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Efficiencies of the Cryoprotectants N-Methylacetamide, N-Methylformamide and Dimethyl Sulfoxide, and the Cell Protectants Trehalose and Hydroxyethyl Starch in Cryopreservation of Swine Sperm

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Abstract

Polar molecules protect against freeze-induced damage by interfering with the polarised water arrangement during ice nucleation inside cells. Insufficient protection from freeze-induced damage (acting during freezing) and cryopreservative-induced cytotoxicity (acting before and after freezing) causes cell death due to cryopreservation. Although dimethyl sulfoxide (DMSO) is a useful cryopreservative, the oxidation and lone pair electrons of the sulfinyl group cause cytotoxicity. The dipole moment of the sulfinyl group in DMSO and those of the amide groups in N-methylacetamide(NMA) and N-methylformamide (NMF) are similar. Furthermore, NMA and NMF retain the methyl group present in DMSO, which confers permeation ability to DMSO. Thus, using NMA and NMF rather than DMSO may attenuate cell death due to cryopreservation, both by ensuring cryopreservation action consisting (molecular polarity and cell permeation) and by reducing cytotoxicity both before freezing and after thawing. In this study, we examined the efficiencies of different cryoprotectants in swine sperm. Swine sperm were cryopreserved in six combined solutions of three cryopreserves, i.e. NMF (7.5% [w/v], 1286 mM), NMA (7.5% [w/v], 964mM), and DMSO (7.5% [w/v], 1056mM), and two cell protectants, i.e. trehalose (5.8% [w/v]) and hydroxyethyl starch (HES; 6% [w/v]). Addition of NMF and NMA resulted in similar increases in cell activity compared with that in DMSO-treated cells before freezing and at 5 and 30 min after thawing. The ratio of cell activity resulting from the addition of trehalose to that of HES was about 1.5 before freezing but ranged from 2 to 5 at 5 and 30 min after thawing. These results showed that NMF and NMA may be more effective cryopreservatives than DMSO and that trehalose may be a better cell protectant than HES.

Keywords: Freeze-induced damage; Cryopreservative-induced cytotoxicity; Cell permeation; Cell protectant; Hydroxyethyl starch; Trehalose

Introduction

Cryopreservation is performed by adding a cryoprotectant (cryopreservative) to living cells before freezing; this is necessary because hard ice crystals crush living cells, and frost damage (freeze-induced damage) is generated when living cells are frozen [1,2]. Polar molecules, such as dimethyl sulfoxide (DMSO; H_3C-S(O)-CH_3; Figure 1), are considered effective cryopreservatives owing to their ability to permeate into the cells and reduce freeze-induced damage by distorting the polarised water arrangement in ice nucleation inside the cells. Because the molar concentration of the cryopreservative is several times higher than that of saline (0.9% [w/v] NaCl) [3], regardless of toxic chemical reactivity, cryopreservatives can cause cryopreservative-induced cytotoxicity in cells. Thus, cell protectants, which are hydratable molecules that promote water retention, such as albumin, hydroxyethyl starch (HES), and trehalose, are required to protect cells by maintaining colloid osmotic pressure [4-7].

Two factors influence cell death due to cryopreservation, namely protection from freeze-induced damage due to the formation of ice crystals inside the cells during freezing, and cryopreservative-induced cytotoxicity acting before freezing and after thawing at room temperature. Thus, cryopreservation solution is composed of the cryopreservative and cell protectant. The cryopreservation solution CP-1 (Kyokuto, Tokyo, Japan), which has been used for cryopreservation of umbilical cord blood, contains water, 0.9% (w/v) NaCl, the cryopreservative DMSO, and the cell protectants human serum albumin (HSA) and HES [8].

DMSO, which consists of one sulfinyl group [-S(O)-] bound to two methyl groups [-CH_3], is a useful cryopreservative for living cells. The higher electronegativity of sulfur, which is related to the higher polarity of DMSO, is useful in the sense that DMSO acts as a scavenger of radicals generated by inflammation and radiation [9,10]. Additionally, oxidation of luciferin for bioluminescence assays is carried out with DMSO [11]. The electron configurations of carbon $_6$C, nitrogen $_7$N, oxygen $_8$O, and sulfur $_{16}$S are $1s^2 2s^2 2p^2 3s^0 3p^0$, $1s^2 2s^2 2p^3 3s^0 3p^0$, $1s^2 2s^2 2p^4 3s^0 3p^0$, and $1s^2 2s^2 2p^6 3s^2 3p^4$, respectively, where s and p indicate s- and p-orbitals, respectively [12]. Although the d-orbitals of $_6$C, $_7$N, $_8$O, and $_{16}$S are unoccupied in their ground states, the (unoccupied) d-orbital effect of $_{16}$S is much stronger than those of $_6$C, $_7$N, and $_8$O because the four electrons occupy the 3p-orbital of $_{16}$S, for which the energy level is closer to that of the 3d-orbital. Thus, the sulfur atom can adopt various valence states and flexible bonding angles owing to the existence of unoccupied d-orbitals (d-orbital effect), similar to the behaviours of transition metals. DMSO is metabolised to dimethyl sulfide (DMS; H_3C-S-CH_3; Figure 1) with release of reactive oxygen species (oxidation). Two carbons and one sulfur in DMSO form a plane, and the oxygen in DMSO is not included in the plane due to the d-orbital effect (Figure 1) [13,14]. The oxygen atom protruding from the plane exposes the lone pair of electrons, which is related to various chemical reactions due to flexible bonding angles of sulphur [15]. Electronegativity, oxidation reactivity, and the lone pair electrons of the sulfinyl group in DMSO often lead to cytotoxicity [9,10,16,17].

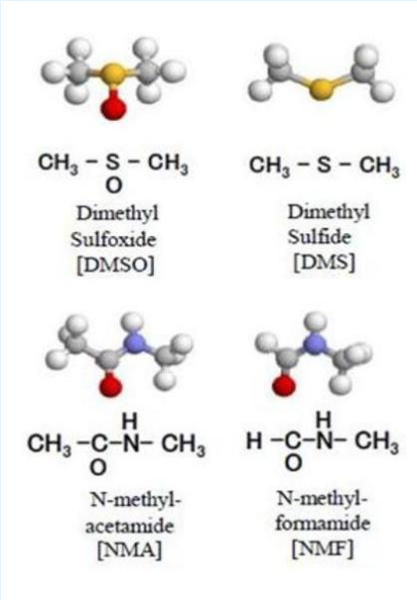

Figure 1: Chemical structures of (A) DMSO, (B) DMS, (C) NMA, and (D) NMF. Models were created using RasMol. The lone pair electrons in the sulfinyl group [-S(O)-] in DMSO, as well as the release of oxygen (oxidation) in the transition from DMSO to DMS, may lead to cytotoxicity.

N-methylacetamide (NMA; H_3C-C(O)-N(H)-CH_3; Figure 1) is yielded by replacement of the sulfinyl group [-S(O)-] in DMSO with an amide group [-C(O)-N(H)-]. Because $_6$C, $_7$N, and $_8$O in the amide group have no d-orbital effects [12] and the amide group is naturally involved in bioprocesses [18,19], the cytotoxicity of NMA is lower than that of DMSO in cryopreserved human cells [3]. Replacement of the methyl group [CH_3] in NMA with a

hydrogen [H] yields N-methylformamide (NMF; [H-C(O)-N(H)-CH_3]; Figure 1). The addition of organic polar molecules, such as DMSO, NMA, or NMF, to the culture medium for murine erythroleukaemia cells successfully induces erythropoietic differentiation [20,21]. Because the high permeation ability of DMSO is associated with its methyl groups, which are also present in NMA and NMF, NMA and NMF may also be able to pass through cell membranes and prevent the formation of ice crystals within the cell. However, DMSO and glycerol cannot easily pass through sperm membranes. The dipole moments of the sulfinyl group in DMSO and amide groups in NMA and NMF are similar [22,23]. Therefore, NMA and NMF have higher permeation ability for the membrane of sperm cells than DMSO or glycerol, and dipole moments similar to that of DMSO are expected to yield better cryopreservation activity for sperm.

NMA is used in the cryopreservation of bovine spermatozoa [24] and fowl semen [25,26].Although the cryopreservation activity of NMA is higher than that of NMF for human HL-60 and RPMI8226 cells [3], NMF has been shown to be an effective cryopreservative for Asian elephant [27], Mangalarga Marchador [28], and Colossoma [29] semen. The higher permeability of amides, such as NMF, in sperm can be explained by the lower molecular-weight and lower viscosity compared with glycerol [27-29]. Thus, enhanced permeation ability and lower cytotoxicity are required for effective cryopreservation. Trehalose has been reported to have advantages as a cell protectant in cryopreservation solution [5-7].

Accordingly, in this study, we aimed to compare the efficiencies of NMA, NMF, and DMSO as cryopreservatives and HES and trehalose as cell protectants in the cryopreservation of swine sperm.

Methods and Materials

Collected umbilical cord blood (100 mL) was mixed with CP-1 (100 mL; Kyokuto, Tokyo, Japan) [8]. The final concentrations of the cryopreservation agents in an equivolume mixture of blood and CP-1 (200 mL) were 0.9% (w/v) NaCl, 5% (w/v) DMSO, 4% (w/v) HSA, and 6% (w/v, molecular weight [MW]: 66000 Da) HES. Umbilical cord blood was cryopre served in CP-1 at -80°C. The final concentration of HSA was adjusted to 4% (w/v) in the blood. When starch is decomposed with amylase, HES of various MWs is produced. The MW of HES was fixed to that of HSA (66000 Da) in CP-1.

Bovine serum albumin (BSA; Sigma-Aldrich, USA), HES (Sigma-Aldrich), and trehalose (Tokyo-Kasei, Japan) were used as cell protectants in this study. Aqueous solutions of trehalose and HES, which are the second diluents, were stored in a water bath maintained at 15°C; the components are shown in Tables 1 and 2, respectively. Both solutions contained 3% (w/v) BSA. Powdered HES was used to produce the HES solution, and the MW of HES was not fixed. All aqueous solutions were made with pure water (18.2 MΩ·cm at 25°C). The first diluent (Modena diluent) was stored at 15°C; the components are shown in Table 3 [30].

Trehalose・$2H_2O$	115.0 g/L
Bovine serum albumin	3.0g/L
Gentamicin sulfate	0.1% (v/v)

Table 1: Components of the trehalose aqueous solution (second dilution).

Hydroxyl ethyl starch	120.0g/L
Bovine serum albumin	3.0g/L
Gentamicin sulphate	0.1% (v/v)

Table 2: Components of the hydroxyl ethyl starch (HES) aqueous solution (second dilution).

Glucose	27.5g/L
Sodium citrate	6.9 g/L
Sodium hydrogen carbonate	1.0 g/L
Disodium EDTA	2.35 g/L
Citric acid (monohydrate)	2.9 g/L
THAM	5.65 g/L
Polyvinyl alcohol	0.6 g/L
Gentamicin sulfate	0.1 % (v/v)

Table 3: Components of the first dilution (Modena).
EDTA = ethylene diamine tetra acetic acid
THAM = tris (hydroxymethyl) amino methane

Three cryopreservatives, i.e. NMA (MW: 73.1 Da; Tokyo-Kasei), NMF (MW: 59.1 Da; Tokyo-Kasei), and DMSO (MW: 78.1Da; Wako, Japan), were prepared. Because the melting points of NMA, NMF, and DMSO are 28°C, -4°C, and 19°C, respectively, bottles of NMA and DMSO were stored at 30°C and 20°C, respectively, for 3 h. The process for preparing the six solutions including cryopreservatives and cell protectants involved the following three steps. 1) Six 100-mL beakers were

marked from A to F. 2) Three trehalose solutions of 85 mL were injected into beakers A-C, and three HES solutions of 85 mL were injected into beakers D-F. 3) NMA (14.1 g), NMF (15.2 g), or DMSO (16.5 g)was injected with a 15 mL volume into beakers A and D, B and E, and C and F, respectively.

Sperm from a swine (male, 19 months of age, weighing 220-250 kg) was collected. The activity of semen just after collection was 70∼75++, as shown in Table 4 [31]. The collected sperm cells were injected into the first diluent at 15°C. The activity of semen just after injection into the first diluent was 65++ (Table 4). Six 10-mLcentrifuge tubes were marked A-F, and six diluted semen samples (10 mL) were injected into the six tubes. The six centrifuge tubes were stirred and then centrifuged at 250 × *g* for 10 min at 15°C, allowing the semen to precipitate at the bottom. The supernatants were discarded by decantation, and the centrifuge tubes were filled with 10 mL of fresh first diluent and stirred to resuspend the semen. The centrifuge tubes were then centrifuged at 250 × *g* for 10 min at 15°C. The supernatant was discarded by decantation, and the centrifuge tubes were filled with 1 mL fresh first diluent and stirred to resuspend the semen.

The following two processes were performed in a room at 5°C, and the liquid was maintained in a water bath at 15°C. 1) The six centrifuge tubes (A-F) were placed in the water bath for 2 h.2) Six 1-mL solutions in beakers A-F were injected into the centrifuge tubes and stirred gently. The final concentrations of cryopreservatives and cell protectants were defined at this stage before freezing.

The final concentrations of NMA in tubes A and D, NMF in tubes B and E, and DMSO in tubes C and F were 7.1%(w/v), 7.6% (w/v), and 8.3%(w/v), respectively, and those of trehalose in tubes A-C, HES in tubes D-F, and BSA in tubes A-F were 5.8%(w/v), 6.0% (w/v), and 0.15%(w/v), respectively, as shown in Table 4. The concentration of HES was adjusted to that of CP-1 for umbilical cord blood. Because the final molar concentrations of NMA, NMF, and DMSO were 964, 1286, and 1056 mM, respectively, and were more than six times of higher than that of saline (154 mM), the cell protectants of trehalose, HES, and BSA were used protect swine sperm from cryopreservative-induced cytotoxicity. The activities of swine sperm in the six centrifuge tubes were measured before freezing (Table 4).

Just after collection						
1. Sperm activity	70∼75++					
In the first diluent (Modena)						
2. Sperm activity	65++					
	□ Concentrations in six second diluents just before freezing					
Before freezing	A	B	C	D	E	F
Cell protectant in % [w/v]	THL (5.8 %)	THL (5.8 %)	THL (5.8 %)	HES (6.0 %)	HES (6.0 %)	HES (6.0 %)
Cryopreservative in % [w/v]	NMA (7.1 %)	NMF (7.6 %)	DMSO (8.3 %)	NMA (7.1 %)	NMF (7.6 %)	DMSO (8.3 %)
Before freezing	A	B	C	D	E	F
3. Sperm activity	60+	60+	50∼60+	40+	40+	30∼40+
◇ Concentrations in six mixtures of the first and second diluents after thawing						
After thawing	A	B	C	D	E	F
Cell protectant in % [w/v]	THL (1.9 %)	THL (1.9 %)	THL (1.9 %)	HES (2.0 %)	HES (2.0 %)	HES (2.0 %)
Cryopreservative in % [w/v]	NMA (2.4 %)	NMF (2.5 %)	DMSO (2.8 %)	NMA (2.4 %)	NMF (2.5 %)	DMSO (2.8 %)
After thawing	A	B	C	D	E	F
4. Sperm activity at 5 min	30∼40+	30∼40+	20∼30+	10∼20+	10∼20+	5+
5. Sperm activity at 30 min	30∼40+	30∼40+	20+	10+	10+	5+

Table 4: Change in the activities of swine sperm before freezing and after thawing.
Trehalose is abbreviated as THL.

The process for preparing the six frozen straws involved the following five steps (performed at 5°C). 1)Six 0.5-mL straws were filled with the six second diluents of A-F. Approximately 5 × 10^8 cells were in each straw. 2) A plastic cradle for sustaining straws was placed in an adiabatic cube made of Styrofoam, the bottom of which was filled with an appropriate volume of liquid nitrogen at -196°C. 3) The heights of the six straws over the surface of the liquid nitrogen were set to 35-40 mm. 4) The box was then covered and left undisturbed for 30 min. 5) The box was stored in a liquid nitrogen storage container. The samples were maintained in the frozen state in liquid nitrogen for 1 week.

The process for thawing the frozen straws involved the following five steps (performed at 25°C). 1) The six first diluents (1 mL) were injected into the six test tubes (A-F) and maintained in a water bath at 37°C. 2) The adiabatic Styrofoam cube box was removed from the liquid nitrogen storage container. 3) The six frozen straws were removed from the adiabatic cube and maintained in the room for 10 s. 4) The six straws were then immersed in a water bath at 37°C for 10 s. 5) The six sperm samples in the second diluents were injected into the six test tubes filled with the first diluents at 37°C. This time is defined as 0 min after thawing. The mixture volumes of the straw and first diluent were 0.5 and 1.0 mL, respectively. Thus, after thawing, the concentrations of NMA in tubes A and D, NMF in tubes B and E, and DMSO in tubes C and F were 2.4%(w/v), 2.5% (w/v), and 2.8%(w/v), respectively, and those of trehalose in tubes A-C, HES in tubes D-F, and BSA in tubes A-F were 1.9%(w/v), 2.0% (w/v), and 0.05%(w/v), respectively, as shown in Table 4. The activities of the sperm in the test tubes were measured at 5 and 30 min after thawing.

Results

As shown in Table 4, the sperm activity was the highest just after collection and decreased after injection into the first diluent. The sperm were placed in the first diluent for 20 min (centrifuged twice for 10 min each), and the activities in the three trehalose solutions (A-C) were then distinctly higher than those in the three HES solution (D-F) just after mixing of the first and second diluents before freezing. Although the activities in the second diluent containing DMSO (C and F) were lower than those in the second diluent containing NMA (A and D) and NMF(B and E) in both trehalose (A-C) and HES(D-F) solutions, the differences in sperm activities were found to be mainly caused by differences in the cell protectant (e.g. trehalose or HES).

A comparison of the sperm activities in tubes A-F provided results that were consistent with the previous finding that the cytotoxicity of DMSO was higher than that of amides, such as NMA and NMF, because the cytotoxic sulfinyl group in DMSO was replaced with the amide group in amides, and the amides were replaced with methyl groups to maintain cell permeation, similar to the process observed for DMSO. However, the choice of the appropriate cell protectant was considered the most essential factor when the molar concentration of the cryopreservative (NMA, NMF, or DMSO) was more than six times higher than that of saline [3,4]. The cytotoxicity of DMSO was higher than those of NMA and NMF, and the efficiency of the cell protectant of trehalose was higher than that of HES from comparison of sperm activities just before freezing (Table 4).

The molar concentration of the cryopreservative was approximately double that of saline after thawing (Table 4). The activities in the three trehalose solutions (A-C) were higher than those in the three HES solutions (D-F) after thawing. Because the sperm activities were lower in HES solutions(D-F) than in trehalose solutions (A-C) before freezing, differences in freeze-induced damage between trehalose and HES solutions could not be determined from the finding that the activities in the trehalose solutions (A-C) were higher than those in the HES solutions (D-F). However, the activities did not decrease from 5 to 30 min after thawing in trehalose solutions (A,B) containing NMA or NMF, but did decrease from 5 to 30 min after thawing in HES solutions (D,E) containing NMA or NMF. Thus, the efficiency of trehalose as the cell protectant was greater than that of HES, even when the molar concentration of the cryopreservative after thawing was decreased to one-third of that before freezing. The sperm activity in the HES solution containing DMSO(F) decreased to the lower observable limit of 5+ at 5 and 30 min after thawing (Table 4), indicating that the efficiency of trehalose and HES solutions containing DMSO could not be compared, even when the molar concentration of DMSO after thawing decreased to one-third of those before freezing.

Discussion

The activity of sperm is a useful index for evaluating cryopreservation activity, combined with cell viability and cell death analyses. Cryopreservation solutions should be

composed of a cryopreservative and cell protectant because the cryopreservation activity is determined by protection against freeze-induced damage and cryopreservative-induced cytotoxicity. In this study, freeze-induced damage and cryopreservative-induced cytotoxicity were evaluated based on the activity of swine sperm before and after cryopreservation. It is difficult to distinguish freeze-induced damage and cryopreservative-induced cytotoxicity because sperm activity changes just after injection into the cryopreservation solution before freezing. However, adoption of trehalose solution as a cell protectant was more efficient than that of HES solution in the cryopreservation of swine sperm.

All cryopreservatives cause cryopreservative-induced cytotoxicity in cells, regardless of the presence of toxic atomic groups, such as the sulfinyl group in DMSO, because the molar concentration of the cryopreservative in the cryopreservation solution is several times higher than that of saline. Thus, the lowest concentration of any cryopreservative required to yield sufficient cryopreservative activity should be determined for all cell types [3]. In this study, we examined the effects of the cryopreservatives NMA, NMF, and DMSO at only two ratios (six and two times higher than that of saline); therefore, further studies are needed to determine the efficiencies of the cell protectants trehalose and HES in the cryopreservation of swine sperm in the presence of various concentrations of cryopreservatives. Freeze-induced damage and cryopreservative-induced cytotoxicity for any cell type will be clearly discriminated under the best conditions with the appropriate choice and concentration of cell protectant. Under optimal cell protectant conditions, the lowest concentration of each cryopreservative will be used, and this value may differ for each cell type. The use of the optimal conditions will lead to the fewest side effects, even if complete removal of the cryopreservative is not achieved after thawing before transplantation of cells into the body, e.g.in the clinical setting.

HES, which can have a wide range of MWs, is created during the decomposition of starch. In order to avoid kidney and liver damage during metabolism of HES in the body, the appropriate MW of HES is determined and extracted among the decomposed starches; this HES is then used as a cell protectant in the cryopreservation solution. Molecules of HES with MWs similar to that of albumin are chosen and used in the cryopreservation solution of umbilical cord blood [8]. Notably, in this study, the MW of HES was not fixed. Although the cell protectant effects of HES are thought to be related to its hydratability and not to the MW, the appropriate MW of HES as a cell protectant in cryopreservation solution should be determined with regard to abnormal metabolism in organs, such as the kidneys and liver, in future studies.

BSA was used as a cell protectant in both the trehalose and HES solutions in this study, similar to has, which is used in the cryopreservation of umbilical cord blood. Considering the risk of viral infection through albumin, cell protectants without albumin are necessary. Therefore, methods for cryopreservation without albumin as a cell protectant should be developed.

Conclusion

In this study, we found that NMF and NMA were better cryopreservatives than DMSO, and trehalose was a more effective cell protectant than HES in the cryopreservation of swine sperm. All cryopreservatives cause cryopreservative-induced cytotoxicity in cells, regardless of the presence of toxic atomic groups, because the molar concentration of the cryopreservative is several times higher than that of saline. Thus, the choice of an appropriate cell protectant is the most important factor in the cryopreservation of swine sperm.

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