Original Research Article

Effect of elapsed time after blood collection on the viability and mitotic index of human lymphocytes during karyotype analysis

Running title: ***Mitotic index of cultured human lymphocyte at variable time***

Abstract

Background: Chromosome sating using G banding is a commonly used technique during karyotyping however, a limited number of laboratories carries out the test. Blood samples must be send to the laboratory within 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 were either processed for chromosome analysis within 2h of sample collection or stored at 4C then processed at 24h and 48h. Lymphocytes viability was determined by Trypan blue and mitotic cells were visualized by the lighted microscope at 40x objective. Mitotic index was calculated per 1000 cell count. **Results:** Delay in sample processing more than 24h have a deleterious effect on lymphocyte viability with significant reduction in mitotic index relative to the freshly processed sample. **Conclusion:** Culturing of cell with in 24h of sample collection is highly recommended whenever possible and delay more than 48h should be avoided.

Key word: Karyotype, Mitotic Index

Introduction

Karyotype analysis of human chromosomes is a widespread cytogenetic technique used in screening and diagnosis of inherited genetic diseases and in cancer diagnosis and research [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].

Human karyotyping examination is typically performed on peripheral blood, bone marrow or amniotic fluid samples with viable cells capable of division to get into the metaphase of 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 [8, 9].

Because cytogenetic laboratories performing Karyotype analysis are only present in large centers, it necessitate physicians, clinics and affiliated laboratories to draw blood samples and amniotic fluid sample and send them to these centers. Throughout the transportation period which may take up to 24h after sample collection the cell proliferation and viability may be seriously compromised, causing reduction in the mitotic quality or even absence of mitosis, depending on the elapsed time and the number of survival cells [10-12].

This aim of this study was to assess the effect of time lapsed from sample withdrawal to the beginning of cell culture on the growth and viability, the quality of metaphase spread and the mitotic index (MI) of cultured human lymphocyte.

2. Material and Methods

The study was carried out in the period from March 2017 to July 2017, in the department of the Biochemistry and Molecular diagnostic at the National liver institute, Menoufia University, Egypt. The research ethics committee of the institute approved the study, and 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±2.5y) were used to carry out karyotype analysis. 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 the blood collection time. The second and the third parts were stored in the refrigerator at 4C and processed in the same way at 24h and 48h respectively. For all samples, the viability of the harvested lymphocytes were tested by Trypan blue before starting the culture process and the percent of viable cells were determined.

2.1 Chemicals and Reagents: RPMI1640, lymphocyte Separation medium were purchased from Lunza, (Lunza, Bio Whittaker Germany). Trypsin, Colcemid (10 µg/ml), Penicillin-Streptomycin, (10,000 U/ml; 10,000 µg/ml), L-glutamine, Giemsa stain (Life Technologies, Gibco,USA) Fetal bovine serum, Phytohemagglutinin (PHA) (BioChrom-Germany), Glacial acetic acid, Methanol and KCl from (Thermo Fisher Scientific, Waltham, MA USA).

2.2 Preparation of metaphase chromosome spread and GTG banding

Lymphocyte were collected using Ficoll-Paque method. Lymphocytes were counted and evaluated for viability by Trypan blue staining. Harvested lymphocyte were cultured in flask containing RPMI 1640 medium, supplemented with 10 % FBS, Penicillin/Streptomycin and 100 µg/ml Phytohemagglutinin. Culture flasks were incubated in 5% CO_2 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 0.75M KCL for 10 minutes. Cells were fixed using freshly prepared ice cold Carnoy's fixative solution for 15 min at -20C. Chromosome spreads were prepared by gently dropping the cell suspension on a clean glass slide followed by overnight incubation at 60C. 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. 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 slide were prepared from each blood culture; cells were visualized under a light microscope at 400 magnification 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, 13]. 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

Harvested lymphocyte cells suspension (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 clean dried coverslip. Living and dead (blue) cells were visualized and counted using light microscope. Cell Viability % = Number of live Cells / Number of Live Cells + Number of Dead Cells.

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.

Result

***Satisfactory chromosomal spread with adequate metaphase plates of sample processed 2h after 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. Culture of the lymphocytes at 2 h produced sufficient mitotic spread with good number of mitotic cells showing typical chromosomal plate consisted of 23 pairs of sister chromatids with classic appearance of four arm attached to each other at the centromere indicating a satisfactory karyotype result (**Fig.1)**.

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, 24 h and 48h are summarized in the (Table1). Cytogenetic analysis of samples cultured 2h after sample collection were satisfactory in 21 (84%) samples and inadequate in 2 (8%) samples due to poor morphology or scarcity of metaphases; 2 (8%) other samples had complete absence of metaphases. Samples cultured at 24h had 20 (80%) satisfactory; three (12%) inadequate and two (8%) complete absence of metaphase relative to 12 (48%), seven (28%), and six (24%) samples cultured at 48 h. Delay in sample processing >24h was associated with significant increase in the number of cells with reduced mitosis, inadequate mitosis or complete absence of mitosis **(Fig.2).**

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 sample processed at 2h, 24 and 48h respectively **(Table2)**. 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 24, and 48h was subjectively the same **(Fig.3B)**.

Sample storage more than 24h reduced lymphocyte viability

Similarly the effect of the elapsed time on viability of lymphocyte were 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. 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).

Discussion

The goal of the present study was to examine the potential time a blood sample can be stored before starting culture process on the viability and mitotic index during karyotype testing. Cultures of 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 phase and G2/M phases of the cell cycle. Cell cycle studies 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 [14, 15]. 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 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 within the same day of sample collection. 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 4C.

The current study found that a delay more than 24h in starting culturing process have detrimental effects on lymphocyte viability and proliferating ability with reduction in the mitotic index of the proliferating lymphocyte. As mitotic index represents the percent of cells in metaphase of the cell cycle in a population of proliferating cells [16, 17], 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 [18]. Despite the delay was associated with decrease in MI%, yet the morphological aspect of the chromosomes in fresh and stored cells for 24, and 48h was subjectively the same.

Conclusion: The culture time of venous peripheral blood after collection of the sample for karyotype test is crucial for successful karyotype analysis. Sample should be processed within 24h of blood collection; prolongation of this time significantly decreases the cellular viability.

Table1: Numbers of metaphases, karyotype result, and MI % of the cultured cells at indicated time.

	2h			24h			48h		
Sex	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.

Table2: Mitotic index (MI) values of cultured human blood lymphocytes.

	Mean	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

Figure Legend:

Fig.1. Metaphase spreads of human peripheral blood lymphocytes cultured at 2h of sample collection. Photomicrograph of metaphase spread prepared from human lymphocytes viewed at 10x and 20x objectives (top panel) with arrows pointed at mitotic chromosomes. Photomicrograph of metaphase spread viewed at 100x objective (lower panel).

Fig.2. Effect of time passed before processing blood sample for lymphocyte culture on the quality of mitotic spread. Bar graph of total of 25 blood samples processed at the indicated time. *p< 0.05.

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.

Conflict of Interest

We do not have a conflict of interest.

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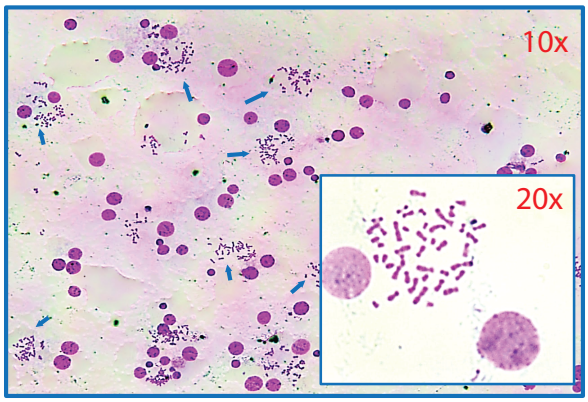

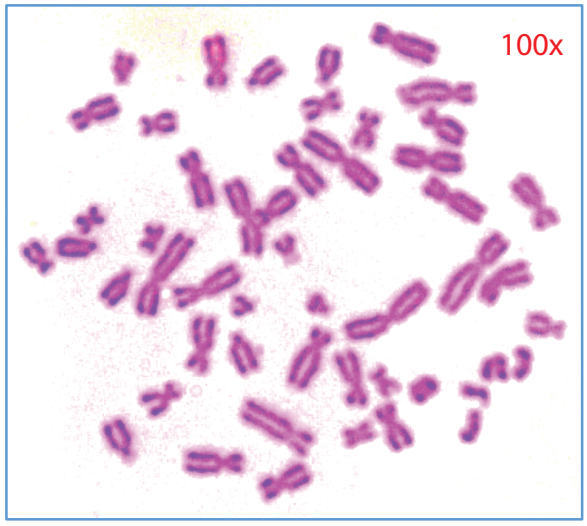

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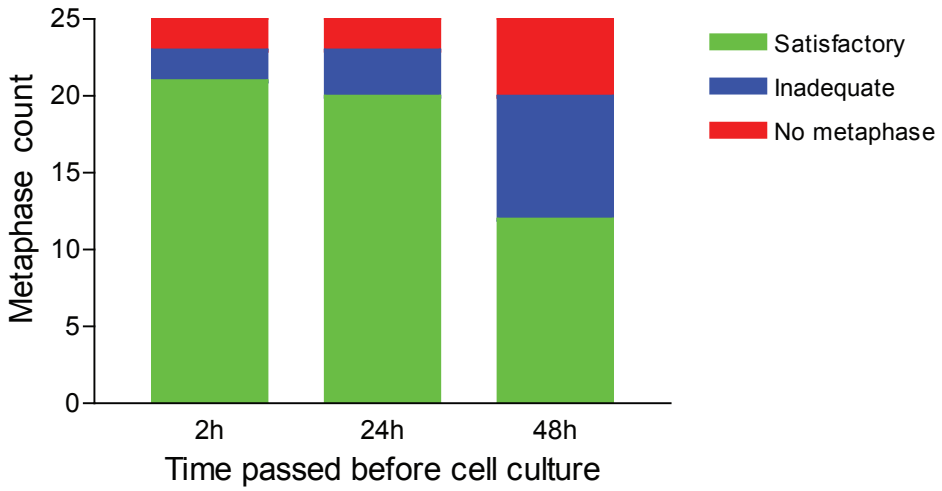
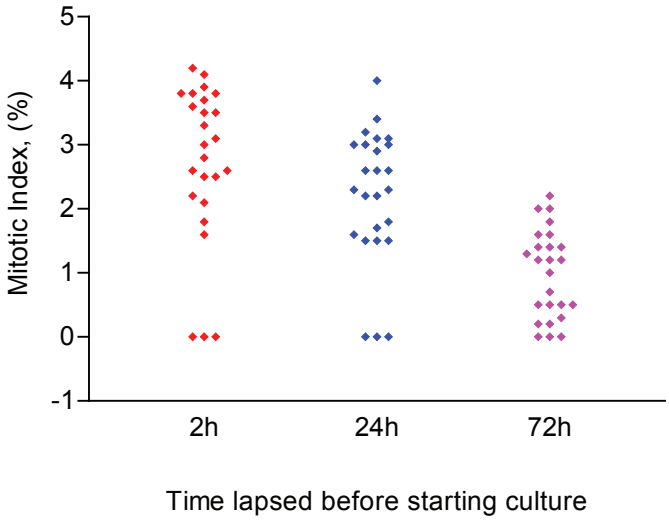


El Khateeb et al Figure 2

A



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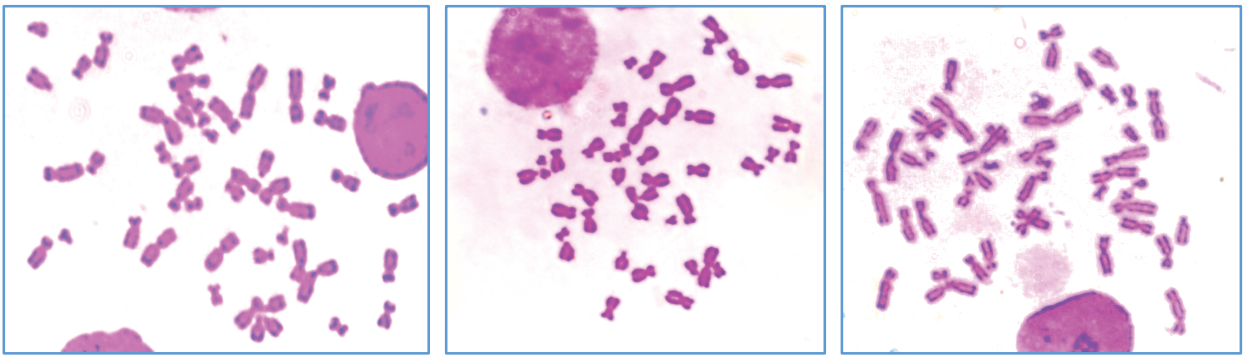

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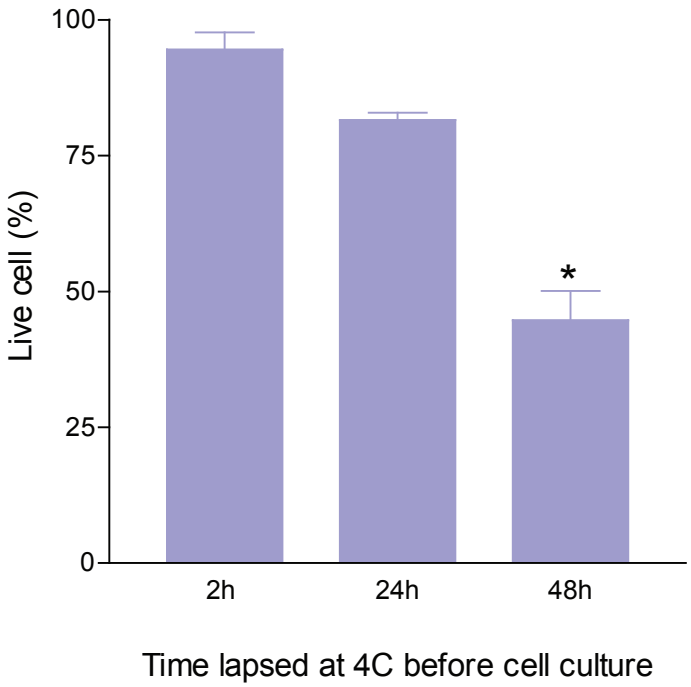

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