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METHODOLOGY

A LEUKOCYTE CRYOPRESERVATION TECHNIQUE FOR CYTOGENETIC STUDIES

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ABSTRACT

Several culture, cryopreservation, freezing and thawing methods were tested to develop an efficient technique for storing cervid and ovine leukocytes. The best results were obtained with McCoy or Vega y Martinez (VYM) solution with dimethyl sulfoxide (DMSO), horse serum and polyvinylpyrrolidone (PVP) as cryopreservatives. The best protocol for freezing was 4°C for 30 min followed by 15 min in liquid nitrogen vapor. To thaw, the still frozen material was placed onto cultivation medium for propagation.

INTRODUCTION

Cellular cryopreservation is an important mechanism to store genetic material. During the freezing process, cells suffer injuries that most times result in death. According to Grout *et al.* (1990), the main problems during cryopreservation are cellular exposure to low temperatures, mechanical and physical effects of the ice crystals and property alterations of the extracellular solutions, which consequently alter the intracellular environment. Mazur (1970) indicated that damage caused by solution effects could be controlled by increasing the freezing speed, thereby decreasing the time between freezing the extra and intra-cellular solutions. On the other hand, the intracellular freezing effect could be minimized by decreasing freezing speed, and thus suppressing crystal formation. Therefore, every cell type has an optimum freezing speed that counterbalances the extra- and intra-cellular effects.

Some cryoprotective substances have been used to minimize cellular damage occurring in liquid nitrogen storage (-196°C). There are two types of cryoprotectors: those that permeate the cells, like dimethyl sulfoxide (DMSO), glycerol, sucrose, trehalose, methanol, glucose, 1.2 propanediol, proline, glycine betaine, fructose, galactose and lactose, and those cryoprotectors that do not permeate the cells, such as hydroxy ethyl starch, dextran and polyvinylpyrrolidone (PVP) (Grout *et al*., 1990). The exact protective mechanism of these substances is still not clear (Mazur, 1970).

Speed, not just of freezing but also of thawing, possibly interferes with cell viability. Rall *et al.* (1980) found ice crystals formed in the cytoplasm of mouse embryos that were slowly heated. These crystals did not form during freezing. Furthermore, the critical temperature for formation of these crystals was -65°C. If the samples were heated slowly until -85°C and then heated quickly, these crystals did not form.

Protozoa (*Babesia*) were successfully frozen by Vega *et al.* (1985), using Vega y Martinez (VYM) solution with 10% PVP. After thawing, the material was washed with VYM and deposited in culture.

Nachimuthu *et al*. (1992) worked with baby hamster kidney (BHK), Mardin-barby bovine kidney (MDBK), chicken embryo-related (CER) and Vero cells. The BHK, MDBK and CER lineages had approximately 90% protection with DMSO (10%), while the Vero cells were only protected by 20%. Association of 5% DMSO and 5% glycerol in the freezing medium was capable of protecting 66% of the Vero cells.

Heszky *et al*. (1990) worked with plant cells (*Puccinellia distans* (L.) Parl.). They tested three cryoprotectors and five pre-nitrogen temperatures. Samples were cooled at 1°C/min until -10°, -20°, -30°, -40° or -50°C, before being submerged in liquid nitrogen. The best results were obtained with 12.5% proline as a cryoprotector, at a temperature of -30°C, which resulted in 78% cell protection. Concentrated glycerol (12.5%) at -40°C protected 58% of the cells.

Cryopreservation protocols vary greatly with cell type. The objective of the present study was to standardize a leukocyte storage technique for use in cytogenetics, which would facilitate material collection from animals in the field.

MATERIAL AND METHODS

Ten milliliters of blood was collected in heparin-treated tubes from *Ovis aries* or *Mazama* sp. (Cervidae, Mammalia). The blood was centrifuged at 1,000 rpm for 5 min, and then maintained at 4°C for 15 to 30 min so that there would be agglutination of the leukocyte ring. The leukocyte ring was removed (± 1 ml) with a Pasteur pipette and deposited in 4 ml culture medium with the cryoprotector. The following media were tested: VYM (Vega *et al*., 1985), Hank's, McCoy's, Ham's F10 and heat-inactivated horse serum with

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DMSO (6 to 12%), PVP (10%), serum and antibiotics. The cellular solutions were then stored in 0.5-ml straws for 0, 10 or 30 min in the refrigerator, 0 or 15 min in the freezer or 0 or 15 min in liquid nitrogen vapor (1 to 2 cm above the nitrogen level). Afterwards, the material was submerged in liquid nitrogen, where it remained for one week to six months.

Thawing was achieved by one of two procedures: immersing the straws in water at room temperature until completely thawed or opening the straw and depositing the still frozen material onto the culture medium. The objective of the latter was to dilute the potentially toxic cryoprotector before the cell initiated metabolism. The medium used was Ham's F10 with 20% heat-inactivated horse serum, 5% phytohemagglutinin, 100 IU/ml penicillin and 0.1 mg/ml streptomycin. The cells were incubated at 37.5°C for 72-120 h, and subsequently conventional cytogenetic techniques were used for blocking metaphase cells through colchicine, hypotonization, fixation and mounting on slides.

Results were evaluated by the occurrence or not of metaphase nuclei in the cultivated material. The quantity of whole nuclei on the slides was also an evaluation factor.

RESULTS AND DISCUSSION

Of the basic culture mediums tested, the best results were obtained with McCoy's and VYM. DMSO as the only cryoprotector in the freezing medium was able to protect cells from cryogenic effects, but the best result was obtained with the association of DMSO (6.25%) and PVP (10%). The addition of 20% heat-inactivated horse serum in the freezing medium also improved the final results.

The best freezing protocol was 4°C for 30 min followed by 15 min in liquid nitrogen vapor before immersion in liquid nitrogen.

The best thawing protocol was the placement of the still frozen material onto the culture medium at room temperature.

Freezing medium: 3 ml McCoy's medium, 0.8 ml heat-inactivated horse serum, 0.25 ml DMSO, 400 mg PVP, 0.4 mg streptomycin and 400 units penicillin.

Protocol:

1. Collect blood in heparin-treated tubes
2. Centrifuge at 1,000 rpm for 5 min
3. Refrigerate (4°C) for 30 min
4. Deposit 1 ml of the leukocyte ring onto 4 ml freezing medium (4°C)
5. Store media with the cells in 0.5-ml straws
6. Refrigerate for 30 min (4°C)
7. Leave 15 min in liquid nitrogen vapor (2 cm above liquid level)
8. Submerge in liquid nitrogen

This technique has been used with approximately 1,000 cell cultures, with an average success rate of 20%. The cultivated material had adequate growth in these samples, though the quality was inferior to that of cultures realized with fresh material. To increase the number of cells per slide, cell culture time was increased from 72 to 120 h. According to Tanaka *et al.* (1979), the freezing process can cause mutations in *Escherichia coli*; nevertheless, the authors dissected the bacteria after freezing, and the present work did not. It is still unknown the real influence of freezing on the chromosome morphology. If any break or rearrangement in the chromosome happens, the cell would be unable to propagate *in vitro*. Continuing the typical cellular cycle, the normal cells will proliferate perfectly, and the ones with many chromossomic rearrangements will become unstable and will die. Some metaphases with a large number of breaks appeared in the material, but were very rare.

This technique could be used for storage of cytogenetic material for long periods of time. Furthermore, collection in the field would be facilitated. The material could then be transported to a laboratory and propagated under more aseptic conditions and with better equipment for long-term cultivation.

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RESUMO

O presente estudo teve como objetivo desenvolver uma técnica eficiente de estocagem de leucócitos de cervídeos e ovinos para posterior cultivo e obtenção de preparações citogenéticas. Vários meios de cultura, crioprotetores e protocolos de congelação e descongelação foram utilizados. Foram obtidos os melhores resultados utilizando-se meio de cultura McCoy ou VYM, acrescidos de DMSO, soro equino e PVP como crioprotetores. O melhor protocolo de congelação foi submeter as células a 30 minutos em 4°C e 15 minutos em vapor de nitrogênio líquido, e para descongelação, o lançamento do material ainda congelado no meio de cultivo para propagação.

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