Aneuploidy-Inducing Effect of Fenamiphos, Dimethoate, and Chlorpyrifos in Human Peripheral Blood Lymphocyte Cultures

The running title: Organophosphate-Induced Aneuploidy

Keywords: Chromosome gain, Chromosome loss, FISH, and Organophosphate.

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Abstract

For many years, organophosphate (OP) pesticides have been considered an attractive choice for pest control around the world. Excessive use of OPs is a concerning issue for human health. Although the genotoxic effect of these pesticides has been reported, studies that examined their aneuploidy-inducing effect are limited or absent. Therefore, we sought to investigate the potential of organophosphate pesticides, which are extensively used in the Gaza Strip, to induce aneuploidy in human peripheral blood lymphocyte (PBL) cultures. To achieve this goal, we first assessed the cytotoxic effect of different concentrations of Nemacur (fenamiphos), Rogor (dimethoate), and Dursban (chlorpyrifos) on human peripheral blood lymphocytes by the MTT assay. Then, we treated human PBL cultures with different concentrations of these OPs. Fluorescence *in situ* hybridization (FISH) technique was used to determine the frequency of induced aneuploidy. All the examined concentrations did not have a significant cytotoxicity effect except for the employed concentrations of Nemacur. Increase in frequencies of aneuploidy, chromosome loss, and chromosome gain were observed after each treatment and this was significantly evident for the 0.050 mg/mL concentration of Nemacur. It was also noticed that chromosome loss is more frequent than chromosome gain for each concentration of the three types of OPs. We conclude that all the examined OPs have the potential to induce aneuploidy in cultured human PBL. In light of the obtained results, we believe that the aneuploidy-inducing capacity of OPs requires further attention.

Introduction

Pesticides cover a wide range of chemicals including fungicides, insecticides, herbicides, nematicides, etc. (Mužinić et al., 2019; Sharma et al., 2019). Organophosphate (OP) compounds are one of the major components of these chemicals. For many years, OPs have

been considered an attractive choice for pest control around the world because they possess rapid degradation rates under natural conditions; air, sunlight, and soil (Adesiyan et al., 2020; Sidhu et al., 2019). In the Gaza Strip, organophosphates are the most common insecticides used among farmworkers (Yassin et al., 2002).

Excessive use of OPs is a concerning issue for human health because many studies have previously found an association between pesticide exposure and the increased incidence of different diseases, including cancer, Alzheimer's disease, Parkinson's disease, birth defects, and reproductive disorders (Kaur & Kaur, 2018).

During the past three years, the most extensively used organophosphates in the Gaza Strip were Nemacur E.C. (400g/L fenamiphos), Rogor E.C. (400g/L dimethoate), and Dursban E.C. (480g/L chlorpyrifos) according to the report of the Palestinian Ministry of Agriculture.

The findings of *in vitro* genotoxic studies of fenamiphos stated that positive results for clastogenicity were observed in the chromosomal aberration assay in human lymphocytes (Authority et al., 2019) at cytotoxic concentrations in the presence and absence of exogenous metabolic activation (Herbold-B, 1987). Fenamiphos was however negative in all the *in vivo* studies, for example, fenamiphos was negative in micronucleus and dominant lethal tests in mice (Authority et al., 2019). Information obtained from genotoxic previous studies of Dimethoate (Rogor) on human lymphocyte cultures showed that dimethoate was genotoxic *in vitro* since it induced a dose-related increase in the frequency of sister-chromatid exchanges (SCE) (Dolara et al., 1992), a statistically significant elevation of micronuclei (MNs) frequency in a nondose-related manner (Bianchi-Santamaria et al., 1997), and a significant increase in DNA damage using the comet assay in human lymphocyte cultures (Ündeğer & Başaran, 2005).

Although several studies confirmed the genotoxic effect of fenamiphos and dimethoate *in vitro*, no previous studies investigated their effect on the induction of aneuploidy either *in vitro* or *in vivo*. In the case of Chlorpyrifos, there are a limited number of studies that reported the induction of aneuploidy after exposing cultured human lymphocytes to this pesticide (Mužinić et al., 2019; Sultana Shaik et al., 2016). Therefore, we sought to investigate the aneuploidy-inducing capacity of those three organophosphate pesticides in human peripheral blood lymphocyte (PBL) cultures using fluorescence *in situ* hybridization (FISH) analysis.

Finding an association between exposure to organophosphate pesticides and the induction of aneuploidy in human PBL cultures may contribute to the interpretation of the increasing incidence of birth defects in the Gaza Strip that result from chromosomal abnormalities.

Materials and Methods

Ethical approvals from the Helsinki ethical committee and the Ethical Research Committee at the Islamic University of Gaza were obtained before the initiation of this work. Informed consent was taken from the blood donor who was acquainted with the purpose of this study. Prior to investigating the aneuploidy-inducing capacity of the three organophosphate pesticides, their cytotoxicity on human peripheral blood lymphocyte cultures was assessed using the MTT assay.

Human peripheral blood lymphocyte separation

Peripheral blood mononuclear cells (PBMCs) were separated from whole heparinized blood of a healthy donor by centrifugation through a density gradient using Leucoprep™ lymphocyte separation medium (iNtRON Biotechnology, Korea). Cells were counted using Trypan blue solution (0.5%) (Biological industries, USA) and a hemocytometer and then resuspended in Dulbecco's Modified Eagle's Medium (DMEM) (Biological Industries, USA)

supplemented with 10% Fetal Bovine Serum (Biological Industries, USA), 10 mg/ml penicillin and 10 mg/ml streptomycin (Biological Industries, USA).

MTT assay

After counting cells by using a hemocytometer, the cells were planted onto 96-well plates at a density of 40,000 cells/well (El-Burai et al., 2020) in 100 µl of full DMEM and treated with dimethyl sulfoxide (DMSO) (Sigma, Steinheim, Germany) alone (solvent control) or different amounts of Nemacur E.C. (Bayer AG, Leverkusen, Germany), Rogor E.C. (Agrimag Bio, Italy), Dursban E.C. (Darbouco S.A., Haïti), and Taxol (Paclitaxel Injection) (Indiamart, Uttar Pradesh, India) (positive control) dissolved in DMSO. The selected concentrations of these chemicals are according to previous studies as shown in Table I. Wells containing only medium and cells served as controls (untreated cells) and those containing only the medium served as blank samples. Each assay was performed in 6 replicates, three of them for zero time incubation and the others for 18 hours incubation at 37°C in 5% CO2 incubator.
Ten µl of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) (MP Biomedical, LLC) at 0.45 mg/ml was added to all the microtiter plate wells either immediately (zero time incubation) or after 18 hours of incubation. The plate was reincubated for 3 hours in a CO_2 incubator at 37ºC, then 100 µl of DMSO were added to dissolve the formazan crystals that were formed followed by 15 min incubation at 37°C. Exposure to laboratory lights was limited after the addition of MTT solution. The plate was agitated on orbital shaker for 30 seconds. Then, the Optical Density (OD) of the MTT formazan was detected at 550 nm and 620 nm in a microplate reader (Multiskan FC, Republic of Korea). The 620 nm OD background was subtracted from the value of OD at 550 nm to get a more accurate measurement by correcting for background noise. Cell viability was calculated as the ratio (%) of OD of treated cells to untreated cells (control) after subtraction of the blank reading (El-Burai et al., 2020; Gautam et al., 2016; Qu et al., 2016).

Blood Sampling and Cell Culture

Venous blood samples were drawn into heparinized tubes from a healthy, young (less than 35 years of age), non-smoking donor without previous exposure to any of the tested OPs. One-half mL of peripheral blood was introduced into five mL of PB-MAX™ Karyotyping Medium (Thermo Fisher Scientific, Waltham, MA, USA). The PBL cultures were incubated at 37^{o} C, 5% CO_2, for 24 h.

Treatment schedule

After 24 h of incubation, the cultures received dimethyl sulfoxide (DMSO) alone or different concentrations of the three types of organophosphate pesticides dissolved in DMSO as in Table I. The positive control cultures received Taxol (Paclitaxel Injection) dissolved in DMSO as a well-known aneuploidy-inducing agent, to assess the performance of the test system (Mattiuzzo et al., 2006). Every treatment was performed in duplicate. The PBL cultures were harvested after 18 h from the beginning of the treatment where each PBL culture was centrifuged at 3500 rpm for 5 minutes and the supernatant was discarded except about 1.0 mL to resuspend the cell pellet. About 8.0 mL of prewarmed hypotonic solution (0.075M KCl) (Sigma, Steinheim, Germany) was added with proper mixing by a vortex mixer. The PBL culture tube was incubated for 15-20 minutes at 37^{o}C in the incubator. The cells were collected by centrifugation and supernatant removal as presented above and then fixed by adding about 5.0 mL of freshly prepared fixative solution "1 part glacial acetic acid with 3 parts absolute methanol" (Sigma, Steinheim, Germany), with gentle agitation. The cells were then collected by centrifugation as described above and the fixation steps were repeated additional times until all debris was removed. The cells were then resuspended with a small amount (about 0.5 mL) of a fixative solution.

Fluorescence *in situ* hybridization (FISH) with chromosome-specific probes

To determine the frequency of aneuploidy of chromosomes 21 in Taxol and Nemacur-treated lymphocyte cultures; slides were hybridized with either a translocation, dual fusion probe; TEL/AML1 (LPH 012) (FITC-green), or 21 qter subtelomere specific probe (LPT 21QR) (TRITC-red) (Cytocell, Cambridge, UK). And to determine the frequency of aneuploidy of chromosomes 16 in Rogor and Dursban-treated lymphocyte cultures, slides were hybridized with a translocation, dual fusion probe; CBFβ/MYH11 (LPH 022) (FITC-green) (Cytocell, Cambridge, UK). Chromosomes 21 and 16 are among the most frequent autosomal chromosomes involved in aneuploidy (Franasiak et al., 2014). Each probe was applied separately to slides. Slide preparation, pre-denaturation, denaturation, hybridization, post-hybridization washes were done according to the manufacturer's recommendations (Cytocell, Cambridge, UK). Slides were counterstained with 10.0 µL of DAPI-Antifade (Cytocell, Cambridge, UK). Analysis of FISH signals was carried out on the Olympus Bx51 fluorescent microscope (Olympus, Tokyo, Japan), under 1,000x magnification. A total of 400 interphase cells were scored per slide. Every treatment was at least performed in duplicate. The frequency of aneuploidy was determined by counting the number of aneuploid interphase cells that contain one probe signal (Chromosome loss) of the relevant autosomal chromosome, or more than two probe signals (Chromosome gain).

Data Analysis

The results were entered in the Statistical Package for Social Sciences (SPSS Inc., US) 23.0 software and different statistical analyses were performed. Results of MTT assay were presented as mean±SD and the independent samples *t*-test was performed to compare the mean of each concentration used with the mean of solvent control (DMSO). For FISH analysis, values of total aneuploidy, chromosome loss, and chromosome gain per each concentration were compared to respective solvent control values by χ^2 test to asses

significance of difference toward solvent control. All results were accepted as significant when p-value < 0.05.

Results

The results of the MTT assay indicated that there is no significant change in the viability of PB lymphocytes when treated with Taxol or different concentrations of Rogor, and Dursban either after 0 h or 18 h incubation compared to solvent control (0.50% (v/v) DMSO) samples. However, a significant decrease in cell viability was observed for Nemacur-treated cells after 18 h incubation as shown in Table II.

Tables III and IV show the frequencies of aneuploidy, chromosome loss, and chromosome gain for chromosome 21 in PB lymphocyte cultures treated with Taxol and different concentrations of Nemacur (fenamiphos), and for chromosome 16 in PB lymphocyte cultures treated with different concentrations of Rogor (dimethoate) and Dursban (chlorpyrifos) as detected by FISH analysis, respectively. Some increases in frequencies of aneuploidy, chromosome loss, and chromosome gain were observed for each treatment compared to the respective solvent control (DMSO 0.50 % (v/v)) cultures, but the χ^2 test showed a significant difference in the frequency of aneuploidy ($P < 0.05$) between treated and solvent control cultures only with a concentration of 0.050 mg/mL of Nemacur.

Tables III and IV also show that chromosome loss is present in higher frequencies than chromosome gain for each concentration of the three types of organophosphates, as well as in Taxol treated cultures. As shown in Table III, the concentration of 0.025 mg/mL of Nemacur significantly induced chromosome loss in human PBL cultures and the concentration of 100 nM of Taxol and 0.025 mg/mL of Nemacur significantly (p-value <0.05) induced chromosome gain. Figure 1 shows fluorescent micrographs of normal diploid and aneuploid interphase cells.

Discussion

Organophosphate pesticides are extensively used in various agricultural practices around the world. Nemacur E.C. (400g/L fenamiphos), Rogor E.C. (400g/L dimethoate), and Dursban E.C. (480g/L chlorpyrifos) organophosphates are the most widely used in the Gaza Strip among farmworkers. Although several studies have reported their genotoxic effect, studies that investigated their aneuploidy-inducing effect are scarce or absent. Therefore, we sought to assess the aneuploidy-inducing activity of these pesticides in human peripheral blood lymphocyte (PBL) cultures using fluorescence *in situ* hybridization (FISH) analysis.

The results obtained using FISH analysis of a large number of interphase cells after treatment with the three types of organophosphate pesticides showed that all those types regardless of their concentrations have aneuploidy inducing capacity in human PBL cultures. These results correspond to the results of studies that have investigated the ability of organophosphate pesticides to induce aneuploidy in human cell lines or cultured human lymphocytes (Mattiuzzo et al., 2006; Sultana Shaik et al., 2016).

In a previous study, exposure of human PBLs to low levels of three types of insecticides, including chlorpyrifos as a representative of the OP insecticides, elicited a significant induction of chromosome loss (Mužinić et al., 2019). Those findings are similar to what we observed in our study, where chromosome loss was more frequent than chromosome gain for each concentration of the three types of the tested pesticides. These observations suggest that aneuploidy may arise from a particular mechanism(s) of loss rather than a mitotic nondisjunctional event(s), the latter of which would be predicted to result in equal frequencies of monosomies and trisomies.

Jackson- Cook, after reviewing many FISH studies of interphase nuclei, found increased frequencies of loss for autosomal chromosomes "1, 2, 6, 9, 15, 16, 17, 18, and 21", and

decreased frequencies of loss for chromosome 3 in both uncultured and cultured cells. Interestingly, 6 of these autosomes are heterochromatin-rich chromosomes (Jackson-Cook, 2011). Based on the above, the increased frequency of chromosome loss in our tested chromosomes (21 and 16) was previously seen by investigators who studied frequencies of acquired aneuploidy in the nuclei of interphase cells using the FISH methodology. The chromosome-specific differences in acquired, somatic cell autosomal aneuploidy frequencies are explained by a non-random loss hypothesis which postulates that "different chromosomes have a different propensity for abnormality occurrence and that the genetic make-up or structure of the chromosome is the primary determining factor for its acquisition of abnormalities (Jackson-Cook, 2011). For example, a heterochromatin-rich chromosome with less actively transcribed genes is more frequently missegregated since huger parts of heterochromatin between homologs may result in asynchrony in replication, which may contribute to misalignment and malsegregation. Alternatively, epigenetic changes (primarily hypomethylation) may arise in pericentromeric heterochromatin leading to changes in chromatin conformation that compromise the ability of the chromosomes to attach to the mitotic spindle, increasing missegregation" (Jackson-Cook, 2011; Mužinić et al., 2019). The hypothesis offered above of aneuploidy induction and chromosome malsegregation would result in the increased micronuclei (MN) formation (Jackson-Cook, 2011), which may be due to concomitant delays with these mechanisms in the separation of centromere that lead to the preferential exclusion of these chromosomes into micronuclei. This corresponds to the findings of several studies that showed an increased frequency of micronuclei (MN) after exposure to organophosphate pesticides (Ayed-Boussema et al., 2012; Mattiuzzo et al., 2006; Omari, 2011).

Based on the results of the present study we conclude that all the three types of the applied organophosphate pesticides have aneuploidy inducing capacity in human PBL cultures

regardless of their concentrations. The induction of chromosome loss was present at higher frequencies than chromosome gain for each examined concentration. Further investigations on larger number of samples are needed to confirm the association between exposure to OPs and induction of aneuploidy and other chromosomal abnormalities.

Author Contributions

Prof. Dr. Fadel Sharif is the principal investigator and supervisor of this study. Mr. Shadi Alashi and Ms. Hadeer Abuwarda carried out the experimental part of this work. Ms. Hadeer Abuwarda carried out statistical analysis of results, prepared tables and figures, and wrote the manuscript. All authors approved the final manuscript.

Acknowledgments

This work was performed at the genetic diagnosis laboratory of the Islamic University of Gaza.

Conflict of interest

The authors declare no conflict of interest.

Tables

Table I: Concentrations of chemicals used for *in vitro* treatments of PBL cultures

Chemicals	Final concentrations in cultures	References
DMSO (Solvent control)	0.50 % (v/v)	(Mattiuzzo et al., 2006)

Taxol (6mg/mL) **(Positive control)**	85.39 ng/mL (100.00 nM)	
Nemacur E.C. **(400g/L fenamiphos)**	0.025 mg/mL 0.050 mg/mL 0.100 mg/mL	(Herbold-B, 1987)
Rogor E.C. **(400g/L dimethoate)**	0.025 mg/mL 0.050 mg/mL 0.100 mg/mL	(Dolara et al., 1992; Ündeğer & Başaran, 2005)
Dursban E.C. **(480g/L chlorpyrifos)**	1.00 µg/mL 12.00 µg/mL	(Gautam et al., 2016)

Table II: Cell viability (%) of human peripheral blood lymphocytes after exposure to 100 nM Taxol, 0.50% (v/v) DMSO, and different concentrations of Nemacur, Rogor, and Dursban after zero and 18 h of incubation

Final concentrations of chemicals in culture	**After 0 h incubation**	**After 18 h incubation**
	Cell viability (%) **Mean ± SD**	
Control (Untreated cells)	100.00 ± 0.00	100.00 ± 0.00

DMSO (0.50% (v/v)) (Solvent control)	101.44 ± 8.55	94.64 ± 1.78
Taxol (100.00 nM) (Positive control)	94.90 ± 1.96	88.92 ± 8.32
Nemacur (fenamiphos)		
0.025 mg/mL	86.24 ± 0.00	78.38 ± 0.52*
0.050 mg/mL	86.98 ± 4.21	78.93 ± 0.78*
0.100 mg/mL	85.68 ± 1.83	82.41 ± 2.07*
Rogor (dimethoate)		
0.025 mg/mL	102.59 ± 5.25	98.89 ± 0.52
0.050 mg/mL	99.48 ± 7.04	102.67 ± 11.03
0.100 mg/mL	108.17 ± 10.51	112.72 ± 12.30
Dursban (chlorpyrifos)		
1.00 µg/mL	97.02 ± 1.05	104.39 ± 2.07*
12.00 µg/mL	99.44 ± 0.79	94.13 ± 4.65

Statistically significant compared to the solvent control (0.50 % DMSO) * P < 0.05

Table III: Frequencies of aneuploidy, chromosome loss, and chromosome gain of **chromosome 21** in lymphocyte cultures treated with Taxol and Nemacur as detected by FISH analysis

Final concentrations of chemicals in culture	Number of analyzed cells	Total Aneuploidy n (%)	Chromosome Loss (Monosomy) n	Chromosome Gain (Trisomy) n
Taxol (100.00 nM) (Positive control)	400	9 (2.25)	7 (1.75)	2 (0.50)
	400	10 (2.50)	6 (1.50)	4 (1.00)*
Nemacur (fenamiphos)				
0.025 mg/mL	400	12 (3.00)	12 (3.00)*	0 (0.00)
	400	12 (3.00)	8 (2.00)	4 (1.00)*
0.050 mg/mL	400	13 (3.25)*	10 (2.50)	3 (0.75)
	400	12 (3.00)	10 (2.50)	2 (0.50)
	400	13 (3.25)*	10 (2.50)	3 (0.75)
0.100 mg/mL	410	10 (2.44)	9 (2.20)	1 (0.24)
	400	11 (2.75)	10 (2.50)	1 (0.25)

Statistically significant compared to the solvent control (0.50 % DMSO) * P < 0.05

Table IV: Frequencies of aneuploidy, chromosome loss, and chromosome gain of **chromosome 16** in lymphocyte cultures treated with Rogor and Dursban as detected by FISH analysis

Final concentrations of chemicals in culture	Number of analyzed cells	Total Aneuploidy n	Chromosome Loss,(Monosomy) n	Chromosome Gain, (Trisomy) n
Rogor (dimethoate)				
0.025 mg/mL	400	7 (1.75)	7 (1.75)	0 (0.00)
	400	8 (2.00)	8 (2.00)	0 (0.00)

0.050 mg/mL	**600**	15 (2.50)	14 (2.33)	1 (0.17)
	600	14 (2.33)	13 (2.16)	1 (0.17)
0.100 mg/mL	400	5 (1.25)	5 (1.25)	0 (0.00)
	400	7 (1.75)	7 (1.75)	0 (0.00)
Dursban (chlorpyrifos)				
1.00 µg/mL	400	5 (1.25)	4 (1.00)	1 (0.25)
	400	6 (1.50)	6 (1.50)	0 (0.00)
12.00 µg/mL	400	9 (2.25)	8 (2.00)	1 (0.25)
	400	8 (2.00)	8 (2.00)	0 (0.00)

Statistically significant compared to the solvent control (0.50 % DMSO) * $P < 0.05$

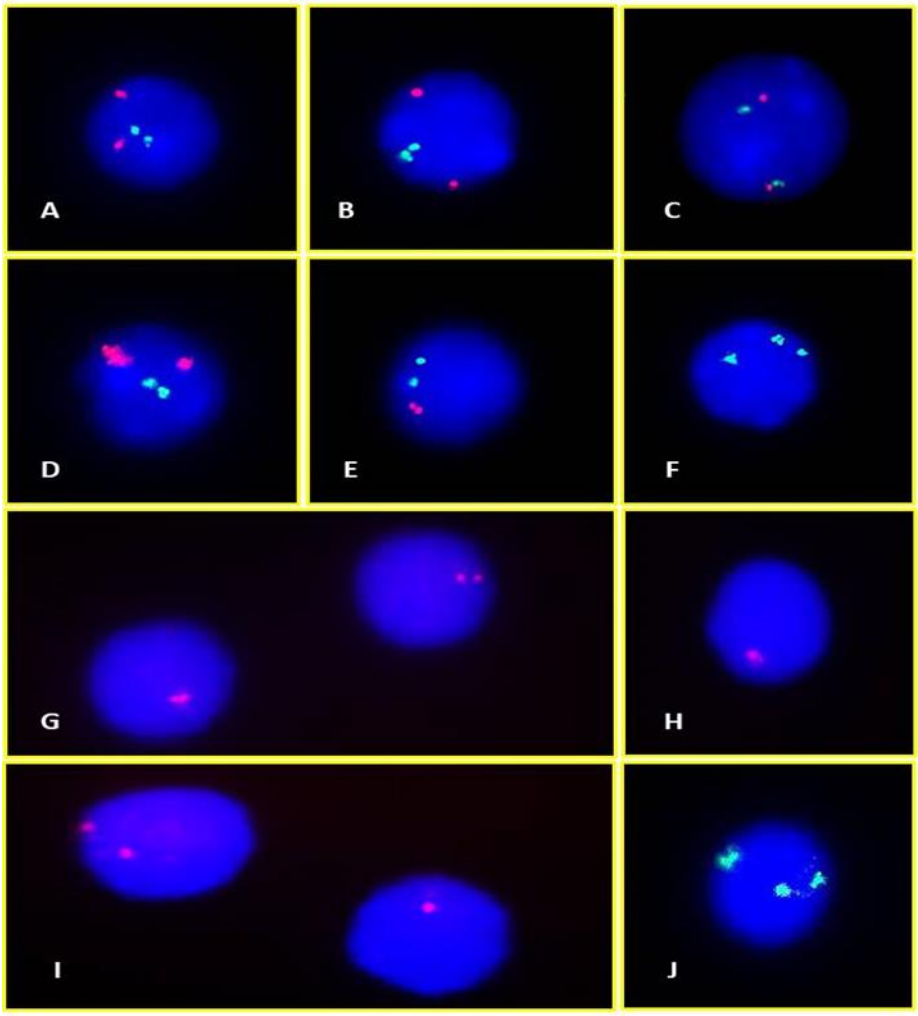

Figure 1: Fluorescent micrographs of normal diploid and aneuploid interphase cells by FISH technique: (A, B, C, D, and E) normal diploid cells with 2 signals for chromosome 21 (green) and two signals for Chr. 12 (red); (F) a trisomic cell with 3 signals for Chr. 21; (G) a monosomic cell with one signal for Chr. 21 and a normal diploid cell with 2 signals for Chr. 21; (H) a monosomic cell with one signal for Chr. 21; (I) a normal diploid cell with 2 signals

for Chr. 21 and a monosomic cell with one signal for Chr. 21; and (J) a trisomic cell with 3 signals for Chr. 16.

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