Three live births after human embryo vitrification with the use of aluminum oxide as an intermediate cooling agent: a case report

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Objective: To study the possibility of increasing the cooling rates of the vitrification procedure in a closed system with the use of aluminum oxide as an intermediate coolant.
Design: Case report.
Subjects: Six patients undergoing procedures for assisted reproduction.
Intervention: Comparative studies of cryopreservation of donor embryos with aluminum oxide as an intermediate cooling agent (experimental group) and without it (control group) have been performed. After thawing, the embryo morphology and its potential to develop to the blastocyst stage have been assessed. The methodology was then applied to clinical practice.
Main Outcome Measures: Twenty embryos of 6 patients have been vitrified on day 4 after fertilization with the use of aluminum oxide as an intermediate coolant. Fourteen of them have been thawed. All have displayed normal morphology and 10 have formed blastocysts after 24 hours of culture. Four of the patients received embryo transfer with 2 embryos and the other 2 with single embryos.
Results: After preliminary comparative studies of embryos frozen with aluminum oxide and a control group, the results showed no statistically significant difference between their quality and potential to reach to blastocyst stage. That gave us ground to apply the methodology in clinical practice. After the embryo transfer, 3 clinical pregnancies with successful live births have been obtained.
Conclusions: Our experience shows that preimplantation embryos can be cryopreserved aseptically, in closed systems, with the help of aluminum oxide as an intermediate coolant. (Fertil Steril Rep® 2024;5:145–51. ©2024 by American Society for Reproductive Medicine.)
Key Words: Human embryo, vitrification, survival rate, intermediate cooling agent, case report

Cryopreservation of preimplantation embryos is a routinely used technique in programs for assisted reproduction. In recent years, vitrification is the method of choice over slow freezing for cryostorage for elective embryo transfer (ET). It has been shown that over 90% of the embryos retain their viability after thawing and the percentage of clinical pregnancies is not significantly different from fresh ET (1–3). Perinatal and neonatal outcomes after vitrification are also comparable to those after fresh ET (4). Similar rates of congenital malformations in children born after vitrified/warmed ET when compared to those born from fresh ET also have been reported (5). In 2019, according to the Centers for Disease Control and Prevention, 78.8% of all cycles were frozen ETs (6).

The main parameters that determine the overall survival rates of different biological objects during vitrification are the cooling and thawing rates, viscosity and volume of the sample (7, 8). The procedures include the exposure of cells to relatively high concentrations of the cryoprotectants, but for a limited time to avoid their cytotoxicity, and small sample size coupled with rapid cooling rates. In brief, the technology comprises of stepwise increase of the concentration of cryoprotectants in the freezing medium (3).

After the discovery of vitrification at the beginning of the implementation of the procedure in laboratories, different types of carrier devices for both embryo and oocyte freezing were used. At present, as more data have been accumulated for their efficacy, most of them have similar size and shape, which allows for the highest possible cooling rates to be achieved with minimal volume of the

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cryoprotective media (9–11). Up until now, in literature approximately 30 different carrier tools have been described, and approximately 15 versions are commercially available (12). Two types of carrier devices are used in the vitrification procedures—open and closed. Among the most popular are the commercially available modifications of the first carrier tools like the open-pulled straws (13), Cryoloop (14, 15) and Cryotop (16, 17). These devices are open systems (3), and they allow the achievement of higher cooling rates and give better overall parameters than the closed, sterile systems.

During the process of vitrification, initially in the equilibration phase the oocytes and embryos are placed for 12–15 min in a medium containing 7.5% ethylene glycol (EG) and 7.5% dimethyl sulfoxide (DMSO). Afterward, they are transferred for approximately 1 min in the vitrification medium supplemented with 15% EG, 15% DMSO, and 0.5 M sucrose. The embryos are then placed on an open carrier device, which is plunged into liquid nitrogen. The high cooling rates achieved in such a way, together with the high concentrations of the cryoprotectants lead to the freezing of water molecules in a glass-like state, without crystal formation. Finally, the carrier is sealed and kept in super-low temperatures. The use of "open systems" allows for high cooling rates to be achieved (>20,000°C/min). Ready-to-use media for vitrification and carriers are commercially available.

On the other hand, the contact with the liquid nitrogen leads to a risk of exposure to bacteria, fungi, viruses, heavy metals, etc. (18–24). Investigators report high contamination levels by microorganisms and fungi in carrier devices immersed in liquid nitrogen (25, 26).

Regrettably, a convenient method for use in clinical practice for the sterilization of liquid nitrogen still has not been developed. To avoid the risk of contamination, some investigators suggest the freezing to be performed in sterile liquid air (27), a possibility which has been confirmed to be efficient by our investigations as well (28). In 2016, the use of liquid helium, instead of liquid nitrogen was suggested by Chinese investigators (29). However, it is important to be noted that these technologies are not readily available for application in clinical practice.

All mentioned above determines the necessity to research other alternatives as aseptic technologies for cryopreservation in closed carrier systems, such as CryoTip (30), and the development of new closed devices to protect from direct contact and contamination in the liquid nitrogen. Furthermore, 2 of the leading organizations in the field of assisted reproductive technologies—the American Society for Reproductive Medicine (31), and the European Society for Human Reproductive Embryology (32) have recommended performing freezing and storage of reproductive cells and tissues in closed carrier systems. The European Union Directive concerning the packaging of cells and tissues states that "Following procurement, all recovered tissues and cells must be packaged in a manner which minimizes the risk of contamination and must be stored at temperatures that preserve the required characteristics and biological function of the cells/tissues" (33, 34).

The possibility to increase the speed of cooling in those cases definitely attracts the attention of the investigators in the field. The main decrease in the cooling rate when the devices are plunged into liquid nitrogen is due to the formation of bubbles at the boundary biological object—medium due to the nitrogen "boiling". A layer of vapor is created, which hampers the direct contact of the liquid nitrogen with the cryopreservation device or carrier, the so-called Leidenfrost effect (35). In liquid nitrogen, it can lower significantly the heat transfer coefficient and lead to reduced cooling rates (36). To avoid this phenomenon, some investigators suggest a different approach—vitrification in liquid nitrogen slush, which is at −210°C (37–39). Such slush is obtained by the application of negative pressure on the liquid nitrogen, under which it freezes without vaporization (40). It should be noted that this procedure is quite hard to perform, and moreover, a significant difference in the overall results has not been detected (41, 42). This suggests that alternative approaches should be developed to increase the cooling rates.

It is also possible to avoid the nitrogen boiling with plunging of the carrier in preliminary equilibrated to the same temperature intermediate coolant with dense consistency, for example, aluminum oxide (Al_2O_3) or other metal oxides in a powdered state. The small size and the structure of the particles allow for the direct dipping of the closely sealed embryo carrier into the Al_2O_3. Due to its high density, the use of the precooled Al_2O_3 leads to increased cooling rates, because no bubbles are created. The technique has been developed at the Institute of Cryobiology and Cryomedicine in Kharkiv, Ukraine and has proven its efficacy in the cryopreservation of spermatozoa and other cell suspensions (43).

The aim of the current study was to investigate the possibility of vitrifying embryos with the use of Al_2O_3 as an intermediate coolant, equilibrated to −196°C.

Case Report

All the investigations in the present study were performed after permission from the relevant Medical Ethics Committee had been obtained. All the patients involved both in the preliminary and the clinical studies have signed an informed consent.

MATERIALS AND METHODS

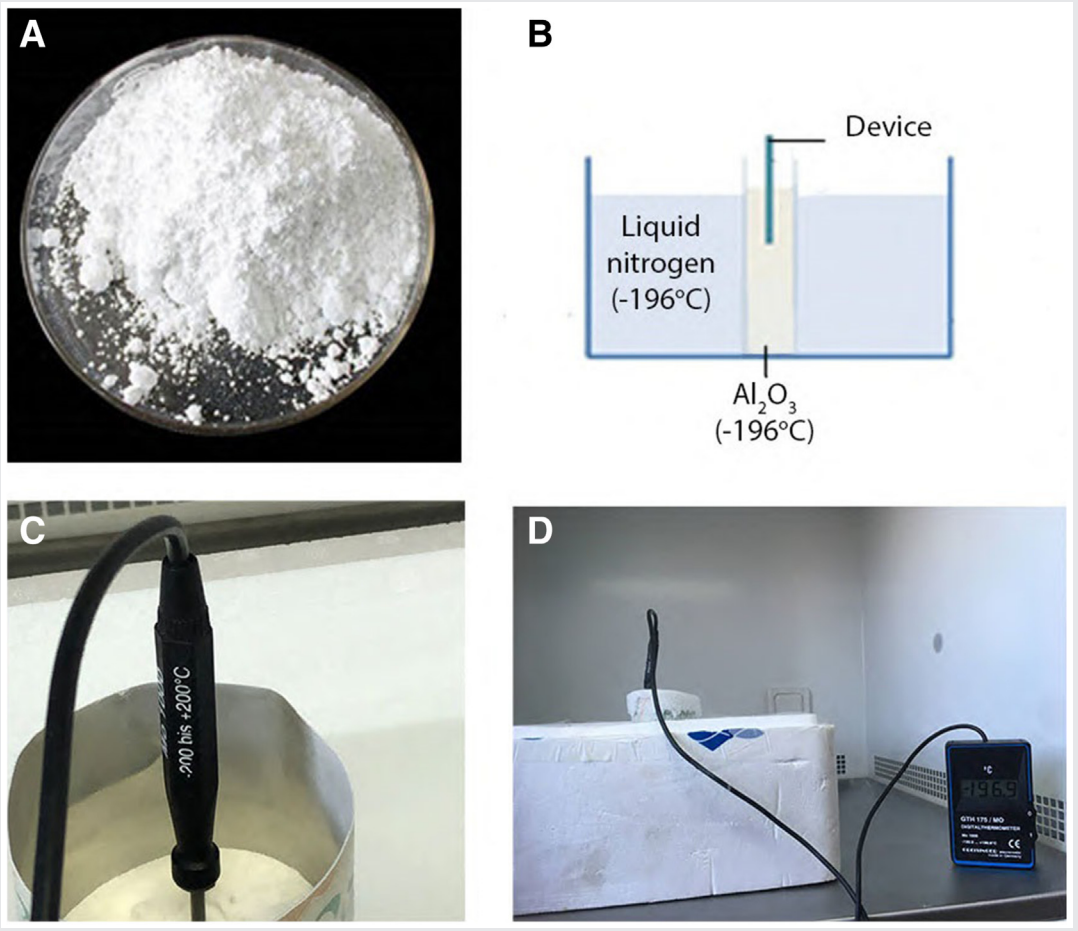
Preliminary Investigations

Initially, comparative studies on donated embryos (n = 48) were performed, 12 of them were vitrified under the classic protocol (plunging the carrier directly into liquid nitrogen)—a control group and 36 with the use of Al_2O_3 (Merck)—study group. The embryos were obtained from 21 women (aged 26–35 years), undergoing assisted reproductive technologies procedures. The semen used for insemination was from their partners. The embryos were frozen either on day 3 or 4 after fertilization and stored in liquid nitrogen for not less than a week. After thawing, the morphology of the embryos was assessed and they were cultured until the stage of expanded or hatched blastocyst. On day 5, the stage of blastocyst was reported and the expanded/hatched blastocyst on day 5/6.

Vitrification

Commercially available vitrification media was used (Kitazato), containing EG, DMSO, trehalose, and hydroxypropyl cellulose. First, the embryos were placed into the equilibration

FIGURE 1



Freezing with the use of aluminum oxide (Al_2O_3) as an intermediate cooling agent: (**A**) Al_2O_3 powder; (**B**) schematic representation of the experimental design; and (**C** and **D**) measuring of the temperature of Al_2O_3.

Todorov. Vitrification of human embryos with Al_2O_3. Fertil Steril Rep 2024.

solution for 14 min (7.5% EG and 7.5% DMSO) at room temperature. Afterward they were transferred into a vitrification solution (15% EG and 15% DMSO) for 30 s and then placed into a new vitrification solution again for 30 s at room temperature. The embryos from both groups were put onto closed carriers (CBS High Security Vitrification Straw, Cryo Bio System). Then the control group embryos were plunged into liquid nitrogen (direct vitrification).

Vitrification with Al_2O_3

Approximately 600 g of Al_2O_3 (enough to cover the closed carrier device) were placed into a metal container with high conductivity walls and then placed into liquid nitrogen to equilibrate. After the temperature of the Al_2O_3 had reached the temperature of the liquid nitrogen, the vitrification was performed. The embryos sealed into the closed carrier systems were put in the Al_2O_3 for approximately 1 min and then transferred into the liquid nitrogen at maximum speed (Fig. 1).

Thawing was performed with a warming kit (Kitazato), which contains trehalose and hydroxypropyl cellulose. In the first step, the embryos were placed into prewarmed to 37°C thawing solution (1 M trehalose) for 1 min. Then they were transferred into 300 μL diluent solution (0.5 M trehalose) for 3 min at room temperature. Afterward, the embryos were put into 300 μL washing solution for 5 min and finally in another well containing 300 μL new washing solution for 1 min at room temperature.

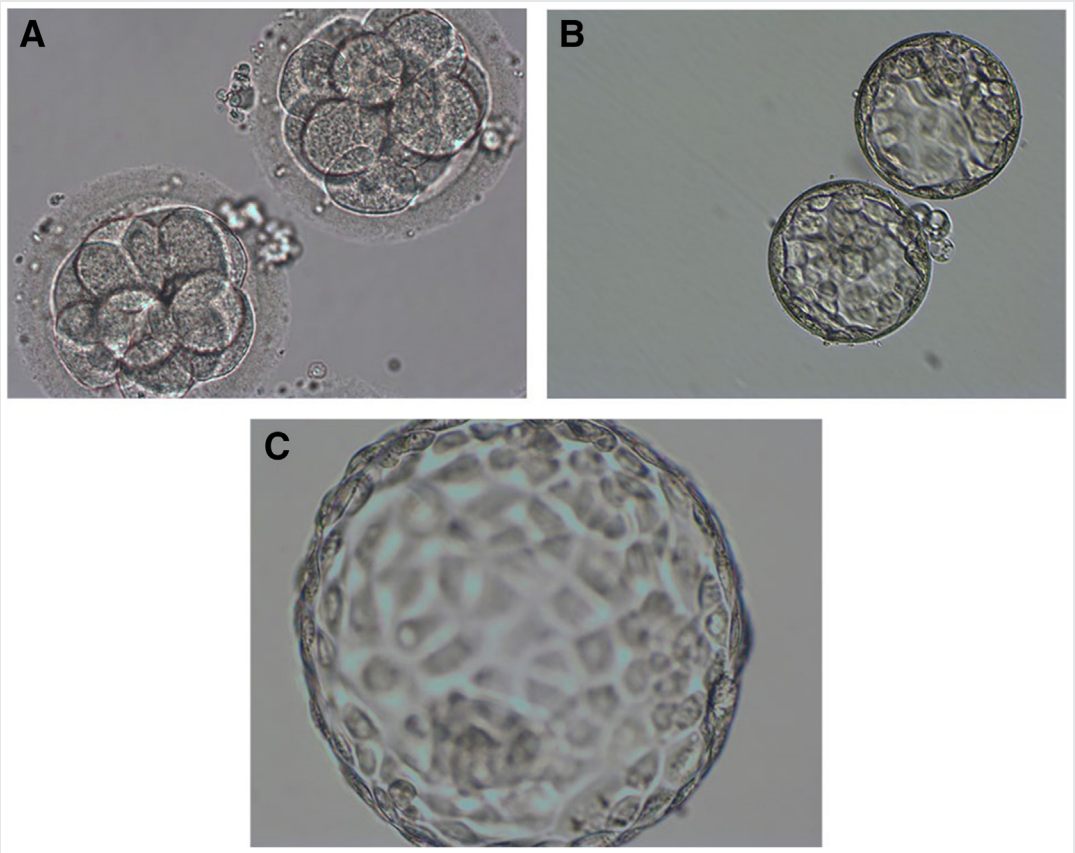
Clinical Data

On the basis of the above-described preliminary studies, the technology was introduced into clinical practice.

Obtaining of the Oocytes

Standard ovarian stimulation protocols have been used with the application of antagonists (44). Oocyte pick-up was performed between 35 and 36 hours after triggering of the ovulation.

FIGURE 2



Representative images from embryos frozen with the use of aluminum oxide (Al_2O_3); (**A**) day 3 before cryopreservation; (**B**) blastocyst developed from the same embryos on day 5 postthaw; and (**C**) hatched blastocyst (1 of the same embryos) on day 6 after cryopreservation.

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Fertilization and Embryo Culture

The obtained oocytes were fertilized by conventional in vitro fertilization and/or intracytoplasmic sperm injection. The embryos were cultured in sequential media (VitroLife or Origio).

Cryopreservation in Clinical Practice

The vitrification procedure was performed as previously described. Only good quality embryos (45, 46) were selected for freezing with closed carriers (CBS High Security Vitrification Straw, Cryo Bio System). One or 2 embryos were loaded onto each device. Commercially available vitrification media was used (Kitazato). After thawing, the embryos have been cultured until day 5 and then transferred.

Statistical Analysis

The statistical significance was assessed using a 2-tailed *t* test, with the SPSS software. A probability of $P<.05$ was considered significant.

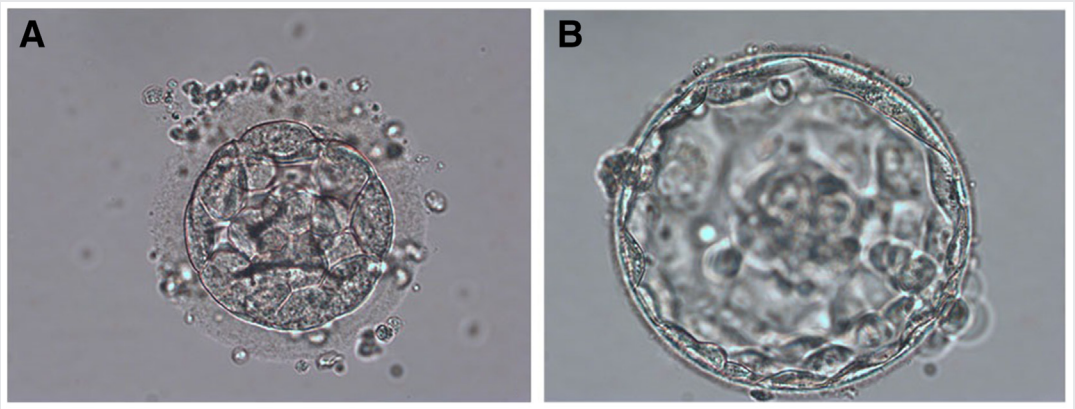
RESULTS

Preliminary Studies

After thawing no difference between the qualities of the embryos in both groups—11 of 12 embryos (91.7%) of the control and 33 of 36 embryos (91.7%) in the experimental group have retained their morphological integrity. During postthaw culture, a major part of the embryos reached the blastocyst stage—63.63% (7 of 11 embryos) in the control and 84.84% (28 of 33 embryos) in the experimental group, respectively, showing a tendency for better blastocyst rate. The quality of the blastocysts was also comparable. Further, in the control group, 3 of 7 embryos (42.9%) have reached expanded blastocyst (1 of them has hatched—33.3%). In the experimental group, more of the obtained embryos have developed to the stage of expanded blastocyst—16 of 28 (57.1%) and 6 of them have hatched (37.5%) (Fig. 2). No contamination of any of the samples was observed by visual examination.

The obtained results from the preliminary studies, gave us ground for our laboratory group to propose the method of vitrification in Al_2O_3 to a group of patients.

FIGURE 3



Representative images of an embryo used in clinical practice and with the use of aluminum oxide (Al_2O_3); (**A**) day 4, 2 hours after thawing; and (**B**) expanded blastocyst, obtained from the same embryo on day 5 before transfer.

Todorov. Vitrification of human embryos with Al_2O_3. Fertil Steril Rep 2024.

Clinical Data

In 2021, 20 embryos of 6 patients (2 in each carrier device) have been vitrified in Al_2O_3 on day 4 after fertilization. Of them, 14 have been thawed and showed intact morphology. After 24 hours of culture, 10 of the 14 embryos (71.43%) have reached to the blastocyst stage (Fig. 3). Four patients received ET with 2 embryos and 2 single ET. The observed clinical pregnancies were 3 (50%) all with successful child births (Table 1).

DISCUSSION

The current report describes the process of implementation of a method for vitrification (closed system) of human embryos, on the basis of the increase of cooling rates due to the plunging of the device into preliminary cooled Al_2O_3. The results from the investigation point out that the technique is safe and easily accessible for laboratories. The carrier device implemented in the investigation is popular in clinical practice and easily accessible. The straws are individually packed and sterile. Each device has an outer sheath and a capillary tube, where the embryos are loaded.

Chemically, aluminum oxide, or aluminum trioxide is a chemical compound comprising of aluminum and oxygen, with the chemical formula Al_2O_3. Its appearance is of a white powder and it has high thermal conductivity (30 $Wm^{-1}K^{-1}$), which characterizes the heat transfer capacity of compounds and materials. Thermal conduction is the main method for heat transfer in solids and it is the amount of heat flow per unit time per unit area at a certain temperature of the material (47, 48). For example, the thermal conductivity of the liquid nitrogen is approximately 25 $Wm^{-1}K^{-1}$, which means that, theoretically, the cooling rates should be lower. Moreover, the heat transfer in precooled metal powder is faster than in liquids. In our experiments, it was important that the selected cooling agent would be able to lower the temperature of the samples to that of the liquid nitrogen with high speed. Because it is a chemically inert compound and stable over a wide range of temperatures (49), the Al_2O_3 is appropriate to be used as an intermediate coolant. At the same time, Al_2O_3 is available from chemical and biological suppliers, which grants easy access to it for laboratories. Being readily obtainable, the Al_2O_3 might prove to be more suitable for application in clinical practice than the liquid nitrogen slush, although the latter also shows good results in avoiding the Leidenfrost

TABLE 1

Number of embryos frozen, thawed, and transferred for each of the patients, participating in the clinical investigation.

Patient	Age (y)	Frozen embryos (number)	Thawed embryos (number)	Transferred embryos (number)	Embryos left frozen (number)
1	31	6	4	2 (pregnant)	2
2	33	4	2	1 (pregnant)	2
3	29	2	2	2 (pregnant)	0
4	34	4	2	1	2
5	31	2	2	2	0
6	35	2	2	2	0

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effect and therefore improving the heat transfer. Because as mentioned above, the slush is quite hard to form and requires the use of negative pressure. However, the Al_2O_3 powder is not appropriate to be used in open carrier systems, as it may potentially stick to the samples. In the closed device used in our experiments, the compound has no contact with the bio-objects, because during thawing the outer straw is removed and in the thawing media only the capillary tube with the embryos is placed. There is no possibility for the Al_2O to enter into the thawing media.

In the present report, the obtained experimental and clinical data show that the results can be compared to those achieved with an open system, but the proposed technique is considerably safer in terms of the requirements for aseptic cryopreservation and embryo storage. Future optimization of the method can be achieved by measuring the cooling rates, investigating of other types of closed carriers (for example, CryoTip), and attempts to increase the thawing speed. This is the first demonstration of the application of the method for vitrification of embryos, since in the available literature we could not find any reports regarding the matter. Fundamental research in the field of cryobiology has shown that the thawing rates should be equal to or higher than the cooling ones. The possibility to vitrify oocytes and embryos at a different stage of development with this technology also represents an interesting aspect of investigation.

Another intriguing application of the method is the cryopreservation of ovarian tissue. In such cases, the process of vitrification is hindered by the relatively large sizes of the ovarian fragments, which prevent the achievement of higher cooling and thawing rates.

CONCLUSION

Vitrification with the use of Al_2O_3 as an intermediate coolant was found to be an effective method for cryopreservation of cleavage stage embryos in a closed, aseptic system, but further investigations into the cooling rates are needed to safely apply the technique in clinical practice. We speculate that further studies will substantiate its usefulness in the cryopreservation of other reproductive tissues.

CRediT Authorship Contribution Statement

Plamen Todorov: Writing – original draft, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Elena Hristova:** Writing – review & editing, Writing – original draft, Visualization, Methodology, Conceptualization. **Nadya Petrova:** Resources, Methodology, Investigation, Data curation. **Tanya Milachich:** Validation, Supervision, Resources, Investigation, Data curation, Conceptualization.

Declaration of Interests

P.T. has nothing to disclose. E.H. has nothing to disclose. N.P. has nothing to disclose. T.M. has nothing to disclose.

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