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Effect of Vitrification on Mature Buffalo Oocytes and Their Fertilization Rates using Different Concentrations of Cryoprotectants

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ABSTRACT

Background: Buffalo is an integral part of livestock agriculture in Asia since many centuries, because they provide draught power, milk, meat and hide to millions of people, particularly small-scale farmers. In addition, female buffaloes have few primordial follicles and a higher rate of follicular atresia. Therefore, the emphasis has now shifted to *in vitro* embryo production (IVEP). Oocyte cryopreservation expands the potential of IVF (*in vitro* fertilization). Cryopreservation of oocytes by vitrification, especially with the use of very high cooling rates for oocytes suspended in extremely small volumes of various additives, seems the most appropriate method. The present investigation was aimed to test the efficacy of different concentrations of cryoprotectants like EG (Ethylene glycol), DMSO (Dimethyl sulphoxide), their combinations and their effect on mature oocytes and IVF of vitrified mature buffalo oocytes and their comparison with the control/non-vitrified oocytes.

Methods: A total of 2,127 matured oocytes were used in the present study which was undertaken during the year 2015-17. For the process of vitrification, different concentrations of EG (10, 20 and 30%), DMSO (10, 20 and 30%) and EG+DMSO (15% and 20%) in eight treatments were used.

Result: Highest morphological survival rate was noticed in 30% EG (90.55±0.79) while lowest was seen in 10% DMSO (77.56±1.59 per cent). The proportion of damage was significantly higher in 10% DMSO (22.44±1.59 per cent) than 20% (14.40±2.93 per cent), 30% EG (9.44±0.79 per cent) and 20% EG+20% DMSO (10.60±2.19 per cent). The cleavage rate was significantly ($P \leq 0.05$) higher in 30% EG (41.65±0.53 per cent), 20% EG+20% DMSO (43.29±0.65 per cent) and 30% DMSO (39.75±0.69 per cent) than 20% EG (30.95±0.81 per cent), 10% EG (27.71±0.56 per cent), 20% DMSO (33.22±0.52 per cent), 10% DMSO (25.38±.60 per cent) and 15% EG+15% DMSO (38.01±1.24 per cent).

Key words: Buffalo, Hemi straw, *In vitro* Fertilization, Mature oocytes, Vitrification.

INTRODUCTION

Cryopreservation of oocytes is difficult in most animal species because of their large volume, high sensitivity to cooling, low surface area to volume ratio, high water content and low hydraulic conductivity (Leibo, 1980). Cryobiology in reproduction has revolutionized applications to preserve the genetic material of rare breeds, endangered species or transgenic animals (Dua *et al*., 2021). Various protocols for cryopreservation involved slow cooling, rapid cooling and ultra rapid cooling. In the past decade, various new methods for embryo cryopreservation have been published (Niemann, 1991; Rall, 1992). Among these methods, vitrification have been widely used and is now regarded as a potential alternative to traditional slow-rate freezing. Vitrification which is defined as a physical process by which a highly concentrated solution of cryoprotectants solidifies during cooling, without formation of ice crystals (Niemann, 1991). The success of vitrification was determined by subjecting vitrified-warmed oocytes to IVF and comparing fertilization and subsequent embryo development to those of fresh oocytes. Laboratory production of embryos allows mass scale production of embryos for practical and scientific purposes as well as for the exploitation of oocytes from donors (Pitroda *et al*., 2024).

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MATERIALS AND METHODS

A series of experiments were carried out to standardize the best concentration and combination of cryoprotectants.

Various concentrations and combinations of cryoprotectants using EG and DMSO for cryopreservation of mature oocytes and their effect on IVF were studied.

Group I (n=2,127): Cryopreservation of mature oocytes following *in vitro* maturation.

Treatment I: 10% EG (n=266/5) Treatment II: 20% EG (n=265/5) Treatment III: 30% EG (n=266/5) Treatment IV: 10% DMSO (n=265/5) Treatment V: 20% DMSO (n=266/5) Treatment VI: 30% DMSO (n=267/5) Treatment VII: 15% EG+15% DMSO (n=266/5) Treatment VIII: 20% EG+20% DMSO (n=266/5.

Group II (n=261/5): Control (fresh oocytes) without cryopreservation.

*n=Number of oocytes/no. of replicates. All the experimental groups were subjected to IVF.

Vitrification of mature buffalo oocytes

Vitrification was done as per the standard protocol (Dutta *et al*., 2013). The mature oocytes were exposed to vitrification solution 1 (half of the original concentration i.e 5, 10, 15 per cent EG; 5, 10, 15 per cent DMSO and 7.5, 10 per cent EG+DMSO) for equilibration upto 3 minutes followed by 25-30 seconds in vitrification solution II (original concentration *i.e* 10, 20, 30 percent EG; 10, 20, 30 per cent DMSO and 15, 20 per cent EG+DMSO) at room temperature (22-25°C). The oocytes in vitrification solution II were immediately loaded into hemi-straw and plunged into liquid nitrogen (LN_2). Straws were stored for a period of 7 days.

Hemi-straw (HS) method

It was carried out according to the method used by Vandervorst *et al*. (2001) with slight modifications. The open end of a 0.25 ml straw (6.5 cm) was cut with a surgical blade, so that the end of the straw was open (about 1 cm in length) and it was easy to pipette a small droplet (<1.0 µ diameter) onto the open inner surface of the straw. The 0.25 ml straw with the cut end was inserted into a 5 cm length of 0.5 ml straw. The cut end of the straw (0.25 ml) was pressed with an artery forceps to become flat at 1.5 cm below the cotton plug to avoid the slipping from the covering (0.5 ml straw). Prior to loading, the COC's were exposed to vitrification solutions. After placing a drop (0.75 µl) of VS II with COC's into the inner surface of the cut end of the straw, the HS was directly dipped into the LN_2. Then the 0.25 ml straw was pulled back (1.5 cm) to cover the open end of the straw with 0.5 ml straw. Similarly, for warming the 0.25 ml straw was pushed 1.5 cm into the 0.5 ml straw in sucrose solution, so that the cut end of the straw comes out of the covering.

Thawing/warming of vitrified oocytes

After 7 d, the straws were warmed and the oocytes were rehydrated in serial dilutions of sucrose (0.3 M and 0.15 M in holding medium) each for 1 minute and washed in holding medium (filtered Dulbecco's phosphate buffered saline with 20% estrus buffalo serum, 0.4% bovine serum albumin and 50 µg/ml of gentamicin) at the end.

Assessment of oocyte survival

The survival of oocytes post-thaw and embryos after IVF was evaluated by morphological examination. Oocytes with homogenous ooplasm, intact membranes and zona pellucida post-thaw were recorded as living oocytes (Men *et al*., 2002) which were observed under stereo zoom microscope.

***In vitro* fertilization (IVF)**

Two frozen thawed murrah buffalo semen straws were used for each experiment.

Preparation of motile sperm

By swim-up method described by Parrish *et al*. (1995) was used to separate the motile fraction of sperm. 0.5 ml semen was diluted with 4.5 ml of SOF (Synthetic oviductal fluid) and centrifuged at 200g for 5 min. Then the supernatant was removed and the sperm pellet was resuspended with SOF and recentrifuged. Lymphocyte tubes containing 1 ml of swim up medium was layered with 0.2 ml of sperm pellet at the bottom. After 1 h incubation at 39°C at 5% CO_2 under humidified air, the top most 0.5 to 0.8 ml of medium containing the motile fraction of sperm was removed from each tube and pooled in a sterile centrifuge tube. This sample was centrifuged at 200 g for 5 min. After washing, sperm concentration was assessed by using Neubaeur counting chamber and diluted to a final sperm concentration of 2×10^6 sperm/ml progressive motile spermatozoa.

***In vitro* fertilization of oocytes**

After maturation, oocytes were denuded off cumulus cells by repeated pipetting in SOF medium and were washed thrice with IVF medium. Subsequently, mature oocytes were selected for IVF and placed in 75 µl droplets (10 oocytes/ droplet) of IVF medium (SOF supplemented with 20% (v/v) estrus buffalo serum, 2 mM glutamine, 2% BME- essential amino acids and 1% MEM-non essential amino acids) in 35 mm tissue culture dishes. The droplets were overlaid with pre equilibrated light weight mineral oil. 5µl aliquot of sperm suspension was transferred into each 75 µl fertilization droplet and incubated for 24 hours at 38.5°C in 5% CO_2 under humidified atmosphere in air.

***In vitro* culture following fertilization (Day 0)**

Oocytes were washed with SOF and cultured in SOF for another 6 days at 38.5°C in 5% CO_2 under humidified atmosphere in air. During culture, the embryos were transferred to fresh SOF medium every 48 hours.

Evaluation of embryos

The cleavage was assessed at 24 and 48 hours post insemination and the number of embryos at each stage was recorded under stereo zoom microscope.

Statistical analysis

Fertilization rates of vitrified oocytes were analyzed statistically and compared with the non-vitrified oocytes

(control). Comparisons of fertilization rates of cryopreserved mature and control groups were analyzed by one-way analysis of variance (ANOVA) using software (SPSS, 2005, Version 16).

RESULTS AND DISCUSSION

A total of 2400 immature oocytes were taken for maturation. Out of which, 2,127 matured oocytes were vitrified using different concentrations and combinations of EG and DMSO. Various post-thaw/warming oocyte quality parameters (Fig 1 Table 1) for different concentrations of cryoprotectants were observed.

Post-thaw recovery rate

The post-thaw recovery rate in the present study ranged between 92.19±0.73 to 95.31±1.51 per cent. These were in agreement to the findings of Sharma and Loganthasamy (2007) (89-92%), Dolakasaria *et al*. (2013) (91.06%), Loganathasamy and Sharma (2014) (89-92%) using French mini straw and higher than Mishra *et al*. (2012) (81.35%) using straw. The post thaw recovery rate was noticed immediately after warming/thawing the oocytes which were placed in hemi-straw in serial dilutions of sucrose. The recovery rate of oocytes for different concentrations of cryoprotectants were placed in Table 1. There was no significant difference in post-thaw recovery rate among different treatment groups. The recovery rate of vitrified thawed oocytes has been reported to vary from 80-100% in different species (Fuku *et al*., 1992 and Nowshari *et al*., 1994) which was in agreement with the present study. The loss of oocytes oocur due to sticking of oocytes on inner wall of straw adhering to rough surface or oocyte disintegration due to improper vitrification. Furthermore, it was demonstrated that warming is also a critical step of procedure and that the efficiency of vitrification may be improved by utilizing higher concentration of sucrose and multistep dilution approach. Step wise dilution might be helpful to reduce osmotic injury of vitrified oocytes after thawing.

Morphological survival rate of vitrified mature oocytes post-thawing

The morphological survival rate of oocytes for different concentrations of cryoprotectants were shown in Table 1. Highest survival rate was noticed in 30% EG (90.55±0.79 per cent) while lowest was seen in 10% DMSO (77.56±1.59 per cent). In the current study, the morphological survival rate in 20% EG+20% DMSO (88.45±1.95 per cent) was in

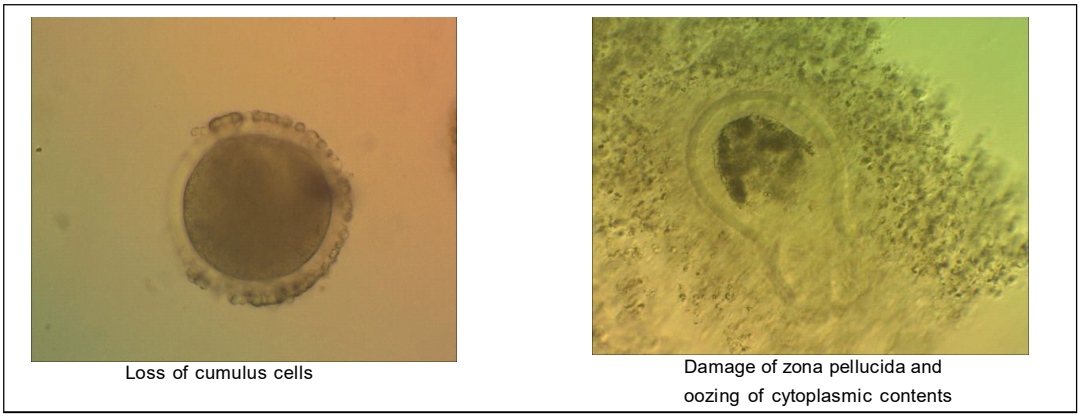

Fig 1: Post warming quality parameters.

Table 1: Effect of vitrification on maturation status of *in vitro* matured oocytes using different concentrations of cryoprotectants.

Treatment groups	Concentration of cryoprotectant	No. of mature oocytes	Post thaw* ecovery rate	Normal morphological survival rate*	Damaged percentage*	Maturation rate*
I	10% EG	266/5	249(93.57±0.41)	203 (81.37±0.92^{ab})	46 (18.62±0.92^{ab})	76.14±1.19^{a}
II	20% EG	265/5	252 (95.35±1.22)	221(85.59±2.93^{bcd})	31(14.40±2.93^{a})	80.88±3.73^{bc}
III	30% EG	266/5	251(94.13±.56)	228(90.55±.079^{d}	23(9.44±0.79^{a})	85.2600±1.01^{c}
IV	10% DMSO	265/5	245(92.19±.73)	191(77.56±1.59^{a})	54(22.44±1.59^{b})	71.51± 1.7^{a}
V	20% DMSO	266/5	253(95.31±1.51)	211(83.28±1.45^{abc})	42(16.72±1.45^{a})	79.3±0.68^{bc}
VI	30% DMSO	267/5	250(93.73±1.17)	217(87.42±3.02^{bcd})	33(14.57±2.35^{ab})	80.04±2.25^{bc}
VII	15% EG+15% DMSO	266/5	249(93.32±1.75)	216(85.44±3.17^{bcd})	33(14.55±3.17^{ab})	79.84±3.8^{bc}
VIII	20% EG+20% DMSO	266/5	250(93.24±1.56)	227(88.45±1.95^{cd})	23(10.60±2.19^{a})	83.48±3.29^{bc}

*Figures in paranthesis are mean%±SE. Figures with different superscripts with in the column are significantly different. One way ANOVA followed by Duncan's multiple range test (P≤0.05).

Table 2: Effect of vitrification on *in vitro* fertilization and embryonic development of vitrified mature oocytes using different concentrations of cryoprotectants.

Concentration of cryoprotectant	No. of mature oocytes/replicates	No. of oocytes morphologically survivived	Cleavage rate*	2 cell stage*	4 cell stage*	8 cell stage*	Morula stage*
10% EG	266/5	203	56(27.71±.56^{b})	28(13.90±.53^{a})	23(11.39±.42^{a})	18(8.88±.36^{ab})	17(8.37±.19^{ab})
20% EG	265/5	221	71(30.95±.81^{c})	35(16.36±.82^{b})	29(13.35±.72^{ab})	25(10.91±.76^{bc})	24(10.63±.77^{bcd})
30% EG	266/5	228	95(41.65±.53^{fg})	48(21.27±.76^{d})	39(17.33±.57^{c})	31(13.69±.88^{cd})	29(12.72±.31^{d})
10% DMSO	265/5	191	49(25.38±.60^{a})	26(13.45±.48^{a})	22(11.74±.82^{a})	16(7.47±1.99^{a})	16(7.48±1.99^{a})
20% DMSO	266/5	211	70(33.22±.52^{d})	34(16.14±.50^{b})	28(13.31±.40^{ab})	24(11.41±.82^{bc})	21(9.88±.41^{abc})
30% DMSO	267/5	217	87(39.75±.69^{ef})	44(20.23±1.09^{cd})	36(17.02±.97^{c})	32(14.97±1.33^{d})	26(11.68±.34^{cd})
15% EG + 15%DMSO	266/5	216	83(38.01±1.24^{e})	42(19.23±.65^{c})	40(14.99±.80^{b})	26(11.58±1.09^{bc})	21(9.58±.35^{abc})
20% EG + 20%DMSO	266/5	227	97(43.29±.65^{g})	49(21.64±.33^{d})	43(18.71±.49^{c})	34(14.71±.56^{d})	29(12.13±0.85^{cd})
Control group (Freshmature ooctes)	261/5	261	132(50.34±.82^{h})	72(27.69±.56^{e})	61(23.32±0.78^{d})	55(20.96±.32^{e})	39(15.36±.36^{e})

*Figures in paranthesis are mean%±SE. Figures with different superscripts with in the column are significantly different. One way ANOVA followed by Duncan's multiple range test (P≤0.05).

agreement to the findings of Attanasio *et al*. (2007) (83.6-92.8%) using cryotop, Sharma and Loganathasamy (2007) (92.1%), Gautam *et al*. (2008) (96%) using straw and higher than findings of Luna *et al*. (2001) (72.6%), Morato *et al*. 2008 (60.3%- 60.6%) using cryotop and OPS and Mishra *et al*. (2012) (74.28%) using straw. The survival rate noticed in 30%EG (90.55±0.79 per cent) was correlated with the findings of Boonkusol *et al*. (2007) (89.3%), Gasparrini *et al*. (2007) (95.8%) and Gautam *et al*. (2008) (85%) using 35% EG and 40% EG, respectively.

Damage of vitrified mature oocytes post-thawing

The proportion of damage was significantly higher in 10% DMSO (22.44±1.59 per cent) than 20% (14.40±2.93 per cent), 30% EG (9.44±.79 per cent) and 20% EG+20% DMSO (10.60±2.19 per cent) (Fig 1). Mishra *et al*. (2012) reported proportion of morphologically survived and damaged oocytes as 74.28% and 25.71%, respectively using 20% EG+20% DMSO which were lower and higher, respectively than the present study.

Fertilization rate and embryonic development of vitrified mature oocytes

The cleavage/fertilization rate in the present study was significantly higher in 30% EG (41.65±0.53 per cent), 20% EG+20% DMSO (43.29±0.65 per cent) and 30% DMSO (39.75±0.69 per cent) than 20% EG (30.95±0.81 per cent), 10% EG (27.71±0.56 per cent), 20% DMSO (33.22±0.52 per cent), 10% DMSO (25.38±0.60 per cent) and 15% EG+15% DMSO (38.01±1.24 per cent). Among all the treatment groups, cleavage rate was significantly less in 10% DMSO (25.38±0.60 per cent). The cleavage rate in 30% EG (41.65±0.53 per cent) was similar to the findings of Sripunya *et al*., 2010 (41.65%) using SSV, Ocampo *et al*., 2014 (40.0%) using 40% EG. In the present study, the cleavage rate in 20 % EG +20% DMSO (43.29±0.65 per cent) was correlated with Attanasio *et al*., 2007 (36.3-55.3%), Morato *et al*., 2008 (31.5-46.1%). In the current study, the cleavage rate in 15% EG+15% DMSO (38.01±1.24 per cent) was correlated with the reports of Zhou *et al*., 2010 (35.2%). After culturing, different stages of embryonic development Fig 2 like cleaved oocytes, 2 cell, 4 cell, 8 cell and morula stage embryos were shown in Table 2.

Fertilization rate and embryonic development of buffalo oocytes in control (Non-Vitrified) group

The fertilization rate (50.34±0.82 per cent) recorded in this group was like observations made by Madan *et al*., 1994 (54.4%), Samad *et al*., 1998 (55.38%), Hegab *et al*. (2009) (50%) using TALP medium, Sadeesh *et al*. (2014) (44.1± 2.9-64.8±3.8 per cent) using TALP and BO medium. This is higher than reports of Hammam *et al*. (2010) (11-24%), Mehmood *et al*. (2011) (34%) using sperm TALP medium, Khandoker *et al*. (2012) (23.28-30.52%) using BO medium, Singh *et al*. (2012) (20.16%) using modified TALP medium, Rahman *et al*. (2015) (19.63±3.11 and 29.52±1.98 per cent) and Ruhil and Purohit (2015) (16.4%). In the present study,

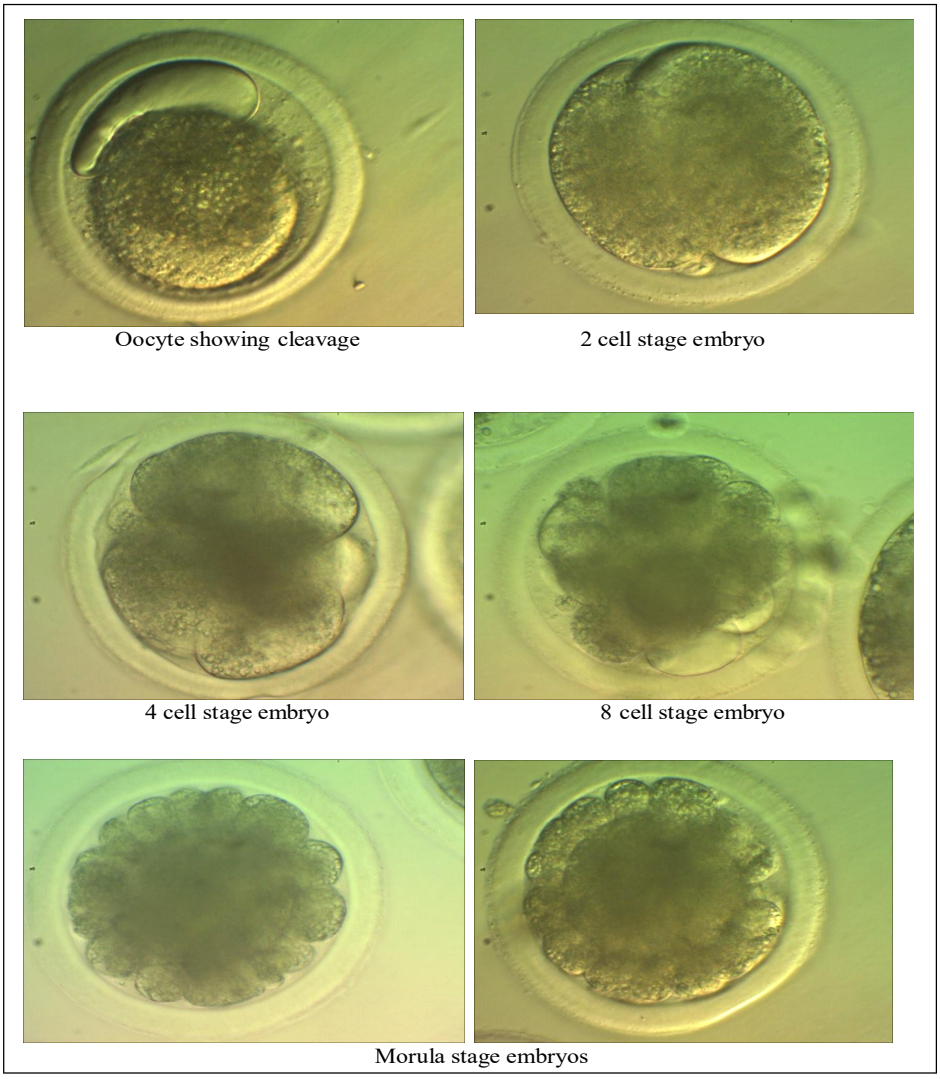

Fig 2: *In vitro* embryonic development of oocytes in vitrified group.

swim up method in modified SOF medium was found to be the best method for preparation of buffalo bull spermatozoa, as determined by significantly higher recovery of motile spermatozoa. On contrary, the present fertilization rate recorded was lower than the findings of Abdoon *et al*. (2001) (63.0±0.5%), Tatham *et al*. (2001) (67.2%), Patil and Totey (2003) (78.4%), Neglia *et al*. (2003) (65%), Jamil *et al*. (2007) (63.75±2.81%) using Ca++ tyrodes medium, Mehmood *et al*. (2007) (75%) using hypotaurine, epinephrine and Kadoom *et al*. (2014) (64.5%) using IVF-TALP medium. The reduced embryonic development in the present study may be due to high sensitivity of buffalo oocytes and embryos to oxidative stress because of their high lipid content. Increased oxidative stress appears to be a major factor impairing *in vitro* mammalian development.

CONCLUSION

The mature oocytes are more resistant to cryopreservation. This might be because of the cytoskeleton of the first meiotic division in immature oocytes is particularly susceptible to damage. Mature oocytes display a more flexible cytoskeleton, which may be one reason that they are less subjected to cryo damage (Allworth and Albertini, 1993). The results of the present study showed that concentrated solutions of permeating cryoprotectants like EG and DMSO were required for successful cryopreservation of oocytes when rapid cooling and warming rates are used which was supported by Arav (2014). Therefore, it was suggested that cryopreservation of buffalo oocytes can be best achieved with 30% EG, 30% DMSO and 20% EG+20% DMSO with least damage to the oocytes.

Conflict of intrest

The authors have no conflict of interest.

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