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Trehalose and Ascorbic Acid improves the Cryopreservation of Umbilical Cord Blood Hematopoietic Stem Cells (CD34+) with Low Concentrations of Dimethylsulfoxide

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Abstract :

Cryopreservation of umbilical cord hematopoietic stem cells (HSCs) is essential step in stem cell transplantation. The Dimethylsulfoxide (DMSO) mostly used as a cryoprotective agent, associated with toxic effects to stem cells. In order to minimize the effects of DMSO, low concentrations of DMSO with additional cryoprotectants should used. In this study mononuclear cells (MNCs) isolated by ficoll from umbilical cord blood (UCB), which contain hematopoietic stem cells (CD34+), were used for cryopreservation.

Cryopreservation of UCB-derived MNCs was done for period of one month by using uncontrolled-rate freezing technique at -196˚C in liquid nitrogen. Cryopreservation solution was used which consisted of minimum essential medium (MEM) and 20% fetal calf serum (FCS) supplemented with the cryoprotectant dimethylsulfoxide (DMSO) in two concentrations 2.5% and 5% DMSO, lower than usually used 10%, with and without 25µg/ml trehalose or 80µg/ml ascorbic acid to improve cryopreservation process. The addition of trehalose and ascorbic acid improved cryopreservation process in comparison with control. Addition of 5% DMSO alone and with additives showed a better result than 2.5% DMSO alone or with additives for cryopreserving CD34+ cells as indicated by immunocytochemistry.

Washing out DMSO also affected the count and viability of MNCs. These results indicated that it could use low concentrations of DMSO in cryopreservation of HSCs by association with trehalose and ascorbic acid.

Key words: *cryopreservation, DMSO, trehalose, ascorbic acid, hematopoietic, stem, CD34+.*

Introduction:

Cord blood is rich in stem cells, as it contains hematopoietic stem cells (HSCs) and mesenchymal stem cells (MSCs) (1,2) so, the use of UCB stem cells has been increasing interest because both kinds of stem cells which are regarded as a valuable source for cell transplantation and therapy. Cryopreservation of HSCs is the backbone of clinical stem cell transplantation (SCT).

The DMSO has been the most used cryoprotective agent for human stem cells (9), Since DMSO is highly soluble in water and readily crosses cell membranes, the addition of DMSO to a cell suspension results in a high DMSO osmolality both outside and inside the cell, offsetting the salt gradient established a cross the membrane (7).

Virtually the free water existing within the cell will diffuse out of the cell, thereby preventing intracellular ice crystals formation (9). DMSO associated with side effects to stem cells. Zambelli et al. (11) showed that DMSO is associated with a clinically significant side effect when infused in patient and assumed to have toxic effect to stem cells.

Several investigators showed that addition of ascorbic acid and trehalose with 10% DMSO improves the cryopreservation of HSCs. Limaye L. and, Kale V. concluded that cryopreservation of HSCs with medium contain 10% DMSO supplemented with 25µg/ml trehalose

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and 80µg/ml ascorbic acid affords better cryoprotection as evidenced by significantly increased colony formation as compared to 10% DMSO alone (1).

The purpose of this study was to determine the affects of using the cryoprotectans trehalose and ascorbic acid with lower concentrations of DMSO, 2.5% and 5%, in order to minimize the effect of 10% DMSO during the cryopreservation of hematopoietic stem cells (CD34+).

Materials and Methods:

Umbilical cord blood collection:

Umbilical cord blood (UCB) sample were obtained mainly Fattema AL-Zahrraa Hospital in Baghdad. All UCB specimens were freshly collected from discarded placentas of full term normal vaginal deliveries.

Immediately after delivery of the baby, the umbilical cord clamped then breaking the link between the baby and placenta, the baby separation from the cord, and the cord then cleaned a 5-8 cm area of umbilical cord with antiseptic solution and inserted the blood bag needle in the umbilical cord vein (fig.1).

The blood was flow by gravity into the bag containing citrate phosphate dextrose adenine-1(CPDA-1) anticoagulant approximately 25 ml, since total collections was approximately 100-120 ml. During collection the blood bag was shaken gently, so that the anticoagulant freely mixed with UCB (reference).

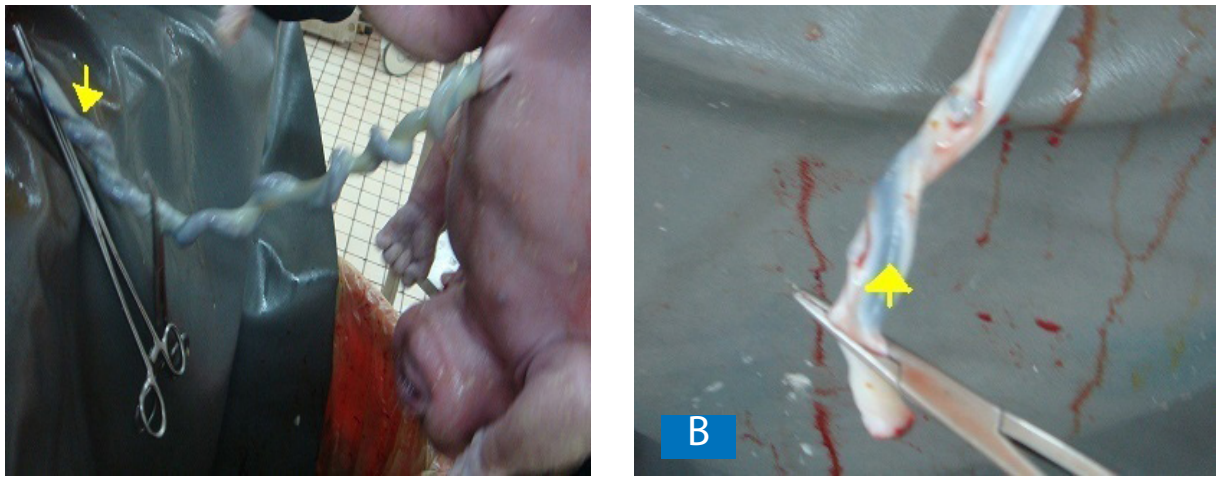

Figure (1): Collection of umbilical cord blood. (A): The place of umbilical cord cut (yellow arrow) in order to separate baby from placenta. (B): The blood was collected from the vein (yellow arrow) of umbilical cord, while the placenta in uterus and the umbilical cord out of vagina.

Cord blood cells mononuclear cells separation:

After successful collection of UCB, it was kept in anticoagulant treated bag and kept at 4°C and processed within 24hr. The mononuclear cells (MNCs) were separated from UCB by density gradient centrifugation using Ficoll according to the protocol described by (7) with some modification. The viable cell count was performed using trypan blue method (8). Viable Cells (%) = [No. of viable cells/Total No. of cells (dead and viable)] ×100

CD34+ immunocytochemistry:

The isolated MNCs were fixed on precaoted charged microscope slide for determination of percentage of CD34+ expression stem cells. Ten µL of cell suspension was added on slide and kept for air dry. Twenty µL of fixation solution was added on dry cells and kept for 10 min at room temperature, after that slides were tapped and left for 3min at room temperature then infolded with aluminum foil and stored at -20°C until use. Immunocytochemistry staining of fixed cells was performed according to the manufactured company instruction (DAKO).

Cryopreservation

Cells were cryopreserved under sterile condition as follows: the MNCs were isolated from UCB by density gradient centrifugation method according the protocol described by Erices et al. (2000) with some modification, suspended with MEM culture medium contain DMSO (2.5% or 5%) supplemented with or without 25µg/ml trehalose or 80µg/ml ascorbic acid and considered ready for cells count, viability and percentage of HSCs CD34+.

The cryotubes were fulled with 1ml of cells suspension and labeled with number and date of freezing. One ml of chilled sterile cryoprotective media were carefully layered

on cells suspension in cryotubes, the 1:1 mixture was slowly inverted to promote mixing, then placed on ice for 1min allow equilibrium between the cells and the cryoprotective media. The cryotubes were placed in refrigerator at 4°C for 10min then placed in the bottom of a freezer at -80°C for 24 hr; the cryotubes were quickly transferred to a container holding liquid nitrogen for period one month.

Thawing and washing:

Cryopreserved cells were thawed by rapid immersion of cryotubes into water bath at 37°C for 5min then cells viability was directly measured after thawing and 1ml of aliquots of cells were quickly after thawing transferred into 10 ml tubes and diluted (1:4) with MEM medium supplemented with 10% FCS and kept for 1hr then centrifuged at 1000 rpm for 10 min and resuspended in MEM medium supplemented with 10% FCS. Cells viability was assessed by trypan blue dye exclusion test.

Statistical analysis:

The statistical analysis system-SAS (2004) was used to data analysis in study parameters. The least significant difference test- LSD was used to compare between means (28).

Results and discussion:

The collection strategy is the first step for collecting good-quality cord blood units and varies among banks and among collection sites for the same cord blood bank (12). In this sense, different collection methods have been proposed to optimize volume and total nucleated cells content of cord blood units.

In this study the close system was used and found effortlessness, easy, quick and no contamination was observed during cord blood collection process, collection yielded a significant higher volume and total number of mononuclear cells (13), with higher median concentrations of nucleated cells and total colony-forming units (CFU) (14), and reduction blood clotting problems (15) in comparison with other methods.

After a successful separation of the MNCs, characteristics of cell surface antigen of the specific HSCs represented by CD34+ was investigated by using immunocytochemistry technique. The result showed that the deep brown color for DAB stain represent the positive cells while the blue color for the haematoxylin stain represent the negative cells (Fig. 2). The CD34+ antigen is a defining hallmark of HSCs/ progenitor cells (16).

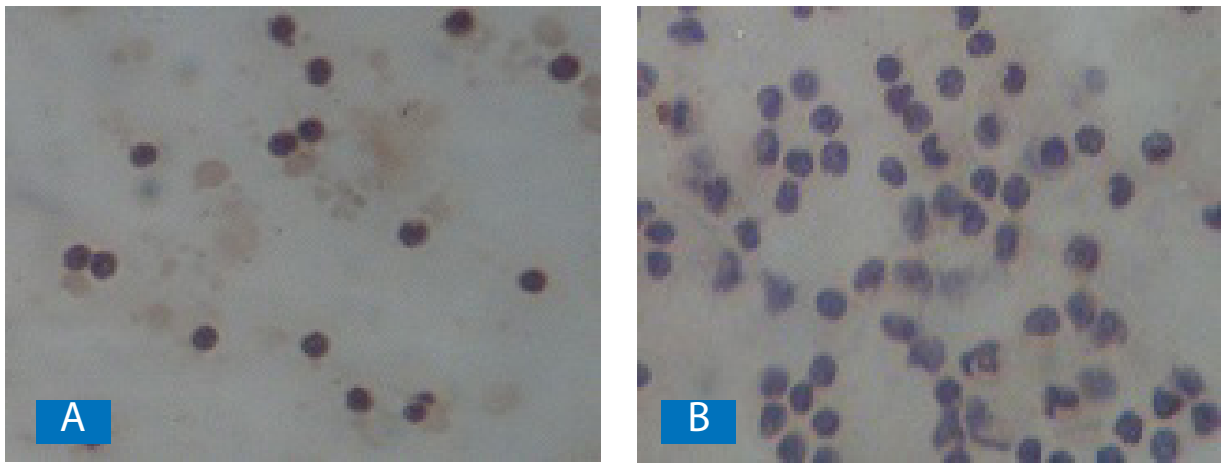

Figure (2): The expression of cell surface marker that showed on HSCs after isolation of cells (A): The deep brown color (DAB stain) represent the positive cells (X40). (B): The blue color (haematoxylin stain) represent the negative cells (X40).

In this study uncontrolled-rate freezing technique was used, this technique represents an attractive alternative to controlled-rate cryopreservation procedures which are time-consuming and require high-level technical expertise.

The main advantage of uncontrolled-rate freezing which translate in markedly labor and costs but the main disadvantage might be the lack of a record documenting the cooling rate (8).

The MNCs were cryopreserved in liquid nitrogen for a period of one month. HSCs are routinely cryopreserved in liquid nitrogen at -196˚C; at these low temperature almost all biologic functions are halted (17).

To improve cryopreservation process and decrease the concentration of DMSO to 2.5% and 5%, trehalose was used as a membrane stabilizer and ascorbic acid as an antioxidant. Figure (3) showed that although the use

of trehalose and ascorbic acid, a significant (P>0.05) reduction in the number of MNCs was noticed after freezing in both concentrations of 2.5% DMSO and 5% DMSO, but 5% DMSO with and without trehalose and ascorbic acid produced a significant (P>0.05) improvement in comparison with concentration 2.5% DMSO with and without trehalose and ascorbic acid and best result was with 5% DMSO plus trehalose

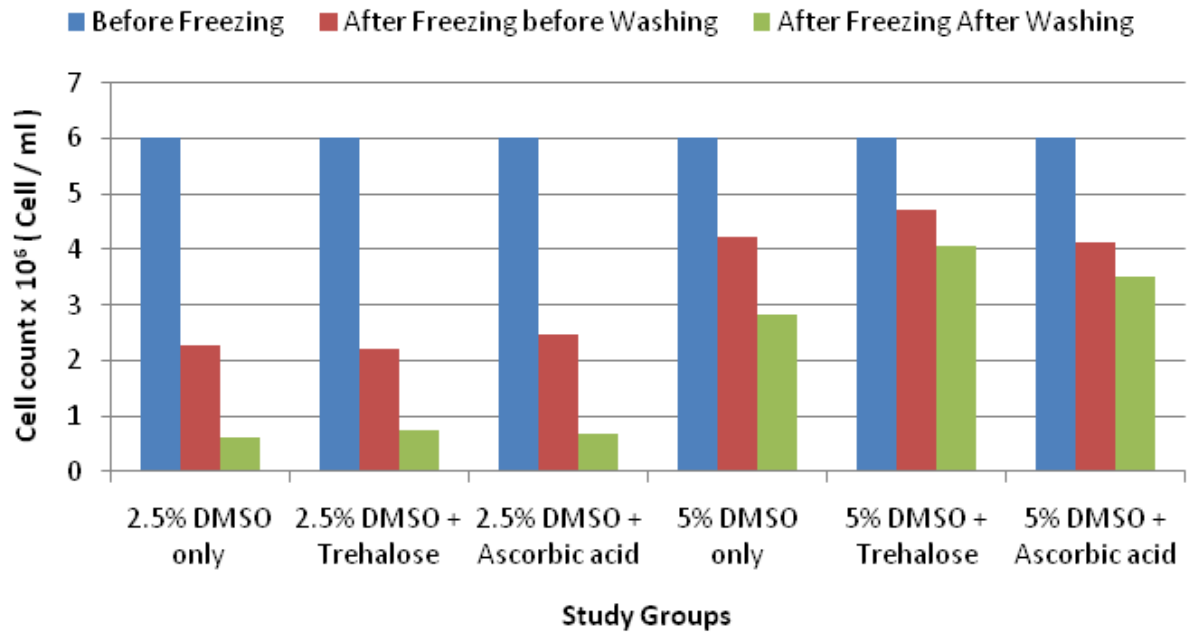

Figure (3): Effect of DMSO and additives on cell count (cell/ml).

Although extracelluler trehalose can reduce cryodamage to cells, the trehalose is usually required on both sides of the membrane for maximum protection efficient (18).

Buchanan et al. (19) demonstrated that 1-2M DMSO+0.2M trehalose with loaded it in cells can produced haematopoietic progenitors cells from freezing injury, it also improves the survival of cryopreserved other mammalian cells (Eroglu et al.,2000). Scheinkonig et al. (20) found that the total number of CFU was highest for cryopreservation solutions containing 0.5mol/l trehalose in 10%DMSO with HSCs. Zhang et al. (21) found similar result with umbilical cord blood. Limaye (1) found that the addition of membrane stabilizers as trehalose and antioxidant as ascorbic acid with 10%DMSO to the conventional freezing medium helps to post thaw recovery of HSCs.

When ascorbic acid was used with other antioxidants it gave good results. Limaye (22) showed that when 10%DMSO was used with 80µg/ml ascorbic acid alone or in combination with other antioxidants such as catalase and α-tocopheryl acetate it helped to preserve the functionality of BM cells.

Figure (3) showed that a significant (P<0.05) reduction of MNCs after washing of the cells in all concentration of DMSO with and without trehalose and ascorbic acid. Broxmeyer et al. (23) showed that any washing of the cells to remove DMSO leading to decrease the recovery of nucleated cells. The wash out of the most popular cryopreservants has conceivable benefits for recipient and reduction of toxicity. It was also suggested that wash out of DMSO can enhances engraftment (24).

Figure (4) also indicates that significant reduction (P<0.05) of percentage of CD34+cells after freezing at all concentrations of 2.5% DMSO and 5% DMSO with and without additives and concentration of 5% DMSO with and without additives significantly rather than concentration of 2.5% DMSO with and without additives and best results were 5% DMSO plus trehalose and 5% DMSO plus ascorbic acid.

Yang et al. (25) demonstrated that removing of DMSO by centrifugation leading to decrease in viability and recovery of CD34+ cells in both UCB and BM.

The results suggested that inspite of reduction of count and viability of MNCs, the percentage of CD34+ cells were not significantly affected (8).

Further work is necessary to investigate other concentrations for DMSO and the cryoprotectants trehalose and ascorbic acid for improvement of CD34+ cryopreservation.

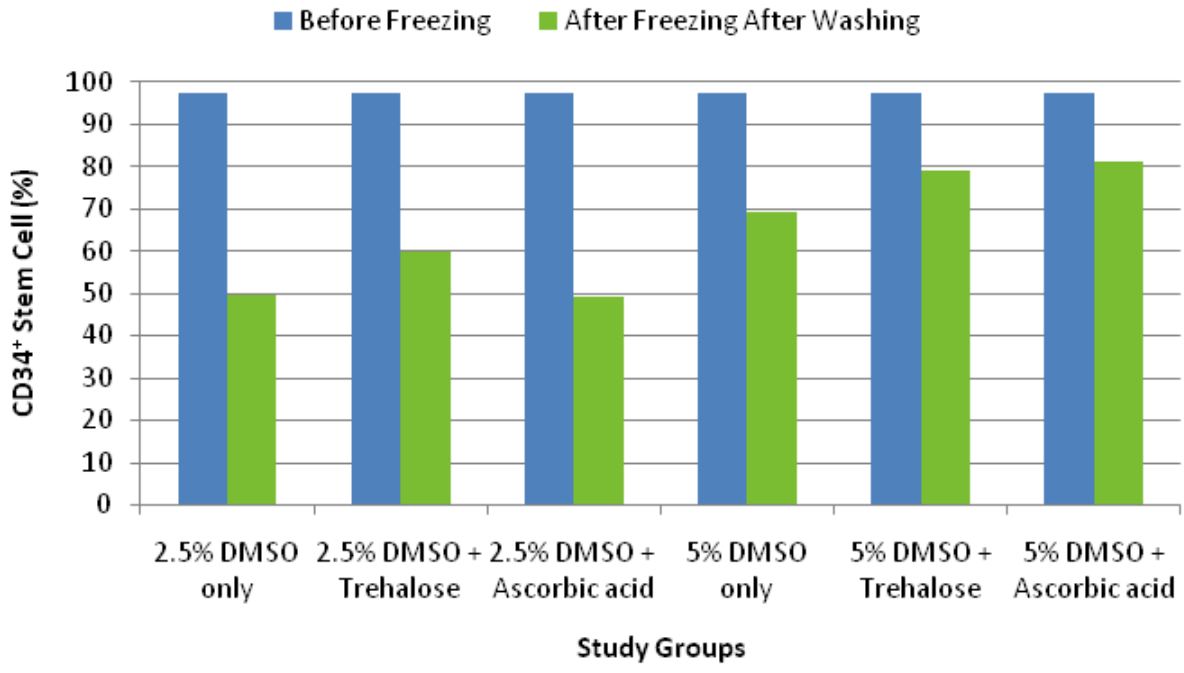

Figure (4): Effect of DMSO and additives on Percentage of CD34+ stem cells.

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التريهالوز وحامض الاسكوربيك يحسنان الخزن بالتجميد لخلايا دم الحبل السري الجذعية $CD34^+$ مع التراكيز القليلة لـ Dimethylsulfoxide

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الخلاصة:

الخزن بالتجميد لخلايا الحبل السري الجذعية خطوة اساسية في نقل الخلايا الجذعية. ال Dimethylsulfoxide (DMSO)عادة يستخدم كعامل حماية من التجميد يرافقه بعض السمية للخلايا الجذعية. لاجل تقليل تأثير ال DMSO يستخدم تراكيز اقل منه مع مواد تحمي من التجميد. في هذه الدراسة تم عزل الخلايا وحدة النواة والحاوية على الخلايا الجذعية باستخدام الفيكول لغرض التجميد. تم التجميد لخلايا وحيدة النواة والمعزولة من دم الحبل السري لمدة شهر باستخدام تقنية التجميد المباشر بدرجة -١٩٦ م° بالنايتروجين السائل. يتألف محلول الخزن بالتجميد من وسط MEM و ٢٠٪ من سيرم جنين الابقار بالاضفة الى تراكيز اقل من ١٠٪ من DMSO وهي ٢,٥٪ و ٥٪ مع و بدون ٢٥ مايكروغرام/مل من التريهالوز أو ٨٠مايكروغرام/ مل من حامض الاسكوربيك لتحسين عملية الخزن بالتجميد. ان اضافة التريهالوز وحامض الاسكوربيك حسن عملية التجميد بالقارنة بالسيطرة.

ان اضافة ٥٪ DMSO لوحده او مع الاضافات اظهرت نتائج افضل من تركيز ٢,٥٪ لوحده او مع الاضافات للخلايا الجذعية المخزونة بالتجميد كما اثبت بتقنية الكيمياء المناعية. غسل ال DMSO أثر على عدد وحيوية الخلايا الوحيدة النواة. هذه النتائج اكدت امكانية استخدام تراكيز اقل من DMSO في الخزن بالتجميد للخلايا الجذعية باضافة التريهالوز وحامض الاسكوربيك.