Original Article

Cryobiological Characteristics of L-proline in Mammalian Oocyte Cryopreservation

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Abstract

Background: L-proline is a natural, nontoxic cryoprotectant that helps cells and tissues to tolerate freezing in a variety of plants and animals. The use of L-proline in mammalian oocyte cryopreservation is rare. In this study, we explored the cryobiological characteristics of L-proline and evaluated its protective effect in mouse oocyte cryopreservation.

Methods: The freezing property of L-proline was detected by Raman spectroscopy and osmometer. Mature oocytes obtained from 8-week-old B6D2F1 mice were vitrified in a solution consisting various concentration of L-proline with a reduced proportion of dimethyl sulfoxide (DMSO) and ethylene glycol (EG), comparing with the control group (15% DMSO and 15% EG without L-proline). The survival rate, 5-methylcytosine (5-mC) expression, fertilization rate, two-cell rate, and blastocyst rate *in vitro* were assessed by immunofluorescence and *in vitro* fertilization. Data were analyzed by Chi-square test.

Results: L-proline can penetrate the oocyte membrane within 1 min. The osmotic pressure of 2.00 mol/L L-proline mixture is similar to that of the control group. The survival rate of the postthawed oocyte in 2.00 mol/L L-proline combining 7.5% DMSO and 10% EG is significantly higher than that of the control group. There is no difference of 5-mC expression between the L-proline combination groups and control. The fertilization rate, two-cell rate, and blastocyst rate *in vitro* from oocyte vitrified in 2.00 mol/L L-proline combining 7.5% DMSO and 10% EG solution are similar to that of control.

Conclusions: It indicated that an appropriate concentration of L-proline can improve the cryopreservation efficiency of mouse oocytes with low concentrations of DMSO and EG, which may be applicable to human oocyte vitrification.

Key words: Cryoprotective Agentant; Mouse Oocyte; Vitrification

Introduction

L-proline is an important amino acid in terms of its protective effect against freezing injury. A role for L-proline in freeze tolerance has been demonstrated in many species including plants, yeast, and fly larva.[1-4] Cryopreservation of living cells is now indispensable. Because of its protective role against freezing stress *in vivo*, L-proline is used for cryopreservation in biological, medical, and agricultural research fields. For example, 10% (weight/volume) L-proline has been used to cryopreserve maize cells.[5] In addition, 27.00 mmol/L L-proline has been used to preserve ram sperm.[6] Furthermore, human stem cells are successfully frozen using a low level of L-proline (1%) in combination with other solutes.[7] To our knowledge, L-proline has not been used as a cryoprotectant for mammalian oocytes.

Oocyte cryopreservation is the only option for the preservation of reproductive potential in single women who lose their fertility. Although oocytes cryopreserved by slow

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freezing were first used to give rise to offspring, vitrification is widely using in oocyte cryopreservation because it is a quick, easy, and effective freezing technique. Cryoprotective agents (CPAs) such as dimethyl sulfoxide (DMSO) and ethylene glycol (EG) are beneficial for the cryopreservation of oocytes because they protect them against damage caused by the intracellular formation of ice during freezing and thawing. However, there are problems associated with currently used CPAs. Although DMSO is the most effective CPA, it is highly cytotoxic and affects the differentiation of somatic cells.[8] A study by Liang *et al*.[9] reported that a reduction in global genomic methylation due to vitrification of metaphase II (MII) oocytes may compromise the *in vitro* developmental potential of early mouse embryos. Furthermore, residual DMSO inside oocytes needs to be eliminated after thawing because intracellular DMSO cannot be metabolized. Thus, it is necessary to develop other CPAs that can be used to cryopreserve oocytes with a high efficiency and low toxicity. L-proline is a membrane-permeable nontoxic cryoprotectant, and it may therefore be an alternative CPA instead of DMSO and EG for oocyte vitrification.

In this study, we explored the effects of L-proline on mouse oocyte vitrification.

Methods

Oocyte collection

This study was approved by the Institutional Animal Welfare and Ethics Committee of Peking University (No. LA2012-12). Eight-week-old B6D2F1 female mice (Vital River Laboratory Animal Technology Co., Ltd., Beijing, China) were superovulated by treatment with 10 U of pregnant mare serum gonadotropin (Hua Fu Biotechnology Company, Tianjin, China) and 10 U of human chorionic gonadotropin (Hua Fu Biotechnology Company, Tianjin, China) 48 h apart. After 14 h, denuded MII oocytes were obtained from the oviduct ampulla followed by a short exposure to 0.2% hyaluronidase. Oocytes with a single polar body and cytoplasm without inclusions were used for further study.

Raman spectroscopy

For Raman spectroscopy, a frozen sample was prepared using a cryostage system (Linkam Scientific, Surrey, UK). Briefly, an MII oocyte was immersed in 1.00 mol/L L-proline for <1 min. Then, the oocyte was immediately transferred to a droplet of phosphate buffered saline (PBS) on the silver block in the cryostage. The temperature of the cryostage was controlled via the TMS94 unit at rates between 0.01°C/min and 130°C/min. Liquid nitrogen flows through the block to achieve stable and accurate temperature control. The prepared oocyte was placed in the center of a circular glass coverslip (16 mm in diameter), the circumference of which had previously been lightly coated with a layer of silicon grease. A second coverslip was gently pressed on top of it to form a seal, which prevents water evaporation from the sample. Samples were then transferred to the cryostage with a sample holder and cooling at a rate of 50°C/min until down to −40°C. The frozen oocyte was scanned with a Raman spectrometer (Horiba JY HR800, Japan) coupled to a DM IRB inverted microscope (Nikon Microsystems, Japan). For each oocyte, individual spectra were obtained from within the zona pellucida through the center of the oocyte. Raman spectra of 1.00 mol/L L-proline were used as the control of outside the zona pellucida. Each spectrum was integrated for 37 s over the spectral range of 1500–5000 cm^{-1} using a laser power at the sample of 35 mW. Measurements were performed by recording a 100 μm long parallel line scan across the oocyte with 1.5 μm step size for each oocyte. Raman spectrum of each oocyte was obtained by averaging the point spectra of the three line scans. Raman spectra were cut in the 2500–4000 cm^{-1} range, vector-normalized, and baseline-corrected.

Osmotic pressure and pH measurement

The pH value and osmotic pressure of all vitrification solutions were measured by a pH meter (Mettler Toledo, USA) and an Osmometer (OSMOMAT030 Cryoscopic Osmometer, Germany), respectively. Each sample was measured three times.

Treatment of oocytes with vitrification solution containing L-proline

Oocytes were treated with vitrification solutions supplemented with EG (293237; Sigma, USA) and DMSO (D2650; Sigma), and divided into the following four groups: 10% DMSO plus 10% EG, 10% DMSO plus 7.5% EG, 7.5% DMSO plus 10% EG, and 7.5% DMSO plus 7.5% EG. In each group, the vitrification solution was supplemented with 0.00, 0.25, 0.50, 1.00, 2.00, or 4.00 mol/L L-proline (P5607, Sigma, USA). The control vitrification solution consists of 15% DMSO plus 15% EG without L-proline.

Oocyte vitrification and thawing

Three hundred mature oocytes in each group were vitrified by a standard procedure. Oocytes were first equilibrated in a solution containing 7.5% EG and 7.5% DMSO for 5 min at 25°C. Then, oocytes were exposed to the different concentrations of L-proline vitrification solution in four groups for <1 min at 25°C and immediately loaded into a sterile straw (RBC-S-008, Reprobiotech Corp., USA). The straw was plunged directly into liquid nitrogen and stored at −190°C for at least 1 month.

Thawing was carried out by several steps using sucrose solutions prepared using PBS containing 20% fetal bovine serum at 25°C. The oocytes were first expelled from the straws into 1.00 mol/L sucrose, incubated for 3 min, and then sequentially transferred to 0.50, 0.25, and 0.00 mol/L sucrose solutions each for 3 min. Finally, the thawed oocytes were cultured in human tubal fluid (HTF) medium (LGGF-100, LifeGlobal, Belgium) at 37°C in 5% CO_2 and humidified air, and the survival rate was assessed. After incubation for 2 h, the oocytes were ready for further experiments.

Immunofluorescence

Fresh MII oocytes and vitrified-thawed oocytes from groups of 10% DMSO plus 10% EG with 2.00 mol/L L-proline, 10% DMSO plus 7.5% EG with 2.00 mol/L L-proline, 7.5% DMSO plus 10% EG with 2.00 mol/L L-proline, 7.5% DMSO plus 7.5% EG with 2.00 mol/L L-proline, and 15% DMSO plus 15% EG without L-proline were fixed in 4% paraformaldehyde for 30 min. For 5-methylcytosine (5-mC) staining, DNA of the oocytes was denatured with 4.00 mol/L HCl at 25°C for 10 min and subsequently neutralized by treatment with 100 mmol/L Tris-HCl buffer (pH 8.0) for 10 min. After washing, samples were blocked in blocking solution (3% bovine serum albumin [Sigma] prepared in PBS) at 25°C for 1 h, and then incubated with an anti-5-mC antibody (1:50; BIMECY-0500; Eurogentec, Belgium) for 2 h at 25°C. After washing three times, oocytes were incubated with Alex Fluor 568-conjugated goat anti-mouse IgG (1:500, A-11004; Invitrogen, USA) for 2 h at 25°C. After rinsing, samples were incubated with a monoclonal anti-α-tubulin-FITC antibody (1:100, F2168; Sigma, USA) for 1 h. Finally, nuclei were counterstained with 10 mg/ml Hoechst 33342 (Molecular Probes, H3570, Life Technologies, USA) for 15 min. For negative controls, the primary antibody was omitted. All groups of oocytes were observed with a confocal laser scanning microscope (LSM710 Carl Zeiss, Oberkochen, Germany).

In vitro fertilization and embryo culture

Only the experimental group of 7.5% DMSO plus 10% EG with 2.00 mol/L L-proline was used for *in vitro* development. The sperm suspensions retrieved from the cauda epididymis of 10-week-old ICR male mice were capacitated in HTF medium for 1 h at 37°C in a humidified atmosphere of 5% CO_2 in air. The frozen-thawed oocytes from the experimental group (7.5% DMSO plus 10% EG with 2.00 mol/L L-proline) and the control group (15% DMSO plus 15% EG without L-proline) were co-cultured with the capacitated sperm (final density of 2×10^6/ml) in HTF medium for 6 h. The fertilized oocytes with a second polar body or two pronuclei were removed and cultured in GM media (LGGG-50, LifeGlobal, Belgium) to the blastocyst stage for 4.5 days in 5% CO_2 and humidified air at 37°C. The zygotes were assessed every 24 h. Each experiment was repeated five times. Each experiment was repeated five times.

Statistical analysis

Differences among groups were analyzed with Chi-square test for categorical data and an analysis of variance (ANOVA) for numerical data using SPSS 20.0 software (Statistical Product and Service Solutions, IBM, Chicago, IL, USA). Results were expressed as a mean ± standard error (SE). A $P < 0.05$ was considered statistically significant. For all results, the examples shown are representative of at least three replications.

Results

Use of Raman spectroscopy to detect the permeability of L-proline

The Raman spectrum of a mouse frozen oocyte exposed to L-proline displayed a significant difference between the intracellular [Figure 1a, black line] and extracellular [Figure 1a, red line] regions. It is possible to identify prominent spectral peaks in characteristic spectral regions. The spectral peak around 2800–3000 cm^{-1} represented cytoplasm components [Figure 1a, black arrow], which corresponded to the region of the intracellular cytoplasm of the oocyte. The spectral peaks around 2950–3050 cm^{-1} and 3100–3200 cm^{-1} represent L-proline [Figure 1a, red arrow] and ice [Figure 1a, blue arrow], respectively. A sharp peak around 2950–3050 cm^{-1} inside the oocyte was clearly observed after exposure to 1.00 mol/L L-proline [Figure 1a, red arrow, red line]. To determine whether L-proline was present inside cells, the Raman spectra of oocytes in the 2950–3050 cm^{-1} spectral range were measured by line scans [Figure 1b]. The peak at 3000 cm^{-1} was obvious inside oocytes treated with 1.00 mol/L L-proline [Figure 1b]. The intensity of the peak in the central region of the oocyte was much stronger than that in the cortical portion. Therefore, L-proline may play a role in vitrification by penetrating the oocyte within 1 min.

Osmotic pressure and pH determination

To evaluate the biophysical and biochemical properties of the vitrification solutions containing L-proline, we measure the pH values and osmotic pressure. The pH values were similar in all solutions (pH 6.8–7.3) [Figure 2, blue diamond]. The osmotic pressure in the several L-proline-containing solutions [Figure 2, red diamond with asterisks] was similar to that in the control ($P > 0.05$) [Figure 2].

Assessment of the survival rate of vitrified-thawed oocytes

To assess the efficiency with which oocytes were vitrified using the L-proline-containing vitrification solutions, we analyze the survival rates of the oocytes in all groups. Compared with the control group, the survival rate of vitrified oocytes in 2.00 mol/L L-proline + 7.5% DMSO + 10% EG group was significantly higher (93.8% vs. 88.4%, $\chi^2 = 5.381$, $P < 0.05$) [Figure 3]. The survival rates of thawed oocytes vitrified with a mixture of 2.00 mol/L L-proline + 10% EG + 10% DMSO; 2.00 mol/L L-proline + 10% DMSO + 7.5% EG; or 2.00 mol/L L-proline + 7.5% DMSO + 7.5% EG did not differ from that of the control group (86.9%, 86.3%, and 86.2% vs. 88.4%, $\chi^2 = 4.852$, $P > 0.05$) [Figure 3]. These groups (10% DMSO + 10% EG + 2.00 mol/L L-proline, 10% DMSO + 7.5% EG + 2.00 mol/L L-proline, 7.5% DMSO + 10% EG + 2.00 mol/L L-proline, and 7.5% DMSO + 7.5% EG + 2.00 mol/L L-proline) of L-proline-treated oocytes with survival rates that were higher or similar to that of control oocytes were used for further experiments. The survival rate gradually increased as the L-proline concentration increased up to 2.00 mol/L and then decreased.

DNA methylation analysis

Next, we compared global DNA methylation between these specific L-proline-treated groups (10% DMSO + 10% EG + 2.00 mol/L L-proline, 10% DMSO + 7.5%

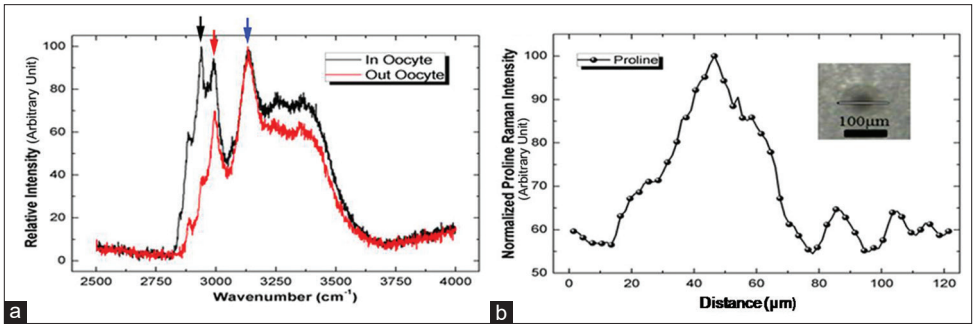

Figure 1: Raman spectrum of frozen oocyte treated with 1.00 mol/L L-proline. (a) The intracellular Raman spectrum (black line) of frozen oocyte treated with 1.00 mol/L L-proline and the extracellular Raman spectrum (red line) of 1.00 mol/L L-proline after freezing. (b) Line scans of the frozen oocyte in the 2950–3050 cm^{-1} spectral range. Black arrow: The peak around 2800–3000 cm^{-1}; Red arrow: The peak around 2950–3050 cm^{-1}; Blue arrow: The peak around 3100–3200 cm^{-1}.

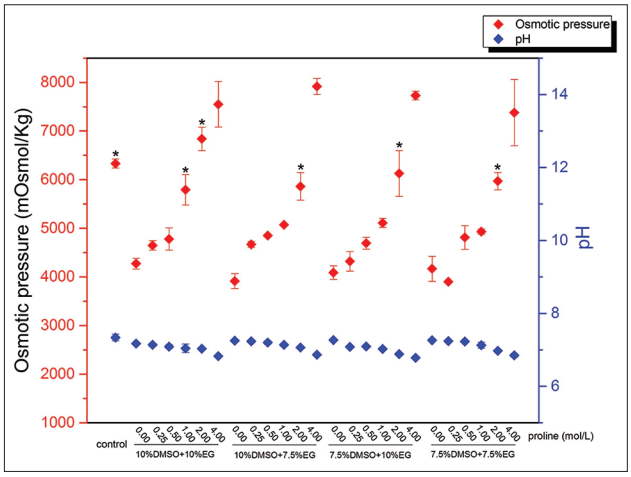

Figure 2: Osmotic pressure (red diamond) and pH (blue diamond) of the vitrification solutions containing different concentrations of L-proline. *There is no difference between values with asterisks and control (15% DMSO and 15% EG without L-proline) ($P > 0.05$). DMSO: Dimethyl sulfoxide; EG: Ethylene glycol.

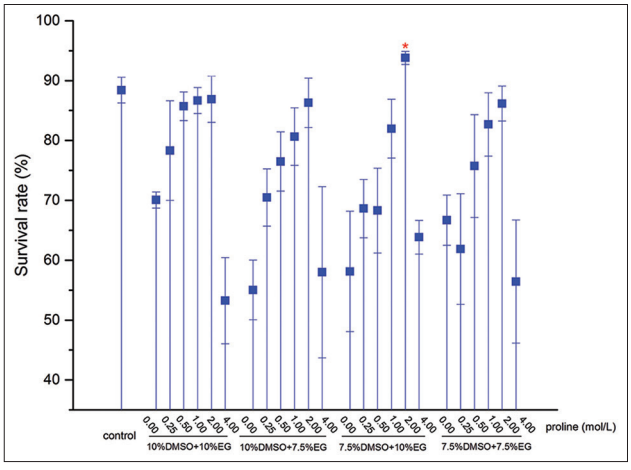

Figure 3: The survival rate of vitrified-thawed oocytes treated with different concentrations of L-proline. *Value is significantly higher than the control group (15% DMSO and 15% EG without L-proline) ($P < 0.05$). DMSO: Dimethyl sulfoxide; EG: Ethylene glycol.

EG + 2.00 mol/L L-proline, 7.5% DMSO + 10% EG + 2.00 mol/L L-proline, and 7.5% DMSO + 7.5% EG + 2.00 mol/L L-proline) and the control group (15% DMSO + 15% EG) by performing 5-mC staining [Figure 4a]. The relative fluorescence of 5-mC staining in thawed oocytes of these L-proline-treated groups was similar to that in the control group and fresh group ($P = 0.543$) [Figure 4b].

Early *in vitro* embryonic development

Based on the above assessment, we decided to observe the embryonic development of vitrified oocytes from the L-proline-containing group (2.00 mol/L L-proline + 7.5% DMSO + 10% EG) with the highest survival rate compared with the control group (15% DMSO + 15% EG) and fresh oocytes. After *in vitro* fertilization, the fertilization rate of the L-proline-treated group was equivalent to that of the control group and fresh oocytes (93.3%, 92.3%, and 95.1%, respectively, $\chi^2 = 0.817$, $P > 0.05$) [Table 1]. The two-cell and blastocyst formation rates of the L-proline-treated group were similar to those of the control group but were lower than those of fresh oocytes (two-cell formation rate: 69.5% and 76.9% vs. 93.5%, respectively, $\chi^2 = 22.314$, $P < 0.05$; blastocyst formation rate: 50.5% and 51.3% vs. 73.2%, respectively, $\chi^2 = 16.223$, $P < 0.05$) [Table 1].

Discussion

L-proline may play a role in oocyte vitrification for three reasons. First, L-proline can penetrate the oocyte, consistent with the biophysical and biochemical properties of L-proline. L-proline is a membrane-permeable cryoprotectant that can penetrate the cell membrane and stabilize it to confer freezing tolerance.[10] We also demonstrated that L-proline can infiltrate the oocyte cytoplasm through the zona pellucida and oocyte membrane [Figure 1].

Second, a mixture of 2.00 mol/L L-proline with low concentrations of DMSO and EG was most effective for oocyte vitrification. In terms of the characteristics of L-proline, osmotic pressure increased along with the concentration of L-proline, whereas pH was not related to the L-proline concentration [Figure 2]. This is because L-proline is a small osmolyte, which means that osmolality increased as the concentration of osmolytes increases. However, the survival rate of thawed oocytes decreased when

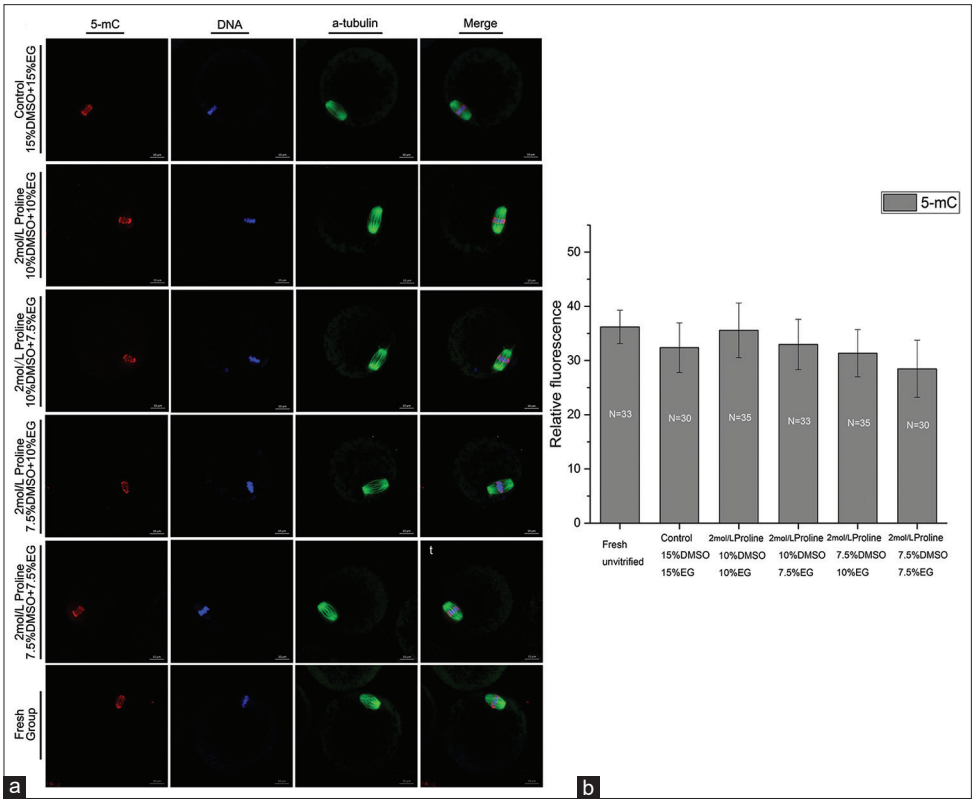

Figure 4: Expression of 5-mC in vitrified-thawing oocytes treated with 2.00 mol/L L-proline. (a) The immunofluorescence photograph of 5-mC (red color), DNA (blue color), and a-Tubulin (green color) in these groups (original magnification, × 100). (b) Comparison of the fluorescence intensities detected by 5-mC. 5-mC: 5-methylcytosine; DMSO: Dimethyl sulfoxide; EG: Ethylene glycol.

Table 1: *In vitro* fertilization and cleavage developments of vitrified-thawed oocytes treated with 2.00 mol/L L-proline

Groups	Percentage (*n*/*N*)			
	Survival rate	Fertilization rate	Two-cell rate	Blastocyst rate
Fresh group	–	95.1 (117/123)*	93.5 (115/123)*	73.2 (90/123)*
Control group: 15% DMSO + 15% EG	88.4 (266/301)	92.3 (108/117)	76.9 (90/117)	51.3 (60/117)
2.00 mol/L L-proline + 7.5% DMSO + 10% EG	93.8 (285/304)	93.3 (98/105)	69.5 (73/105)	50.5 (53/105)

*Values in the same column are significantly different with fresh group (P<0.05). DMSO: Dimethyl sulfoxide; EG: Ethylene glycol; *n*: Survival number; *N*: Total number.

the concentration of L-proline was higher than 2.00 mol/L. This illustrates that high osmolality has negative effects on cells. Oocytes treated with high concentrations of L-proline could be injured by the stress of high osmolality before freezing. Galinski's group[11] used concentrations of up to 5.00 mol/L when they tested the effect of L-proline on protein stability because osmolality does not have the same impact on proteins as on cells. The osmotic pressure of a mixture of 2.00 mol/L L-proline and low concentrations of DMSO and EG were close to that of the control solution, and the survival rate of oocytes treated with this mixture was higher than that of oocytes treated with the control solution. Similar results were reported in a study by Sun *et al*.,[4] which demonstrated that L-proline is the most effective CPA, and it improves the efficiency of recultivation of human endothelial cells by more than 100% in the presence of a low concentration of DMSO. Successful cryopreservation using a reduced DMSO concentration was also shown by other authors. By adding sucrose or EG, the concentration of DMSO can be reduced to 7.5% or 5%.[12,13] In the current study, the addition of L-proline allowed the concentrations of DMSO and EG to be reduced to 7.5% and 10%, respectively, without reducing the recultivation efficiency. Therefore, a mixture of 2.00 mol/L L-proline with low concentrations of DMSO and EG is considered to be a suitable cryopreservation solution for mouse oocyte vitrification.

Finally, L-proline did not change global DNA methylation and had no adverse effect on *in vitro* embryonic development. There was no significant difference in 5-mC expression or the parameters of *in vitro* embryonic development between oocytes cryopreserved using L-proline and control vitrified oocytes [Figure 4 and Table 1]. This indicates that L-proline is a useful cryoprotectant for mouse oocyte vitrification. Ogawa *et al*.[14] reported that L-proline does not affect gene expression in cryopreserved cells. Although L-proline treatment did not perturb DNA methylation and embryonic

development *in vitro*, the effects of L-proline on epigenetic modifications and the *in vivo* development of vitrified oocytes need to be studied further.

In conclusion, this study suggests that an appropriate concentration of L-proline can improve the cryopreservation of mouse oocytes with low DMSO and EG concentrations, and this may be applicable to human oocyte vitrification. Further investigations are required to confirm the efficiency and safety of treatment with a mixture of L-proline and low DMSO and EG concentrations in terms of *in vivo* embryonic development. Despite the higher survival rate of L-proline-treated vitrified oocytes, more indicators such as the spindle configuration, mitochondrial function, and live birth rate need to be investigated in the future. Further attempts should be made to verify the efficacy and safety because more in-depth research of a new cryoprotectant is indispensable for human oocyte applications.

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Conflicts of interest

There are no conflicts of interest.

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