

Production of live offspring from testicular tissue cryopreserved by vitrification procedures in Japanese quail (*Coturnix japonica*)¹

Running title: Recovery of cryopreserved testes of Japanese quail

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¹Supported by a grant from the Canadian Poultry Industry Council, Canadian Poultry Research Council and Egg Farmers of Canada. Agassiz Research Center Contribution Number 814. Presented in part at the Annual Meeting of Poultry Science Association, 22-25 July, 2013, San Diego, California.

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ABSTRACT

Cryopreservation of testicular tissue can be used for ex situ conservation of male germplasm of avian species. The possibility of using vitrification and transplantation of testicular tissue for fertility preservation and recovery was tested in Japanese quail. Testes were removed from week-old Japanese quail, transfixed on acupuncture needles, equilibrated with dimethyl sulphoxide, ethylene glycol and sucrose, plunged into liquid nitrogen, and stored in 2 ml straws.

Cryopreserved tissue was warmed in sucrose solutions at room temperature or at 40°C. Fresh and cryopreserved tissue was transplanted subcutaneously into castrated, week-old recipients. Twenty of 21 recipients survived the surgery and 18 had viable transplants at maturity, with no difference in transplantation success between fresh and cryopreserved tissue. Fluid extrusion from 11 of the transplants was collected and inseminated surgically into the magnum of 22 quail hens and ten inseminations included foam from the proctodeal gland of the same recipients. Egg production in the two weeks after insemination was reduced and none of the hens inseminated with foam produced fertile eggs. Five hens inseminated without foam produced a total of eight live offspring, and four of these were inseminated with fluid extrusion from cryopreserved tissue. Histological examination showed spermatogenesis in the transplants, and the tubules, lumens, and epithelium of the seminiferous tubules were of comparable size to those of testicular tissue from intact males. These results demonstrate that testicular tissue of Japanese quail can be preserved using vitrification procedures and recovered through transplantation.

Keywords: *testicular tissue, cryopreservation, vitrification, transplantation, intramaginal insemination, Japanese quail*

Summary sentence: The function of Japanese quail testicular tissue can be cryopreserved by vitrification procedures and recovered by ectopic allogeneic transplantation.

INTRODUCTION

Cryopreservation of testicular tissue preserves the enclosed spermatogonial stem cells which can produce functional spermatozoa in their normal cellular environment. It is a promising strategy for preservation of fertility of human patients in situations when semen is not available [1], such as before the donor is sexually mature. Restoration of spermatogenesis in testicular tissue that has been cryopreserved and subsequently recovered by transplantation has been reported in various mammalian species [2] with the production of live offspring in laboratory rodents [3]. Cryopreserved testicular tissue of newly-hatched chickens can survive transplantation and can produce live chicks [4]. This provides a practical approach to ex situ germplasm conservation and the management of male germplasm of avian species such as Japanese quail for which semen cryopreservation has not been successful [5].

In most previous studies, slow freezing procedures have been used to preserve testicular tissue. This usually includes induction of extracellular ice nucleation at a particular seeding temperature and controlling the cooling rate to minimize lethal intracellular ice formation. Cryoprotective agents are used to ameliorate the detrimental solution effects which are associated with slow cooling [6]. However, this strategy provides limited protection to multicellular structures [7] such as testicular tissue, and vitrification procedures have been investigated. Vitrification is the process of solidification of a liquid without crystallization, resulting in an amorphous solid called glass [8]. This process can be approached by using ultra-rapid cooling combined with sufficiently concentrated cryoprotective agents so that intracellular and extracellular water is converted to glass [6]. The application of vitrification for preservation of testicular tissue has been attempted in mammalian species [9, 10], including humans [11], and fish [12], with encouraging results although no offspring have been produced. The effectiveness of vitrification procedures in cryopreserving ovarian tissue of Japanese quail has been demonstrated [13]. Testicular tissue of Japanese quail has also been cryopreserved using vitrification procedures and shows normal macro- and microscopic structures after short-term in ovo culture [14]. Whether these findings can be extended to functional recovery requires confirmation.

Functional recovery of mammalian testicular tissue cryopreserved by slow freezing could be achieved by transplantation and subsequent intracytoplasmic sperm injection using sperm extracted from the testicular transplants [2]. Although intracytoplasmic sperm injection is available for birds such as Japanese quail [15], it is unlikely to be practical for conservation programs. Intramaginal insemination using fluid extrusion retrieved from testicular transplants has been used in chickens [4], but has not been described for Japanese quail. In this study, testicular tissue of week-old Japanese quail was cryopreserved using a vitrification protocol [14]. Functional recovery of the cryopreserved testicular tissue was evaluated by ectopic transplantation and intramaginal insemination using the fluid extrusion retrieved from the transplants after maturation of the recipients.

MATERIALS AND METHODS

Birds, Chemicals and Tissue Preparation

One-week old male chicks of the QO and White-breasted (WB) lines [13] were used as testicular donors and recipients, respectively. Adult hens from the QO line and the JWT line (the UBC-N line described by Pisenti et al. [16]) were inseminated intramaginally. All of these lines are maintained at Agassiz Research Center. The research protocol was approved by the Animal

Care Committee of the Agassiz Research Center following principles described by the Canadian Council on Animal Care [17]. All chemicals were purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada) unless otherwise indicated.

Testes were removed from the donor birds immediately after euthanasia by cervical dislocation and immersed in a handling medium (HM) composed of Dulbecco phosphate buffered saline (DPBS) supplemented with 20% fetal bovine serum (FBS) on ice. The tunica vaginalis and tunica albuginea were cut to expose the testicular tissue.

Vitrification and Warming Procedures

The cryopreservation procedures used in this study followed a protocol that has recently been adapted for avian testicular tissue [14]. Five testes from different males were transfixed on an acupuncture needle (Seirin Corporation, Shizuoka, Japan). At room temperature, testes carried by the needle were submerged in an equilibration solution of HM with 7.5% (v/v) dimethyl sulphoxide (DMSO) and 7.5% (v/v) ethylene glycol (EG) for 10 min, then a vitrification solution of HM with 15% (v/v) DMSO, 15% (v/v) EG, and 0.5 M sucrose (VWR, Mississauga, ON, Canada) for 2 min. This protocol has been successfully used for ovarian tissue of mice [18, 19] and Japanese quail [13]. The testes were blotted on a piece of gauze, plunged into liquid nitrogen, and inserted into a modified pre-cooled 2 ml straw with one end pre-sealed with a sealing ball (Minitüb GmbH, Tiefenbach, Germany). The other end was sealed with a sealing ball and the straws were stored in liquid nitrogen.

For warming, the straws were opened with the end containing tissue still immersed in the liquid nitrogen. The needles carrying tissue were removed from the straws and immersed in HM with 1 M sucrose for 5 min at either room temperature or at 40°C. Tissue was transferred to 0.5 M, 0.25 M and 0 M sucrose solutions in sequence for 5 min each at room temperature, and the testes were suspended in HM on ice before further use within 4 h.

Testicular Allografting

Surgical procedures used for ovariectomy of day-old chickens [20] were adapted for castration of week-old quail. Recipient WB chicks were anesthetized by administration of isoflurane gas. The chick was placed on its back on a heated surgical surface. The feathers were removed from the left abdominal area, the skin was disinfected, and a 1.5-cm incision was made 1 cm left of the medial plane. The abdominal organs were displaced to the right to expose the testes, which were removed whole by cutting the mesorchium with a pair of fine forceps, and the incision was closed by simple interrupted stitches. Immediately after castration, two testes from two different males that were cryopreserved and warmed were inserted together under the dorsal skin of each recipient through a small incision, which was closed by a single stitch. Fresh transfixed testicular tissue was also transplanted into castrated recipients using the same procedures. After surgery, the recipient chicks were administered an immunosuppressant, mycophenolate mofetil (CellCept, Hoffmann-La Roche Ltd., Mississauga, ON, Canada), orally at 100 mg/kg per day for 2 wk. Numbers of recipients in each treatment group are shown in Table 1.

Histological Examination

At the age of 22 or 31 wk, 11 of the recipients with visible transplants were euthanized by cervical dislocation and the transplants were isolated. For each recipient, the transplant appeared

to be one piece and whether each transplant was derived from one of the transplanted testes or from both of them is unknown. Pieces of transplant tissue of approximately 3 mm³ were fixed in Bouin solution and the rest of the transplant tissue was used for intramaginal insemination (see below). Similar samples of testicular tissue from three sexually mature males were also fixed and used as controls. The tissue was embedded in paraffin, sectioned at 5 µm and stained with hematoxylin and eosin for histological examination. Images were captured using a digital camera (QICAM; QImaging Corp., Surrey, BC, Canada) mounted on a microscope (EL-Einsatz; Carl Zeiss Group, Jena, Germany). Ten seminiferous tubules from ten different sections for each transplant or control testes were sampled. The shortest diameters of the sampled tubules and their lumens were measured using an ocular micrometer, and half of the difference between the two was considered to be the depth of the seminiferous epithelium.

Intramaginal Insemination

The width (lateral) and the height (dorsoventral) of the proctodeal gland were measured for each recipient sacrificed and the product of the width and the height was used as an index of gland size [21]. Each of the 11 transplants was minced with a scalpel in a separate petri dish. The resultant fluid extrusion was collected in a syringe equipped with a 16-gauge needle. Intramaginal insemination procedures that have been used in chickens [22, 23] were adapted for Japanese quail. The hen was anesthetized with isoflurane gas and placed on its right side on a heated surgical surface. The left leg was stretched caudally to expose the lateral abdominal wall, feathers were removed from the area, and the skin was disinfected. A 2-cm incision was made behind the last vertebral rib, and the incision was spread using a retractor. A section of the magnum was externalized and 0.15 to 0.30 ml of the fluid extrusion, depending on the amount available which varied among transplants, was injected using a 1-ml syringe and an 18-gauge needle. For some hens, the testicular extrusion was mixed with foam produced by the proctodeal gland from the same recipient immediately before injection. The magnum was then released and the incision was closed by interrupted stitches. Eggs were collected for 2 wk before and after insemination and incubated. Numbers of hens inseminated and their performance are shown in Table 4.

Statistical Analysis

All statistical analyses were conducted with SAS Server Interface 2.0.3. The Chi-square analysis in the FREQ procedure was used to test the significance ($P \leq 0.05$) of differences in the numbers of surviving recipients with viable transplants, recipients producing live offspring, and hens producing live offspring. It was also used to test the correlation between the frequency of recipients that showed viable transplants and the frequency of recipients that showed functional proctodeal foam glands. The GLM procedure was used to compare the differences among groups in the transplant weight, proctodeal gland index, and fluid extrusion volume, as well as the seminiferous tubules, lumens, and epithelium. The TTEST procedure was used to compare egg production before and after insemination.

RESULTS

Survival and Growth of Testicular Transplants

Fresh testicular tissue and that which had been cryopreserved and warmed at room temperature or 40°C was transplanted into 21 recipients (Table 1), of which 20 survived the surgical transplantation procedures. At the age of 17 weeks, 18 of the surviving recipients showed

healthy testicular transplants growing on the back (Fig. 1 A-C). No significant difference was observed among groups in the number of surviving recipients or recipients showing viable transplants. A significant correlation was seen between the frequency of recipients showing viable transplants and the frequency of recipients showing functional proctodeal foam glands (Table 1).

Production of Offspring by Intramaginal Insemination

One of the three transplants derived from fresh tissue and two of the four transplants from each cryopreservation group that were used for intramaginal insemination produced live offspring (Table 2). No significant difference was seen among groups. The weight and volume of extrusion obtained from the transplants from the cryopreserved tissue were not different from those of the fresh tissue, and the proctodeal gland index was similar among groups. Seminiferous tubules containing spermatogenesis were seen in the transplants (Fig. 2 B-C), which was confirmed by the presence of motile sperm (Fig. 2 D). The average size of the seminiferous tubule, lumen and epithelium of the transplants was similar to that of normal testes (Table 3).

Five of the 12 hens that were inseminated with the fluid extrusion without proctodeal foam produced live offspring whereas the 10 hens inseminated with extrusion mixed with foam were not fertile (Table 4). Overall, egg production was significantly lower after insemination than before. Table 5 summarizes the performance of those hens that produced live offspring.

DISCUSSION

This study demonstrates that testicular tissue of an avian species can be cryopreserved using a simple vitrification method and recovered using allogeneic ectopic transplantation. Live offspring were obtained by intramaginal insemination using the fluid extrusion retrieved from the transplants.

Cryopreservation of testicular tissue is an important strategy for preserving male fertility, especially in situations where semen is not available or cannot be cryopreserved. Production of live offspring using this strategy has been limited to rodents and chickens [2, 4] and these studies used slow-freezing procedures. Slow-freezing has been used to preserve various types of cells and simple cell aggregations but it is limited for preserving complex multicellular structures such as tissue. The optimal values of the variables for a specific slow-freezing protocol are dependent on cell-specific properties such as surface-to-volume ratio and membrane permeability [7]. In addition, the extracellular ice formation induced by slow-freezing procedures is detrimental to the interstitial tissue compartments which are essential for functional recovery of testicular tissue [1]. These limitations can be circumvented by vitrification procedures, and their advantage in preserving testicular tissue viability and integrity has been demonstrated in pigs [9], mice [10], humans [11], and fish [12].

The vitrification protocol used in this study has been effective for preserving ovarian tissue in mice [18, 19] and Japanese quail [13]. It has recently been optimized for cryobanking avian testicular tissue using a simple and secure storage system [14]. Tissue culture using the chicken chorioallantoic membrane showed that it preserved testicular tissue viability. The efficiency of this protocol was confirmed in the current study by the high percentage of recipients that grew

testicular transplants and the similarity of macroscopic and microscopic properties of the transplants derived from cryopreserved tissue to those of fresh tissue and normal testicular tissue. The production of healthy offspring further validated the feasibility of using vitrification for cryobanking of avian testicular tissue. The beneficial effect of rapid warming seen in a previous study [14] was not apparent in this study, which could be attributed to the small sample size or the difference in the culture systems (in ovo versus in vivo) used. More rigorous assessments of warming procedures could be conducted in the future.

Testicular allografting is a means of in vivo maturation which is essential for recovery of the reproductive potential preserved in the tissue. The high viability of the recipients and the transplanted tissue demonstrated the efficiency of the refined surgical castration and transplantation procedures applied to Japanese quail. In addition, the presence of testosterone-dependent proctodeal glands [24] in most recipients indicated that steroidogenesis of the transplants was properly established. In this study, each testis was removed from the body cavity in one piece. In chickens, this reduced the chance of fatal exsanguination and increased the success rate of castration [25] compared to previous methods [4, 23]. Castration appears to be crucial to the survival and functional recovery of testicular transplants in chickens [4, 23] and some mammals [3], possibly because it creates a favourable endocrine milieu through the recipient's hypothalamic-pituitary-gonadal axis [26]. These data showed that complete castration was not essential in Japanese quail, as was seen in the rabbit-mice xenotransplantation model [27]. Whether the necessity of complete castration is species-specific needs more investigation.

The production of offspring is the ultimate objective of testicular cryopreservation and this was achieved in this study using transplantation and intramaginal insemination. Egg production of the hens was lower after insemination, which is consistent with a previous study in chickens [22]. This disturbance in egg production was ascribed to the use of ketamine and xylazine as anaesthetics [22] and the volatile anaesthetic isoflurane used here may have had a similar effect on quail egg production, or the surgery itself could have a negative effect.

The proctodeal gland foam had an adverse effect on the fertility of the sperm from transplants used for intramaginal insemination. Cheng et al. [28] found that the deposition of foam into the female proctodeum during copulation had a positive effect on the percentage of females fertilized and the duration of fertility. However, Chełmońska et al. [29] reported that mixing foam with ejaculated semen diluted with extender and dimethylacetamide was detrimental to sperm morphology and fertility. In the present study, foam was added to the testicular exudate and the mixture was deposited in the magnum. The foam may favor the sperm only in certain physiological situations, such as when combined with seminal plasma of ejaculated semen or when it is in the distal part of the oviduct, or it could have differential effects on ejaculated or testicular sperm.

The subcutaneous testicular transplants were not connected to the epididymis and vas deferens and the enclosed sperm likely resembled testicular sperm. Chicken testicular sperm can fertilize eggs when deposited into the magnum but not into the vagina of the hen [30], probably because of selective mechanisms in the uterovaginal junction of the oviduct [31]. Ahammad et al. [32, 33] proposed that chicken sperm experienced morphological and biochemical maturation as they

passed through the male reproductive tract, and that this was critical for their survival in the sperm storage tubules in the oviduct. The fertility of one quail hen extended to the second week after insemination, indicating that the sperm from the transplants can survive in the sperm storage tubules as long as that of ejaculated sperm [34], posing the question of whether maturation is necessary for quail sperm to survive in the oviduct and to fertilize eggs. On the other hand, the microenvironment of the oviduct could induce maturation and a third possibility is that intrinsic factors could trigger maturation, making it dependent on time alone. Ectopic subcutaneous transplantation of testicular tissue could provide a useful tool to test these hypotheses.

In conclusion, the production of normal chicks demonstrates that cryopreservation of testicular tissue using vitrification procedures and subsequent transplantation is a feasible option for ex situ conservation of male germplasm in Japanese quail. The vitrification procedures could be used for cryobanking of male germplasm of other avian species and systematic studies of species-specific questions with respect to fundamental reproductive physiology are needed.

ACKNOWLEDGEMENTS

We would like to thank the poultry crew of Agassiz Research Center for taking care of the experimental birds. Dr. Tom Forge and Carol Koch are also acknowledged for providing devices and help for microphotography.

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Figure Legends

Fig. 1. Testicular transplants and chicks produced by intramagnal insemination using the extruded fluid. **A)** A recipient with testicular transplant growing under the skin (feathers removed). **B)** A transplant with the covering skin removed. **C)** A transplant isolated from the recipient. **D)** Chicks hatched from the eggs collected during the first week after insemination.

Fig. 2. Histological examination of testicular tissue. **A)** Intact male. **B)** Testicular transplant. **C)** Seminiferous tubule with spermatogenesis in transplanted tissue. **D)** Sperm (indicated by arrows) in the fluid extrusion of cryopreserved and transplanted testicular tissue. Bars = 50 μ m.

TABLE 1. Transplantation of cryopreserved and warmed testicular tissue in Japanese quail.

Groups	Recipients	Surviving recipients (4 wk after transplantation)	Surviving recipients showing viable transplants (17-wk old) ^{ab}	Surviving recipients showing functional proctodeal foam glands (17-wk old) ^b
Fresh tissue	3	3	3	3
Warming at 40°C	9	9	7	5
Warming at RT	9	8	8	8

^a No significant difference was seen between any two groups.

^b Variables are significantly correlated ($P \leq 0.05$).

TABLE 2. Production of live offspring by intramagnal insemination using sperm retrieved from the testicular transplants (mean \pm SEM).

Groups	Recipients used for insemination	Recipients producing live offspring ^a	Recipients with complete castration	Transplant weight (g) ^a	Proctodeal foam gland index (mm ²) ^a	Fluid extrusion volume (ml) ^a
Fresh tissue	3	1	2	1.50 \pm 0.27	177.59 \pm 1.69	0.40 \pm 0.05
Warming at 40°C	4	2	1	1.79 \pm 0.43	141.52 \pm 21.71	0.59 \pm 0.15
Warming at RT	4	2	3	1.93 \pm 0.21	165.16 \pm 22.58	0.63 \pm 0.03

^a No significant difference was seen between any two groups.

TABLE 3. Histological examination of the testicular transplants.

Groups	Recipients sampled	Diameter of seminiferous tubule (μm) ^a	Diameter of lumen (μm) ^a	Depth of seminiferous epithelium (μm) ^a
Control	3	231.1 \pm 9.9	59.3 \pm 5.4	85.9 \pm 4.4
Fresh tissue	3	280.4 \pm 9.1	67.0 \pm 3.3	106.7 \pm 3.3
Warming at 40°C	4	239.1 \pm 34.4	87.5 \pm 7.2	75.8 \pm 13.9
Warming at RT	4	242.7 \pm 17.6	98.4 \pm 22.2	72.1 \pm 19.1

^a No significant difference was seen between any two treatments.

TABLE 4. Performance of hens inseminated with sperm retrieved from testicular transplants (mean \pm SEM).

Groups	Hens inseminated	Hens producing live offspring	Two-week egg production	
			Before insemination	After insemination
With foam	10	0 ^a	13.5 \pm 0.4	9.1 \pm 1.4
Without foam	12	5 ^b	13.2 \pm 0.5	9.4 \pm 1.2
Total	22	5	13.3 \pm 0.3 ^a	9.3 \pm 0.9 ^b

^{a, b} Values are significantly different.

TABLE 5. Production of eggs and chicks from hens producing live offspring.

Groups/hen ID	Fertile eggs produced	Live chicks produced		Two-week egg production after insemination
		The first week	The second week	
Fresh tissue				
63593	5	2	1	12
Warming at 40°C				
63583	2	2	0	13
63594	2	1	0	13
Warming at RT				
63580	1	1	0	12
63586	1	1	0	5

Figure 1

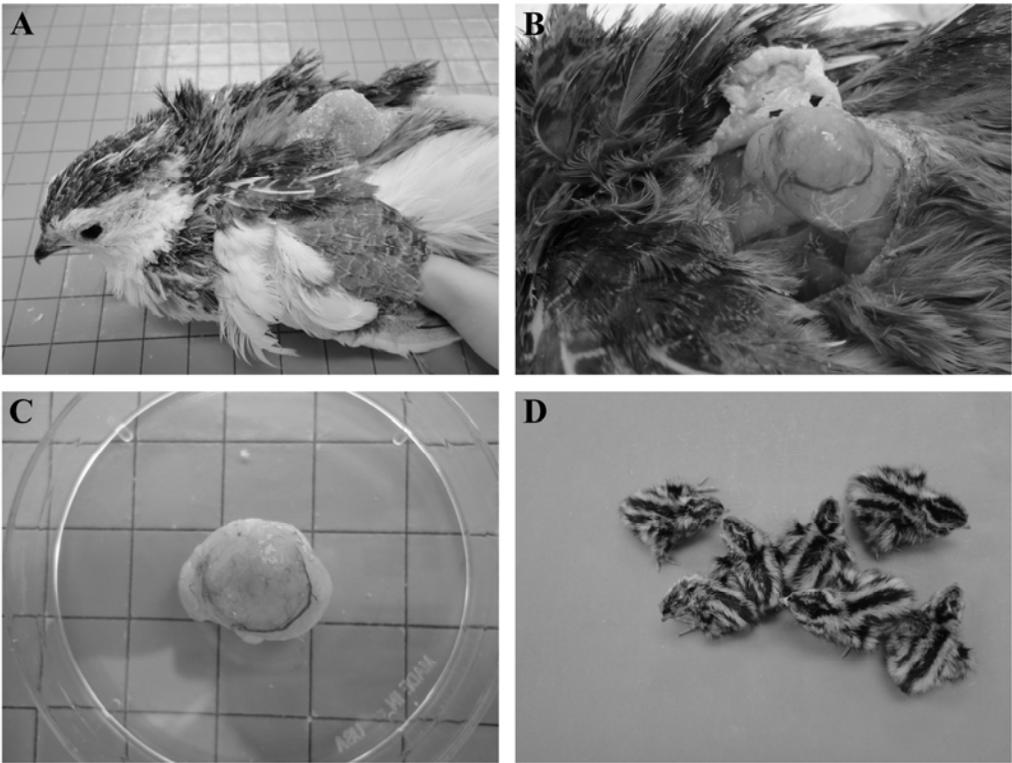


Figure 2

