Cytogenetic behavior of cryoprotectant DMSO

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ABSTRACT: IVF (in vitro fertilization) is now used worldwide to overcome female or male infertility. Cryopreservation of human embryos provides the clearest opportunity to improve the clinical results obtained with IVF. Cryoprotective agents (CPA) are used to minimize freezing injuries. DMSO has been the most widely used CPA, however, high concentrations of CPAs in the vitrification solution have been shown to be detrimental to the cell. In order to determine the effect of DMSO solutions (5%, 10% and 20%) on genetic stability and/or subsequent DNA repair, we have investigated its ability to induce Sister Chromatid Exchanges (SCEs) and Proliferation Rate Index (PRI) in normal human lymphocyte cultures of peripheral blood, due to the fact that the study cannot be conducted on embryos and to the limited number of spare available embryos, the corresponding accessible experimental material was T lymphocyte. The blood samples were taken from three different healthy donors (conducting experimental procedure in triplicate). After the effect of DMSO solutions on blood according to the instructions of kit K-SIBV-500, lymphocytes are harvested and cultured with suitable technique to assess SCEs and PRI. The results show that all three DMSO concentrations cause a statistically dose depended significant increase of SCE frequency of the lymphocytes ($p<0.001$) and raise the need for more research regarding the safe and effective use of cryoprotectant methodologies for the vitrification of cells, gametes and embryos.

Key words: DMSO, Cytogenetic effect, Sister Chromatid Exchanges.

INTRODUCTION

Robert G. Edwards was awarded the Nobel Prize in Physiology/Medicine in 2010 because of his scientific work in reproductive medicine. He is the scientist who developed *in vitro* fertilization (IVF), the process by which an egg is fertilized by sperm in vitro and is now used worldwide to overcome female or male infertility.

Cryopreservation, a freezing technique, of human embryos provides the clearest opportunity to improve the clinical results obtained with IVF and sustain life for later use.[6]

Vitrification, an alternative form of cryopreservation, is defined as the solidification of a solution brought about not by crystallization but by extreme elevation in viscosity during cooling[18,19]. It is a clinical practice that allows preservation of fertility potential in women, involves quick cooling using high concentrations of cryoprotectants to minimize freezing injuries. It is an attractive ultrarapid cryopreservation technique.[4] The extremely high concentrations of cryoprotective agents (CPA) allow the solidification of a solution below the glass transition temperature, without ice crystal formation.[7,8]

Vitrification has been successfully applied to both cleavage and blastocyst stage embryos and clinical trials have shown high survival rates and promising implantation rates following transfer of thawed embryos at all stages.[8]

CPAs can be classified as penetrating and no penetrating agents. Penetrating agents protect the cell at slow freezing rates and are more likely to cause cell

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damage by osmotic imbalance and direct toxicity[7]. Non penetrating agents form a 'shield' around the cell, thereby reducing freezing injury by minimizing the effects of dehydration.

Dimethylsulfoxide (DMSO), a synthetic penetrating CPA is the best studied CPA.[11,20]

DMSO is an aprotic solvent. It has a very strong affinity for water and on exposure to air pure DMSO is rapidly diluted. Therapeutic and toxic agents that are not soluble in water are often soluble in DMSO.[2,11]

DMSO as a cryoprotective agent (CPA) is developed to substitute a portion of water inside and around cells, reducing the size of ice crystals and limiting overconcentration of salts during freezing. However, high concentrations of CPAs in the vitrification solution have been shown to be detrimental to the cell; they can cause cell injuries owing to toxicity.[15,23] Despite the protective effect of CPAs during freezing, they are toxic to live cells, tissues, and organs (bio-samples) and CPA toxicity increases with concentration and contact time in liquid state. CPAs bind to proteins and other molecules, disrupt multiple bio-chemical pathways inside the cells, and cause osmotic imbalance.[12,13,14]

In order to determine DMSO influence on genetic stability and/or subsequent DNA repair, we have investigated its ability to induce Sister Chromatid Exchanges (SCEs) and Proliferation Rate Index (PRI) in normal human lymphocyte cultures of peripheral blood. SCE frequency has been identified as one of the most sensitive indices among sensitive biomarkers of genotoxicity, such as chromosomal aberrations, comet assay and micronuclei. SCEs can provide insight in the cytogenetic damage induced by various genotoxic agents at very low concentrations. The determination of PRI in lymphocyte cultures has been proved to be a very valuable and sensitive indicator of the cytostatic action of various environmental hazards or therapeutic agents.[9,17]

MATERIALS AND METHODS

The vitrification kit K-SIBV-500 is designed for the vitrification of embryos and oocytes. In particular, embryos/oocytes are placed for specific time in 3 solutions that contain 5%, 10% and 20% DMSO respectively, and then put on a carrier device and plunged into liquid nitrogen. Due to the fact that the study cannot be conducted on embryos due to the limited number of spare available embryos, the corresponding accessible experimental material was T lymphocyte cultures from peripheral blood of healthy donors. After a series of pilot experiments in order to improve and adapt the process as much as possible close to the conditions of IVF embryo/oocyte vitrification and given the fact that there hasn't been a corresponding survey, peripheral blood was established as experimental material.

The blood samples were taken from three different healthy donors 18 to 28 years old, who were non-smokers, not receiving any drugs, not consuming considerable quantities of alcohol, or not having suffered any kind of infection for the last 15 days.

IN VITRO SCES AND PRI ASSAYS

1ml heparinized whole blood and 1ml buffer solution containing respectively 0%, 5%, 10%, and 20% DMSO were placed in four (4, experimental) falcon tubes. 1ml of heparinized whole blood without any other solution was placed in one falcon tube, which was used as control. Each of the experimental tubes was kept at room temperature for different period: for 0min, 5min, 2-4min and 40sec respectively (equivalent to the cryopreservation protocols in IVF).

Just after the end of the respective period, in each tube 5ml of RPMI (RPMI-1640, Biochrome, Berlin) solution was added in order to minimize the action of DMSO, centrifugation of the tubes followed for 5min at 1800 rpm and the supernatants were discarded and sediments consisted of lymphocytes which were treated by the cryoprotectants remained in the bottom of the tubes.

Human lymphocyte cultures were prepared by adding in each tube (containing the corresponding whole sediment) 5ml chromosome medium (RPMI-1640, Biochrome, Berlin, supplemented with 20% FCS, 0.63% L-glutamine, 0.63% Penicillin/Streptomycin and 2% Phytohaemagglutinin), and 0,05ml of 5-Bromodeoxyuridine (BrdU) water solution (400 μg/ml) at the beginning of culture life.

The cultures were incubated at 37°C for 72 hours in the dark, in order to minimize photolysis of BrdU. Colchicine (0.3μg/ml) was added 2h before the collection of the cultures. The cells were, then, collected by centrifugation and exposed to 0.075 M KCl for 10 minutes. The hypotonic solution spreads the chromosomes and hemolyses the red blood cells. The pellet was fixed three times with methanol:acetic acid (3:1). Drops of concentrated suspension of cells were placed on microslides that allowed to air dry. For SCEs and PRI analysis, the slides were stained by a modification of the Fluorescence Plus Giemsa procedure to obtain harlequin chromosomes.[16]

The same procedure was repeated for the other 2 donors.

STATISTICAL ANALYSIS

For SCEs, more than 30 suitably spread 2nd division cells from each culture were blindly scored. For PRI, at least 100 cells in the 1st, 2nd, 3rd and higher divisions from each culture were blindly scored. PRI is estimated according to: PRI=M1+2M2+3M3+/100, where M1, M2, and M3+, are the percent values of cells in the 1st, 2nd, 3rd and higher divisions respectively. For the statistical evaluation of the experimental data, Student's t-test was performed to determine whether any SCE values differed significantly from the controls and the X2 test was used for the cell kinetic comparisons (PRI).[9,10]

RESULTS

Table 1 and Figure 1 illustrate the cytotoxicity of DMSO, presented as dose-dependent increase of SCE frequency. The results show that all three DMSO concentrations cause a statistically significant increase of SCE frequency of the lymphocytes ($p<0.001$). This increase is proportional to the increase of the concentration of DMSO. Whereas no statistically significant difference of SCE frequency was found after comparing the effect of the same concentration of DMSO (10%) on two different times (4min and 2min) ($p>0.001$).

Table 2 and Figure 2 represent the cytostatic effect of DMSO on cultured human lymphocytes as PRI values. DMSO has caused a dose-dependent reduction of PRI values, but this reduction was not statistically significant ($p>0.001$).

Table I. Effect of DMSO solutions (5% 10% and 20%) on SCE frequency in human lymphocyte cultures from three different healthy donors.

	1st Donor SCEs±SE/cell	2nd Donor SCEs±SE/cell	3rd Donor SCEs±SE/cell
Control 1st (H_2O)	7.96±0.49	7.67±0.41	7.67±0.4
Control 2nd (buffer)	7.1±0.51	7.33±0.424	7.23±0.497
DMSO 5% for 5min	7.98±0.416	7.98±0.402	8.12±0.370*
DMSO 10% for 2min	11.5±0.574*	11.68±0.418*	10.77±0.444*
DMSO 10% for 4min	11.4±0.518*	10.37±0.375*	10.74±0.353*
DMSO 20% for 40sec	13.03±0.511*	13.14±0.462*	12.81±0.447*

*Statistically significant ($p<0.001$) increase over the corresponding control (t-test)
SE= standard error of the mean
A minimum of 20 cells was scored for SCEs from each culture.

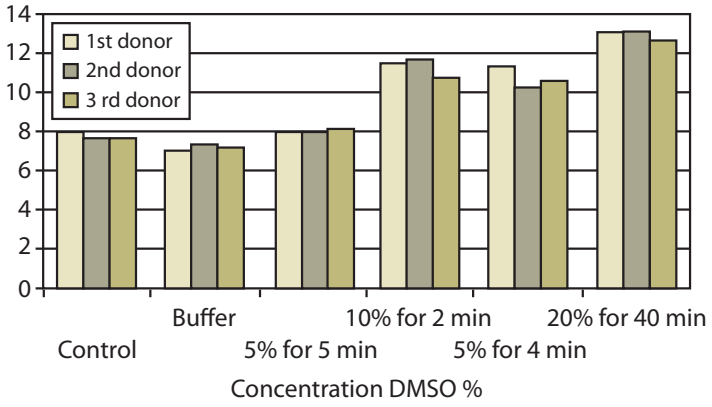

Figure 1. Effect of DMSO solutions (5% 10% and 20%) on SCE frequency in human lymphocyte cultures from three different healthy donors.

Table 2. Effect of DMSO solutions (5% 10% and 20%) on PRI values in human lymphocyte cultures from three different healthy donors.

	1st Donor	2nd Donor	3rd Donor
Control 1st (H_2O)	2.34	2.23	2.2
Control 2nd (buffer)	2.3	2.12	2.13
DMSO 5% for 5min	1.78	2.04	1.88
DMSO 10% for 2min	1.71	1.99	1.8
DMSO 10% for 4min	1.7	1.99	1.79
DMSO 20% for 40sec	1.67	1.83	1.67

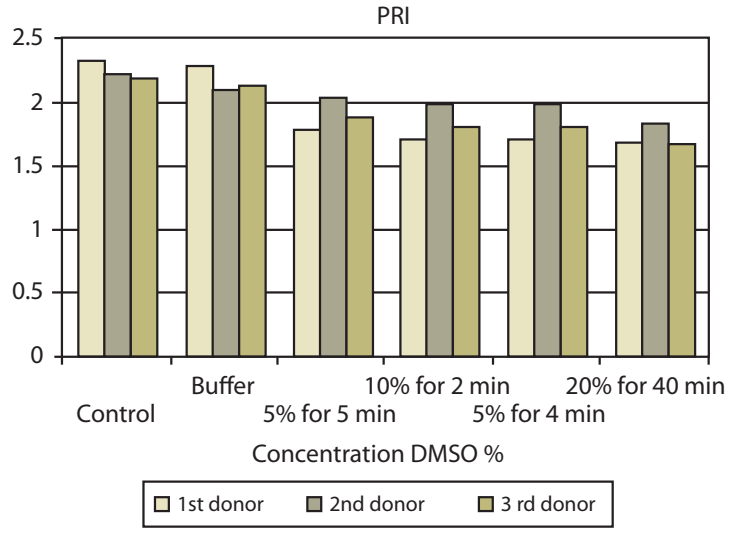

Figure 2. Effect of DMSO solutions (5% 10% and 20%) on PRI values in human lymphocyte cultures from three different healthy donors.

DISCUSSION

DMSO is a clear odorless and colorless organic liquid that freezes at 18.50° C. It is an inexpensive element produced as a by-product of the paper industry.[11] DMSO has been particularly promoted as an analgesic and anti-inflammatory agent, whilst it is a compound that has stimulated much controversy in the scientific literature.[1,2] Adverse reactions of DMSO are common, though they are usually minor and related to the concentration of DMSO in the medication solution.[3]

As IVF has become more widely available and the need for the cryopreservation of human embryos has become apparent, pressure for most effective cryoprotectants has increased.[6]

While physiologic and pharmacologic properties and effects of DMSO are incompletely understood, the research of Branch DR et al has concluded that DMSO has been the most widely used cryoprotective agent for human peripheral blood stem cells. It readily crosses cell membranes, prevents intracellular formation of ice crystals and disruption of cell membranes under freezing.[21]

Kartberg AJ et al 2008 have studied vitrification on early mouse and human embryos comparing two vitrification protocols with similar osmolarities, one containing DMSO and another without DMSO. They have concluded that vitrification with DMSO protects embryo membrane integrity better than solutions without DMSO, but morphological assessment of em-

bryos directly after vitrification was not a useful tool for assessing survival in that study.[5]

Recently Seet VY et al 2013 have investigated the detrimental effects of cryoprotectants on oocyte quality based on the morphological appearance of the oocyte. The results of their study indicate that observations based purely on the morphological appearance of the oocyte to assess the cryosurvival rate are insufficient and sometimes misleading.[4]

Chatzimeletiou et al., 2011 have provided the first cytoskeletal analysis of human vitrified blastocysts comparing the type and incidence of spindle abnormalities, to those observed in fresh blastocysts. The authors concluded that vitrification does not adversely affect embryo development and the ability of spindles to form and continue normal divisions. The majority of spindle/chromosome configurations analyzed were indeed normal, but the incidence of spindle abnormalities (including abnormal shape, chromosome lagging, bridging and multipolarity) were higher in the vitrified group compared to the fresh group. It is unclear whether the increase in spindle abnormalities following vitrification is due to toxicity or mechanical stress following exposure to the high concentrations of cryoprotectants or due to the submersion into liquid nitrogen.[8]

Chatzimeletiou et al., 2010 suggested that the exposure to the cryoprotectants causes the cells to shrink and gives rise to the abnormally shaped spindles which are observed in the vitrified blastocysts. If this abnormality in morphology is superficial and these abnormally shaped spindles complete mitosis normally it is likely that the derivative cells will be normal. However, if this abnormality in the shape of the spindle is conjugated with a function abnormality, it is possible that the spindle will either fail to progress further or if it progresses through mitosis the derivative cells may have an abnormal chromosomal constitution. This is certainly the case for the multipolar spindles that can lead to chaotic chromosomal divisions and the spindles that are associated with chromosome lagging, in which the derivative cells will be affected by chromosome loss, and consequently will become monosomic. Both of these cases may reflect mechanisms that can lead to chromosomal mosaicism in early human development.[22]

Whereas DMSO has received intense lay publicity sporadically over the last four decades studies have not been performed on its effect on DNA, indicating that cytogenetic activity of this effective cryoprotectant has been ignored so far, to the best of our knowledge.

So in order to investigate the cytogenetic behavior of DMSO in the concentrations that are used during vitrification of embryos, we have conducted the present research. Lymphocytes from peripheral blood of three different young healthy donors have been treated with DMSO solutions contained in the embryo vitrification kit K-SIBV-500 according to the manufacturer's instructions. Then these lymphocytes were cultured in the presence of Brdu and processed for Sister Chromatid Exchanges (SCEs) and Proliferation Rate Index (PRI).

All tested DMSO concentrations, 5%, 10% and 20%, have induced the SCE frequency of cultured lymphocytes and the induction has been presented as dose-dependent increase of SCEs, statistically significant ($p<0.001$). The cytogenetic effect of 10% DMSO solution on lymphocytes has been tested for two different times (4min and 2min) of treatment. The results have revealed that the effect of DMSO for an additional 2 minutes treatment does not alter the cytogenetic behavior of the cryoprotectant. The statistically significant increase of SCE frequency of human lymphocytes which has been induced by DMSO solutions suggests that at least these specific concentrations of the cryoprotectant exert cytotoxic activity. In other words, the effect of DMSO on lymphocytes appear to cause instability or/and damages to their DNA molecule that the repair mechanisms of the cell are not able to repair.

The results of the study also suggest that the 5%, 10% and 20% DMSO solutions after 5min, 2min and 40 exposure on lymphocytes respectively have caused a dose-dependent reduction of PRI values, but this reduction was not statistically significant ($p>0.001$). Diminution of PRI values means decrease of Proliferation Rate of cultured lymphocytes. So the specific DMSO solutions could be characterized as mild cytostatic.

This is the first study to investigate the cytotoxicity of the DMSO concentrations used in IVF vitrification protocols, by mimicking the exposure times and concentrations in blood lymphocytes. Our results show an increase SCEs and raise the need for more research regarding the safe and effective use of cryoprotectant methodologies for the vitrification of cells, gametes and embryos.

Κυτταρογενετική επίδραση διαλυμάτων του κρυοπροστατευτικού DMSO

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ΠΕΡΙΛΗΨΗ: Στην μείωση της υπογονιμότητας συμβάλλει σημαντικά η εξωσωματική γονιμοποίηση, κατά την διαδικασία της οποίας για την συντήρηση και εμβρυομεταφορά απαιτούνται διάφορα κρυοπροστατευτικά διαλύματα. Στην πλειοψηφία των διαλυμάτων αυτών δραστική ουσία αποτελεί το DMSO (διμεθυλοσουλφοξείδιο) σε συγκεντρώσεις 5%, 10% και 20%, η κυτταρογενετική δράση των οποίων δεν έχει μελετηθεί. Στόχος της παρούσας έρευνας είναι η διερεύνηση των συγκεκριμένων συγκεντρώσεων DMSO στο γενετικό υλικό καλλιεργημένων λεμφοκυττάρων περιφερικού αίματος φυσιολογικών ατόμων, επειδή η μελέτη δεν είναι δυνατόν να διεξαχθεί σε ανθρώπινα έμβρυα.

Για τις καλλιέργειες των λεμφοκυττάρων χρησιμοποιήθηκε ηπαρινισμένο αίμα από 3 φυσιολογικά νεαρά άτομα (διεξαγωγή πειραματικής διαδικασίας εις τριπλούν). Μετά την επίδραση των διαλυμάτων DMSO (5%, 10% και 20%) στο ολικό αίμα, σύμφωνα με τις οδηγίες του kit K-SIBV-500, συλλέγονται τα λεμφοκύτταρα και καλλιεργούνται με την κατάλληλη τεχνική ώστε να εκτιμηθούν οι SCEs (συχνότητα χρωματιδιακών ανταλλαγών), από τους πιο ευαίσθητους δείκτες κυτταροτοξικότητας και ο PRI (δείκτης ρυθμού πολλαπλασιασμού κυττάρων), αξιόπιστος δείκτης κυτταροστατικότητας.

Τα αποτελέσματα που προέκυψαν δείχνουν στατιστικά σημαντική αύξηση των SCEs ($p<0.001$) των λεμφοκυττάρων αντίστοιχη με την αύξηση της συγκέντρωσης του DMSO, εύρημα που ενοχοποιεί τις συγκεκριμένες συγκεντρώσεις για κυτταρογενετική δράση, γεγονός που πρέπει να ληφθεί σοβαρά υπ' όψιν στην προσπάθεια ελαχιστοποίησης των βλαπτικών συνθηκών κατά την εξωσωματική γονιμοποίηση.

Λέξεις κλειδιά: DMSO, κυτταρογενετικές επιδράσεις, χρωματιδιακές ανταλλαγές

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