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Impact of Presence or Absence of Trehalose during Vitrification on Viability and Development of Vitrified/Warmed Immature Dromedary Camel Oocytes

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Abstract

Vitrification of immature oocytes at the germinal vesicle (GV) stage is important to preserve female gametes. The standard formula for vitrification solutions has long been a debate. Herein, we investigated the effect of the presence or absence of trehalose in vitrification solution on viability, *in vitro* maturation (IVM) rates, and development of vitrified/warmed immature dromedary camel oocytes. Cumulus oocyte complexes (COCs) obtained at slaughter from the ovaries of mature she-camels were randomly allocated into three groups; namely, control group, oocytes were directly subjected to IVM without vitrification, vitrification solution 1 (VS1) group, oocytes were vitrified in a solution composed of 25% ethylene glycol (EG) plus 25% dimethyl sulfoxide (DMSO) + 0.5 M trehalose; and vitrification solution 2 (VS2) group, oocytes were vitrified in a solution composed of 25% EG plus 25% DMSO. Vitrification of COCs was conducted by open pulled straws (OPS) method. Following vitrification and warming, morphologically viable oocytes were matured *in vitro* for 36 h. COCs were then fertilized and cultured *in vitro* for 7 days. The percentage of viable oocytes was significantly higher ($P < 0.05$) in VS2 than VS1 group (80.0% vs. 63.3%, respectively). Nuclear maturation, cleavage (48 h post-insemination; pi), and blastocyst rates (7 days pi) were significantly higher ($P < 0.05$) in VS2 than in VS1 groups. No significant differences were observed in oocyte maturation and development rates between VS2 and control groups. In conclusion, vitrification of immature dromedary camel oocytes in

trehalose-free solution (VS2) was more advantageous than that in trehalose supplemented media since it did not reduce viability and development.

Keywords

Camel, GV, Oocyte, IVM, Trehalose, Vitrification

1. Introduction

Cryopreservation of immature oocytes is an essential approach for the preservation of female germline, providing a non-seasonal, easily accessible source for reproduction and research [1]. Although oocyte cryopreservation was developed more than 40 years ago [2], embryo production rates after *in vitro* fertilization (IVF) from cryopreserved oocytes are still low. Various factors are known to influence the viability and development of cryopreserved oocytes, including species, oocyte quality, stage of maturation, characteristics of the plasma membrane, types of cryoprotectants (CPAs), and cryopreservation techniques [3] [4]. Cryoprotectants are chemicals used during cryopreservation to regulate the water dynamics during cooling and warming procedures [5]. Furthermore, they reduce the freezing point of the solution and subsequently minimize or eliminate ice crystal formation [5]. They are categorized according to their ability to penetrate the cell membrane into penetrating CPAs [e.g., ethylene glycol (EG), glycerol (GLY) and dimethyl sulfoxide (DMSO)], and non-penetrating CPAs, including sugars, and other high molecular weight polymers [6] [7]. Such compounds increase the extracellular osmolality that induces cellular dehydration and reduces the intracellular formation of ice crystals [6] [7]. They also act as osmotic buffers to maintain the structural and physiological integrity of cell membranes and proteins during freezing, as well as safely remove the penetrating CPAs during the warming procedures of vitrified embryos and oocytes [7] [8]. Sucrose and trehalose are the most commonly used non-penetrating CPAs during vitrification and warming of oocytes and embryos. It has been reported that the inclusion of 0.5 M of either sucrose or trehalose in the vitrification solution improved the percentage of viable oocytes post-warming in sheep as compared to those vitrified in sugar-free solutions [9]. However, other studies showed that vitrified/warmed immature ovine, mouse, and buffalo oocytes in sugar-free vitrification solutions could be matured, fertilized, and develop *in vitro* up to the blastocyst stage with high developmental rates [6] [10] [11] [12]. Recently, it has been suggested that exposure of immature buffalo oocytes to sucrose-free vitrification solutions was less detrimental compared to those exposed to a sucrose-containing solution [10].

The dromedary camel, *Camelus* dromedarius, is an important livestock species with high meat and milk production. However, the reproductive potential in camelids is low, partly due to the late onset of puberty, early embryonic mortali-

ty, seasonality, and the length of the gestation period (13 months). Although recent reproductive technologies such as IVF [13] [14] [15] [16] [17] and somatic cell nuclear transfer (SCNT) [18] have been successfully applied to camelids and the birth of live offspring following these technologies has been reported [18] [19], *in vitro* embryo production (IVP) is still not well-developed in this species compared with other domestic species. One of the main reasons contributing to this situation is the limited availability of oocytes due to the scarcity of abattoirs slaughtering female camels as well as the lack of a standardized protocol for *in vitro* maturation (IVM) and IVF of dromedary camel oocytes. Cryopreservation of oocytes would be an alternative way to overcome the limited availability of oocytes, allowing for improvements in IVP in this species. Few studies have been reported on the cryopreservation of dromedary camel oocytes [14]. However, no studies evaluated the impact of the inclusion of sugar during vitrification of dromedary camel oocytes on their developmental potential. Therefore, the aim was to investigate the effect of the presence or absence of trehalose in vitrification solution on viability, IVM rates, and development of vitrified/warmed dromedary camel immature oocytes.

2. Materials and Methods

Unless stated otherwise, all chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.1. Collection of Dromedary Camel Cumulus-Oocyte Complexes (COCs)

Dromedary camel ovaries collected from a local slaughterhouse (Cairo, Egypt) were kept in a thermos flask filled with pre-warmed (30˚C), sterile, normal saline solution (NSS, 0.9% NaCl) until processing. COCs were aspirated from 2 to 8 mm follicles using a 20-gauge needle attached to a 20-mL syringe. The follicular fluid containing the COCs was placed into 50-mL conical tubes with washing medium (HEPES buffered-TCM 199 (H-TCM 199) supplemented with 10% (vol/vol) fetal calf serum (FCS) and maintained at 39˚C for 10 minutes, causing the COCs to settle into the bottom of the tubes. The follicular fluid containing the COCs was poured into a 100-mm Petri dish. COCs with at least one to three layers of compact cumulus cells and a homogenous ooplasm were selected for further experiments. All experiments were approved by Institutional Animal Care and Use Committee (Vet CU12102021340), Cairo University, Egypt.

2.2. Vitrification and Warming of COCs

Selected COCs were equilibrated for 3 min at 37˚C in base medium (BM; TCM 199 + 10% (v/v) FCS) plus 12.5% (v/v) EG and 12.5% (v/v) DMSO. Equilibrated COCs were then exposed for 60 sec to one of the following vitrification solutions (VS), namely: VS1 group, COCs were vitrified in a solution composed of 25% EG plus 25% DMSO + 0.5 M trehalose, and VS2 group, COCs were vitrified in a

solution composed of 25% EG plus 25% DMSO. Groups of five COCs were transferred to 100 µL drop of either VS1 or VS2 before being loaded into open pulled straws (OPS). OPS was prepared according to the method described by [1], briefly, after the cotton plug was removed, the conventional 0.25 mL French straws (IMV, l'Aigle, France) were heated over a hot plate to 200˚C - 250˚C for 10 - 15 sec, the straws were then pulled manually until the inner diameter of the wall reduced to half of their original sizes. After the straws were cooled in the air for 10 sec, they were cut in the middle by a razor plate. OPS was loaded by touching the narrow tip of the straw with a VS droplet containing the oocytes, the loaded OPS was directly plunged into the LN_2. For warming, OPS-loaded ends were submerged into 1 mL of warming solution (1 M of trehalose solution in BM) for 3 min at 37˚C. The COCs were then moved to drops of decreasing concentrations of trehalose solutions (0.5 M and 0.25 M) and then to BM for 3 min each at room temperature.

2.3. Evaluation of the Viability of Vitrified/Warmed COCs

Following vitrification and warming, the oocytes viability was assessed morphologically under a stereomicroscope; oocytes with a spherical, symmetrical shape and no signs of degeneration were considered viable whereas oocytes with losing cumulus cells and/or ruptured zona pellucida, fragmented cytoplasm, or degenerative signs were classified as non-viable. Only viable COCs were selected for IVM [20]. To confirm oocyte viability, a portion of morphologically viable COCs was stained with trypan blue exclusion dye (0.05%). Trypan blue solution (0.05%) was prepared by dissolving trypan blue powder in phosphate buffer saline (PBS; pH = 7.0) and the staining procedures were performed at room temperature for 2 min according to the method previously described [10]. COCs were examined under a phase contrast microscope, and they were categorized as live if they did not take the stain while the fully or partially stained COCs were classified as dead.

2.4. Oocyte IVM and Evaluation of Cumulus Expansion and Nuclear Maturation

IVM of COCs was performed as previously described [17]. Briefly, after washing twice in washing medium and once in maturation medium (TCM-199 with Earle's salts, supplemented with 10% FCS, 10 µg/mL FSH, 50 µg/mL sodium pyruvate, 2.6 mg/mL sodium bicarbonate, and 50 µg/mL gentamycin), groups of 10 to 15 COCs were cultured in 100 µL of pre-warmed maturation medium under mineral oil for 36 h at 39˚C in 5% CO_2 in the air. Following IVM, the proportions of COCs with expanded and loosened cumulus cell layers were identified using a stereomicroscope. Nuclear maturation was assessed using aceto-orcein staining according to the method described by [14]. In brief, 36 h post onset of maturation (hpm), cumulus cells were removed by repeated pipetting in H-TCM 199/PVP containing 300 IU/ml hyaluronidase. Groups of 10 completely denuded oocytes were mounted on a clean glass slide in a small drop of medium.

The slide was prepared by placing four spots of Vaseline (96%) and paraffin wax mixture (4%) in a position equal to the four corners of a cover slip to be used for holding the oocytes. The cover slip was placed onto the Vaseline wax spots and gently pressed down until the oocytes were slightly compressed and not able to roll but remained intact. Slides were placed into a jar containing fixative (ethanol: acetic acid 3:1) for 24 h. Fixed oocytes were stained with 1.0% orcein by drawing the solution under the cover slips using a piece of filter paper. The solution was prepared by boiling 1 gm of orcein in 45 ml acetic acid for two hours, after cooling, the solution was filtrated. The filtrate was then diluted with 55 mL of distilled water to give a 100 mL of a 45% of solution. The solution was stored at room temperature and only upper clear area of the stain was used. Excess orcein was removed by drawing ethanol under the cover slip again using filter paper. The slides were examined under a phase contrast microscope to assess the state of nuclear maturation. Based on the chromatin configuration, oocytes at the metaphase II (MII) stage were recorded as mature.

2.5. *In Vitro* Fertilization (IVF)

Mature oocytes were fertilized *in vitro* using epididymal spermatozoa [15]. Briefly, testicles of mature male dromedary camels were collected and transported to the laboratory in normal saline at 37˚C. The testes were washed twice with normal saline and the spermatozoa were collected from the epididymis by a flushing technique. A small incision was made in the body of the epididymis using a sterile sharp scalpel. Afterward, a 20-gauge sterile needle attached to a 5 mL syringe, filled with flushing medium, namely, Sperm-TALP medium [21] was inserted into the incision. The flushing medium was pushed gently toward the cauda epididymides, and a slight digital pressure was applied all over the epididymis. Another small incision was made in the cauda epididymides. The droplets of the flushing medium containing the spermatozoa were collected in a 100-mm Petri dish. The medium containing the spermatozoa was kept at 39˚C under 5% CO_2 in the air for 10 minutes before being transferred to a 15 mL centrifuge tube. After centrifugation and removal of the supernatant, sperm pellet was re-suspended in 1 mL of sperm-TALP medium supplemented with 5 mM caffeine. Sperm were then incubated for 1 hour at 39˚C for swim up and then sperm motility was evaluated. For IVF, the *in vitro* matured oocytes were washed three times in fertilization medium (TALP supplemented with 6 mg/mL BSA, 50 µg/mL gentamycin, and 5 mM caffeine) and then oocytes were inseminated with motile spermatozoa at a concentration of 2×10^6 spermatozoa/mL. Oocytes and spermatozoa were co-incubated for 18 h at 39˚C in 5% CO_2 in air.

2.6. *In Vitro* Culture and Embryo Evaluation

Eighteen hours post-insemination (pi) the presumptive zygotes were washed three times in H-TCM 199 supplemented with 5% FCS and then twice in embryo culture medium (modified KSOMaa) and groups of five zygotes were cultured in 50 µL drops of embryo culture medium under mineral oil at 38.5˚C in a

humidified atmosphere of 5% CO_2, 5% O_2, and 90% N_2 until Day 7 (Day 0 = day of insemination). Cleavage, and blastocyst development were recorded on Days 2, and 7 pi, respectively.

2.7. Statistical Analysis

Three replicates were used for each experimental group. The post warming viability, nuclear maturation, cleavage, and blastocyst development data were presented as percentages and analyzed by the Chi-squared test. Cumulus cell expansion data were presented as means ± S.E.M. and analyzed by one-way ANOVA followed by Tukey's test. The Statistical Package for Social Sciences SPSS® version 26.0 (SPSS Inc., Chicago, Illinois, USA) was used and the results were statistically significant at $P \leq 0.05$.

3. Results

3.1. Effects of Presence or Absence of Trehalose in Vitrification Solution on the Viability of Vitrified/Warmed Dromedary Camel COCs

No significant difference was observed in the percentage of morphologically viable oocytes between VS1 and VS2 groups. However, when oocytes were stained by trypan blue; vitrification of dromedary camel COCs in VS2 (trehalose-free) solution significantly ($P \leq 0.05$) increased the proportion of viable oocytes compared to those vitrified in VS1 (trehalose-based) solution (80.0% vs. 63.3%) (Table 1).

3.2. Effects of Presence or Absence of Trehalose in Vitrification Solution on Cumulus Cell Expansion and Nuclear Maturation of Vitrified/Warmed Dromedary Camel Immature Oocytes

Vitrification of dromedary camel COCs in a trehalose-free solution (VS2) significantly increased ($P \leq 0.05$) the percentage of oocytes with fully expanded cumulus cells compared to those vitrified in the presence of trehalose (VS1). No significant difference was observed in the percentage of oocytes with expanded cumulus cells between VS2 and the control group (Figure 1). As shown in Table 2,

Table 1. Post warming recovery and viability rates of vitrified/warmed dromedary camel immature oocytes with and without trehalose in vitrification solutions.

Treatment	Total number of COCs	Number of recovered oocytes (%)	Number of morphologically viable oocytes (%)	Number of viable oocytes by trypan blue (%)
VS1	194	136 (70.1)	111 (81.6)	19/30 (63.3)[a]
VS2	193	138 (71.5)	120 (86.9)	24/30 (80.0)[b]

VS1-Oocytes vitrified in vitrification solution composed of 25% EG plus 25% DMSO and 0.5 M trehalose. VS2-Oocytes vitrified in vitrification solution composed of 25% EG plus 25% DMSO. Dissimilar superscripts in the same column are significantly different at $P \leq 0.05$ (n = 3).

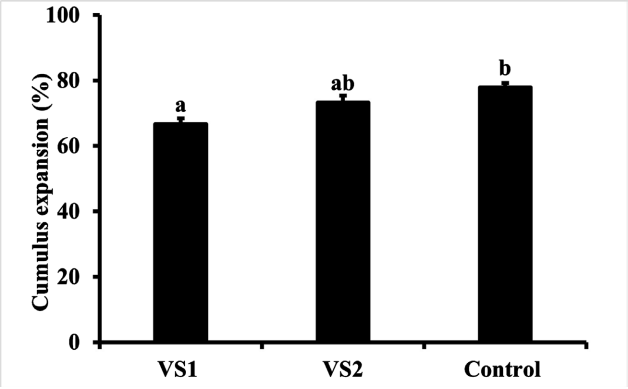

Figure 1. Cumulus cell expansion following *in vitro* maturation of dromedary camel oocytes vitrified at the germinal vesicle stage using Open Pulled Straws (OPS) in vitrification solution either supplemented with or without trehalose. Percentages of IVM oocytes showing expanded cumulus cells after vitrification with VS1 (20% EG plus 20% DMSO plus 0.5 M trehalose) or VS2 (25% EG plus 25% DMSO). Control are those oocytes that were subjected to *in vitro* maturation without vitrification. Data are presented as the means ± S.E.M. Different small letters indicate significant differences at $P \leq 0.05$.

Table 2. *In vitro* maturation of camel oocytes vitrified at the germinal vesicle (GV) stage using trehalose and trehalose free vitrification solutions.

Treatment	Number of oocytes	MII Oocytes (%)
VS1	30	16 (53.3)[a]
VS2	30	22 (73.3)[b]
Control	30	23 (76.7)[b]

VS1-Oocytes vitrified in vitrification solution composed of 25% EG plus 25% DMSO and 0.5 M trehalose. VS2-Oocytes vitrified in vitrification solution composed of 25% EG plus 25% DMSO. Control-fresh oocytes subjected to *in vitro* maturation without vitrification. Dissimilar superscripts in the same column are significantly different at $P \leq 0.05$ (n = 3).

the nuclear maturation (the percentage of MII oocytes) rate was lower ($P \leq 0.05$) in the VS1 group than in the control and VS2 groups (53.3 % vs. 73.3 % and 76.7%, respectively).

3.3. Effects of Presence or Absence of Trehalose in Vitrification Solution on Preimplantation Embryo Development Following IVM and IVF of Vitrified/Warmed Dromedary Camel Immature Oocytes

Cleavage and blastocyst rates were higher ($P \leq 0.05$) in the trehalose-free (VS2) than in trehalose containing (VS1) groups (36.7% and 21.7% vs. 25.5% and 13.7%, respectively). No significant differences were observed in the cleavage and blastocysts rates between trehalose-free and control groups Table 3.

4. Discussion

In the present study, we investigated the impact of the inclusion of trehalose during vitrification on the viability and development of vitrified/warmed dromedary camel

Table 3. Cleavage rates of vitrified/warmed germinal vesicle (GV) stage camel oocytes in presence and absence of trehalose in vitrification solutions after IVM/IVF.

Treatment	Oocytes (n)	Cleavage 48h-pi n (%)	Blastocysts/oocyte n (%)	Blastocysts/cleaved n (%)
VS1	51	13 (25.5)[a]	7 (13.7)[a]	7 (53.8)
VS2	60	22 (36.7)[b]	13 (21.7)[b]	13 (59.1)
Control	74	30 (40.5)[b]	18 (24.3)[b]	18 (60.0)

VS1-Oocytes vitrified in vitrification solution composed of 25% EG plus 25% DMSO and 0.5 M trehalose. VS2-Oocytes vitrified in vitrification solution composed of 25% EG plus 25% DMSO. Control-fresh oocytes subjected to *in vitro* maturation without vitrification, pi-post-insemination. Dissimilar superscripts in the same column are significantly different at $P \leq 0.05$ (n = 3).

GV-oocytes. The results showed that omitting trehalose from vitrification solution enhanced viability, maturation rate and developmental competence of vitrified/warmed dromedary camel immature oocytes. These results highlight the critical roles played by non-penetrating cryoprotectants during vitrification and warming of COCs.

Vitrification is widely used in scientific, and clinical research to keep cells alive for long periods. To achieve this, water must be vitrified on both the inside and outside of the cells. Vitrification is typically accomplished by implementing relatively high concentrations of CPAs into the media and using high cooling and warming rates [22]. Several procedures, including cryotop [23], cryoloop [24], solid surface vitrification (SSV) [25], nylon mesh [26], and OPS [27], have been utilized to accomplish high cooling rates by decreasing the volume of the vitrification solutions. Exposure of oocytes to high concentrations of CPAs during vitrification is toxic and can cause zona hardening and parthenogenetic activation, which harmfully affect the fertilization ability and development of vitrified/warmed oocytes [28]. These toxic effects could be overcome by several approaches including pretreatment of oocytes with lower concentrations of cryoprotectants before exposure to the final vitrification solutions, controlling the time of exposure and choosing the least toxic permeating cryoprotectant agents [28]. Exposure of oocytes to sugars during vitrification could perhaps result in a hyperosmotic gradient across the cell membrane, resulting in a withdrawal of intracellular water, reducing the likelihood of intracellular ice formation, and minimizing freezing injuries [29]. Sugars may also increase the viscosity of intracellular solutes, reducing the intracellular toxicity of penetrating cryoprotectants [22]. Sugars have long been used in vitrification media for cryopreservation of reproductive cells in most mammalian species, particularly mature oocytes [30] [31] [32]. The inclusion of sugars during vitrification of immature COCs at the GV-stage remains a debate. Previous research showed that vitrification of GV-oocytes in sugar-free vitrification media had high survival and development rates following IVM and IVF [10] [11] [12] [20] [33] [34] [35]. In the present study, we found that the post warming viability as evaluated by trypan blue exclusion dye

(Table 1) and nuclear maturation rates (Table 2) of dromedary camel oocytes vitrified in absence of trehalose improved by about 20% compared to those vitrified in presence of trehalose. Moreover, the percentage of oocytes with expanded cumulus cells after IVM of COCs vitrified in the absence of trehalose in the vitrification solution was comparable to those seen in the fresh control oocytes without vitrification (Figure 1). Our results suggest that the inclusion of trehalose during the vitrification of immature COCs may has deleterious effects on their viability, maturation, and subsequent development. A recent study showed that exposure of immature dromedary camel oocytes to 40% EG + 40% DMSO (without inclusion of trehalose) resulted in a high percentage of survival rate (90.16%) and maturation rate (58.95%) [36]. On the other hand, another study demonstrated that dromedary camel GV-oocytes could be successfully matured *in vitro* when they vitrified in presence of trehalose [14]. Our results showed a higher nuclear maturation rate of vitrified warmed camel oocytes in absence of trehalose upon using the same concentration of CPAs (EG 25% + DMSO 25%) and the same cryodevice (OPS) than Fathi *et al.*, 2018 [14]. In buffalo, the viability and maturation rates of COCs vitrified at the GV-stage in sugar-free media were higher compared to those vitrified in the presence of sugar [10]. In cattle, previous studies showed that exposure of MII oocytes to a solution containing high level of sugar; namely, sucrose induced high frequencies of meiotic spindle abnormalities, mainly due to the induced osmotic stress [37]. To the best of our knowledge, our study is the first to compare the effect of the presence or absence of trehalose during vitrification on the developmental competence of dromedary camel immature oocytes. Trehalose is a naturally occurring non-toxic disaccharide that has a similar chemical structure and effect on water activity as sucrose. It is a stable sugar that does not interact with proteins, even under low pH conditions when other disaccharides are hydrolyzed into their monosaccharide state [28]. The influences of sugar types used during vitrification on the developmental potential of oocytes and embryos are still in dispute. For instance, similar blastocyst rates were reported following culture of pronuclear mouse embryos vitrified in either sucrose or trehalose containing vitrification solutions [38]. Similar findings were also observed when porcine mature oocytes were vitrified in sucrose or trehalose-based media [8]. However, previous studies showed that exposure of bovine mature oocytes to trehalose was less harmful than exposure to sucrose [39]. These findings were suggested to be due to the ability of trehalose to prevent changes to the cell membranes during reduced water states [40]. We demonstrated that vitrification of dromedary camel immature oocytes at the GV stage in trehalose-free solution resulted in better cleavage and blastocyst rates after IVM/IVF and embryo culture than those vitrified in a solution containing trehalose. The cleavage and blastocyst rates (36.7% and 21.7%, respectively) reported in the current study using VS2 (trehalose-free solution) were higher than those reported previously when dromedary camel immature oocytes were vitrified with the same cryo-carrier (OPS) and the same concentrations of permeating cryoprotectants (25% EG and 25% DMSO) in addition to trehalose (22.9%

and 6.1%, for cleavage and blastocyst rates, respectively) [14]. These findings indicate that omitting the sugar from vitrification solution has positive influences on viability and development of vitrified/warmed dromedary camel immature oocytes. The negative impact of trehalose on maturation rates of dromedary camel COCs reported in our study could be due to the high viscosity caused by both penetrating and non-penetrating cryoprotectants, which may harmfully affect the bidirectional communication between cumulus cells and oocytes. It is well known that this communication is critical for oocyte maturation and development *in vitro*. Previous studies showed that removal of sugar during vitrification of mouse blastocysts was beneficial for their development [41]. Moreover, sugar-free vitrification solutions were found to be optimal for cryopreservation of human blastocysts without compromising survival rate and pregnancy outcome [42].

5. Conclusion

Vitrification of immature dromedary camel oocytes in trehalose-free solution (VS2) was more advantageous than that in trehalose supplemented media since it did not reduce viability and development.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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