezing of fowl semen were used for evaluating the

optimal diluent. Collected sperm suspension was diluted directly with the diluent containing glycerol and then frozen in 0.25 ml straws.

4. Results

a *Cervus nippon*

Five male deer were anesthetized with xylazine administered by blow dart. Ejaculated semen were obtained from three of them by electro-ejaculation.The color of semen was yellow or dark yellow and the volume of semen was 0.1 - 0.3 ml. The motility before and after freezing-thawing are given in table 2. The rate of live sperm was 50-60% and the motility was very active before freezing. After freezing- thawing 35-45% active sperm was recovered, therefore, the rate of recovery was 67-70%. The motility of post-thaw sperm declined gradually during incubation at 37C. These results indicated that the freezability of deer sperm was very well.

b. *Capricornis crispus*

The average weight of the testes collected from hunted serow was 21.6g with the range of 16.3-33.4 g and varied between right and left testes. A wide variation in the motility of epididymal sperm just after collection was detected amongst individuals and also found between right and left epididymides in individual male.

The motility of post-thaw sperm of serow are shown in table 3. There were no differences between percentage of live sperm before and after freezing, however some little variation in incubated sperm motility occurred amongst samples.

C. *Phascolarcts*

Phascolarcts's epididymal sperm were collected 0.2 ml of 1.5 x 10 sperm /ml and survival rate of the sperm was 30%. A few spermatozoa revived similar in all diluents after freezing-thawing, but the motility was slightly active in the GLR and LAKE compared to that in the NF-3.

5. Discussion

Electroejaculation method was safe and easy for collecting semen from deer. The volume of ejaculate was similar to those of goat and sheep in the breeding season. Around 60% motile sperm just after collection may be average level in nondomesticated animals. Although the second diluted semen was immediately frozen without glycerol equilibration, percentage of active motile spermatozoa after thawing was high. This result indicates that glycerol equilibration may be not important to freeze for deer semen as well as bull semen. Overall, the decline of sperm motility during incubation after thawing was very slow. Those results showed that the NF-3 diluent and freezing method developed for bull semen were available for freezing of deer semen. Takagish et al. (1986)2) reported that egg yolk-citrate extender was good for freezing of the deer semen.

Serow sperm were obtained from epididymides which were collected from males spent several hours after death and transferred to the laboratory at 5C. Although 15 hrs spent until collecting epididymal spermatozoa after hunt, active motile spermatozoa were obtained after freezing-thawing. This result showed that spermatozoa having superior freezability will be able to obtain even if it spent several hours until getting epididymides from a dead male. Serow sperm stored for 16-18 hrs at 5C after the first dilution and then frozen immediately after the second dilution without glycerol equilibration maintained similar active motility after thawing to prefreezing. The length of exposure to cold(5C) prior to freezing was important for getting the best survival rate of bull spermatozoa after freezing-thawing(Masuda and Watanabe, 1991)3). This experiment showed that the prolongation of exposure to coldness prior to freezing was also useful for freezing of serow spermatozoa.

Some motile sperm were obtained from epididymides of a dead male *Phascolarctos* of illness at the zoo but sperm recovery after freezing-thawing was poor, because the sperm activity before freezing was very weak. Cryopreservation of gametes from rare animals that die abruptly could be one of the useful methods for preserving genetic diversity and for breeding artificially to maintain certain species.

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Table 1. Composition of NF-3(in 100 ml)

Components	A	B
Sodium citrate	1.44 g	1.20 g
Sodium phosphate,dibasic	0.17	0.14
Potassium phosphate, monobasic	0.04	0.03
Potassium sodium, tartrate	0.08	0.07
glucose	2.12	1.77
lactose	0.42	0.35
glycerol	-	14.0 ml
egg yolk	15.0 ml	15.0 ml

Table 2. Motility index of deer sperm after freezing-thawing

Sample No.	Fresh semen	Min after thawing at 37C				
		0	30	60	120	180
1	60.0	40.0	40.0	26.3	22.5	12.5
2	50.0	35.0	35.0	15.0	15.0	5.0
3	60.0	45.0	35.0	26.3	12.5	5.0
4	50.0	30.6	26.3	9.4	7.5	7.5
5	55.0	30.6	26.3	12.5	12.5	3.8

Table 3. Motility index of serow sperm after freezing-thawing

Sample No.	Fresh semen	Min after thawing at 37C				
		0	30	60	120	180
1	35.0	35.0	26.3	26.3	22.5	7.5
2	35.0	35.0	35.0	35.0	26.3	12.5
3	30.0	30.0	30.0	22.5	15.6	5.0
4	39.4	39.4	30.0	26.3	15.6	10.0
5	30.0	30.0	30.0	22.5	15.6	10.0

(2) Culture of bovine embryos with endometrium cell monolayer derived from Tammar Wallaby

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1. Introduction

Tammar Wallaby (*Macropus engenii*) living in Australia, has presented the valuable of reproductive endocrinology for research of Marsupial reproductive physiology, especially kangaroo, and control their number.

Tammar Wallaby having fetus into her pouch cow stop developing embryo on monoblastocyst stage by sucking stimulation and keep the embryo in her uterus for a long time.

2. Research Objective

In this experiment, we examined to apply this embryonic diapause function to mammalian embryos for preservation without freezing.

3. Research Method

Tammar Wallabies used in this experiment had raised in the University of Newcastle, Australia. Endometrium were collected from Wallabies in embryonic diapause state.

These cells were separated in trypsin-EDTA solution and cultured in TCM199 +10%FCS' at 35.5℃, in 5% CO_2 in air. After that these cells were frozen with 10% DMSO solution in liquid nitrogen and were brought to Japan and were kept in liquid nitrogen until use.

Bovine oocytes were collected from ovaries derived from slaughterhouse and were matured, fertilized and cultured in vitro followed by the method of Shioya(1). IVF morula and blastocyst in day -6 and -7 were cultured with Tammar Wallaby's endometrium cell monolayer for 48hr and were observed their development.

Culture of bovine embryos on the endometrium cell monolayer of Tammer Wallaby

	Stage of embryos	No. of embryos cultured	developed	degenerated
Co-culture	Morula	2	1	1
	Blastocyst	3	2	1
Control	Morula	2	1	1
	Blastocyst	3	3	0

4. Result

Bovine IVF morula and blastocyst cultured with endometrium cell monolayer of Tammar Wallby in embryonic diapause state did not indicate embryonic diapause. The development rate of these embryos after 48hr culture did not differ from control embryos cultured with bovine cumulus cells (Table)

5. Discussion

Tammar Wallaby is comparatively small in the kind of kangaroo, so they have used generally research of this kind .

It is valid for investigation of the preservation of mammalian embryos without freezing to determine to mechanism of Tammar Wallaby's embryonic diapause. In the present study, bovine morula and blastocyst were not induced in embryonic diapause only by co-culture with Tammar Wallaby's endometrium monolayer in embryonic diapause state. It is to connect platelet-activity factor secretion from endometrium to embryonic diapause and reactivation of Tammar Wallaby. It needs to examine other

method for induction of embryonic diapause to mammalian embryos.

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(3) In vitro fertilization of Japanese serow

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1. Introduction

Habitat destruction has been the primary factor in reducing biodiversity and reduction of wild animals increases risks of inbreeding and reproductive dysfunction and decreases fecundity and survival of species. Traditional preservation system of natural habitat and innovative methods for captive propagation must be developed to prevent from the threats of extinction of endangered animals.

The domestic livestock industry has demonstrated the utility of semen extension, artificial insemination, embryo transfer and in vitro fertilization enhancing the reproductive capabilities of valuable male and female animals.
Methods that have been established to increase reproductivity of farm animals will be applied to nondomestic species. These methods require modification, adaptation and refinement before they can be properly applied to nondomestic animals.

2. Research Objective

In this experiment we tried to establish in vitro maturation and in vitro fertilization of oocytes of Japanese serow.

3. Research Method

Animals : Japanese serow(Capricornis crispus), a special natural monument in Japan which were killed for adjustment to habitat under the strict control of the government were donated for collection of ovaries and testis by the courtesy of Agematsu town in Nagano Prefecture. Ovaries and testis were taken out from hunted serow.

Collection of oocytes : Ovaries were transported in Ringer solution at 25 C to Tsukuba spending 5hr. Small follicles were incised with a surgical scalpel and the contents of the follicle was scrubbed out with a small spoon into phosphate buffered solution supplemented with bovine serum albumin. Then the surface of the ovaries were cut into small pieces and washed oocytes out in PBS.

Collected oocytes were selected and classified according to the cumulus cells ; A-oocytes with compact and thick cumulus cells, B-oocytes with thin cumulus cells ; C-oocytes without cumulus cells, D-oocytes with expanded cumulus cells. Oocytes without cumulus cells were classified by diameter into ordinary (over 150 μ) and small size (below 150 μ)

Oocytes were cultured for maturation according to the procedure of bovine IVM/IVF system(Shioya et al,1988).

Collection and induction of capacitation of sperm: Testis were transported to Tsukuba spending 5hr in a vinyl bag at 4C. Epididymal sperm were collected by injection of air into distal end of dissected epididymides. Collected sperm were diluted with NF-3 diluents and kept 5 C during 16 hr until freezing. Sperm were induced capacitation by the method as same as bovine.

In vitro insemination and in vitro culture for cleavage : Oocytes after maturation culture were inseminated with capacitation-induced sperm. Oocytes and sperm were co-incubated 10 hr and then oocytes were transferred to cleavage medium,TCM199 added with 5% calf serum.

Several oocytes were examined for penetration of sperm 10 hr after the insemination. The rest of the oocytes were cultured and examined cleavage after 72 hr after the insemination (Shioya, 1993).

4. Results

Six male and 3 female were killed and the reproductive organs were dissected out during 30 minutes to 6 hr and 20 minutes(Table 1). Seventy (9 to 41) oocytes were collected from ovaries by incising the follicles and thirty (7 to 12) oocytes were collected by incising the ovaries as shown in table 2.

A microscopic observation of oocytes before maturation culture showed that most of them possessed germinal vesicles. The most of the oocytes cultured 21 hr in maturation medium did not undergo germinal vesicle breakdown. The maturation rate of the serow oocytes was very low in this experiment. Three oocytes 10 hr after insemination were examined by a microscopy and all of them had penetrated sperm heads although the nuclear stage was not matured.

It was demonstrated well that the method of inducing capacitation in the experiment was effective to Japanese serow.

5. Discussion

Japanese serow is the only wild bovine ruminant preserved as a special natural monument in Japan(Miura et al.,1987). There are informations in the literature regarding to the reproductive organs (Kita et al.,1987) but the physiology of reproduction is not clear yet. They are seasonal breeders in the autumn and usually uniparous. The females may conceive at 1.5 years old and the gestation period is estimated 210 to 220 days. They usually have a single offspring as shown as only one case of twins and triplets in the autopsy data of 200 pregnant serow. The birth weight is 3.4 to 4 kg. The longevity is 20 years.

Table1. Hunting of Japanese serow of adjustment to habitat.

Individual number	Sex	Time at death	collection of organ	collection of gametes
367	M	9:30	14:00(4:30)	0:30
368	M	11:00	14:10(3:10)	0:30
369	M	13:30	14:15(0:45)	0:40
329	F	11:00	17:20(6:20)	0:30
378	F	16:40	18:00(1:20)	1:30
394	F	13:20	16:30(3:10)	2:00
376	M	11:00	17:20(6:20)	0:30
395	M	14:50	16:30(1:40)	0:40
377	M	12:00	12:30(0:30)	0:40

Table 2. Oocytes collection from Japanese serow.

Individual number	Oocytes collected by follicle-incision			slicing			
	A	B	C	A	B	C	D
329	8	3	9(5)	0	0	12(4)	0
378	23	5	13	0	3	7(2)	1
394	5	2	2	0	0	7(3)	1

( ) Number of small oocytes with diameter below 150 μ

In Vitro fertilization (IVF) offers a powerful tool for assessing gamete functionality. We have used IVF protocol to produce viable embryos in cattle. This protocol only needs oocytes collection and sperm capacitation. When we will try to embryo transfer, sufficient informations about reproductive physiology of the animals are necessary to succeed an attempt. IVF is highly attractive because it requires neither detection of overt estrus nor direct interaction between the male and female (Wildt et al.,1992). Recent advances in IVF allow recovering and culturing immature oocytes and this approach is useful for salvaging genetic material from rare animals that die abruptly or be hunted. This experiment is the demonstration of this approach.

We collected Japanese serow oocytes and sperm from hunted serow and cultured the oocytes for maturation and induced sperm to capacitation for fertilization. Maturation rate was low but we could first reported the success of penetration of sperm into oocytes.

The procedures in this experiment are almost the same as a previously developed IVM/IVF system of cattle. This system was demonstrated effective to Japanese serow but the maturation and the fertilization rate was not high. Bovine ovaries maintained in cold temperature showed low maturation rate of oocytes (Abe and Shioya. 1993). The business of adjustment in the habitat of Japanese serow was done in the mountainous area and in the winter. The period between the death of serow and collection of the ovaries was at least 80 minutes. So the ovaries were exposed to the winter temperature after the heat release by death. In case of male, sperm are tolerable to coldness. It is very important to take ovaries out from hunted serow to get good results of IVM/IVF.

The technology of reproduction in farm animals must be tested more widely to wild and zoo animals to increase the reproduction or save the genetic resources of endangered species.

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(4) Application of the freezing methods of fowl spermatozoa to the Japanese Copper Pheasant (*Phasianus soemmerringi*)

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1.Introduction

The techniques for reproduction in livestock are useful for conservation of endangered wild species. The methods of cryoperservation of spermatozoa and artificial insemination using such preserved spermatozoa are essential to multiply of wild animal species.

2. Research Objective

There are few information about sperm cryopreservation in wild birds, so, we examined cryopreservation medium to use the sperm of Japanese copper pheasant.

3. Research Methods

The sperm of Japanese copper pheasant were obtained by the method of abdominal region massage and mixed together some individuals. The obtained sperm was cool down at 5 ℃ and diluted 4 times at each experimental cryopreserved medium (Table 1). The diluted semen was packed with 0.25 ml plastic straw and preliminary cryopreservation with vapor of liquid nitrogen was done during 10 minutes, after that the straws were thrown into the liquid nitrogen. The cryopreserved semen were thawed at 5℃ in water, and examined sperm viability and motility. The morphology of pre and after cryopreservation were also examined after staining of giemsa.

4.Results

The volume of semen was collected about 50 μ l / head, and the number of spermatozoa is about 21×10^8 /ml. The semen just after collection showed highly activity and the index of sperm viability indicated above 90. The index of sperm viability after dilution of Lake, modified Lake, protamin and modified protamin were 90,40,90 and 60, respectively. As shown in Fig.1, the index of sperm viability after thawing which cryopreserved except Lake were ranged 10 to 20. The morphology of sperm just after collection judged as a normal moreover 85% and the percentage of morphological normality by the used Lake and modified Lake showed moreover 80% just after thawing. However, such percentage by cryopreserved of protamin and modified protamin were 20% and less than 2%.

5.Discussion

Lake solution has been widely used to cryopreserve the fowl spermatozoa, since the mobility and the morphology after thawing are keeping well. At present, cumulative fertilization rate showed about 70% by artificial insemination. However, Lake solution contains highly glycerol, so, it need that highly contents of glycerol was removed step by step before artificial insemination. It is impossible to get the fertilized egg without removed glycerol less than content of 2%. This step is very complicatedness especially in the filed. To do the direct insemination after thawing without removed glycerol, some modified medium were examined to use the sperm of Japanese copper pheasant. The viability and morphological normlity showed highest in

the Lake, but such values which cryopreserved to use modified Lake and protamin solution are also indicated satisfactory. There is problem that the fertilization ratio is not profile only sperm viability, so examination of artificial insemination to use such cryopreserved semen remains.

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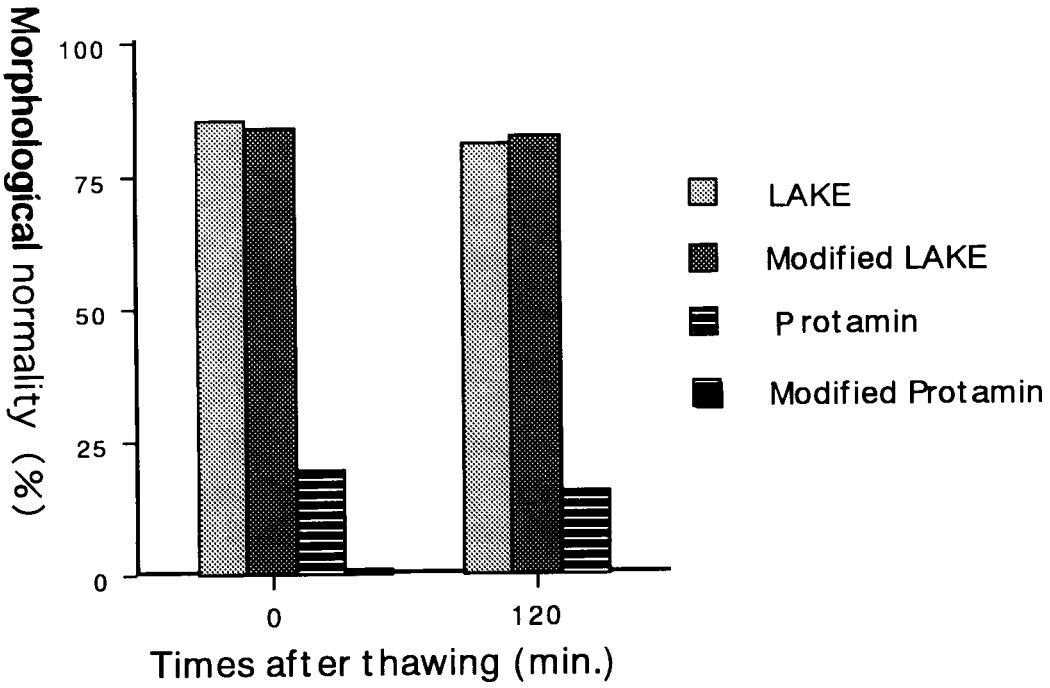

Fig1. The changes of morphological normality of sperm after thawing

Table 1 The composition of each cryopreserved medium (in 100ml)

	LAKE	Modified Lake	Protamin	Modified Protamin
Sodium glutamate (g)	1.920	1.920	1.920	1.920
Fructose (g)	0.800	0.800	0.800	0.800
Raffinose (g)	0	0.800	0	0.800
Torehalose (g)	0	0.800	0	0.800
Protamine sulfate (g)	0	0	0.032	0.032
Magnesium acetate (g)	0.080	0.080	0	0
Potassium acetate (g)	0.500	0.500	0.500	0.500
Polyvinyl pyrrolidone (MW.10,000, g)	0.300	0.300	0.300	0.300
Glycerol (ml)	14.0	2.0	0	0
Dimethyl acetamide (ml)	0	0	6.0	6.0
Penicilin G (IU)	$1x10^5$	$1x10^5$	$1x10^5$	$1x10^5$
Streptomycin (mg)	100.0	100.0	100.0	100.0
Water (ml)	up to 100	up to 100	up to 100	up to 100