

Effect of Elapsed Time after Blood Collection on the Viability and Mitotic Index of Human Lymphocytes during Karyotype Analysis

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Authors' contributions

This work was carried out in collaboration between all authors. Author DHEK performed the experiments- developed the methodology. Author AAEK conceived and designed the experiments, analyzed the data, wrote the manuscript. Author HHES contributed reagents, materials, analysis tools. Author ITES approved the study, revise the methods. All authors read and approved the final manuscript.

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ABSTRACT

Background: Chromosome staining using G banding is a commonly used technique during karyotyping, however, a limited number of laboratories carries out the test. Blood samples must be sent to the laboratory on the same day of sample collection.

Aim: To assess the effect of time passed from sample withdrawal to the beginning of lymphocyte culture on lymphocyte viability and the mitotic index of chromosomal spread.

Methods: Collected peripheral venous blood samples were either processed for chromosome analysis within 2h of samples collection or stored at 4°C then processed at 24h and 48h. Lymphocytes viability was determined by trypan blue and mitotic cells were visualized by the lighted

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microscope at the 40x objective lens. Mitotic index was calculated per 1000 cell count.

Results: Delay in sample processing more than 24h have a deleterious effect on lymphocyte viability with a significant reduction in mitotic index relative to the freshly processed sample.

Conclusion: Culturing of cells within 24h of sample collection is highly recommended whenever possible and delay more than 48h should be avoided.

Keywords: Karyotype; mitotic index; lymphocytes; chromosomes analysis.

1. INTRODUCTION

Karyotype analysis of human chromosomes is a widespread cytogenetic technique used in screening and diagnosis of inherited genetic diseases and cancer [1-4]. Chromosomal aberrations as translocations, inversions or changes in chromosomal number are common finding associated with bone marrow malignancy, leukemia, lymphoma or sarcoma which makes the cytogenetic results crucial for providing diagnostic, prognostic, and predictive information [5,6].

Karyotype examination in human is typically performed on peripheral blood, bone marrow or amniotic fluid samples with viable cells capable of division to get into the metaphase of the cell cycle and subsequently can be used for chromosomal imaging [7].

Metaphase is the stage of cell division in which the chromosomes are most suitable for karyotype analysis. G banding is the most widely used technique in karyotype analysis due to its low cost and short preparation time. It is achieved by digesting the chromosomes with the proteolytic enzyme trypsin for a short period followed by Giemsa staining to acquire the characteristic banding pattern of each chromosome [8,9]. The main challenges in the cytogenetic analysis are that satisfactory karyotype results cannot be achieved in every analysis due to a low mitotic index in some samples or an ambiguous chromosomal pattern that cannot be analyzed. Many technical efforts have been made in order to increase the mitotic index by improving the sample preparations as well as the cell culture condition [10].

Because cytogenetic laboratories performing karyotype analysis are only present in large centers, it necessitates physicians, clinics and affiliated laboratories to draw blood samples or amniotic fluid samples and send them to these centers as soon as possible. Throughout the transportation period which may take up to 24h after sample collection cell proliferation and

viability may be seriously compromised, a situation that may result in a reduction or even complete absence of mitosis [10-12].

The aim of this study was to assess the effect of elapsed time from sample withdrawal to the beginning of cell culture on the growth and viability of the cultured human lymphocyte and to assess its effect on the quality of the metaphase spread and the mitotic index during karyotype analysis.

2. MATERIALS AND METHODS

The study was carried out in the period from March 2017 to July 2017, in the department of the Biochemistry and Molecular diagnostics at the National liver institute, Menoufia University, Egypt. The research ethics committee of the institute approved the study and written informed consent was obtained from all participants. The authors declare there is no conflict of interest. Peripheral venous blood samples collected from 25 healthy volunteers, (14 male and 11 female mean age 29 ± 2.5 y) were used to carry out karyotype analysis. Any participant who was under medication such as antihypertensive, oral hypoglycemic; or suffering from any chronic illness were excluded from the study. According to the protocol, each sample was divided into 3 equal parts. The first part of the sample was processed for lymphocyte separation and culture within 2h of blood collection. The second and the third parts were stored in the refrigerator at 4°C and processed in the same way at 24h and 48h respectively. For all samples, the viability of the harvested lymphocytes was tested by Trypan blue before starting the culture process and the percent of viable cells was determined.

2.1 Chemicals and Reagents

RPMI1640, lymphocyte separation medium were purchased from Lonza, (Lonza, Bio Whittaker Germany). Trypsin, Colcemid (10 µg/ml), Penicillin-Streptomycin, (10,000 U/ml; 10,000 µg/ml), and Giemsa stain from (Life Technologies, Gibco, USA). Fetal bovine serum,

and Phytohemagglutinin from (PHA) (BioChrom-Germany). Glacial acetic acid, Methanol and Potassium chloride from (Thermo Fisher Scientific, Waltham, MA USA).

2.2 Preparation of Metaphase Chromosome Spread and GTG Banding

Lymphocytes were collected using Ficoll-Paque method [13]. Lymphocytes were counted and evaluated for viability by Trypan blue staining. Lymphocytes were cultured in a flask containing RPMI 1640 medium, supplemented with 10 % FBS, Penicillin/Streptomycin and 100 µg/ml phytohemagglutinin. The culture flasks were incubated in 5% CO₂ at 37°C for 72 h before colcemid 100 µg/ml was added to the culture flask for 45 min to arrest cells at metaphase. Cells were swollen by hypotonic treatment using 0.075M KCL for 10 minutes. The cells were fixed using freshly prepared ice-cold Carnoy's fixative solution for 15 min at -20°C. Chromosome spreads were prepared by gently dropping the cell suspension on a clean glass slide followed by overnight incubation at 60°C. The slides were immersed in a 0.05% trypsin solution at room temperature for 30 sec, then immediately immersed in 2% FBS for 10 sec to inactivate the trypsin. The slides were rinsed in PBS and stained with 2% freshly prepared Giemsa in modified Gurr' buffer for 8 min.

2.3 Karyotype Analysis and Calculation of the Mitotic Index

Karyotyping and number of metaphases were determined using an Olympus BX 43 microscope (Olympus Corp., Tokyo, Japan) connected to Nikon JENOPTIK, model ProgRes MF camera using Lucia-Cytogenetics software. For mitotic index determination; five slides were prepared from each blood culture; cells were visualized under a light microscope using the 40x objective lens and 1000 cell were counted. The mitotic index was calculated as follows: Mitotic Index = Number of lymphocytes in metaphase/ Total number of lymphocytes counted x 100 [10,14]. Per culture, at least 20 mitotic cells with good chromosomal spread were analyzed to consider a satisfactory karyotype result.

2.4 Trypan Blue Cell Count

Lymphocyte cell suspensions (100 µl) were mixed with 100 µl of 0.4% trypan blue and incubated for 1 min at room temperature. 10 µl

were loaded in a Hemocytometer covered with a clean dried coverslip. Living and dead (blue) cells were visualized and counted using the light microscope. Cell Viability % = Number of live Cells / Number of Live Cells + Number of Dead Cells [15,16].

2.5 Statistical Analysis

Statistical analysis was performed using GraphPad Prism, version3 software (California, CA, USA). Data were presented as mean and SEM. Data compared by one-way analysis of variance (ANOVA) followed by post-hoc Bonferroni test when appropriate. P < 0.05 considered as the level of significance.

3. RESULTS

3.1 Satisfactory Chromosomal Spread with Adequate Metaphase Plates of Samples Processed within 2h of Sample Collection

As the 2h time point is the earliest time for processing blood sample during karyotype analysis, slides prepared from human lymphocytes cultured within 2h of sample collection were evaluated for adequate metaphase spread. The culture of the lymphocytes at 2h produced sufficient mitotic spread with a good number of mitotic cells. These mitotic cells showed typical chromosomal plate consisted of 23 pairs of sister chromatids with classic appearance of four arms attached to each other at the centromere indicating a satisfactory karyotype result (Fig. 1).

3.2 Delay in Sample Processing Resulted in a Significant Variation in the Cytogenetic Criteria of the Chromosomal Plates

For evaluating the effect of the time passed before sample processing on the quality of the karyotype and mitotic index, lymphocytes were processed for cytogenetic analysis as described in "Material and Methods". The numbers of metaphases, karyotype result, and MI% of cells cultured at 2h, 24h, and 48h are summarized in the (Table 1). Cytogenetic analysis of samples cultured 2h after sample collection was satisfactory in 21 (84%) samples and inadequate in 2 (8%) samples due to poor morphology or scarcity of metaphases; 2 (8%) other samples had a complete absence of metaphases.

Samples cultured at 24h had 20 (80%) satisfactory; 3 (12%) inadequate and 2 (8%) complete absence of metaphase relative to 12 (48%), 7 (28%), and 6 (24%) samples cultured at 48h. Delay in sample processing >24h was associated with a significant increase in the number of cells with reduced mitosis, inadequate mitosis or complete absence of mitosis (Fig. 2).

3.3 Delay in Sample Processing >24h Reduced the Mitotic Index Without Affecting the Morphological Appearance of the Chromosomal Spread

The average number of metaphases were 25 ± 2.4 , 21 ± 2.2 , and 6.7 ± 1.3 for a sample processed at 2h, 24h and 48h respectively (Table 2). The mitotic index for fresh cultures at 2h did not differ from that cultures at 24h ($P > 0.05$), however, there was a significant difference between cultures at 2h and 48h ($p < 0.05$) (Fig. 3A). The morphological aspect of the chromosomes in fresh and stored cells at 24h and 48h was subjectively the same (Fig. 3B).

3.4 Sample Storage More than 24h Reduced Lymphocyte Viability

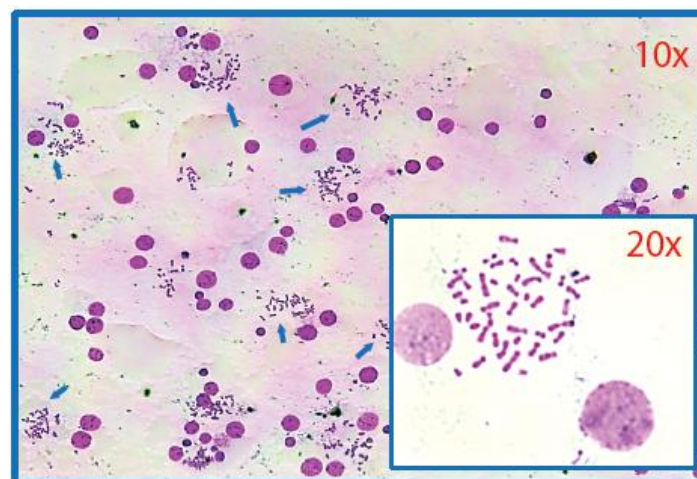
Similarly, the effect of the elapsed time on the viability of lymphocyte was assessed at 2h, 24h, and 48h after sample collection by trypan blue assay. Trypan blue is a dye exclusion test based on live cells possess intact cell membranes that exclude the blue dyes, thus only dead cells allow

the dye to permeate and appear blue under the microscope. The culture of the lymphocyte stored for 48h after sample collection revealed a significant reduction in the lymphocyte viability. Storage of the sample for 24h before processing to culture had little effect on the viability of the cells (Fig. 4).

4. DISCUSSION

Karyotype analysis is a multistep process started with obtaining viable cells, allowing them to grow and proliferate for a certain period of time by culturing them in a growing media in tissue culture incubators. Then the dividing cells are arrested in the metaphase stage of the cell cycle, stained and examined by light microscope. This is followed by acquiring images of the chromosomal spread for chromosomal painting and further analysis [17]. Karyotype analysis is a fundamental test in parental screening, sex determination, identification of chromosomal abnormalities such as Down's syndrome, Turner's syndrome, Klinefelter' syndrome, various malignancies, and leukemia [17-19].

The goal of the present study was to examine the potential time a blood sample can be stored before starting the culture process on the viability and mitotic index of the growing lymphocyte during karyotype testing. Cultures of the peripheral venous blood lymphocytes are most frequently used for human chromosome analysis. Blood lymphocytes are mainly in the G₀ or the quiescent stage of the cell cycle, exposure of the lymphocyte to phytohemagglutinin, stimulates the cells to proliferate and to enter the G₁/ S and G₂/M phases of the cell cycle. Cell cycle studies



A

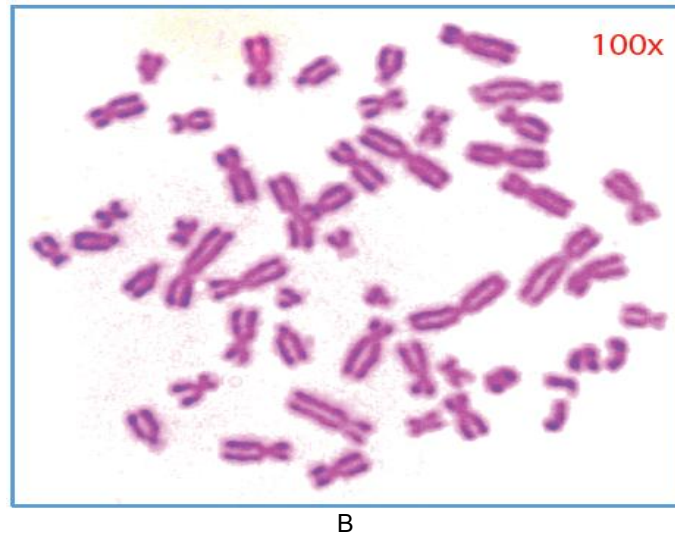


Fig. 1. Metaphase spreads of human peripheral blood lymphocytes cultured 2h after sample collection. (A) Photomicrograph of the metaphase spread viewed at the 10x, and the 20x objectives lens with arrows pointed at mitotic chromosomes. (B) Photomicrograph of the metaphase spread viewed at the 100x objective lens.

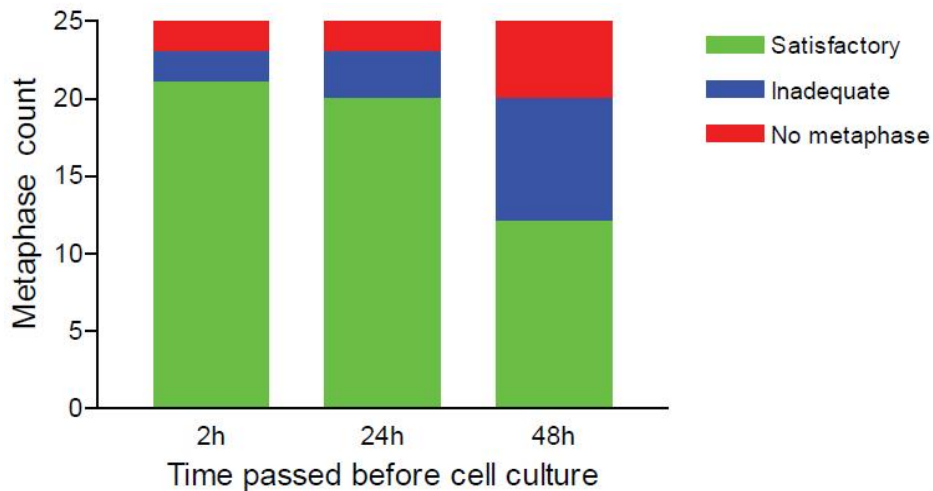


Fig. 2. Effect of time passed before processing a blood sample for lymphocyte culture on the quality of mitotic spread. Bar graph of a total of 25 blood samples processed at the indicated time.

showed that after 48h in culture the majority of lymphocytes are in their first mitotic division however extended cultures for 72h are required to get more cell in the mitotic stage [20,21].

Despite the apparent simplicity of the procedures, a limited number of public and private laboratories carries out the karyotype examination, as it demands special experience with the banding patterns of each chromosome,

beside it is a time consuming and labor-intensive test requiring special cell culture and microscopic facilities. Most of the laboratories performing karyotype testing require blood samples for karyotyping to be delivered as soon as possible and should arrive on the same day of sample collection. The sample must not be frozen nor fixed and for any circumstances that delay sample delivery, blood sample or tissue should be stored in a refrigerator at 4°C.

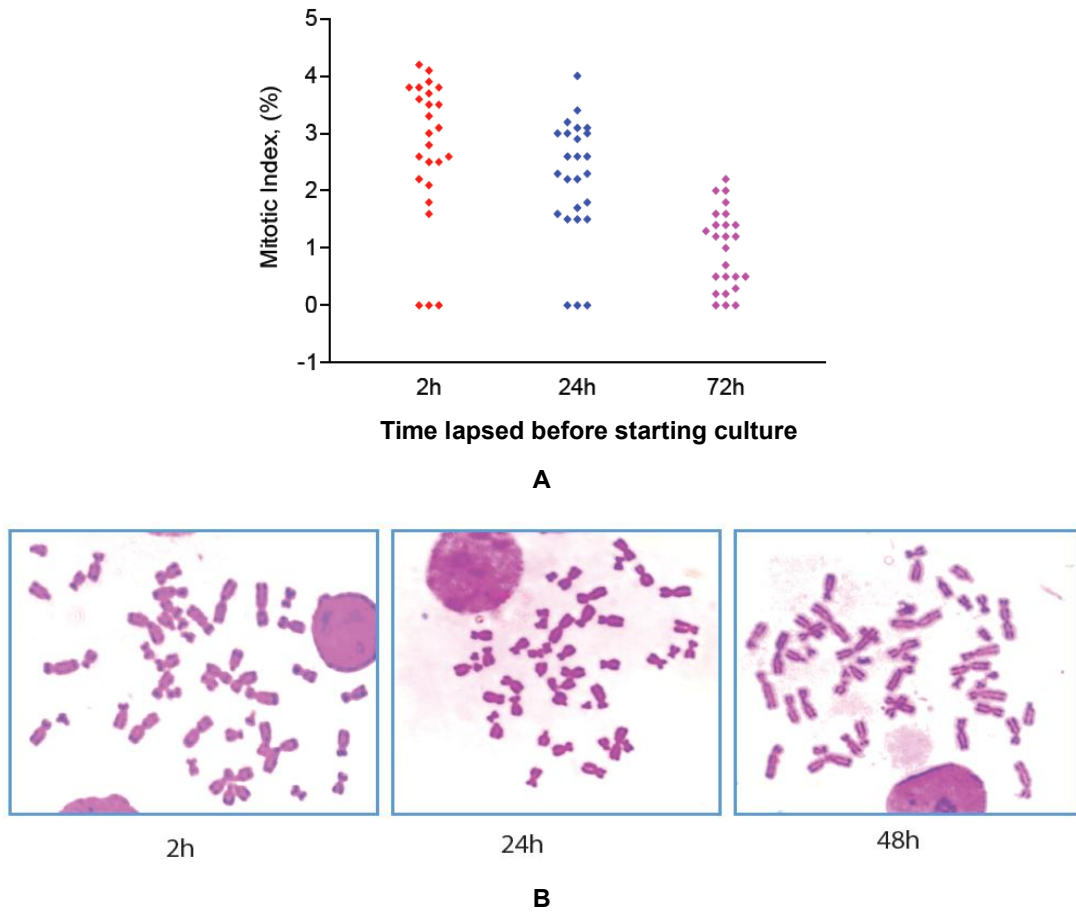


Fig. 3. Effect of time passed before processing blood sample on the MI% and morphological aspect of chromosomes. A. Column scattered graph of the MI% of lymphocyte processed for karyotype cultured at the indicated time. B. Photomicrograph of chromosomal plates stained with Giemsa prepared from sample cultured at 2h, 24h and 48 h of sample collection.

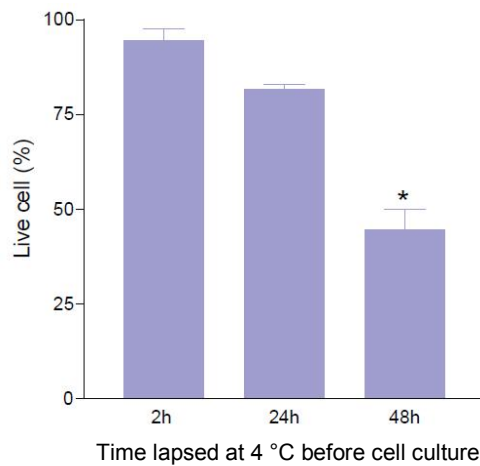


Fig. 4. Effect of culture time on the viability of lymphocyte: Cell viability by trypan blue assessed at 2h, 24, and 48h after blood sample collection

Table 1. Numbers of metaphases, karyotype result, and MI % of the cultured cells at the indicated time

Sex	2h			24h			48h		
	No. of metaphases	Karyotype	MI%	No. of metaphases	Karyotype	MI%	No. of metaphases	Karyotype	MI%
M	35	46, XY	3.5	25	46, XY	2.5	5	Inadequate	0.5
M	18	46, XY	1.8	4	Inadequate	0.4	12	46, XY	1.2
M	33	46, XY	3.3	30	46, XY	3	5	Inadequate	0.5
F	22	46, XX	2.2	16	46, XX	1.6	2	Inadequate	0.2
F	25	46, XX	2.5	15	46, XX	1.5	5	Inadequate	0.5
F	30	46, XX	3	29	46, XX	2.9	18	46, XX	1.8
F	36	46, XX	3.6	30	46, XX	3	16	46, XX	1.6
M	38	46, XY	3.8	35	46, XY	3.5	0	No metaphase	0
M	40	46, XY	4	29	46, XY	2.9	2	Inadequate	0.2
M	39	46, XY	3.9	26	46, XY	2.6	7	46, XY	0.7
M	2	Inadequate	0.2	2	Inadequate	0.2	0	No metaphase	0
F	33	46, XX	3.9	31	46, XX	3.1	14	46, XX	1.4
F	0	No metaphase	0	0	No metaphase	0	0	No metaphase	0
M	37	46, XY	3.7	22	46, XY	2.2	13	46, XY	1.3
M	30	46, XY	3	26	46, XY	2.6	14	46, XY	1.4
F	32	46, XX	3.2	23	46, XX	2.3	12	46, XX	1.2
F	31	46, XY	3.1	32	46, XY	3.2	4	Inadequate	0.4
M	28	46, XY	2.8	31	46, XY	3.1	5	46, XY	0.5
F	26	46, XX	2.6	18	46, XX	1.8	0	No metaphase	0
M	0	No metaphase	0	5	Inadequate	0.5	0	No metaphase	0
M	18	46, XY	1.8	22	46, XY	2.2	16	46, XY	1.6
M	21	46, XY	2.1	26	46, XY	2.6	12	46, XY	1.2
F	25	46, XX	2.5	26	46, XX	2.6	5	Inadequate	0.5
F	26	46, XX	2.6	18	46, XX	1.8	22	46, XX	2.2
M	5	Inadequate	0	0	No metaphase	0	0	No metaphase	0

"Inadequate" indicates poor morphology or scarcity of metaphases. "No metaphase" indicates a complete absence of metaphases.

Table 2. Mitotic index (MI) values of cultured human blood lymphocytes

	M±SEM	Minimum	Maximum	P1, P2
2h	31±7.7	16	42	>0.05
24h	25±7.0	15	40	
48h	11±6.2	2	22	<0.05

P1: 2h vs24h, P2: 2h vs 48h

The current study found that a delay more than 24h in starting the culturing process have detrimental effects on lymphocyte viability and proliferating ability with a reduction in the mitotic index of the proliferating lymphocytes. As mitotic index represents the percent of cells in metaphase of the cell cycle in a population of proliferating cells [22,23], the decrease in mitotic index values signifies inhibition of cell cycle progression and/or loss of ability to proliferate due to cell death or delayed cell cycle [24]. Despite the delay was associated with a decrease in MI%, yet the morphological aspect of the chromosomes in fresh and stored cells for 24, and 48h was subjectively the same. The results of this study are in agreement with Della Rose et al. who experimental similar effect of the storage time on the viability of peripheral blood lymphocyte during karyotyping analysis [25]. Interestingly Chauffaille et al. showed that cytogenetic analysis of cryopreserved bone marrow cells was a reliable alternative when fresh cell analysis cannot be performed. Nevertheless, the cryopreservation of the cells was associated with reduced viability and lower percentages of successful analysis than these associated with fresh cell [10].

5. CONCLUSION

The culture time of venous peripheral blood after collection of the sample for karyotype test is crucial for successful karyotype analysis. The samples should be processed within 24h of blood collection; prolongation of this time significantly decreases the cellular viability and the number of the cells in mitosis.

CONSENT AND ETHICAL APPROVAL

The research ethics committee of the institute approved the study and written informed consent was obtained from all participants.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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