Cryopreservation of Rat Peripheral Nerves and Schwann Cells: Role of Ferric Sulfate.

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Cryopreservation has now become an important tool to store the tissues/cells under ultra low temperatures either till their use or for the purpose to have sufficient tissue in hand. To search for optimal cryopreservative conditions under which glia tissue can be preserved without any alterations to their ultrastructural integrity, we cryopreserved the peripheral nerves & Schwann Cells(SC), obtained from more than 200 rats, under different concentrations of glycerol with or without micrograms quantities of ferric sulfate [$Fe_2(SO_4)_3x3H_2O$] for periods ranging from one to four months. The cells viability was tested in minced nerves & cultured SC & it was observed that while the former retained exceptional cells viabilities of 91.11% & 80.58%, when kept at -196°C & -40°C respectively, in the cryopreservative medium containing 20% of glycerol with 2μg/ml of ferric sulfate, the SC demonstrated significantly better viabilities of 84.36% & 81.82% at -196°C & -40°C in the freezing medium cotaining 20% glycerol with 2μg/ml & 5μg/ml of ferric sulfate respectively. It is concluded that the ultrafreezing coditions for minced tissue & dissociated cells differ at different low temperatures & that ferric sulfate plays a vital role in this regard.

Key Words: Cryopreservation, Schwann Cells, Ferric Sulfate

Cryopreservation (Vitrification) has become an important method of stabilizing biological structures & is an inherent part of techniques like freeze-cleaving, replication or cryo-etching, *in vitro* fertilization, transplantation & ultracryotomy. Primarily adopted for ultrastructural studies, vitrification has gradually emerged from its narrow constraints & is now widely used in various fields of the science. How long we can stop the biological clock optimally & reversibily? is a puzzling question for investigators of this field. Cryopreservation attempts were made more than a century back by Montegazza in 1866[1], & since then the unaccomplished struggle continues to achieve the maximum post-cryo viability with maximum long duration & minimal undesirable impacts. To cryopreserve purified cells or minced tissues, many successful & excellent studies have been made on a variety of tissues[2,3,4,5]. Human & animal nervous tissues & cells have been successfully cryopreserved for prolonged periods without significant loss in cells viability[4,6]. Silani et al[7] successfully recultured human cortical neurons from frozen tissue without any compromise on the cells ultrastructural integrity or immunocytochemical marking. However critical review of the available literature indicates that we still lack a well established procedure for optimal cryopreservation of nervous tissue in general & glia cells in particular due to multiple parameters affecting the cryopreservation.

A variety of cryprotectant including dimethly sulfoxide (DMSO, a dipolar aprotic solvent), glycerol (DNA synthesis inhibitor) & related compounds, Ficoll, methylcellulose (Methocel), & urea have been used in the past in various cocentrations & combinations depending upon experimental parameters[1,2,3,8]. A large number of factors like tissue size & site, species differences, cryoprotectant concentrations[9] & type, speed of cryopreservation & thawing, duration of storage, temperature during storage, pre-cryopreservation treatment and medium composition have been reported to determine the post-cryopreservation cellular survival. 5% glycerol when used for human spermatozoa gave excellent results but proved to be highly toxic for rabbit spermatozoa under identical conditions[10].

Successful freezing depends upon protecting the cells against ice crystal damage, cell dehydration, pH changes, protein denaturation & changes in electrolytes concentrations, so even a minimal experimental change may profoundly effect the final outcome. Reviewing the previous studies, it can be concluded that tissue storage is not a problem but the question is how to get best results for a specific tissue or cell type. Mazur[1] seems to be fully justified in his remarks "We can store a tissue for more than 1000 years, however, the question is not storage stability but how to get cells down to -196°C, in which environment, & how to get them back without killing them". It has been reported that iron contents affects the storage evironments, we attempted to find its optimal concentrations at ultrafreezing using various quantities along with glycerol as cryoprotectant & equal amounts of FCS & DMEM.

Materials and Methods

Peripheral nerves & SC used for this study were removed & dissociated from neonatal (PN1-3: SD albino) rats. The

nerves were aseptically collected in DMEM (D 6780, Hybri Max: Sigma Ch Co, St Louis, MO, USA) from 6-10 litters for one experiment. For the nerves to be cryoprotected, they were debrided, were cut into small pieces of about 0.5-1mm^3& stored in flame sealed glass tubes in one of the following cryprotectant fluids prepared just before tissues introduction: a) Glycerol (sterilized) 20%, FCS & DMEM 40% each, b) As(a) with 2 µg/ml Ferric sulfate(AR: Beijing Biochem Co, PRC), c) as (a) with 5µg/ml of Ferric sulfate, d) Glycerol 10%, DMEM & FCS 45% each. The sealed tubes were stepwise placed at 4^{0}C, -10^{0}C, & -40^{0}C with a total time ranging up to 120min to lower the temperture at the rate of about 0.3^{0}C/min. Finally the tubes were placed in small cloth bags prepared for this purpose & were plunged immersed in the liquid nitrogen cylinder at -196^{0}C.

To prepare the SC cultures, the nerves were enzymatically & mechanically dissociated in culture medium using trypsin & collagenase (T 2271, type XII-s & C 9407, type XI: Sigma Ch Co) at concentrations of 0.125% & 0.06% (prepared in DHBSS) respectively. Maximum amount of the medium was removed after the final wash by repeated observations under the phase contrast microscope. SC were diluted with fresh culture medium & viability test was carried out by trypan blue exclusion test[7] (also see Sigma Catalogue, Technical Information). The cells (with plating density as 4-5x10^4 cells/ml) were cultured on acid treated, sterilized & poly-l-lysin (PLL: P 5899 Sigma Ch Co) pre-coated coverslips. The cells were partially purified by differential adhesion method & two pulses of cytosine β-D-arabinofuranoside (24Hrs postculture: AraC: C 1768: Sigma Ch Co) each with 48Hrs duration with 12Hrs recess in between. The cultures were detached by combined treatment of 0.02% trypsin & 0.1% EDTA at 5 div. The cells were washed twice in growth medium & the final cell count used for cryostorage was kept near 10^{6}cells/ml by trypan blue exlusion test. Before storage in the cryoprotectant medium, the culture medium was reduced to its minimum. They were stored in the same combinations of agents as mentioned for nerves. At the end of the storage periods, which differed from 1 month to 4 months, the tissues were removed & their cell viability & cellular characteristics were examined as follows:

(a) For nerves, the tubes were removed & immediately placed in water bath at 37-40^{0}C. They were opened under sterilized conditions, contents were washed 4-5 times in growth medium to get rid of the cryoprotectant fluid, & were then dissociated with gentle enzymatic digestion (0.03% collagenase & 0.125% trypsin) in growth medium at 37^{0}C for a time period of 15-20min with constant observation. The enzymatic reaction was stopped when no tissue fragment was apparently visible. To avoid cellular damage no mechanical dissociation was attempted. The cells were washed 2-3 times at minimum centrifuge speed for 5min & viability was assessed by trypan blue exclusion test. The cells were plated on PLL pre-coated coverslips & immunostained for S100 (PAP method).

(b) The SC tubes were removed as mentioned for nerves. The contents were mixed with growth medium & centrifuged for 5min. The step was repeated with growth medium thrice. Cell viability was assessed: Trypan -ive cells/Total cell count x100. SC were immunostained with S100 after 3-10div under standard conditions.

Some of the cultures were processed for Scanning Electron Microscope & Transmission Electron Microscope[11] to see any morphological abnormalities.

Results and Observations

We observed that dissociation of nerves yielded good results in growth medium rather than in CMF-DHBSS. Trypsin proved to be more damaging if used in concentrations exceeding the amounts mentioned. In case of SC, effective removal of the cryopreservative fluid was of critical importance as it significantly decreased the plating efficiency & viability. Moreover, the period between tissue removal & its plating also affected the results fatally. Good results were obtained if this time period was kept within the range of 1/2 and 1hour for SC& nerves respectively. The comparatively abundant debris /cell fragments didn't pose much problem to cells survival or attachment as the cultures usually became clear after first change of medium.

Analysis of the cells viability (see Table) from nerves cryopreserved under different conditions revealed that addition of 2µg/ml of ferric sulfate to 20% of glycerol significantly improved the cells counts of peripheral nerves kept at -196^{0}C ($P<0.05$ to 0.001). A similar addition did prove significantly beneficial ($P<0.05$) when tissues were kept at deep freezer for up to 2 months, although not to the extent as was in the case of Liq. N_2. Using 10% glycerol (as most of the commercially available freezing medium contain) gave very low cell viability & seemed to be of little value for practical purposes. The 65% & 68% cells yield at -40^{0}C when tissues were kept for 6-8Wks indicated that for short durations tissues can evenly be stored at this temperature with acceptable cell counts. However, the 80% count was significantly low from storage under ultrafreezing ($p<0.01$). These results also indicate that the freezing medium compositional requirements differ under different temperatures.

The data analysis of SC viability is different from that of the intact nerves. While 20% glycerol medium with 2µg of ferric sulfate gave significantly higher counts

(P<0.05 to 0.01) when stored at -40°C, the same medium proved to be significantly inferior (P<0.05 to 0.01) than the medium devoid of any addition or when 5µg of ferric sulfate is added at –196°C.

Table: Showing the % cell viabilities of cryo-preserved nerves & SC.

Freezing temp. (Nerves)	Glycine 20%+No iron (A)	Glycine 20%+2µg/ml iron(B)	Glycine 20%+ 5µg/ml iron (C)	Gly10%+ No iron(D)
-196°C	63.65±2.04	91.11±1.07	54.18±2.34	37.91±0.71
-40°C	65.82±2.82	80.58±2.95	68.90±1.13	20.67±1.21
Dissociated SC	(A)	(B)	(C)	(D)
-196°C	72.52±1.97	69.13±2.37	84.36±1.99	15.83±1.86
-40°C	62.46±1.91	81.82±2.63	52.37±2.08	07.77±1.50

Note: (a) The P values for peripheral nerves cryopreserved at -196°C are: B vs A, C, D (P<0.001), A vs C (P<0.05)
(b) The P values for peripheral nerves cryopreserved at -40°C are: A vs B (P<0.05), A vs C (NS), A vs D (P<0.001), B vs C (P<0.05)
(c) The P values for SC cryopreserved at -196°C are: A vs B (NS), A vs C (P<0.05), A vs D (P<0.001), B vs C (P<0.001)
(d) The P values for SC cryopreserved at -40°C are: B vs A, C (P<0.01), A vs C (P<0.05), A vs D (P<0.001)
(e) P value between groups B of peripheral nerves cryopreserved at -196°C & -40°C is <0.05
(f) In one case of peripheral nerve cryopreserved at -196°C (B Group) the viability was 93.12% after 12Wks of storage.
NS: Non-Significant

Examinations of the post-cryopreserved cells at the time of trypan blu exclusion test showed many intensely trypan blue +ive cells (Fig I) with some of the unstained cells also looking unhealthy. The dissociated cultures even after 24Hrs showed many rounded cells not yet either fully attached or perhaps because of "cryo-after effects" still unable to extend their processes. The fibroblasts (FB) in this case also seemed to be in advantage, as some of the FB were well pasted to the coverslips within 24Hrs. The majority of cryopreserved SC gave slightly different picture than those of the nerves with most of them seemed to be well attached & extending their processes giving bipolarity to the cells. After 8-10 div, the cultured cells looked completely indifferentiable from normal cultures showing long extending processes (Fig II). We also attempted to see whether the nerve segment can grow on collagen or not. When the cryopreserved nerves were explanted on collagen, they like the fresh nerve explants, expanded their FB first followed by SC. Cultures were immunostained from both type of tissues at differnt time periods & all of them exhibited immunoreactivity for S100.

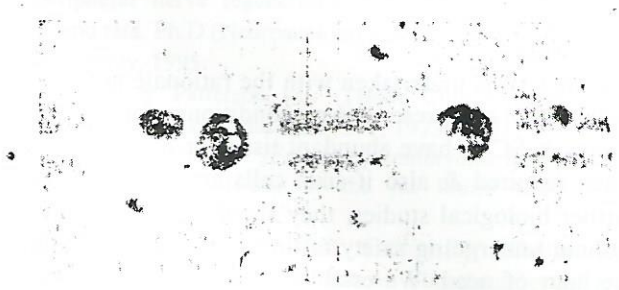
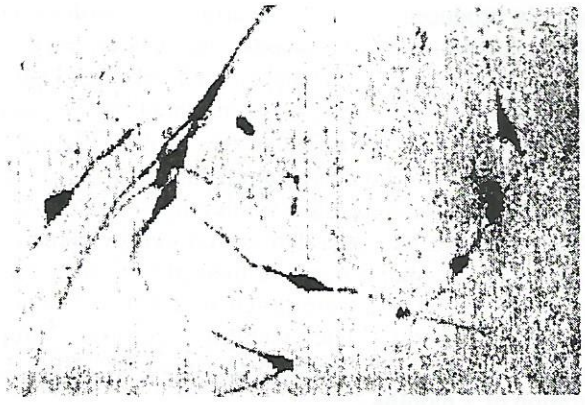


Fig I: Tryphan exclusion test of dissociated cryopreserved nerve (8Wks: 20%glycerol+2µg/ml of F. Sulfate). OMx200



FigII :Cryopreserved SC showing S100+ive immunostaining (S100-ive FB: 7div). OMx200

SEM & TEM observations showed the normal cellular architecture with all cell organelles. Some of the SC were unusually loaded with myelin inclusions (Fig III).

Fig III: A TEM photomicrograph of two bipolar SC from

cryopreserved nerve (8Wks: 20%glycerol+2μg/ml of Ferric.Sulfate) showing normal cell features but loaded with myelin inclusions. OM x6K

Discussion

The study was undertaken with the rationale to find more favourable cryopreservative conditions for peripheral nerves & SC to have abundant tissue or SC in hand as & when required & also if such cells are to be utilized for further biological studies, they should be "at ready hand" without undergoing safety testing & critical evaluation at the hour of need. We established a successful procedure for nerves & SC preservation using the cryoprotectant agent glycerol alongwith micrograms quantities of ferric sulfate. This combination has been previously used for cryopreservation of many other tissues[12]. Our procedure not only produced completely normal SC with excellent cells viabilities. We didn't analyse our data on the basis of cells/unit in fresh & cryopreserved tissues. However, Mason et al[4] data indicate a loss of about 55% of cells in their final count when sciatic nerves from 2 days rat pups were stored in the liquid N_2 (study lacks to give the exact storage period) in a medium containing 50% FCS & 25% DMSO & DMEM each. Our initial cells viability from group B of peripheral nerves climaxed to 93.12% (mean: 91.11%) when nerves were stored at -196°C for a duration up to 4 months. The viability, however, dropped significantly ($P<0.05$) in nerves stored at -40°C for a duration of 2 months.

Various studies have reported various % of survival but there are gross differences for different tissues & species. Mazur[1] reported 75% survival for mouse pre-implanted embryos cryopreserved at -196°C but he failed to get +ive results for pig embryos under the same conditions. The same author also indicated developmental stage differences for cryopreserving morula, blastocyst & its earlier stages. Polge et al[10] successfully cryopreserved human spermatozoa at -79°C with 5% glycerol & reported that concentrations lower than this (their concentration ranged from 5% to 30%) were not of more benefit. According to these workers, concentrations more than 20% resulted in progressive loss of spermatozoa mobility. Houle` & Das[13] used 10% DMSO,with EMEM & Ringer's solution or amniotic fluid to cryofreeze rat embryonic neural tissue for few Hrs at -70°C, while same medium was successfully used for cryopreservation of other tissues. Zhang & Qiu[5] reported successful cryopreservation of human fetal SC at 4°C & -196°C. Kawamoto & Berret[9] had reported successful neurons culture from cryopreserved (using 5-10% DMSO) rat embryonic cortical tissues for 4-88 days, while Kim et al[14] reported 55-70% viability from adult human oligodendrocytes (dissociated autopsy material) stored in boiling liquid N_2 in a medium consisting of 10% DMSO & 90% FCS at a cell concentration of 2-5x10^{6}cells/ml. Mattson & Ryetlik[15] used a medium containing EMEM, FBS & 8% DMSO for storage of human fetal brain tissue in liquid N_2 & obtained 85% & 36% of viable cells from 14 & 16 weeks of fetuses respectively (indicate age importance). Robbins et al[16] & Kontur et al[6] reported no morphological or biochemical changes in the human fetal brain tissue cells cryopreserved at -196°C. These workers adopted the new method of gradually increasing the DMSO concentrations which resulted into the excellent 90% viability. Our results of peripheral nerve cells viability, however can't be for obvious reasons, compared with these studies. But our more than 91% initial cell viability looks for better beyond any doubts when compared with any of the above studies on the basis of cells viability overlooking the difference in species, tissue, age of animal & duration of storage. In this regard the cells viability reported by Mason et al[4] are far low than ours.

The 84% SC viability in 20% glycerol with 5μg/ml of ferric sulfate can be compared with the data of Kim et al[14]disregarding the species & cell type difference. Our data demonstrate for better SC viability.

Although glycerol & DMSO have been reported to affect various biological aspects of cells[17,18] in cell cultures studies but our attempts to squeeze any remnants of these agents in our cultures curtailed any possible harmful effect(s) on cells, & so we didn't observe any change in cells morphology under light & electron-micrscope or alterations in immunostaining.

In conclusion we can claim that our cryopreservation procedure offers an acceptable method for long term cryopreservatin & isolation of copious quantities of SC as & when desired with a minimal loss of cells without morphological alterations. We have successfully used these cryopreserved SC in our nerve regeneration studies (article in preparation) to see the combined effects of SC & LMN & have obtained the same results as with the fresh SC.

We conclude that while 2μg/ml of $Fe_2(SO_4)_3x3H_2O$ significantly improve the glial cells viability from peripheral nerves stored in 20% glycerol concentrations at -196°C & -40°C, the requirements differed for the dissociated SC from the same source at the same temperatures & that the crypreservative conditions differ for nerves & SC.

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