Experimental Animals
Exp. Anim. 72(1), 30–37, 2023

Original

Rescue of oocytes recovered from postmortem mouse ovaries

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Abstract: It is well known that the survivability of gametes of postmortem carcass was decreased as time passes after death. In this study, it was examined whether cytoplasmic replacement rescues the survivability of germinal vesicle stage (GV) oocytes of postmortem carcass in the mouse. Reactive oxygen species (ROS) levels and mitochondria numbers in GV oocytes of the dead mice stored at 4 degrees were significantly impaired after 44 h postmortem compared to the control (0 h). However, when kayoplasts of GV oocytes of postmortem carcass was transferred to recipient ooplasts (GV transfer), proportion of *in vitro* maturation (IVM), normal spindle morphology, *in vitro* and *in vivo* developmental ability after *in vitro* fertilization (IVF) of reconstituted oocytes was improved. Moreover, secondary follicle oocytes of postmortem carcass were developed, matured and fertilized *in vitro* and developed to go to term, when GV transfer was conducted at the GV phase. Thus, transfer of GV karyoplast recovered from postmortem carcass, which viability was decreased, into fresh GV recipient ooplasm, rescues survivability of reconstituted oocytes. It suggested the effective use of oocytes of dead animals in the mouse and this achievement must apply to other rare animal species, especially animals under control by human.
Key words: germinal vesicle (GV) oocyte, GV transfer, *in vitro* maturation (IVM), nuclear transfer

Introduction

For research use, the ovaries and oviducts are usually immediately collected after the death of animals. However, there are possibilities when the ovaries and oviducts are not collected in time due to either an unanticipated death of the animal or a careless timing including genetically modified individuals. Therefore, effects of the delayed removal of ovaries and oviducts on oocyte quality should be studied to obtain the better results of experiments. Schroeder *et al.* [1] showed the rate of intact, cumulus cell-enclosed oocytes decreased as the interval between death of the animal and removal of the ovary increased. There was no difference, however, in developmental potential to blastocyst stage from 2-cell stage embryos between the control and 3–9 h postmortem groups, but embryos in the 12 h postmortem group did not develop to blastocysts. From 6 h postmortem group, 36% of live young was obtained after transfer of 2-cell stage embryos to foster mothers. Miao *et al.* [2] demonstrated that when oviduct excision was delayed 10 min, many ovulated oocytes lysed or spontaneously activated, and this postmortem effect aggravated with the extension of postmortem interval and oocyte aging. Zhang *et al.* [3] showed that delayed excision of ovary not only decreases the number of surviving germinal vesicle (GV) oocytes but also reduces the germinal vesicle break down (GVBD) and maturation percentages after culture *in vitro*. They also suggested that the mouse carcass can be preserved at 4°C for 10 h without oocyte evident cytoplasmic damage, but as the preservation time longer, the quantity and quality of oocytes at the GV stage are all

(Received 28 March 2022 / Accepted 17 July 2022 / Published online in J-STAGE 15 August 2022)
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reduced. As shown above, the relationship between postmortem time and egg viability is clear, but methods for regenerating the viability of oocytes have not been investigated.

Nuclear transfer at the GV stage (GV transfer) has been used as a tool to study the interaction between the nucleus and the cytoplasm [4]. It is also reported that GV transfer can reduce chromosomal abnormalities caused by aging [5–7] and damage caused by external factors [8, 9]. Furthermore, it has been reported in some animal species that reconstructed oocytes after GV transfer have normal maturation ability and can be fertilized by intracytoplasmic injection (ICSI) [10, 11].

It can be inferred that oocytes collected from the ovaries of dead animals have a reduced *in vitro* maturation rate, with deterioration of ooplasm being one of the major factors. The ooplasm is more sensitive to external factors than the nucleus. It has been shown that the cytoplasm is more sensitive to low temperatures than the nucleus and can be rescued by nuclear transfer [12].

In this study, GV nuclei obtained from preserved mouse carcasses were transferred to fresh GV stage egg cytoplasm, which had been enucleated, to create reconstructed eggs. The *in vitro* and *in vivo* developmental ability of the eggs collected from preserved mouse carcasses were compared with reconstituted eggs.

Materials and Methods

The animal experiments in this study were carried out in compliance with the laws and regulations established by the government of Japan and the Kindai University Animal Experiment Regulations. The experiments were carried out after obtaining permission from the university. Reagents were purchased from SIGMA except for special notes.

In this study, GV oocytes and secondary follicles were recovered from carcass mice that had been slaughtered and stored for 20 to 68 h at 4°C. Karyoplasts of GV oocytes collected from carcass mice were immediately transferred to fresh GV recipient ooplast (GV transfer). For secondary follicles, *in vitro* growth culture (IVG) was first performed, and then GV transfer was performed after IVG. After GV transfer, reconstituted oocytes were matured (IVGM) and fertilized (IVGMF) *in vitro*. Experimental design was shown in Fig. 1.

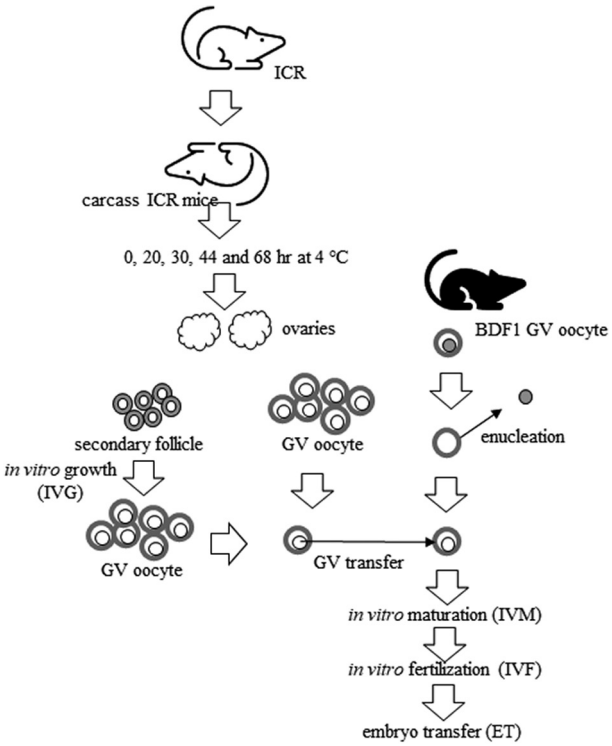

Fig. 1. Experimental design. Secondary follicle and germinal vesicle stage (GV) oocytes were recovered from the ovaries of ICR mice stored for 0–68 h after sacrifice. Secondary oocytes were grown to the GV phase by *in vitro* growth culture (IVG). GV oocytes were GV transferred to the same stage oocyte immediately after collection from BDF1 mice. GV transferred eggs were cultured *in vitro* for maturation (IVM). Eggs matured to the MII stage were fertilized *in vitro* (IVF) and embryo transfer (ET) to a foster mother was performed in the 2-cell stage to examine their development potential for full-term development. The control group was that without GV transfer group.

Collection of non-growth oocytes and IVG

After slaughtering 12–16 week old ICR female mice (CLEA Japan Inc., Osaka, Japan), secondary follicles were recovered from the ovaries. Secondary follicles were transferred to α-MEM (alpha Modified Eagle Minimum Essential Medium, Nacalai Tesque, Kyoto, Japan) supplemented with 0.1% collagenase I and treated in an incubator for 1 h, respectively. *In vitro* growth culture was performed for 10 days according to the previous report [13, 14]. Briefly, secondary follicles were placed on Millicell membrane (Millipore PIHP03050, Merck Millipore, MA, USA) on a culture dis and cultured in α-MEM medium supplemented with 2% PVP (360 kDa), 5% FBS (fetal bovine serum (FBS, COSMO Bio, Tokyo, Japan), and 0.1 IU / ml FSH. Culture medium was exchanged in half every other day. Egg with a clear GV was regarded as a grown egg, and GV transfer, *in vitro* maturation (IVM) and *in vitro* fertilization (IVF) were performed as described later.

Collection of GV oocytes

12–16 weeks old ICR female mice were injected intraperitoneally with 5 IU of equine chorionic gonadotropin (eCG, ASKA Animal Health Co., Ltd., Tokyo,

Japan) and were sacrificed 46 h later and stored at 4°C for 5 different time durations: 0, 20, 30, 44, and 68 h. The 0 h storage group was taken as control. The ovaries from these 5 groups were collected to obtain cumulus-oocyte-complexes (COCs) and GV oocytes in COCs were extracted.

GV transfer

GV oocytes were subjected to cytoplasm replacement, GV transfer. GV transfer was performed according to the previous reports [5]. Briefly, denuded GV oocytes from individual groups were pre-cultured in α-MEM medium supplemented with 0.33 mM dibutyryl cyclic AMP (dbcAMP) and 5μg/ml cytochalasin B (CB) for 1 h, and a cut was made in the zona pellucida using a micromanipulator. A micropipette was inserted through this incision to collect the karyoplast containing the GV nucleus. These karyoplasts were then injected along with a small amount of inactivated HVJ − Hemagglutinating Virus of Japan (HVJ-E; Ishihara Sangyo, Osaka, Japan) into the perivitelline space of the enucleated recipient oocytes, which were freshly collected from BDF1 mice (CLEA Japan Inc.) using a micromanipulator. After culturing the karyoplast transferred recipient oocytes for 30–60 min, the fused-reconstructed oocytes (reconstituted GV oocytes) were cultured for IVM at 37°C, 5%CO_2 in air as shown below.

IVM

Reconstituted GV oocytes were cultured *in vitro* for 16 to 18 h in-α-MEM supplemented with 10% FBS, 0.1 IU/ml Follicle stimulating hormone (FSH) and 4 ng/ml epidermal growth factor (EGF) at 37°C, 5% CO_2 in air.

IVF and *in vitro* culture (IVC)

The epididymal tail from 10 to 18 weeks male ICR mice were collected and the sperm mass was slowly released into a 200 μl modified Human Tubal Fluid Medium (mHTF) drop and pre-cultured for 1 h. After the sperm motility was confirmed, the sperm suspension (10^6 sperm/ml) was added to the mHTF drops containing MII oocytes for insemination. Six h later, fertilized oocytes were further cultured (IVC) in potassium-supplemented SOM supplemented with amino acids (KSOM AA) medium at 37°C, 5% CO_2 in air until blastocyst stage.

Embryo transfer

In vitro fertilized embryos at the 2-cell stage were transferred into the oviducts of 0.5–1.0 day old pseudo-pregnant recipient mice and their development until full-term was examined.

Reactive oxygen species (ROS) level and mitochondria membrane potential

GV oocytes were processed for analysis of ROS levels using ROS Detection Cell-Based Assay Kit (K936-100; Funakoshi, Tokyo, Japan) and mitochondria membrane potential using MitoTracker Green FM (Thermo Fisher Scientific, M7514, MA, USA) and were examined under a fluorescent microscope and camera system (Nikon ECLIPSE E1000 or E800M, Tokyo, Japan, Keyence VB-7000, Osaka, Japan), at Excitation filter/ Emission filter (Ex/Em): 495/529 nm or at Ex/Em: 490/516 nm respectively according to the manufacturer's instructions. Then the images were analyzed using ImageJ software. The amount of fluorescence was normalized with the fluorescence of control oocytes as 1.

Immunostaining for tubulin

According to the previous report [15], MII oocytes were treated with 2.5% Triton X-100 microtubule-stabilization buffer solution at 37°C for 1 h. After fixing with 2% paraformaldehyde (PFA) for 30 min, oocytes were treated with 0.1 M glycine for 10 min and then treated with anti-α-tubulin mouse monoclonal antibody: 0.1% triton supplemented PBS (PBT) (1:200) for 1 h at 4°C. After washing several times with 0.1% PBT, oocytes were treated with Fluoresceinisothiocyanate (FITC) conjugated affinity-purified goat anti-mouse IgG: 0.1% PBT (1:20) for 1 h. After washing with 0.1% PBT, the cells were mounted on a slide glass with glycerol containing 0.5μg/ml Hoechst 33342, and covered with a cover glass. Then they were examined under a fluorescent microscope at Ex/Em: 450/520 nm for FITC, Ex/Em: 365/400 nm for Hoechst and the images were analyzed using ImageJ software.

Statistical processing

The experimental results were statistically processed using the χ^2 test for the *in vitro* maturation and the *in vitro* development rates, and the *t*-test for the ROS and mitochondria analyses. It was considered significant when $P<0.05$.

Results

Effects of GV transfer on the *in vitro* and *in vivo* developmental ability of GV oocytes collected from postmortem carcass mice

As shown in Table 1, GV oocytes collected from mice 20 h after death were matured *in vitro* similar to control, but was significantly reduced in those collected after 30 h. However, when karyoplast from these GV oocytes were transferred into the enucleated fresh oocytes, the

 doi: 10.1538/expanim.22-0043

Table 1. *In vitro* maturation of germinal vesicle stage (GV) oocytes collected from dead mice with or without GV transfer

Time after death (storage time) (h)	GV transfer (cytoplasm replacement)	Number of GV oocytes	
		cultured (%)	matured (%)
0 (control)	−	507	421 (83)
	+	72	61 (85)
20	−	207	175 (85)
	+	54	50 (93)
30	−	137	61 (45)*
	+	25	24 (96)
44	−	124	12 (10)*
	+	8	6 (75)
68	−	54	0
	+	0	0

*Significantly different from control (0 h) and GV transfer group within the same column (P<0.05).

Table 2. *In vitro* developmental potential of *in vitro* fertilization (IVF) MII oocytes matured *in vitro* which derived from with or without germinal vesicle stage (GV) transfer

Time after death (storage time) (h)	GV transfer	Number of MII oocytes			
		cultured	fertilized (%)	cleaved to 2-cell stage (%)	developed to blastocyst stage (%)
0 (control)	−	136	111 (82)	83 (75)	53 (48)
	+	61	55 (90)	39 (71)	32 (58)
20	−	101	86 (85)	50 (58)*	14 (16)*
	+	50	46 (92)	41 (89)*	25 (54)
30	−	46	37 (80)	29 (78)	8 (22)*
	+	24	22 (92)	17 (77)	12 (55)
44	−	9	2 (22)*	0	-
	+	6	3 (50)*	2 (67)	1 (33)

*Significantly different from control (0 h) and GV transfer group within the same column (P<0.05).

Table 3. *In vivo* developmental potential of *in vitro* for maturation (IVM) oocytes after embryo transfer at the 2-cell stage derived from germinal vesicle stage (GV) transfer

Time after death (storage time) (h)	No.of oocytes			
	used for IVF	fertilized (%)	used for embryo transfer (cleaved to 2-cell stage) (%)	developed to full-term young after embryo transfer (%)
0 (control)	48	38 (79)	35 (92)	10 (29)
20	43	37 (86)	28 (76)*	9 (32)
30	47	41 (87)	36 (88)	5 (14)*
44	23	20 (87)	16 (80)*	1 (6)*

*Significantly different from control (0 h) (P<0.05). IVF; *in vitro* fertilization.

viability of reconstituted oocytes was improved. The rate of *in vitro* maturation was significantly increased compared to control (non-transplanted) oocytes at 30 and 44 h post death. In the 44 h group, GV transfer was difficult to achieve as the ooplasm was degenerated. Therefore, the number of GV transfers at 44 h was smaller than that of other storage time zones.

Table 2 shows the developmental ability of MII oocytes matured from reconstructed or non-reconstructed GV oocytes from dead mice. After IVF, developmental potential *in vitro* was significantly reduced in oocytes collected from mice 20 h after death. But, when karyoplast from GV oocytes obtained from dead mice were transferred into the enucleated fresh oocytes, reconstructed oocytes development to blastocysts increased in all groups. The blastocyst rate in reconstructed oocytes was significantly higher compared to the non constructed oocytes in 20 and 30 h post death groups.

Table 3 shows the *in vivo* developmental potential of reconstructed oocytes after embryo transfer of IVF em-

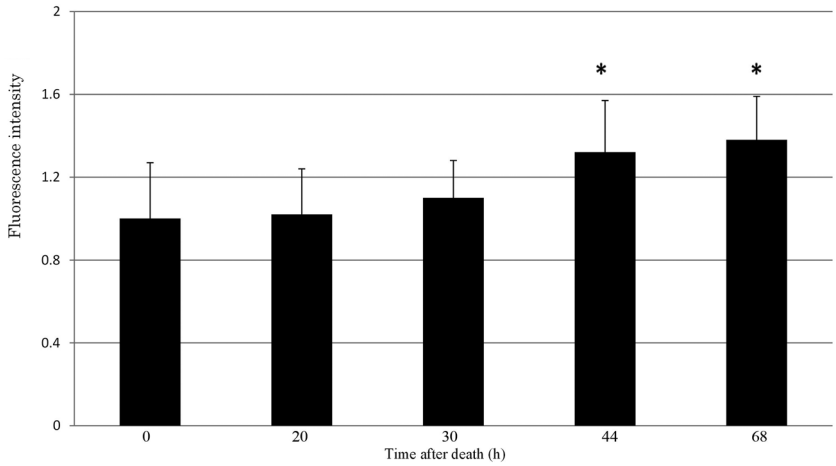

Fig. 2. Effect of postmortem time on ROS levels in germinal vesicle stage (GV) oocytes. *Significantly different from control (0 h) group. The number of oocytes examined (n) are 20–30 in each group. Data are presented as mean ± SE from 3–4 independent experiments.

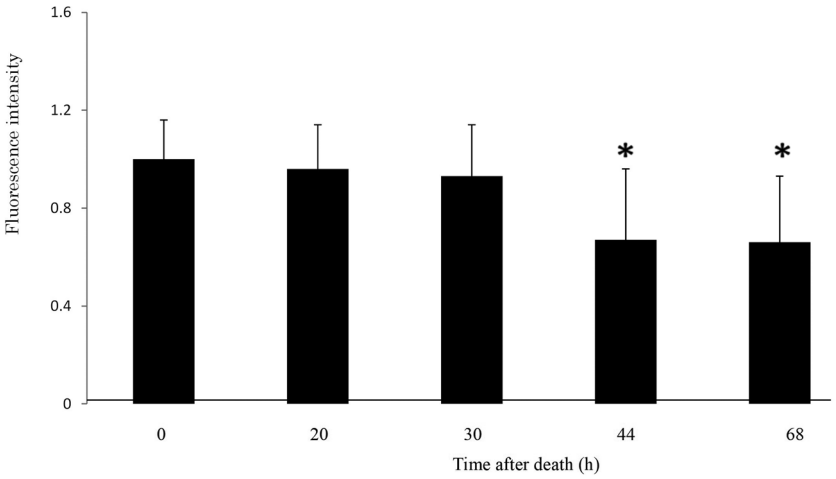

Fig. 3. Effect of postmorterm time on mitochondria in germinal vesicle stage (GV) oocytes. *Significantly different from control (0 h) (P<0.05). The number of oocytes examined (n) are 20–30 in each group. Data are presented as mean ± SE from 3–4 independent experiments.

bryos. As shown in the table, full-term developmental potential was not different in reconstructed embryos 20 h after death. Though after 30 h post death, developmental potential of reconstructed embryos was impaired, the reconstructed embryos were capable of developing into pups even at 44 h after death.

Effect of postmortem time on ROS level and mitochondria activities

As shown in Fig. 2, ROS in GV oocytes from the dead mice stored at 4 degrees were significantly increased at 44 h and 68 h postmortem compared to the control (0 h) group. As shown in Fig. 3, fluorescence intensity of mitochondria in GV oocytes from the dead mice stored at 4 degrees were significantly reduced at 44 h and 68 h postmortem compared to the control (0 h) group.

Effects of GV transfer on normal spindle morphology and chromosome alignment of GV oocytes collected from postmortem carcass mice

Chromosomes arranged in a row on the equatorial plane of the bale-shaped spindle seen in *in vivo* eggs are judged to be normal, and those with abnormal morphology and those with scattered chromosomes and those for which the spindle could not be confirmed were regarded as abnormal. As a result, it was found that the abnormal rate of the mitotic apparatus increased with 20 h after death. But GV transfer rescues the normality of spindle and chromosomes until 30 h after the death (Fig. 4). Figure 5 showed images of oocyte chromosomes and

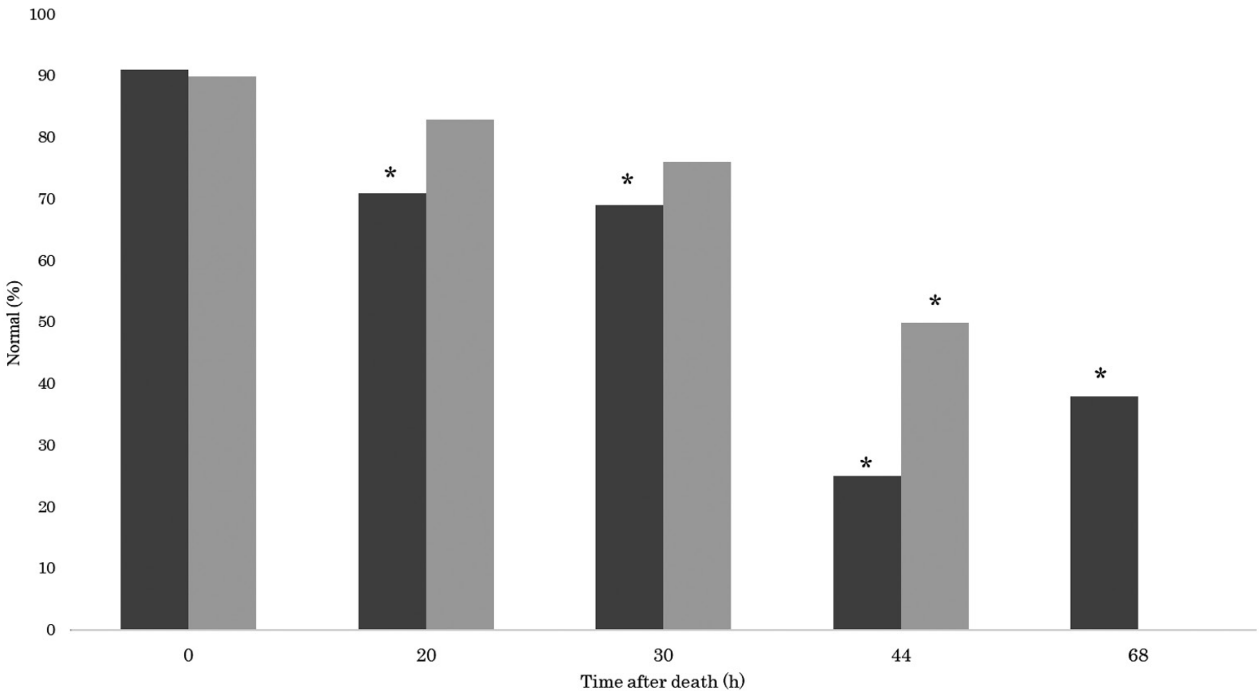

Fig. 4. Effect of postmortem time on normal spindle morphology. Proportion of normal spindle morphology with (gray) or without (black bar) germinal vesicle stage (GV) transfer. *Significantly different from control (0 h) (P<0.05).

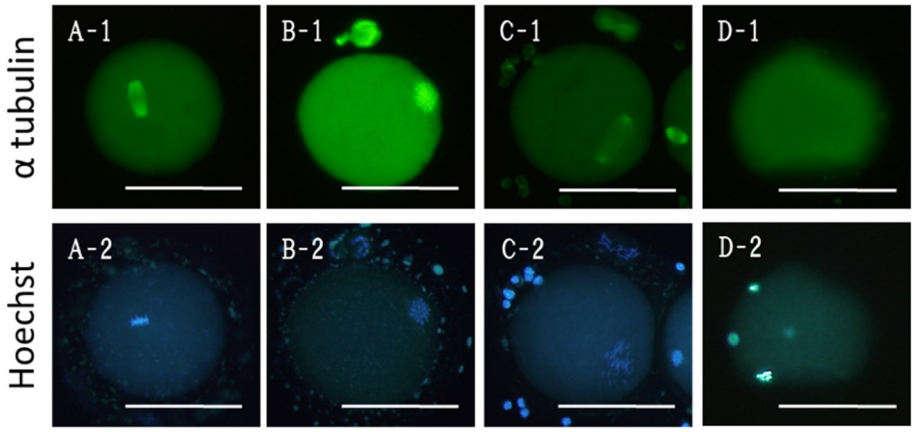

Fig. 5. Images of oocyte chromosomes and spindles stained with Hoechst 33342 and anti-tubulin antibodies, respectively. The upper row (A1, B1, C1, D1) shows the image stained with αtubulin (green), and the lower row (A2, B2, C2, D2) shows the image stained with Hoechst 33342 (blue). The blue dots outside the ooplasm are the nuclei of cumulus cells. A-1, 2: Normal morphology. B-1, 2: Morphological abnormality. C-1, 2: Chromosome dispersion. D-1, 2: Disappearance of splitting device. Scale bar: 100 μm.

spindles stained with Hoechst 33342 and anti-tubulin antibodies, respectively.

Effects of GV transfer on the *in vitro* and *in vivo* developmental ability of IVGMF oocytes collected from postmortem carcass mice

Table 4 shows the *in vitro* development rate of IVGMF eggs. GV transfer of IVG eggs significantly improved the *in vitro* maturation rate (61 vs. 80 and 81%) and cleaved rate (52 vs. 87 and 76%) compared to the non-GV transfer group. No non-GV transfer embryos developed into blastocysts. Storage of carcasses for 20 h significantly reduced the incidence of blastocyst formation (24 vs. 43%). When the reconstituted oocytes derived from carcasses were stored for 20 h and then IVGMF was done, 2% developed to full-term after embryo transfer to foster mother, but it was significantly lower than that of the control group (Table 5, 2 vs. 11%).

Table 4. *In vitro* developmental potential of IVGMF reconstituted oocytes which cytoplast were replaced at germinal vesicle stage (GV) phase

Time after death (storage time) (h)	Number of oocytes (%)							
	used for IVG	formed COCs	grow to GV phase (used for GV transfer)	used for IVM	matured to MII phase	fetilized	cleaved	developed to blastocyst
0*	102	66 (65)	-	66	40 (61)	21 (53)	11 (52)	0
0 (control)	165	95 (58)	50 (53)	50	40 (80)	30 (75)	26 (87)	13 (43)
20	255	121 (47)	54 (45)	54	44 (81)	38 (86)	29 (76)	9 (24)**

*No-GV transfer. **Significantly different from control (P<0.05). IVG; *in vitro* growth culture, COCs; cumulus-oocyte-complexes, IVM; *in vitro* maturation.

Table 5. *In vivo* developmental potential of IVGMF reconstituted oocytes after embryo transfer

Time after death (storage time) (h)	Number of oocytes (%)							Number of 2-cell embryos developed to full-term
	used for IVG	formed COCs	used for GV replacement	used for IVM	matured	fertilized	cleaved / transferred	
0 (control)	190	126 (66)	82 (65)	82	68 (83)	54 (79)	44 (81)	5 (11)
20	332	195 (59)	112 (57)	103	86 (83)	75 (87)	57 (76)	1 (2)*

*Significantly different from control (0 h) (P<0.05). IVG; *in vitro* growth culture, COCs; cumulus-oocyte-complexes, IVM; *in vitro* maturation.

Discussion

In this study, we showed that GV transfer is useful for effective utilization of eggs obtained from carcass. This study provided a useful method when the ovaries are not collected in time due to either an unanticipated death of the animal or a careless timing. It seems reasonable to store the carcasses at 4 degrees in this study. This is because ice crystals are likely to form in all cells when stored in temperatures below freezing, and it is unlikely that the viability of GV oocytes will be maintained. Moreover, in order to maintain the viability of the eggs of dead animals, it is necessary to store the carcasses at 4°C [3]. Therefore, in this study, carcasses were stored at 4°C. Applying this method to wildlife is considered difficult in common sense, but it is by no means unrealistic for animals under human control.

Although the female ovaries of mammals contain a large number of eggs at various growth stages, most of them disappear without ovulation. Therefore, in the field of livestock and medical treatment, IVM and IVG technology are being developed. Recently, it has become possible to induce the differentiation of functional eggs from mouse pluripotent stem cells [14].

The ROS levels of GV eggs significantly increased 44 h after death. At the same time, the mitochondria numbers decreased significantly. In addition, the abnormal rate of the mitotic apparatus had dramatically increased 44 h after death. On the other hand, when GV transfer was performed, the normality of the mitotic apparatus could be maintained up to 30 h after death. Since tubulin is supplied from the cytoplasm, it is considered that the mitotic apparatus was normally formed with materials from fresh ooplasm. Shilpa *et al.* [16] demonstrated that physiological level of ROS modulates oocyte functions, while its accumulation leads to oxidative stress. Oxidative stress triggers apoptosis in majority of germ cells and even in ovulated oocytes [16]. It was shown that the accumulation of spontaneous damage to the mitochondria arising from increased ROS in oocytes in aged oocytes, generated by the mitochondria themselves during daily biological metabolism. Moreover, mitochondrial dysfunction reduces ATP synthesis and influences the meiotic spindle assembly responsible for chromosomal segregation [17]. In addition, this result is consistent with the improvement in *in vitro* maturation rate and *in vitro* development rate by GV substitution, and it was speculated that abnormalities in the mitotic apparatus cause a decrease in the development rate. GV transfer did not reduce the developmental potential to the blastocyst stage after IVF until 44 h after death. But 68 h after death, the cell membrane of the oocytes became significantly fragile, and it became impossible to collect karyoplast to perform GV transfer, even though it was an apparently intact egg. GV transferred eggs up to 44 h after death yielded mature fetuses after IVF and ET. Our findings are consistent with previous works in which the oocytes recovered from ovaries of endangered animals [18, 19] and mice [1] immediately postmortem can be successfully matured to MII stage. It was suggested that by combining with GV transfer, it is possible to regenerate eggs obtained from carcasses over time, not limited to immediate use.

In this study, 20 h of storage of carcasses did not re-

duce the IVM rate, but the *in vitro* development potential after IVF was significantly reduced. On the other hand, it means that 16% of oocytes can develop to blastocysts when stored for 20 h and 22% of oocytes can develop to blastocysts when stored for 30 h, and it is worth considering a method for selecting eggs that retain the developmental ability.

Furthermore, it was found that the secondary follicle eggs of ICR strain carcass mice still could mature to the GV phase. Then, it was clarified that GV transfer of karyoplast of GV stage egg to a recipient egg derived from BDF1 strain mouse improves the maturation rate and the development rate after fertilization. Furthermore, when IVF eggs derived from secondary follicle eggs and from GV transfer were transferred to the foster mother, full-term development was observed. It may be necessary to consider GV transfer using a combination of other mouse strains.

If the other germ cell, sperm and egg, is compared and discussed, for males, ICR or BDF1 male mice could fertilize oocytes (21–39%) after being stored for 5–7 days at 4–6°C. Live mice were produced by *in vitro* fertilization with spermatozoa retrieved after 24 h of holding at 22°C after death, although the fertilization rate was low (19%) [20]. The reason why sperm survive longer than egg is considered to be due to the difference in cytoplasmic size and cell membrane structure between egg and sperm.

In summary, transfer of GV karyoplast recovered from postmortem carcass, which viability was decreased, into fresh GV recipient ooplasm, rescues survivability of reconstituted oocytes. For livestock and zoo animals, dead bodies can be placed at 4–10°C at night in cool seasons or in cool places. This study can propose a new approach that has the potential to revive such rare animals under control.

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