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ORIGINAL ARTICLE

Semen Analysis

Clinical efficacy of a combination of Percoll continuous density gradient and swim-up techniques for semen processing in HIV-1 serodiscordant couples

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To evaluate the clinical efficacy of a procedure comprising a combination of Percoll continuous density gradient and modified swim-up techniques for the removal of human immunodeficiency virus type 1 (HIV-1) from the semen of HIV-1 infected males, a total of 129 couples with an HIV-1 positive male partner and an HIV-1 negative female partner (serodiscordant couples) who were treated at Keio University Hospital between January 2002 and April 2012 were examined. A total of 183 ejaculates from 129 HIV-1 infected males were processed. After swim-up, we successfully collected motile sperms at a recovery rate as high as 100.0% in cases of normozoospermia (126/126 ejaculates), oligozoospermia (6/6), and asthenozoospermia (36/36). The recovery rate of oligoasthenozoospermia was 86.7% (13/15). In processed semen only four ejaculates (4/181:2.2%) showed viral nucleotide sequences consistent with those in the blood of the infected males. After using these sperms, no horizontal infections of the female patients and no vertical infections of the newborns were observed. Furthermore, no obvious adverse effects were observed in the offspring. This protocol allowed us to collect HIV-1 negative motile sperms at a high rate, even in male factor cases. We concluded that our protocol is clinically effective both for decreasing HIV-1 infections and for yielding a healthy child.

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INTRODUCTION

Currently, around 33 million people are reportedly infected with the human immunodeficiency virus type 1 (HIV-1) worldwide, and 80% of these people belong to the reproductive age group.[1] Since the introduction of highly active antiretroviral therapy (HAART) in 1996, HAART use by the infected person was accompanied by a 92% reduction in risk of HIV-1 transmission to their partner.[2] Although sexual contact serves as the major transmission route for HIV,[3] it has been reported that HIV can also be transmitted by artificial insemination,[4,5] indicating that the infection can be spread horizontally from infected sperm. Because of its high prevalence among youth, preventing secondary infection in couples consisting of an HIV-1-positive male partner and an HIV-1 negative female partner (serodiscordant couples) is important.

Because HIV-1 in sperm can be removed by processing semen, current therapeutic approaches for infertility, including intrauterine insemination (IUI)[6] and *in vitro* fertilization (IVF) with intracytoplasmic sperm injection (ICSI),[7] now involve semen processing to reduce the risk of horizontal and vertical infection. However, Garrido *et al.*[8] reported 10% of the samples positive by nested polymerase chain reaction (nested-PCR). We have developed a sperm recovery method that combines Percoll continuous density gradient and modified swim-up techniques. We investigated the clinical efficacy of this processing technique in terms of sperm recovery, HIV-1 detection in the washed sperm suspensions and embryo culture media, and pregnancy outcomes.

MATERIALS AND METHODS

We performed assisted reproductive technology (ART) for 129 HIV-1 serodiscordant couples treated at Keio University Hospital between January 2002 and April 2012. Because of the decrease in the ovarian reserve, couples in which the female was 42 years of age or older were excluded. Cases with azoospermia in the ejaculate were also excluded. Some patients had complicating infections, including hepatitis B, hepatitis C, and/or syphilis. Many patients in this study were hemophiliacs, who were to become infected with HIV-1 through

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repeated infusions of plasma-derived products. Before starting the treatment, women partners were confirmed as being HIV-1 seronegative at the initial diagnosis based on antibody screening using an HIV-enzyme linked immunosorbent assay (HIV-ELISA). All the participating couples provided their written informed consent after receiving a thorough explanation of the risk of HIV-1 infection and the risks and benefits of IVF and ICSI. This study was approved by the Institutional Review Board of Keio University.

Semen collection, processing, and freezing

After approximately 3–5 days of abstinence, semen samples were collected. In accordance with the World Health Organization (WHO) laboratory manual for the Examination and Processing of Human Semen (5th Edition),[9] semen analysis was begun after the sample liquefied, usually within 15–60 min at room temperature. The specimens were divided into two aliquots. For both aliquots, HIV-1 was removed through our Percoll continuous density gradient and modified swim-up method.

The semen processing protocol has been described elsewhere.[10] Briefly, to prepare the Percoll continuous density gradient, 2 ml of Percoll (80%) was overlaid with 2 ml of Hanks balanced salt solution in a sterilized disposable centrifuge tube (Round tube, #2059, Falcon) and the tube was rotated to maintain a 76° vertical tilt. The semen from the infected male was diluted by adding 1 ml of Hanks solution, then overlaid on the prepared continuous gradient before centrifugation at 1600 ×*g* for 10 min. After centrifugation, a sterilized thumbtack with a silicone protector was used to punch a small hole at the bottom of the tube, and an aliquot of medium containing motile sperm was recovered through this hole, washed once with culture medium, then re-suspended in 0.3 ml quantities.

To introduce the sperm suspension to the bottom of the swim-up tube without allowing it to come in contact with the HIV-1-free medium of the upper layer, outer and inner embryo transfer (ET) tubes were utilized (**Figure 1**). First, the outer sheath of the ET tube was set near the bottom of the centrifuge tube in the Percoll continuous density gradient (**a**). Second, the inner ET tube was introduced inside the outer sheath (**b1**). The sperm suspension prepared by density gradient centrifugation was loaded into the inner tube, which was then placed beneath the medium (**c2** and **c3**) so that the potentially contaminated inner tube would not come directly in contact with the culture medium. After 45 min, the swim-up sperm (0.5 ml) was recovered from the surface of the medium (**d4**). Each resultant sperm suspension was then divided into two portions: one portion was used for the detection of HIV-1 using nested-PCR targeted at the *gag* genes, and the other was frozen for future use in ART. Cryopreservation of the sperm suspension was performed using liquid nitrogen vapor with KS-II cryo-medium.[11]

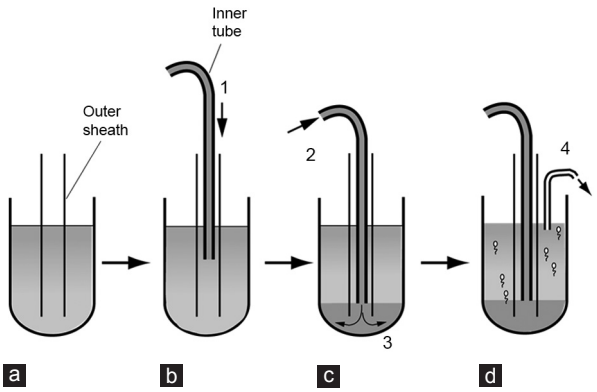

Figure 1: Preparation of the swim-up apparatus using an embryo transfer tube. (**a**) First, the outer sheath of the ET tube was set near the bottom of the centrifuge tube in the Percoll continuous density gradient. (**b1**) Thereafter, the inner ET tube was introduced inside the outer sheath. (**c2** and **c3**) The sperm suspension prepared by density gradient centrifugation was loaded in the inner tube, which was then placed beneath the medium so that the potentially contaminated inner tube would not come in direct contact with the culture medium. (**d4**) After 45 min, the swim-up sperm was recovered from the surface of the medium. ET: embryo transfer.

To prevent the processed HIV-1 containers from contaminating other semen samples, they were stored using standard cryopreservation in a storage tank with liquid nitrogen vapor for 1 or 2 months until the results of the nested-PCR analysis were ascertained. After confirming that the semen sample was HIV-1 negative, it was transferred to a liquid nitrogen container for subsequent ICSI.

To reduce bias, we attempted to use the same methods for semen processing and swim-up in all the cases, regardless of the semen parameters, blood viral load, or history of HAART. In addition, HIV-1 testing was performed using the same protocol for all the subjects in this series. The viral load in unprocessed semen was not measured.

Assisted reproductive technology

Ovarian stimulation was conducted using either the gonadotropin-releasing hormone (GnRH) agonist Buserelin (Suprecur®: Mochida Pharmaceutical) long protocol or the GnRH antagonist cetrorelix/ganirelix (Cetrotide®: Shionogi, Ganirest®: MSD) protocol, depending on the age and hormonal status of the female patient, as well as recombinant follicle stimulation hormone (FSH) (Follistim®: Schering-Plough Corporation, Gonal F®: Serono Pharmaceutical) or human menopausal gonadotrophin (HMG) (Ferring®: Ferring Pharma, HMG Teizo®: Aska Pharmaceutical). The administration of a GnRH agonist was started during the midluteal phase of the previous cycle, and the administration of a GnRH antagonist was started when one or more follicles reached 14 mm in diameter. The GnRH agonist and the GnRH antagonist were administered until the day of human chorionic gonadotrophin (HCG) administration. When three or more follicles reached 18 mm in diameter, intramuscular HCG (10 000 IU, hCG Mochida®; Mochida Pharmaceutical) was administered 34 h before egg collection to trigger ovulation. ICSI was used as the insemination method for all the collected sperms. After embryo culture, the culture mediums were examined for the presence of HIV-1 in each of the culture dishes using HIV-RNA or proviral DNA analysis with nested-PCR. Only embryos from apparently HIV negative culture medium dishes were replaced, and surplus eggs were frozen. Sequence analysis was conducted in PCR-positive specimens. ET was performed using abdominal ultrasonography assistance on days 2–6.

Implantation was defined as the increase in serum HCG levels to >25 IU l^{-1} and/or detection of the gestational sac. Implanted embryos were subsequently monitored using transvaginal ultrasonography. Clinical pregnancy was ascertained once a gestational sac was detected. Births at <22 weeks were classified as abortions and births at 37–42 weeks were classified as full-term infants.

Examination of processed sperm suspensions and culture media using nested-PCR

The samples of sperm suspension, culture medium, or plasma were centrifuged at 35 500 ×*g* for 1 h at 4°C. RNA and DNA were extracted from the precipitate using QIAamp UltraSens Virus Kit (Qiagen, Tokyo, Japan). Nested-PCR targeted the *gag* gene was performed using our previously reported method.[12] This protocol has proven capability of

detecting a single virion or infected cell even in the presence of up to 8×10^6 spermatozoa per sample. Throughout the procedure, the medium used for washed sperm or fertilized eggs was the negative control, and the medium with 10 virions added was a positive control. We defined HIV-1-positive specimens as those in which a positive band was detected using nested-RT-PCR. When the virus was detected using the nested-PCR assay in any of the sample aliquots, sequencing analysis of the PCR products was performed using second-round primers and the BigDye Terminator (ver. 1.1, Applied Biosystems, Foster City, CA, USA).[13] In cases with washed sperm, the HIV-1 analysis was performed within 1 month; in cases with culture media, the analysis was performed within 48 h. After 2007, the DNA sequencing analysis was performed after the virus sequence had been detected using nested-PCR.

Evaluation of horizontal infection

Cases that did not result in pregnancy were subjected to a peripheral blood HIV-1 antibody test, at least, 3 months after ET. For cases that resulted in pregnancy, HIV-1 antibody testing of maternal blood was performed at 36 weeks of gestation, and maternal and neonatal or infantile blood samples were analyzed at delivery and/or 6 months after birth using nested-PCR.

Statistical analyses

Using SPSS (ver. 11.5, SPSS Inc., Chicago, IL, USA), the Mann–Whitney U-test and Chi-square test were conducted to compare the two groups that underwent either fresh or frozen ET. $P < 0.05$ were considered significant.

RESULTS

At the time of presentation, the average ages of the male and female subjects were 37.2 ± 4.3 years and 35.6 ± 6.1 years, respectively. The mean basal FSH levels of the females were 6.11 ± 2.72 mIU ml^{-1}. At the time of semen collection, the mean CD4 count of the infected males was 444 ± 220 μl^{-1}, and the serum viral load (VL) was <40 copies ml^{-1} in 62 cases (**Table 1**).

Sperm washing and recovery

A total of 183 ejaculates from 129 HIV-1-infected males were processed. The mean values of semen volume, sperm concentration, and sperm motility rate were 2.49 ± 1.42 ml, 46.48 ± 24.04 × 10^6 ml^{-1}, and 50.2% ± 18.5%, respectively. Motile sperms were collected from 181 ejaculates (181/183:98.9%) after swim-up (**Table 2**). Approximately, 15 cases were diagnosed with oligoasthenozoospermia and 36 cases with asthenozoospermia as per the new WHO criteria.[9] Among them, the collectible cases in oligoasthenozoospermia and asthenozoospermia were 13 (86.7%) and 36 (100.0%), respectively.

Table 1: Characteristic of HIV-1 serodiscordant couples (*n*=129)

Variables	*Mean±s.d.*
Female partner	
Age (year)	35.6±6.1
Basal FSH (mIU ml^{-1})	6.11±2.72
Male partner	
Age (year)	37.2±4.3
CD4 count ×10^6 (ml^{-1})	444±220
VL <40 copies (ml^{-1}), *n* (%)	62 (48.1)
HAART, *n* (%)	84 (65.1)

Values are listed as mean±s.d. CD4: cluster of differentiation 4; FSH: follicle-stimulating hormone; HAART: people who received highly active anti-retroviral therapy; HIV: human immunodeficiency virus; VL: viral load; s.d.: standard deviation

Nested-PCR and sequence analyses of semen suspension and culture medium

The specimens were divided into two aliquots. When the virus was detected by nested-PCR on aliquots of either, DNA sequencing analysis was performed. Only four ejaculates were HIV-1 positive on the nested-PCR analysis. One ejaculate was positive in both aliquots, and three were positive in one aliquot. These aliquots showed viral base sequences consistent with those in the blood of the male partner (**Table 2**). Two of the four ejaculates were from the same patient. This patient was not receiving HAART and had a VL of 7000–8000 copies ml^{-1} and a CD4 count of 826 cells ml^{-1} at the time of semen collection; the patient also had complications involving chronic hepatitis B and syphilis. Semen re-collected and re-processed from the same patient was HIV-1-positive in one aliquot, indicating a high viral load. Third of the four ejaculates was from a patient who received HAART and had a VL of <50 copies ml^{-1} and a CD4 count of 1 162 cells ml^{-1} at the time of semen collection; this patient also had chronic hepatitis C. The hepatitis C viral load was 4.3×10^6 IU ml^{-1}. Semen re-collected and re-processed from the same patient was HIV-1 negative. Forth ejaculate was from a patient who did not receive HAART had a VL of 4 400 copies ml^{-1} and a CD4 count of 580 cells ml^{-1} at the time of semen collection; this patient also had chronic hepatitis C. The nucleotide sequence of the amplified DNA found in the swim-up sperm suspension of this patient was the same as that of a positive control and also the same as the DNA amplified from the peripheral blood mononuclear cells (PBMCs) of this patient. Consequently, it is unclear whether the amplified DNA from the swim-up suspension in one aliquot was the result of contamination of control HIV-1 RNA or unremoved HIV-1 after sperm washing. Semen recollected and re-processed from the same patient was HIV-1 negative.

In the 11 culture medium specimens from nine cases, the nested-PCR results for the culture media were positive and the fertilized embryos were suspected of being infected with HIV-1. Of these, the nested-PCR amplification products of six cases were subjected to a gene sequence analysis. Five cases were considered false positive based on the consistency of their sequence with that of positive controls. In only one case, the sequence was consistent with that of the virus in the blood of the male partner. The collected sperms, in this case, were HIV-1 negative on nested-PCR testing. This patient was being undergoing HAART, and the VL and CD4 count were <50–100 copies ml^{-1} and 400–632 cells ml^{-1}, respectively. The remaining three cases (five media samples) were processed before 2007, and gene sequencing was not performed for the PCR products.

Clinical outcomes

The implantation, live birth rates per ET were 36.1% and 26.3%, respectively (**Table 3**). Similar to cases in other countries, the use of frozen-thawed embryo transfer has increased in our hospital in recent years because of the reduced risks associated with many prenatal problems, such as multiple gestation, low birth weights, and prematurity.[14] Multiple pregnancies occurred in seven cases. We identified 91 cases of live births; no horizontal infections in the female patients and no vertical infections in the newborns were observed. One hydrocephalus related abortion and one hereditary genetic disorder (glucose-9-phosphate dehydrogenase deficiency) were observed.

DISCUSSION

HIV-1 can propagate through semen, and the horizontal infection risk per sexual intercourse is 0.1%–0.2% in the absence of preventive

Table 2: Semen analysis

	Total	*Normozoospermia*	*Oligozoospermia*	*Asthenozoospermia*	*Oligozoospermia and asthenozoospermia*
Ejaculates, *n*	183	126	6	36	15
Sperm volume (ml)	2.49±1.42	2.53±1.33	2.92±1.50	2.35±1.11	2.99±1.82
Sperm concentration ($\times 10^6$ ml^{-1})	46.48±24.04	53.88±18.64	11.73±5.39	47.64±23.11	6.07±5.27
Sperm mobility rate (%)	50.2±18.5	60.7±8.2	61.7±17.7	32.1±8.2	23.8±12.4
Sperm concentration post swim up ($\times 10^6$ ml^{-1})	2.45±2.94	2.85±3.00	1.10±0.66	1.18±1.41	1.46±3.62
Collectable ejaculates, *n* (%)	181 (98.9)	126 (100.0)	6 (100.0)	36 (100.0)	13 (86.7)
HIV-1-positive ejaculates using nested-PCR after processed semen, *n* (%)	4 (2.2)	4 (3.2)	0 (0.0)	0 (0.0)	0 (0.0)

Values are listed as mean±s.d. HIV: human immunodeficiency virus; PCR: polymerase chain reaction; s.d.: standard deviation

Table 3: Clinical outcomes of ART procedures conducted on serodiscoradant couples

	Total	*Fresh ET*	*Freeze ET*	*P**
Number of OPU cycles, *n*	334	-	-	-
Number of oocytes retrieved per retrieval, *n*	8.79±5.43	-	-	-
Number of 2 pronuclei embryos per retrieval, *n*	3.95±3.12	-	-	-
Number of embryo transfer cycles, *n*	319	179	140	-
Number of embryo transferred per transfer, *n*	1.72±0.60	1.67±0.50	1.78±0.69	0.394
Implantations per embryo transfer, *n* (%)	115 (36.1)	57 (36.3)	58 (39.3)	0.077
Live-birth per embryo transfer, *n* (%)	84 (26.3)	32 (18.8)	52 (37.1)	<0.001
Abortions per embryo transfer, *n* (%)	29 (9.1)	23 (12.8)	6 (5.2)	0.004
Ectopic pregnancies per embryo transfer, *n* (%)	2 (0.6)	2 (1.1)	0 (0.0)	0.506
Delivered pregnancies: Singleton, *n*	77	27	50	0.004
Delivered pregnancies: Twin, *n*	7	5	2	0.404
Term birth weight (g)	3082±465	3025±474	3115±463	0.345
Estimated gestational age at delivery (week)	38.6±2.31	38.9±1.4	38.5±2.7	0.513

Values are listed as mean±s.d. *Fresh ET versus Freeze ET (Mann–Whitney U-test). ET: embryo transfer; OPU: oocyte pick up; ART: assisted reproductive technology; s.d.: standard deviation

measures.[15,16] Furthermore, HIV-1 infection can even occur by artificial insemination if the semen is inseminated without proper processing.[17] Consequently, a semen processing protocol to eliminate HIV-1 prior to IUI, IVF, or ICSI is important for HIV-1 serodiscordant couples opting for fertility treatment.

Although the HIV-1 content in the seminal plasma and cellular component of sperm can be reduced considerably by washing and the swim–up technique, HIV-1 can still be detected in some cases. Marina *et al.*[18] and Savasi *et al.*[19] reported that 5.6% (6/107) and 4% (96/2400), respectively, of analyzed washed semen samples, were HIV-1 positive after RT-PCR. Garrido *et al.*[20] reported that HIV-RNA was present in approximately 11.5% (9/78) of washed semen specimens using nested-PCR. Nicopoullos *et al.*[21] reported that they conducted sperm washing by combining 45%/90% colloidal silica density gradient and swim-up techniques to obtain a collected sperm concentration of 15.7×10^6 ml^{-1} and an HIV-RNA-positive rate of approximately 3.7% (16/437) using RT-PCR; they subsequently used IUI with the collected sperm. The HIV-1-positive rate after washing using nested-PCR in our study was 2.2% (4/181), showing that this method is significantly successful in eliminating HIV-1. Out of four ejaculates, three ejaculates were HIV-1 positive for only one aliquot; the remaining aliquot was HIV-1 negative on nested-PCR and could be used for ICSI.

Motile sperm in a continuous density gradient is thought to move more easily to the bottom than in a discontinuous gradient because of the absence of a boundary for each concentration gradient. Kobayashi *et al.*[22] reported the efficacy of semen processing using continuous density for oligozoospermic men. Recently, Edmond *et al.*[23] reported that a continuous gradient increased equine sperm recovery rate as compared with the discontinuous gradient. In our study, motile sperms were recovered using the Percoll continuous density gradient and swim-up method, with a recovery rate as high as 100.0% (126/126) in patients with normozoospermia, oligozoospermia, and asthenozoospermia; even in oligoasthenozoospermia patients, the recovery rate was 86.7%. Although testicular sperm extraction (TESE) and microsurgical epididymal sperm aspiration (MESA)[24–29] are usually necessary in cases of extremely low numbers of motile sperms, our method has the potential to decrease the requirement of these procedures.

The high success rate of HIV-1 elimination in our study might also be the result of our use of Percoll as the density gradient medium. Although Percoll has not been used for ART in many countries since 1996, Percoll-based density gradients are still used clinically for sperm separation in some countries[30] including Japan, despite the fact that there are some concerns about their limitations.[31,32] Since we previously reported that Percoll might be superior as a density gradient medium for HIV-1 viral elimination, compared with the more widely used silane-coated density medium,[33] we have been using Percoll with the consent of our patients. However, whether Percoll is indeed superior to silane-coated medium remains to be confirmed.

In this study, there was only one case in which the result of the HIV-1 gene sequence analysis from the embryo culture medium was consistent with that of the male partner-derived HIV-RNA. Whether the HIV-1 gene was actually present in the fertilized eggs, in this case, is unknown because the patient did not opt for a nested-PCR analysis of the fertilized eggs. However, it is possible that HIV-1 was present inside or outside (e.g., sperm suspensions) the injected sperm. In this situation, it seemed appropriate to check for HIV-1 in the embryo culture medium, even if HIV-1 was not detected in the processed semen.

There are two circumstances under which the culture medium could contain HIV-1 virus even when the result for the washed sperm suspension is negative: (1) when a minute amount of the virus remains in the washed sperm suspension, the detection system could fail to recognize the existence of the virus; (2) even if one-half of the resulting sample did not contain the virus, a minute amount of the virus could have contaminated the other half that was frozen for the

ICSI procedure. Since the replication, transcription, and translation of the HIV-1 *gag* gene is reportedly maintained through at least one cycle of embryonic cell division when injected into hamster eggs,[34] there is a possibility that amplification could occur in the culture. Thus, it appears that HIV RNA/DNA can amplify during the culture period.

In this study, the false-positive rate of HIV-1 analysis for the culture media was higher than that for the washed sperm suspensions. One possible reason for this difference might be the limited time available for the analysis when analyzing the culture media. Culture media are mostly sampled on day 2 after insemination and are analyzed within 24 h for the presence of HIV while up to a month is available when analyzing sperm suspensions.

Although some concern exists regarding the safety of ICSI for the embryo and future offspring, Melo *et al*.[35] reported that fertilization with ICSI using sperms from HIV-1-positive males does not appear to have a significantly negative impact on embryo development. In addition, other studies suggest that ART is safe and effective for avoiding horizontal and vertical transmission in HIV serodiscordant couples.[36] In this study, we observed no obvious increase in abnormalities in the children, except for one case of hydrocephalus and one case of glucose-6-phosphate dehydrogenase deficiency; the latter case was considered to be hereditary. In the future, however, it might be more reasonable to perform IUI in cases where plenty of motile sperm can be recovered, and the HIV-1 PCR analysis is negative, especially for the male partner whose viral load in the blood remains almost zero for a long time.

CONCLUSION

Our semen processing protocol using a combination of Percoll continuous density gradient and swim-up methods allowed us to collect HIV-1 negative motile sperms at a high rate, even in cases of low numbers of motile sperm, after the application of a sensitive nested RT-PCR analysis. We believe that our semen processing protocol is clinically effective.

AUTHOR CONTRIBUTIONS

OI and NK carried out the study design and execution, analysis and interpretation, manuscript preparation, and critical discussion. SK, AO, and HH carried out the execution of study, acquisition and analysis of data, and critical discussion. HI, MY, HT, KI, and MT carried out the design assistance, data analysis, interpretation and critical discussion. All the authors examined the data and approved the final manuscript.

COMPETING INTERESTS

All authors declare no competing financial interests.

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