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Viability of guinea pig (*Cavia porcellus*) spermatozoa diluted in Tris-buffer extenders stored at 5.00 ˚C

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Abstract

The cooling procedure markedly diminishes the quality of guinea pig (*Cavia porcellus*) sperms, primarily because their membranes are highly susceptible to this process. This susceptibility triggers the generation of reactive oxygen species and free radicals, ultimately leading to lipid peroxidation in the sperm membrane. Surprisingly, there has been a lack of research on the use of Tris-based extenders to safeguard guinea pig sperm under refrigeration conditions. This study aimed to assess the viability of guinea pig spermatozoa diluted in Tris buffer-based extenders during storage at 5.00 ˚C. Sperm collection was carried out through castration of the animals. For this study, 10 adult male guinea pigs were utilized, being divided into four groups including phosphate-buffered saline (PBS), human tubal fluid (HTF), Tris-citric-glucose (TCG), and Tris-fructose-yolk (TFY) cultures. Evaluations including sperm motility, morphology, plasma membrane integrity, viability, and total count were conducted at 0, 24, and 48 hr after sampling. The results obtained indicated that at the 24-hr and 48-hr marks of the experiment, both overall and progressive motility percentages, viability, plasma membrane integrity, and morphology of sperms in the PBS and HTF cultures exhibited a significant increase in comparison with the TCG and TFY cultures. Consequently, it can be inferred that PBS and HTF cultures are highly effective in preserving the quality of guinea pig spermatozoa.

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Introduction

The reproductive system plays a vital role in ensuring the continuity of generations. Any malfunction within its components contributing to sperm production, development, or function can hinder the achievement of the ultimate goal, *i.e.*, the continuation of family lines in human populations and preservation of generations in animal species. Unconventional pets encompass a diverse range of animal species spanning various orders, such as carnivores, Lagomorpha, rodents, and more. Among these orders, rodents have garnered heightened interest compared to the rest, mainly because of their lightweight stature, tranquil disposition, and manageable care requirements.[1] The guinea pig, scientifically known as *Cavia porcellus*, stands out as the most well-known member among caviomorph rodents. Historically, this species has often symbolized scientific experimentation. However, in recent years, guinea pigs have transitioned from primarily serving as research models in studies related to tuberculosis, asthma, scurvy, and more to popular pets.[2] Until now, there has been a limited amount of information concerning the morphological characteristics of the reproductive systems of uncommon pet or laboratory species. Moreover, within the categories of rodents and lagomorphs, mice, rats, and rabbits have garnered more research focus, primarily due to their extensive use in medical studies.[3] The reproductive components in all these species exhibit striking similarities, encompassing testes, spermatozoa, and accessory glands.[1] The growth and development of these components are influenced by a multitude of factors, including hormonal regulation and androgenic stimulation.[4] With the increasing popularity of keeping guinea pigs as pets, there is a growing interest in preserving high-quality sperm for potential use in artificial insemination. This becomes achievable through the study and identification of appropriate sperm preservation

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methods tailored to this species. In essence, having the capability to store sperm for extended durations enhances the efficiency of artificial insemination processes in farm animals, reducing the necessity to maintain male animals continuously.[5,6] In the preparation of chilled semen, a common recipe entails combining 2.40 g of non-fat dry milk, 4.90 g of glucose, 0.15 g of sodium bicarbonate, and adding water until the total volume of 100 mmol. To enhance preservation, anti-microbial agents like 150,000 IU of penicillin and 150,000 IU of streptomycin can be included.[7] Several studies have examined the impact of various substances on safeguarding sperm when stored in the refrigerator. However, as far as we are aware, no research has been undertaken to identify the optimal conditions and preservatives for the extended preservation of guinea pig semen. The primary objective of our study was to contribute to the breeding and perpetuation of superior strains within this species.

Materials and Methods

Animals. For this study, a cohort of 12 male guinea pigs was utilized, comprising both Peruvian and American breeds, aged between 10 months and one year, to obtain sperm samples. To ensure optimal sperm activity, these males were housed in a close proximity to female guinea pigs. The presence of male and female guinea pigs was alternated, as prolonged separation from females was believed to potentially reduce sperm activity. These guinea pigs, sourced from pet stores, underwent a 2-week acclimation period at the Laboratory Animal Facility of Urmia University's specialized clinic. During this period, they all received an oral dose of 5.00 mg kg^{-1} of praziquantel (Zagros Pharmed Pars, Burujerd, Iran). Throughout the storage period, the ambient temperature for the animals was maintained between 19.00 and 22.00 ˚C. The storage area was designed to provide access to both natural and artificial lighting. Regarding their diet, the guinea pigs were fed a combination of alfalfa, fresh vegetables, fruits, and MoFeed plate-nuts (Kimiya Daneh Company, Isfahan, Iran). To obtain sperm samples, a sterile surgical procedure was performed, meticulously extracting samples from the testes, being subsequently examined for sperm parameters. This study was conducted in strict adherence with international ethical standards for animal research. The study protocol was reviewed and approved by the Research Deputy Office of the Faculty of Veterinary Medicine, Urmia University, Iran (Ref: IR-UU-AEC-3/9).

Surgical procedure. A sterile method was employed to retrieve sperm from guinea pigs, utilizing a closed surgical approach. Anesthesia was induced by administering 85.00 mg kg^{-1} ketamine (Alfasan, Woerden, The Netherlands) and 12.00 mg kg^{-1} xylazine (Bioveta, Ivanovice na Hané, Czech Republic) intramuscularly. Animals received intramuscular enrofloxacin 5.00 mg kg^{-1} (Erfan Darou, Tehran, Iran) prophylactically. The surgical procedure involved making a midline incision on the abdomen, guiding the testicles into the abdominal cavity, ligating the vas deferens, removing the testicles, and suturing the abdominal wall and skin layers. Following surgery, the animals were placed in an oxygen chamber for recovery. Oxytetracycline spray (Vetaque, Tehran, Iran) was used topically, and flunixin Meglumine (Royan Darou, Tehran, Iran) was administered for post-operative care and alleviate pain and inflammation.[8,9]

Sperm extraction and slide preparation. To retrieve sperm from the excised testicles, the epididymis tail was opened and separated from the testicle. The extracted sperm was then placed into various culture media including Tris-citric-glucose (TCG; Sigma, St. Louis, USA) comprising 0.845 g citric acid, 1.514 g Tris, 0.423 g glucose, and 5.00 mL of egg yolk in a 50.00 mL solution, Tris-fructose-yolk (TFY; Sigma) containing 2.495 g fructose, 0.909 g citric acid, 1.816 g Tris, and 7.00 mL of egg yolk in a 50.00 mL solution, phosphate-buffered saline (PBS), and human tubal fluid (HTF; Sigma) culture media.

Categorization. In the present study, the sperm samples prepared earlier were allocated into distinct experimental groups as follows: 1) The 1st group consisted of samples preserved in TCG medium. 2) The 2nd group encompassed samples stored in the TFY medium. 3) The 3rd group included samples placed in PBS.4) The 4th group comprised samples kept in HTF. Following collection, these samples were refrigerated and subjected to the analysis at three time points including the outset, as well as 24 and 48 hr later, to assess sperm parameters under varying conditions.

Sperm count. To determine the sperm count, a hemocytometer slide was employed. Initially, the semen sample was diluted with physiological serum at a ratio of 1.00:50.00. Then, the number of sperm *per* mL was calculated using the following procedure: First, the hemocytometer slide was disinfected using 70.00-degree alcohol and securely positioned in its designated spot. Next, 10.00 - 15.00 µL of the diluted semen was carefully placed between the slide and cover slip. With the aid of a microscope set at 40.00 × magnification, the sperm heads were systematically counted in five large squares, each of which contained 16 smaller squares.

Sperm motility. The computer-assisted semen analysis (CASA; Test Sperm 3.2; Videotest, St. Petersburg, Russia) was utilized to assess sperm motility. Sperm motility indicators, including curvilinear velocity, straight-line velocity, average path velocity, linearity, straightness, and beat-cross frequency were evaluated at three time points including the outset, as well as 24 and 48 hr after storage in a refrigerator at 5.00 ˚C.

Sperm viability and morphology. Two methods were employed for preparing sperm smears. The 1st method

involved utilizing a slide for undiluted samples, while the 2nd one employed a pipette for diluted samples. To assess the proportion of non-viable and abnormal sperms, Eosin-Nigrosin staining was utilized. The staining solution was prepared by dissolving 1.67 g of Eosin-Y (Merck, Darmstadt, Germany), 10.00 g of Nigrosin (Merck), and 2.90 g of sodium citrate in 100 mL of distilled water. This staining technique operates on the basic principle that damaged sperms with compromised plasma membranes become permeable to the above-mentioned dye. It enables the differentiation between live and non-viable sperms. Sperm cells showing any coloration in their head, neck, or tail regions were classified as non-viable. Moreover, sperm cells displaying cytoplasmic remnants or other morphological irregularities were categorized as abnormal sperms. A meticulous examination of 200 sperm cells from each sample was conducted under 400 × magnification, and the results were expressed as percentages.[10]

Sperm plasma membrane integrity. The hypo-osmotic swelling test (HOST) was used to evaluate the integrity of sperm plasma membrane. For this method, the HOST solution, consisting of 0.735 g of sodium citrate and 1.351 g of fructose dissolved in 100 mL of distilled water, was prepared. Subsequently, 50.00 µL of seminal fluid was combined with 500 µL of HOST solution and incubated at 37.00 ˚C in a water bath for 60 min.

Statistical analysis. The research data were analyzed using the SPSS Software (version 26.0; IBM Corp., Armonk, USA) through two-way ANOVA. The data were presented as the mean and standard error of the mean. The significance of differences was denoted as $p < 0.05$.

Results

Sperm count. The sperm count using a hemo-cytometer slide indicated that the average count in guinea pigs was 43.40 ± 4.78 × 10^6 *per* mL.

Sperm motility. When we examined the total sperm motility, we observed marked differences among the treatment groups, particularly at the initial time point. Notably, our findings indicated that, at the outset of the experiment, the percentage of total sperm motility in the TCG and TFY groups significantly decreased compared to the other groups. After 24 hr, it became evident that the PBS group exhibited significantly higher total sperm motility ($p < 0.05$) than the other groups. However, at the 24-hr time point, there was a notable decrease in motility percentages in the TCG and TFY groups compared to the other groups. As we reached the 48-hr time point in our experiment, we observed that the PBS and HTF groups exhibited the highest percentages of sperm motility, surpassing all other treatment groups significantly ($p < 0.05$). Notably, our study revealed that both the TCG and TFY groups showed a complete absence of total sperm motility. Also, our research consistently demonstrated that, in all treatment groups, the percentage of total sperm motility significantly declined over time ($p < 0.05$), as indicated in Table 1.

Sperm progressive motility. Significant variations were noted among the treatment groups, particularly at the initial stage. At this time, our findings highlighted that the PBS and HTF groups exhibited the highest percentage of progressive motility. As we reached the 24-hr time point in our experiment, it became evident that the PBS and HTF groups maintained significantly higher percentages of progressive mobility ($p < 0.05$) compared to the other groups. Furthermore, at the 48-hr time point, we found that the PBS and HTF groups consistently maintained the highest percentage of progressive sperm motility, significantly surpassing the other treatment groups ($p < 0.05$). Our study consistently revealed that, in all treatment groups, the percentage of sperm progressive motility significantly decreased over time ($p < 0.05$), as presented in Table 1.

Table 1. Guinea Pig's sperm characteristics (mean ± SE) in different diluents.

Parameters	Storage period (hr)	HTF	PBS	TCG	TFY
Total motility (%)	0	66.52 ± 1.67 ^{aA}	65.58 ± 1.67 ^{aA}	52.38 ± 1.63 ^{bA}	53.79 ± 1.12 ^{bA}
	24	50.38 ± 1.55 ^{bB}	54.16 ± 1.46 ^{aB}	0.00 ± 0.00 ^{cB}	0.00 ± 0.00 ^{cB}
	48	37.69 ± 1.73 ^{bC}	43.28 ± 1.73 ^{aC}	0.00 ± 0.00 ^{cB}	0.00 ± 0.00 ^{cB}
Progressive motility (%)	0	32.17 ± 1.87 ^{aA}	34.72 ± 1.55 ^{aA}	26.35 ± 1.66 ^{bA}	27.68 ± 1.54 ^{bA}
	24	23.96 ± 1.35 ^{bB}	28.55 ± 1.38 ^{aB}	0.00 ± 0.00 ^{cB}	0.00 ± 0.00 ^{cB}
	48	14.32 ± 1.94 ^{bC}	21.24 ± 1.75 ^{aC}	0.00 ± 0.00 ^{cB}	0.00 ± 0.00 ^{cB}
Viability (%)	0	71.25 ± 1.49 ^{aA}	73.14 ± 1.85 ^{aA}	66.78 ± 1.26 ^{bA}	64.51 ± 1.66 ^{bA}
	24	56.82 ± 1.45 ^{bB}	61.39 ± 1.21 ^{aB}	18.00 ± 1.75 ^{cB}	20.00 ± 1.12 ^{cB}
	48	44.57 ± 1.84 ^{bC}	49.51 ± 1.27 ^{aC}	0.00 ± 0.00^{cB}	0.00 ± 0.00 ^{cB}
Plasma membrane integrity (%)	0	67.80 ± 1.11 ^{aA}	66.58 ± 1.52 ^{aA}	66.62 ± 1.79 ^{aA}	65.23 ± 1.06 ^{aA}
	24	55.27 ± 1.30 ^{aB}	57.43 ± 1.18 ^{aB}	15.54 ± 1.30 ^{bB}	16.35 ± 1.64 ^{bB}
	48	38.78 ± 1.58 ^{bC}	46.15 ± 1.89 ^{aC}	0.00 ± 0.00^{cB}	0.00 ± 0.00 ^{cB}
Normal morphology (%)	0	93.80 ± 1.11 ^{aA}	92.58 ± 1.52 ^{aA}	93.62 ± 1.79 ^{aA}	92.23 ± 1.06 ^{aA}
	24	92.27 ± 1.30 ^{aA}	91.43 ± 1.18 ^{aA}	91.54 ± 1.30 ^{aA}	90.35 ± 1.64 ^{aA}
	48	90.78 ± 1.58 ^{aA}	90.15 ± 1.89 ^{aA}	90.00 ± 0.00 ^{aA}	89.00 ± 0.00 ^{aA}

HTF: Human tubal fluid; PBS: Phosphate-buffered saline; TCG: Tris-citric-glucose; TFY: Tris-fructose-yolk.

[abc, ABC] Different lower and uppercase letters indicate significant differences in a row and column at $p < 0.05$, respectively.

Sperm viability. In this investigation, we observed significant differences in sperm viability among various treatment groups. Initially, at the start of the experiment, it was evident that the TCG and TFY groups exhibited a significant reduction in sperm viability compared to the other treatment groups ($p < 0.05$). As the study progressed to the 24-hr time point, we observed that the PBS group displayed a significant increase in sperm viability ($p < 0.05$) in comparison with HTF group. However, both the TCG and TFY groups experienced a substantial decrease compared to these two groups. Moving forward to the 48-hr interval, it was found that the PBS group consistently held the highest percentage of sperm survival, being significantly higher than that of the other treatment groups (Fig. 1). Notably, our study revealed that the TCG and TFY groups had a survival rate of 0.00% at 48 hr of storage (Table 1).

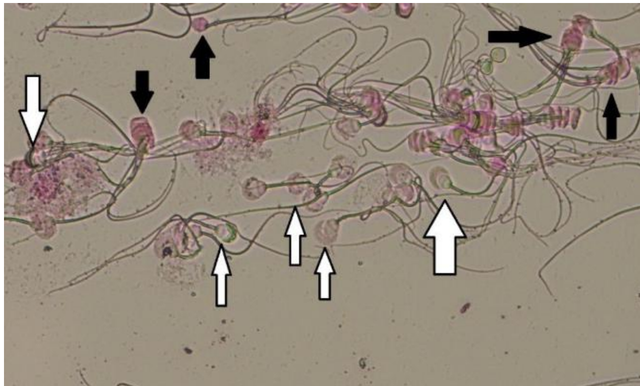

Fig. 1. Sperm viability assessment. Live spermatozoa are denoted by their colorlessness appearance (white arrows), while deceased spermatozoa are identified by their red colour (black arrows; Eosin-Nigrosin staining, 400×).

Sperm plasma membrane integrity. There were no significant differences ($p > 0.05$) at the 1st time point among the treatment groups considering the sperm plasma membrane integrity. However, as we reached the 24-hr time point in our experiment, it became evident that the percentage of sperm plasma membrane attachment had significantly increased in the PBS and HTF groups ($p < 0.05$) compared to the TCG and TFY groups, respectively (Fig. 2). Notably, there were no significant differences between the PBS and HTF groups in this regard. At the 48-hr time point, we found that the PBS and HTF groups consistently exhibited the highest percentage of sperm plasma membrane adhesion, significantly surpassing the other treatment groups (Table 1).

Sperm morphology. No significant differences were observed among the treatment groups at the initial time point ($p > 0.05$). However, at both the 24- and 48-hr time points of the experiment, there were no significant differences in the percentage of normal morphology between the PBS and HTF groups and the TCG and TFY groups (Table 1).

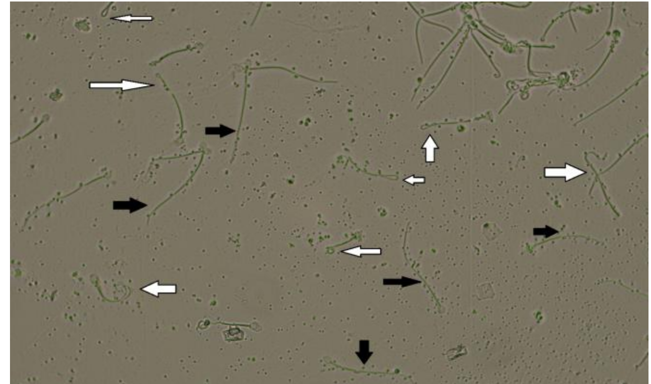

Fig. 2. Sperm plasma membrane integrity analysis. Sperms with bent tails (white arrows) exhibit intact plasma membrane integrity. Conversely, sperms with straight tails (black arrows) display plasma membrane disintegration (200 ×).

Discussion

This research aimed to assess the quality attributes of guinea pig spermatozoa when diluted with Tris buffer-based preservatives. The findings from this study indicated that Tris buffer-based diluents may not be ideal for storing guinea pig sperm. Instead, two alternative diluents, HTF and PBS, were found to effectively preserve sperm quality parameters at an acceptable level. Notably, prior investigations exploring sperm quality parameters in a Tris buffer environments for rabbits have revealed varying effects, highlighting the potential species-specific differences in the impact of these diluents.[11-14] Sperm motility and viability stand out as pivotal parameters when gauging fertilization potential and the robustness of the sperm membrane. The connection between heightened oxidative stress and reduced sperm motility can be elucidated through two theories. Firstly, a series of pathological processes results in diminished sperm motility by reducing the phosphorylation of axonemal proteins. Secondly, the sperm membrane is exceedingly sensitive to free radicals and peroxidation generated by various stressors. This heightened sensitivity is attributed to the composition of the sperm membrane, featuring unsaturated fats containing docosahexaenoic acid with six double bonds *per* unsaturated fatty acid molecule.[15] In a former study examining how sound exposure impacts the sperm characteristics of mice, the research explored various aspects of sperm quality, encompassing sperm count and motility (progressive, non-progressive, and immobile), as well as the viability rate or the percentage of viable sperm following exposure to a physical stimulus, namely sound. This investigation aimed to assess the repercussions of sound-induced damage. The study findings illuminated a marked influence of sound on the percentage of motile sperm within the experimental group. Conclusively, the research indicated that sound, particularly in the 100 dB frequency range, can diminish

the quantity of progressively motile sperm in mice. Simultaneously, it can lead to an increase in the number of non-motile sperms. The research suggested that sound interferes with sperm maturation and motility by exerting adverse effects on the secretions within the epididymal wall. These secretions are pivotal for sperm maturation. Furthermore, the study implied that sound may contribute to hormonal alterations, which, in turn, affect sperm parameters. This aligns with the findings of Noorafshan *et al.*, demonstrated that curcumin has a remedial impact on sperm tail length, count, and motility, as well as testosterone levels in mice exposed to metronidazole-induced toxicity.[16] In the current study, we observed a gradual decline in the motility of guinea pig sperm during refrigerated storage. Among the various diluents utilized, PBS and HTF diluents demonstrated the capacity to sustain sperm motility at an optimal level. Specifically, when employing a Tris-yolk preservative, we determined that sperms subjected to cooling and preservation at 5.00 ˚C for 24 hr exhibited an average motility rate of 45.00%. However, this measure notably decreased to 25.00% after a subsequent 48-hr storage period.[17] In an investigation examining the influence of storage at 15.00 ˚C on rabbit semen, the findings highlighted the effectiveness of preserving rabbit sperm in a solid-state at this temperature. Remarkably, this storage condition maintained sperm motility and fertility for up to five days.[12] Our study revealed that TCG and TFY diluents were unable to sustain the viability of guinea pig sperm within a refrigerator environment. However, two novel diluents, PBS and HTF, introduced for the first time in guinea pig sperm preservation, exhibited the capacity to maintain sperm survival. In a relevant study conducted by Roca *et al.*, the viability and fertility of rabbit sperm were investigated when stored in a diluted Tris buffer-based preservative at 15.00 ˚C. Their results revealed that Tris buffer-based preservatives are suitable for short-term storage, up to 6 hr, of chilled rabbit semen. However, there remains a lack of research examining their efficacy in prolonging sperm viability and fertility over longer periods.[14] Reportedly, it was demonstrated that the preservation of rabbit sperm using a Tris-based diluent could be attributed to the influence of low temperature. Although the precise threshold temperature for rabbit sperm deterioration remains undetermined, a comparative analysis of multiple studies suggests that 15.00 ˚C proves more conducive for storing chilled rabbit semen than the conventional 5.00 ˚C. Furthermore, this research underscores the effectiveness of employing Tris buffer-based preservatives in maintaining the quality of cold-stored rabbit semen at a temperature of 15.00 ˚C.[14] An investigation regarding the viability and fertilization potential of rabbit sperm stored at 5.00 ˚C has revealed that, in contrast to glucose-yolk-citrate, the utilization of TFY buffer for diluting and preserving rabbit semen at 5.00 ˚C is notably more efficacious.[17] The research conducted by Martins-Bessa and colleagues highlighted that the most effective semen preservative comprises 4.00% glycerol and 10.00% egg yolk.[18] Dimethyl sulfoxide (DMSO) serves as a cryoprotectant renowned for its swift penetration into the sperm cells. It finds application in maintaining the quality of frozen sperm from various animals like bulls, boars, goats, and dogs. In stallions, DMSO, often in combination with methylformamide and glycerol, proves advantageous when used alongside skim milk preservatives, offering an alternative to glycerol-based preservatives. In a comparative study involving dogs, the efficacy of three cryoprotectants including glycerol, ethylene glycol, and DMSO was assessed. The findings revealed that glycerol exhibited superior post-thaw motility and fertilization capacity compared to ethylene glycol and DMSO. Notably, ethylene glycol has been predominantly employed in the preservation of semen from buffaloes, bulls, and sheep.[19] Our study under-scored that the plasma membrane integrity of guinea pig sperm was not effectively upheld in the TCG and TFY environments. However, PBS and HTF environments exhibited competence in preserving the plasma membrane integrity of guinea pig sperm. In a related study conducted by Nagy *et al.*, the focus was on rabbit sperm survival and acrosomal integrity within a preservative supplemented with gelatin.[13] Their findings revealed that the percentage of sperm survival and acrosomal integrity in the gelatin-supplemented preservative surpassed that of the control samples, particularly after a short storage period. Their analysis revealed that buffers are integrated into preservatives with the primary aim of mitigating pH fluctuations resulting from the sedimentation of sperm cells during storage. The inclusion of gelatin serves to inhibit sedimentation, promoting a more uniform distribution of sperm cells. Consequently, buffers can more efficiently counteract pH variations in this context.[13]

This study suggests that PBS and HTF storage conditions are effective in preserving guinea pig spermatozoa while upholding the quality of various sperm parameters within a refrigerated environment. In contrast, Tris-based media, particularly TCG and TFY, are not deemed suitable as storage media for guinea pig sperm. Additionally, future research should aim to investigate the fertility rates of diluted guinea pig spermatozoa across various preservatives.

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Conflict of interest

None of the authors have any conflict of interest to declare.

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