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Preservation of Avian Cells at Sub-zero Temperatures

N. Ratnamohan[A] *and P. B. Spradbrow*

Department of Veterinary Pathology and Public Health,
University of Queensland, Brisbane, Qld 4067.
[A] Present address: Virology Department, Fairfield Hospital,
Yarra Bend Road, Fairfield, Vic. 3078.

Abstract

The cryoprotective agents dimethyl sulfoxide (DMSO), glycerol, polyvinylpyrrolidone (PVP) and dextran were evaluated for their ability to protect avian cells during storage at sub-zero temperatures. DMSO was the most effective cryoprotective agent for the short- and long-term storage of avian cells and glycerol was also effective when used at low concentrations. PVP and dextran did not protect avian cells during storage in our experiments. Primary chicken cells and avian cells at higher passage levels were successfully recovered after storage with DMSO for periods ranging from 4 to 12 months.

Whole embryos were frozen with varying concentrations of DMSO, and the survival rates and cell yields were determined for chicken embryo fibroblasts (CEF) prepared from the frozen embryos. Frozen embryos did not yield as large a number of cells as the fresh ones, but storage with 25% DMSO gave over 50% cell yields and survival rates.

The ability of frozen CEF and chicken embryo kidney cells (CEK) to support virus growth was also investigated. Assays of infectious laryngotracheitis, Sindbis, fowlpox, Newcastle disease, adeno- and turkey herpes viruses agreed within 0·5 $\log_{10}$ (50% tissue culture infective doses) when conducted in frozen and fresh CEF and CEK.

Introduction

Avian cell cultures are extensively used for the cultivation of avian viruses and some arboviruses, and for the production of avian viral vaccines. Established virus-free avian cell lines are not available and this necessitates the frequent preparation of primary cultures, which may be contaminated with reoviruses, adenoviruses and other agents (Mustaffa-Babjee and Spradbrow 1973).

Although mammalian cells are routinely stored at sub-zero temperatures, little use had been made of this technique for preserving avian cells. Porterfield and Ashwood-Smith (1962), Dougherty (1962) and McManus *et al.* (1974) established the feasibility of sub-zero storage of avian cells, while the latter authors also showed that cultures produced from the preserved cells were as sensitive to many viruses as were freshly prepared cultures.

The present paper describes the freeze storage of chicken, turkey and goose cells in suspension with various cryoprotective agents and the use of frozen whole chicken embryos to establish cell cultures. The susceptibility to avian viruses of cultures prepared from fresh and frozen cells was also compared.

Materials and Methods

Cell Cultures

Chicken embryo fibroblast (CEF) cultures were prepared as described by Mustaffa-Babjee (1970). Cultures of turkey embryo fibroblasts (TEF) and goose embryo fibroblasts (GEF) were prepared in a similar manner. Chicken embryo kidney (CEK) cultures were prepared by trypsinization of the kidneys of 18- or 19-day-old chicken embryos. Explant cultures were prepared from cartilage, liver and retina (which were taken from 14- or 15-day-old embryos) and cut into pieces approximately 1 mm by 1 mm and placed in Petri dishes. The cell culture medium was medium 199 additionally buffered with 0·01 M tricine (T199) and supplemented with 10% foetal calf serum (FCS). Closed culture vessels were incubated in a hot room at 37°C and Petri dishes in an atmosphere of 5% CO_2 in air at 37°C.

Monolayer cultures and the growth from explant cultures were harvested when necessary by treating with a mixture of trypsin and versene.

Freezing, Storage and Thawing of Cells

Freshly prepared cells and cells harvested from monolayer cultures were suspended at a concentration of 2×10^6 viable cells per millilitre in cell culture medium to which was added a cryoprotective agent. The cell suspensions were placed in 1-ml volumes in plastic ampoules which were then stored at −70°C for 2–4 h in a polystyrene box. The polystyrene box was 1 cm thick with inside dimensions of 6 by 4 by 12 cm and had a cover of the same material. These were held together by rubber-bands. For prolonged storage the ampoules were then transferred to a liquid nitrogen container.

Cell suspensions were thawed by placing the ampoules in a water bath at 37°C. The suspension was then centrifuged at 100 *g* for 5 min and the cells resuspended in fresh growth medium. Viable counts were done on cells excluding trypan blue dye and cell suspensions were used to initiate cell cultures.

Comparison of Cryoprotective Agents for Storage of CEF and CEK Cells

Primary CEF and CEK cells were frozen with different concentrations of dimethyl sulfoxide (DMSO)* (B. D. H., Poole, U.K.), glycerol* (E. Merck Ag., Darmstadt, Germany), polyvinylpyrrolidine (PVP)† (B. D. H., Poole, U.K.) or dextran† (B. D. H., Poole, U.K.). DMSO and glycerol were found to be sterile and used as 5, 10, 15, 20, 25 or 30% solutions in T199 containing 10% FCS. Five, 10 or 20 g of PVP or dextran were dissolved in 50 ml of distilled water, sterilized by autoclaving at 15 lb pressure for 15 min, mixed with an equal volume of double-strength T199 containing 10% FCS and used as 5, 10 or 20% solutions. The cells were recovered 24 h later and the survival rate of the frozen cells was determined by a method similar to that described by Dougherty (1962). For CEK cells, frozen and freshly prepared cells were seeded in Petri dishes at a concentration of $0{\cdot}2 \times 10^6$ cells/ml of growth media. Forty-eight hours after incubation the plates were fixed in methanol, stained with Giemsa stain and the number of healthy colonies of cells in 20 randomly selected fields were counted. The percentage survival is expressed as the ratio of the number of healthy colonies formed by the frozen cells to the number formed by the fresh cells.

For CEF, a similar method was used except that the cells were seeded at a concentration of $0{\cdot}1 \times 10^6$ cells/ml and the cells were counted after 18 h incubation.

Effect of Time of Storage on the Survival of CEF and CEK Cells

Primary CEF and CEK cells were frozen with 5% or 10% glycerol or DMSO and stored either at −70°C or in liquid nitrogen (−196°C). The cells were recovered at regular intervals over a period of 12 months, plated and survival rates determined as previously described.

* Percentage expressed (v/v).
† Percentage expressed (w/v).

Storage of Other Avian Cells

Subcultured TEF, GEF, chicken cartilage, chicken liver and chicken retina cells were stored with 10% DMSO in liquid nitrogen. The passage level and the approximate cell-doubling number of the cells are shown in Table 3. The cells were recovered 4–7 months after storage. The percentage viability was determined by the trypan blue dye exclusion test and the cells were seeded in prescription bottles at a concentration of $0{\cdot}5 \times 10^6$ cells/ml. The bottles were observed daily for monolayer formation. The survival rates were not determined by the method described previously as unfrozen cultures at the appropriate passage levels were not available for comparison.

Storage of Embryos and Preparation of Cell Cultures from Frozen Embryos

The method of sub-zero storage was essentially similar to that described for storage of cells. Ten-day old chicken embryos were placed in 1 oz screw-capped bottles, suspended in 4 ml of T199 containing 10% FCS and varying concentrations of DMSO and then frozen to −70°C. The frozen embryos were taken out after 48 h, thawed quickly in a water bath at 37°C, washed twice in phosphate-buffered saline (PBS) and used for the preparation of primary CEF cultures (Mustaffa-Babjee 1970). The cell yield per embryo and survival rates were determined. The cell yields from frozen and fresh embryos were also compared.

Growth of Avian Viruses in Cultures Prepared from Fresh and Frozen CEF and CEK Cells

Virus stocks available in this laboratory were assayed for their infectivity in fresh and frozen cells. Frozen primary CEF and CEK cells were recovered and plated in test tubes at concentrations of $0{\cdot}5$ and $1{\cdot}0 \times 10^6$ viable cells/ml respectively. Freshly trypsinized primary CEF and CEK cells were plated similarly. Serial ten-fold dilutions of the viruses were made in T199 containing 2% FCS and 0·2 ml of the virus was added to each tube. Fresh and frozen cells were inoculated simultaneously and four tubes were used per dilution. The titres were calculated by the method of Reed and Muench (1938) and expressed as 50% tissue culture infective doses/0·2 ml ($TCID_{50}$/0·2 ml). The standard error was calculated by the method of Pizzi (1950).

Results

Comparison of Cryoprotective Agents for Storage of CEF and CEK Cells

Percentage survival values of primary CEF and CEK cells when stored with DMSO, glycerol, PVP and dextran are shown in Table 1. The results show that DMSO was

Table 1. Survival of primary chicken embryo cells after storage with different cryoprotective agents for 24 hours

Survival rates determined as described in Materials and Methods. The results are the means of two experiments and in each experiment two plates were used for assay

Agent	Concn (%)	Mean survival rate (%) ± s.d. CEF	CEK	Agent	Concn (%)	Mean survival rate (%) ± s.d. CEF	CEK
Glycerol (v/v)	5	59·2±2·6	63·1±1·7	DMSO (v/v)	5	84·2±2·9	88·2±2·0
	10	48·8±2·7	42·9±2·1		10	87·6±2·0	86·5±2·8
	15	1·7±2·8	0·6±2·6		15	68·5±2·5	63·8±3·8
	20, 25 and 30	0	0		20	42·3±1·7	36·9±1·7
					25	21·2±1·5	24·2±4·3
					30	0	0
PVP (w/v)	5, 10 and 20	0	0	Dextran (w/v)	5, 10 and 20	0	0

superior to the other cryoprotective agents and was most effective at concentrations of 5–15%. Glycerol was effective only at low concentrations. PVP and dextran did not offer protection to chicken cells during storage.

Effect of Time of Storage on the Survival of CEF and CEK Cells

Survival of primary CEF and CEK after long-term storage with glycerol or DMSO at −70°C or in liquid nitrogen is shown in Table 2. When stored with 10% DMSO, survival rates of 50% or more were obtained for primary CEF after storage for 9–12 months at −70°C or in liquid nitrogen. Though slightly lower values were

Table 2. Survival of primary chicken embryo cells after long-term storage with glycerol or DMSO at −70°C or in liquid nitrogen (−196°C)

Survival rates determined as described in Materials and Methods. The results are the means of two readings. n.d., not done

Agent	Concn (% v/v)	Cell type	Percentage survival rate after storage at −70°C for (months): 2	5	7	8	9
Glycerol	5	CEF	50·1	45·4	40·6	32·5	30·1
DMSO	10	CEF	64·6	48·5	56·2	59·1	53·5
DMSO	10	CEK	60·4	48·6	n.d.	n.d.	n.d.

			Percentage survival rate after storage at −196°C for (months): 2	4	6	8	10	12
Glycerol	5	CEF	47·1	n.d.	48·5	46·2	45·6	n.d.
DMSO	10	CEF	63·2	n.d.	64·5	58·6	60·2	57·5
DMSO	10	CEK	55·8	57·2	n.d.	n.d.	n.d.	n.d.

obtained for primary CEK, over 50% of cells survived after storage for 4 months in liquid nitrogen. Primary CEF stored with 5% glycerol showed lower survival rates than those stored with DMSO. A slight decline in the survival rates of CEF and CEK was apparent with increased storage time.

Table 3. Survival of other avian cells after long-term storage with 10% DMSO in liquid nitrogen (−196°C)

P, passage level; N, cell doubling number

Cell strain	P	N	Duration of storage (months)	Viability by trypan blue staining (%)	Monolayer formation
TEF	12	(24)	4	72	+
GEF	3	(3)	6	48	+
Chicken cartilage	4	(6)	7	36	+
Chicken liver	8	(12)	7	20	±[A]
Chicken retina	8	(12)	7	32	+

[A] Monolayer was incomplete and could not be passaged any further.

Storage of Other Avian Cells

Survival of other avian cells after long-term storage with 10% DMSO in liquid nitrogen is shown in Table 3. TEF, GEF, chicken cartilage and chicken retina cells recovered 4–7 months after storage with DMSO and formed monolayers after 4–7 days.

Storage of Embryos and Preparation of Cell Cultures from Frozen Embryos

Table 4 shows the survival rates of CEF prepared from frozen embryos. Only embryos stored with 25% DMSO gave more than 50% survival rates. The viable cell yield per frozen embryo compared unfavourably with that from a single fresh embryo. Embryos stored with 25% DMSO gave the highest viable cell yield per embryo, about 55% of that from a fresh embryo.

Table 4. Survival of primary chick embryo fibroblasts prepared from embryos frozen with DMSO at −70°C

Survival rates determined as described in Materials and Methods. The results are the means of four separate experiments. n.d., not done

DMSO concn (%)	Mean survival rate (%) ± s.d.	Mean cell yield ± s.d.
10	*31·2±3·5	6·6±1·4 × 10^6
15	34·6±4·7	10·3±0·8 × 10^6
20	n.d.	31·8±2·0 × 10^6
25	66·9±2·7	46·2±3·2 × 10^6
Fresh embryo	—	84·1±5·6 × 10^6

Growth of Avian Viruses in Cultures Prepared from Fresh and Frozen CEF and CEK Cells

As shown in Table 5, the avian viruses assayed in frozen and fresh CEK and CEF gave comparable results. All the viruses tested agreed within 0·5 $\log_{10}$ TCID when assayed in frozen and fresh CEF and CEK cells.

Table 5. Comparative infectivity assay in frozen and fresh primary chicken cells

n.d., not done

Virus	Titre[A] ± s.d.			
	CEK (fresh)	CEK (frozen with 10% DMSO)	CEF (fresh)	CEF (frozen with 10% DMSO)
Infectious laryngotracheitis	5·8±0·48	5·8±0·48	2·5±0·40	2·2±0·48
Sindbis	5·2±0·48	5·7±0·36	8·2±0·30	8·3±0·36
Adeno virus	6·6±0·36	6·5±0·40	6·0±0·52	6·2±0·24
Fowlpox	3·2±0·48	3·5±0·40	n.d.	n.d.
Newcastle disease virus	6·6±0·36	6·5±0·54	3·2±0·31	3·0±0·34
Turkey herpes virus	n.d.	n.d.	3·8±0·48	3·5±0·40

[A] Titre expressed as the negative logarithm to base 10 of 50% tissue culture infective doses per 0·2 ml (see text).

Discussion

The results of these experiments show that primary CEF, CEK and other avian cells can be preserved for long periods with the cryoprotective agents DMSO or glycerol. The preservation of CEK cells at sub-zero temperatures has not been

reported previously. The polymers PVP and dextran did not protect avian cells during storage in our experiments. PVP was found to be an effective cryoprotectant for erythrocytes (Bricka and Bessis 1955), bone marrow and tissue culture cells (Mazur *et al.* 1969). Dextran, an effective cryoprotectant for erythrocytes (Bricka and Bessis 1955; Ashwood-Smith and Warby 1971*a*, 1971*b*), did not protect mammalian cells during freeze-storage (Ashwood-Smith *et al.* 1972). The mechanism of action of PVP and dextran is believed to be different to that of glycerol and DMSO (Mazur *et al.* 1970) and higher molecular weight PVP was suggested to be better than the lower molecular weight PVP (Ashwood-Smith *et al.* 1972). The reason for the lack of protection to avian cells in our experiments is not clear. Possibly, cooling rates for the efficient freezing of avian cells with PVP and dextran are different from those for DMSO and glycerol.

A critical factor in the survival of frozen cells is the rate of cooling when they are frozen, and Dougherty (1962) found 1°C per minute to be the optimum cooling rate for CEF. Such cooling rates can be obtained by the use of a controlled-rate freezing apparatus which is not available in many laboratories. However, simple devices have been described for the slow freezing of cells (Dougherty 1962; Grant 1976). Grant (1976) found that lymphocytes frozen in an insulated polystyrene box placed directly in a deep-freezer at −65°C performed as well as those stored in a controlled-rate freezing apparatus. In our experiments, good survival rates were obtained by the use of an insulated polystyrene box similar to that described by Grant (1976). Though a slight decrease in the survival rates was evident with increased storage periods, 50% or greater survival rates were achieved for CEF after storage for one year.

Frozen embryos did not yield as large a number of cells as the fresh ones, but storage with 25% DMSO gave over 50% cell yields and survival rates. The survival rates and cell yields could possibly be increased by adopting multiple trypsinization procedures as described for the preparation of chicken kidney monolayers by Churchill (1965). In multiple trypsinization the tissue fragments are subjected to four or five cycles of trypsinization for relatively short periods of time, and this may be less injurious to the DMSO-preserved embryonic tissue. The use of frozen avian embryos to initiate cell cultures has not been reported previously.

Frozen CEF and CEK cells were found to be nearly as sensitive as fresh cells to the avian viruses tested. These results agree with the findings of McManus *et al.* (1974), who found frozen CEF and duck embryo fibroblasts to be good substitutes for fresh cells in plaque neutralization assays and virus growth quantitations.

There are obvious advantages in the preservation of avian cells at sub-zero temperatures. They can be substituted for fresh cells in the event of a crisis. Eggs from specific pathogen free (SPF) flocks are not always available. Some laboratories have no SPF flocks available, but can purchase SPF eggs or chickens from vaccine manufactorers at times of the year when the manufacturer's production of eggs exceeds requirements. These cells could be stored frozen for future use. McManus *et al.* (1974) have also suggested that extensive quality control and standardization are possible with cells preserved at sub-zero temperatures.

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