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Original Article

The effect of methylxanthines on motility, membrane integrity and DNA damage of frozen-thawed buffalo spermatozoa

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ABSTRACT

Methylxanthine supplementation has resulted in better seminal characteristics in fresh and cryopreserved spermatozoa. The objective of this study was to determine the effect of methylxanthines such as pentoxifylline, theophylline and caffeine on post-thaw quality of buffalo bull spermatozoa. The semen was collected from four mature regular donor buffalo bulls. The ejaculates having more than 80% motility were pooled, split into four aliquots and then diluted in Tris-citric acid-based extender having different concentrations of pentoxifylline (3.5 mM), caffeine (10 mM), theophylline (25 Mm), and control (without additives). Of the three additives, only supplementation of pentoxifylline in cryopreservation extender significantly improved total and progressive semen motility relative to that of untreated control ($p<0.05$). Pentoxifylline also increased plasma membrane integrity and some motion patterns such as curvilinear velocity (VCL), average path velocity (VAP), and straight-line velocity (VSL) when compared to theophylline, caffeine or control ($p<0.05$). No significant differences were observed for acrosomal integrity and DNA damage of frozen thawed buffalo spermatozoa in extender containing methylxanthines. The findings of this study showed that supplementation of methylxanthines such as pentoxifylline in semen cryopreservation extender has more potentiality to elevate motility and membrane integrity of buffalo frozen thawed spermatozoa.

تاثیر متیل گزانتین ها بر روی تحرک ، یکپارچگی غشای و آسیب وارده بهDNA اسپرماتوزوئید های منی منجمد گاومیش متعاقب یخ گشایی

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چکیده

افزودن ترکیبات متیل گزانتین باعث بهبود کیفیت منی تازه ومنجمد می گردد. هدف از انجام این تحقیق بررسی تاثیر متیل گزانتین هائی نظیر پنتوکسی فیلین، کافئین و تئوفیلین بر روی اسپرماتوزوئید های منی منجمد متعاقب یخ گشائی بود. نمونه انزال چهار راس از گاومیش نا کیفیت عالی و با داشتن بیش از ۸۰ درصد اسپرماتوزویید با تحرک رو به جلو با رقیق کننده تریس سیترات- زرده تخم مرغ ترکیب گردید و پس از طی مرحله خنک کردن در دمای ۴ درجه سانتی‌گراد به مدت ۲ ساعت، به چهارگروه آزمایشی حاوی ۳/۵ میلی‌مول پنتوکسی فیلین ، ۱۰ میلی‌مول تئوفیلین و ۲۰ میلی‌مول کافئین به ازای هرمیلی‌لیتر ونیزکنترل تقسیم گردید از بین سه افزودنی، فقط افزودن پنتوکسی فیلین به رقیق کننده مورد نظر باعث افزایش میزان تحرک اسپرماتوزوییدها پس از ذوب گردید ($p<۰/۰۵$). همچنین پنتوکسی فیلین باعث افزایش برخی از الگوی حرکتی نظیر سرعت در مسیر میانگین، سرعت در مسیر منحنی، سرعت در مسیر مستقیم ونیز یکپارچگی غشای اسپرم متعاقب یخ گشائی در مقایسه با گروه کافئین، تئوفیلین و نیز کنترل گردید ($p<۰/۰۵$). در این راستا، درصد اسپرماتوزوییدهای با آکروزوم نرمال و نیز با DNA آسیب دیده متعاقب یخ گشائی در تمامی گروه‌های آزمایشی حاوی متیل گزانتین ها نسبت به گروه کنترل تفاوت معنی‌داری را نشان نداد. به طور کلی نتایج به دست آمده نشان داد که افزودن ترکیبات متیل گزانتین نظیر پنتوکسی فیلین به رقیق کننده منی در فرآیند انجماد، توانائی افزایش میزان تحرک و یکپارچگی غشای اسپرماتوزوئید های منجمد متعاقب یخ گشائی را دارد.

واژه های کلیدی: متیل گزانتین، اسپرماتوزوئید، انجماد منی ، تحرک ، گاومیش

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INTRODUCTION

Methylxanthines are frequently used as additives to sperm suspensions in order to improve sperm characteristics. Among methylxanthines, pentoxifylline (3,7dimethyl-1-(5-oxo-hexyl)-xanthine), caffeine (1,3,7-trimethylxanthine), theophylline (1,3-dimethylxanthine) and theobromine (3,7-dimethylxanthine)) have commonly been employed [1,2]. In several species, caffeine and pentoxifylline promote sperm motility by increasing energy production [3]. In numerous studies, methylxanthine supplementation resulted in better motility characteristics in fresh and cryopreserved spermatozoa [1]. The addition of these derivatives to sperm suspension seems to improve sperm function leading to better sperm fertilizing ability [4]. Caffeine and theophylline have similar properties with pentoxifylline and they are methylxanthine derivative phosphodiesterase inhibitors. These compounds positively affect sperm function by regulating glycolysis via a buildup of cyclic adenosine monophosphate (cAMP) through inhibition of the enzyme cAMP phosphodiesterase [5]. Elevated cAMP levels increase the rate of glycolysis, which generates adenosine triphosphate (ATP), used to power sperm movement [5]. Therefore, diluting semen with methylxanthines increases cellular ATP levels, which gives rise to elevated motility [6]. Methyl xanthine supplementation has resulted in better seminal characteristics in fresh and cryopreserved spermatozoa viz., motility and curvilinear velocity [7,8]. Pentoxifylline and caffeine have been shown to improve motility and longevity of fresh and frozen semen in a variety of mammalian species. Motility as well as fertility of spermatozoa can be improved by incorporating various motility enhancing agents like pentoxifylline [9]. In-vitro treatment of semen with pentoxifylline leads to marked augmentation of sperm motility, enhancement of acrosome reaction, increase of sperm penetration into zona-free hamster oocytes, and protection of the sperm plasma membrane. Such properties indicate that the drug may be a useful tool for semen preparation in assisted reproduction. However, its real effectiveness in improving fertilization rates has not been confirmed, mainly in association with intrauterine insemination [10]. The use of caffeine has been explored previously [11], while theophylline, a similar additive, has not been studied in depth with buffalo frozen semen. Pentoxifylline has been used with mixed results in a number of species, although it has never been applied for buffalo cryopreserved semen. Therefore, the objective of this study was to determine the effect of using methylxanthines such as pentoxifylline, theophylline and caffeine on post thaw motility, membrane integrity, acrosome intact and DNA damage of buffalo frozen semen.

MATERIALS AND METHODS

Semen collection and freezing

The experiment was conducted at the Buffalo Breeding and Extension Training Center, Urmia, West Azerbaijan, Iran (Latitude:38′23″ N; Longitude 47′ 40″ E; Altitude:1568.5 M) during October and December 2014. Two consecutive ejaculates were collected using artificial vagina (IMV, France; at 42°C) from three adult buffalo bulls (*Bubalus bubalis*) of known fertility and similar age (4–5 years) for a period of six weeks (replicate). Ejaculated semen from each bull was immediately transferred to the laboratory. Sperm progressive motility was determined microscopically (×400; Olympus BX20, Tokyo, Japan) and sperm concentration was

determined by the digital photometer (IMV, France). At least, one ejaculate of each bull at each replicate always passed the criteria (Motility>70%). To eliminate individual differences, semen samples from the four bulls were pooled. Each pooled sample was split into four equal aliquots at a concentration of 50×10^6 motile spermatozoa/1 ml at 37 °C and embedded with different levels of mehylxanthins in tris-citric egg yolk extenders. These extenders were prepared by using 3.0 g tris (hydroxymethyl -amino methane) and 1.56 g citric acid, fructose 0.2% w/v, glycerol 7.0 ml (Merck, Germany), and egg yolk 20% in 74 ml distilled water. All chemicals used in this study were obtained from Sigma–Aldrich (St. Louis, MO, USA) unless otherwise indicated. Antibiotics namely benzyl penicillin (1000 IU/ml, Pharmacia & Upjohn, Belgium) and streptomycin sulphate (1000µg/ml, Pharmacia & Upjohn, Belgium) were added to tris-citric egg yolk extender. Concentrations for pentoxifylline (3.5 mM), theophylline (25 mM), and caffeine (10 mM) were determined based on previous similar studies in cows and buffaloes [2,11,12]. Antioxidant-free extender was used as a control. After dilution, it was cooled from 37 to 25◦C in approximately 2 hours at room temperature (22–25 ◦C). The samples were filled in 0.5 ml French straws, sealed with filling and sealing device (IMV, France), and frozen in programmable cell freezer before plunging into liquid nitrogen. After a week, the frozen straws were thawed at 37 °C for 40 s in water bath and prepared for evaluation.

Semen evaluation

1. Motility

An aliquot of semen (5µL) was placed on a pre-warmed (37˚C) Makler chamber and analyzed for sperm motion characteristics using a computer-assisted sperm analyzer (Sperm Class Analyzer, version 5.1, Microptic; Barcelona, Spain). The CASA-derived motility characteristics were analyzed immediately after thawing and four hours of incubation at 37˚C four microscopic fields were analyzed in each sample using a phase-contrast microscope (Nikon, Tokyo, Japan) supplied with a pre-warmed stage at 37 ˚C and at ×100 magnification. Four microscopic fields with 400 spermatozoa were analyzed. The CASA derived motility characteristics included percentages of motility (%) and progressive motility (PM, %), straight-line velocity (VSL, µm/s; the straight-line distance from beginning to end of track divided by time taken), average path velocity (VAP, µm/s; the spatial averaged path that eliminated the wobble of the sperm head) ,curvilinear velocity (VCL, µm/s; total distance traveled by a sperm during the acquisition divided by the time taken), lateral head displacement (LHD, µm; deviation of the sperm head from the average path), linearity (LIN, %; VSL/VCL × 100), and straightness (STR, %; VSL/VAP × 100) [13].

2. Sperm plasma membrane integrity

Sperm membrane integrity was determined using hypo-osmotic swelling (HOS) assay. HOS solution consisted of 0.73 g sodium citrate and 1.35 g fructose dissolved in 100 ml distilled water (osmotic pressure -190 mOsmol/Kg). An aliquot of 50 µl of frozen–thawed semen was mixed with of HOS solution (500 µl) and incubated for 30 minutes at 37 °C before examining with a phase contrast microscope (×400; Olympus BX20, Tokyo, Japan). Two hundred spermatozoa were assessed for their swelling ability in HOS. The swollen spermatozoa characterized by coiling of the tail were considered to have an intact plasma membrane [14].

3. Normal acrosomes

To assess sperm acrosomal integrity, 100 µl of semen sample was fixed in 500 µl of 1% formal citrate (2.9 g tri-sodium citrate dihydrate, 1 ml of 37% solution of formaldehyde, dissolved in 100 ml

of distilled water) for 15 minutes. One hundred spermatozoa were examined with a phase contrast microscope (×1000; Olympus BX20, Tokyo, Japan) under oil immersion. A normal acrosome was characterized by a normal apical ridge [15].

4. DNA damage

Assessment of DNA integrity was carried out with sperm chromatin structure assay (SCSA). This technique is based on the susceptibility of the sperm DNA to acid induced denaturation in situ shown by the meta chromatic shift in Acridine Orange (AO) stain from green (dsDNA) to red (ssDNA) fluorescence depending on the degree of DNA denaturation. Frozen–thawed semen was centrifuged at 500 g for 5 min. Supernatant was removed, and pellets were suspended with tris Null EDTA buffer (0.15mol NaCl, 1mmol EDTA and 10mmol tris at pH 7.2) with concentration of 5 9 106 sperm ml1. Afterwards, µl of acid/detergent solution (0.1% v/v Triton X-100 in 0.08 mol HCl and 0.15 mol NaCl) was added. After 40 s, 1200 µl of acridine orange solution, containing 6 µl/ ml of acridine orange hydrochloride solution (A8097 in 0.15 mol NaCl, 1mmol EDTA, 0.2 mol Na2HPO4 and 0.1 mol citric acid at pH 6.0), was added, and finally, after 30 min, spermatozoa were subjected to flow cytometry. Green fluorescence of double-stranded DNA (intact DNA) and red fluorescence of single-stranded DNA (damaged DNA) were detected with FL1(500– 530 nm) and FL3 (620 nm) detectors respectively (Figure 1) [16].

Statistical analysis

Each treatment was replicated six times. For each replicate, four straws were thawed and pooled for evaluation of sperm parameters. One-way analysis of variance (ANOVA) along with Duncan's multiple range tests was used to evaluate differences between the groups. Results are presented as the mean percentage standard error of mean (SEM). The statistical analysis was carried out using SPSS computer program (Version 16.0; SPSS Co, Chicago, IL, USA). The value of $p<0.05$ was considered statistically significant.

RESULTS

Motility

The type of extender had a significant effect on overall post-thaw sperm motility as determined subjectively (Table 1). Supplementation of cryopreservation extender with 3.5Mm pentoxifylline improved motility of post-thawed spermatozoa as compared to the control group ($p<0.05$). Kinematic parameters such as VSL, VCL, VAP and LIN in the pentoxifylline-based extender were comparatively superior to others additives (Table 1). Concerning sperm motion characteristics, supplementation of cryopreservation extender with caffeine and theophylline did not show significant differences with control groups (Table 1).

Plasma membrane integrity

The data on plasma membrane integrity of post-thawed spermatozoa are given in Table 2. Supplementation of buffalo cryopreservation extender with 3.5 pentoxiphylline significantly increased plasma membrane integrity of spermatozoa as compared to the control group ($p<0.05$). Supplementation of cryopreservation extender with caffeine and theophylline did not show significant differences in control groups (Table 2).

Acrosomal integrity and DNA damage

As shown in Table 2, after thawing, the proportion of spermatozoa with normal apical ridge for post thaw samples did not show significant differences in treatment and control extenders. The type of additives as well as the interaction between extenders did not have significant effects ($p>0.05$). No significant differences were observed in DNA damage of spermatozoa among the groups ($p>0.05$).

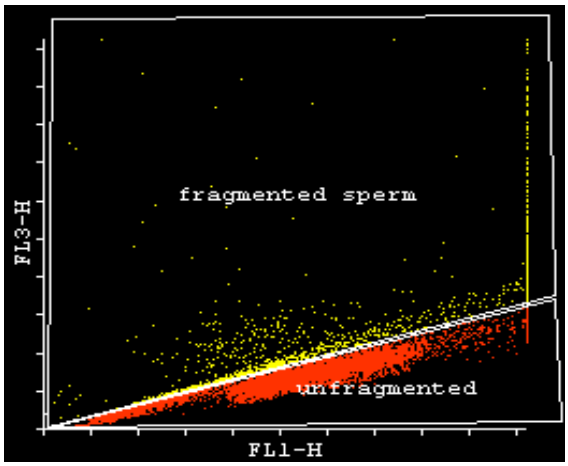

Figure 1. SCSA cryptogram of frozen thawed buffalo sperm cells. Each cell's position is based on theamount of native DNA satiability (green fluorescence; FL 1) vs. fragmented DNA (red fluorescence; FL 3).

Table 1. Mean (±SE) Motility characteristics of buffalo semen extender supplemented with methylxanthines following freeze/thawing

Parameters	Groups				
	control	Pentoxifylline	Theophylline	Caffeine	P value
Motility (%)	61.79 ± 2.47 [a]	73.43 ±2.25 [b]	64.43 ±1.05[a]	65.17 ±1.12 [a]	0.015
Progressive motility (%)	48.37 ± 3.27 [a]	59.76 ±3.25 [b]	51.66 ±1.91[a]	52.66 ±2.02 [a]	0.006
Curvilinear velocity (VCL; µm/s)	42.78± 2.61 [a]	56.12± 1.63 [b]	45.90± 2.16[a]	45.37± 3.82[a]	0.032
Straight line velocity (VSL; µm/s)	23.37±1.32[a]	28.66 ± 0.75[b]	23.11± 1.85 [a]	24.61± 0.75[a]	0.017
Average path velocity (VAP; µm/s)	23.41± 1.88 [a]	30.05± 2.65[b]	26.38± 2.41[a]	26.52± 2.63[a]	0.009
Linearity (LIN; %)	34.96± 1.88	39.18± 1.92	35.49±2.79	37.96± 3.19	0.417
Straightness (STR; %)	58.89± 1.79	62.85 ± 3.13	61.48± 1.06	61.48± 1.06	0.121
Wobble (WOB; %)	42.41± 0.61	45.37± 2.39	45.21± 1.75	42.34± 2.77	0.359
Beat cross frequency (BCF; Hz)	4.95± 0.48	5.68± 0.29	5.36± 0.487	4.96± 0.43	0.992
Lateral head displacement (ALH; µm)	2.06± 0.12	2.30± 0.11	2.09± 0.06	2.02± 0.12	0.877

[a, b] Values in the same row with different superscripts have significant difference ($p<0.05$)

Table 2. Mean (±SE) plasma membrane integrity, Normal apical ridge and DNA damage of buffalo semen extender supplemented with methylxanthines following freeze/thawing

Parameters	Groups			
	control	Pentoxifylline	Theophylline	Caffeine
Plasma membrane Integrity (%)	61.54± 0.62[b]	69.65± 0.82[a]	62.65± 3.62[b]	60.56± 1.46[b]
Normal apical ridge (%)	79.01± 2.68	79.16± 1.12	82.16± 3.12	83.79± 1.02
DNA damage (%)	5.91± 0.79	5.58± 0.49	6.18± 0.51	6.23± 0.63

[a, b] Values with different superscript in the same row have significant difference ($p< 0.05$)

DISCUSSION

Pentoxifylline and caffeine have been shown to improve motility of fresh and frozen semen in a variety of domestic animals such as cows [12,17], ewes [18], goats [16,19], equine [6,8] and dog [7]. The additives vary in efficacy based on species and concentration and whether fresh, cooled or frozen semen is used(6). In a few previous studies , caffeine has been used to enhance the post thaw motility of buffalo frozen semen as well as fresh semen of other mammals [11]. However, there is no study on the effects of pentoxifylline or theophylline in frozen thawed buffalo semen. Results from the present study indicated that among methylxanthines only pentoxifylline has been effective in increasing post-thaw motility and kinematics parameters such as curvilinear velocity (VCL), average path velocity (VAP), and straight-line velocity (VSL) when compared to theophylline, caffeine or control groups. Earlier reports have indicated the use of pentoxifylline as a sperm movement enhancer for post thaw cryopreserved semen. Pentoxifylline significantly increased percentage of sperm motility, average path velocity (VAP), curvilinear velocity (VCL), straight line velocity (VSL), and amplitude of lateral head displacement (ALH) of post thaw cryopreserved semen in human [20,21] and dogs [7]. Data related to the motility in our study are completely in agreement with different studies all indicating that the inclusion of pentoxifylline with a Tris-based medium in ram increased the motility of frozen thawed spermatozoa [18]. In addition, no significant differences were observed for motion patterns between groups with different concentrations of caffeine or theophylline. It seems that the additional pentoxifylline as an ATP generator increases the activity of motility and some kinematic parameters of the sperm cells. Why pentoxifylline but not caffeine or theophylline was associated with improvement motility patterns is not clear. Moreover, the concentration of these derivative used in tris citrate-based extender may have been inadequate to elicit desired result. It seems that further studies with different extenders should be carried out. In similar studies, Pankaj et al. (2012) reported that supplementation of pentoxifylline and theophylline with fortified plant based extender significantly improved semen quality such as sperm motility, live-dead count, hypo-osmotic swelling and acrosomal integrity at room (22-25 °C) and refrigerated temperature (4-7°C) for various incubation periods [2]. In another study, the addition of caffeine or pentoxifylline on pooled turkey semen either during or after storage does not affect sperm mobility [22]. Similarly, Esteves et al. (2007) showed that pre-freeze treatment of poor quality human sperm with pentoxifylline did not improve post-thaw motility or viability nor did it prevent acrosomal loss during the freeze-thaw process [21]. In another study, the addition of 3mmol pentoxifylline to human fresh semen during incubation period prior to cryopreservation significantly decreased progressive and total motility compared with controls [23]. Similar results were found in studies on motility and acrosome integrity of ram semen after treatment with caffeine and pentoxifylline [18]. In another study, Sinha et al. (1995) reported the effects of two methylxanthines (caffeine and theophylline) at 2 and 5 mMol concentrations on goat sperm motility and on the percentage of acrosomal damage. However, our findings are in contrast with these studies in that the addition of caffeine and theophylline had a stimulatory effect on goat spermatozoa; even among the two methylxanthines used, caffeine was found to be the more effective in improving motility than theophylline. In our study of the three additives such as pentoxifylline, theophylline and caffeine, only supplementation of pentoxifylline in cryopreservation extender significantly improved semen motility relative to that of untreated control [19]. Lecewicz et al. (2019) showed that both phosphodiesterase inhibitors, caffeine and pentoxifylline, as well as 2'-deoxyadenosine a concentration of 10 mM after different post-thaw incubation times (30, 60, 120 min) increased the motility and selected kinematic parameters of thawed canine spermatozoa [24]. Moreover, fertility and viability of post-thawed spermatozoa may be affected by membrane integrity (Mahmoud et al., 2009). In this study supplementation, pentoxifylline in tris citrate- egg yolk extender were enhancement protection of the

sperm plasma membrane during cryopreservation. During freezing, the hypertonic extracellular environment causes intracellular dehydration as water leaves the cell to balance the osmotic gradient, causing cell shrinkage. Post-thaw motility and longevity are reduced due to membrane damage caused by osmotic and oxidative stress during freezing and thawing events [25]. Previous studies have indicated that vitro treatment of bull semen with pentoxifylline leads to marked augmentation of sperm motility, enhancement of acrosome reaction and protection of the sperm plasma membrane [10]. Similarly, Vega (1997) reported that the addition of pentoxifylline had a protective effect on sperm membrane prolonging the viability of post-thaw bovine semen. This effect may be ascribed to neutralization of reactive oxygen species (ROS) and reduction of lipid peroxidation (Vega, 1997). Pal et al. (2005) found that the addition of pentoxifylline with sperm cryopreservation medium not only provided a comparatively higher yield of spermatozoa with motility and forward progression on thawing but also minimized the lipid peroxidation of sperm membrane on cryopreservation [9]. Contrary to our results, post-thaw hypo osmotic swelling (HOS) test scores were not improved with the addition of pentoxifylline in frozen thawed human spermatozoa compared with the control group in frozen thawed human spermatozoa [23]. In a similar study, no differences were observed when the caffeine (5 mM), pentoxifylline (3.5 mM), or both additives were added prior or after stallion semen cooling. Pentoxifylline added after cooling reduced significantly motility during incubation. Viability and membrane functionality were also significantly impaired by additives. Pentoxifylline when added after cooling improved sperm motility and kinematic parameters for a short period of time [3]. In the present study, supplementation of cryopreservation extender with pentoxifylline, caffeine or theophylline revealed no significant effects on the prevention of DNA damage in frozen thawed spermatozoa. Kadirvel et al. (2009) reported that chromatin integrity of buffalo spermatozoa had a negative correlation with intracellular reactive oxygen species (ROS) but remained unaffected during cryopreservation. Extracellular ROS can easily permeate through the sperm membrane, elevate intracellular ROS and cause DNA damage and lipid peroxidation (Mahfouz et al., 2010). Koonjaenak et al. (2007) maintained that previously frozen thawed buffalo sperm chromatin integrity was not seriously damaged by cryopreservation [26]. However, Mahmoud et al. (2015) reported that highly significant differences were observed in chromatin integrity between fresh and frozen thawed buffalo semen [27].Our results are concordant with a study on human sperm, in which supplementation of methylxanthins did not improve post-thawed sperm DNA damage, even after 4 h incubation with induction of oxidative stress. However, our findings are in contrast with studies in which the addition of pentoxifylline led to increased DNA damage in human after thawing [4]; pentoxifylline, as a supplement when added to the freezing extender, has a deleterious effect and that it did not improve the survival of frozen–thawed boar spermatozoa [28].

CONCLUSION

In this study, among methlxanthins only pentoxifylline supplementation in tris- citrate egg yolk extender significantly improved the motility of buffalo frozen thawed semen as compared to caffeine or theophylline. Pentoxifylline can theoretically increase motility and therefore improve breeding efficiency and decrease the costs associated with frozen semen use. Further studies need to be conducted to evaluate the effect of methylxanthines on the fertility of cryopreserved buffalo semen.

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experiment. There is no conflict of interest in this study.

ETHICS

Approved.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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