Maintenance of developmental competence of porcine cumulus–oocyte complexes during 72-hour ambient storage in chemically defined solutions

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Journal of Livestock Science (ISSN online 2277-6214) 17: 484-490
Received on 14/5/26; Accepted on 9/7/26; Published on 13/7/26
doi. 10.33259/JLivestSci.2026.484-490

Abstract

This study aimed to establish a protocol for the short-term preservation of *in vitro*-derived porcine cumulus-oocyte complexes (COCs) at ambient temperatures for up to 72 h. The COCs were stored at 25°C for 24 and 72 h in Cell-W, Cell-S (chemically defined cell preservation solution), or maturation culture (MC) medium. Stored and fresh (control) COCs were evaluated for nuclear maturation, DNA fragmentation, fertilization, and development. After 24 h of storage, the maturation rates of COCs stored in Cell-S and MC media after *in vitro* maturation (IVM) culture significantly decreased compared to the controls. No significant differences were found in DNA-fragmented nuclei rates, fertilization rates, blastocyst formation rates, or embryo quality between the groups. After 72 h of storage, significantly fewer COCs stored in MC remained at the germinal vesicle stage compared to the controls. The DNA-fragmented nuclei rate was significantly higher in MC-stored COCs than in controls before IVM culture. The maturation rates of all stored COCs were significantly lower than those of the controls, but blastocyst formation rates in Cell-W (4.9%) and Cell-S (3.6%) were similar to controls (8.4%). These findings indicate that Cell-W and Cell-S better preserved developmental competence than MC during prolonged storage and maintained blastocyst formation rates comparable to those of fresh controls, suggesting that chemically defined cell preservation solutions may maintain oocyte development when stored at 25°C even for three days.

Keywords: ambient temperature, embryo development, immature oocyte, pig, short-term preservation

Introduction

In regions where acquiring oocytes from livestock is challenging, oocytes or ovaries must be collected from slaughterhouses and transported to *in vitro* fertilization facilities. When the facility is located far away, long-distance ovary transport is impractical because porcine ovaries stored for more than 4 h show decreased oocyte maturation (Tellado *et al.*, 2014). Moreover, as storage time increased and storage temperature rose, ROS levels in immature oocytes increased, while oxidative activity and survival rates decreased (Tellado *et al.*, 2014). Thus, transport requiring one day inevitably compromises oocyte quality, making reliable preservation techniques essential for maintaining developmental competence. Although significant progress has been made in the cryopreservation of oocytes and embryos in livestock species, porcine oocytes are particularly sensitive to cryopreservation primarily because of their high lipid content and susceptibility to chilling injury (Somfai *et al.*, 2012; Morado *et al.*, 2023). Therefore, an alternative method is needed to preserve porcine oocytes without the harmful effects of cryopreservation.

Recent studies have demonstrated that storage at ambient temperatures (e.g., 25°C) effectively preserves oocyte quality, meiotic competence, and fertilization ability, suggesting that ambient-temperature preservation is a promising approach for porcine oocytes (Tellado *et al.*, 2014; Lin *et al.*, 2022a; Murray and Gibson, 2022). Unlike embryos, oocytes are arrested at the germinal vesicle (GV) stage before maturation and thus may be more vulnerable to oxidative stress and metabolic disruptions during storage (Agarwal *et al.*, 2022). Moreover, our previous study confirmed that one-stage porcine embryos retained developmental competence when stored at 25°C for 24 h (Lin *et al.*, 2022a), suggesting that porcine oocytes may benefit from similar preservation conditions.

The choice of the preservation solution plays a crucial role in determining the success of short-term preservation. Traditionally, serum-based media (e.g., follicular fluid or fetal bovine serum) have been used to maintain the viability of mammalian embryos during storage at ambient temperatures (Ideta *et al.*, 2013; Martinez *et al.*, 2018). Serum contains a variety of nutrients, hormones, and growth factors that support oocyte and embryo survival; however, it also introduces batch-to-batch variability and the potential risk of pathogen transmission (van der Valk *et al.*, 2018). Therefore, chemically defined media offer safer and more reproducible alternatives. For example, Tyrode's lactate solution with polyvinyl alcohol (TL-PVA) has been successfully used to store in vivo-derived porcine morulae at 25°C (Martinez *et al.*, 2019). Given these findings, chemically defined preservation solutions should be explored for the short-term preservation of porcine oocytes to improve their viability and streamline the storage process.

Cell-W (cell wash and preservation solution) and Cell-S (cell suspension and preservation solution) are commercially available protein-free preservation solutions designed for the short-term storage of mammalian cells at ambient temperature. Cell-W contains trehalose, a disaccharide known for its membrane-stabilizing and osmoprotective properties, whereas Cell-S additionally includes dextran, a high-molecular-weight polysaccharide that enhances structural stability under stress conditions. Chemically defined solutions containing trehalose have previously been reported to support short-term ambient preservation of porcine embryos without impairing their developmental competence (Lin *et al.*, 2022a; Lin *et al.*, 2022b). Based on these mechanistic and empirical considerations, we hypothesized that these preservation strategies could also be applied to immature porcine cumulus–oocyte complexes.

The present study aimed to evaluate the applicability of two chemically defined, protein-free preservation solutions (Cell-W and Cell-S) for maintaining the developmental competence of porcine COCs at 25 °C for up to 72 h.

Materials and Methods

Ethical approval

All animal experiments were approved by the Institutional Animal Care and Use Committee of Tokushima University (approval number: T2025-6).

Study area

This study was conducted at the Bio-Innovation Research Center, Tokushima University, Tokushima, Japan (34.0642° N, 134.4546° E). Ovaries were collected from a local slaughterhouse, Nippon Food Packer Shikoku Plant, located approximately 2 km north of the research center.

Oocyte collection and storage

Porcine ovaries were obtained from prepubertal crossed gilts (Landrace ×Large White ×Duroc breeds) at a local slaughterhouse and transported to the laboratory in physiological saline at 30°C. The ovaries were washed three times with pre-warmed physiological saline supplemented with 100 IU/ml penicillin G potassium and 0.1 mg/ml streptomycin sulfate. Follicles (3–6 mm in diameter) on the ovarian surface were sliced into dishes containing modified phosphate-buffered saline (m-PBS) using a surgical blade, and cumulus-oocyte complexes (COCs) were collected under a stereomicroscope. After collection, COCs were randomly transferred into 2-ml tubes, each containing 700 µl of the following preservation solutions; Cell-W solution, Cell-S solution (Otsuka

Pharmaceutical Factory, Inc., Tokushima, Japan) and maturation culture (MC) medium. The composition of each storage solution is summarized in Table 1. Subsequently, the tubes containing the COCs (70 COCs per tube) were stored at 25°C in a cool incubator (Cool-Incubator A1201; Ikuta Sangyo, Nagano, Japan) for 24 h or 72 h.

***In vitro* maturation (IVM) and assessment of oocyte quality**

IVM of oocytes was performed as described by Lin *et al*. (2022a). After storage at 25°C, 70 COCs were cultured in 700 µl MC medium of TCM 199 with Earle's salts supplemented with 10% porcine follicular fluid, 0.6 mM cysteine, 50 µM β-mercaptoethanol, 50 µM sodium pyruvate, 2 mg/mL d-sorbitol, 10 IU/mL equine chorionic gonadotropin, 10 IU/mL human chorionic gonadotropin, and 50 µg/mL gentamicin for 22 h in 4-well dishes. The COCs were then transferred to hormone-free maturation medium for 22 h. Incubation was performed at 39°C in 5% CO_2. Unstored fresh COCs were used as controls.

To determine the meiotic status and DNA fragmentation before and after IVM, oocytes were fixed and analyzed by nuclear staining with DAPI and terminal deoxynucleotidyl transferase nick-end labeling (TUNEL), as described by Do *et al*. (2015). Briefly, COCs were denuded from cumulus cells using 150 IU hyaluronidase and mechanical pipetting. Denuded oocytes were fixed at 4°C in 3.7% paraformaldehyde in PBS and permeabilized in PBS containing 0.1% Triton-X100 for 40 min. Oocytes were incubated overnight at 4°C in PBS with 10 mg/mL bovine serum albumin, followed by incubation in fluorescein-conjugated 2′-deoxyuridine-5′-triphosphate and terminal deoxynucleotidyl transferase (TUNEL reagent) for 1 h at 38.5°C. After TUNEL staining, the oocytes were counterstained with 1 µg/mL DAPI for 10 min and examined under an epifluorescence microscope. The oocytes were determined to be either in the germinal vesicle (GV), condensed chromatin (CC), metaphase I (MI), anaphase I–telophase I (AT), or metaphase II (MII) stage according to the chromatin configuration based on DAPI staining. Unidentifiable chromatin configurations were classified as degenerated (DG). To assess DNA damage in all oocytes before and after IVM, the nuclei labeled with TUNEL were counted.

***In vitro* fertilization and assessment of fertilization**

In vitro fertilization (IVF) of oocytes was performed according to the method described by Lin *et al*. (2022a). After IVM, the COCs were washed with porcine fertilization medium (PFM), and approximately 60 COCs were transferred to a 4-well dish containing 600 µL of PFM containing frozen-thawed spermatozoa at a final concentration of 1×10^6 sperm/mL and co-cultured for 5 h at 39°C in a humidified incubator containing 5% CO_2, 5% O_2, and 90% N_2 in air.

To evaluate fertilization, some oocytes were fixed with acetic acid: ethanol (1:3 v/v) for 48–72 h at 10 h after insemination. Fixed oocytes were stained with acetic orcein (1% orcein in 45% acetic acid) and examined by phase-contrast microscopy. Oocytes containing both female and male pronuclei were considered fertilized and categorized as normal or polyspermic, based on the number of swollen sperm heads and/or pronuclei in the cytoplasm (Do *et al.*, 2015). The proportion of monospermic fertilization was calculated by dividing the number of monospermically fertilized oocytes by the number of fertilized oocytes.

***In vitro* culture of embryos and assessment of blastocyst quality**

After co-incubation, the putative zygotes were denuded from the cumulus cells and attached spermatozoa by mechanical pipetting, transferred to porcine zygote medium (PZM-5), and cultured for three days. All the cleaved embryos were then transferred into 500 µl of porcine blastocyst medium (PBM) and cultured for an additional four days. Zygotes and embryos were incubated at 39°C in a humidified incubator containing 5% CO_2, 5% O_2, and 90% N_2.

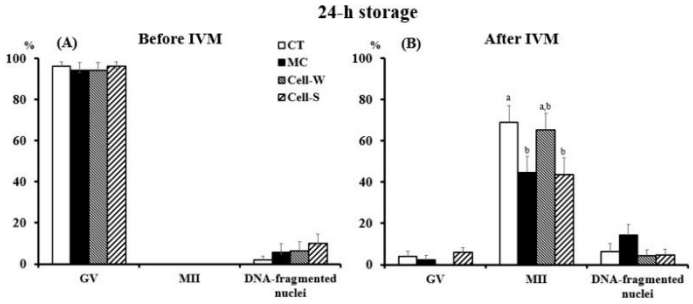
To evaluate the cell number and apoptotic cells, blastocysts and expanded blastocysts were fixed and analyzed by the TUNEL assay, as described above. The apoptotic index was calculated by dividing the number of cells containing apoptotic nuclei (labeled with TUNEL) by the total number of cells.

Statistical analyses

The experiments were repeated five times for each storage period. Statistical significance was inferred using analysis of variance (ANOVA), followed by Fisher's protected least significant difference (PLSD) test using STATVIEW (Abacus Concepts, Inc., Berkeley, CA, USA). Percentage data were subjected to arcsine transformation before statistical analysis. Data were expressed as the mean ± standard error of the mean (SEM). Differences with $P \leq 0.05$ were considered significant.

Results

When COCs were stored in Cell-W, Cell-S, and MC media for 24 h, the rates of oocytes remaining at the GV stage before IVM culture did not differ from those of fresh control COCs, irrespective of the preservation solution (Fig. 1A). However, the maturation rates of COCs stored in Cell-S and MC media after IVM culture were significantly lower ($P < 0.05$) than those of the control COCs (Fig. 1B). There were no differences in the rates of oocytes with DNA-fragmented nuclei before and after IVM culture between the groups. The rates of total fertilization, monospermic fertilization, cleavage, and blastocyst formation of stored COCs did not differ from those of control COCs, irrespective of the preservation solution (Fig. 2A and Table 2). Moreover, the mean total



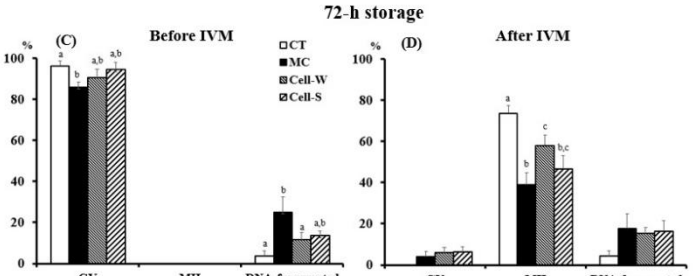


Fig. 1. Effect of storage solution on meiotic maturation and quality before and after in vitro maturation (IVM) of porcine oocytes stored at 25 °C for 24 h (A and B, respectively) and 72 h (C and D, respectively). As control, fresh oocytes were examined before and after IVM. CT: control, MC: maturation culture medium, Cell-W: cell wash and preservation solution, Cell-S: cell suspension and preservation solution. The DNA-fragmented nuclei were defined as the ratio of the number of oocytes containing an apoptotic nucleus to the total number of examined oocytes. GV: germinal vesicle, MII: metaphase II. Five replicate trials were performed (number of examined oocytes per group: 46–52). Error bars represent the mean ± standard error of the mean (SEM). Groups identified by different superscripts in the meiotic stage are significantly different ($P < 0.05$).

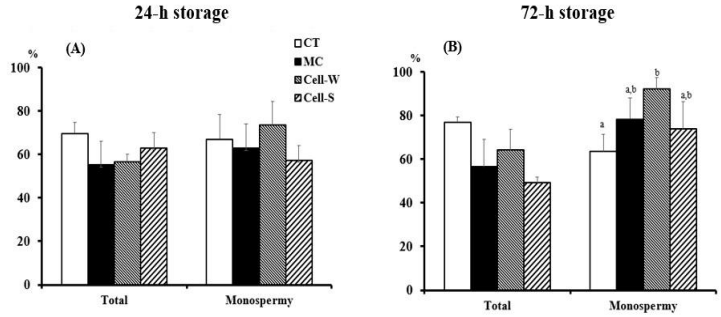


Fig. 2. Effect of storage solution on the fertilization before and after in vitro maturation （IVM） of porcine oocytes stored at 25℃ for 24 h (A) and 72 h (B). As control, fresh oocytes were examined before and after IVM. CT: control, MC: maturation culture medium, Cell-W: cell wash and preservation solution, Cell-S: cell suspension and preservation solution. The monospermic fertilization rate was defined as the ratio of the number of monospermic oocytes to the total number of fertilized oocytes. Five replicate trials were performed (number of examined oocytes per group: 41–57). Error bars represent the mean ± standard error of the mean (SEM). Groups identified by different superscripts in the meiotic stage are significantly different ($P < 0.05$).

Table 1. Composition of storage solutions and maturation culture (MC) medium

Storage solution	Main components
Cell-W	3% trehalose hydrate, 0.02% calcium chloride hydrate, 0.03% potassium chloride, 0.6% sodium chloride, pH adjuster, 0.3% L-sodium lactate
Cell-S	Cell-W + 5% dextran-40
MC medium	TCM199 with Earle's salts + 10% pFF + 0.6 mM cysteine + 50 μM β-ME + 50 μM sodium pyruvate + 2 mg/mL d-sorbitol + 10 IU/mL eCG +10 IU/mL hCG + 50 μg/mL gentamicin

Abbreviations: Cell-W, cell wash and preservation solution; Cell-S, cell suspension and preservation solution; TCM199, tissue culture medium 199; pFF, porcine follicular fluid; β-ME, β-mercaptoethanol; eCG, equine chorionic gonadotropin; hCG, human chorionic gonadotropin.

Table 2. Effect of storage solution on the development of porcine oocytes stored at 25℃ for 24 h*

Storage solution**	No. of examined oocytes	No. (%) of embryos		Total cell number of blastocyst	Apoptotic nucleus index***
		Cleaved	Developed to blastocysts		
Control	229	189 (82.2 ± 3.7)	24 (9.8 ± 2.1)	48.5 ± 3.5	2.3 ± 0.5
MC	233	173 (75.7 ± 3.9)	15 (6.8 ± 2.3)	44.9 ± 3.6	2.5 ± 0.8
Cell-W	225	164 (73.3 ± 3.6)	17 (7.1 ± 1.5)	50.7 ± 5.3	2.4 ± 0.7
Cell-S	223	162 (73.9 ± 4.0)	11 (4.1 ± 1.8)	58.3 ± 9.9	3.5 ± 1.1

*Five replicates were performed. Percentages are expressed as mean ± SEM. **As control, fresh oocytes were matured, fertilized, and then cultured for 7 days. MC, maturation culture medium; Cell-W, cell wash and preservation solution; Cell-S, cell suspension and preservation solution.***The apoptotic index was defined as the ratio of the number of cells containing an apoptotic nucleus and the total number of cells in a blastocyst.

Table 3. Effect of storage solution on the development of porcine oocytes stored at 25°C for 72 h*

Storage solution**	No. of examined oocytes	No. (%) of embryos		Total cell number of blastocyst	Apoptotic nucleus index***
		Cleaved	Developed to blastocysts		
Control	250	217 (86.8 ± 3.2)[a]	21 (8.4 ± 2.1)[a]	43.4 ± 3.8	3.1 ± 0.7
MC	252	147 (58.2 ± 6.2)[b]	4 (1.6 ± 1.6)[b]	49.0 ± 5.8	0.9 ± 0.5
Cell-W	246	184 (74.6 ± 3.2)[c]	12 (4.9 ± 0.8)[a,b]	49.9 ± 9.7	1.4 ± 0.5
Cell-S	251	171 (68.1 ± 4.2)[b,c]	9 (3.6 ± 1.7)[a,b]	38.9 ± 4.9	3.7 ± 1.6

*Five replicates were performed. Percentages are expressed as mean ± SEM. **As control, fresh oocytes were matured, fertilized, and then cultured for 7 days. MC, maturation culture medium; Cell-W, cell wash and preservation solution; Cell-S, cell suspension and preservation solution.***The apoptotic index was defined as the ratio of the number of cells containing an apoptotic nucleus and the total number of cells in a blastocyst. [a-b]Values with different superscript letters in the same column are significantly different ($P < 0.05$).

cell number and apoptotic nucleus index in the resulting blastocysts did not differ between the groups. Overall, Cell-W maintained developmental competence comparable to that of fresh control COCs, whereas Cell-S and MC showed reduced maturation rates after 24 h of preservation. When COCs were stored in Cell-W, Cell-S, and MC media for 72 h, the rate of oocytes remaining at the GV stage before IVM culture was significantly lower ($P < 0.05$) in COCs stored in MC than in fresh control COCs (Fig. 1C). Moreover, the rate of oocytes with DNA-fragmented nuclei before IVM culture was significantly higher ($P < 0.05$) in COCs stored in MC than in Cell-W and control COCs. The maturation rates of all stored COCs after IVM culture significantly decreased ($P < 0.05$) compared to those of the control COCs (Fig. 1D). The monospermic fertilization rate of COCs stored in Cell-W was significantly higher ($P < 0.05$) than that of the control oocytes, but there were no significant differences in the total fertilization rates among the groups (Fig. 2B). The cleavage rates of all stored COCs decreased compared with those of the control (Table 3). The blastocyst formation rate of embryos derived from COCs stored in the MC was significantly lower ($P < 0.05$) than that of the control; however, the blastocyst formation rates in the Cell-W (4.9%) and Cell-S (3.6%) groups were similar to those of the control (8.4%). The mean total cell number and apoptotic nucleus index in the resulting blastocysts did not differ among the groups. Overall, among the three preservation media, Cell-W and Cell-S maintained developmental competence similar to fresh control after 72 h of preservation, showing better results than MC.

Discussion

The application of latest techniques Crispr/Cas9 in pig breeding is increasing still the IVF/IVM technique for breed improvement hold great importance (Tinh et al., 2020). In the present study, the results demonstrated that chemically defined, protein-free solutions, although neither Cell-W nor Cell-S produced better outcomes than the fresh control, both media preserved developmental competence more effectively than MC during prolonged storage and maintained blastocyst development comparable to that of the fresh control. Cell-W and Cell-S solutions, can effectively preserve the developmental potential to the blastocyst stage of porcine COCs stored at 25°C even for 72 h. When COCs were stored for 24 h, the maturation rates of oocytes stored in MC and Cell-S solutions decreased compared to those of fresh control oocytes. Extended storage of COCs for 72 h led to a significant decline in maturation rates, irrespective of the preservation solution, which is consistent with previous observations that storage duration critically affects the viability of porcine zygotes (Lin *et al.*, 2022a). These findings suggest that the decline in the maturation rate after storage may be attributed, at least in part, to the cumulative subcellular damage incurred during prolonged ambient storage. Moreover, Cell-W and Cell-S effectively maintained the GV stage before IVM culture in COCs stored for 72 h, but MC reduced the rates of oocytes remaining at the GV stage and increased DNA fragmentation. DNA damage, as evidenced by TUNEL staining, likely reflects early apoptotic processes or chromatin instability, which can impair meiotic progression during IVM (Lin *et al.*, 2014). In porcine oocytes, oxidative stress and mitochondrial dysfunction trigger meiotic arrest and spindle abnormalities (Zhang *et al.*, 2020). Therefore, metabolic imbalances induced by extended storage might disrupt cytoplasmic maturation processes, resulting in reduced MII formation.

While the blastocyst formation rate of embryos derived from COCs stored for 72 h in MC solution decreased compared to that of the control, the blastocyst formation rates and quality (total cell number and apoptotic nucleus index) of embryos from Cell-W or Cell-S solutions were comparable to those of the control group. This indicates that short-term preservation for 72 h in Cell-W and Cell-S solutions was effective in maintaining the ability of oocyte development. The underlying mechanisms contributing to the protective effects of Cell-W and Cell-S solutions may be attributed to their composition. Both media were supplemented with trehalose, a disaccharide with membrane-stabilizing properties. Trehalose enhances cryotolerance in various cell types by protecting cellular proteins through hydrogen bonding and osmotic regulation (Stewart and He, 2019; Murray *et al.*, 2024). The Cell-S solution contained 5% dextran, which has been suggested to stabilize cell membranes during cryopreservation (Murray and Gibson, 2022). Moreover, since the mechanisms of trehalose and dextran are similar, it has been proposed that the simultaneous addition of trehalose and dextran may produce an additive

complementary effect on cell cryopreservation (Fujita *et al.*, 2021). However, compared to the Cell-W solution, the Cell-S solution, containing both trehalose and dextran, had no apparent effects on maturation, fertilization, embryo development, or embryo quality, irrespective of the storage duration. In the present study, considering that COCs were stored at ambient temperature (25°C) and not frozen, the additive complementary effect of dextran may not have been observed.

Interestingly, the monospermic fertilization rate of COCs stored in Cell-W for 72 h was significantly higher than that of the control group, despite similar total fertilization rates. This observation suggests that oocytes preserved in Cell-W acquired an enhanced ability to prevent polyspermy. One possible explanation is that Cell-W may have induced structural or functional modifications in the oolemma or zona pellucida, which promoted a more efficient block to sperm entry. Trehalose, a key component of Cell-W, may stabilize phospholipid membranes and membrane-bound proteins through hydrogen bonding and osmotic regulation (Stewart and He, 2019; Murray *et al.*, 2024), which helps to maintain oolemma integrity and responsiveness during fertilization. Additionally, the absence of dextran in Cell-W results in a lower osmotic pressure than that in Cell-S, which may facilitate better membrane homeostasis and reduce cellular stress during storage. These physicochemical properties may contribute to more effective zona hardening or cortical granule exocytosis upon fertilization, thereby favoring monospermy.

Conclusion

Our findings indicate that Cell-W and Cell-S better preserved developmental competence than MC during prolonged storage and maintained blastocyst formation rates comparable to those of fresh controls, suggesting that chemically defined cell preservation solutions may maintain oocyte development when stored at 25°C even for three days. While storage for 24 h maintains oocyte viability and developmental potential, longer storage durations lead to a decline in maturation capacity. Further refinement of storage protocols, including the adjustment of media components, may improve long-term preservation outcomes and broaden the applicability of these solutions to reproductive technologies.

Acknowledgments

We thank the Nippon Food Packer and K. K. Shikoku (Tokushima, Japan) for supplying the pig ovaries. This study was supported in part by KAKENHI grants JP21KK0124, JP22H02499, JP22K19896, and JP23K23805 from the Japan Society for the Promotion of Science (JSPS).

Conflict of interest

The authors declare no conflict of interest.

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