PRELIMINARY STUDIES ON CRYOPRESERVATION OF EMBRYONIC CATTLE FIBROBLASTS*

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The major obstacle in using primary cells as experimental material in long term studies is that, unlike cell lines, they have finite lifespan and lose their viability after a few sub cultivations. Survival of cells at low temperatures with all their original characteristics, if achieved, will ensure almost unlimited supply of the cell strain opening new vistas in cellular studies. Hayflick and Moorhead (1961) revived human diploid cells to form healthy confluent culture after preservation in growth medium with 10 per cent sterile glycerol at -70^0 C. Later investigators preferred storage in liquid Nitrogen (LN_2). Silani *et. al*. (1988) attempted cryopreservation of human neuronal cell with 10 per cent DMSO at -196^0 C and obtained an average recovery rate 62 per cent in culture. Dong *et. al.* (1993) reported primary human foetal brain cells were better stored at -196^0 C. In this study, the cell revival rates of primary foetal fibroblasts isolated from cattle kidney cryopreserved at -80^0 C and -196^0 C were compared.

Primary fibroblast cultures were established from embryonic kidneys collected from six cattle foetuses in Dulbecco's modified Eagles' Medium (DMEM) following the procedure proposed by Ephrussi and Weiss (1969) with modifications. Sub confluent monolayer fibroblast cultures in mid or late log phase were supplemented with fresh growth medium (DMEM + 10% FCS) 24- 48 hours before freezing attempts. Healthy confluent cultures thus obtained were then treated for 30 sec with just enough trypsin versene glucose (TVG) medium containing 0.1 per cent trypsin to cover the monolayer. The excess TVG medium was removed and the cultures were incubated for 10 min. Free cells were suspended in 5 ml of fresh growth medium containing 20 per cent FCS to neutralise the action of trypsin. The concentration of viable cells in each sample culture was determined by Trypan blue dye exclusion count. Only healthy cultures with 90 per cent viable cell count were selected for freezing. Freezing medium was prepared by incorporating DMSO at 10 per cent level in growth medium containing 50 per cent FCS. The cells were collected by centrifugation at 1000 rpm for 10 min and resuspended at a seeding concentration of 2 x 10^6 cells/ ml in chilled freezing medium. A volume of 1.8 ml of the prepared fibroblast suspension was transferred into labelled and paired 2 ml cryotubes (Nunc). Slow freezing procedure was followed from 4 to 0^o C. All cryotubes were held at 4^o C for 2 h and 0^o C for an hour. Later the tubes were rapidly frozen to -20^o C in a freezer for an hour. One set of the paired samples (six numbers) were transferred to -80^o C freezer. The other set of tubes were kept in LN_2 at -196^o C. The survival rate of cryopreserved fibroblast after seven days of storage was assessed by determining the post thaw viability. The post thaw cell viability was determined using trypan blue dye exclusion staining. The frozen cryotubes were rapidly thawed by placing in a water bath of 37^o C. The cells were retrieved from the suspension by centrifugation at 1000 rpm for 10 min in complete growth medium. The cells from each cryotube were resuspended in 2 ml complete growth medium to remove the cryoprotectant. The mean viability of fibroblast cryopreserved at -80^{o}C and -196^{o}C were statistically compared using one way analysis of variance. The revival rate was also assessed by ability of the cyropreserved cells to establish and proliferate to form confluent monolayers when seeded into sterile 25 cm^2 culture flasks containing growth medium.

The results of the study are summarised

Table1. Viability variation during cryopreservation (Dye exclusion test)

Freezing Temperature	Mean viability (%)	SE	CV (%)
Before freezing	92.54[a]	0.77	2.04
-80°C	40.72[c]	2.8	16.84
-196°C	73.36[b]	1.6	5.34

Means bearing different superscript differ significantly (P< 0.01)

Table 2. Analysis of variance for effect of two freezing temperatures on cell viability (arcsin $\sqrt{p}$)

Sources of variation	Degrees of freedom	Mean sum of squares
Between methods	2	1809.52**
Error	15	9.18

Critical difference at 1% = 5.07 ** Highly significantly (P< 0.01)

in tables1 and 2. The observations indicate that freezing significantly decrease cell viability. But the viability of fibroblasts was better preserved at -196°C (73.36± 1.6%) than at -80°C (40.72± 2.8%). When the fibroblast samples cryopreserved at -196 °C were inoculated into culture flasks containing growth medium five out of six samples grew to confluence within five days. But none of the six samples stored at -80°C could establish as monolayers.

The cryopreservation at -196°C involved sudden deep freezing and holding at constant temperature. This might have led to the formation of smaller and regular intracellular ice crystals, causing very little damage to cell membrane and resulting in higher percentage of viable cells. A minimum seeding concentration of cells is required for cell adhesion prior to formation of monolayers. It was observed in this study that primary fibroblasts at a minimum seeding concentration of 10^6 cells/25cm^2 were optimum to establish into monolayer. Significantly higher percentage of viable fibroblasts in the samples stored at -196°C must have ensured this minimum seeding concentration. Insufficient viable cell numbers might have prevented the adhesion and establishment of fibroblasts in cultures in case of samples stored at -80°C.

Summary

Preliminary studies were conducted to study the feasibility of prolonged storage of cattle primary fibroblast cultures through cryopreservation at -80^0 C and -196^0 C. The cells were suspended in the freezing medium containing 50per cent foetal calf serum (FCS) and 10 per cent Di methyl sulfoxide (DMSO). The reduction in viability of cells after freezing was highly significant. On the basis of trypan blue staining, it was shown that better post thaw viability was obtained at -196^0 C(73.36± 1.6%) than at -80^0 C(40. 72± 2.8%).

References

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