EFFECTS OF CRYOPROTECTANTS ON FREEZING, CULTURE BEHAVIOUR OF BUFFALO UMBILICAL CORD MATRIX CELLS

SINGH, P., ROSE, M. K., YADAV, P. S., SHARMA, R. AND SINGH, J.

Department of Veterinary Physiology and Biochemistry, College of Veterinary Sciences, Lala Lajpat Rai University of Veterinary and Animal Sciences, Hisar 125004, Haryana

E.mail: preeti0829@gamil.com

Cell 09697025323

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Abstract: Umbilical cord represents the link between mother and fetus during pregnancy. Postnatally umbilical cord is a discarded organ and the collections of umbilical cord cells do not require an invasive procedure with ethical concerns. It is composed of a special embryonic mucous connective tissue called Whartons jelly and its cells have the properties of stem cells. Umbilical cord matrix cells are cryopreserved to study their future applications. Cryopreservation is a method to protect cells from freezing injury. In the present study confluent cultures from buffalo umbilical cord matrix cells were frozen at three different passages (P3, P6, P9) in three different combinations of cryoprotectants viz. DMEM culture medium containing 20% FBS and 10% DMSO, 1.5 M ethylene glycol and 0.2 M sucrose in PBS and 4% DMSO, 6% trehalose and 90% FBS. Pre and post freezing cell viability (%) was assessed using trypan blue dye exclusion method. The cells in (FBS-DMSO) and (EG-Sucrose) had more post thaw viability than (DMSO-trehalose –FBS) cryoprotectant combination. In post thaw culture behavior studies cells cryopreserved in (FBS-DMSO) started adhering to the surface of flask within 4 hrs and could proliferate well with similar morphology as that of continuous culture. Most of the cells cryopreserved in (EG-Sucrose) could not adhere to the surface of flask even after 24 hrs. The cells cryopreserved in (DMSO-trehalose –FBS) could adhere to surface of flask after 10-12 hrs and could grow well without any marked change in morphology.

Key words: Cryoprotectant, Stem cell, Umbilical cord.

INTRODUCTION

Stem cells are defined as undifferentiated cells that have the ability to self renew and differentiate into multiple cells or tissues [1]. There are two broad categories of stem cells: embryonic stem cells and adult stem cells. Adult stem cells, such as hematopoietic stem cells and bone marrow-derived mesenchymal stem cells (MSC), also known as marrow stromal cells are harvested from adult bone marrow [2]. The umbilical cord, found in amniotes, is a cord that connects fetus to the placenta. The umbilical cord consists of two umbilical arteries, and an umbilical vein and surrounding connective tissue matrix called Whartons jelly. The umbilical cord helps in transport of nutrients and oxygen rich blood between fetus and placenta. Cryopreservation is the technique of freezing cells and tissues at very low temperatures to preserve structurally intact living cells and tissues [3]. In cryopreservation the biological material remains genetically stable and metabolically inert, while minimizing ice crystal formation. In general, when a tissue is subjected to low temperatures, ice crystals will eventually form. These crystals may disrupt the cell membrane leading to the death of the cell.
**MATERIALS AND METHODS**

Gravid buffalo uteri were brought from the slaughter house at 4°C. The uterus was washed thoroughly with clean water and then rinsed twice with normal saline solution. After sterilizing with 70% ethanol, an incision was made on the uterine horn bearing fetus with the help of dissecting scissors to expose the fetus. Then, the umbilical cord was held with the help of two special holding artery forceps and was cut with the dissecting scissors. Age of each buffalo fetus was estimated by measuring curved crown –rump (CVR) length using the following formula: \( Y = 28.66 + 4.496 \times \) (If CVR is less than 20 cm), Where \( Y \) is the age of fetus in days and \( X \) is the CVR length of fetus in centimeters.

**Primary culture initiation:** The umbilical cord was placed in a sterile solution and the segments of 1-3 cm in length were cut longitudinally to expose and remove two umbilical arteries and two umbilical veins. The remaining tissue, containing Wharton’s jelly was diced into small explants. Tiny drops of re-calciﬁed plasma were made on 25 cm² culture flasks and tiny pieces of chopped umbilical tissue were ﬁxed on the drops. Then culture ﬂasks were kept at 37°C for an hour for ﬁxing of tissue. The tissues were observed for their fixing on the surface of the ﬂask in coagulated re-calciﬁed plasma. After ﬁxing the tissues, 5ml of D-10 culture medium with composition as given in Table 1.1 was added to each ﬂask. The cultures were maintained in CO₂ incubator at 38.0°C, 5% CO₂ and 85% humidity. On reaching conﬂuence (80-90%) the cultures were trypsinised and subcultured further with a split ratio of 1:2.

**Sub culturing of cells:** For sub culturing of cells, ﬁrstly the medium was decanted out from each ﬂask and then the cells were washed twice with 5 ml of 1 × phosphate buffer saline (PBS). Afterwards, the cells were trypsinised with 1 ml of 1x trypsin at 38°C. Trypsinisation of cells was observed at a regular interval of 1-2 min under phase contrast inverted microscope (Nikon Eclipse Ti, Japan). Trypsinised cells got detached from surface and became rounded. 1 ml of the culture medium was added to these trypsinised cells in each culture ﬂask to stop further trypsinization. Then the cellular content of each ﬂask was taken in 2 ml eppendorf tubes and centrifuged at 1000 rpm so as to remove trypsin. The cell pellet thus obtained was suspended in 2 ml of culture medium. This cell suspension was added in new flasks (1ml in each flask) and 4 ml of culture medium was added further to each ﬂask. Then the ﬂasks were placed in CO₂ incubator.

**Cryopreservation of cells:** Three cryoprotectants alone or in combinations were used for the freezing of cells. Detail of cryoprotectants used for freezing is given in Table 2. The confluent cultures were treated with 0.25% trypsin-EDTA and washed twice with cell culture medium by centrifugation (1500 rpm, 5 min) to remove trypsin-EDTA. The cell pellets thus obtained were suspended in 6 ml of culture medium. 1 ml from each cell suspension was taken for cell count and the remaining 5 ml was centrifuged again. The cell pellets thus obtained were suspended in 1ml of pre-cooled freezing media and transferred into 1 ml cryovials (Iwaki). Then the cryovials were placed at -20°C for 24 hours. After 24 hours, the cryovials were transferred to liquid nitrogen cryocan. Finally, cells were cryopreserved in 1 ml cryopreservation vials in liquid nitrogen at -196°C for one week.

**Thawing of cells:** After one week of cryopreservation, cryovials were taken out from liquid nitrogen cryocan and thawed by placing at 37°C in a water bath. The cell content of each cryovial were transferred in 15 ml centrifuge tubes and washed twice with culture medium. The cell pellets thus obtained were suspended in 6 ml of culture medium, vortexed gently and 1 ml was taken for cell count and remaining cell suspension were added to culture flasks and placed in CO₂ incubator to check the post thaw culture behaviour.

**Cell viability:** To ascertain the before freezing and post thaw cell viability in fresh and frozen thawed cultures, 1 ml cell suspension was taken for cell count as described above was vortexed gently. 100 µl from this cell suspension was mixed with equal volume of trypan blue dye and vortexed again. Neubar counting chamber of haemocytometer was charged with 10 µl of cell suspension on both sides and placed under microscope for cell counting. The viable (small, unstained and refractile) and non viable (large and dark blue) cells were counted. The average taken of the cells counted from both sides of the counting chamber was used as final reading.

**RESULTS**

Gravid buffalo uteri were brought from the slaughter house at 4°C and umbilical cord matrix cells were
Fig. 1: Cells with Spikes
Fig. 2: Post thaw cells in (DMSO-FBS)
Fig. 3: Post thaw cells in (EG-Sucrose)
Fig. 4: Post thaw (DMSO-Trehelose-FBS)
cultured from three different fetuses. Age of fetus was determined by curved crown rump (CVR) length method (Table 3). Cultures from fetus 1, 2 and 3 were considered as trial 1, 2 and 3.

**Primary culture initiation and culture behavior:**
Cells started emerging out of the tissue explants in 2 days. Cells during culture had fibroblast like morphology with clear spikes (Fig. 1) during primary culture. After 70-80% confluency, the cells were sub cultured and this was considered as passage 1 (P1). Confluent cells at passage 1 were further passaged with a split ratio of 1:2 upto P9. Spike initiation time in days and average passage time for each trial is shown in table 4. No significant difference in passage interval during culture of the three trials was observed. Cell remained homogenous with embedded fibroblast like morphology throughout the culture.

**Cell freezing:** Confluent cultures at 3 different passages (P3, P6, P9) from all the 3 trials were cryopreserved in 3 different combinations of cryoprotectants viz. DMEM culture medium containing (FBS –DMSO), (EG –Sucrose) and (DMSO-Trehalose-FBS). Pre freezing % viability of cells was assessed using trypan blue dye exclusion method. After minimum one week freezing post thaw cell viability was also assessed. Statistical analysis was performed using SPSS software. One way ANOVA was performed to test the difference in prefreezing and post thaw viability of cells among the different cryoprotectant. The result in Table 5 shows that prefreezing cell viability was similar in all the three cryoprotectants. But the post thaw cell viability was significantly (P<0.05) lower (50.11%) in (DMSO-Trehalose-FBS) than the (FBS –DMSO), and (EG –sucrose). However, combinations of (FBS –DMSO) and (EG –sucrose) did not differ significantly from each other. The paired T test was also used to compare the prefreezing and post thawing values of cell viability for each cryoprotectant. The results in table 6 revealed that, the post thaw cell viability was decreased in comparison to prefreezing values significantly (P < 0.01) in all the three cryoprotectants.

**Post thaw culture behavior:** Cells cryopreserved in different combinations of cryoprotectant at P3, P6 and P9 of each trial were thawed and seeded in 25cm² culture flasks to check their post thaw culture behavior. The cells cryopreserved in (FBS –DMSO) started adhering to the surface of flask within 4 hrs and could proliferate well with similar morphology as that of continuous culture and become confluent in 3-4 days (Fig. 2). The cells cryopreserved in (EG –sucrose) could not adhere to the surface of flask even after 24 hrs and few cells could adhere after 2-3 days. These cells become enlarged with expanded cytoplasm; formed cell aggregates and become confluent after 7-9 days (Fig. 3). The cells cryopreserved in (DMSO-Trehalose-FBS) could adhere to the flask surface after 10-12 hrs and normal growth without any change in morphology. (DMSO-Trehalose-FBS) got confluent in 5-6 days (Fig. 4). But the time of confluence in later combination was more (5-6 days) than that of cells preserved in (FBS –DMSO).

**DISCUSSION**
A wide range of cells having biological, medical and agricultural importance can be cryopreserved at -196°C for many years in a stable state. The survival of different types of cells after freezing and thawing varies markedly and depends on the ability of the cells to withstand a variety of stresses imposed by the physical and physiochemical changes occurring in the bathing medium during cooling to and warming from the storage temperature [4]. The greatest challenge during cryopreservation of cells is the lethality due to cooling and thawing processes. Therefore, it is important to evaluate the cell potential before and after cryopreservation [5]. Hematopoietic stem cells can be stored for prolonged period at cryogenic temperatures [6]. The major need is to cryopreserve stem cells for long term, so that they can be used in future. Umbilical cord blood contains hematopoietic and mesenchymal stem cells so it must be frozen and banked [7]. Thus, optimization of cryopreservation protocol to maintain the quality of stem cells is a critical task for their banking [5]. Cryopreservatives are necessary additives in the freezing medium since they inhibit the formation of intracellular and extracellular ice crystals and hence cell death. The standard cryoprotectant is DMSO, which prevents freezing damage to living cells [8]. To enhance the effect of cryopreservative, the combination of DMSO and extracellular cryoprotectant hydroxyl ethyl starch has been used with success in bone marrow grafting and umbilical cord blood [9]. Alternative cryoprotectants used in cryo-preservation methods are propylene glycol, a
combination of alpha tocopherol, catalase and ascorbic acid and the glucose, trehalose as intra cellular and extracellular cryoprotectants combinations [10-12]. Cells isolated from porcine umbilical cord matrix expresses genes responsible for pluripotency [13]. Buffalo umbilical cord matrix cells have also been reported to express pluripotency marker genes which make them important candidate cells for freezing [14].

So, keeping in view that umbilical cord is a good, non ethical and non controversial source of stem cells, buffalo cord matrix cells were frozen in this study. The umbilical cord was processed by explant method and cells were cultured. Finally, cells were cryopreserved in 3 different combinations of cryoprotectants - culture medium containing 10% DMSO and 20% FBS (combination I), 1.5 M ethylene glycol and 0.2 M sucrose in PBS (combination II) and 4% DMSO, 6% trehalose and 90% FBS (combination III). Among the three combinations, highest post thaw viability (66.93 ± 3.20) was observed in combination I. But statistically no significant difference was observed in the cryopreservation ability of combination I and II. A similar post thaw survival was observed by using 10% DMSO and 90% FBS [15]. The above results were supported by freezing EG-sucrose in a study [16] and found ethylene glycol and sucrose combination gave highest success in caprine ovarian tissue.

For human follicles, mixture of sucrose with DMSO is reported to be best cryoprotectant. Researchers had observed that the combination of 4% DMSO, 6% trehalose revealed good results after post thaw in a study [5] but in the present study, this combination had lower post thaw viability and more confluency time after post thaw culture of frozen cells. Trehalose combination was used as one of the combination as it is a nontoxic disaccharide of glucose [11]. Alternative to DMSO such as glycerol, proline or ectoin have been used in a study [17] and found that cells cryopreserved in ectoin led to high post-thaw cell survival of up to 72% , where as cells in glycerol and proline were completely dead (glycerol) or had only poor cell survival (proline, 22%). The explant fixation method for primary culture initiation was performed in porcine umbilical cord cells. These cells were cryopreserved in freezing medium consisting of 93% FBS and 7% DMSO, but results obtained from fresh and frozen were indistinguishable [18].
REFERENCES