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The Effect of Phytohaemagglutinin and Colcemid on the Reliability of the Chromosome Aberration Analysis after Ionizing Radiation Exposure

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THERE are some correlations between cell's ability to remove DNA damage and proliferative activity. Another phenomenon was observed that the frequency of radiation-induced aberration can be influenced according to the concentrations of colcemid or colchicine which used for arresting the cell cycle in metaphase.

The aim of this study is to improve the technical accuracy of the chromosome aberration analysis and demonstrate the dose-dependent effect of colcemid and colchicine which may be of interest to physicians encountering irradiated patients.

So, the study examined the effect of phytohaemagglutinin (PHA) and colcemid or colchicine dose concentrations on DNA repair capacity and chromosome aberration yields in human blood lymphocytes exposed to ionizing radiation. Whole blood samples were irradiated with (2 Gy) γ-rays. PHA (0.2 ml) was added to blood cultures either immediately after irradiation or after a certain recovery period (1 & 2 h). In the second part of the experiment, we used both colcemid and colchicine with 2 different concentrations (0.025 & 0.05 μg/ ml).

The number of dicentric chromosomes and acentric fragments were significantly increased in lymphocytes stimulated by PHA immediately after the irradiation. On the other hand, the use of colcemid with dose range 0.025 to 0.5 μg/ ml is better than colchicine with the same doses in the accurate estimation of the absorbed radiation dose.

Keywords: Ionizing radiation, chromosome aberration, phytohaemagglutinin, colcemid, colchicine.

Extensive studies have demonstrated that living cells react in different ways when subjected to radiation injury causing DNA damage, depending on the cell type, cell cycle and radiation dose (Cory and Adams, 2002). If the damage is

modest, it may be completely handled by the specific survival-response network, while if the damage is excessive and/or irreparable, the cell undergoes apoptosis through activation of the programmed cell-death pathway. In case of escape from these mechanisms, a transformed tumour cell is possibly generated (Martina *et al.,* 2006). Since the outcome of genotoxic damage in cells resistant to apoptosis will be entirely different from that found in apoptosis-prone cells, apoptosis appears to play an important role in physio-pathological processes, particularly within the blood system. In fact, human peripheral blood lymphocytes provide a convenient and readily available source of human material and are routinely used experimentally to assess the extent of cytogenetic damage induced by physical and chemical agents (Carloni *et al.,* 2001). Peripheral blood lymphocytes represent the most common source in radiation-biodosimetry studies. It is known that culture conditions, cell-cycle phase and differentiation stage can influence their sensitivity to radiation. In particular, addition of mitogens or growth factors can enhance their radio-resistance (Feng *et al.,* 2007 and Meijer *et al.,* 1999). Furthermore, mitogen-stimulated cells immediately after irradiation have a higher frequency of chromosome aberrations than cells resting in G0-phase before the addition of mitogens (Belloni *et al.,* 2005).

It is known that ionizing radiation deposits its energy in cellular structure through discrete ionization events that are essentially randomly distributed in space. Unlike chemical agents, whose damaging potential is strongly dependent on diffusion processes and thus may be affected by sub-cellular structures, ionizing radiation is highly penetrating: the physics and subsequent chemistry associated with the photon absorption and the ionizing events that occur along fast electron track are complete within a few microseconds (Pfeiffer *et al.,* 2002). Ionizing radiation causes a wide spectrum of chemically different types of lesions in DNA of which the so-called locally multiply damaged sites (LMDS) are assumed to be biologically most important (Ward, 1998). LMDS may consist of single-strand breaks on opposite strands that, if located close to each other, may give rise to double strand breaks (DSB) **(**Friedberg *et al.,* 2001). Thus, DSB induced by ionizing radiation may arise as a direct consequence of one or more ionizing events or indirectly as a base or sugar damage on opposite

strands. Ionizing radiation in physicochemical interaction with cellular DNA also produces a variety of primary lesions, alkali-labile sites, DNA-DNA and DNA-protein crosslinks, and damage to purine and pyrimidine bases (Garaj-Vrhovac *et al.*, 2002, Natarajan, 1993 and Wilson *et al.*, 1993).

DNA-DSB are the most serious form of DNA damage, there are two pathways for the repair of DSB , one is homologous recombination which occurs during late S and G2 phase of the cell cycle and the other pathway is non homologous DNA end joining . It is predominant during G0, G1 and S phase of the cell cycle. If not repaired, DNA lesions could cause cell death. If misrepaired, DSBs contribute to chromosomal aberrations and genomic instability (Liang *et al*., 1998 and Sonoda *et al*., 1998). Ionizing radiation produces chromosome aberrations (involving two chromatids) at the S phase and the chromatid aberrations at G2. To detect genetic alterations at the chromosome level using chromosome aberration and micronucleus assay the cells should be induced to enter the G1 phase and undergo division (Carrano and Natarajan, 1988 and Maluf, 2004). For biodosimetry based on the scoring of chromosome aberrations (dicentrics, rings and fragments), it is important to consider only first-division (M1) lymphocytes. However, following partial-body irradiations, the irradiated fraction of cells may not have enough time to reach the first metaphase in traditional 48 hr cell cultures because they may be selectively delayed or held for longer at check points during the cell cycle (Heimers *et al*., 2006). So, the usual time of cell culture is 48 h, because with longer times many cells enter into their second or later cell cycles so that selective elimination of chromosomal damage starts to occur by mitotic non-disjunction. Nevertheless, if culture time is prolonged to 72 h to compensate for the delay, there is the possibility of slow growing irradiated T cells or other sub-populations, e.g., B-cells with differing radio sensitivities, coming to M1 (Han and Daday,1978 and Wuttke *et al.*, 1993).

PHA selectively stimulates T-lymphocytes to enter mitosis. The widespread popularity of peripheral blood culture as means of chromosome analysis has been largely dependent on that mitogen (Boerrigter and Vijg, 1992, Maluish and Strong, 1986 and Savoldi-Barbosa *et al.*, 1999). It is well established that unstable aberrations like dicentric chromosomes, chromosome breaks and acentric fragments can be eliminated during the cell division. It was

suggested that the regulation of DNA repair is dependent on cell cycle. It involves the expression of DNA repair enzymes within the defined program of gene control during the cell cycle. Some authors have shown that immediately after the irradiation mitogen stimulated cells have a higher frequency of chromosome aberrations than the cells resting in G0 phase before the addition of mitogens (Santos Mello *et al*., 1974). The phenomenon that the change in colcemid or colchicine concentrations influenced on the aberration yield was confirmed by repeating the experiment. One possible explanation for this phenomenon is that the fraction of escaping cells from arrest may be higher in normal cells than in aberrant cells in the culture with 0.025 ug/ ml of colcemid, whereas at 0.05 ug/ ml of colcemid mitoses are completely blocked both in normal and aberrant cells. Consequently, the ratio of normal metaphases could be lower in the culture at the lower colcemid concentration. Another hypothesis could be that the colcemid exerts a certain inhibitory effect on the repair or replication of some kind of latent lesions of DNA during S phase which are expressed as aberrations at metaphase, and that the higher the colcemid concentration the less the resultant aberrations (Hayata *et al*., 1992).

The aim of the present study was to examine the influence of PHA and colcemid concentration on the accuracy of the chromosome aberration analysis for biodosimetry.

Materials and Methods

Chemicals

The chemicals of the blood cultures were purchased from GIBCO-BRL, USA, heat-inactivated foetal calf serum (FCS) and other chemicals were purchased from Sigma/ Aldrich Chemical Co., St. Louis, USA.

Blood sampling

To overcome possible inter-individual variability in response to treatments, blood sample was obtained from one healthy male donor (age 35 years, non-smokers) who gave an informed consent for participation in the study. The donor was selected according to current International Programme on Chemical Safety (IPCS) guidelines for the monitoring of genotoxic effects of carcinogens in humans (Albertini *et al.,* 2002). Venous blood was collected under sterile

conditions in heparinised vacutainer tubes (V= 5 ml, Becton Dickinson, USA) containing lithium heparin as anticoagulant.

Irradiation of the blood samples

Blood samples were exposed to ^{137}Cs γ-irradiation source which provided by the NCRRT, Egypt, manufactured by the Atomic Energy of Canada. The dose rate was 0.52 Gy/ min. The adjusted dose was 2 Gy. The samples were kept at 37 °C after irradiation immediately to allow possible damage-repair, a process simulating the *in-vivo* situation.

Experimental design

The experiment was divided into two parts: the first one was to estimate the PHA affects which added (0.2 ml) to the blood cultures either immediately after the irradiation or after a certain recovery period (1 or 2 h). The second part was to estimate the effects of the colcemid and the colchicine with two different concentrations (0.025 or 0.05 μg/ ml) on the chromosomal aberration expression in the irradiated blood samples. Meanwhile cultures were held at 37°C (Natarajan and Obe, 1980). The analysis was done in duplicate, for each point under investigation, so two different cultures were started for the chromosomal aberration analysis. Non-irradiated samples were be cultured under the same circumstances for each part of the experiment.

The analysis of structural chromosome aberrations

The structural chromosome aberration analysis test was performed according to current IAEA guidelines (IAEA, 2001). Cultures for the chromosome aberration test were set up in the same manner. Duplicate cultures per sample were set up and incubated at 37 °C for 48 h.

Cultures were centrifuged at 1000 rpm for 10 min, the supernatant was carefully removed, and the cells were re-suspended in a hypotonic solution (0.075M KCl) at 37 °C for 20 min. After the second centrifugation, the cells were fixed with a freshly prepared fixative of ice-cold methanol/glacial acetic acid (v/v 3:1). Fixation and centrifugation were repeated several times until the supernatants were clear. The cell suspension was dropped onto microscope slides and left to air-dry. Slides were stained with 5 % Giemsa solution (Merck). For each stimulation analysis was done in duplicate, a total number of 200 metaphases was scored. Only metaphases containing 46 centromeres were

analyzed. A total number of each type of aberrations, as well as the percentage of aberrant cells per subject were evaluated.

Statistical analysis

Data are presented as distribution analysis and analysed using two ways analysis of variance *"F"* test according to Abramowitz and Stegun (1972). The level for statistical significance was $P< 0.05$.

Results

Types and frequencies of chromosome aberrations which induced by irradiation in cultured human blood lymphocytes are recorded in Table 1& 2.

TABLE 1. Distribution of chromosome types aberrations in human lymphocytes stimulated to proliferate after the indicated post-irradiation periods (the analysis done in duplicate for each stimulation time).

Irradiated samples / Time after irradiation	No. of structural Chromosome aberrations/ 200 cells					% of aberrant cells
	Total No. of aberrations	Chromatid breaks	Chromosome breaks	Acentric fragment	Dicentric	
0 h	51 [a]	4	5 [a]	27 [a]	15 [a]	15 %
1 h	31 [a,b]	3	2	17 [a]	9 [a]	11 %
2 h	38 [a]	4	3	19 [a]	12 [a]	13 %
Non-irradiated samples						
0 h	5	1	0	4	0	1.5 %
1 h	2	0	1	1	0	1.0 %
2 h	3	1	0	2	0	1.5 %

The analysis was done in duplicate for each stimulation time.

a= Significant when compared with the non-irradiated sample 0 h.

b= Significant when compared with the irradiated sample 0 h.

In Table 1, there are remarkable increment in the total number of aberrations for the cultures which stimulated with PHA immediately after irradiation compared to the cultures with recovery periods 1 h and 2 h post-irradiation. The total count of dicentric and acentric fragments was found to be decreased in cultures stimulated with PHA 1 and 2 h after irradiation compared to the cultures where mitogen was added immediately after irradiation.

The number of dicentric chromosomes, acentric fragments and the total number of aberrations were found to be significantly increased in all irradiated

lymphocytes regardless of the start point of PHA stimulation compared to non-irradiated lymphocytes ($P< 0.05$). Whereas, there was insignificant different for all types of chromosome aberrations and its total number between the irradiated sample 0 h and the irradiated samples I h and 2 h except the total number of aberrations in cultures stimulated with PHA after 1 h post-irradiation. There was no observed difference in count of chromosome aberrations between the non-irradiated cultures which stimulated with PHA in 0, 1 and 2 h post-irradiation periods.

TABLE 2. Distribution of chromosome types aberrations in human lymphocytes metaphases arrested by two different concentrations (0.025 & 0.05 µg/ ml) of colcemid and colchicine (the analysis done in duplicate for each stimulation time).

Aberration types	Colcemid 0.25µg/ ml		Colchicine 0.25µg/ ml		Colcemid 0.05µg/ ml		Colchicine 0.05µg/ ml	
	Irrad. Sample	Control I	Irrad. Sample	Control II	Irrad. Sample	Control III	Irrad. Sample	Control IV
Chs Brk	4	1	2	1	5	1	5	1
Chd Brk	2	1	4	2	4	1	3	1
Acentric fragment	22 [c]	3	19 [c]	2	23 [c]	4	22 [c]	2
Dicentric	11 [c]	0	7 [c]	0	12 [c]	0	11 [c]	0
Total No. of aberration	39 [c]	5	32 [c]	5	44 [c]	6	41 [c]	4
% of aberrant cells	15%	2%	12.5%	1.5%	18%	2.5%	17.5%	2%

The analysis was done in duplicate for each concentration test. Irrad.= Irradiated.
c= Significant when compared with control sample. Chs= Chromosome.
d= Significant when compared with irradiated sample(colcemid 0.025µg/ml). Chd= Chromatid.
e= Significant when compared with irradiated sample(colcemid 0.05µg/ml). Brk= Break.

Table 2. show that the total number of the chromosome aberrations, the frequencies of acentric fragments and the dicentric chromosomes was increased by colcemid at concentrations 0.05 µg/ ml.

Whereas, the other cultures treated with colcemid or colchicine at the concentration 0.025 µg/ ml recorded less number aberrations compared to colchicine with concentration 0.05 µg/ ml. There was insignificant difference in count of chromosome aberrations between all irradiated samples treated with colcemid or colchicine with the two different concentrations. But, there is

significant difference in count of chromosome aberration between irradiated and non-irradiated samples. There was no significant differences in counts of chromosome aberrations between the metaphases of the non-irradiated samples which arrested by either colcemid or colchicine with its different doses.

Discussion

Damage from occupational or accidental exposure to ionizing radiation is often assessed via the monitoring of chromosome aberrations in peripheral blood lymphocytes, and these procedures have in several cases helped physicians in the management of irradiation victims (Voisin, 1997). Circulating lymphocytes, which are in the G0 stage of the cell cycle are stimulated with a mitogenic agent, usually PHA, to replicate their DNA in vitro and enter cell division, and are then observed for abnormalities. Comparison with dose response relationships obtained in vitro allows an estimate of exposure based on scoring of chromosome aberrations (Albertini *et al.*, 2000).

This study presented the possible influence of PHA and concentrations of colcemid or colchicine on DNA-repair scoring the number of chromosome aberrations. The results obtained in (table 1) discussed by Martina *et al.* (2006) and Rothkamm and Lobrich (2002) that DSB repair mechanisms are efficient in G0 phase of the cycle and/or that the stimulation of lymphocytes to undergo division without having time to eliminate the majority of the DNA lesions in G0 phase increases a misrepair rate resulting in the increased number of chromosome type aberrations. Moreover, the results supported by the facts that the formation of unstable aberrations is cell cycle dependent and the most of DSB can be fixed in first 24 hrs after the irradiation. Mayer *et al.* (2002) stated that a higher level of the DNA repair events in stimulated cells does not necessarily reflect a higher DNA repair capacity. Additionally, they showed that all repair proteins needed for the repair of irradiation induced DNA-damage are already present in G0 cells at sufficient amounts and do not need to be induced once lymphocytes are stimulated to start cycling. Only specific DNA repair genes were found to be up-regulated after the PHA stimulation of which most have an additional function in the DNA replication. The mitogen stimulation of lymphocytes may result in an increased removal of only specific types of DNA lesions as it was reported by other authors. This observation might be explained

by the cell cycle dependent regulation of specific DNA repair enzymes, that are more active in proliferating than in resting cells or by differences in the availability of deoxyribonucleotides which are necessary for the DNA excision repair which is not involved in DSB repair (Green *et al.,* 1994). Mayer *et al.* (2002) identified only 12 genes that responded with a more than 2-fold increase of transcripts to the mitogenic stimulus, with a maximum induction for each of the genes 72 h after the PHA treatment. A decrease in the number of chromosome type aberrations with the delay of PHA stimuli could indicate the gradual activation of additional repair capacities, but still the decrease was not to found to be significant. This observation is in the correlation with findings that more than 70% of all evaluated genes had constant expression levels within a twofold range compared to not stimulated. Table 2. showed that the count number of the metaphases with chromosome aberrations in the irradiated samples arrested by colcemid concentration from 0.025 to 0.05 μg/ml was remarkable increased compared with colchicine with the same concentrations.

The phenomenon that the change in colcemid concentration influenced on the aberration yield was confirmed by repeating the experiment. One possible explanation for this phenomenon is that the fraction of escaping cells from arrest may be higher in normal cells than in aberrant cells in the culture with 0.025 ug/ ml of colcemid, whereas at 0.05 ug/ ml of colcemid mitoses are completely blocked both in normal and aberrant cells. Consequently, the ratio of normal metaphases could be lower in the culture at the lower colcemid concentration. Another hypothesis could be that the colcemid exerts a certain inhibitory effect on the repair or replication of some kind of latent lesions of DNA during S phase which are expressed as aberrations at metaphase, and that the higher the colcemid concentration the less the resultant aberrations (IAEA,1986). On the other hand, Hayata *et al.* (1992) reported that the concentration higher than 0.05 ug/ ml is thought to be inappropriate for the continuous treatment with colcemid, since it tends to increase over condensed chromosomes which interfere with the analysis .In addition, it was concluded that colchicine could not be substituted for colcemid, since the continuous treatment of colchicine did not obtain as high mitotic index as colcemid treatment did. Moreover, it must be take attention on the purity of colcemid used and the means for measuring the amount of colcemid both in preparing the stock solution and in applying it to the culture medium in order to maintain a defined culture conditions used in a series of experiments.

Purrott *et al*. (1981) reported that the proliferative effect of PHA and the cell cycle status of the human lymphocytes stimulated by the types of the synthetic culture media and the concentration of the colchicine. Fernandes *et al*. (2008) stated that it has been cautioned that reducing the concentration of Colcemid could lead to an insufficient amount for effective mitotic arrest. Then, cells will progress into second and further cycles, diluting the dicentric frequency and particularly for partial-body exposures, leading to an underestimation of absorbed dose. Therefore, it is important to find a window of colcemid concentration that is low enough to avoid chromosome contraction but high enough to accumulate exclusively M1 metaphases. This possibility requires more investigation.

Conclusion

In order to maximize the sensitivity of the chromosomal aberration analysis PHA has to be added immediately after the irradiation. Colcemid (0.025 to 0.05 μg/ ml) is shown to be better than colchicine and suited for estimating dose of ionizing radiation exposure.

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تأثير الفيتوهيموجلوتانين و الكولسيميد على موثوقية تحليل التشوهات الكروموسومية بعد التعرض للإشعاع المؤين

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قسم البيولوجيا الإشعاعية ، المركز القومي لبحوث وتكنولوجيا الإشعاع ، هيئة الطاقة الذرية ، ص. ب. ٢٩ مدينة نصر، مصر.

هناك بعض العلاقات بين قدرة الخلية للتخلص من التلف الحادث في المادة الو راثية و نشاطها الانقسامي. وهناك ظاهرة أخرى هي التأثر العددي للتشوهات الكروموسومية و الناتج عن تعرض اشعاعى طبقا لتركيز الكولسيميد أو الكولشيسين المستخدمين في إيقاف الانقسام الخلوي عند الطور الاستوائي .

تهدف الدراسة إلى تحسين الدقة التقنية لاستخدام تحليل التشوهات وكذلك إيجاد الجرعة المؤثرة للكولشيسين و الكولسيميد خلال العلاج الاشعاعى لبعض المرضى.

لذلك تتناول الدراسة اختبار تأثير جرعات الفيتوهيموجلوتانين و الكولسيميد أو الكولشيسين على الجهاز الاصلاحى للمادة الوراثية و عدد التشوهات الكروموسومية الناتج عن التعرض الأشعاعى في خلايا الدم الليمفاوية المعرضة للإشعاع.

يتم تشعيع عينات الدم بجرعة ٢ جراى لأشعة جاما مع إحداث عملية حث للانقسام إما مباشرة بعد عملية التشعيع أو بعد عملية التشعيع بساعة أو ساعتين بإضافة ٢ ميللى للفيتوهيموجلوتانين.

و في الجزء الثاني من التجربة استخدمنا الكولسيميد و الكولشيسين بتركيزين مختلفين و هما (٠,٠٢٥، و٠,٠٥٠ ميكروجرام/ ميللى) لكل منهما على حدة.

تشير النتائج إلى أن هناك زيادة في أعداد بعض أنواع الشذوذ الكروموسومى مثل الكر وموسومات الثنائية السنترومير و الكسور الطرفية للكر وموسومات عند استخدام الفيتوهيموجلوتانين مباشرة بعد عملية التشعيع .

وعلى الجانب الأخر توضح النتائج أن استخدام الكولسيميد بتركيز يتراوح بين ٠,٠٢٥و٠,٠٥٠ ميكروجرام/ميللى أفضل من استخدام الكولشيسين بنفس التركيزات و ذلك لزيادة دقة تحليل التشوهات الكروموسومية لمعرفة قيمة الجرعة الممتصة للتعرض الاشعاعى.