VNIVERSITAT DE VALÈNCIA

Facultat de Farmàcia

Departament de Medicina Preventiva i Salut Pública, Ciències de l'Alimentació,

Toxicología i Medicina Legal

MARCADORES CITOGENETICOS APLICADOS EN BIOMEDICINA CYTOGENETIC BIOMARKERS APPLIED IN BIOMEDICINE

Doctorat en Ciències de l'Alimentació

TESIS DOCTORAL INTERNACIONAL

Presentada por

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Dirigida por

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VNIVERSITAT DE VALÈNCIA Facultat de Farmàcia

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INFORMAN QUE:

La Licenciada en Ciencia y Tecnología de los Alimentos ha estado trabajando bajo nuestra dirección en la elaboración de la Tesis Doctoral "MARCADORES CITOGENETICOS APLICADOS EN BIOMEDICINA", razón por la cual autorizamos su presentación para optar al Grado de Doctora por la Universitat de València.

En Valencia, a 5 de Marzo de 2013.

Fdo. Dr. José Miguel Soriano del Castillo

Fdo. Dra. Alegría Montoro Pastor

La presente Tesis Doctoral Internacional se engloba dentro de los siguientes proyectos de investigación:

- (2696/SRO) "Estudio de radioprotectores de origen alimentario para pacientes y trabajadores en procedimientos de tratamiento o diagnóstico médico con radiaciones" enmarcado en el "Programa de ayudas para la realización de proyectos de investigación y desarrollo sobre seguridad nuclear y protección radiológica. Plan de I+D del Consejo de Seguridad Nuclear 2008-2011".
- Plan de Vigilancia Sanitaria de los trabajadores afectados por la exposición a la aerografía textil, Conselleria de Salut, Generalitat Valenciana 2003.
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Para la realización de la Tesis Doctoral Internacional presentada, la estudiante de doctorado Natividad Sebastià Fabregat ha disfrutado de:

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Para la obtención de la Tesis Doctoral presentada con mención de "Tesis Internacional", la estudiante de doctorado ha realizado una estancia internacional en el:

- "Institute of radioisotopes and radiodiagnostic products, radiobiology and biodosimetry laboratory" del National Centre for Scientific Research "Demokritos", Atenas, Grecia.

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Uno mismo nunca se da cuenta de lo que ha hecho, sino que solo puede ver lo que queda por hacer.
(*Maria Salomea Skłodowska, 1894*)

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AAPH: (2,2'-azobis (2-amidinopropane) hydrochloride

ADN: Ácido desoxirribonucleico

BER: Base Excision Repair

BP: Benzo(a)pyrene

BrdU: Bromodeoxiuridina

CAs: Chromosome Aberrations

CAT: Catalase

CHL: Chinese hamster cell line

CHO: Cell Hamster Ovary

CI: Confidence interval

CPK: Cell Proliferation kinetics

CSCs: Cigarette Smoke Condensates

CSN: Consejo de Seguridad Nuclear

Ctb: Chromatid breaks

DPC: Dímero de Pirimidina Ciclobutano

DSB: Double Strand Break

EJ: End Joining

EPIs: Equipos de protección individual

FBS: Fetal Bovine Serum

FDA: Food and Drug Administration

FISH: Fluorescence In Situ Hybridization

FP: Fotoproducto

FPG: Fluorescent Plus Giemsa

GPx: Glutathione Peroxidase

GSH: Glutathione

Gy: Gray

HFCs: High Frequency Cells

HRR: Homologous Recombination Repair

IAEA: International Atomic Energy Agency

IRS: Individual Radiosensitivity Parameter

ISH: In Situ Hybridization

IU: International Units

LD_{50}: Letal dose 50

LDL: Low Density Lipoprotein

MMR: Mismatch Repair

MN: Micronucleus

MPF: Mitotic Promoting Factor

NER: Nucleotid Excision Repair

NF-κB: Nuclear Factor κB

NHEJ: Non-Homologous End-Joining

NRF2: Nuclear factor (erythroid-derived 2)-like 2

ODAC: Oncologic Drugs Advisory Committee

OMS: Organización Mundial de la Salud

PARP: Poly (ADP-ribose) polymerase

PBS: Phosphate Buffered Saline

PCC: Premature Chromosome Condensation

PEG: Polyethylene Glycol

PHA: Phytohaemagglutinin

PKCδ: Protein Kinase Complex

RNS: Reactive Nitrogen Species

ROS: Reactive Oxigen Species

SCEs: Sister Chromatid ExcHanges

SOD: Superoxide Dismutase

SSB: Single Strand Break

SSC: Standard Saline Citrate

TBARS: Thiobarbituric Acid Reactive Substances

TPM: Total Particulate Matter

XRCC1: X-ray repair cross-complementing protein 1

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SUMMARY

A. SUMMARY

Several biomarkers are used to identify and confirm human exposure to exogenous compounds. In cases where the exposure has an adverse effect on the genetic material, the interest is focused on the genotoxic biomarkers. If this effect on the genetic material results in changes in the chromosomal structure or number, which are observable and quantifiable by cytogenetic techniques, these changes can be used as biomarkers. Nowadays, cytogenetic biomarkers are endpoints frequently used in population studies; their sensitivity for measurement of exposure to genotoxic agents and the role of some of the cytoenetic biomarkers as early predictors of cancer risk have contributed to this success. Different cytogenetic biomarkers can be used according to the purpose of the study. Those generally used are micronucleus, sister chromatid exchanges and chromosomal aberrations.

Among the wide variety of chemicals to which humans can be exposed, tobacco is a very important. A large number of compounds from tobacco and tobacco smoke have been classified by the International Agency for Research on Cancer (IARC) as carcinogens or probably carcinogens for humans. The DNA damage assessed by cytogenetic techniques is a useful tool in order to confirm the harmful effects of tobacco on the genetic material.

Other types of chemicals, present in aerosol paints, caused a severe health impact in some workers in the textil painting factories in the region of Valencia (Spain). This outbreak was classified as the “Ardystil syndrome” and subsequently it was decided to carry out a health surveillance program in affected people. In order to evaluate a persistent alteration in chromosomes, the sister chromatid exchange technique was carried out.

Ionizing radiation is another agent responsible for genetic damage. In specific situations these lesions in the DNA are an expected effect of radiation, such as radiotherapy. However, these methods of clinical treatment or medical diagnosis involving ionizing radiation can cause undesired effects involving an impact on human health. In order to avoid or mitigate these undesired effects many synthetic or natural products have been proposed, with an increasing interest in the latter. Cytogenetic biomarkers such as chromosomal aberrations can highlight the DNA damage in cells

caused by radiation and therefore the possible damage reduction provided by compounds usually called radioprotectors. Similarly, the genotoxicity of these compounds which are under study can be evaluated. One of the groups of natural compounds with a major interest for application as radioprotectors is the polyphenols group.

Related to ionizing radiation exposure, cytogenetic techniques can be used as a useful tool to evaluate the individual radiosensitivity because it is known that humans exhibit a range of individual variation in the frequency and severity of effects occurring after ionizing radiation exposure. This measure could help to assess the individual risk of subjects exposed to ionizing radiation, thus a better dose adjustment in case of medical intervention or an additional preventive measure in subjects working with radiation. In addition, the cytogenetic technique for the assessment of individual radiosensitivity can be used to evaluate, *in vitro*, whether certain compounds, which may be present simultaneously with radiation, are capable of modifying the cell response.

In genotoxicity studies, the biomarkers analyzed were the chromosomal aberrations, sister chromatid exchanges, mitotic index and the proliferation index. From the analysis of sister chromatid exchanges, in the tobacco and "Ardystil syndrome" study, it was calculated the parameter of cells with a frequency of exchanges higher than the 95 percentil and a ratio which analyzed the clustering of exchanges in one chromosome instead of being uniformingly distributed among the metaphase. In these studies it is essential to differentiate the number of cell cycles of the lymphocytes; for this reason, at the beginning of the cell cultures a substance should be added which allows for the differentiation of the first, second and third cell divisions with the Fluorescence plus Giemsa staining. For the tobacco and "Ardystil syndrome" studies, human peripheral blood samples were processed for the cytogenetic analysis of the sister chromatid exchanges biomarker. In the study of the genotoxicity of the polyphenols curcumin and *trans*-resveratrol, blood samples were incubated with different concentrations of both compounds before starting the culture. Cytogenetic analysis included the study of biomarkers such as chromosomal aberrations, sister chromatid exchanges and mitotic and proliferation index.

In the radioprotection studies, two different techniques were carried out. On the one hand, the dicentric chromosome assay, which is based on the curcumin and *trans*-resveratrol pre-incubated human peripheral blood irradiation with ionizing radiation, samples culture and cytogenetic analysis of chromosomal aberrations. Analysis was based in dicentric chromosomes wich are considered biomarders of radiation-induced damage. On the other hand, the premature chromosome condensation technique, which is based on the curcumin and trans-resveratrol pre-incubated lymphocytes isolated from human peripheral blood, fusion with mitotic hamster ovary cells, culture and cytogenetic analysis. Analysis was focused in the counting of chromosomal fragments radio-induced.

For the radiosensitivity studies, the G2-assay was applied in two different experiments. On the one hand, study of the radiosensitivity induced by curcumin and *trans*-resveratrol, aimed at evaluating the *in vitro* radiosensitivization ability of both compounds by modifying the lymphoyctes radiosensitivity in G2-cell cycle phase. On the other hand, the G2-assay was implemented in a case report in order to evaluate the individual radiosensibility in a patient undergoing interventionist radiology who suffered adverse secondary effects. With this assay, the patient can be classified as “hiperradiosensitive”, “radiosensitive”, “normal” or “radioresistant” according to the classification of Terzoudi and Pantelias (2011).

Possible alterations in the chromosomal material caused by different chemical and physical agents can be evaluated by using cytogenetic biomarkers. The analysis of biomarkers for the assessment of the genotoxicity of tobacco and some components used in textile airbrushing with non adequate preventive measures ("Ardystil Syndrome") allowed to observe a statistically significant genetic affectation increase in smokers and check whether this affectation was no longer present in subjects affected by "Ardystil syndrome" after ten years of the outbreak.

The *in vitro* study of the genotoxic, radiosensitizing and radioprotective properties of polyphenolic compounds, curcumin and *trans*-resveratrol, demonstrates the versatility of these compounds depending on the concentrations used, the cell cycle phase in which they are absorbed by cells and the conditions to which samples are subjected.

SUMMARY

The application of the individual radiosensitivity G2-assya in a patient with secondary effects after a medical intervention with ionizing radiation constitutes a first step for the future application of this cytogenetic technique in preventive medicine. Patients and workers subjected to ionizing radiation treatment would have an additional tool to improve the individualization of treatments as well as improving the prevention of occupational hazards.

A. RESUMEN

Cuando se pretende identificar una determinada exposición a una sustancia exógena al organismo se utilizan biomarcadores que nos permitan confirmar esta exposición. En el caso de que la exposición provoque un efecto nocivo en el material genético, el interés se centra en biomarcadores genotóxicos. Si el efecto en el material genético se traduce en cambios en la estructura o en el número de cromosomas observables y cuantificables mediante técnicas citogenéticas, estos cambios se podrán emplear como biomarcadores citogenéticos. De hecho, los biomarcadores citogenéticos son criterios de valoración frecuentemente utilizados en estudios poblacionales. Su sensibilidad para la medición de exposición a agentes genotóxicos y el papel de algunos biomarcadores como indicadores tempranos del riesgo de cáncer han contribuido a este éxito. Existen distintos biomarcadores citogenéticos según el propósito del estudio, entre ellos, los generalmente más utilizados son los micronúcleos, los intercambios de cromátidas hermanas y las aberraciones cromosómicas.

Entre los agentes químicos a los que los humanos pueden estar expuestos se encuentran aquellos que componen el tabaco. Un elevado número de compuestos presentes en el tabaco han sido clasificados por la Agencia Internacional de Investigación sobre el Cáncer (IARC) como cancerígenos o posibles cancerígenos para el ser humano. La evaluación mediante técnicas citogenéticas del daño causado en el ADN por la exposición a estos compuestos es de gran utilidad para confirmar los efectos perjudiciales del tabaco sobre el material genético.

Otro tipo de agentes, concretamente los presentes en las pinturas usadas como aerosoles, causaron un grave impacto en la salud de ciertos trabajadores de industrias textiles in la región de Valencia (España). Este suceso fue clasificado como el “syndrome de Ardystil” y después del accidente se pensó en llevar a cabo un plan de vigilancia de la salud de la gente afectada. Con el fin de evaluar la posible persistencia de alteración en los cromosomas, se realizó la técnica de intercambio de cromátidas hermanas.

La radiación ionizante es otro agente que provoca lesiones en el material genético de las células. En determinados casos estas lesiones forman parte del efecto

deseado de las radiaciones, como en la radioterapia, sin embargo, en estos procedimientos de tratamiento o en determinadas situaciones de diagnóstico médico con radiaciones ionizantes se pueden ocasionar efectos indeseados con repercusiones sobre la salud de los individuos. Con el propósito de evitar o mitigar estos efectos indeseados de la radiación ionizante se han investigado diversos productos sintéticos y naturales, con un interés creciente por estos últimos. Mediante los biomarcadores citogenéticos como las aberraciones cromosómicas podemos evaluar el daño que la radiación causa en el ADN de las células y la posible disminución ofrecida por parte de los compuestos llamados radioprotectores. De forma semejante podemos evaluar la toxicidad que los compuestos objeto de estudio pueden ejercer por sí mismos. Uno de los grupos con más interés para su aplicación como radioprotectores es el grupo de los polifenoles.

En relación con la exposición a la radiación ionizante, las técnicas citogenéticas pueden ser utilizadas como una herramienta para evaluar la posible radiosensibilidad de cada individuo ya que se sabe que en los seres humanos se exhibe un rango de variación interindividual en la frecuencia y severidad de los efectos aparecidos después de la exposición a las radiaciones ionizantes. Esta medida podría ayudar a evaluar el riesgo individual de sujetos expuestos a tratamientos con radiaciones ionizantes con la posibilidad de un ajuste más personalizado de las dosis o como una medida preventiva adicional en trabajadores expuestos a radiación ionizante. Además, la técnica citogenética desarrollada para la evaluación de la radiosensibilidad individual permite evaluar *in vitro*, cómo determinados compuestos, que pueden estar presentes de forma simultánea a la radiación, son capaces de modificar la respuesta de las células a la misma.

En los estudios de genotoxicidad, se analizan los biomarcadores de aberraciones cromosómicas, intercambio de cromátidas hermanas, índice mitótico e índice de proliferación. Concretamente, en el estudio de individuos fumadores y afectados por el síndrome Ardystil, del análisis del intercambio de cromátidas hermanas se extrajeron dos parámetros, las células con un número de intercambios superior al percentil 95 y un ratio que analiza la concentración de intercambios en un cromosoma en lugar de distribuilos uniformemente en los 46 cromosomas. Resulta imprescindible la diferenciación del número de ciclos celulares que han experimentado los linfocitos, por

lo que al principio del cultivo se debe añadir una sustancia que permitirá diferenciar la primera, segunda y tercera división mediante la técnica de tinción concreta de Fluorescencia más Giemsa. Para los estudios del tabaco y síndrome de Ardystil se procesaron las muestras de sangre periférica obtenidas de los sujetos estudiados y se procedió al análisis citogenético para el estudio del biomarcador de intercambio de cromátidas hermanas. Para el estudio de la genotoxicidad de los polifenoles de la curcumina y el *trans*-resveratrol, ambos compuestos se añadieron por separado y en distintas concentraciones un determinado tiempo antes de empezar el cultivo de las muestras. El análisis citogenético incluyó el estudio de los biomarcadores de aberraciones cromosómicas, intercambio de cromátidas hermanas y los índices mitótico y de proliferación.

En los estudios de radioprotección, se utilizaron dos técnicas citogenéticas. Por una parte, el ensayo de cromosomas dicéntricos, basado en la irradiación con radiación ionizante de muestras de sangre periférica humana pre-incubadas con curcumina y *trans*-resveratrol, cultivo de las mismas y análisis citogenéticos de las aberraciones cromosómicas para el recuento de cromosomas dicéntricos considerados biomarcadores de daño radioinducido. Por otra parte, la técnica de condensación prematura de cromosomas, basada en la irradiación con radiación ionizante de linfocitos aislados de sangre periférica humana previamente incubadas con diferentes concentraciones de curcumina y *trans*-resveratrol, fusión con células mitóticas de ovario de hámster chino, cultivo y análisis citogenético del daño radioinducido en forma de fragmentos cromosómicos.

En los estudios de radiosensibiliad, se empleó el ensayo G2 para dos aplicaciones distintas. Por un lado, el ensayo de radiosensibilidad en fase G2 aplicado a la evaluación de la radiosensibilización *in vitro* de compuestos químicos, concretamente curcumina y *trans*-resveratrol, para estudiar si ambas sustancias son capaces de modificar la radiosensibilidad en fase G2 de linfocitos humanos. Por otro lado, el ensayo de radiosensibilidad en fase G2 aplicado a un caso clínico para la evaluación de la radiosensibilidad individual de un paciente con efectos secundarios adversos tras un tratamiento de radiología intervencionista. De esta forma la paciente puede ser clasificada como “hiperradiosensible”, “radiosensible”, “normal” o “radioresistente” según la clasificación propuesta por Terzoudi y Pantelias (2011).

Mediante el empleo de biomarcadores citogenéticos es posible evaluar como diferentes agentes químicos y físicos causan una alteración del material cromosómico. El análisis de los biomarcadores utilizados para evaluar la genotoxicidad del tabaco y de componentes utilizados en aerografía textil sin condiciones de prevención de riesgos laborales ("Síndrome de Ardystil") permitió observar un aumento estadísticamente significativo de la afectación del material genético en individuos fumadores así como comprobar que esta afectación ya no estaba presente en sujetos afectados por el "síndrome de Ardystil" pasados diez años del suceso.

El estudio *in vitro* de las propiedades genotóxicas, radioprotectoras y radiosensibilizantes de los compuestos polifenólicos curcumina y *trans*-resveratrol pone de manifiesto la versatilidad con la que actúan estos compuestos dependiendo de las concentraciones utilizadas, el momento del ciclo celular en el que son absorbidos por las células y las condiciones a las que son sometidas las muestras.

La aplicación del ensayo de radiosensibilidad individual en fase G2 del ciclo celular a una paciente con efectos secundarios tras una intervención mediada por radiación ionizante es un primer paso para la aplicación futura de esta técnica citogenética en medicina preventiva. Tanto pacientes como trabajadores sometidos a tratamientos con radiaciones ionizantes dispondrían de una herramienta adicional para mejorar la individualización del tratamiento o para la mejor prevención de riesgos laborales.

OBJECTIVES

The **overall objective** of this study is to carry out an analysis regardingt the effects of several chemical and physical compounds on the human chromosomes using different cytogenetic techniques which permitted the analysis of several cytogenetic biomarkers:

To achieve this general objective the following specific objectives have been set:

1. To evaluate the effect of tobacco use on the Spanish population group by the Sister Chromatid Exchange (SCEs) technique.
2. To evaluate whether subjects affected by the "Ardystil Syndrome" still show chromosome damage several years after the exposure from the SCEs technique.
3. To assess the genotoxic, radioprotective and radiosensitizer properties of two naturally-occurring compounds, curcumin and *trans*-resveratrol using cytogenetic biomarkers.
4. To apply the individual radiosensitive G2-Test to a clinical case undergoing clinical treatment using ionizing radiation.

WORKING PLAN

To accomplish the objectives proposed a working plan has been designed with the following steps:

1. To evaluate the effect of the tobacco habit on the Spanish population group, with the Sister Chromatid Exchanges technique, blood samples were collected from smokers and non-smoker subjects and a classification within the smoking group was designed in order to differentiate the amount of cigarretes smoked. The parameters evaluated were SCEs and HFCs frequencies and a SCEs distribution pattern ratio.
2. To evaluate the possible residual chromosomal damage in those subjects affected by the "Ardystil Syndrome", with the Sister Chromatid Exchange technique, blood samples were collected from the "Ardystil" subjects and a control group. The tobacco smoking habit was considered in the analysis. The parameters evaluated were the SCEs and HFCs frequencies and a SCEs distribution pattern ratio.
3. To assess the genotoxic, radioprotective and radiosensitizer properties of curcumin and *trans*-resveratrol, blood samples were obtained from healthy subjects. The samples were irradiated when the radioprotective and radiosensitizer properties were studied, and processed according to the different cytogenetic techniques in order to analyze endpoints such as CAs, SCEs, MI and PI indexes.
4. To apply the individual radiosensitive G2-Assay in a clinical case where an individual underwent treatment using ionizing radiation. A blood sample was obtained from the patient and processed according to the assay. The typical CAs caused during the irradiation of cells in the G2-phase cell cycle was analyzed and an Individual Radiosensitivity Parameter was calculated with the aim of assessing the individual radiosensitivity of the patient.

LITERATURE REVIEW

B. ANTECEDENTES BIBLIOGRÁFICOS

B. 1. Citogenética

B.1.1. Concepto y orígenes

El término citogenética hace referencia a la parte de la genética enfocada al estudio de la estructura y el comportamiento de los cromosomas, así como a las implicaciones genéticas derivadas de su estudio (Herrera, 2007). El análisis de los cromosomas es uno de los test de diagnóstico más comúnmente utilizado en citogenética (Lee *et al.,* 2001).

En el desarrollo histórico de la citogenética cabe señalar dos etapas; el período clásico y el molecular. El primero, surgió en los albores de siglo XX con los estudios sobre la estructura y el comportamiento de los cromosomas del maíz, concretamente en 1902 (hipótesis de Sutton-Boveri) y llega hasta mediados de los años 60. En este periodo la investigación citogenética era principalmente descriptiva y estaba basada mayoritariamente en la microscopía óptica y también, a partir de 1956, en la electrónica. A grandes rasgos, en este periodo habría que destacar la labor de varios investigadores, pero es necesario destacar a Cyril Dean Darlington, como el padre de la citogenética, no sólo por su labor de síntesis, sino también por su propia labor de investigación original. Algunas de sus teorías no fueron del todo correctas pero sirvieron de estímulo al tratar de comprobarlas impulsando así el desarrollo de la citogenética. Después del descubrimiento en 1953 del código genético por Watson y Crick, la mayor parte de los trabajos científicos en el área de la citogenética se orientaron a la descripción minuciosa de la forma y el número de cromosomas, así como a la caracterización detallada de las mutaciones. A mediados de los años 50 los grupos de investigación de Joe Hin Tjio (que trabajaba en la estación experimental de Aula Dei, en Zaragoza, España) y Albert Levan (del *Cancer Chromosome Laboratory, Institute of Genetic* de Lund, Suecia) establecieron el número y la estructura de los cromosomas humanos (**Figura 1**). Desde ese momento, las técnicas para la caracterización del cariotipo humano iniciaron un continuo desarrollo en cuanto a su poder de resolución y eficacia (Martínez-Fernández *et al.,* 2010).

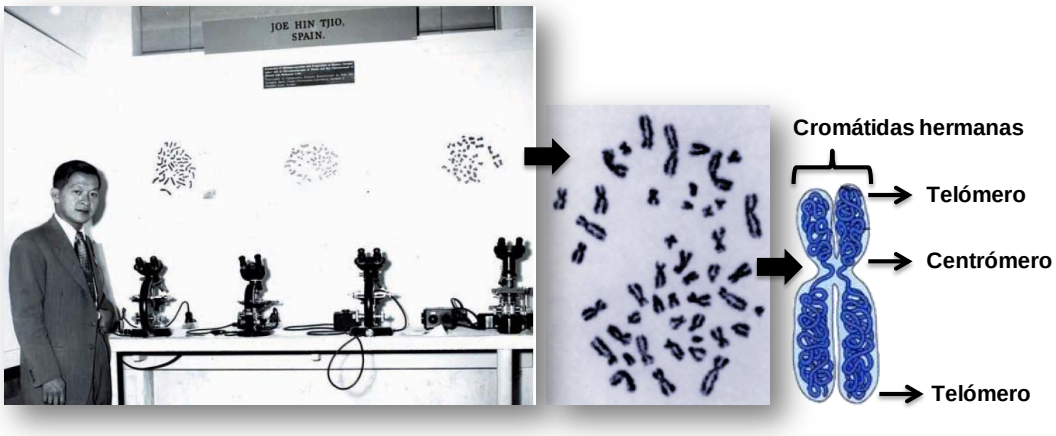

Figura 1. Dr. Joe Hin Tjio junto a fotografías de sus estudios cromosómicos en el primer Congreso Internacional de Genética Humana en Copenhague en 1956. Metafase humana obtenida por Joe Hin Tjio y Albert Levan en 1956.

A partir de la visualización por microscopía óptica convencional del ADN en forma de cromosomas condensados se estableció que, las células humanas somáticas tienen 46 cromosomas (es decir, son diploides, 2n) y las células humanas sexuales tienen 23 cromosomas (es decir, son haploides, n). Los cromosomas, los agrupamos en pares cromosómicos atendiendo a su tamaño y morfología existiendo 23 pares cromosómicos (22 pares de autosomas y los cromosomas sexuales, XX en la mujer y XY en el hombre). Los cromosomas del mismo par se llaman cromosomas homólogos, procediendo cada cromosoma de uno de los progenitores del individuo (Galán, 2002).

La incorporación de nuevas técnicas y metodologías moleculares dio lugar al segundo período (citogenética molecular), iniciándose este periodo en 1957, con un modelo semiconservativo de reproducción cromatídica utilizando timidina tritiada. A partir de mediados de los sesenta la citogenética molecular se expandió con la aplicación de las técnicas de hibridación *in situ* (*in situ hybridization; ISH*) de ácido nucleicos, la visualización de la expresión génica con microscopía electrónica de extensión, las técnicas de bandeo cromosómico, el análisis bioquímico del ciclo de división celular, el análisis de la estructura cromosómica tanto a nivel de cromatina y su organización en nucleosomas como en el papel estructural de las proteínas no histonas.

En la década de los ochenta, se produjo un punto de inflexión en la citogénetica molecular, cuando se profundizó en el análisis molecular y funcional de estructuras cromosómicas como el centrómero y el telómero así como en el control genético del ciclo celular y en el análisis molecular del movimiento cromosómico en la mitosis. Especial importancia tuvo la introducción de la técnica de hibridación *in situ* con fluorescencia (*Fluorescence In Situ Hybridization; FISH*) en la que el marcaje radiactivo de la sonda fue sustituido por otro marcaje no radiactivo como la biotina, la digoxigenina, etc.

La aplicación de las nuevas técnicas ha permitido afrontar viejos problemas citogenéticos pendientes aún por resolver. De hecho, la metodología de la citogenética moderna está permitiendo responder a muchas cuestiones uniendo los estudios bioquímicos y fisiológicos de los biólogos celulares con el diseño experimental genético. De esta forma, el laboratorio de citogenética juega un papel fundamental y complementario a la genética clínica.

B.1.2. Biomarcadores citogenéticos

Un **biomarcador** se define como aquellas alteraciones celulares, bioquímicas o moleculares que son medibles en medios biológicos tales como tejidos humanos, células o fluidos. Más recientemente, la definición se ha ampliado para incluir características biológicas que se pueden medir objetivamente y pueden evaluarse como un indicador de procesos biológicos normales, patogénicos o respuestas farmacológicas a una intervención terapéutica (Mayeux, 2004).

Los estudios de biomonitorización llevados a cabo mediante biomarcadores están siendo cada vez más usados en los últimos años para demostrar cómo la exposición a un determinado xenobiótico puede asociarse con la aparición de un determinado efecto biológico. Por este motivo, a lo largo del tiempo se ha intentado clasificar y definir cuáles son los biomarcadores más adecuados en cada caso y realizar ciertas guías para planificar, llevar a cabo e interpretar aquellos estudios para monitorizar grupos o individuos expuestos a estos agentes (Gil, 2000).

De acuerdo a esto, un **biomarcador de genotoxicidad** se considera aquella respuesta biológica y cuantificable de un organismo vivo, traducida en una alteración

temprana en el material genético, como resultado de una acción provocada por un determinado agente genotóxico (agente físico o químico que produce un daño en el material genético celular). Los biomarcadores de genotoxicidad representan el reflejo de determinados efectos biológicos que pueden ser cuantificados a través de distintas variables (Mudry *et al.,* 2011).

Un **biomarcador citogenético** será aquel que nos permita observar y cuantificar, mediante el estudio de la estructura y comportamiento de los cromosomas, las alteraciones genéticas producidas por los xenobióticos. Los biomarcadores citogenéticos son frecuentemente usados en estudios de biomonitorización de humanos como parámetros para evaluar el impacto de factores ambientales, ocupacionales y médicos en la estabilidad genética (Battershill *et al.,* 2008). Su sensibilidad a la hora de medir la exposición a xenobióticos y su papel como indicadores tempranos de riesgo de cáncer han contribuido a su éxito. Si se seleccionan aquellos biomarcadores citogenéticos que reflejen una alteración genética reconocida, asociada con un deterioro de la salud o posible aparición de una enfermedad, los generalmente más utilizados (Norppa 2004; Bonassi *et al.,* 2005; Mudry *et al.,* 2011; Suspiro *et al.,* 2011) son los micronúcleos, los intercambios de cromátidas hermanas (*Sister Chromatid Exchanges; SCEs*) y las alteraciones cromosómicas (*Chromosome Aberrations; CAs*). Concretamente los dos últimos son objeto de la presente tesis doctoral.

B.1.2.1. Intercambios de cromátidas hermanas (Sister Chromatid Exchanges; SCEs)

Representan el intercambio recíproco y simétrico de productos de replicación del ADN entre cromátidas hermanas en lugares (*locus*) aparentemente homólogos que no conlleva una alteración en el número o estructura de los cromosomas (Norppa *et al.,* 2004; Suspiro *et al.,* 2011). Es probable que el proceso de intercambio comprenda la rotura y la reunión del ADN, aunque se sabe poco sobre su base molecular (**Figura 2**).

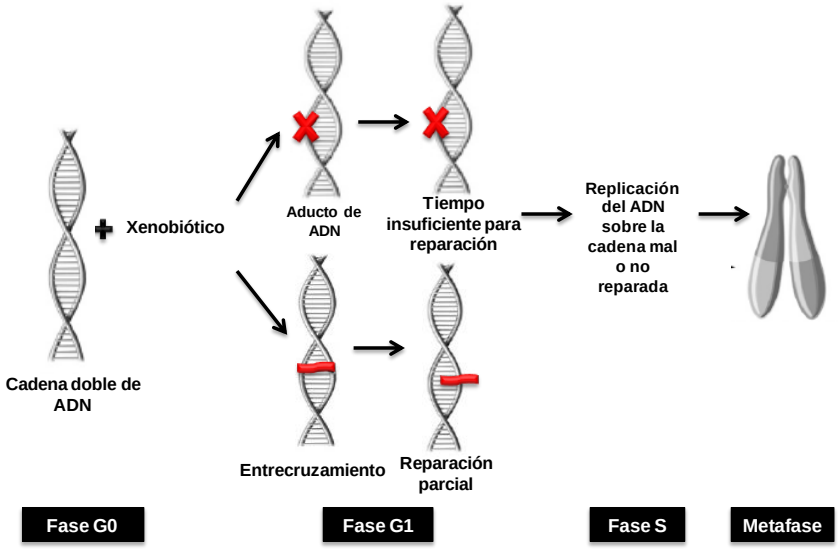

Figura 2. Posibles mecanismos de formación de las SCEs por acción de un xenobiótico.

Debido a que no conllevan ningún cambio númerico o estructural, para poder observar los SCEs, es necesario poder marcar de forma diferente las cromátidas hermanas, hecho que se consigue mediante la incorporación al ADN de un análogo de la timina, la bromodeoxiuridina (BrdU). Si la BrdU se encuentra presente durante dos ciclos celulares, una cromátida tendrá BrdU en una de sus dos cadenas polinucleótidas y la cromátida hermana tendrá BrdU en las dos cadenas. Esta incorporación de la BrdU junto con la tinción con el colorante Hoechst (33258), que se une a la molécula de ADN en los pares de bases adenina+timina, permite diferenciar cromátidas en las que aún hay timina, de las cromátidas en las que en las dos cadenas la timina está sustituida por BrdU (**Figura 3**).

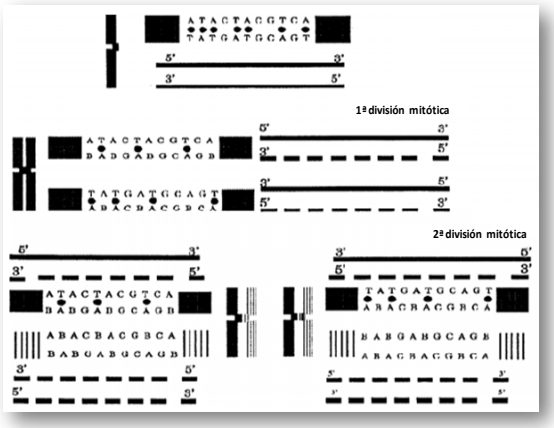

Figura 3. Esquema del fundamento de la técnica Fluorescente Plus Giemsa. (FPG) B= 5´bromo 2´deoxiuridina. T=timina. A=adenina. C=citosina. G=guanina (Barquinero *et al.,* 1991).

Para la evaluación de los xenobióticos mediante SCEs, se exponen células de mamífero *in vitro* (generalmente linfocitos de sangre periférica humana) a la sustancia objeto de estudio y se cultivan durante dos ciclos de replicación en un medio que contiene la BrdU (Andreoli *et al.,* 2003). Después del tratamiento con un inhibidor del huso (p. ej., colchicina) para acumular las células en fase de mitosis de tipo metafásico, se recolectan las células, las cuales se encuentran en su segunda división y se realizan las preparaciones de cromosomas (**Figura 4**).

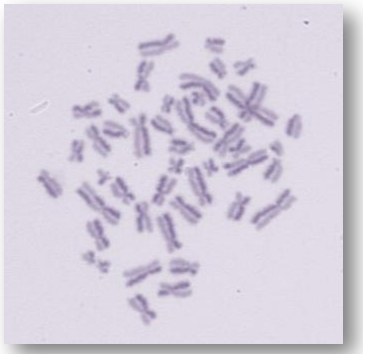

Figura 4. Metafase en segunda división teñida mediante la técnica FGP exhibiendo la diferente tinción de las cromátidas hermanas, conocido como "efecto arlequín" (IAEA, 2011).

En los humanos, existe una frecuencia basal de SCE cuyo valor suele variar debido a diferencias a nivel de comparación entre laboratorios (cantidad de BrdU usada, diferentes reactivos y condiciones de cultivo de las células o distintos métodos de tinción) y debido a diferencias a nivel interindividual (exposición ambiental a tóxicos, edad, etc.). Las diferencias interindividuales afectan en menor medida ya que la distribución de SCE en una población de células se aproxima a la normalidad a excepción de una asimetría en el lado derecho de la distribución determinada por la presencia de células con un número elevado de SCEs, las llamadas células HFCs (*del inglés, High Frequency Cells*). De hecho, las HFCs se definen como células con una frecuencia de SCEs que excede el percentil 95 de una población control de individuos. En determinadas ocasiones es aconsejable calcular este parámetro ya que se considera más sensible para ciertos tipos de exposición como por ejemplo el hábito de fumar. Algunos autores estiman que las células HFCs podrían representar a una sub-población de linfocitos con deficiencias en reparar el ADN, otros, que quizás representen a una sub-población de linfocitos de larga vida que tienen más posibilidades de acumular

lesiones durante su ciclo vital. De cualquier forma, la primera interpretación sería más adecuada para la evaluación de la capacidad individual de reparar el ADN mientras que la segunda lo sería para la evaluación de la exposición a agentes genotóxicos (Ponzanelli *et al.,* 1997).

Existe cierto nivel de incertidumbre a la hora de establecer el significado claro de un aumento en la frecuencia de SCE en las células, por eso el valor predictivo de una frecuencia mayor de SCE con respecto a la aparición de cáncer no está establecido. Podría considerarse más bien un biomarcador de exposición a agentes genotóxicos que un biomarcador de efecto (por ejemplo el cáncer), ya que la frecuencia de SCEs en células eucariotas se ve aumentada por la exposición a agentes genotóxicos que inducen un daño en el ADN (bases alquiladas, entrecruzamientos, etc.) capaces de interferir en la replicación del ADN (Albertini *et al.,* 2001). Esta técnica se sigue usando para la evaluación de exposición al humo del tabaco (Ben Salah *et al.,* 2011; Khabour *et al.,* 2011) y otros compuestos químicos como el benzo(a)pireno, los hidrocarburos aromáticos policíclicos o el butadieno, todos ellos reconocidos como agentes cancerígenos (Andreoli *et al.,* 2003)**.** En la Nota Técnica 192 "Genotóxicos: control biológico" y en la 354 "NTP 354: Control biológico de la exposición a genotóxicos: técnicas citogenéticas" del Instituto de Nacional de Seguridad e Higiene del Trabajo perteneciente al Ministerio de Trabajo y Asuntos Sociales de España, el estudio de la frecuencia de SCEs está incluido en el apartado "Indicadores de efectos biológicos precoces" y contemplado como técnica citogenética para la evaluación de exposición a genotóxicos (NTP 192 1994; NTP 354 1994).

B.1.2.2. Aberraciones cromosómicas (Chromosomal Aberrations; CAs)

Son alteraciones estructurales y numéricas causadas por un xenobiótico que interacciona con el material genético produciendo como consecuencia un daño y unas lesiones (rupturas de cadena simple, rupturas de doble cadena, daños de bases, etc.) reparadas de distinta forma por la célula. Cabe tener en cuenta que los cromosomas humanos contienen una molécula de ADN que puede llegar a los 7,5 cm de longitud la cual está compactada en una estructura fibrilar de unos 1350 µm de longitud durante la interfase del ciclo celular y en un cromosoma de unos 10 µm de largo durante la

metafase. Debido a estas enormes dimensiones, las moléculas de ADN son blancos permanentes de daños químicos o físicos de distinto origen. Este daño inducido al ADN puede ser reparado por los mecanismos de reparación de la célula, pero si no se repara o lo hace de forma incorrecta, puede dar lugar al biomarcador aquí tratado, las CAs. Las alteraciones pueden afectar tanto a la estructura de los cromosomas (clastogénicas) como a su número (aneugénicas). La radiación ionizante y ultravioleta y otros agentes genotóxicos como agentes alquilantes o hidrocarburos aromáticos policíclicos son agentes con toxicidad sobre el material genético de los organismos produciendo unas lesiones sobre el mismo. El análisis de CAs constituye por tanto una de las estrategias para clasificar agentes mutagénicos y/o carcinogénicos y, aunque las CAs son sólo una pequeña fracción de un gran cantidad de cambios en el ADN cromosómico, éstas reflejan la enorme plasticidad del genoma con muchas consecuencias de largo alcance para la evolución (Obe *et al.,* 2002) (**Figura 5**).

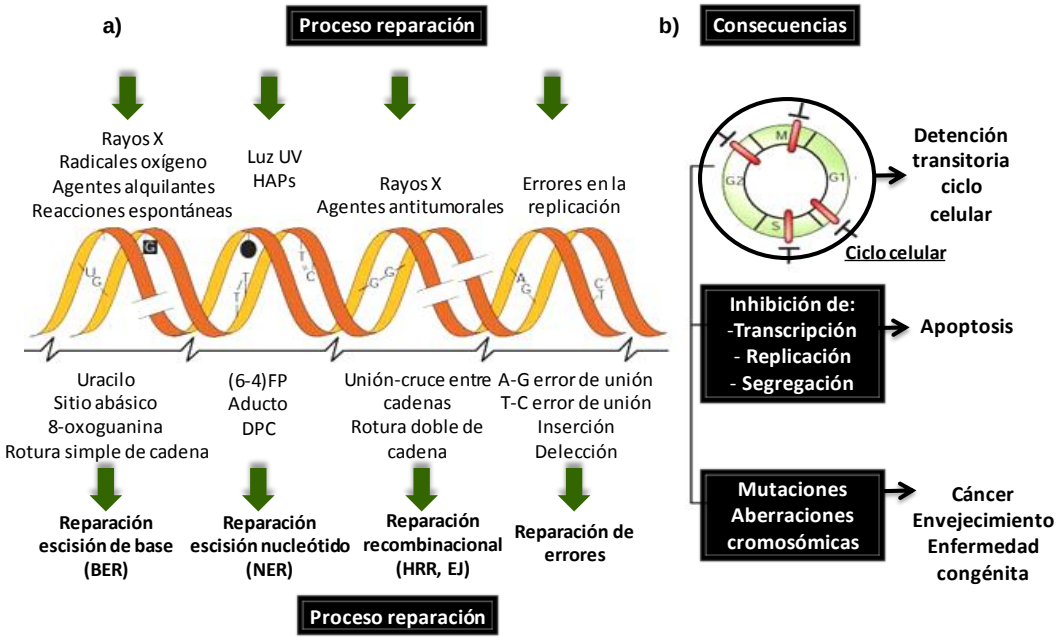

Figura 5. Daño en el ADN, mecanismos de reparación y consecuencias. a) daños más comunes en el ADN (arriba), lesiones inducidas por estos agentes (en medio) y mecanismos más comunes para la reparación de estas lesiones (abajo). b) consecuencias agudas del daño en el ADN en el ciclo celular. HAPs: Hidrocarburos Aromáticos Policíclicos; FP: fotoproducto; DPC: dímero de pirimidina ciclobutano; BER: *del inglés, Base Excision Repair*; NER: *del inglés, Nucleotid Excision Repair*; HRR: *del inglés, Homologous Recombination Repair*; EJ: *del inglés, End Joining*) (Hoeijmakers, 2001).

Los diferentes tipos de lesiones que puede sufrir la molécula de ADN se reparan por diferentes mecanismos de forma más o menos fácil, siendo los daños de base y las roturas de cadena simple (*Single Strand Break;* SSB) las lesiones de más fácil reparación. Sin embargo, las roturas que afectan a las dos cadenas de ADN (*Double Strand Break;* DSB) son las lesiones más difíciles de reparar, por esto, aunque se producen en menor proporción, son de mayor efecto citotóxico para la célula. De hecho, actualmente se considera que la principal responsable de la formación de las CAs es la reparación errónea de los DSB (Galán et *al.,* 2002).

Los diferentes mecanismos de reparación de las lesiones en al ADN se pueden clasificar en (Lehnert, 2008):

- Reparación directa de defectos simples (modificación de 06-alquilguanina).
- NER (*Nucleotide Excision Repair*): reparación por escisión de nucleótidos, lesiones grandes (dímeros de pirimidina).
- BER (*Base Excision Repair*): Reparación por escisión de bases.
- MMR (*Mismatch Repair*): reconoce los fallos de inserción y/o deleción de bases y los corrige.
- Actividad PARP-XRCC1-ligasa III: repara las SSB.
- HRR (*Homologous Recombination Repair*): recombinación homóloga de las DSB.
- NHEJ (*Non-Homologous End-Joining*): recombinación no homóloga de las DSB.

De todos estos mecanismos de reparación, los más importantes son la HRR y la NHEJ (**Figura 6**) ya que son los mecanismos encargados de reparar las DSB, consideradas, como se ha mencionado, las lesiones más difíciles de reparar:

• Mecanismos de reparación homóloga (HRR, *Homologous Recombination Repair*): son mecanismos de reparación de alta fidelidad, basados en la recombinación homóloga entre las cromátidas hermanas. Restituyen la secuencia original del ADN donde se ha producido la DSB utilizando la cromátida hermana no dañada como un molde para sintetizar de nuevo los fragmentos perdidos en la cromátida rota (Shiloh, 2003). La alta fidelidad que caracteriza este proceso hace que raramente produzca mutaciones, y por eso a menudo se le llama reparación “libre de errores” (Shcherbakova *et al.,* 2003).

• Mecanismos de reparación no homóloga (NHEJ, *Non-Homologous End-Joining*): son mecanismos rápidos con tendencia a producir errores los cuales no dependen de la homología y unen directamente los dos extremos rotos, pudiendo crear microdelecciones en el punto donde se ha producido el DSB. La desventaja de la baja fidelidad en la reparación se ve compensada por la rápida actuación y, además, a diferencia de los HRR, no dependen de la presencia de moléculas de ADN homólogas las cuales sólo se encuentran en la célula después de la replicación del ADN (Shiloh, 2003). En la siguiente **Figura 6** se pueden observar los dos mecanismos de reparación de las DSB:

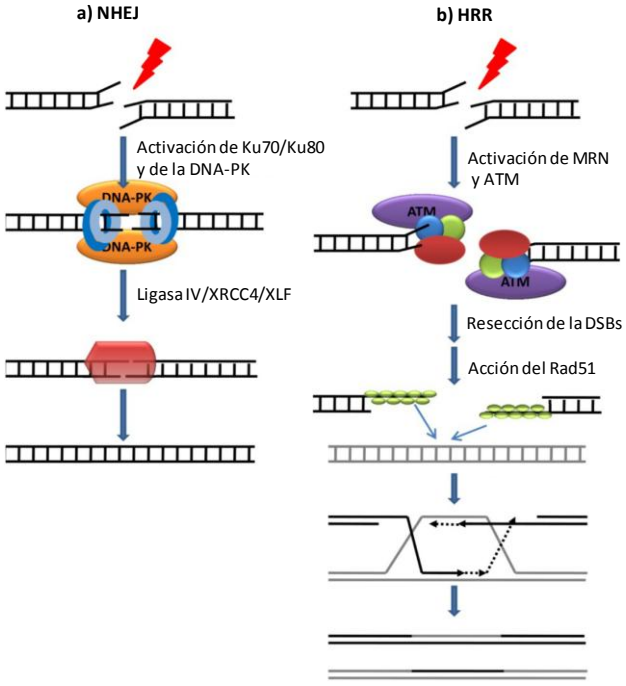

Figura 6. Principales mecanismos de reparación del ADN. a) NHEJ. Ku70/Ku80: heterodímero que se estabiliza los dos extremos de ADN y recluta a la DNA-PK; DNA-PK: fosforila y activa el complejo NHEJ efector (ligasa IV/XRC44/XLF) que finalmente re-úne el ADN cortado. b) HR. ATM: quinasa recrutada por la DSB por una interacción con el complejo MRN (Mre11-Rad50-Nbs1) que fosforila múltiples substratos; las DSBs se seccionan en cadenas de ADN que atraen al Rad51 para que invadan las cadenas no dañadas y formen estructuras homólogas. La HRR es completada con la síntesis de nuevo ADN (López and Fernández, 2012).

Ambos mecanismos son complementarios ya que no actúan durante las mismas fases del ciclo celular. El HRR actúa mayoritariamente después de la replicación del ADN, en la fase G2, mientras que el NHEJ actúa principalmente durante la fase G0, G1 y

al principio de la fase S (ciclo celular en **Figura 5**). En las células de mamíferos los mecanismos de NHEJ son predominantes frente a los HRR (Shiloh, 2003).

En la mayoría de los casos, o al menos en aquellos en el que el número de roturas iniciales es pequeño, es probable que las DSB se reparen correctamente o den lugar a pequeñas alteraciones del ADN de unos pocos pares de bases o kilobases que pueden ser analizados sólo con un mapa de restricción (diagrama de una molécula de ADN que muestra las posiciones relativas de los sitios de escisión de varias enzimas de restricción) o secuenciación (marcaje del ADN con fluorescencia y sistemas de detección adecuados). En algunos casos, las DSBs pueden conducir a alteraciones visibles a gran escala bajo el microscopio de luz como las ACs. Por lo tanto, las ACs no son fenómenos especiales resultado de actividades específicas celulares, son sólo la parte visible al microscopio de una amplia gama de productos generados por diferentes mecanismos de reparación de las DSB. Respecto a la forma en la que las ACs se pueden observar en un microscopio, depende del tipo de tinción utilizada, desde las técnicas clásicas de tinción uniforme de los cromosomas hasta técnicas más específicas para determinadas alteraciones como la FISH (Obe *et al.,* 2002) (**Figura 7**).

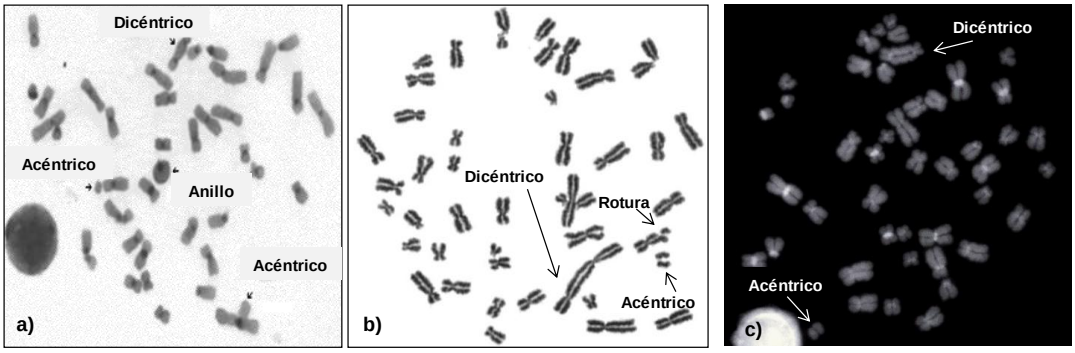

Figura 7. Algunas CAs observadas en el microscopio mediante 3 tipos distintos de tinción: a) tinción de Bandas C; b) tinción uniforme y c) tinción FISH con DAPI (imagen cedida por Barquinero JF).

La clasificación de las ACs puede hacerse de acuerdo a su apariencia o a su método de presunta formación. Resulta lógico pensar que debido a que su identificación y recuento depende de su visualización en el microscopio, la forma más simple de clasificarlas es de acuerdo a su apariencia. Para lograr ver el mayor número de alteraciones posibles y clasificarlas, se procedió a estudiar los acontecimientos que se sucedían en las células tras ser expuestas a radiaciones ionizantes, agentes

clasificados como cancerígenos por la IARC. La terminología adoptada basada en la "descripción" del cromosoma es la más habitual, siendo la clasificación más usada la siguiente (Benison *et al.,* 1986):

- Alteraciones de tipo cromosoma:
  - Delecciones terminales e intersticiales
    - Fragmentos acéntricos (ace) (**Figura 8a**): son alteraciones en las cuales la metafase presenta un fragmento acéntrico. Debido a que es difícil distinguir entre delecciones terminales e intersticiales, se clasifican conjuntamente como fragmentos acéntricos.
    - Rotura de cromosoma (*chromosome strand break*; csb) (**Figura 8b**): Para clasificar una deleción como rotura de cromosoma se debe cumplir que las dos cromátidas del fragmento estén separadas y sigan una dirección diferente a las de las cromátidas del cromosoma roto.

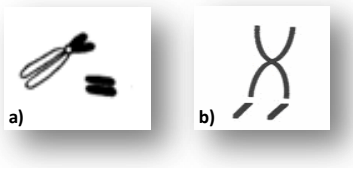

Figura 8. a) fragmento acéntrico; b) rotura de cromosoma.

  - Alteraciones intercromosómicas
    - Intercambios asimétricos: el dicéntrico (dic) (**Figura 9a**). Un dicéntrico se puede producir por la rotura de dos cromosomas y posterior translocación de estos. Un dicéntrico analizado en la primera metafase estará acompañado de uno o dos fragmentos acéntricos. También son intercambios asimétricos los cromosomas tricéntricos y tetracéntricos que se pueden producir por la rotura y posterior reorganización de 3 ó 4 cromosomas respectivamente.
    - Intercambios simétricos: translocaciones recíprocas (others) (**Figura 9b**). Las translocaciones se producen cuando un fragmento de un cromosoma, que no contiene el centrómero, es translocado a otro cromosoma y un fragmento de éste es translocado al primero. Los

intercambios simétricos son particularmente difíciles de observar en preparaciones teñidas uniformemente, a no ser que los intercambios produzcan cromosomas morfológicamente diferentes de los de un cariotipo normal.

- Intercambios asimétricos: inserciones (others) (**Figura 9c**). Las inserciones se producen cuando un fragmento acéntrico de un cromosoma se inserta en un brazo de otro cromosoma. Son particularmente difíciles de observar en preparaciones teñidas uniformemente, a no ser que los intercambios produzcan cromosomas morfológicamente diferentes de los de un cariotipo normal.

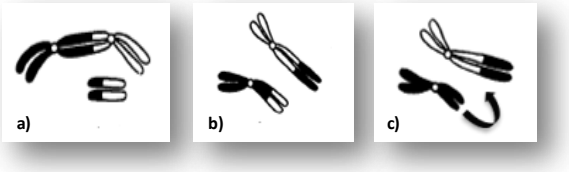

Figura 9. a) dicéntrico con su correspondiente fragmento acéntrico; b) translocación recíproca, c) inserción.

➢ Alteraciones intracromosómicas

- Intracambios asimétricos: anillo + acéntrico (**Figura 10a**). Un anillo se produce al romperse los dos brazos del mismo cromosoma y la posterior unión de estos brazos acompañandose por uno o dos fragmentos acéntricos.

- Intracambios simétricos: inversiones (others) (**Figuras 10b** y **10c**). Se dividen en inversiones pericéntricas y paracéntricas, según se implique la zona centromérica o no, son difíciles de observar en preparaciones teñidas uniformemente.

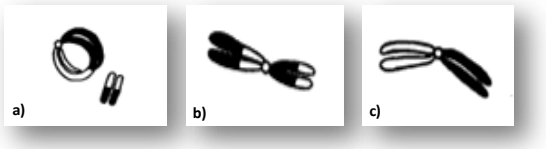

Figura 10. a) anillo con su correspondiente fragmento acéntrico; b) inversión pericéntrica; c) inversión paracéntrica.

- Lesiones acromáticas o "gaps" de cromosoma (*chromosome gap; csg*) (**Figura 11**): estas lesiones son regiones del cromosoma muy poco o nada teñidas presentes en las dos cromátidas hermanas en el mismo nivel. La apariencia bandeada que pueden presentar ciertas metafases hace muy difícil establecer un criterio para diferenciarlas claramente. El criterio utilizado es observar el resto de cromosomas de la metafase: si también presentan una apariencia bandeada, se descarta la anomalía; si, por el contrario, es el cromosoma dudoso el único que presenta una zona poco teñida se contabiliza la alteración.

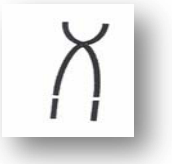

Figura 11. Gap de cromosoma presente en las dos cromátidas.

❖ Alteraciones de tipo cromatídico:

- Lesiones acromáticas o "gaps" de cromátida (*chromatid gap; gap*) (***Figura 11a***): los "gaps" de cromátida son regiones del cromosoma no teñidas o teñidas muy ligeramente, presentes en una cromátida. Se utiliza el mismo criterio que para los csg.
- Rotura de cromátida (*chromatid breaks; ctb*) (**Figura 12b**): Las ctb son el resultado de roturas de una sola cromátida del cromosoma, observándose el fragmento de cromátida asociado al cromosoma. Para diferenciar los ctb de los "gap", el desplazamiento de la región no teñida tiene que ser más ancho que el ancho de una cromátida.
- Radiales (**Figura 12c**): son alteraciones producidas por la rotura de una cromátida de dos cromosomas diferentes y posterior intercambio o intracambio de fragmentos. El intercambio puede ser tanto simétrico como asimétrico. Pueden ser inversiones paracéntricas, si las dos roturas afectan a un mismo brazo cromosómico (no cambia la morfología del cromosoma), o pericéntricas, si cada rotura afecta a un brazo cromosómico (cambia la morfología del cromosoma).

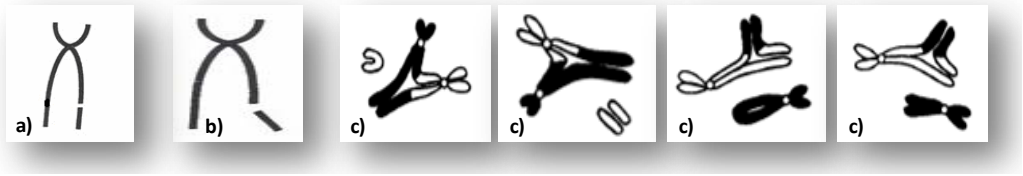

Figura 12. a) Gap de cromátida b) Rotura de cromátida; c) Diferentes formas de cromosomas radiales.

B.2. Marcadores citogenéticos aplicados en biomedicina

B.2.1. Tabaquismo

La genotoxicidad derivada del hábito de fumar tabaco ha sido ampliamente estudiada en las últimas décadas (Khabour *et al.,* 2011). Se sabe que el tabaco posee unos 3800 compuestos y que hay más de 4800 compuestos en el humo de los cigarros de los cuales 60 son carcinógenos para los animales y al menos 10 de ellos lo son también para los humanos (Andreoli *et al.,* 2003), como las aminas aromáticas, las nitrosaminas específicas del tabaco y los hidrocarburos aromáticos policíclicos (HAPs) (Lei *et al.,* 2002). En humanos, fumar provoca mutaciones en los genes y ACs; la mayoría de estos efectos genéticos vistos en fumadores han sido igualmente observados en cultivos de células o en animales de experimentación expuestos al humo del tabaco o al condensado de este humo. Por consiguiente, la IARC declara que el hábito de fumar y el humo del tabaco son cancerígenos para el ser humano (Grupo 1) así como también lo es la exposición involuntaria a los mismos (IARC, 2002). Concretamente en una publicación especial de la IARC se recopilaron aquellos tumores para los cuales la evidencia de causalidad es suficiente y se especificaron la cavidad oral, orofaríngе, nasofaríngе, hipofaríngе, esófago (adenocarcinoma y carcinoma de células escamosas), estómago, colon-recto, hígado, páncreas, cavidad nasal y paranasal, senos nasales, laríngе, pulmón, útero, ovario, vejiga urinaria, riñón, uretra y médula ósea (leucemia mieloide) (Secretan *et al.,* 2009).

En lo que concierne a los estudios citogenéticos realizados para el estudio de la posible genotoxicidad del tabaco, éstos se remontan a la década de los años 70 cuando varios investigadores empezaron a evaluar los efectos adversos que el tabaco y sus principales componentes ejercían en células de mamíferos *in vitro* e *in vivo* (Perry y

Evans 1975; Vogel y Bauknechet 1976; Lambert *et al.,* 1978; Hollander *et al.,* 1978; Murthy *et al.,* 1979).

Las técnicas de citogenética usadas desde el inicio en los años 70 para detectar la posible genotoxicidad del tabaco se basan en la citogenética clásica, siendo el análisis de la frecuencia de SCEs y las CAs unos de los más utilizados. La razón por la cual un gran número de estudios decidieron tomar como referente principalmente el análisis de SCE se debe a que según anunciaron Perry y Evans (1975) y Vogel y Bauknecht (1976) varios químicos, mutágenos y carcinógenos, aumentaban la frecuencia de SCEs en células de mamíferos *in vitro*. Siguiendo este antecedente y apoyándose en el estudio de Rudiger *et al.* (1976) que afirmaba que concentraciones *in vitro* de 1 µM de benzo(a)pireno, un por entonces sospechado pre carcinógeno del humo del tabaco, aumentaban la frecuencia de SCEs al menos el doble del valor control en linfocitos humanos. Lambert *et al.* (1978) estudiaron la frecuencia de SCEs en linfocitos de sangre periférica de sujetos sanos fumadores no expuestos ocupacionalmente a agentes mutágenos y los resultados demostraron que los individuos pertenecientes al grupo de fumadores tuvieron frecuencias de SCEs más altas que los no fumadores. Los autores afirmaron que sus resultados proporcionaban una evidencia de que el tabaco quizás causara un daño que resultara en un aumento en el número de SCEs en linfocitos de sangre periférica en individuos no expuestos ocupacionalmente a mutágenos.

El Instituto Nacional de Seguridad e Higiene del Trabajo del Ministerio de Trabajo y Asuntos Sociales de España publicó la Nota Técnica 354 (NTP 354, 1994) correspondiente a las técnicas citogenéticas utilizadas en el control biológico de la exposición a genotóxicos, y se afirmó en dicho documento que:

1. Los cambios en la estructura de los cromosomas fueron una de las primeras formas conocidas de lesión genética producida por la exposición a agentes físicos o químicos.

2. El control biológico es una metodología especialmente interesante para la evaluación de la exposición a agentes potencialmente cancerígenos (para los que no se conoce la existencia de niveles umbral de seguridad, el

tiempo de latencia entre la exposición, el efecto suele ser muy largo y los efectos finales dependen en gran medida de variaciones interindividuales).

3. La base para la aplicación de las técnicas citogenéticas para el control biológico de exposición a genotóxicos es el principio de que el grado de lesión genética en tejidos no diana (en este caso, linfocitos de sangre periférica), refleja lo que ocurre en las células verdaderamente importantes en el potencial desarrollo de un proceso cancerígeno. De esta forma, los linfocitos de sangre periférica pueden ser contemplados tanto como indicadores de la exposición como del efecto producido en otras células del organismo.

4. Las ACs estructurales son indicadores de exposición a genotóxicos y de efectos mutagénicos. Aunque en sí no constituyen mutaciones porque, normalmente, conducen a la muerte de la célula o de su progenie, pueden originarse cuando los cromosomas rotos se restituyen o intercambian fragmentos entre ellos. Por esto se puede asumir que todos los agentes que producen roturas cromosómicas también inducen mutaciones.

5. Frente a determinados agentes químicos, la inducción de SCEs puede presentar una mayor sensibilidad que las ACs, pudiendo manifestarse efectos genotóxicos a concentraciones hasta 100 veces menores a las necesarias para producir las aberraciones cromosómicas.

6. Se ha observado que la frecuencia de SCEs aumenta cuando las células son expuestas a agentes mutágenos y cancerígenos conocidos.

Por tanto, esta Nota Técnica concluye que la exposición a una variedad de agentes, tanto físicos como químicos, puede resultar en un incremento en la frecuencia de ACs y/o SCEs en linfocitos de sangre periférica de individuos expuestos. Por ello, las técnicas citogenéticas pueden ser usadas para establecer, en una población determinada, el daño derivado de una exposición y para estimar el potencial riesgo para la salud.

B.2.2. Síndrome Ardystil

El llamado "Sindrome de Ardystil" está caracterizado por una enfermedad pulmonar causada por la inhalación de vapores tóxicos y/o sustancias en industrias de aerografía textil que no cumplieron las regulaciones de seguridad y salud laborales de la Comunidad Europea.

Este síndrome fue definido en 1993 a partir de los desórdenes de salud que sufrieron los trabajadores de varias empresas de aerografía textil de la comarca de Alcoi (España), concretamente, el nombre de "Síndrome de Ardystil" se debe a que el primer fallecimiento por este síndrome tuvo lugar en una industria con este nombre. Durante el año 1992, 6 trabajadores fallecieron debido a una insuficiencia pulmonar restrictiva por una neumonía asociada en algunos casos con una fibrosis pulmonar y otro trabajador se sometió a un transplante doble de pulmón (Solé y Cordero, 1995; Turuguel *et al.,* 1996).

Se piensa que aproximadamente 90 trabajadores de la Comunidad Autónoma de Valencia fueron afectados por esta exposición, de los cuales 27 padecieron los síntomas clínicos más severos. Los síntomas más frecuentemente detectados fueron tos, respiración entrecortada y sangrado de nariz. Además de los síntomas respiratorios, 14 pacientes experimentaron dolor abdominal, diarrea o nauseas. Alteraciones cognitivas como la pérdida de memoria y manifestaciones neurológicas menores (dolor de cabeza, parestesias y temblores) fueron detectadas en 18 pacientes entre varios meses y un año después de diagnosticar la enfermedad pulmonar (Solé *et al.,* 1996).

Para detectar los mecanismos de toxicidad de esta pintura y sus principales componentes poliméricos: Acramin FWR, Acramin FWN, Acrafix FHN y Acramoll W, un grupo de investigadores (Hoet *et al.,* 1999) llevo a cabo algunos estudios usando una batería de diferentes tipos de células para evaluar la citotoxicidad *in vitro*. El estudio mostró que, al igual que en los estudios *in vivo*, los tres componentes policatiónicos de la pintura, Acramin FWR (una poliurea), Acramin FWN (una poliamida-amina) y Acrafix FHN (una poliamina) exhibieron una citotoxicidad considerable (DL_{50} por debajo de 100 µg/ml en incubación de 20 a 24h) *in vitro*, mientras que Acramoll W no fue tóxico al menos para las concentraciones probadas. La citotoxicidad fue comparable en cultivos

primarios de rata y en pneumocitos humanos de tipo II, en macrófagos alveolares, en la línea celular pulmonar A549 y en las células hepáticas HepG2. En los eritrocitos humanos, la toxicidad fue menos pronunciada.

En España, las investigaciones llevadas a cabo por el Ministerio de Salud Pública establecieron que la enfermedad pulmonar fue causada por técnicas de spray que emitían un aerosol respirable de Acramin FWN (obtenida de la reacción entre una dietilenetriamina y un ácido adípico). Los datos clínicos sugirieron que el mecanismo del daño en el pulmón fue por toxicidad directa, con una pequeña evidencia en el sistema inflamatorio, en la fase aguda de la respuesta, en la sangre periférica o anormalidades extra pulmonares en la necropsia (Solé *et al.,* 1996) (**Figura 13**).

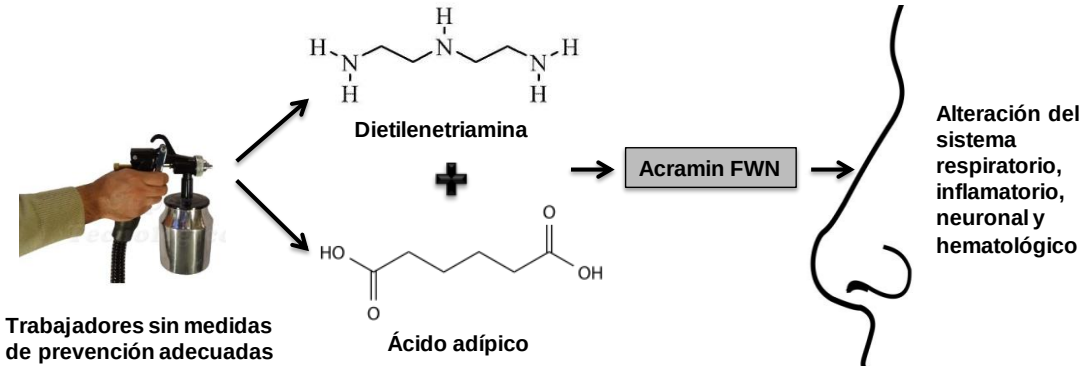

Figura 13. Representación de la formación de la Acramin FWN.

Esta enfermedad con una tendencia a evolucionar hacia la fibrosis intersticial progresiva a pesar del uso de corticosteroides (Cordero y Solé, 1995) provocó, en 1993, que el Gobierno español añadiera el "síndrome Ardystil" como una nueva enfermedad profesional y permitió la aplicación, en 1995, de la Ley 31/1995 de Prevención de Riesgos Laborales, que se basaba en la Directiva marco (89/391/CEE) de 1989 a cerca de la salud y la seguridad en la Unión Europea (Cortés, 1994). En el Artículo 22 de dicha ley, concretamente en el punto e) se concreta que, del Artículo 37.3 RD 39/1997, de 17 de enero, Reglamento de los Servicios de Prevención, se establece: “En los supuestos en que la naturaleza de los riesgos inherentes al trabajo lo haga necesario, el derecho de los trabajadores a la vigilancia periódica de su estado de salud deberá ser prolongado más allá de la finalización de la relación laboral a través del Sistema Nacional de Salud”, y en el Artículo 37.3 c) además de que: “La vigilancia de la salud

estará sometida a protocolos específicos u otros medios existentes con respecto a los factores de riego a los que esté expuesto el trabajador". En base a este Artículo 22, el Servicio de Salud Laboral de la Dirección General de Salud Pública elaboró una propuesta de plan de actuación específico destinado a optimizar las actuaciones sanitarias relacionadas con el colectivo de trabajadores del "síndrome Ardystil". A partir de dicha propuesta, la Administración Sanitaria Valenciana se propuso establecer una serie de actuaciones coordinadas para mejorar aspectos desde el punto de vista sanitario para este colectivo de trabajadores, profundizando en la vigilancia de su salud y tratando este tema como una actividad de medicina preventiva, paralela a la asistencial en curso. Con dicho plan se pretendía detectar todos los posibles daños a la salud derivados de la exposición a tóxicos laborales, especialmente disolventes y colorantes textiles de personas que estuvieron expuestas. Así pues, el Plan para la Vigilancia de la salud post-ocupacional de los trabajadores que estuvieron expuestos a la aerografía textil debía de incluir la realización de diversas pruebas médicas con el fin de obtener la mejor y más completa información de la situación de salud individual. La evaluación de la situación de los trabajadores en el momento de realizar el Plan de vigilancia se realizó a partir de un protocolo determinado por un grupo de expertos, teniendo en cuenta los posibles problemas de salud que deberían de identificarse. En el protocolo se evaluaron un amplio listado de problemas de salud (insuficiencia respiratoria, alteraciones serie roja y blanca, alteración emocional, nefropatía, osteoporosis, etc.) mediante la realización de distintos procedimientos (espirometría forzada, hemograma completo, miniexamen cognitivo, proteinuria, SCEs, contenido mineral óseo, etc.).

B.*2.3. Cito-y genotoxicidad de compuestos polifenólicos*

El concepto de **compuesto fenólico** engloba una gran cantidad de compuestos orgánicos aromáticos, derivados del benceno, con grupos hidroxilo como sustituyentes. El compuesto base es el fenol pero la mayoría son polifenólicos. La mayoría de los compuestos fenólicos provienen de las plantas. De hecho, son uno de los grupos fitoquímicos más grande y más ubicuo ya que se encuentran en la gran mayoría de plantas, frutas y vegetales de la dieta. En la naturaleza son formados en las plantas para protegerlas del estrés fotosintético y de las especies reactivas del oxígeno

actuando también como señales químicas en la polinización de las plantas y en los procesos de simbiosis y parasitismo (Gil, 2002; Lambert *et al.,* 2005).

La formación de los compuestos fenólicos se realiza a partir de metabolitos primarios a través de dos rutas: la vía del ácido acético que comienza con la acetil-CoA y la vía del ácido sikímico con origen en los carbohidratos (Bruneton 2001). Algunos compuestos proceden de ambas vías, por lo que su clasificación no resulta sencilla. Una clasificación resumida sería la siguiente:

❖ Vía del ácido acético:

- Quinonas: antraquinonas, naftoquinonas, macrólidos (**Figura 14**).

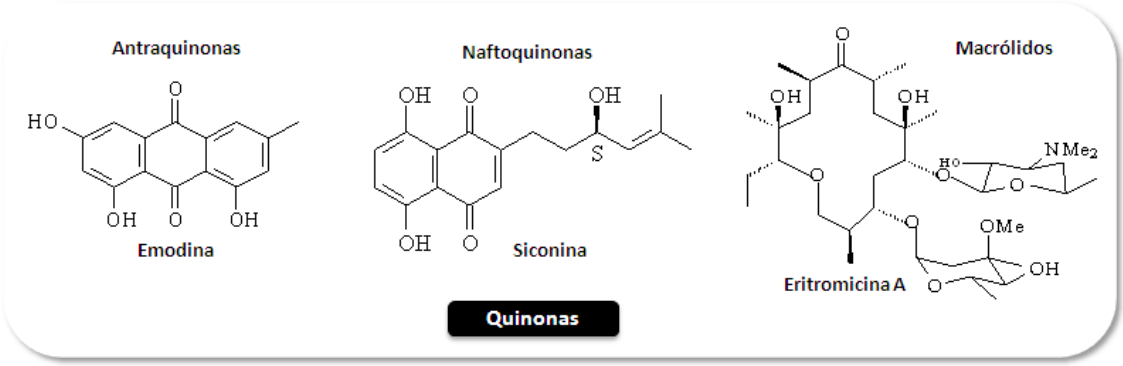

Figura 14. Principales ejemplos de compuestos fenólicos procedentes de la vía del ácido acético.

❖ Vía del ácido sikímico:

- Fenoles y ácidos fenólicos: fenoles simples, ácidos fenólicos derivados del ácido benzoico y del ácido cinámico, ésteres heterósidos fenilpropanoicos (**Figura 15**).
- Cumarinas: umbeliferona, herniarina, esculetol, fraxetol, etc.
- Xantonas: bellidifolina, mangostina, etc.
- Estilpironas: kavaína, yangonina, etc.
- Lignanos y neolignanos: pinorresinol, matairresinol, secoisolaricirresinol, etc.
- Estilbenoides: *trans*-resveratrol.
- Diarilheptanoides y arilalcanonas: curcumina, zingibereno, etc.
- Flavonoides: flavonoles, flavanolas, flavanoles, antocianidinas, etc.

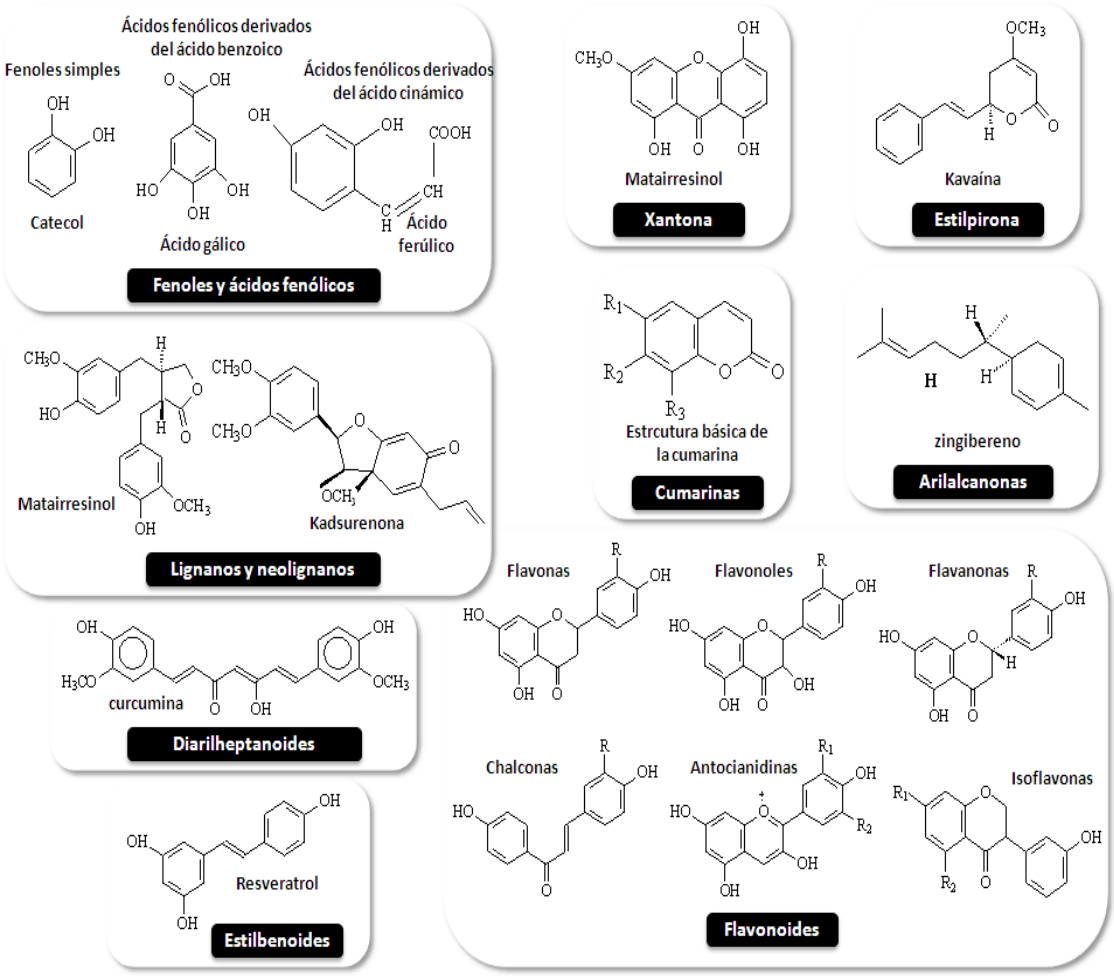

Figura 15. Ejemplos y estructuras básicas de los compuestos fenólicos procedentes de la vía del ácido sikímico.

Los compuesto fenólicos han demostrado su capacidad para secuestrar radicales libres, regular el óxido nítrico, inducir apoptosis, inhibir la proliferación celular y la angiogénesis, etc., por todo ello se les atribuyen propiedades antioxidantes, anticancerígenas y antiinflamatorias (Arts y Hollman, 2005; Lamber *et al.,* 2005).

Entre estas propiedades terapéuticas, la posibilidad de actuar como anticancerígeno reside, entre muchas otras, en la capacidad prooxidante de los polifenoles a la hora de inducir la apoptosis de las células cancerígenas (Hadi *et al.,* 2007). Sin embargo, esta acción prooxidante se debería considerar también en las células normales o no cancerígenas. De hecho, son varios los estudios que han observado que otros componentes de origen natural con propiedades beneficiosas por sus efectos antioxidantes, como los flavonoides o el ácido tánico, también pueden comportarse como prooxidantes en distintos tipos de células y ser capaces de generar

radicales del oxígeno en presencia o no de iones metálicos (Ahsan *et al.,* 1999; Perron *et al.,* 2011).

Además de poder actuar como prooxidantes tanto en células cancerígenas como normales, los flavonoides y otros compuestos polifenólicos han demostrado ser capaces de inhibir enzimas encargadas de mantener la estructura del ADN a través de la inhibición o disminución de su actividad y de la estabilización de complejos formados con la molécula de ADN. Además se ha observado que las células son muy sensibles a este tipo de daño relacionado con los enzimas estabilizadores de la estructura del ADN. Esta propiedad conduce a que los compuestos polifenólicos puedan actuar como agentes clastogénticos causando roturas dobles de cadenas que podrían conducir a la formación de aberraciones cromosómicas (Webb and Ebeler, 2004).

Este conjunto de estudios ponen de manifiesto que las actividades antioxidantes/prooxidantes o protectoras/destructoras de la estabilidad del material genético de muchos compuestos naturales aún no estan claramente definidas (Cemeli *et al.,* 2009).

Por este motivo, antes de apuntar que los compuestos naturales, entre ellos los polifenoles, pueden tener efectos beneficiosos es necesario evaluar sus efectos tóxicos debidos a otro tipo de propiedades como las prooxidantes o el efecto sobre determinados enzimas implicados en la estabilidad de la molécula de ADN. El objetivo de la presente tesis contempla dos compuestos fenólicos: la curcumina y el *trans*-resveratrol.

La **curcumina** (1,7-bis (4-hidroxi-3-metoxifenil)-1,6-heptadieno-3,5-diona) es un compuesto fenólico de bajo peso molecular perteneciente al grupo de los diarilheptanoides. Se incluye en el grupo de los curcuminoides presentes en la raíz de la especie *Curcuma* spp. (**Figura 16**), siendo la planta *Curcuma longa* la más conocida (comúnmente llamada cúrcuma). La fracción de color amarillo de la cúrcuma contiene los curcuminoides (3-5%) relacionados químicamente con su principal compuesto, la curcumina. Los principales curcuminoides presentes en la cúrcuma son la demetoxicurcumina (curcumina II), bisdemetoxicurcumina (curcumina III) y ciclocurcumina. Si consideramos las preparaciones comerciales de curcumina, estas contienen aproximadamente un 77% de curcumina, un 17% de curcumina II y un 3% de

curcumina III. La mezcla de estos curcuminoides es llamada de distintas formas como azafrán de la India, jengibre amarillo, raíz amarilla, o amarillo natural 3. Aunque su cultivo se puede extender por todo el planeta, las zonas principales son la India, el Sudeste de China y otras regiones o países tropicales (Goel *et al.,* 2007).

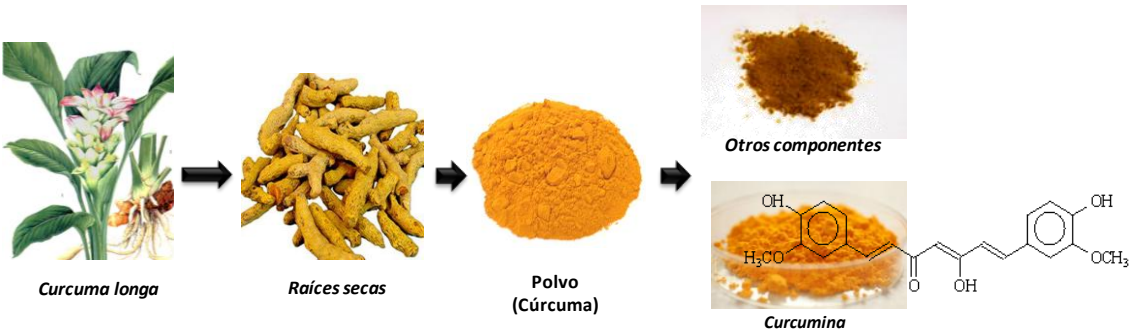

Figura 16. Esquema de la obtención de la curcumina desde la *Curcuma longa.*

La estructura de la curcumina se caracterizó en 1910 y es considerada como el componente más activo de la especie. Químicamente, es considerada una β-dicetona bis-α,β -insaturada la cual actúa como un potente donador de átomos de hidrógeno entre un pH de 3-7 y como donador de electrones a pH superior de 8 (Sharma *et al.,* 2005). Su elevada lipofilia permite una rápida absorción gastrointestinal por difusión pasiva; tras la administración de curcumina ésta se biotransforma primero a dihidrocurcumina y tetrahidrocurcumina, y estos compuestos son convertidos, posteriormente, a conjugados monoglucurónidos, de forma que los principales metabolitos de la curcumina *in vivo* son los glucurónidos de curcumina, de dihidrocurcumina y tetrahidrocurcumina (Mesa *et al.,* 2000).

Tradicionalmente, las preparaciones de cúrcuma se han utilizado en cosmética, como ingredientes alimentarios principalmente como especia para dar color y sabor (forma parte del curry) y como colorante para quesos, mantequillas y otros alimentos. El interés en la especie de la *Curcuma* spp. en medicina tradicional se mantiene desde hace miles de años, tanto, que es descrita en la medicina ayurvédica, el conocido sistema médico holístico asiático basado en compuestos naturales, como agente anti-inflamatorio. Aunque la planta es conocida desde hace tiempo y su uso más común es el de especia alimentaria, el interés por el uso del principio activo de la curcumina en medicina ha empezado a crecer en las últimas décadas. Su estudio se ha extendido de

forma que cientos de investigaciones científicas se han publicado en los últimos treinta años, estudiando sus propiedades antioxidantes, antiinflamatorias, anticancerígenas y quimioterapéuticas. Estas propiedades se deben principalmente a que la curcumina posee un gran cantidad de dianas moleculares como los factores de transcripción, factores de crecimiento y sus receptores, proteínas, citoquinas, enzimas o genes reguladores de la proliferación celular y de la apoptosis (Goel *et al.,* 2007).

Como contraposición a todas estas propiedades beneficiosas, algunos estudios sugieren que la curcumina, entre otros polifenoles, es capaz de mostrar también propiedades genotóxicas. Esta cualidad se debería tal vez a la posibilidad de ejercer actividad anti y prooxidante en diferentes tipos de células. De hecho, la apoptosis mediada por la curcumina en células cancerígenas está íntimamente relacionada con el aumento intracelular de especies reactivas del oxígeno (ROS) (Banerjee *et al.,* 2008). A modo de ejemplo, la curcumina provocó estrés oxidativo a altas dosis dañando el ADN en un estudio en el que se utilizaban células de leucemia HL60 (Yosihno *et al.,* 2004) y en otro trabajo en el que se aplicaba a células de hepatocarcinoma celular HepG2 (Cao *et al.,* 2006).

La toxicidad celular, atribuida a la viabilidad celular, de la curcumina también se ha observado en líneas celulares no cancerígenas; unos estudios observaron que la curcumina reducía la viabilidad de células de ovario de hámster (*del inglés Cell Hamster Ovary*; CHO) o células de osteoblasto humano (Mendonça *et al.,* 2009). Aunque estudios en individuos humanos han demostrado que la curcumina resultó segura y las reacciones graves aparecieron a partir de una dosis de hasta 12g/día (Anand *et al.,* 2007), su doble acción anti y prooxidante en estudios *in vitro* sobre determinadas líneas celulares hacen necesario el estudio de su posible efecto genotóxico.

El ***trans*-resveratrol** (3,5,4'-trihidroxiestilbeno) es una forma isomérica del resveratrol, un compuesto fenólico que pertenece al grupo de los estilbenos. El resveratrol fue descubierto por primera vez en 1940, en la planta del eléboro. Se sintetiza en las plantas y microorganismos pero no en animales. La fuente más rica de resveratrol es la raíz de *Polygonum cuspidatum* (Ko-jo-kon), cultivada principalmente en China y Japón (Catalgol *et al.,* 2012). Una de las fuentes más importantes para los humanos es la uva; de hecho su piel puede contener alrededor de 50-100 mg de

resveratrol, llegando a pasar al vino una cantidad aproximada de 0.2-7 mg/L (Marques *et al.,* 2009) (**Figura 17**).

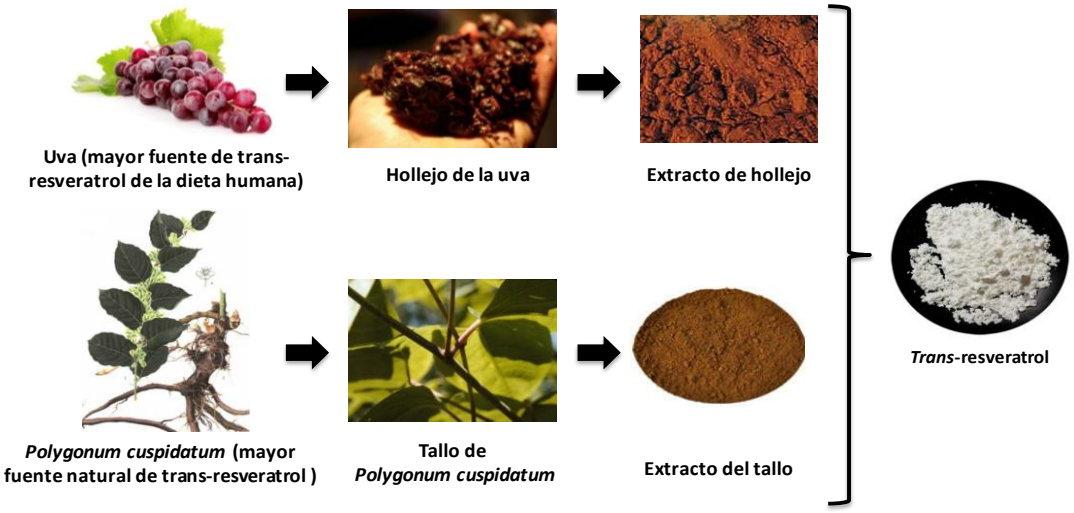

Figura 17. Esquema de la obtención del *trans*-resveratrol desde la uva y el *Polygonum cuspidatum.*

Además de la uva, una gran variedad de frutas como la mora, arándano rojo y azul, fresa, sandía o cacahuete, y una amplia variedad de flores y hojas, incluyendo el lirio, alguna orquídea, eucalipto, abeto o pino silvestre, también contienen resveratrol. Su nombre deriva del hecho de que este polifenol está presente en gran cantidad de plantas de la especie *Veratrum* (Lançon *et al.,* 2007). Su producción por parte de las plantas se aumenta en respuesta a problemas microbianos o a otros estímulos externos como el ataque de patógenos, radiación ultravioleta o heridas. Normalmente, el resveratrol es el estilbeno más común de las bayas y se presenta en dos formas isoméricas: *trans*- y *cis*-resveratrol (Signorelli and Ghidoni, 2005). Además de las fuentes naturales se produce también mediante síntesis química y biotecnológica y se comercializa como suplemento nutricional mediante la extracción de la planta japonesa conocida por el público como planta de Itadori (*Polygonum cuspidatum)* (Catalgol *et al.,* 2012).

El principal problema de este compuesto es su baja biodisponibilidad y rápido aclaramiento que experimenta en la circulación sanguínea. Estudios humanos y animales revelan que el resveratrol es principalmente metabolizado en conjugados glucurónidos y sulfatos (Delmas *et al.,* 2011).

El *trans*-resveratrol, como muchos otros compuestos fenólicos, se considera como un componente de la dieta "preventivo" (Delmas *et al.,* 2011). El interés de la comunidad científica por este polifenol aumentó hace unos años a raíz de unos estudios que indicaban una relación inversa entre el consumo moderado de vino y la enfermedad coronoria en la población francesa, la llamada "Paradoja francesa". Este término se acuñó en 1992 y se basó en estudios epidemiológicos de la población francesa la cual presentaba una baja incidencia de enfermedades coronarias a pesar de consumir una dieta rica en grasas saturadas. Después de algunas observaciones, fueron Renaud y de Lorgeril quienes en ese mismo año propusieron que el consumo moderado de vino explicaba esta aparente contradicción y sugirieron que la disminución de la agregación plaquetaria podría ser el principal factor de su efecto en la enfermedad coronaria. Una investigación llevada a cabo por la OMS en 2009 observó que la mortalidad asociada a la enfermedad coronaria era tres veces menor en Francia que en otros países como Estados Unidos, Reino Unido o Suecia. Y adicionalmente, un estudio en Copenhague con 6051 hombres y 7234 mujeres de edades entre 30 y 70 años demostró que el consumo bajo-moderado de vino se asociaba a una menor mortalidad por enfermedades cardiovasculares y cerebrovasculares (Catalgol *et al.,* 2012).

A partir de ese momento los estudios científicos aumentaron considerablemente y una gran cantidad de beneficios para la salud han sido atribuidos a esta molécula; anti-carcinogénica, antiinflammatoria, antidiabética y neuroprotectora (Stervbo *et al.,* 2004; Bisht *et al.,* 2010; Chakraborty *et al.,* 2010). Estos beneficios se atribuyen a su gran número de actividades biológicas, como la inhibición de la peroxidación lipídica, quelante del cobre, secuestrador de radicales libres, alteración de la síntesis de eicosanoides, inhibición de la agregación plaquetaria, actividad vaso-relajantes, modulación del metabolismo lipídico, etc. (Frémont *et al.,* 2000).

Respecto a las propiedades anticancerígenas del *trans*-resveratrol, éstas se deben en gran parte a su capacidad para inhibir el crecimiento de varias líneas de células cancerígenas humanas y a estudios con modelos de carcinogénesis animal (Joe *et al.,* 2002). Al igual que la curcumina, cabe pensar que esta propiedad anticancerígena se debe a sus efectos cito- y genotóxicos en las células.

Existen varios estudios enfocados a la afectación de la viabilidad celular por parte del *trans*-resveratrol. Trabajos como el de Surgh et al. (1999) ya observaban la inducción de la apoptosis por parte de este polifenol y analizaron la causa por la que el *trans*-resveratrol disminuía la viabilidad de células de leucemía humanas HL-60 de forma dosis-dependiente; los análisis mostraron núcleos rotos en fragmentos de cromatina de varios tamaños, células hinchadas y pérdida de material cromosómico. En otro trabajo, la inducción de la apoptosis también en células leucémicas fue debida a la inducción de la actividad de enzimas caspasas (mediadores esenciales de los procesos de apoptosis) (Chen and Wang, 2002) y a la detención del ciclo celular en fase G2/M. En el trabajo con células cancerígenas de Joe et al. (2002) el *trans*-resveratrol también ejerció actividades antiproliferativas en células de ratón epidérmicas preneoplásicas JB6, células colorectales CaCo-2 y células de carcinoma epidérmico A431. Además, en este mismo estudio, se consideró no sólo las células cancerígenas sino también las normales y se observó que el *trans*-resveratrol disminuía el crecimiento de los linfocitos humanos normales circulantes en sangre periférica (Ferry-Dumazet *et al.,* 2002).

Además de estudios centrados en la apoptosis y viabilidad celular, otros autores indican que el *trans*-resveratrol es capaz de inducir CAs y SCEs en células de pulmón de hámster chino (*del ingés Cell Hamster Lung*; CHL) (Matsuoka *et al.,* 2001; 2002; 2004), hechos que sugieren la posible genotoxicidad de este polifenol.

Debido a los efectos opuestos observados que ponen en una balanza los beneficios e inconvenientes del uso terapéutico del *trans*-resveratrol y de la curcumina, y a la incertidumbre general que existe en torno a la actividad de los compuestos fenólicos, resulta fundamental realizar ensayos de cito- y genotoxicidad relacionados con el objetivo concreto del estudio.

Como se ha mencionado anteriormente, los biomarcadores citogenéticos centrados en alteraciones del número o de la forma de los cromosomas de linfocitos de sangre periférica humana han sido usados ampliamente en la vigilancia de la exposición de los individuos a agentes genotóxicos u otras moléculas y/o en la evaluación de los efectos que estos agentes pueden tener a corto o largo plazo, debido

a su capacidad de provocar daño cromosómico. Entre los biomarcadores citogenéticos más utilizados se encuentran:

- Las ACs: se han usado como biomarcadores en una gran cantidad de estudios, tanto *in vivo* como *in vitro,* de la evaluación de efectos mutagénicos de diferentes químicos (Bonassi *et al.,* 2008) (*Ver sección B.1.2.2*).

- Los SCEs: son un parámetro altamente sensible para la evaluación ocupacional y medioambiental de exposición a agentes carcinogénicos y mutagénicos (Norppa *et al.,* 2006) (*Ver sección B.1.2.1*.).

Además de la evaluación de la genotoxicidad, cuando se pretende introducir nuevos compuestos sintéticos o drogas para fines terapéuticos es necesaria la evaluación de su citotoxicidad. Normalmente, en el estudio de la actividad antitumoral de compuestos sintéticos o naturales, el cribado preclínico se lleva a cabo evaluando un único parámetro, la muerte celular. Además de este tipo de marcador, resulta útil emplear otro tipo de marcadores para obtener información acerca de cómo influyen estos compuestos en la cinética del ciclo celular. Concretamente, para monitorizar la evaluación de la proliferación de células tumorales o células normales, como los linfocitos de una población. Dos biomarcadores son comúnmente utilizados, la cinética de proliferación celular medida mediante el índice mitótico y el índice de proliferación (Hatzi *et al.,* 2007; Gomes and Colquhoun, 2012; Montoro *et al.,* 2012; Masood and Malik, 2012; Toydemir *et al.,* 2013).

- El índice mitótico: permite cuantificar la relación que existe entre el número de células que se hallan en estado de mitosis respecto a un total de células que pueden estar en cualquier otra fase de su ciclo celular (interfase) (**Figura 18**). Una inhibición del índice mitótico se puede interpretar en términos de muerte o de detención del ciclo celular en algún momento durante la interfase (Rojas *et al.,* 1993).

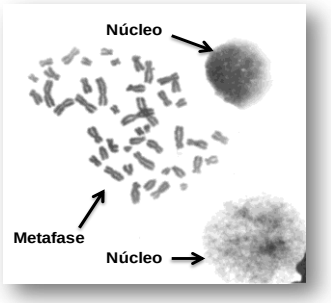

Figura 18. Ejemplo de células para el cálculo de IM: de un total de tres células, una se encuentra en metafase (por tanto en mitosis) y las otras dos no (en cualquier otra fase del ciclo celular).

- El índice de proliferación: permite cuantificar la relación que existe entre células que se encuentren en su primera, segunda o tercera división (**Figura 19**). Este índice resulta útil para diferenciar compuestos que tienen un efecto citostático por detención de alguna fase del ciclo celular (Rojas *et al.,* 1993).

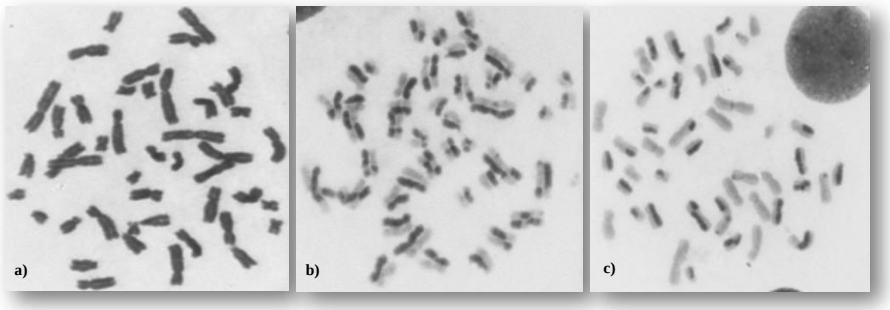

Figura 19. Imagen con 3 divisones consecutivas de un linfocito teñido con la tinción de Fluorescencia más Giemsa. En la imagen se observa a) linfocito en primera divisón; b) linfocito en segunda división y c) linfocito en tercera división.

B.2.4. Radioprotección con compuestos polifenólicos

B.2.4.1. La radiación y sus efectos

Llamamos radiación a la energía que se propaga en forma de onda a través del espacio. Dentro de la radiación encontramos un amplio rango de energías diferentes que forman el conocido espectro electromagnético en el que se ordenan el conjunto de ondas electromagnéticas según su distribución energética. Habitualmente la radiación se clasifica en radiación ionizante (rayos X, rayos γ y luz ultravioleta de alta energía) y no ionizante (luz visible, luz ultravioleta, microondas, rayos infrarrojos y ondas de radio). Los usos de la radiación ionizante, al contrario que las no ionizantes,

se alejan más de las actividades de la vida cotidiana, y se utilizan para procesos específicos y complejos como son la producción de energía eléctrica, medicina (radioterapia contra células tumorales, el diagnóstico médico, intervencionismo, etc.), la elaboración de ciertos productos alimentarios, etc. La población está expuesta a diversas formas de radiación ionizante, sin embargo, cabe mencionar que la exposición con propósitos médicos es la mayor fuente de radiación artificial que recibe el ser humano. Todos estos avances y nuevas aplicaciones de las radiaciones ionizantes suponen ventajas para la sociedad pero se deben tener en cuenta posibles efectos perjudiciales como es el posible daño que la radiación ionizante puede provocar en los organismos vivos (CSN, 2013).

Cuando la radiación ionizante incide en la materia viva produce un daño sobre las células el cual puede ser producido a través de procesos directos o indirectos (Hall and Giaccia, 2012).

- *Efecto directo*: tiene lugar cuando la interacción de la radiación se produce con una macromolécula biológica alterando estructuras atómicas y produciendo cambios biológicos, químicos y físicos. Cuando la radiación incide en la molécula, ésta se ioniza, se libera un electrón y se generan radicales libres que posteriormente continúan reaccionando entre ellos y con otras moléculas. Aunque el efecto directo de la radiación más estudiado es la interacción con el ADN, otras biomoléculas también pueden ser dañadas por la trayectoria de la radiación. Un ejemplo de efecto directo sería una ruptura en la doble hélice de ADN causada por la ionización de la propia molécula tras recibir la radiación ionizante (**Figura 20**). Estudios biofísicos han observado que el daño en la doble hélice de ADN puede producirse en forma de ionizaciones en grupo localizadas con un sola trayectoria de un electrón (radiación de baja transferencia lineal de energía) o en forma de muchas ionizaciones extendidas por la hélice (radiación de alta transferencia de energía), dando lugar a varios tipos de lesiones en el ADN.

- *Efecto indirecto*: está producido por un conjunto de reacciones químicas entre las células y radicales libres u otros productos generados por la propia radiación. El efecto indirecto viene pues causado por la interacción de la

radiación con las moléculas del organismo, principalmente las de agua, ya que las células de los mamíferos están constituidas por un 70-85% de agua. Debido a la abundante presencia de agua en nuestro organismo, el efecto indirecto predomina sobre el directo. Por ejemplo, se estima que la rotura del ADN se produce en un 40% debido a interacciones directas y en un 60% debido a los procesos indirectos (**Figura 20**).

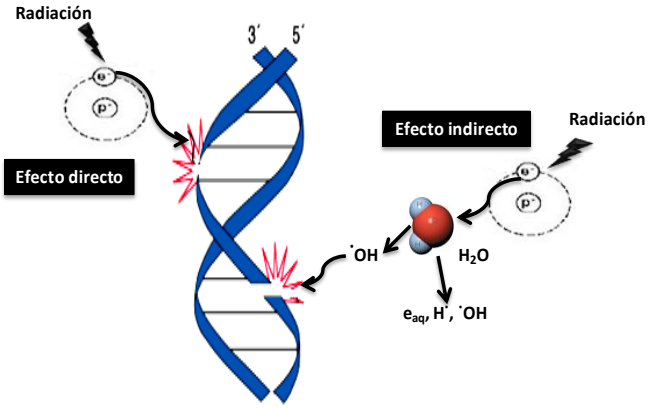

Figura 20. Representación del efecto directo e indirecto de la radiación ionizante sobre el ADN.

Los fenómenos indirectos más importantes de la radiación ionizante son la radiólisis del agua y sus productos resultantes así como la generación de especies reactivas del oxígeno (ROS) y del nitrógeno (RNS) por la enzima óxido nítrico sintasa. Estas reacciones se llevan a cabo en cuatro fases temporales (física, pre-química o físico-química, química y biológica) dentro de una determinada escala de tiempo. En la **Figura 21** se muestran algunas de las reacciones de radiólisis del agua además de los productos más importantes de éstas que producirán los efectos sobre las células en etapas posteriores.

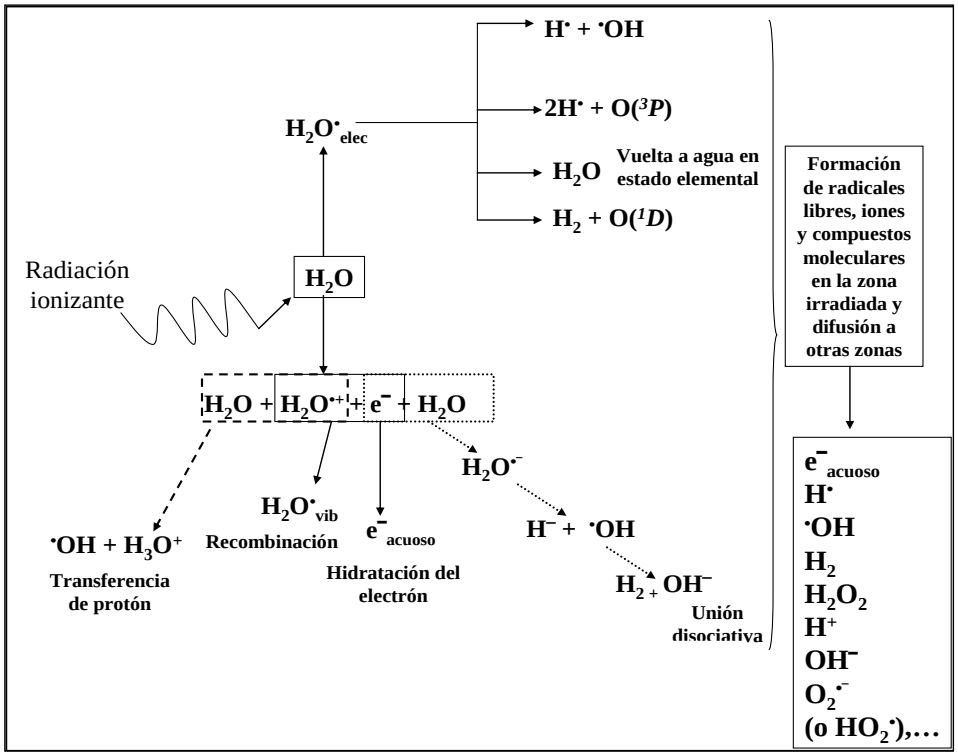

Figura 21. Reacciones más frecuentes de radiólisis y sus productos más importantes.

Una vez producidos los productos de la radiación, los daños biológicos ocasionados pueden ser somáticos (en el individuo) o hereditarios (en la descendencia), deterministas (no estocásticos) o estocásticos (al azar), o producirse sobre moléculas diana o sobre células y componentes que no funcionan como diana por mecanismos intercelulares (Azzam *et al.,* 2012). Tras realizarse este daño biológico, se suceden una serie de mecanismos bioquímicos y moleculares de señalización que culminan en la reparación del daño causado, o bien pueden producirse cambios fisiológicos permanentes e incluso muerte celular. Entre las moléculas y estructuras biológicas dañadas por la radiación se pueden destacar las proteínas (desnaturalización), las membranas biológicas (peroxidación lipídica) y los ácidos nucleicos (ADN) cuyo daño resulta determinante en los efectos biológicos de la radiación ionizante.

B.2.4.2. La radioprotección en medicina

Algunos de los campos de aplicación de la radiación ionizante es la radioterapia, el radiodiagnóstico, la medicina nuclear y la investigación biomédica. En estos procedimientos tanto los pacientes como los trabajadores son expuestos a diferentes

tipos y dosis de radiación (Arora *et al.,* 2008). En el caso de la radioterapia por ejemplo, es frecuente su uso como un componente de la terapia contra un gran número de enfermedades malignas como el cáncer (Citrin *et al.,* 2010). El propósito de la radioterapia en el caso del cáncer es destruir las células cancerígenas causando el mínimo daño posible a las células normales. Siempre se intenta dar la dosis necesaria para dañar al máximo las células tumorales sin perjudicar a las no tumorales, sin embargo, se suele causar cierto daño a las células normales y, a partir de ahí, pueden derivar los efectos colaterales adversos. La naturaleza y el grado de ese daño indeseado depende de la dosis de la radiación ionizante y de la sensibilidad de los órganos irradiados (Hosseinimehr, 2007). Para minimizar el daño producido, se busca mejorar el índice terapéutico por diferentes vías pero pese a los esfuerzos un pequeño volumen de células no tumorales resulta afectado por la radiación. También, en el caso del personal dedicado al radiodiagnóstico, se dispone de toda una serie de medidas preventivas (EPIs: delantales plomados, guantes, etc.) para evitar al máximo la exposición directa a las radiaciones ionizantes. Pese a todos estos esfuerzos es un hecho ya conocido que las radiaciones ionizantes al interaccionar con la materia viva provocan un efecto adverso.

Por esta razón, cobra especial importancia la utilización de sustancias radioprotectoras para la protección de los tejidos sanos, de modo que se disminuyan los efectos secundarios producidos por la radiación. Los radioprotectores se engloban dentro de los compuestos llamados radiomoduladores, es decir, que modifican la respuesta a la radiación en los sistemas biológicos e incluyen compuestos químicos y naturales (Arora *et al.,* 2008). El concepto de **radioprotector** se podría definir como agente químico o droga que reduce el efecto dañino de la radiación, cuando se administra a organismos vivos (Arora, 2006). Pero las definiciones de radioprotectores han ido cambiando con el tiempo según el ámbito clínico donde se pretendían utilizar o por otras diversas razones.

La primera tentativa acerca del uso de compuestos químicos como radioprotectores con el fin de proteger de los efectos nocivos de la radiación fue llevada a cabo después de la Segunda Guerra Mundial ya que se pensó en la posible necesidad de salvaguardar a los humanos del uso militar de las radiaciones ionizantes

(Jagetia, 2007). El grupo de Patt *et al.* (1949) fue el primero en investigar el efecto del aminoácido cisteína en ratas expuestas a dosis letales de rayos X. De esta forma, en los últimos 60 años, como resultado de la gran necesidad clínica de agentes radioprotectores efectivos, muchos han sido sintetizados y estudiados para encontrar el más efectivo a la vez que menos tóxico (Hosseinimehr, 2007). Para ser útiles en la radioterapia clínica, los radioprotectores deberían poseer diferentes características ideales relacionadas con su habilidad para mejorar el ratio terapéutico. Primero, deben de ser selectivos y proteger a los tejidos normales de la radiación sin proteger al tejido tumoral, de otra forma el índice terapéutico no mejorará. Segundo, estos compuestos deben proteger a los tejidos normales a los que se les considere sensibles de desarrollar una toxicidad a corto o largo plazo que comprometa una reducción en la calidad de vida (mucositis, pneumonitis, mielopatía, xerostomía, proctitis y leucoencefalopatía) (Citrin *et al.,* 2010). Se puede definir un decálogo del radioprotector ideal que contendría las siguientes características:

1. Protección significativa contra los efectos de la radiación.
2. Amplio espectro de protección sobre tejidos sanos y órganos.
3. Disposición en una vía de administración sencilla, si es posible no invasiva.
4. Adecuado perfil de estabilidad de la masa activa y del compuesto final.
5. Compatible con el amplio rango de otros fármacos y alimentos/nutrientes que puedan estar utilizando los pacientes o personal sometido a radiaciones.
6. Debe poseer un perfil de toxicidad aceptable.
7. No debe poseer toxicidad propia.
8. Largo tiempo de protección.
9. Si es utilizado para terapia antitumoral, debe preservar su eficacia, permitiendo que no se observen efectos positivos sobre la masa tumoral.
10. Relación coste/efectividad razonable que permita su uso en la medicina clínica.

En general, aquellos agentes biológicos o químicos utilizados para modificar la toxicidad que provoca la radiación sobre los tejidos pueden clasificarse en tres categorías en función del momento en el que se proporcionan en relación a la emisión

de la radiación: radioprotectores químicos, mitigadores y de tratamiento de los efectos secundarios. Se denominan **radioprotectores** a aquellos agentes proporcionados antes o en el momento de la irradiación con la intención de prevenir o reducir el daño a los tejidos normales. Aquellos que se administran en el momento de la irradiación o después del fin de la misma, pero siempre antes de que se manifieste cualquier toxicidad en el tejido normal, se describen como **mitigadores** de las lesiones de los tejidos normales. Finalmente, los agentes facilitados para mejorar la toxicidad ya manifiesta en el tejido normal se consideran **agentes de tratamiento** (Stone *et al.,* 2004) (**Figura 22**).

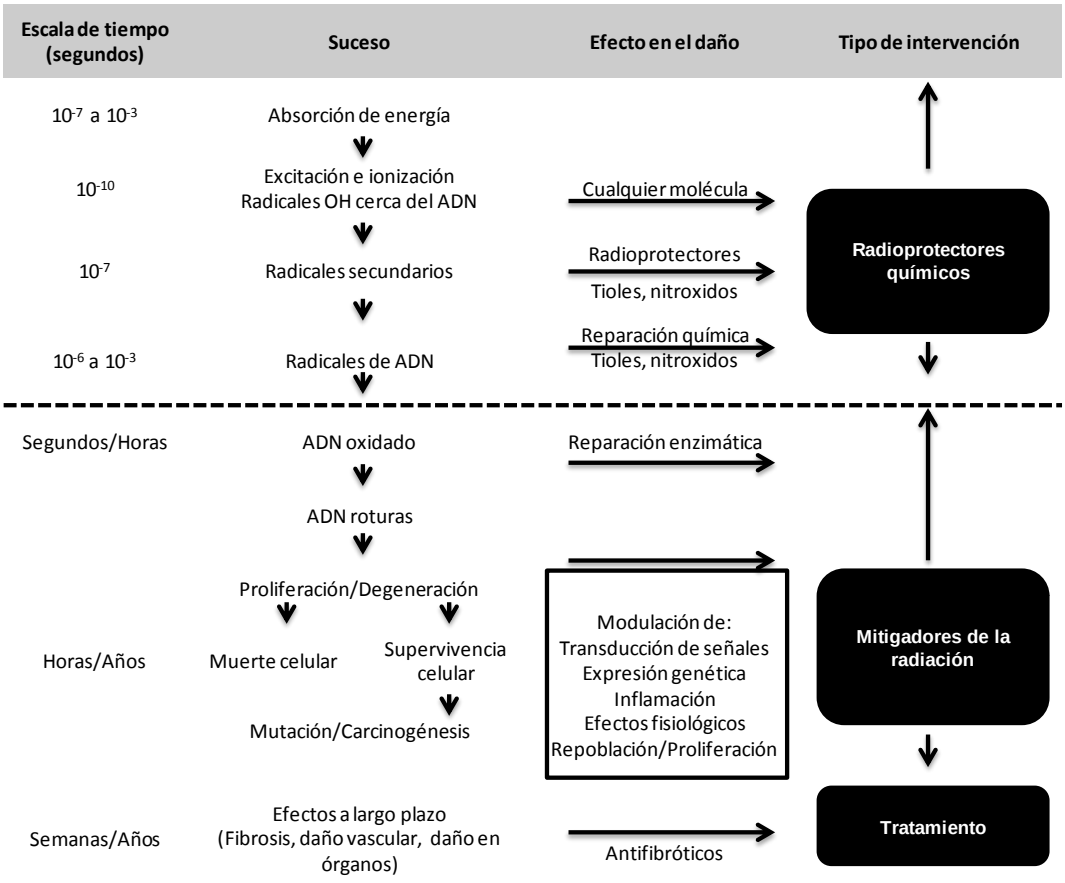

Figura 22. Secuencia de sucesos después de una irradiación que pueden ser modificados por la acción de los radioprotectores, mitigadores de la radiación o agentes destinados al tratamiento de los efectos.

Dependiendo del tipo de radioprotector, su mecanismo de acción puede ser diferente, desde impedir la formación de radicales libres, detoxificar las especies reactivas inducidas por la radiación, estimular a los radioprotectores celulares como la

superóxido dismutasa, el glutatión, las prostaglandinas y la interleucina-1, aumentando los mecanismos de reparación propios del ADN o retrasando la división celular e inducir hipoxia en los tejidos (Maurya *et al.,* 2006).

De la familia de los radioprotectores más estudiados, la Amifostina® (un compuesto con un grupo tiol) es el único radioprotector que ha sido clínicamente aprobado por la Agencia de Alimentos y Medicamentos para mitigar los efectos adversos en pacientes sometidos a radioterapia (ODAC, 2003). Este medicamento ofrece una buena protección pero es relativamente tóxico (nauseas, vómitos y hipotensión, son algunos de los efectos adversos más comunes) (Koukourakis *et al.,* 2000). Así que, el uso de la Amifostina como radioprotector se debe realizar con precaución y debe de evaluarse para cada tipo de enfermedad (Brenner *et al.,* 2003).

Así pues, los tioles, aunque son muy efectivos desde el punto de vista de protección de los tejidos normales de la radiación y no a los cancerígenos, poseen una alta toxicididad que ha hecho necesaria la búsqueda de agentes alternativos, que pudieran ser menos tóxicos y altamente eficaces. Se pensó que los productos o compuestos aislados de fuentes naturales podrían ser usados sustancialmente como radioprotectores no tóxicos (Jagetia, 2007). Muchos de los compuestos presentes en la naturaleza son conocidos por sus propiedades antioxidantes, como la gran mayoría de compuestos presentes en las plantas, y han sido usados desde la antigüedad para tratar gran cantidad de enfermedades humanas. Además, en la medicina moderna un gran número de compuestos procedentes de plantas se usa para mitigar y tratar los radicales libres mediados por enfermedades como el cáncer, la diabetes y los desórdenes neurodegenerativos (Arora, 2006). Teniendo esto en cuenta, resulta lógico pensar que los daños provocados por los radicales libres producidos por la radiación ionizante también pueden ser modificados por compuestos naturales con propiedades antioxidantes presentes, por ejemplo, en plantas. El motivo por el que los compuestos antioxidantes son capaces de neutralizar estos radicales libres es por su afinidad química por los grupos hidroxilo (OH^-).

Los criterios para evaluar y buscar radioprotectores en los compuestos antioxidantes se basan en la premisa de que la radiación ionizante es extremadamente efectiva a la hora de producir CAs debido a la inducción de las roturas de doble cadena

del ADN bien por su efecto directo o indirecto. Aquellos compuestos que puedan secuestrar a los radicales libres quizás también reduzcan la aparición de las roturas de cromátida en el ADN y por tanto las CAs. Por consiguiente, los agentes que puedan prevenir la formación de los radicales libres o que los destruyan reaccionando con ellos, inhibiendo por tanto su reacción con las biomoléculas, pueden funcionar como radioprotectores (Maurya *et al.,* 2008). De hecho, se estima que, por ejemplo, sobre dos terceras partes del daño de los rayos X en el ADN de células humanas es causado por el radical hidroxilo ($OH^{\cdot}$), comprobado por estudios en los que usan secuestradores de radicales libres capaces de reducir a una tercera parte el efecto biológico causado por la radiación (Hall and Giaccia, 2006).

Los agentes antioxidantes más comunes de la dieta son probablemente las vitaminas; entre ellas la vitamina A, C y E han demostrado ser radioprotectoras. Por ejemplo, la vitamina E, administrada a una dosis de 400 IU/kg de peso corporal antes de irradiar a ratones, mostró un aumento en el ratio de supervivencia de hasta un 79% frente a un 4% en el grupo control (Hosseinimehr, 2007). Y la vitamina A, a una dosis de 150000 IU/kg de dieta administrada a ratones ofreció protección contra una exposición a radiación del esófago o del intestino (Weiss and Landauer, 2003). Además de los antioxidantes comunes, un gran número de plantas contienen fitoquímicos antioxidantes que han demostrado ser radioprotectores en varios modelos. Algunos ejemplos son las preparaciones herbales chinas, las preparaciones ayurvédicas, los vegetales de la familia de las crucíferas, el té verde, los compuestos de la soja, el extracto *de Gingko biloba*, la *Mentha arvensis,* el *Panax gingeng,* la *Spirulina platensis,* el *Podophyllum hexandrum* (manzana del Himalaya), el *Syzygium cumini* (ciruela negra), los extractos del triphala, las semillas de uva, el *Aspalathus linearis* (té rooibos), la cistona, el geriforte, el propóleos, (Jagetia and Baliga, 2002; Jagetia *et al.,* 2004; Montoro *et al.,* 2005). Dentro de estas preparaciones o extractos se encuentran los principios activos que contribuyen en mayor o menor medida al poder radioprotector del conjunto. De entre los principios activos que han demostrado su capacidad radioprotectora se puede nombrar a modo de ejemplo la melatonina (Vijayalaxmi *et al.,* 1995), la naringina (Jagetia *et al.,* 2003), el eugenol (Tiku *et al.,* 2004), el sesamol (Prasad *et al.,* 2005), el ácido ferúlico (Prasad *et al.,* 2006), la quercetina (Devipriya *et*

al., 2008), el licopeno (Cavusoglu and Yalcin, 2009), al ácido rosmarínico (Sánchez-Campillo *et al.,* 2009), entre otros.

Como se ha mencionado en la sección ***B.2.3.***, las investigaciones biomédicas sobre la **curcumina** han provisto evidencias suficientes de amplio rango de actividades moleculares y celulares, la mayoría de ellas relacionadas con reacciones redox y transducción de señales (Aftab and Vieira, 2010). Estas actividades son las de antioxidante, antiinflamatorio, antiproliferativo, proapoptótico, antibacteriano y anticancerígeno (Epstein *et al.,* 2009). La curcumina es un potente antioxidante que puede proteger contra el daño inducido por los radicales libres en varios tejidos. Puede también proteger de la inducción de roturas simples de cadena por radicales del oxigeno en plásmidos, y reduce el número de roturas de cadena inducido por agentes genotóxicos en el ADN de levaduras (Polasa *et al.,* 2004).

Asimismo, al ***trans*-resveratrol** también se le atribuyen muchas actividades biológicas (apartado ***B.2.3***), entre ellas, la prevención de la aparición de enfermedades coronarias por inhibición de la oxidación de las lipoproteínas de baja densidad y de la agregación plaquetaria y coagulación o la prevención de la aparición de cáncer por inhibición de los procesos de iniciación, promoción y progresión tumoral (Sgambato *et al.,* 2001). Con respecto a su capacidad antioxidante, el mismo grupo de trabajo evaluó la habilidad del *trans*-resveratrol para prevenir la formación de ROS en fibroblastos normales de ratas expuestos a estrés oxidativo por peróxido de hidrógeno (H_2O_2). La pre-incubación de las células con *trans*-resveratrol durante 24 horas causó una disminución de los niveles de ROS en las células expuestas al agente oxidante de entre un 26,4% a un 57,3% respecto a las células control. Además también demostraron que este polifenol era capaz de disminuir los niveles de ROS intracelulares causados por los condensados del humo de cigarrillo.

Teniendo en cuenta que la reducción de los radicales libres radioinducidos proporciona cierto grado de radioprotección contra el daño causado por la radiación ionizante (Hosseinimehr, 2007), los polifenoles curcumina y *trans*-resveratrol con conocida capacidad antioxidante son candidatos para el estudio de su capacidad radioprotectora.

Con el fin de evaluar la radioprotección alcanzada en presencia de compuestos de origen natural se emplean ensayos tanto *in vivo* como *in vitro*. Los indicadores utilizados para esta evaluación pueden ser de tipo bioquímico (peroxidación), de tipo clínico (daño intestinal o hematopoyético), medida de la apoptosis celular, de los niveles de antioxidantes, etc. (Weiss *et al.,* 2003). Las técnicas citogenéticas permiten también cuantificar la reducción de daño cromosómico radioinducido mediante el análisis de los cromosomas y así evaluar la radioprotección ofrecida por un compuesto.

Las técnicas objeto de estudio son:

- *Frecuencia de cromosomas dicéntricos*. La relación de causalidad existente entre la formación de aberraciones cromosómicas y la exposición a las radiaciones ionizantes ha dado lugar a que el análisis de determinadas CAs como los cromosomas dicéntricos (**Figura 23**) se considere una técnica citogenética de referencia en dosimetría biológica (IAEA, 2011). El recuento de cromosomas dicéntricos presentes en linfocitos de sangre periférica, se considera el biomarcador más específico y sensible de dosis en caso de sobreexposición a radiación a corto plazo para estudios de dosimetría biológica (Voisin *et al.,* 2004; Romm *et al.,* 2009) aunque existen otros biomarcadores de exposición a radiación ionizante especificados por la IAEA (2011) que son igualmente utilizados en los laboratorios.

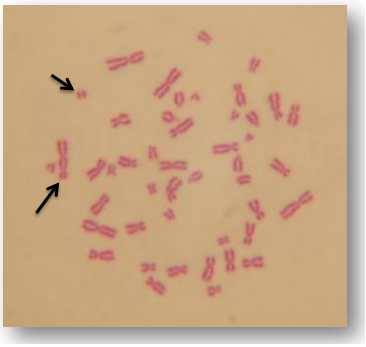

Figura 23. Metafase en primera división con un cromosoma dicéntrico acompañado de su fragmento acéntrico (IAEA, 2011).

Dado que la exposición a radiaciones ionizantes provoca un aumento en la frecuencia del biomarcador por excelencia, los cromosomas dicéntricos, el estudio del efecto radioprotector que pueden ofrecer determinadas sustancias cuando están presentes en sangre durante la irradiación se puede llevar a cabo mediante el estudio

de la reducción en la frecuencia de dicéntricos. Este estudio se puede realizar *in vivo*, con la administración de las sustancias radioprotectoras a animales que serán irradiados y estudio posterior de los linfocitos de sangre periférica o *in vitro* mediante la extracción de sangre periférica de humanos, adición de la sustancia a estudiar, irradiación de la misma y análisis citogenético posterior.

Las sustancias radioprotectoras, debido a su poder antioxidante, serán en un principio capaces de secuestrar los radicales libres causados por la radiación y así se puede disminuir tanto la frecuencia de cromosomas dicéntricos como de otras CAs causadas por la radiación. Además de su capacidad antioxidante, muchos compuestos naturales como lo polifenoles aumentan los niveles endógenos de enzimas antioxidantes como glutatión reductasa, glutatión peroxidasa, superóxido dismutasa y catalasa (Stevenson and Hurst, 2007), niveles que aumentaran la capacidad total para secuestrar radicales libres durante todo el ciclo celular.

- *Técnica de Condensación Prematura de Cromosomas (PCC).* Esta técnica tiene como fundamento la condensación prematura de los cromosomas en un momento del ciclo celular previo a la fase de mitosis para así reducir el tiempo de cultivo y por lo tanto evitar el retraso para entrar en mitosis o incluso la muerte celular. Para provocar la condensación prematura existen dos métodos, por un lado, la PCC por fusión mitótica y por otro lado la fusión por compuestos químicos. Concretamente, la PCC por fusión mitótica se basa en la fusión de linfocitos humanos con células mitóticas CHO en presencia de un agente de fusión, el polietilenglicol (PEG), mediante el cual es posible medir las CAs inmediatamente después de una irradiación sin la necesidad de la estimulación de ningún agente mitógeno o de su cultivo. Esta técnica permite la detección de roturas, cromosomas dicéntricos y anillos así como de translocaciones (IAEA, 2011). En el año 1985, Pantelias y Maillie establecieron un protocolo simple y reproducible para la inducción del PCC mediante fusión celular de los linfocitos humanos con células CHO. Usando el ensayo de PCC por medio de la fusión de células, los cromosomas de las células que están en interfase o que tienen el ciclo celular detenido pueden ser visualizadas y analizadas. En los híbridos formados por la fusión celular, los factores mitóticos presentes en la célula mitótica inductora de mitosis disuelven la membrana nuclear de la célula objeto de estudio y su cromatina se

condensa. Concretamente, las células que se han sometido a PCC asumen una morfología que es característica de la posición de la interfase de forma que la apariencia que toma la PCC puede ser usada para definir la fase del ciclo celular del linfocito en el momento de su tratamiento. Cuando las células están en G0/G1 aparecen cromátidas simples, en fase S aparecen como cromosomas pulverizados sin forma definida y en G2 como 2 cromátidas (**Figura 24**).

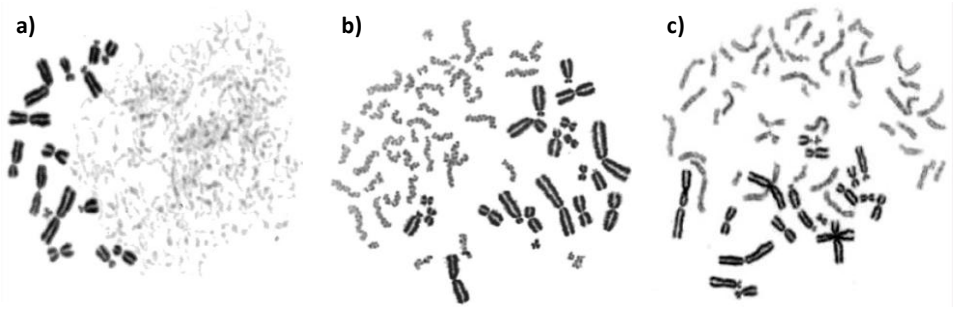

Figura 24. PCC por fusión de linfocitos mediante CHO en mitosis; a) fase G0/G1, b) fase S y c) fase G2 (Hatzi *et al.,* 2006).

De esta forma, cuando los linfocitos humanos son analizados por ejemplo en G0/G1 cada elemento que aparece tras la fusión representa a un cromosoma humano (**Figura 24 a**). Por lo tanto, en una situación normal, se deberían contar 46 elementos (fragmentos) correspondientes a los 46 cromosomas. Si la radiación ionizante incide en los linfocitos, las roturas producidas en el material genético aumentaran los elementos contabilizados (**Figura 25**).

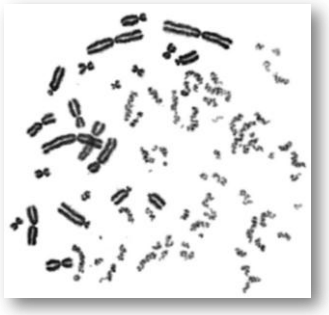

Figura 25. PCC por fusión de linfocitos humanos en fase G0 y CHO en mitosis tras una irradiación (IAEA, 2011).

Una ventaja importante que ofrece la PCC, en comparación con el análisis convencional en la metafase, es que no requiere que las células se dividan para la

evaluación de daño genético con lo que no son necesarias las 48 horas de cultivo de la muestra. Debido a esto, la PCC se ha aplicado con éxito en la observación directa del daño genético inducido por la radiación, por efecto directo, en linfocitos humanos estimulados, en interfase o en células CHO. Esta aplicación es de particular importancia, ya que permite la visualización y la valoración del daño inducido por la radiación en cromosomas en fase G1 y G2 inmediatamente tras la irradiación. Además, la inducción de la PCC permite la medición directa de la cinética de unión de los fragmentos rotos del ADN en interfase, así como de la cinética de formación de anillos y cromosomas dicéntricos debidos a la exposición a la radiación ionizante.

B.2.5. Radiosensibilidad

La radiosensibilidad es la respuesta de un sistema biológico frente a la radiación. Este concepto genérico incluye sistemas biológicos con distintos grados de organización: células, tejidos e individuos.

El método original de evaluar la radiosensibilidad en cultivos irradiados en fase G2 del ciclo celular fue desarrollado por Sanford et al. (1989), quién observó que las células en fase G2 de ciertos cánceres heredables exhibían valores de daño cromosómico inducido por rayos X mucho más altos que aquellos encontrados en la mayoría de controles sanos. Su metodología se modificó por Scott et al. (1996) para minimizar los problemas asociados con la reproducibilidad y obtener una ligera mejor discriminación a la hora de realizar estos trabajos. Sin embargo, estas ligeras modificaciones han permitido vislumbrar que la sensibilidad del ensayo G2, en determinados grupos (por ejemplo personal sometido a radiaciones), es significativamente más alta que en individuos normales, y el valor de punto de corte para la radiosensibilidad al método G2 se calcula desde la población control. De hecho en algunos estudios, se toma el percentil 90 de la distribución de los resultados de una población normal como valor de punto de corte.

Uno de los factores que juegan un papel fundamental en la radiosensiblidad son los mecanismos de reparación como el HRR o el NHEJ que constituyen un respuesta importante al daño cromosómico y a las dobles roturas del ADN causadas por la radiación. No obstante, la regulación del ciclo celular es tal vez uno de los factores más

determinantes de la radiosensibilidad, concretamente en la fase G2 de las células (Terzoudi *et al.,* 2009). En la regulación del ciclo celular intervienen los llamados puntos de control o "checkpoints" que son los responsables de que la proliferación celular se lleve a cabo bajo condiciones aceptables (Thomas *et al.,* 2013). El concepto de "checkpoint" lo introdujeron Hartwell and Weinert en 1989 cuando hipotetizaron que debían de existir unas vías metabólicas por medio de las cuales si un determinado evento dentro de una fase del ciclo celular estaba incompleto, una señal inhibitoria debería de enviarse a la siguiente fase para que la célula no progresase hacia ella. Por ejemplo, cuando una célula recibe daño en su ADN por la radiación, ésta detiene su división hasta que el daño haya sido reparado. El ciclo celular de una célula eucariota es vigilado por tres puntos de control (**Figura 26**):

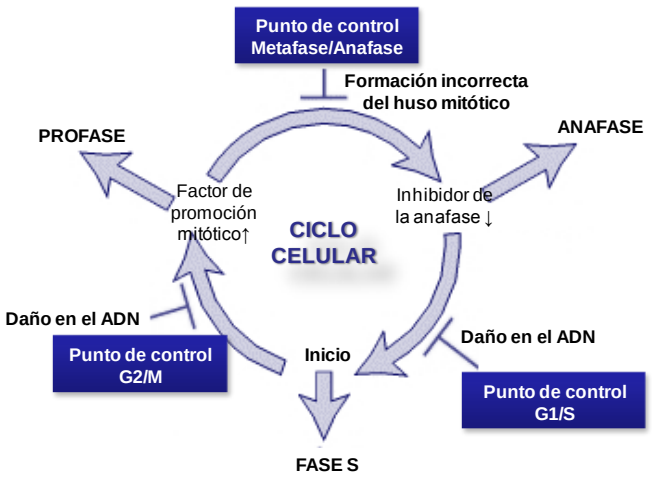

Figura 26. Etapas en las que los puntos de control pueden detener el paso a través del ciclo celular (Lodish *et al.,* 2000.)

- Punto de control en G1/S: las células comprueban si su entorno favorece la proliferación y si su genoma está listo para ser replicado. Este punto de control se denomina punto de restricción en los mamíferos.
- Punto de control en G2/M: Antes de someterse a condensación de los cromosomas y a la división nuclear, las células tienen que estar seguras de que su ADN es replicado completamente y sin daños. Si no es así, este punto de control retrasa la célula en S-G2.
- Punto de control en metafase/anafase: la separación de las cromátidas hermanas durante la anafase debería tener lugar solamente cuando todos los

cromosomas están unidos al huso mitótico. Si esta unión es incorrecta el punto de control impide que la célula se separe.

Si las condiciones para una división celular correcta no se cumplen en algún punto durante el progreso del ciclo celular, éste podrá detenerse en uno de estos tres puntos de control (Novák *et al.,* 2002).

El grupo de Terzoudi (Terzoudi *et al*., 2009; Panthelias and Terzoudi 2011) desarrolló un método basado en el análisis de determinadas alteraciones cromosómicas bajo la influencia de determinados puntos de control del ciclo celular (**Figura 26**). Esta técnica se basa principalmente en la irradiación *in vitro* de linfocitos de muestras de sangre periférica cuando estos se encuentran en la fase G2 de su ciclo celular con la posterior determinación de daño cromosómico usando técnicas de citogenética tradicional. La irradiación en la fase G2 se debe a que en esa fase del ciclo celular se sintetizan las proteínas necesarias para la mitosis y es más radiosensible que la fase G1 y la de síntesis (S). Después de la irradiación, las muestras se procesan durante la transición de la fase G2 a la fase de mitosis donde entra en juego el papel del punto de control en G2/M, permitiendo así visualizar y cuantificar el daño cromosómico mediante el recuento de las denominadas roturas cromosómicas y cromatídicas. Esto permite obtener dos valores; el primero de ellos representa el daño cromosómico bajo los efectos del punto de control (**Figura 27a**) y el segundo es el valor de la abrogación o eliminación del punto de control de daño cromosómico en G2 por la presencia de cafeína (**Figura 27b**).

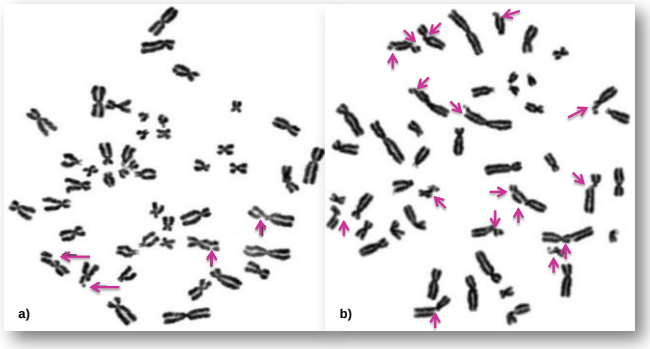

Figura 27. Roturas cromosómicas después de 1 Gy de rayos γ visualizadas en una metafase de linfocitos de sangre periférica humana, a) G2 convencional, b) G2 aplicando la abrogación del punto de control mediante la cafeína (Flechas señalan las roturas o ctb).

B.2.5.1. Ensayo de radiosensibilidad en fase G2 aplicado a la evaluación de la radiosensibilización in vitro de compuesto químicos

El test G2 se ha utilizado para evaluar *in vitro* la radiosensibilización cromosómica en fase G2 inducida por determinados compuesto químicos (Hatzi *et al.,* 2009). La aplicación de esta metodología puede contribuir a evaluar, *in vitro*, el potencial radiosensibilizante en fase celular G2 de determinados compuestos químicos utilizados en entornos profesionales donde pueda existir una exposición simultánea a la radiación ionizante ayudando así a determinar el posible riesgo de exposición combinada a estos compuesto químicos y a la radiación. Además, la evaluación de la capacidad de un determinado compuesto para inducir radiosensibilización cromosómica aporta nuevos datos sobre su riesgo y/o potencial carcinogénico. En el campo de la terapia del cáncer, la aplicación del ensayo de radiosensibilidad en fase G2, para dilucidar el mecanismo de acción de los compuestos radiosensibilizadores utilizados en el tratamiento del cáncer, puede contribuir a la modulación de la administración de las dosis, tanto del compuesto químico como de la radiación, por lo tanto la optimización de protocolos de radioterapia. En este sentido, se han estudiado algunos compuestos químicos como el glutaraldehído, presente en un amplio espectro de aplicaciones en el campo de la medicina, ciencia e industria o como la hidroquinona, el principal metabolito del benceno (Hatzi *et al.,* 2007; Hatzi *et al.,* 2008); ambos han resultado ser agentes inductores de radiosensiblidad en modelos *in vitro*.

La metodología a emplear es idéntica a la del test G2 del grupo de Terzoudi and Pantelias (2011). El parámetro nuevo a incluir en el procedimiento es el momento en el que el compuesto a estudiar debe ser incorporado al cultivo de sangre para garantizar su absorción a nivel del núcleo de los linfocitos según los mecanismos de absorción propios del compuesto. Para este estudio, se compara estadísticamente el valor de CAs obtenidas con y sin la adición del compuesto a evaluar. Mediante este ensayo se podrá evaluar si el compuesto tiene un efecto radiosensibilizante en células que se encuentren en fase G2 del ciclo celular.

B.2.5.2. Radiosensibilidad individual

En los seres humanos se exhibe un rango de variación interindividual en la frecuencia y severidad de los efectos que presentan luego de la exposición a las radiaciones debido a diferencias en su radiosensibilidad individual. Por lo general entre un 5 y un 7% de los pacientes sometidos a esquemas convencionales de terapia radiante desarrollan reacciones severas. En estos pacientes la respuesta se expresa a través de reacciones precoces y/o tardías aún con dosis por debajo de las dosis de tolerancia para tejidos sanos. En menos del 1% del total de pacientes estas reacciones pueden llegar a comprometer el pronóstico vital (pacientes hiper-respondedores; *over-reactors*). Estas diferencias en la radiosensibilidad individual pueden reflejarse en la severidad de las lesiones radioinducidas tanto en el caso de las exposiciones programadas (radiología intervencionista, radioterapia) como en las sobreexposiciones accidentales. Ciertos mecanismos, como la disminución de la muerte celular programada (apoptosis), la alteración en el nivel de la reparación genómica y el aumento de transcripción de mediadores inflamatorios han sido involucrados en lesiones radioinducidas. Estados de alteración de la microvasculatura (diabetes, senescencia, ateroesclerosis) y alteraciones de la respuesta inmune (lupus eritematoso, esclerodermia, dermatomiositis) pueden asimismo modificar la respuesta individual frente a una irradiación.

En los últimos años se ha buscado una técnica de evaluación individual de la radiosensibilidad que permita una mejor discriminación y una reproducibilidad intraexperimental. La identificación de individuos entre la población que tengan un incremento inherente de la radiosensibilidad es de vital importancia para la protección de los efectos adversos de radiación, tanto en pacientes como en personal sanitario, si bien, la potencial utilidad de los tests predictivos de radiosensibilidad individual se vincula particularmente con exposiciones planificadas. Estos tests, permitirían evaluar el riesgo individual y, en el caso de las exposiciones médicas, posibilitarían un ajuste personalizado de las dosis (Borgmann *et al.,* 2008). Esto resultaría de gran valor en la planificación de esquemas personalizados de radioterapia y en la toma de decisiones a cerca de no tratar al paciente con radioterapia o en derivarlo a cirugía. Por otra parte, en el caso de lesiones ya constituidas facilitarían la mejor comprensión de los factores

que predisponen y eventualmente podrían llegar a tener un valor médico-legal (Terzoudi *et al.,* 2009). En oncología se conoce que la dosis máxima de radiación terapéutica es limitada por la incidencia de los efectos adversos sobre el tejido no cancerígeno, pero resultaría interesante individualizar un protocolo de terapia en la radiación la cual prediga la radiosensibilidad individual (Bentzen, 1997). Además, es bien conocido que la radiosensibilidad individual está relacionada con la predisposición al cáncer, de forma que es de vital importancia la identificación de la hipersensibilidad a la radiación entre la población. Esta afirmación se llevó a cabo a partir del estudio de Scott (2004) en el que se observó que las células de pacientes con cáncer de pecho, cuello y cabeza presentaban una radiosensibilidad superior a individuos sin cáncer. Por esto, en ese trabajo se propuso que tan elevada sensibilidad a la radiación era un marcador de baja penetración para la predisposición al cáncer.

En los últimos años, son varias las evidencias que se han obtenido para apoyar la hipótesis a cerca de que ciertas variantes en los genes, que juegan un papel clave en la respuesta a la radiación, son predictivas de la radiosensibilidad individual y el desarrollo de efectos adversos después de exposiciones a radiación ionizante (como es el caso de la radioterapia) (Alsner *et al.,* 2008).

La técnica citogenética de evaluación de la radiosensibilidad cromosómica conocida como ensayo G2 es usada por algunos laboratorios para la evaluación de la radiosensibilidad individual (Terzoudi *et al.,* 2009). De acuerdo con la metodología de Terzoudi and Pantelias (2011), los cultivos son irradiados en la fase G2 del ciclo celular para ser tratados en paralelo con y sin un compuesto que abroga o elimina el punto de control en fase G2/M y de este modo obtenemos dos valores de daño cromosómico. El primer valor representa el daño cromosómico bajo los efectos del punto de control y el segundo valor representa el daño obtenido tras la abrogación o eliminación del checkpoint del punto de control G2/M.

La diferencia entre los dos valores aporta una nueva medida conocida con el nombre de parámetro de radiosensibilidad individual, que expresa, el potencial del punto de control en G2 para facilitar el reconocimiento de daño genético y la reparación del daño genético inducido por la radiación durante la transición de la fase G2 a la M.

MATERIAL AND METHODS

C. MATERIAL AND METHODS

C.1. Tobacco study

C.1.1. Human subjects

A sampling frame of 101 adults (55 females and 46 males) aged 18-49 years was considered for the study. The control group (non-smokers) included 48 subjects and the smoking group 53.

According to Laugesen and Swinburn (2000) subjects were categorized into three categories: light smokers (who smoke less than 10 cigarettes per day), heavy smokers (defined as those who smoke 10 or more cigarettes per day) and non-smokers **(Table 1)**.

Table 1. Characteristics of human subjects in the tobacco study.

	Smokers (n= 48)		Non smokers (n=53)
	«Light smokers»	«Heavy smokers»	
Women	13	7	35
Men	17	11	18

C.1.2. Culture conditions

Human peripheral blood samples were collected using sterile BD Vacutainer® blood collection tubes (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) containing lithium heparin as anticoagulant.

For each subject, separate cultures were set up by mixing 0.75 mL of whole blood with 5 mL of PB-MaxTM Karyotiping medium (Gibco, Barcelona, Spain) and incubated 72 h at 37 ºC. In order to differentiate the first, second and third division metaphases, a final concentration of 12 mg/mL of BrdU (Sigma, Madrid, Spain) was present for the setting up of the cultures. As BrdU is photosensitive the tubs were rolled in aluminum foil. One hundred and fifty µL of Colcemid® (Gibco) from a stock solution of 10 µg/mL were added 2 h before harvesting to stop the cell culture in metaphase.

After blood cultures were stopped. Cells were collected by centrifugation at 1200 rpm for 10 min and the supernatant was poured off. The remaining cells were treated with 75 mM KCl (Merck, Darmstadt, Germany) for 30 min in a water bath at 37 ºC, centrifuged once more at 1200 rpm for 10 min and the supernatant was poured off. The cells were then fixed in the so-called lymphocytes fixative, which ismethanol:glacial acetic acid (3:1 v/v) (Panreac, Barcelona, Spain) until the tube was filled; tubes were centrifuged at 1200 rpm for 10 min and the supernatant was poured off, leaving a pellet of lymphocytes in the bottom. The fixation was made several times (2-3) in order to fix the chromosomes in the cells and to clean those cellular compounds not needed for the analysis. In the first fixation it is important to add the fixative very slowly in small drops since during fixation the hemolysis of erythrocytes occurs and if this happens abruptly, the suspension can agglutinate. Once the pellet was considered as clean enough, lymphocytes were re-suspended in a small amount of fixative solution and were placed on cleaned frosted slides (2 to 3 drops per slide).

C. 1.3. Staining technique

Two- to three-day-old slides were stained with Fluorescence plus the Giemsa stain technique (Wolf and Perry, 1974). Old slides were briefly treated for 20 min in Hoechst 33258 (bisbenzimide) (Sigma Chemical Co., St. Louis, MO, USA) at room temperature. The slides were washed with distilled water and allowed to dry at room temperature for approximately 30 min. The slides were covered with 2x standard saline citrate (SSC) (Gibco) and with a coverslips, and treated with UV supplied from the high pressure mercury lamp of 300 W (Eikosha Co. Model PIH 300) for approximately 2 min until the appearance of bubbles. Once washed with distilled water and dried 30 min, the slides were stained with Leishman dye (Merck) for 5 min being useful in the identification of first-, second- and third-division metaphases (see **Figure 19**). Dried slides were embedded with cover slips and coded for analysis.

C. 1.4. Cytogenetic analysis

The frequency of SCEs, HFCs and the SCE distribution pattern parameter ratio was determined from the analysis of 50 second division metaphases for each person.

The evaluation of SCE scores in lymphocytes included on the one hand, scoring total exchanges in the total number of analyzed cells for each treatment to establish its frequency (Y_{SCE}) in 46 chromosomes per cell and to establish the HFCs parameter. On the other hand, for the SCE distribution pattern, the analysis identified and quantified the number of chromosomes with one, two or three exchanges per chromosome **(Figure 28).** Cytogenetic analyses were carried out by using a conventional microscope (Izasa, Barcelona, Spain) and an image analysis system with the IKAROS-software (MetaSystems, Altlussheim, Germany).

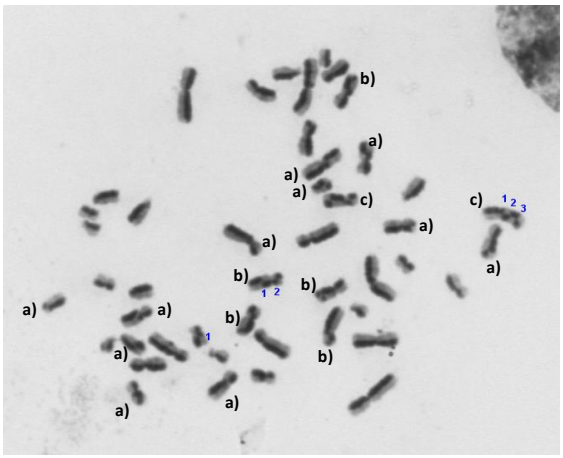

Figure 28. Metaphase in second division showing chromosomes containing: a) 1 SCE, b) 2 SCE, c) 3 SCE. The ratio is calculated according to this formula: Ratio = ax1 + bx2 + cx3 (total SCEs)/a + b + c (affected chromosomes). For this metaphase: Ratio = 29/20 = 1.45. Adjusted ratio = 1.45 – expected ratio for 29 SCE (1.35) = 0.1

C.1.5. Statistical analysis

For each cell, the SCE frequency was calculated. Alternatively, the number of HFCs per individual was also computed according to the method proposed by Carrano and Moore (1982); a HFC cell was defined as a cell which exceeds the 95th percentile of the SCE distribution from control individuals. As a measure of the differences in SCE distribution patterns, we estimated the number of SCEs per affected chromosome for each cell. The formula for this estimate was:

$$\frac{\text{nº of SCE}}{\text{nº of affected chromosomes}}$$

A higher ratio represented a more clustered SCE pattern. Since the estimated ratio for a random distribution of SCEs in a cell grows as a function of the number of SCEs (**Figure 29**), we subtracted the expected ratio from our observed ratio as a measure of deviation from a random distribution pattern.

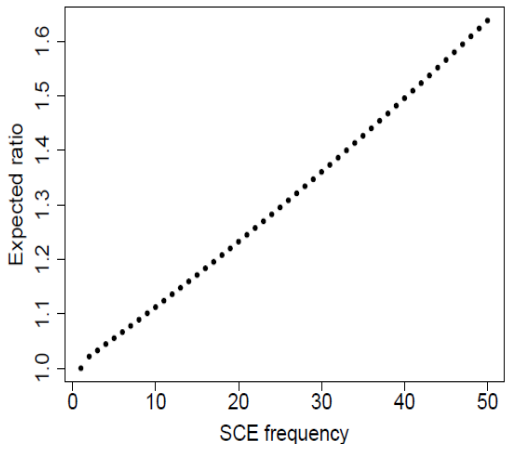

Figure 29. Estimated ratio for a random distribution of SCEs in a cell.

SCE frequencies and ratio distribution patterns were modeled in terms of age, sex and smoking status using a linear mixed model approach. These models regarded individuals as a random factor to correct for intra-individual correlation among cells. Counts of HFCs per individual were modeled with the same explicative variables using a negative binomial distribution. The negative binomial distribution was preferred over the Poisson distribution due to the presence of overdispersion in the data.

In the statistical model all the interactions between variables were assessed (age-sex, age-smoking, sex-smoking). After fitting of the saturated models with all the variables and their interactions, a model selection procedure was performed using second-order Akaike Information Criterion (AICc) to find the best model. All statistical analyses were carried out using the R software (version 2.15.2).

C.2. Ardystil syndrome

C.2.1. Human subjects

The Valencian Community surveillance program in 2003 included a total of 125 subjects, although only 52 of them participated in the program. The control sampling frame consisted of 48 subjects who were not classified as having the "Ardystil

syndrome"**.**

C.2.2. Culture conditions

The culture conditions carried out for this study were exactly the same as those described in section *C.1.2*.

C.2.3. Staining technique

The staining technique for this study was the Fluorescence plus Giemsa stain technique (described in section *C.1.3*).

C.2.4. Cytogenetic analysis

The cytogenetic analysis in this study for the SCE analysis was the same as that described in section *C.1.4.*

C.2.5. Statistical analysis

The statistical analysis was the same as carried out for tobacco study because of the same parameters were considered: SCE frequency, HFCs and the chromosome aggregation pattern ratio (See section ***C.1.5***).

Given that some subjects were smokers and that tobacco smoke is a SCE inducer (Khabour *et al.,* 2011), we decided to consider this variable and categorized the groups according to Laugesen and Swinburn (2000).

Table 2. Characteristics of human subjects in the "Ardystil syndrome" study.

	Ardystil (n=52)		Control (n=48)	
	Women	**Men**	**Women**	**Men**
Non-smokers	19	8	16	9
Light smokers	7	9	6	8
Heavy smokers	3	6	4	5

SCE frequencies and ratio distribution patterns were modeled in terms of age, sex, smoking status and ardystil exposure using a linear mixed model approach. These

models regarded individuals as a random factor to correct intra-individual correlation among cells. Counts of HFCs per individual were modeled with the same explicative variables using a negative binomial distribution. The negative binomial distribution was preferred over the Poisson due to the presence of overdispersion in the data.

After fitting the saturated models with all the variables and interactions, a model selection procedure was performed using second-order Akaike Information Criterion (AICc) to find the best model. All statistical analyses were carried out using the R software (version 2.15.2).

C.3. Cyto- and genotoxicity of curcumin and *trans*-resveratrol.

C.3.1. Chemicals preparation

Standards of *trans*-resveratrol and curcumin were supplied by Sigma-Aldrich (St. Louis, MO, USA). From the stock solution and using ethanol 95% (Panreac) as solvent, *trans*-resveratrol and curcumin dilutions were prepared in order to reach the concentrations of 1.4, 2.8, 5.6, 14, 28 and 140 µM of curcumin and 2.2, 22 and 220 µM of *trans*-resveratrol in 5 mL of human peripheral blood samples, adding in all cases a volume of 100 µL from the stock solution dilution.

C.3.2. Culture conditions

After informed consent was obtained human peripheral blood samples were collected in sterile vacutainer tubes (Becton, Dickinson and Company) containing lithium heparin as anticoagulant. This study was approved by the Ethical Committee of the University of Valencia (see Annexes).

For each concentration, a sample of 5 mL of blood was incubated with curcumin or *trans*-resveratrol for 1 hour in a water bath at 37 ºC. Separate cultures were then set up by mixing 0.75 ml of whole blood with 5 ml of PB-Max^{TM} Karyotiping medium and incubated at 37 ºC, 48 h for chromosome aberrations (CAs) and 72 hours for Mitotic Index (MI), Proliferation Index (PI) and Sister Chromatid Exchanges (SCEs). Two samples were incubated and cultured as controls; one with peripheral blood alone and the other with peripheral blood in the presence of the solvent alone (100 µl of 95% ethanol). A final concentration of 12 µg/ml of BrdU was present from the setting

up of the cultures in order to differentiate first-, second- and third-division metaphases. One hundred and fifty µL of Colcemid® (Gibco) from a stock solution of 10 µg/mL were added 2 h before harvesting to stop the cell culture in metaphase. At 48 and 72 h cells were collected by centrifugation, treated in 75 mM KCl, centrifuged once more and lymphocytes were fixed and processed for cytogenetic analysis (see section C.1.2.).

C.3.3. Staining technique

The stain technique carried out was exactly the same as in the Section ***C.1.3.***

C.3.4. Cytogenetic analysis

- *Chromosomal aberrations (CAs).* The CAs were analyzed using samples from the 48 h cultured lymphocytes. Chromosomal analysis was carried out exclusively on the first-division metaphases containing 46 centromeres. For each curcumin and *trans*-resveratrol treatment and for the controls, a total of 100 cells were analyzed. CAs analyzed were classified in chromosome- or chromatid type aberrations. For chromosome type aberrations, dicentric chromosomes and rings were only considered when an acentric fragment was present, acentric fragments not associated with dicentric and ring chromosomes were classified as extra acentric fragments. Other chromosome-type aberrations like translocations and inversions were only recorded when the morphology of the derivate chromosome was clearly abnormal. The chromatid-type aberrations were chromatid breaks and inside the gaps included chromosome and chromatid gaps.

- *Sister chromatid exchanges (SCEs).* The SCEs were analyzed using samples cultured during 72 h. The frequency of SCEs was determined from the analysis of 25 second division metaphase for each concentration.

- *Mitotic Index (MI).* It provides information about the proportion of cells that have followed normal cell cycle. MI was calculated according to Rojas *et al.* (1993). For each concentration, the MI was obtained from the 72 h cultured samples, as the proportion of the number of metaphases found in a total of 500

cells (cells in metaphase/total cells) **(Figure 30)**. The relative mitotic index (RMI) was evaluated as: RMI = [MI treated/MI control] x 100.

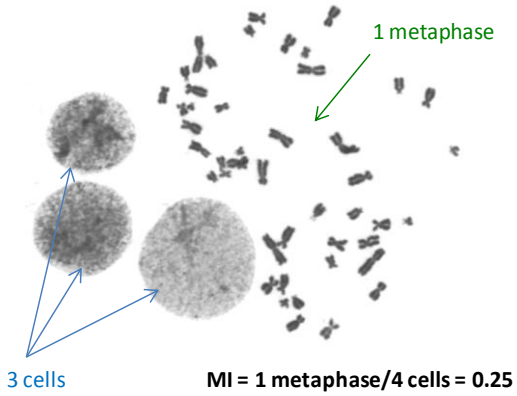

Figure 30. Example of MI calculation.

- *Proliferation Index (PI).* In order to assess the cell proliferation kinetics, the PI using the 72 h cultured samples was analyzed. For each concentration, the proportion of cells in first (M1), second (M2) and third (M3) division was obtained from 100 consecutive metaphases (See **Figure 19**). The proliferation index (PI) was calculated according to the formula: PI = (M1 + 2M2 + 3M3)/100 and the relative proliferation index (RPI) (Rojas *et al.,* 1993) was evaluated as: RPI = [PI treated/PI control] x 100.

C.3.5. Statistical analysis

For statistical analysis, a Student test was used and p-values <0.05 were considered significant. Correlation was assessed using Spearman's rank correlation coefficient. All statistical analyses were carried out using SPSS software (SPSS, Chicago, IL, USA) version 10.0 for Windows.

C.4. Radioprotection of curcumin and *trans*-resveratrol

C.4.1. Radioprotection assessed by dicentric chromosome assay

C.4.1.1. Chemical preparation and samples

Stock solutions of curcumin and *trans*-resveratrol (Sigma-Aldrich) were prepared the day before irradiation procedure from the pure standard reagents and stocked at -21ºC in darkness. From the stock solution using ethanol 95% (Panreac) as solvent,

curcumin and *trans*-resveratrol dilutions were prepared in order to reach the concentrations of 1.4, 14, 140 and 1400 µM of curcumin and 2.2, 22 and 220 µM of *trans*-resveratrol in 12 mL of human peripheral blood samples, adding in all cases a volume of 250 µL from the stock solution dilution. After informed consent was obtained human peripheral blood samples were collected, in sterile vacutainer tubes (Becton, Dickinson and Company) containing lithium heparin as anticoagulant.

C.4.1.2. Irradiation conditions

The different concentrations of curcumin and *trans*-resveratrol were added to blood samples 1 h before irradiation and incubated at 37 ºC in a water bath. Samples were irradiated at 2 Gy (dose rate 50 cGy/min) using a Cobalt Teletherapy Unit (Theratron Phoenix, Otawa, Canada) located at Hospital La Fe (Valencia) (**Figure 31**).

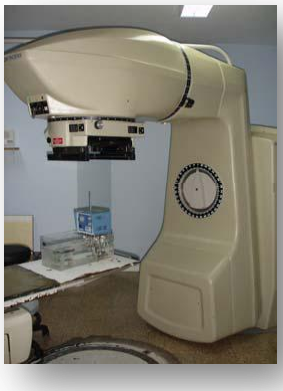

Figure 31. Cobalt Teletherapy Unit (Hospital La Fe, Valencia).

International Atomic Energy Agency (IAEA, 2001) recommendations were followed during irradiation to ensure a homogeneous irradiation. The parameters considered during the irradiation procedure were the following:

- To ensure that the sample is always included within the beam, the radiation field was 13 x 24 cm^2.
- In order to emulate the conditions *in vivo*, the blood sample was stored at 37°C during irradiation.
- The irradiation was performed within a water phantom with the sample placed in its center and ensuring a complete electronic balance (**Figure 32)**.

- The absorbed dose was assessed by dosimetry protocol used at the University Hospital of La Fe (TRS-398 IAEA) considering the decay of the source in the corresponding days of irradiation.
- To achieve a uniform irradiation dose all material was used from two opposing sides 90/270 degrees of the drive arm. This sample was placed in a depth of 6.3 cm lateral fields.

Figure 32. Water phantom with the blood sample submerged

Incubation in a water bath for 1 h at 37 ºC soon after irradiation was required to facilitate the repair mechanisms. Two tubes were irradiated at 2Gy to serve as controls; one tube with peripheral blood alone and the other with peripheral blood in the presence of the solvent alone (250 µl of 95% ethanol). A schematic representation of the basic steps of dicentric assay is shown in **Figure 33**.

C.4.1.3. Culture conditions

For each curcumin and *trans*-resveratrol concentration, separate cultures were set up by mixing 0.75 mL of whole blood with 5 mL of PB-MaxTM Karyotiping medium and incubated during 48 h at 37 ºC. To analyse exclusively first-division metaphases (only the first division is considered in a dicentric assay), a final concentration of 12 µg/mL of BrdU (Sigma-Aldrich) was present from the setting up of the cultures. One hundred and fifty µL of Colcemid® (Gibco) from a stock solution of 10 µg/mL were added 2 hours before harvesting to stop the cell culture in metaphase. At 48 h cells were collected by centrifugation, treated in KCl, centrifugated once more and lymphocytes were fixed and processed for cytogenetic analysis (see section *C.1.2.*). A schematical procedure for the dicentric assay is shown in **Figure 33**.

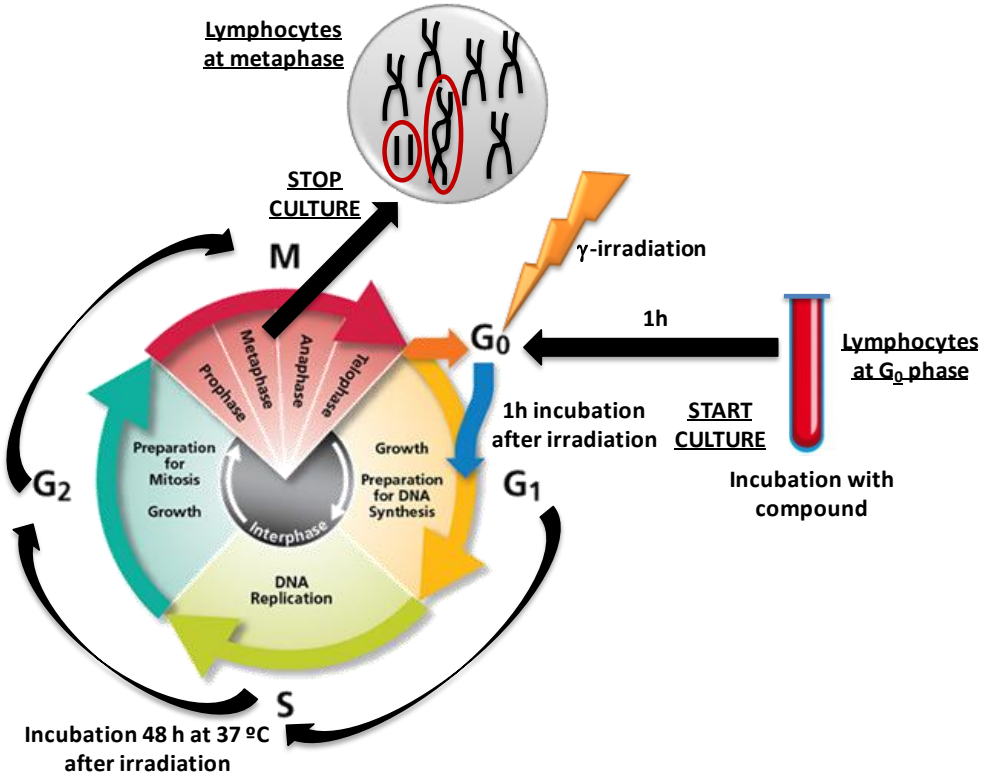

Figure 33. Basic steps for dicentric assay.

C.4.1.4. Staining technique

For the analysis of CAs, two- to three-day-old slides were stained with C banding stain technique (Fernandes *et al.,* 2008). Slides were placed in a staining rack and covered with 0.2N HCl (Carlo Erba Reagents, Val de Reuil, France) in room temperature for 30 minutes. Then, HCl was removed and slides were washed in triplicate with distilled water changing which was changed after each wash in order to discard waste acid. The slides were incubated without drying, in a 5% solution of BaOH (Merck), prepared at the time of use, in a Coplin jar for 1 min at 60 °C. The solution should be in continuous agitation so that the salt is well distributed among all the slides. The slides were then placed back into the grid and covered again with 0.2N HCl for two minutes, the acid was removed and the operation was repeated with distilled water. In another Coplin jar, a solution of 2 x SSC at 60 °C was prepared to submerge the slides for 45 min. After that, the slides were removed from this solution, washed with distilled water and allowed to dry in room temperature.

C.4.1.5. Cytogenetic analysis

Chromosomal analysis was developed on first division metaphases containing 46 centromeres. CAs were classified as follows: dicentric chromosomes and rings only scored when an acentric fragment was present. Other abnormalities like chromatid breaks and gaps were also recorded. Taking into account that the dicentric chromosomes is the gold standard biomarker for radiation dose assessment according to the IAEA (2011), the frequency of dicentric chromosomes were recorded for blood cultures without any treatment, 250 µL of ethanol 95% and different concentrations of curcumin and *trans*-resveratrol. The number of scored cells was 100.

C.4.1.6. Statistical analysis

For statistical analysis, the Student-t test was used and p-values <0.05 were considered significant. Correlation was assessed using Spearman's rank correlation coefficient. All statistical analyses were carried out using SPSS version 10.0 for Windows. The Poisson distribution of the dicentrics was checked by the test quantity U of the dispersion index (variance/mean), a value of $U > 1.96$ indicates over dispersion at the 5% level of significance. The Poisson distribution was checked in order to check whether the irradiation of the sample was uniform, because it is known that after homogenous low linear energy transfer irradiations, such as that used in the present study, the distribution of dicentrics per cell follows a Poisson distribution (Montoro, 2006).

C.4.2 Radioprotection with curcumin and *trans*-resveratrol assessed by Premature Chromosome Condensation (PCC) technique.

C.4.2.1. Chemicals preparation and samples

Stock solutions of curcumin and *trans*-resveratrol (Sigma Aldrich) were prepared the day before irradiation procedure from the pure standard reagents and stocked at -21 ºC in darkness. From the stock solution and using ethanol 95% (Merck) as solvent, curcumin and *trans*-resveratrol dilutions were prepared in order to reach the concentrations of 0.14, 1.4 and 7 µM curcumin and 2.2, 22 and 220 µM of *trans*-resveratrol in isolated lymphocytes from the human peripheral blood adding in all the samples a volume of 40 µL of each solution.

C.4.2.2. Chinese Hamster Ovary (CHO) cells preparation

In order to reach a correct PCC fusion a sufficient quantity of CHO cells should fuse in mitosis with the human lymphocytes; in this way, the Mitotic Promoting Factor (MPF) will go from the CHO cells in mitosis to lymphocytes in G0 to initiate the cell cycle and to induce the prematurely chromosome condensation. For this purpose, CHO were growth in McCoy's 5A culture medium (Gibco, Thessaloniki, Greece) supplemented with 10% fetal bovine serum (FBS) (Gibco) and 1% phytohaemagglutinin (PHA) (Biochrom AG, Cambridge, United Kingdom), 1% glutamine and antibiotics (penicillin: 100 U/ml; streptomycin: 100 µg/ml) (Sigma-Aldrich) ("whole medium"), and keep them in exponentially growing monolayer cultures in 75 cm^3 flasks (**Figure 34**); once a sufficient number of mitotic cells were obtained they were used to supply the mitotic cells with a mitotic factor promoter to induce PCC in human lymphocytes. Colcemid® (Gibco) at final concentration of 0.2 µg/mL was added to a CHO culture for 4 h and the accumulated mitotic cells were harvested by selective detachment.

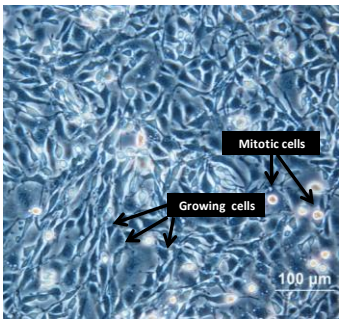

Figure 34. CHO cells, the round forms are the mitotic cells, the rest are the growing cells.

C.4.2.3. Lymphocytes separation

Human lymphocytes were separated from the whole blood according to a slightly modified Ficoll-paque method (Pantelias and Maillie, 1985). In a 15 ml plastic tube, whole blood was layered on the top of the Biocoll Separating solution (Biochrom AG) using the same volume as the blood volume used. Tubes were centrifuged at 1800 rpm for 30 min. The interface enriched with lymphocytes was collected with a Pasteur pipette, washed twice with 10 ml whole RPMI medium (pouring off the medium in each washing) and keeping them in approximately 10 ml of whole medium. In a 15 ml round bottom culture tube a final volume of 2 ml was prepared with half the volume of

lymphocytes diluted in whole RPMI medium and approximately half of the whole RPMI medium alone.

C.4.2.4. Irradiation conditions

The 2 mL solution of lymphocytes and the whole RPMI medium was incubated for 1 h before irradiation in a water bath at 37 ºC with 40 µL of the different concentrations of curcumin and *trans*-resveratrol, one sample with the same volume of the compound solvent (95% ethanol) alone as solvent control and one sample with the lymphocytes and the whole medium alone as a control. Thereafter, irradiation was carried out in a Gamma Cell 220 irradiator (Atomic Energy of Canada Ltd., Ottawa, Canada) at room temperature and at a dose rate of 450 cGy/min (**Figure 35**); different irradiation times with this dose rate were administered in order to reach a dose ranging from 0 to 6 Gy in the samples. For each experiment, control samples without the addition of compounds, were irradiated from 0 to 6 Gy in order to calculate the PCC fragments considering only the effect of the irradiation. These data were used to analyze the possible influence of 95% ethanol, curcumin and *trans*-resveratrol pre-incubation in the radio-induced DNA damage.

After irradiation, tubes were immediately put in ice in order to avoid cell repair mechanisms and see the direct effect of the radiation, and were centrifugated at 1200 rpm for 7 min. Supernatant was discarded and tubes were allowed to dry in order to avoid the presence of the whole RPMI medium with fetal bovine serum.

Figure 35. Gamma Cell 220 irradiator (Institute of Radioisotopes & Radiodiagnostic Products,National Center for Scientific Research, NCSR ‘‘Demokritos’’, Athens).

C.4.2.5. Lymphocytes and CHO fusion for the PCC induction culture conditions

Cell fusion and PCC induction in lymphocytes were carried out by the method of Pantelias and Maillie (1983). Isolated mitotic cells from McCoy's medium culture were mixed with 5-7 times as many lymphocytes available in a 15 ml round-bottom culture tube. After centrifugation of the mixture at 900 rpm for 5 min, the supernatant was poured off and the tubes were always kept inverted in order to completely eliminate the medium and keep the pellet with the cells as dry as possible. While holding the tube in an inverted position, 150 µL of Polyethylene Glycol 1500 (Roche, Basel, Switzerland) were injected into the tube straight to the cell pellet and turning quickly the tube upright. 1.5 mL of RMPI medium without FBS was added 0.5 ml by 0.5ml (3 times) and gently shaken after each addition. The cell suspension was then centrifuged at 900 rpm for 5 min. The supernatant was discarded and the pellet re-suspended in 0.5 ml of RPMI with FBS.

Tubes were incubated at 37 ºC for 75 min in a humidified incubator in an atmosphere of 5% CO_2 and 95% air allowing cell fusion and PCC induction. After the incubation period cultures were stopped and fixated with a lymphocytes fixative solution (methanol:acetic acid, 3:1 v/v). The pellet alone was re-suspended in 10-15 drops of lymphocytes fixative solution and clean frozen slides were prepared by adding 2 drops per slide. **Figure 36** represents schematically the process of human lymphocytes and CHO cell fusion and PCC induction.

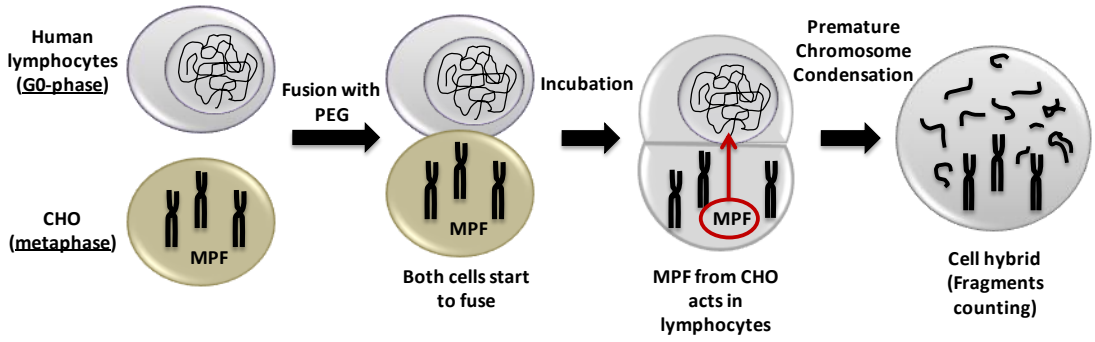

Figure 36. Basic steps occurred during the fusion of the human lymphocytes and CHO cells. (MPF: Mitotic Promoter Factor; PEG: Polyethylene Glycol).

C.4.2.6. Staining technique

The slides were air-dried and stained in a 2% solution of Giemsa dye for 5 min and rinsed with water. Dried slides were embedded with cover slips and coded for analysis.

C.4.2.7. Cytogenetic analysis

Because lymphocytes were treated at G0/G1 cell cycle phase due to the incubation time, chromosomes appeared as a single chromatid. Chromosome damage was quantified scoring "chromosome fragments", as the number of chromosome fragments in excess of 46 per cell. The excess of fragments was counted from 46 because in normal un-irradiated human lymphocytes the number of chromosomes is 46. For each experimental point, 50 lymphocytes in G0-phase were scored.

C.4.2.8. Statistical analysis

Statistical analysis was performed using a linear multiple regression model. The model considered as a response variable the chromosome damage and the radiation dose, and the compound and the concentration of compounds as predictive variables.

C.5. Radiosensitivity

C.5.1. Radiosensitivity induced by curcumin and *trans*-resveratrol

C.5.1.1. Chemical compound preparation

Stock solutions of curcumin and *trans*-resveratrol (Sigma Aldrich) were prepared the day before the irradiation procedure from the pure standard reagents and stocked at -21 ºC in darkness. From the stock solution and using ethanol 95% as solvent, *trans*-resveratrol and curcumin dilutions were prepared in order to reach a final concentration of 2.2 and 220 µM *trans*-resveratrol and 1.4 and 140 µM curcumin adding in all the samples 50 µL of the dilution. Caffeine (Sigma Aldrich) was prepared as a 100 mM stock solution in Phosphate Buffered Saline (PBS) (Biochrom AG).

C.5.1.2. Culture conditions

Peripheral blood lymphocytes were cultured by adding 1 ml of whole blood to 10 ml of McCoy's 5A medium supplemented with 10% FBS, 1% PHA, 1% glutamine and antibiotics (penicillin: 100 U/ml; streptomycin: 100 µg/ml). Cultures were incubated for 72 h before their use for radiosensitivity estimation experiments. At the time of 71h the different concentrations of curcumin and *trans*-resveratrol were added to the samples. Two tubes were used as controls; one with the compound solvent (95% ethanol) and another with only the blood in culture medium. The culture of the samples was interrupt by the irradiation procedure (**Figure 37**).

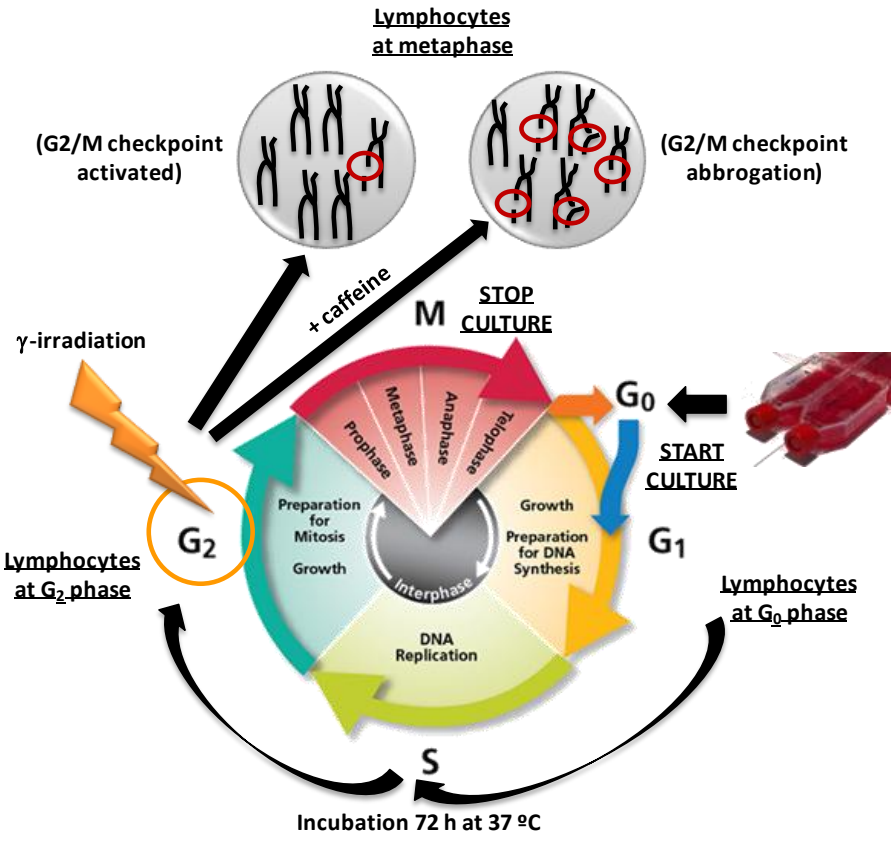

Figure 37. Schematic representation of the keys steps of G2-assay.

C.5.1.3. Irradiation conditions

Irradiation was carried out in a Gamma Cell 220 irradiator at room temperature and at a dose rate of 0.45 Gy/min (**Figure 38**).

Figure 38. Flasks in the Gamma Cell 220 irradiator (Institute of Radioisotopes & Radiodiagnostic Products, National Center for Scientific Research, NCSR ''Demokritos'', Athens).

C.5.1.4. Staining technique

The slides were air-dried and stained in a 2% solution of Giemsa dye (Merck) for 5 min and rinsed with water. Dried slides were embedded with cover slips and coded for analysis.

C.5.1.5. Cytogenetic analysis

Chromosomal damage was visualized and quantified as chromatid breaks (ctb) in cells at metaphase. For each experimental point, 50 cells were scored based on standard criteria. Ctbs and gaps were scored, the latter only when longer than a chromatid width. Light microscopy was coupled with an image analysis system (MetaSystems) to facilitate scoring. Standard deviations of the mean values from two to three independent experiments were calculated. Following this protocol, chromosomes were analyzed and 2 distinct yields were obtained for each individual after G2-phase irradiation. One yield corresponded to that obtained when the conventional G2 assay was applied, and the other represented, for each individual, the maximum yield of chromatid breaks obtained when the G2 checkpoint was inactivated by means of caffeine. The two yields (with and without caffeine) were calculated for each experiment: control without compounds, control with solvent (95% ethanol) and curcumin and *trans*-resveratrol pre-treated samples. The difference between the two yields obtained reflects the efficiency of the G2 checkpoint to prevent chromatid

breakage, so that the G2 checkpoint efficiency for each experiment was calculated as the ratio (G2caf-G2)/G2caf.

C.5.1.6. Statistical analysis

Statistical analysis was performed using a linear multiple regression model. The model considered the mean value of the ctb/cell as the response variable and the ethanol 95%, compound concentration and caffeine as explanatory variables.

C.5.2. Individual radiosensitivity. Case report

C.5.2.1. Patient characteristics and blood sample

The patient was a woman 44 years old. Her medical anamnesis reflected a hepatomegaly with cirrhosis, idiopathic portal hypertension and arteriovenous and porto-systemic shunts. She presented splenomegaly and the blood analysis showed a slight decrease in platelet levels and a tendency in decreasing the red blood cell line. She had been diagnosed with 3 aneurysms located at: splenic artery, posterior communicating artery cerebellar and left posterior inferior cerebellar artery. The patient was admitted to the hospital for embolization of the aneurysm of the left posterior artery by interventional vascular radiology with an accumulated time of fluoroscopy of 55.47 min. After the intervention a complete loss of hair occurred in the irradiated area. The G2-assay was then carried out in order to study whether the patient was in the radiosensitive group of the population. At the same time, the Protection Radiology Service of the hospital estimated the maximum dose and the effective dose skin by physical and biological dosimetry. In addition, a routine medical surveillance was conducted to monitor the patient and blood sample test.

One subject with no known irradiation exposure was taken as a control. After obtaining their informed consent, a heparinized blood sample (2 ml) was taken from the subjects for the individual radiosensitivity G2-assay.

All the results were pooled in order to clarify the main cause for the hair loss in this patient.

C.5.2.2. Culture conditions

Peripheral blood lymphocytes were cultured by adding 1 ml of whole blood to 10 ml of PB-MaxTM Karyotiping medium and incubated at 37 ºC for 72 h before their use for G2-assay. The culture of the samples was interrupt by the irradiation procedure. Caffeine (Sigma Aldrich) was prepared as a 100 mM stock solution in Phosphate Buffered Saline (PBS) (Biochrom AG) in order to be added immediately after the irradiation procedure.

C.5.2.3. Irradiation conditions

Irradiation was carried out in a Lineal accelerator Clinac iX (Varian Medycal Systems, Palo Alto, CA, USA) located at Hospital La Fe (Valencia) at room temperature and at a dose rate of 200 cGy/min (**Figure 39**).

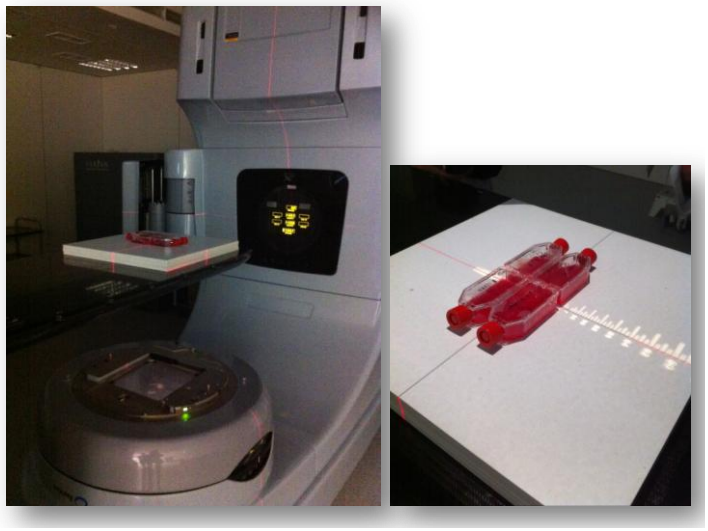

Figure 39. Flasks in the lineal accelerator Clinac iX (Hospital La Fe, Valencia).

Proliferating cells from the patient and the control were irradiated *in vitro* in G2-phase with 1 Gy and the culture was divided immediately after, so that one half was treated with 200 µL caffeine (4 mM) and incubated for 30 min at 37˚C to allow the division of irradiated cells, while the other half was cultured for the same time period without the presence of caffeine (**Figure 37**). Fifty µL of Colcemid® (Gibco) was subsequently added to the cell cultures for 60 min.

At 90 min post irradiation, cells were collected by centrifugation, treated in 75 mM KCl for 10 min, fixed with lymphocytes fixative solution and processed for cytogenetical analysis (See section ***C.1.2.*** for culture detention).

C.5.2.4. Staining technique

The staining technique was exactly carried out as described in section *C.6.1.4.*

C.5.2.5. Cytogenetic analysis

Chromosomal damage was scored as explained in the Section *C.6.1.5.* For each subject 50 cells were scored for chromatid breaks, based on standard criteria. Chromatid breaks and gaps were also scored, the latter only when longer than a chromatid width. Chromosomes were analyzed at the subsequent metaphase and two distinct yields of chromatid breaks were obtained for each individual after G2-phase irradiation. One yield corresponded to that obtained when the conventional G2 assay was applied and the other represented the maximum yield of chromatid breaks obtained when the G2 checkpoint was inactivated by means of caffeine. The two yields (with and without caffeine) was calculated for each subject. Using these two yields, the individual radiosensitivity (IRS) can be evaluated using the formula IRS = (G2/G2caf) x 100%. The difference between the two yields obtained reflects the efficiency of the G2 checkpoint to prevent chromatid breakage, thus, the G2 checkpoint efficiency for each individual is calculated as the ratio (G2caf-G2)/G2caf. The IRS for each subject was compared with the normal distribution obtained after the experiments carried in a healthy population by Pantelias and Terzoudi (2011).

RESULTS AND DISCUSSION

D. RESULTS AND DISCUSSION

D.1. Tobacco study

D.1.1. SCE analysis

The results from the analysis of the SCEs showed a total SCE frequency mean of 8.19 with a 95% CI from 7.88 to 8.50. The best model, according to the model selection procedure, included only the smoking variable, rejecting the effect of age and sex (**Table 3**).

Table 3. Selected linear mixed model for SCE frequency by AIC_C criterion.

	Estimate	Std. Error	t-value	p-value
Intercept	7.46	0.21	35.82	<0.001
Light smoking	1.21	0.34	3.52	<0.001
Heavy smoking	2.02	0.41	4.92	<0.001

According to the model estimates for the coefficients, the mean frequency of SCE in non-smokers was 7.46 (95% CI [7.12; 7.81]), in the light smoking group it was 1.21 (95% CI [0.60; 1.88]) units higher and in the heavy smoking group the mean was 2.02 (95% CI [1.27; 2.78]) units higher. The effect of smoking, with a p-value <0.001, was statistically highly significant. In addition, both, heavy and light smoking status, were associated with a significant increase in the SCE frequency (**Figure 40**).

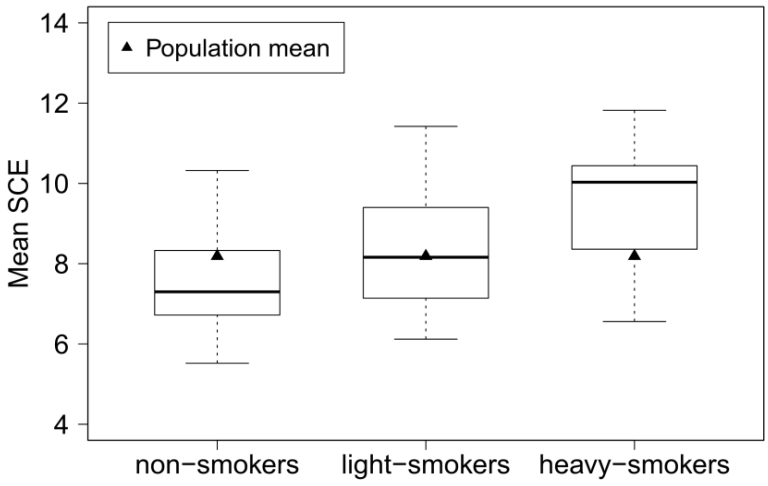

Figure 40. Comparison of SCE frequencies distribution between non-smokers, light and heavy smokers and the population mean. Distribution of the mean frequency of SCEs in the population.

D.1.2. HFC Analysis

The results of the second analysis, regarding count of HFCs per individual, were similar. First, the distribution of SCEs was calculated, which had a 95th percentile of 16 (**Figure 41**), then cells with more than 16 SCEs were considerate as HFCs.

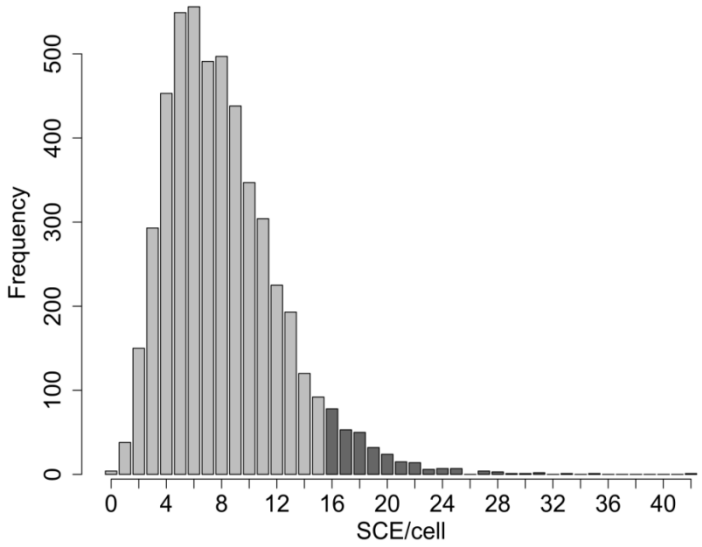

Figure 41. Graphic showing the distribution of SCEs with a 95th percentile of 16 in order to classify the HFCS.

Mean number of HFCs/individual was 3 with a 95% CI from 2.35 to 3.85. The best model, according to the model selection procedure, included only the smoking variable (p-value<0.001), rejecting the effect of age and sex (**Table 4**).

Table 4. Selected negative binomial model for count of HFCs by AIC_C criterion.

	Estimate	Std. Error	t-value	p-value
Intercept	0.33	0.17	1.92	0.055
Light smoking	1.08	0.25	4.30	<0.001
Heavy smoking	1.44	0.29	5.04	<0.001

The effect of smoking, with a p-value<0.001, was statistically highly significant. Both, heavy and light smoking status was associated with a significant increase in HFCs count per individual. According to the model estimates for the coefficients, the mean HFCs count per individual in the non-smoking group was 1.38 ($e^{0.33}$) (95% CI [0.99;

1.93]), in the light smoking group it was 4.07 ($e^{0.33+1.08}$) (95% CI [2.50; 6.69]) and in the heavy smoking group the mean was 5.83 ($e^{0.33+1.08+1.44}$) (95% CI [3.38; 10.36]) (**Figure 42**).

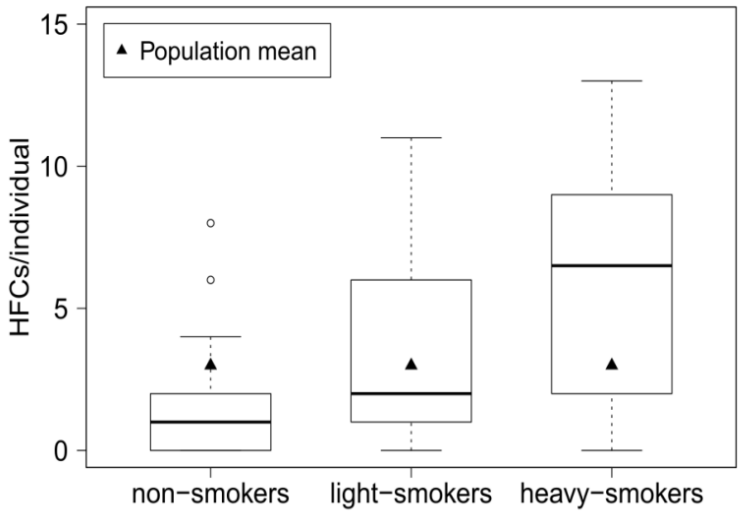

Figure 42. Comparison of HFCs count per individual between non-smokers, light and heavy smokers and the population mean.

D.1.3. SCE distribution pattern

Because of the estimated ratio for a random distribution of SCEs in a cell grows as a function of the number of SCEs (see **Figure 28**), the expected ratio from the observed data was subtracted first of all as a measure of deviation from a random distribution pattern. The best model included only the variable "smoking status" (p-value<0.001) rejecting the effect of age and sex (**Table 5**).

Table 5. Selected linear mixed model for the SCE aggregation pattern ratio by AIC_C criterion.

	Estimate	Std. Error	z-value	p-value
Intercept	0.011	0.003	3.17	0.0015
Light smoking	0.011	0.006	2.04	0.042
Heavy smoking	0.015	0.007	2.26	0.024

Both, heavy and light smoking status was associated with a statistically significant increase in the ratio ($p<0.05$). The light smoking status increased the mean value in 0.011 units and the heavy smoking status increased the mean in 0.015 units.

To the best of my knowledge this is the first study which assesses the tobacco-related genotoxic effect in peripheral blood lymphocytes by SCEs on the Spanish population. It is known that SCEs is a common biomarker to assess the genotoxic potential of several agents because its expression reflects possible alterations of the cell cycle or genetic damaging events at the chromosomal level (Mudry *et al.,* 2012).

It was originally decided to include in the statistical model the age and sex variables, however, the analysis showed that they did not influence the results, and so they were not included in the final model. The presented results match those of Husum *et al.* (1982) and Hedner *et al.* (1983) who studied a group of smokers and non-smokers and observed that, in their study, sex did not significantly influence the SCEs frequency. However, different results have also been found; Anderson *et al.* (1986) observed that females had significantly higher frequencies of SCEs than males, and cigarette smoking significantly increased such values after correction for sex. Lazutka *et al.* (1994) reported similar results, indicating that SCEs increased with age and cigarette smoking intensity, and higher SCE frequencies were observed in females. It is necessary, therefore, to consider whether the age and gender variables can influence the statistical model results.

After discarding the sex and age variables, we only considered in our statistical model the tobacco smoking variable (non-smokers, light smokers defined as less than 10 cigarettes/day- and heavy smokers as more than 10 cigarettes/day-). The results showed that the effect of smoking in general was statistically highly significant than non-smoking. Moreover, smoking classifications, heavy and light smoking status were associated with a significant increase in the frequency of SCEs compared to the non smoking status which means than even the light smokers had a significantly higher SCEs frequency than non-smokers.

Since the 70's, quite a number of studies have attributed the effect of the tobacco habit on the increase of the individual frequency of SCEs. At the beginning, Lambert *et al.* (1978) reported that, among 43 subjects, those who were cigarette

smokers had significantly higher frequencies of SCEs frequencies than non-smokers. They observed a stepwise increase of about 15% in the average SCE frequency among moderate (<10 cigarettes per day) and heavy smokers (≥10 cigarettes per day) and they ascribed their results to benzo(a)pyrene (BP) which, as they stated, increased the frequency of SCEs almost two-fold in human lymphocytes *in vitro*. Actually, the BP is classified as a carcinogen for humans (Group 1) (IARC, 2012). Moreover it has been shown that BP induces disruption of cell cycle kinetics and cell cycle arrest in S-phase, which is a preferential condition for SCEs induction (Matsuoka *et al.,* 2001). Iit has been indeed demonstrated that BP can induce SCEs in human lymphocytes analyzed in metaphase and even in G2-phase prematurely condensed chromosomes (G2-PCCs) (without going through the cell cycle) highlighting the genotoxic activity of this tobacco compound (Hatzi *et al.,* 2011).

Wulf *et al.* (1983) studied a group of healthy human subjects non-exposed to known mutagens, without regular medication, and had suffered no viral or bacterial infections for two months before the study. The subjects were cigarette smokers, cheroot or pipe smokers and in the cigarette group they included smokers of high-tar cigarettes with filter, high-tar cigarettes without filter and low-tar cigarettes with filter, all of them with the same cigarette consumption. The SCE mean values in 3 categories of cigarette smokers were statistically higher than the mean value observed in the non-smokers group. However there was no statistically significant difference between the three groups of cigarette smoking. They also detected that even the mean value of the SCEs for pipe and cheroot smokers were always higher than the non-smoking groupso that all smokers of different tobacco types had an increased frequency of SCEs.

The group of Kao-Shan *et al.* (1987) examined the SCEs in the peripheral blood and bone marrow in 18 smokers with an average cigarette use corresponding to 6 pack years. They found a significant increase in the mean number of SCEs in both bone marrow cells ($p<0.001$) and peripheral blood lymphocytes ($p<0.005$) of the smoker group. When they classified the smoking group in less than 5 pack years and 5 or more they observed in both, bone marrow cells and in peripheral blood lymphocytes, a significantly increased frequency of SCEs in the subgroup of smokers with 5 or more cigarette pack years, but not a significant increase in SCEs for the subgroup of smokers

with less than 5 cigarette pack years. In addition to this, they they provided a more detailed analysis of fragile sites in lymphocytes because of the importance of the relationship of fragile sites to a genetic predisposition for development of human tumors. The peripheral lymphocytes of smokers then demonstrated a significantly higher frequency of fragile sites, an increased number of metaphases with extensive breakage and an expression of fragile sites at some cancer breakpoints and oncogene sites. They found a higher frequency of SCEs in peripheral blood lymphocytes than in bone marrow cells and they stated that chromosomal DNA of peripheral blood lymphocytes is sensitive to cigarette smoking, so that it is not necessary to take the bone marrow cells which is a more invasive technique.

Once more, an increase in the frequency of SCEs in the peripheral lymphocytes of smoker volunteers (6.5 ± 0.3) compared to non-smokers (4.1 ± 0.2) was observed by Sardaş *et al.* (1991) where both the duration of smoking and the number of cigarettes smoked per day appeared to influence the frequency of SCEs specifically, those who smoked more than 10 cigarettes per day and who had habitually smoked for over 10 years had a higher SCE frequency.

An interesting study was performed by Milić *et al.* (2008) which aimed at assessing the genotoxic hazard for workers in the cigarette manufacturing industry because it has been reported that tobacco workers producing cigarettes are exposed to a wide range of chemical compounds that have been shown to be genotoxic or carcinogenic, such as nicotine, nitrosamines, formaldehyde, acetaldehyde, crotanaldehyde, hydrazine, arsenic, nickel, cadmium, benzopyrene and potassium. They divided the groups into exposed and non-exposed to genotoxic agents and both were also divided into smokers and non-smokers. As was expected, smoking subjects had in general a higher frequency of SCEs than non-smokers in both the control and exposed groups. Furthermore, exposed smokers showed a significantly higher frequency of SCEs than the control smokers. Finally, their data showed that exposure to tobacco dust caused a significant increase in the frequency of SCEs.

Recently, Ben Salah *et al.* (2011) carried out a study in Tunisia, even though during recent years it has been established that the prevalence of smoking has declined in many developed countries because of the growing concern about the effects of smoking on health. However, this habit continues to increase in low- and

middle-income countries. The authors mentioned that in their country, an epidemiological study conducted by another research group which took 5696 subjects showed that up to 30–35% of the population were smokers being and was especially prevalent among men (55%). Statistical data published in the Tunisian cancer registry show an alarming 54% increase in lung cancer incidence in the previous 10 years. As expected, they indicated that the mean frequency of SCEs per cell was significantly higher in smokers than in non-smokers. They divided the smoking group into heavy smokers (smoking > 10 years) and in light smokers (smoking ≤ 10 years). The difference was still statistically significant between the group of light and heavy smokers but not between the light and non-smokers. Results from both studies suggest that the mean frequency of SCEs might increases not only because of the number of cigarettes smoked but also by the number of years an individual had been smoking.

Another recent study of Khabour *et al.* (2011) also evaluated the genotoxicity of cigarette smoking in volunteers from Jordan using the SCEs assay. Our results were in agreement with these results since SCE frequencies in the cigarette smoking group significantly increased compared with those of non-smokers. Furthermore, they aimed to compare SCEs value between cigarette and waterpipe smokers and found that SCE frequencies in the waterpipe smoking group were higher than in the control group (non smokers) and even higher than in the cigarette smoking group. In addition, they even found statistically significant differences between SCE frequencies of light, medium and high waterpipe smokers, similar to our results between light and heavy smokers. They concluded that waterpipe smoking was strongly genotoxic to users, regardless of the way in which the waterpipe is smoked and the genetic background of the population, the genotoxicity increases with use and surprisingly is higher than that of regular cigarette smoking.

In an attempt to carry out a more sensitive and accurate analysis, we analyzed the HFCs parameter since HFCs may represent a more sensitive criterion for assessment of exposure, such as the smoking habit, than the mean SCE values (Ben Salah *et al.,* 2011). According to the analysis of HFCs, we found that a heavy and light smoking status were associated with a significant ($p<0.001$) increase in the HFC count per individual. Some authors have postulated that HFCs could represent a subpopulation of DNA repair-deficient lymphocytes. Interpreting SCEs as a signal of

DNA damage and due to the fact that SCE frequency decreases through repeated cell cycles as a consequence of DNA damage removal, it can be inferred that HFCs, although initially damaged more, succeeded in removing most SCEs inducing lesions over three cell cycles, especially after the first cycle. The greater number of SCEs observed, therefore, in HFCs could be attributable to a higher level of initial damage and not to repair deficiencies. Other authors state that HFCs may represent a subpopulation of older lymphocytes which have an increased likelihood of accumulating a larger number of lesions during the G0 phase *in vivo*, thus showing a predisposition to SCEs (Ponzanelli *et al.,* 1997). In the present study by analyzing HFCs we also consider the older lymphocytes that could have been accumulating lesions from tobacco compounds.

From the analysis of the SCE distribution pattern, both a heavy and light smoking status, were associated with a significant increase in the ratio. This means that the SCEs were not uniformly distributed among the 46 chromosomes in the cell, and it seems that a tendency exits to accumulate the SCEs in only one chromosome rather than being distributed among all of them. The mechanisms by which some compounds can induce SCEs are different; some polyphenols damage the DNA and may produce SCEs by arresting S phase through cleaving the DNA (Matsuoka *et al.,* 2001), while others, such as antitumor antibiotics (i.e., mytomycin), damage the DNA in the presence of a redox-active metal ion such as iron (Fe^{2+}) or cooper (Cu^{2+}), as well as molecular oxygen (O_2) (Hecht, 2000). Considering this last mechanism, it can be postulated that among the wide variety of tobacco compounds some of them can act like antitumor antibiotics, this means that they would suffer activation by a redox complex with metal ions from DNA. These active products, usually with a short life, would produce a clustering damage in the same chromosome. In our analysis the fact that one chromosome is more damaged than another cannot be attributed to one specific compound due to the complex tobacco composition (more than 4000 compounds). However, it has been described that some molecules such as nitroimidazole derivates did not affect all the chromosomes equally, supporting the idea of the "specific genomic targets". Even analyzing SCE frequencies by chromosome, these authors observed that certain chromosomes showed higher

susceptibility to these treatments and moreover, they specified which region inside the band was more affected and emphasized the importance of the so-called “hot spots” and the validity of SCE biomarkers as cytogenetic indicators of genomic fragility (Carballo *et al.,* 2009; Mudry *et al.,* 2011). Some compounds from tobacco could affect the chromosomes in the same way.

In vitro toxicology studies of tobacco and tobacco smoke had been a useful tool to understand why tobacco consumtion causes cancer and a plethora of *in vitro* assays are available assessing tobacco smoke modes of action, mostly using non–human cell models. SCEs are one assay considered for this assessment, using mainly cultured CHO cells or human lymphocytes, in part because it is a technical challenge to obtain a high percentage of synchronized metaphases using cell types. Johnson *et al.* (2009) reviewed the literature available for these assays and observed that it had been demonstrated that cigarette smoke condensates (CSCs) and total particulate matter (TPM) are capable of inducing SCEs in *in vitro* studies in a concentration-dependent manner. This means, that they CSCs and/or TPM should have genotoxics agents which can induce SCEs. In a Spanish study covering 10 tobacco brands (Marcilla *et al.,* 2012), the authors carried out an extensive analysis to elucidate the main compounds found in mainstream tobacco smoke. The analysis of vapor fraction showed that the major compounds were principally carbon monoxide (CO) and carbon dyoxide (CO_2) and it is well known that CO is a poison. In addition the small aldehyde molecules present in tobacco smoke are particularly harmful, especially acetaldehyde. Another remarkable compound found in the study was the 1,3-butadiene; this compound presents the highest cancer risk index of all constituents of cigarette smoke and metabolite 1,2:3,4-diepoxybutane was shown to induce SCEs *in vitro* in human lymphocytes (Kligerman and Hu, 2007). Furthermore, in the Spanish study, 85 components were identified in the particulate matter such as nicotine, polycyclic aromatic compounds or nitrosamines which according to IARC (2004) are known carcinogens.

In vitro studies are correlated with those *in vivo* studies mentioned here with our results. Unfortunately the *in vitro* toxicology methods provide data that cannot reliably be extrapolated to infer human cancer risk and were intended primarily as screening methods for chemicals to identify possible human carcinogen (Johnson *et*

al., 2009). *In vivo* studies are necessary therefore to detect and evaluate the impact of the tobacco habit in human health with the use of biomarkers.

1969 was an important date prompting the global concern about the health effects of tobacco consumption, when the IARC initiated a program for the evaluation of the carcinogenic risk to humans of chemicals involving the production of critically evaluated monographs on individual chemicals (IARC, 2004). As a result, in the last few years, evidence has been accumulating regarding the carcinogenicity of tobacco smoke. After extensive research it is now known that both tobacco and tobacco smoke are very complex matrices consisting of thousands of compounds. A total of 3044 constituents have so far been isolated from tobacco and 3996 from the mainstream smoke of cigarettes and a total of 1172 constituents are present both in tobacco and tobacco smoke. Advances in chemical analytical techniques and an increased knowledge of the genotoxic environmental agents brought the number of carcinogens identified in tobacco smoke to 69 by the year 2000. These carcinogens include 10 species of polynuclear aromatic hydrocarbons (PAHs), 6 heterocyclic hydrocarbons, 4 volatile hydrocarbons, 3 nitrohydrocarbons, 4 aromatic amines, 8 N-heterocyclic amines, 10 N-nitrosamines, 2 aldehydes, 10 miscellaneous organic compounds, 9 inorganic compounds and 3 phenolic compounds. 11 compounds (2-naphthylamine, 4-aminobiphenyl, benzene, vinyl chloride, ethylene oxide, arsenic, beryllium, nickel compounds, chromium, cadmium and polonium-210) classified as IARC Group 1 human carcinogens have been reported as present in tobacco smoke (IARC, 2004).

From this present study it can be stated that the frequencies of SCEs and HFCs from the cigarette smoking group significantly increased compared with those of non-smokers. Moreover it seems that there exists a distribution pattern that concentrates the damage in some chromosomes instead of distributing homogeneously, which can be interpreted as a genomic susceptibility to tobacco compounds. The findings are in agreement with other authors when using the SCE assay to confirm that smoking induces chromosome damage and to justify the need to launch campaigns and laws to reduce cigarette consumption.

D.2. Ardystil syndrome

D.2.1. SCE and HFC analysis

Both analyses yielded non-significant results for Ardystil exposure. We found no evidence of an association between SCE frequency and Ardystil exposure (p-value=0.84) and no association between HFCs and Ardystil exposure (p-value=0.13).

D.2.2. SCE distribution pattern

Results of our analysis regarding the SCE distribution pattern showed an effect for Ardystil exposure not found in the other two previous section analyses. The best model included both, the variable "smoking status" (p-value=0.029) and "Ardystil exposure" (p-value=0.008) and rejected the variables sex and age (**Table 6**).

Table 6. Selected linear mixed model for the SCE aggregation pattern ratio by AIC_C criterion.

	Estimate	Std. Error	z-value	p-value
Intercept	0.004	0.004	1.00	0.317
Light smoking	0.011	0.005	2.07	0.041
Heavy smoking	0.015	0.006	2.37	0.020
Ardystil	0.013	0.005	2.69	0.008

Both, heavy and light smoking status, were associated with a significant increase in the ratio (p=0.041 and 0.020, respectively). The light smoking status statistically increased the mean value in 0.011 units and the heavy smoking status statistically increased the mean in 0.015 units. Concerning "Ardystil exposure" the mean value was 0.013 units higher.

It is worth mentioning that the tobacco smoking variable was included because it is known that this habit increases the background level of SCEs (Norppa, 2004). Recent studies of Ben Salah *et al.* (2011) and Khabour *et al.* (2011) indicated that the mean frequency of SCE per cell was significantly higher in smokers than in non smokers. Moreover, it has been reported that smokers exposed to other genotoxic agents have a higher frequency of SCEs than smokers not exposed to this genotoxic

(Hedner *et al.,* 1983; Milić *et al.,* 2008). Therefore the combination of both variables should be considered in our study.

Unfortunately, the evaluation of the Ardystil risk exposure by means of the SCEs biomarker is not discussed by other authors probably due to the fact that there were no studies carried out at the time of the accident. Our study was based on the idea that some authors studied the persistence of SCE frequencies in a population occupationally exposed to the well-known chemical mutagen, the vinyl chloride, and found that increased values of SCE frequencies were still present in the lymphocytes of workers up to 10 years after exposure (Fucic *et al.,* 1990). Vinyl chloride is one of the components of the Acramin F paint system. Bearing in mind that an increased SCEs frequency, caused by one component of the Acramin F system, can be found many years after the exposure, our aim was to evaluate if DNA damage could still exist in subjects affected with the "Ardystil syndrome" which can be measured by SCEs. Because of the length of time since the accident until the SCEs analysis we also evaluated the HFCs parameter which can be interpreted in terms of those lymphocytes with a longer life and thus can represent accumulated DNA damage over a long period (Ponzanelli *et al.,* 1997).

Investigations carried out by the Public Health Department established that lung disease was caused by spraying procedures delivering a respirable aerosol of Acramin FWN (a substance obtained from the reaction between a diethylenetriamine and adipic acid) which belongs to the Acramin F paint system (**Figure 43**). This system has been used since the early fifties for drying textiles. Its components are polymers with no particular "structural alerts" and according to the Material Safety Data Sheets, the paint components were considered, on the basis of standard toxicity testing procedures, to be non-irritants for the skin or eyes and safe after being swallowed (Hoet *et al.,* 1999).

However, an interesting fact is that the Acramin F paint system was intended to be applied as a paste for screen printing and not as an aerosol by air brushing as was used in the textile printing factories affected by the Ardystil syndrome. According to these data, it seems obvious that several other factors play a role in the outbreak. Moya *et al.* (1994) reported that the paints were not applied as recommended but

rather used as aerosols for airbrushing and the hygienic conditions in the factories appear to have been unhealthy since the workers were exposed to very high concentrations (up to 10 mg/m^3) of aerosolized paint.

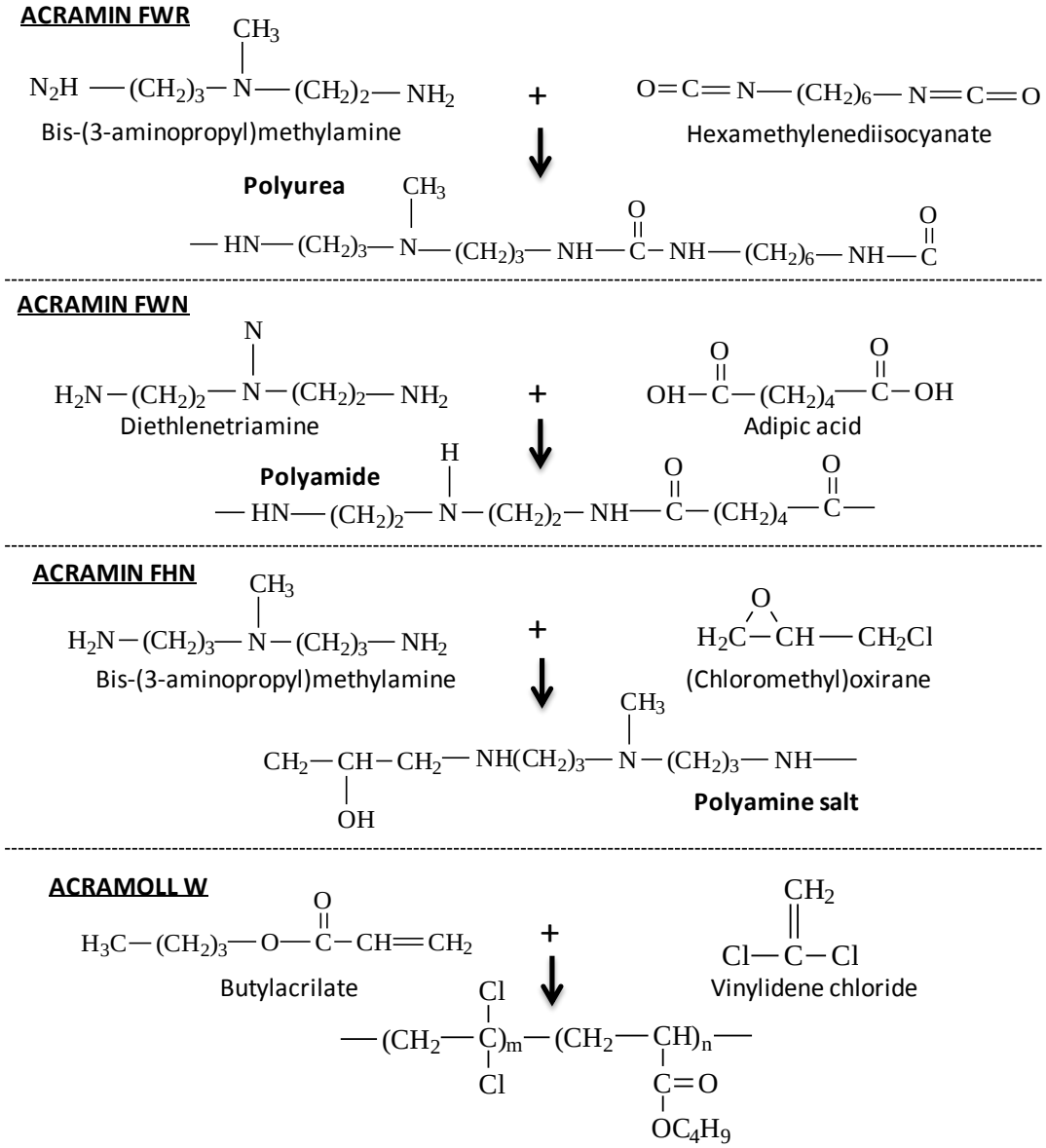

Figure 43. Chemical structure of the components of the Acramin F paint system and their precursors. No free monomers are present in the final products.

Although the Public Health Department showed strongly that the outbreak was caused with a formula change from Acramin FWR to Acramin FWN, several authors (Solé *et al.,* 1996; Clottens *et al.,* 1997; Hoet *et al.,* 1999, 2001) suggested that it was not only the Acramin FWN responsible for the "Ardystil syndrome", but the

concurrence of several toxic substances. Some studies have therefore analyzed the toxicity of the components.

In *in vivo* studies, Clottens *et al.* (1997) carried out studies on hamsters which received a single intratracheal instillation of the relevant components or mixture. The authors indicated that Acramin FWR and FWN, and another component present in the Acramin F paint system called Acrafix FHN or their mixtures, caused lung damage while other component, Acramoll W, did not show toxic effects. Moreover, based on the doses that killed 50% of the animals (LD_{50}), various components were found to be 10 to 1250 times more toxic, intratracheally, than orally, that could confirm the fact that Acramin F was used for many years without reported toxicity but its use as an aerosol, which can be absorbed by inhalation, can cause lung toxicity.

Concerning *in vitro* studies, Hoet *et al.* (1999) observed comparable results using a battery of different cell-types and assessing the *in vitro* cytotoxicity by measuring lactate dehydrogenase leakage. From their data they observed that Acramin FWR, Acramin FWN and Acrafix FHN exhibited considerable cytotoxicity (LD_{50} generally below 100 µg/ml for 24 h incubation) *in vitro*, while Acramoll W was almost non-toxic. FWN was consistently less toxic than the two other components, in human alveolar macrophages, which is contradictory to the Public Health Department statement regarding the main causal agent of the syndrome (Solé *et al.,* 1996), but in general the three compounds exhibited a similar degree of toxicity. Hoet *et al.* (1999) observed that the toxicity of FWR, FWN and FHN appeared to be neither cell-specific, nor purely dependent on a polyamine structure (since FWR is not a polyamine), thus, the authors speculate that the toxicity of these compounds could be based mainly on the fact that at physiological pH they all carry multiple positive charges on the nitrogen atoms, and that it had been shown that polycations exert a severe cytotoxicity on both epithelial and endothelial cells. Accordingly, the pulmonary epithelium would leak more due to the action of the polycations and lung disorders will consequently appear. The main cause seems therefore to be clarified since the new formulation contained significantly more polycations and that this presumably leads to more toxic effect in epithelial cells. However, according to Clottens *et al.* (1997) the new paint mixture was not more toxic than the initial one when intratracheally instillated. Therefore, Hoet *et al.* (1999)

thought that the addition of FWN possibly reduced the particle size of the aerosolized paint mixture and, probably, the penetration was increased in the respiratory tract leading to lung disorders.

A long-running court case over the "Ardystil syndrome" finally reached its conclusion on 30 June 2003, when the Spanish court issued its judgment. The Court reached a guilty verdict and that the serious pulmonary damage suffered by workers was directly related to the lack of preventive measures and continued exposure to mixtures of different chemical products, including Acramin, Acrafix, Acramoll, acetic acid and trichloroethane.

The literature was reviewed in order to discuss why compounds involved in the Ardystil syndrome can be genotoxic and could modify the frequency of SCEs at the time of exposure. On the one hand, Acramin FWN (**Figure 43**) is a polyamide-amine that has as precursors to diethylenetriamine (DETA) and adipic acid. Reisenbichler and Eckl (1993) observed that di(2-ethylhexyl) adipate, which is derived from adipic acid, did not significantly alter the dose response of SCEs. Leung (1994) and Perkowska *et al.* (2001) demonstrated that DETA was inactive in inducing SCEs.

On the other hand, Acramin FWR (**Figure 43**) is a polyurea that has other precursor, called hexamethylenediisocyanate, which has the same functional group; isocyanate (–N=C=O) that methyl isocyanate. This last has demonstrated (Conner *et al.,* 1987) not genotoxic properties measured by SCE analysis.

Acrafix FHN (**Figure 43**) is chemically a polyamine salt; polyamines are compounds which interact with the double helix stabilizing the DNA molecule (Cozzi et *al.,* 1991), thus is not the responsible for the SCE induction. However, (chloromethyl)oxirane of Acrafix FHN is a type of oxirane which has been seen to increases significantly the SCE frequencies (Kostoryz *et al.,* 2007).

Acramoll W (**Figure 43**), a non-polycationic component, has been demonstrated as non-toxic in s*in vitro* studies (Hoet *et al.,* 1999). However, it has two precursors; butyl acrylate and vinylidene chloride. A type of acrylate (ethyl acrylate) was studied by Kligerman *et al.* (1991) observing that this compound is only clastogenic at near toxic concentrations during a specific stage of the cell cycle but any increase in SCEs was observed. No literature reflected studies of SCEs and vinylidene chloride, but Zhao

et al. (1994) studied a compound similar to vinylidene chloride, the vinyl chloride, and observed the results of the analysis of SCEs in a group including workers exposed to 4.1 mg/m^3 for at least a period of 7 years had significantly higher SCE values than the control group or non-workers. Our viewpoint is similar to other authors mentioned in the literature (Solé *et al.,* 1996; Clottens *et al.,* 1997; Hoet *et al.,* 1999, 2001).

Trichloroethane was used to clean aerograph air guns. Chemically, trichloroethane reacts with acetic acid forming chloride and phosgene. For the latter, Mateuca *et al.* (2010) observed that it also significantly increased the frequency of SCEs and HFC values.

Furthermore, according to Ould *et al.* (1994) and Turuguet and Pou (1994), aziridine must be added as another potential compound involved in the "Ardystil syndrome". Aziridine is a crosslinking substance which has the function of increasing the fixation pattern on synthetic tissue in order to prevent it from sticking to the fabric roll; several authors (Ould *et al.,* 1994; Turuguet and Pou, 1994) suggested that it was used in textile paint sprayers and helped to change Acramin powder (Acramin FWR) to liquid (Acramin FWN). Aziridine is classified by the IARC as a possible carcinogen (group 2B) (IARC, 1999) because in Volume 71 of the IARC Monograph “Re-evaluation of some organic chemicals, hydrazine and hydrogen peroxide” it was concluded that it was taken into consideration that aziridine is a direct-acting alkylating agent which is mutagenic in a wide range of test systems and forms DNA adducts that are promutagenic. It could be responsible for the so-called Ardystil paradox in which it is said that the liquid Acramin (FWN) is less toxic than powder (FWR). In fact, Hoet *et al.* (1999) rejected that only Acramin FWN was responsible for this outbreak due to the fact that the formulation used had more polycation content per weight in the final paint mixture but observed, together with Clottens *et al.* (1997), the same toxicological properties in Acramin FWN, Acramin FWR or when an 13% Stoddard solvent (also called white spirit) was included in the mixture. The presence of solvent is very important in the Acramin F paint system such as the Stoddard solvent (Clottens et al., 1997) or aziridine (Ould et al., 1994; Turuguet and Pou, 1994) noted in the bibliography.

Although we did not find a statistically higher frequency of SCEs in Ardystil affected subjects compared with the non-affected, it is supposed that at the time of

the exposure the levels of SCEs could be increased because of the exposure of any or several of the genotoxic compounds previously mentioned.

To sum up the Ardystil paradox toxicity could be hypothetically explained by three factors; i) presence of impurities in the mixture consisting in several precursors, used in the Acramin F paint system, ii) the increase, in the new formulation, of polycation content which exerts interactions with anions as is DNA and, iii) the use of aziridine or other solvents in the final paint mixture, since this helps to reduce the particle size of the aerosolized paint mixture and possibly increased penetration in the respiratory tract. This evaporation would improve hygienic conditions in the factories, where areas are insufficiently ventilated without the use of respiratory protection or fume exhausters which favored evaporation and therefore reducing the particle size of the aerosolized paint mixture and in addition having wood stoves, gas, oil or butane for heating. These facts indicate that the "Ardystil syndrome" must be classified as a multifactorial disease.

This study was carried out in order to analyze the frequency of SCEs in subjects affected by the “Ardystil syndrome” and the non-affected. The initial hypothesis was to find higher frequencies of SCEs in the affected group, but on the contrary, it was found there was no statistically difference. Taking into account two factors: first, the time between the occurrence of the “Ardystil syndrome” and the analysis of blood samples is more than 10 years and second, lymphocytes have a half life of about 3-4 years, so that the data seem logical since lymphocytes present during the exposure may undergo apoptosis in approximately 3-4 years, so that changes in their genetic material could no longer be analyzed. Even the value of HFCs which can be interpreted in terms of those lymphocytes with a longer life (Ponzanelli *et al.,* 1997) was not statistically different in the “Ardystil” group.

However, the analysis regarding SCE distribution patterns showed an effect for Ardystil exposure. The estimated ratio for a random distribution of SCEs in a cell grows as a function of the number of SCEs (**Figure 28**). The expected ratio from the observed ratio as a measure of deviation from a random distribution pattern was first subtracted and it was seen that the ratio for the Ardystil group was statistically higher than the control group. This means that the SCE was not uniformly distributed among the 46

chromosomes in the cell; it seems that there is a tendency in accumulating the SCEs in only one chromosome rather than being distributed among all of them. To the best of our knowledge this is the first time that this parameter is proposed and agreed with the hypothesis expounded in the tobacco study. It is suggested that the non-uniformly distribution of SCEs among the chromosomes can be due to the fact that the mechanisms by which some compounds can induce SCEs are different; for instance, antitumor antibiotics (i.e., mytomycin) can damage the DNA in the presence of a redox-active metal ion such as Fe^{2+} or Cu^{2+}, as well as O_2 (Hecht, 2000). Considering this last mechanism with metal ions, it can be believed that among the compounds present in the Ardystil painting system some of them could be accumulated in the organism and still produce a clustered DNA damage. For instance, plumb (Pb), which has been a common component in painting systems for years, is introduced in the organism either by ingestion or inhalation. The toxic action of Pb is attributed to its affinity for molecular sites of action of calcium (Ca^{2+}), so that the bone tissue, as the main mineral compartment of the human body, has become an important element in the study of the cumulative exposure to Pb. Therefore, Pb, which is accumulated in bones, can be released to the blood stream depending on the physiological circumstances. If Pb could enter into the nuclear region of the lymphocytes, it might suffer activation by reacting with the redox-active metal ions such as Fe^{2+} or Cu^{2+} from DNA (like mytomycin) and these active products, usually with a short life, may produce a clustering damage in the same chromosome. This mechanism may explain the increased SCE aggregation pattern ratio we have detected in subjects affected by Ardystil syndrome.

D.3. Cyto- and genotoxicity of curcumin and *trans*-resveratrol

D.3.1. Cyto- and genotoxicity of curcumin

D.3.1.1. Chromosomal aberrations (CAs)

Results obtained from the analysis of CAs in human lymphocytes cultured with no treatment, 100 µL of 95% ethanol and with different curcumin concentrations are shown in **Table 7**. On the one hand, the results showed that the frequency of acentric fragments increased in the sample treated with 95% of ethanol. In addition, data

reflected a dose-dependent increase in the frequency of acentric fragments in the samples cultured with curcumin concentrations ranging from 1.4 to 140 μM. This increase in the frequency of acentric fragments was statistically significant ($p<0.05$) from 14 to 140 μM. On the other hand, the data did not show a dose-dependent increase nor a difference statistically significant ($p<0.05$) from the control samples for the other chromosomal aberrations (gaps and breaks) recorded.

Table 7. Frequency (Y ± SE) and type of CAs in normal human lymphocyte cultures exposed to different concentrations of curcumin.

Curcumin (μM)	Chromatid-type		Chromosome-type	Total aberrations (Y ± SE)
	Gaps (Y± SE)	Breaks (Y ± SE)	Acentric fragments (Y ± SE)	
0	0.02 ± 0.01	0	0	0.02 ± 0.01
0 + 95% ethanol	0	0	0.04 ± 0.02	0.04 ± 0.02
1.4	0.01 ± 0.01	0	0.03 ± 0.02	0.04 ± 0.02
2.8	0.04 ± 0.02	0	0.05 ± 0.02	0.09 ± 0.03*
5.6	0.02 ± 0.01	0	0.06 ± 0.02	0.08 ± 0.03*
14	0	0	0.13 ± 0.02*	0.13 ± 0.02*
28	0	0	0.19 ± 0.04*	0.19 ± 0.03*
140	0.04 ± 0.02	0.01 ± 0.01	0.19 ± 0.04*	0.25 ± 0.03*

•Difference statistically significant ($p < 0.05$).
SE: Standard Error.

The results reflected a genotoxic action offered by curcumin when the concentration became higher. These results are in accordance with some other studies revealing the possible genotoxic effect of curcumin depending on the concentration.

Concerning *in vitro* studies, in the work of Cao *et al.* (2006) they found that high concentrations of curcumin induced DNA damage in human hepatoma G2 cells. They used other types of markers for detecting DNA damage; quantitative polymerase chain reaction (QPCR) (to detect polymerase-blocking lesions, such as abasic sites, strand breaks, and several damaged bases), immunocytochemistry staining of 8-hydroxydeoxyguanosine (8-OHdG) (a common method to measure oxidative damage to mithocondrial as well as nuclear DNA damage) and Comet Assay (which allows

detection of DNA fragments resulting from a wide variety of DNA damage). With these three experiments they confirmed that concentrations from 28 µM of curcumin were able to induce damage of both, nuclear and mitochondrial genome. Moreover, because some of these three parameters reflect genome damage caused by an increase of oxidative levels in the cells, they measured as well the level of reactive oxygen species (ROS) and lipid peroxidation. They found that curcumin had a strong effect on ROS production, and thiobarbituric acid reactive substances (TBARS) formation (which represent lipid peroxidation) significantly increased at higher concentrations of curcumin. They suggested, therefore, that low concentrations of curcumin did not produce DNA damage but this damage was indeed induced at higher concentrations. The same working group analyzed in another study the genotoxic effect of curcumin but with another biomarker, the micronucleus (MN) assay (a topical assay to detect genotoxic effects, mainly chromosome fragments, of physical and chemical agents). Their results showed that, on the one hand, curcumin at the higher tested concentrations (22.4 and 44.8 µM) displayed a small but significant increase in the frequency of MN and, on the other hand, it was observed that the lowest tested concentration (5.6 µM) significantly reduced the MN formation induced by the chemotherapeutic agent cyclophosphamide. They confirmed that curcumin showed both genotoxicity and antigenotoxicity depending on its concentration (Cao *et al.,* 2007). Similar results regarding genotoxicity were obtained in the study of Mendonça *et al.* (2009) which evaluated the genotoxic effect of curcumin in PC12 cells obtained from rat pheochromocytoma with the MN assay. They observed an induction of chromosome damage in binucleated cells at curcumin concentrations of 28 µM, this difference was statistically significant from the control group. Nevertheless, they observed both, genotoxic and antigenotoxic effects, as mentioned in the study of Cao et al. (2007); when cells were pre-treated with curcumin before adding cisplatin (an antineoplasic pharmaceutical) the frequency of MN was significantly reduced compared with cells without curcumin pre-treatment, confirming the antigenotoxic effect of curcumin. This action against cisplatin DNA damage was explained by Alaikov *et al.* (2007) who showed that curcumin had DNA protective activity in a panel of eight leukemic cell lines by enhancing the scavenging of free radicals induced by cisplatin.

The biphasic response of curcumin for genotoxic and antigenotoxic properties is once more demonstrated.

All the studies mentioned before ascribed the genotoxic effects of curcumin its biphasic antioxidant/pro-oxidant properties which can lead to DNA damage measured by different methodologies. In the study of Banerjee *et al.* (2008) they directly studied the antioxidant or pro-oxidant activity of curcumin in one type of cells. They analyzed the ability of curcumin to prevent oxidation of intracellular glutathione (GSH) in red blood cells but they observed mixed results; at low concentrations of curcumin (<10 µM) it prevented GSH depletion, and at higher concentrations, the GSH levels decreased gradually because of the pro-oxidant activity of curcumin. Hence, it is clear that curcumin is a polyphenol which can exert a dual role with its antioxidant/pro-oxidant properties and the genotoxicity of curcumin caused by this kind of ability can be different according to its concentration.

Regarding the literature, curcumin is not the only pholyphenol which shows pro-oxidant activities, other polyphenols have also shown this, and therefore DNA-breaking, properties. Aydin *et al.* (2005) reported that concentrations above 100 µM of thymol, 100 µM of γ-terpinene and 50 µM of carvacrol significantly induced DNA damage in human lymphocytes, analyzed by the Comet Assay. In the study of Bhat *et al.* (2007) the caffeic acid also showed DNA breakage in human peripheral lymphocytes. Furthermore, Kocaman *et al.* (2011) observed a bicyclic monoterpene alcohol present in the essential oils of many medicinal and aromatic plants also induced CAs on human lymphocytes at concentrations ranging from 80 to 300 µM.

The present results and the previous mentioned show that curcumin, as well as other natural substances, can have a certain genotoxic effect in some concentrations. A possible mechanism for the DNA damaging properties of curcumin is proposed by Kocaman *et al.* (2011) who stated that curcumin contributs to the formation of CAs by breaking the phosphodiester backbone of DNA.

From another point of view, the pro-oxidant activity of curcumin is not the only mechanism by which this molecule can damage the DNA and may finally induces CAs as we found in our study. Another mechanism responsible for the DNA damage is the clastogenic effect that curcumin can exert. A clastogenic agent is an agent able to

induce chromosome breaks leading to sections of the chromosome being deleted, added, or rearranged (Dunn and Curtis, 1985). The reason why curcumin can act as a clastogenic agent was explained in the work of Snyder and Arnone (2002) who reported the clastogenic activity of curcumin which might be due to its poisoning of cellular DNA Topoisomerase II. Topoisomerases are enzymes that regulate the supercoiling of chromosomal DNA, and play pivotal roles in chromosome replication, transcription, recombination, segregation, condensation and repair. Curcumin can act as a topoisomerase poison helping in a deleterious effect on the genomic stability of non-tumorous cells by increasing the incidence of homologous and non-homologous recombination effects. These events result in chromosomal changes such as clastogenic effects. They facilitate the relaxation of supercoiled DNA, essentially through a mechanism involving the breakage of a phosphodiester bond of either one strand (topo I) or both strands (topo II) of the duplex DNA (Webb and Ebeler, 2004). Therefore, compounds that inhibit topo I or topo II can contribute to this DNA fragility. The DNA damage by the ability called "Topoisomerase II poisoning" was also observed in many flavonoid compounds (Webb and Ebeler, 2004).

These studies and the present results indicated that curcumin can exert genotoxic effects at high concentrations which can finally induce CAs, as is observed in this study, or another type of DNA damage. The mechanisms by which curcumin can damage the DNA may be by pro-oxidant activity or by clastogenic effect. However, it must be also considered that low curcumin concentrations are not genotoxic and moreover, in combination with another type of genotoxic agent, curcumin can prevent the DNA damage induced by the agent, mainly by its antioxidant action. Some authors have postulated that the pro-oxidant action may be related to its anticarcinogenic effects (Yoshino *et al.,* 2004).

D.3.1.2. Sister Chromatid Exchanges (SCEs)

Concerning results obtained from the analysis of SCEs in human lymphocytes cultured with no treatment (control sample), 100 µL of 95% ethanol and different curcumin concentrations are presented in **Table 8**. The frequency of SCEs in the

curcumin treatments at the evaluated concentrations were not statistically significant ($p<0.05$) when compared to the sample without treatment.

Table 8. Frequency of SCE (Y_{SCE} ± SE) in human lymphocytes exposed to different concentrations of curcumin.

Curcumin (µM)	Y_{SCE} (±SE)
0	6.90 ± 0.60
0 + 95% ethanol	7.53 ± 0.53
1.4	5.64 ± 0.50
2.8	6.88 ± 0.70
5.6	7.16 ± 0.56
14	6.44 ± 0.41
28	5.56 ± 0.59
140	8.00 ± 0.65

SE: Standard Error.

The SCEs assay in human peripheral lymphocytes is widely used to detect occupational and environmental exposures to genotoxic compounds because it has been shown that it is a highly sensitive parameter for evaluating human exposure to mutagenic and carcinogenic agents (Gadano *et al.,* 2006). Moreover a lot of studies have used in recent years this parameter to assess the anti/genotoxic effects of a great quantity of natural products (Alpsoy *et al.,* 2011; Dirican *et al.,* 2011; Kocaman *et al.,* 2011; Kotan *et al.,* 2011; Buyukleyla *et al.,* 2012; Rencuzogullari *et al.,* 2012).

Our results showed a small variation in the frequency of SCEs, a fact that, according to Stanimirovic *et al.* (2005) can be explained by slight variations caused by some other endogenous factors such as oxidative stress. The fact that curcumin increased the acentric fragments but not the frequency of SCEs seems to indicate, and help to prove the idea mentioned in the previous point, that curcumin could be a clastogenic factor, but S-phase independent; because S-phase arrest is a preferential for the SCE formation (Matsuoka *et al.,* 2001). The literature reflects several studies comparing curcumin with other compounds; Giri et al. (1990) studied SCEs induced by curcumin and tartrazine on bone marrow cells of mice and rats following acute and

chronic exposure via the diet and demonstrated that except for low concentrations in the curcumin treated series, a significant increase in SCEs was observed in all the concentrations of curcumin tested.

D.3.1.3. Mitotic Index (MI) and Proliferation Index (PI)

For the study of the MI, a total of 500 cells were scored for each experiment and for the PI, a total of 100 cells. Data relating to the MI, relative mitotic index (RMI), PI and relative proliferation index (RPI) of the different curcumin concentrations are summarized in **Table 9**. On the one hand, data from the MI revealed that all the curcumin concentrations tested induced a MI higher than those from the control sample without treatment. This increase was statistically significant ($p<0.05$) at curcumin concentrations ranging from 2.8 to 140 µM. On the other hand, concentrations of 5.6 and 14 µM increased the PI in a statistically significant way ($p<0.05$) while concentrations of 1.4 and 140 µM presented a PI lower than the control value but this difference was not statistically significant ($p<0.05$).

Table 9. Results of MI, RMI, PI, RPI in human peripheral lymphocytes pre-treated with various concentrations of curcumin (Mean ± SD).

Curcumin (µM)	MI (Mean ± SD)	RMI	PI (Mean ± SD)	RPI
0	0.04 ± 0.01	100	1.56 ± 0.08	100
0 + 95% ethanol	0.04 ± 0.01	116.28	1.53 ± 0.08	97.74
1.4	0.05 ± 0.01	153.90	1.39 ± 0.06	89.03
2.8	0.08 ± 0.01*	222.56	1.68 ± 0.08	107.44
5.6	0.08 ± 0.01*	232.59	1.89 ± 0.08*	120.87
14	0.09 ± 0.01*	239.74	1.95 ± 0.08*	124.74
28	0.09 ± 0.01*	247.48	1.61 ± 0.07	102.97
140	0.09 ± 0.01*	248.93	1.45 ± 0.06	92.73

•Difference statistically significant ($p < 0.05$).
SD: Standard deviation.

Regarding the RMI it is noteworthy that the value obtained at the concentrations from 2.8 to 140 µM was more than 2-fold the value obtained in the control sample. On

the other hand, the RPI did not differ so much from the control sample RPI as in the RMI case; the maximum RPI reached was at 14 µM curcumin which had a value 24% higher than the control. A graphical representation of the RMI and RPI is shown in **Figure 44**.

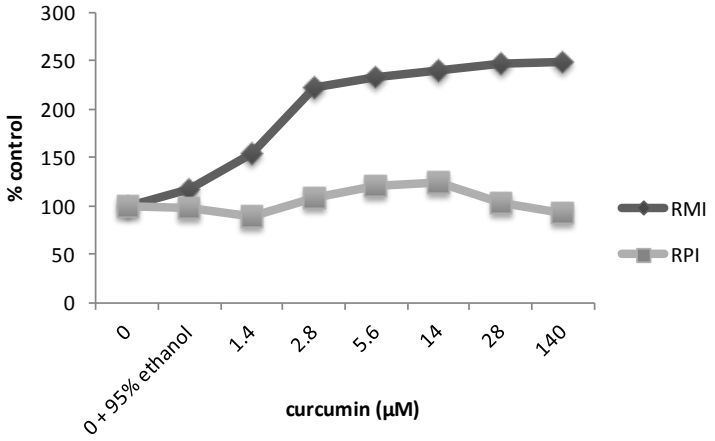

Figure 44. Graphic with RMI and RPI for each lymphocytes curcumin treatment. Results for control cultures are taken as 100%.

Parameters such as MI and cell proliferation kinetics, scored by PI, have been proposed as useful biomarkers for the pre-screening of the potential cytostatic activity of new drugs. The use of the cell proliferation kinetics and the MI are recognized biomarkers in biological monitoring to evaluate lymphocyte proliferation in population as well as to evaluate normal or tumour cells (Rojas *et al.,* 1993).

Our work reflected that MI was significantly ($p<0.05$) increased in almost all the concentrations whereas the PI was statistically modified only in two concentrations. This behavior in the MI indicates that curcumin could stimulate the cell division process, acting as a mitogen such as phytohemaglutinin. Sherr and DePinho (2000) and Stanimirovic *et al.* (2005) suggested that the increase of both indexes is influenced by two factors, the ‘‘culture shock’’ and the ‘‘mitotic clock’’ especially considering that increases in these parameters were not observed in the negative controls.

The findings presented here add to and strengthen the fact that determining the cyto- and geno-toxic effects of curcumin is essential as it is a frequently used anti-oxidant in clinical models. It is important not only to establish its safety, but also to assess possible hazards when combined with other chemical agents, such as the

chemotherapy drugs used in cancer therapy (Mendonça *et al.,* 2009). From this study, we could observe that curcumin, at high concentrations, can exert a genotoxic effect in human lymphocytes in *in vitro* models.

D.3.2. Cyto- and genotoxicity of trans-reveratrol

D.3.2.1. Chromosomal aberrations (CAs)

Different CAs in human lymphocytes with no treatment, 100 µL of ethanol 95% and different concentrations of *trans*-resveratrol are shown in **Table 10**. The most frequent CAs were acentric chromosomes but gaps were also observed. CAs were not induced in a dose-dependent manner, in fact, the highest number of total aberrations recorded (the sum of chromatid gaps and acentric fragments) was observed at the lower concentration of 2.2 µM. Any other chromosomal abnormality (including dicentric, ring, translocation or inversion) were not found in all the different cells treated.

Table 10. Frequency (Y ± SE) and type of CAs in normal human lymphocyte cultures exposed to different concentrations of *trans*-resveratrol.

Trans-resveratrol (µM)	Chromatid-type		Chromosome type	Total aberrations (Y± SE)
	Gaps (Y ± SE)	Breaks (Y ± SE)	Acentric fragments (Y ± SE)	
Control	2.00 ± 0.01	0	0.00 ± 0.00	0.02 ± 0.01
0 + 95% ethanol	0.00 ± 0.00	0	0.04 ± 0.02	0.04 ± 0.02
2.2	0.01 ± 0.01	0	0.26 ± 0.05*	0.27 ± 0.05*
22	0.02 ±0.01	0	0.08 ± 0.03	0.10 ± 0.03
220	0.01 ± 0.01	0.02 ± 0.01	0.10 ± 0.03	0.13 ± 0.04

•Difference statistically significant ($p<0.05$).
SE: Standard Error.

A small amount of CAs, mainly acentric fragments, was found when these cells were treated with this compound. Matsuoka *et al.* (2001) studied the cytogenetic effect of *trans*-resveratrol in Chinese hamster lung cells and found that it induced structural CAs in cells, mainly chromatid breaks and exchanges, whereas in the present study it was found, mainly, acentric chromosomes and gaps.

Studies concerning the possible genotoxic effect of resveratrol started with Fukuhara and Miyata (1998). These authors based their study on the idea that some molecules so-called "DNA-cleaving agents" can cause the DNA strand scission by a Cu^{2+}(copper)-dependent complex. They reported that a molecule, resorcinol, induced a Cu^{2+}-dependent DNA strand scission; the genotoxic effect of resveratrol, since it has a similar molecular structure, could be mediated by the same mechanism. In their study with a plasmid-based DNA cleavage assay, resveratrol was found to have a greatly potentiated ability to mediate Cu^{2+}-dependent DNA cleavage under aerobic conditions (**Figure 45**).

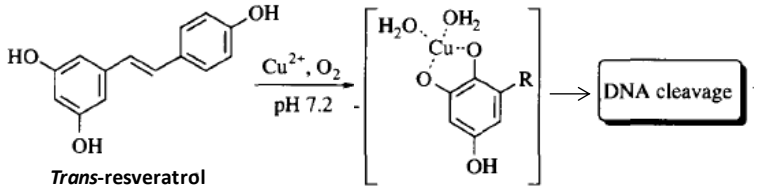

Figure 45. Resveratrol reacts with Cu^{2+} under aerobic conditions (O_2) and the resultant complex can cleave the DNA molecule.

In the study, they tested different metal ions and found that in the presence of others different to Cu^{2+} resveratrol did not induce DNA cleavage, which suggests that Cu^{2+} is the specific metal ion required for DNA degradation mediated by resveratrol. It was also found that, under anaerobic conditions, the efficiency of Cu^{2+}-dependent DNA cleavage was not produced even with increasing the concentration of resveratrol. Thus, their results suggested that the resveratrol DNA cleavage is absolutely dependent on the presence of both Cu^{2+} and O_2.

This mechanism of inducing strand scission of DNA or DNA-cleavage in the presence of O_2 and metal transition ions especially Cu^{2+} has been confirmed by another study; they show that when resveratrol bind Cu^{2+} it reduced the Cu^{2+} to Cu^{+}, and this reaction was accompanied by the formation of oxidized product(s) of resveratrol, which in turn appear to catalyze again the reduction of other ions of Cu^{2+} and thus, producing a high amount of oxidative molecules. They confirmed that resveratrol–Cu^{2+} system was indeed capable of causing DNA degradation in cells such as lymphocytes. Based on their results, they proposed a mechanism for the cytotoxic action of plant

polyphenolics against cancer cells that involves mobilization of endogenous Cu^{2+} and the consequent pro-oxidant action (Azmi *et al.,* 2005).

The ability of *trans*-resveratrol to damage the DNA molecule and finally may induce CAs has been also attributed to the same activity as curcumin (see section *D.3.1.1.),* the so-called topoisomerase poisoning. If *trans*-resveratrol can act as a topoisomerase poison then it will contribute to the deleterious effect on the genomic stability of cells by increasing the incidence of homologous and non-homologous recombination effects. These events result in chromosomal changes such as clastogenic effects. Flavonoids and other polyphenolic compounds have been shown to inhibit human topoisomerase I (topo I) through both inhibition of relaxation activity and through stabilization of the cleavable complex (poisoning). The ability of some flavonoids to act as topoisomerase poisons has been used to explain the results of mutagenicity testing where it was shown that many flavonoids test positive for clastogenicity in mammalian cell systems (Webb and Ebeler 2004). These authors classified *trans*-resveratrol as a topo I inhibitor, which may clarify whether this polyphenol can induce CAs.

Other studies have seen that *trans*-resveratrol can be also cytotoxic by measuring parameters such as cell viability. In the study of Fujimoto *et al.* (2009) the effects of some polyphenols on rat thymocytes were examined by flow cytometric analysis. Resveratrol was the most cytotoxic on rat thymocytes incubated for 24 h with 100 µM of this compound. Resveratrol at concentrationd of 10 µM or higher (up to 100 µM) led to a significant dose-dependent increase in the population of dead cells. The authors attributed the cytotoxic action of resveratrol with cell shrinkage, which occurs during the early stage of apoptosis. According to Szende *et al.* (2000), the induction of apoptosis is a possible explanation for the antiproliferative effect of resveratrol. Usually this antiproliferative effect is adequate for cancerous cells but it must be considered that it can occur in non-cancerous cells as well.

The data presented here shows that *trans*-resveratrol-induced CAs did not follow a dose-dependent manner relation. Actually, the lowest concentration, 2.2 µM, caused the highest number of acentrics fragments and total aberrant cells. Some evidences maintain that polyphenolic compounds can show a biphasic dose response curve

which is called hormetic response (Webb and Ebeler, 2004). Calabrese *et al.* (2010) observed that many effects induced by resveratrol are dose-dependent and that opposite effects occur at low and high doses, being indicative of a hormetic dose response.

D.3.2.2. Sister Chromatid Exchanges (SCEs)

From the study of SCE, results concerning the frequency of SCE (Y_{SCE}) for each concentration are shown in **Table 11**. All tested concentrations, included treatment with 100 µL of ethanol 95%, caused a dose-dependent increase of SCE frequency, the two higher concentrations (22 and 220 µM) which proportioned a SCE statistically significant ($p < 0.05$) compared to cells without *trans*-resveratrol treatment. The peak frequency of SCEs was at the maximum concentration 220 µM.

Table 11. Frequency of SCE (Y_{SCE} ± SE) in human lymphocytes exposed to different concentrations of *trans*-resveratrol.

Trans-resveratrol (µM)	Y_{SCE} (±SE)
0	6.84 ± 0.42
0+ 95% Ethanol	7.50 ± 0.42
2.2	7.96 ± 0.52
22	8.98 ± 0.49*
220	10.02 ± 0.49*

•Difference statistically significant ($p<0.05$).
SE: Standard error

The same tendency in values of SCE as in the present study was observed by Matsuoka *et al.* (2001) when Chinese Hamster lung cells were treated with *trans*-resveratrol. In this study it was observed a dose-dependent induction in the SCEs with a maximum frequency of SCEs per cell at the highest concentration (44 µM). They suggested that *trans*-resveratrol induced SCEs because it is capable of arresting S phase, a preferential condition for SCEs induction. The S phase arrest may be induced by two actions. One of the biological activities which could be responsible for the S phase arrest is the action mentioned in the CAs section (*D.3.2.1.);* resveratrol can

cleave DNA through a Cu^{2+}-complex, ability demonstrated by Fukuhara and Miyata (1998). Another activity is that resveratrol can act like an arrest factor during DNA replication (Matsuoka *et al.,* 2004). In this mechanism, the compound scavenges the tyrosyl free radical in the protein R2 subunit of the ribonucleotide reductase (RNR) enzyme, causing depletion of nucleotides and resulting in a failure in DNA synthesis and consequently resulting in a replication fork arrest. These authors suggested that the new conformation of the RNR may favor recombination of the DNA, resulting in a high frequency of homologous recombination (HR) events. In conclusion, they suggested a correlation between tyrosyl radical scavenging in protein R2 of the RNR by resveratrol and the frequency of HR events. The HR increased events which are indirectly induced by resveratrol are one of the main mechanisms for the SCE formation.

In an attempt to investigate structure toxicity relationships, Matsuoka *et al.* (2002) synthesized six analogues of resveratrol differing in number and position of hydroxyl groups (-OH) and investigated their activity in CAs, MN and SCEs tests in a Chinese hamster cell line. Two of the six analogues (3,4'-dihydroxy-trans-stilbene and 4-hydroxy-trans-stilbene) showed clear positive responses in a concentration-dependent manner in all three tests. Both were equal to or stronger than resveratrol in genotoxicity. The 4'-hydroxy analogue had the simplest chemical structure and was the most genotoxic, confirming that the 4'-hydroxy group, plays an essential role in its cytotoxic activity.

D.3.2.3. Mitotic Index (MI) and Proliferation Index (PI)

For the study of the MI, a total of 500 cells were scored for each experiment and for the PI, a total of 100 cells were scored in each experiment. Data relating to the MI, RMI, PI and RPI of the different curcumin concentrations in human blood samples are summarized in **Table 12**. Concerning the MI, although all the different cell treatments changed this index with regard to cells with no treatment, the only statistically significant difference occurred in those cells treated with ethanol 95% in which the MI decreased almost the half. On the other hand, in order to assess cell proliferation

kinetics, the PI was calculated. Samples with 2.2 and 22 µM *trans*-resveratrol statistically increased the PI.

Table 12. Results of MI, RMI, PI, RPI in human peripheral lymphocytes pre-treated with various concentrations of *trans*-resveratrol (Mean ± SD).

Trans-resveratrol (µM)	MI (Mean ± SD)	RMI	PI (Mean ± SD)	RPI
0	0.07 ± 0.01	100	1.64 ± 0.06	100
0+ 95% Ethanol	0.04 ± 0.01*	58.05	1.53 ± 0.08	93.09
2.2	0.08 ± 0.01	106.91	2.26 ± 0.08*	137.51
22	0.08 ± 0.01	116.00	2.13 ± 0.07*	129.88
220	0.06 ± 0.01	76.42	1.62 ± 0.06	98.57

•Difference statistically significant ($p<0.05$).
SD: Standard deviation

When the relative indexes (RMI and RPI) were compared, the RMI drastically decreased with the 95 % ethanol treatment (RMI=58 %) while the 2.2 and 22 µM of *trans*-resveratrol raised the RMI up to approximately the control values. The RPI was also increased at the same concentration of *trans*-resveratrol. Both RMI and RPI displayed a hormetic biphasic concentration response relationship at the three tested concentrations of *trans*-resveratrol (**Figure 46**).

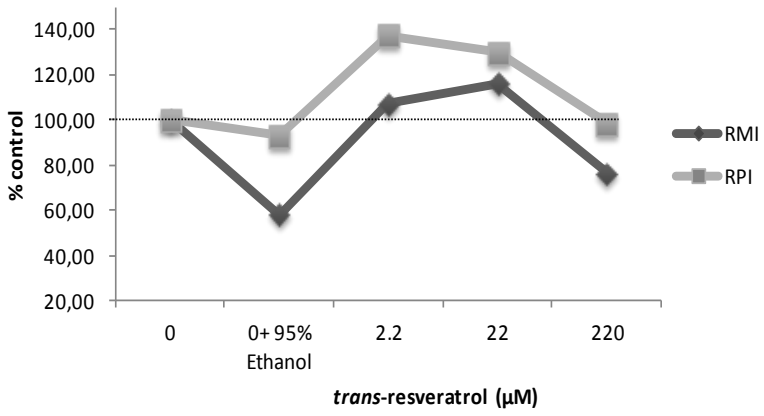

Figure 46. Graphic representing the hormetic effect of *trans*-resveratrol concentrations on the RMI and RPI of human lymphocytes. Results for control cultures are taken as 100%.

The values for the MI and PI are in agreement with the study of Szende *et al.* (2000) in which the lowest amounts of *trans*-resveratrol (0.44 µM and 4.4 µM) enhanced human umbilical endothelial cell culture proliferation and 10 to a 100-fold concentration drastically decreased cell growth. De Salvia *et al.* (2002) demonstrated that the MI was not modified by the lowest concentration they tested, however, in the following higher concentrations this parameter decreased significantly. In the study of Calabrese *et al.* (2010), low doses of *trans*-resveratrol stimulated the proliferation of healthy cells; however, at high doses, an inhibition was found in the cell proliferation. Similarly, we obtained higher values for MI and PI at the lowest *trans*-resveratrol concentration than at the samples without treatment. Moreover, a reduction of both indexes was observed at the highest concentration of *trans*-resveratrol (**Table 12**). Data observed by these studies and also our results agree with this and help to establish the fact that *trans*-resveratrol commonly displays hormesis.

D.4. Radioprotection with curcumin and *trans*-resveratrol

D.4.1. Radioprotection assessed by the dicentric assay

D.4.1.2. Radioprotection with curcumin

The cytogenetic results obtained after the analysis of human lymphocytes exposed at 2 Gy of γ-radiation in the presence of different concentrations of curcumin are shown in **Table 13**. Cytogenetic analysis considered chromosome- and chromatid-type aberration (CAs). However, in order to assess the radioprotective effect of the compounds only the frequency of dicentrics was considered. After 2 Gy of γ-irradiation, the frequency of dicentrics (Y_{dic}) in samples pretreated with curcumin was significantly reduced compared to the control samples. The differences in the frequencies (**Table 13**) were significant ($p<0.05$) for 2.8, 14 and 1400 µM curcumin. Nevertheless, we could not score 100 metaphases at a concentration of 1400 µM curcumin probably due to the fact that the concentration was cytotoxic for the cells. All conditions were exactly the same in every concentration analyzed and we were not able to find 100 metaphases in the totality of prepared slides.

The maximum level of radioprotection reached when lymphocytes were exposed to 2 Gy of γ-rays was around of 52.03 % by 14 µM curcumin. This value was obtained for each concentration of curcumin from the formula:

Y = 100 – [(Dicentrics frequency with curcumin x 100)/Dicentrics frequency in control blood sample)]

Table 13. Chromosomal aberrations and frequency of dicentric chromosomes (Y_{dic}±SE) in human lymphocytes exposed to 2 Gy of γ-rays in the presence of different concentration of curcumin.

Curcumin (µM)	Cells (n)	Chromosomal aberrations				Chromatid aberrations			Tac
		Dic	Y_{dic} (± SE)	R	Ac	Oth	Ctb	Gaps	
2Gy (without substance)	110	32	0.29 ± 0.05	1	5	-	-	-	30
0 + 95% Ethanol	103	25	0.24 ± 0.05	2	23	5	-	-	41
1.4	100	19	0.20 ± 0.02	-	8	-	2	-	23
2.8	100	15	0.15 ± 0.01*	1	6	-	2	-	20
14	107	15	0.14 ± 0.04*	-	4	1	-	-	18
140	104	20	0.19 ± 0.04	-	9	1	-	-	25
1400 [a]	39	6	0.16 ± 0.12*	-	-	-	-	-	2

n: number of analyzed cells; Dic: dicentrics; R: rings; Ace: acentrics; Oth: Other (translocations and inversions) Ctb: chromatid type breaks; Tac: total aberrant cells. SE: Standard Error.
[a] The number of analyzed cells is lower than 100 due to that we could no achieve this number, thus a cytotoxic effect is supposed.
* Difference statistically significant ($p<0.05$).

The distribution of cells containing a different number of dicentrics is shown in **Table 14**. The intercellular distribution of dicentrics follows a Poisson distribution in all cases with or without curcumin (U = ±1.96). Departures from Poisson were assessed in terms of the test quantity U, a value of U > 1.96 indicates over dispersion at the 5% level of significance.

Table 14. Dicentric cell distribution, dispersion index (DI) and normalized unit of this index (U) for each concentration and with 2 Gy of γ-radiation.

Curcumin (µM)	Cells (n)	Dic	0dic[a]	1dic[a]	2dic[a]	3dic[a]	DI	*U*
0	110	32	83	23	3	1	1.10	0.77
0 + 95% Ethanol	103	25	80	23	1	-	0.86	-1.03
1.4	100	19	81	17	1	-	0.92	-0.55
2.8	100	15	85	15	-	-	0.86	-1.03
14	107	15	92	15	-	-	0.87	-1.00
140	104	20	85	18	1	-	0.92	-0.61
1400	39	6	33	6	-	-	0.91	-0.30

[a]: cells with 0, 1, 2 or 3 dicentrics (dic).

It is worth mentioning that 2.8 µM curcumin offered almost the same percentage of radioprotection as the following concentration which offered the maximum percentage (14 µM) while the next one (140 µM) gave a higher frequency of dicentrics. This means that the decrease in the dicentric frequency was dose-dependent up to 14 µM and reached a maximum at this concentration.

The present analysis reflected the radioprotective effect of almost all the tested curcumin concentrations. These results are in agreement with some *in vitro* and *in vivo* studies. Concerning *in vitro* studies, Parshad (1998) was among the first to suggest a radioprotective role of curcumin when they studied radiation induced CAs in human skin fibroblasts and observed a reduction in the damage. The study of Srinivasan *et al.* (2006) proved the radioprotector efficacy directly to isolated human lymphocytes. They pre-treated the lymphocytes with similar concentrations than ours (2.8, 14 and 28 µM), irradiated the samples at 1, 2 and 4 Gy and analyzed the chromosome damage by the dicentric assay and by the increase of micronucleus (MN). Concerning the dicentric assay, they found that all the concentrations of curcumin significantly decreased the dicentric frequency compared to the control samples and the most radioprotective concentration was 28 µM of curcumin for all the radiation doses administered.

Data concerning *in vivo* studies with animals have established that curcumin has a strong radioprotective function. For instance, rodent oral administration of curcumin 2 h before or inmmediately after exposing the animal to whole-body γ-irradiation significantly decreased the frequency of micronucleated polychromatic erythrocytes (Abraham *et al.,* 1993). Some similar effects were observed in mice pre-treated with

curcumin before γ-rays exposure where the polyphenol reduced the number of bone marrow cells with CAs (Thresiamma *et al.,* 1998).

In an interesting study with rodents, the radioprotective action of curcumin was evaluated against acute and chronic effects and mortality induced by exposure of female rats to radiation. They study was based on analyzing 8-hydroxy-2'-deoxyguanosine (8-OHdG) in urine, a marker for acute effects of radiation. By adding curcumin to the diet in a 1% proportion (wt/wt) for 3 days before and/or 2 days after irradiation the elevated 8-OHdG levels were reduced by 50–70%. Moreover, the evaluation of the radioprotective action of curcumin against the long-term effects revealed that curcumin significantly decreased the incidence of mammary and pituitary tumors; unfortunately, the experiments on survival revealed that curcumin was not effective (Inano *et al.,* 2002).

Concerning the mechanisms by which curcumin can exert a radioprotective effect, it has been suggested that curcumin has the ability to reduce oxidative stress and to inhibit transcription of genes related to oxidative stress and inflammatory responses which may afford protection against the harmful effects of radiation (Hatcher *et al.,* 2008).

On the one hand, the antioxidant activity is one of the most important properties. This antioxidant ability can be mediated by the inhibition of oxidation of some molecules such as membrane lipids and by the induction of the activity of some endogenous antioxidant enzimes. For instance, in the study of Srinivasan *et al.* (2006) where the radioprotector effect of curcumin was tested in isolated human lymphocytes, the authors measured the cellular changes due to radiation by using lipid peroxidative by-products like thiobarbituric acid reactive substances (TBARS) and the antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and reduced glutathione (GSH). They concluded that pre-treatment of curcumin resulted in a decreased lipid peroxidation level and an improved antioxidant status of cells, due to the induction of the endogenous antioxidant enzymes because of the antioxidant action of curcumin. In a study with activated rat peritoneal macrophages it was shown that pre-incubation of macrophage with 10 µM curcumin inhibits ROS generation by the macrophages (which play an important role in

initiation of inflammation) (Hatcher *et al.,* 2008). Another study measured expression of several antioxidant enzymes (e.g., hemeoxygenase-1, glutathione peroxidase, modulatory subunit of gamma-glutamyl-cysteine ligase, and NAD(P)H:quinone oxidoreductase 1, increase glutathione (a product of the modulatory subunit of gamma-glutamyl-cysteine ligase)) in normal organs exposed to radiotherapy and chemotherapy agents and observed that curcumin activated all the enzymes (Goel and Aggarwal, 2010).

Concerning another type of enzymes, the induction of the phase II enzyme system is an important event of the cellular response during which a diverse array of electrophilic and oxidative compounds can be eliminated or inactivated before they cause damage to cellular macromolecules. The enzymes of the phase II systems (i.e. methyltransferase, sulfotransferase, glutathione S-transferase, acetyl Co-enzyme) are regulated at the transcriptional level by consensus sequences for the antioxidant response element; when curcumin is present in cells suffering an increment of oxidative stress it triggers the transcriptional factors and induce the gene expression in order to increase the intracellular antioxidant levels (Srinivasan *et al.,* 2006). According to these authors the specific chemical structure may play a crucial role in preferential affinity towards selective cysteine residues of targeted proteins that control the gene expression. Therefore, they suggested that the position of the -OH groups in curcumin may play an important role in the induction of antioxidant enzymes.

The potential protective effect of curcumin, related to the free radical scavenging properties, has not been only demonstrated against radiation injury; it has been indeed confirmed as chemoprotective against the free radical produced by some chemical compounds. For instance, Balasubramanyam *et al.* (2003) studied how curcumin could abolish the oxidative stress produced in diabetic cells (oxidative stress is considered a common endpoint of chronic diseases) and demonstrated that curcumin inhibited ROS generation in cells from control and diabetic subjects.

Protection against chemical induced DNA damage by curcumin has been observed by Polasa *et al.* (2004) who proved that curcumin significantly protect the benzo(a)pirene (a human carcinogen) induced single strand breaks (SSB) in human peripheral blood lymphocytes. Tiwari and Rao (2010) revealed that curcumin efficiently

ameliorates the toxic effect of arsenic and fluoride in human peripheral blood lymphocytes by reducing the frequency of structural aberrations (>60%), hypoploidy (>50%) and primary DNA damage.

Curcumin is a hydrophobic molecule, thus, the ability of curcumin to protect cells from DNA damage could be explained in part by this hydrophobic property which confers two types of action. On the one hand, this compound can pass easily through plasma membranes into cytosol and directly scavenges the free radicals like superoxide anion (O_2^-) and hydroxyl radical ($OH^{\cdot}$). On the other hand, due to its lipidic soluble properties, although curcumin passes through the lipid bilayer membrane it also becomes localized in it; there it acts as a chain terminating antioxidant reacting with lipid peroxyl radicals (**Figure 47**). Concerning its chemical structure, the presence of electron donating groups like phenolic -OH groups and a β-diketone structure is responsible for the free radical scavenging activity and inhibiting lipid peroxidation (Kunwar *et al.*, 2008).

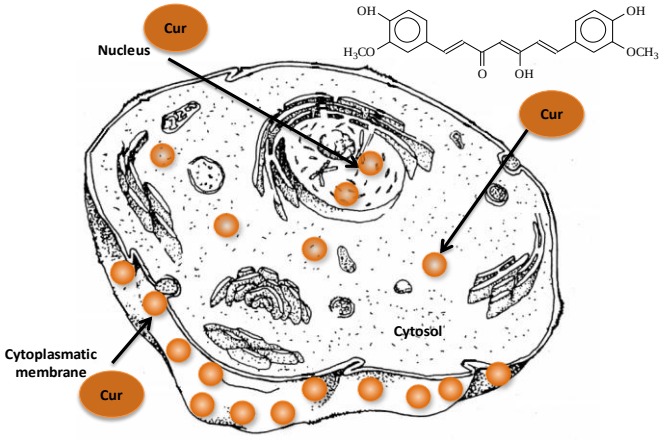

Figure 47. Schematic representation of the curcumin situation in the cell. According to Kunwar et al. (2008), cell membrane is the preferred site of localization for curcumin but it is also localized in the nucleus.

On the other hand, another mechanism by which curcumin may afford protection against the harmful effects of radiation is that this polyphenol is involved in the activation or inhibition of various transcription signaling pathways, which are crucial to the cell survival. It is known that after the initial injury, the cell assesses the damge and activates some signaling pathways, which then determines the fate of the cell. Findings from one study confirmed the radioprotective role of curcumin related to

these mechanisms; it was reported that curcumin activated the expression of the protein kinase complex PKCδ (implicated in cell survival under oxidative stress) and Nuclear Factor NF-κB (implicated in protecting cells from apoptosis and in the transcriptional regulation of pro-inflammatory gene expression) in the splenic cells of irradiated mice (Kunwar *et al.,* 2007), thus it could protect from the irradiation-induced cell damage. Furthermore, Goel and Aggarwal (2010) showed that curcumin protects normal organs such as liver, kidney, oral mucosa, and heart from chemotherapy and radiotherapy-induced toxicity, confirming that the radioprotective effects of curcumin are due, in part, to the ability based on inducing the activation of several transcription factors such as the Nuclear factor 2 (NRF2).

One of the worst consequences of radiation is its related lethality or tumor occurrence, but of course other adverse effects not so severe can also occur. Wound healing following radiation therapy is a frequent undesirable effect, as radiation treatment often disrupts normal response to injury and results in delayed recovery periods. Radioprotective effects of curcumin were investigated on wound healing in mice exposed from 2 to 8 Gy doses of whole-body γ-radiation and it was observed that pre-treatment with curcumin significantly enhanced the rate of wound contraction, shortened wound healing duration and increased collagen synthesis (Jagetia and Rajanikant, 2004).

These preclinical studies, in addition to the fact that curcumin has been reported pharmacologically as safe (Aggarwal e*t al.,* 2003), are expected to lead to clinical trials to prove the potential of this age-old golden spice for treating cancer patients or at least, to reduce the adverse secondary effects of radiation in patients or workers in contact with radiation. These results are particularly interesting in many parts of India due to the high consumption of turmeric (*C. longa*) which may offer protection to individuals staying in areas where the background radiation from natural radioactivity is higher. Considering the available information it could be said that radioprotective effect of curcumin is mainly due to reducing oxidative stress both by free radical scavenging and antioxidant enzymes upregulation and to inhibit transcription of genes related to oxidative stress and inflammatory responses.

D.4.1.2. Radioprotection with trans-resveratrol

The cytogenetic results obtained after the analysis of human lymphocytes exposed at 2 Gy of γ-ray in the presence of different concentrations of *trans*-resveratrol are shown in **Table 15**. Cytogenetic analysis considered chromosome- and chromatid-type aberration (CAs), however, in order to assess the radioprotective effect of the compounds only the frequency of dicentrics was considered. After 2 Gy irradiation, the frequency of dicentrics in samples pre-treated with *trans*-resveratrol was reduced compared to samples irradiated without this compound. The differences in the frequencies were statistically significant ($p<0.05$) for 2.2 µM *trans*-resveratrol where the maximum level of radioprotection, around of 47 %, was achieved. This value was obtained for each concentration of *trans*-resveratrol from the formula:

Y = 100 – [(Dicentrics frequency with trans-resveratrol x 100)/Dicentrics frequency in control blood sample)].

Table 15. Chromosomal aberrations and frequency of dicentric chromosomes (Y_{dic} ± SE) in human lymphocytes exposed to 2 Gy γ-rays in presence of different concentrations of *trans*-resveratrol.

Trans-resveratrol (µM)	Cells (n)	Chromosomal aberrations				Chromatid aberrations			Tac
		Dic	Y_{dic} (± SE)	R	Ace	Oth	Ctb	Gaps	
2Gy (without substance)	110	32	0.29 ± 0.05	-	8	-	-	-	33
0 + 95% Ethanol	103	25	0.24 ± 0.05	2	23	5	-	-	41
2.2	115	18	0.15 ± 0.04*	1	14	4	1	1	34
4.4	100	22	0.22 ± 0.03	1	15	-	5	-	40
22	100	23	0.23 ± 0.05	2	32	2	-	-	47
220	101	21	0.21 ± 0.05	2	9	4	1	1	35

N: number of analyzed cells; Dic: Dicentrics; R: Rings; Ace: Extra acentric fragments; Oth: Other (include translocations, inversions) Ctb: chromatid breaks; Tac: Total Aberrant Cells. SE: Standard Error

*: The difference in the frequency of dicentric chromosomes was statistically significant ($p<0.05$).

The intercellular distribution of dicentrics follows a Poisson distribution in all cases with or without curcumin (U = ±1.96) (**Table 16**). Departures from Poisson were assessed in terms of the test quantity U, a value of $U > 1.96$ indicates over dispersion at the 5% level of significance.

Table 16. Dicentric cell distribution, dispersion index (DI) and normalized unit of this index (*U*) for each conditions and with 2 Gy of γ-radiation.

Trans-resveratrol μM)	Cells (n)	Dic	0dic[a]	1dic[a]	2dic[a]	3dic[a]	DI	*U*
0	110	32	83	23	3	1	1.10	0.77
0 + 95% Ethanol	103	25	80	22	1	-	0.86	-1.03
2.2	115	18	97	18	-	-	0.85	-1.16
4.4	100	22	79	20	1	-	0.88	-0.87
22	100	23	78	21	1	-	0.87	-0.97
220	101	21	86	15	3	-	0.98	-0.17

[a]: cells with 0, 1, 2 or 3 dicentrics (dic).

There are very few reports about the radioprotection of *trans*-resveratrol in *in vitro* conditions. In the study of Koide *et al.* (2012) they tested a commercially available resveratrol product. Although the γ-irradiation-induced cell death could not be mitigated by resveratrol, when 32D cl-3 cells were pretreated with 10 μM resveratrol, an increased cell survival was observed 7 days after irradiation from 0-7 Gy.

Concerning *in vivo* studies, some studies have been carried out in animal models. Carsten *et al.* (2008) evaluated the radioprotective effect of orally administered resveratrol on the frequencies of CAs in irradiated mouse bone marrow cells. They reported that resveratrol daily treatment of 100 mg/kg two days prior to whole-body irradiation at 3 Gy of γ-radiation, gave a reduction in the CAs frequency at cells taken 1 and 30 days after irradiation. In their study, fragments were the most common aberration type observed in the radiation and resveratrol pre-treatment radiation groups, followed by gaps, dicentrics and robertsonian translocations. Velioğlu-Oğünç *et al.* (2009) treated rats with 10 mg/Kg/day of resveratrol during 10 days before and after whole body irradiation to determine whether it could ameliorate ionizing radiation-induced oxidative injury. They found that resveratrol treatment reversed all the biochemical parameters altered by the radiation (decrease in glutathione level, increase in malondialdehyde levels, myeloperoxidase activity and collagen content in the liver and ileum tissues and in the antioxidant-capacity; increase in plasma lactate dehydrogenase and pro-inflammatory cytokine levels, 8-hydroxy-2'-deoxyguanosine, leukocyte apoptosis, DNA fragmentation and histopathological alterations), thus, they suggested the inclusion of resveratrol as an adjuvant of radiotherapy. In one study

where the purpose was to evaluate the radioprotective effects in the ovarian of rats exposed to whole-body irradiation, two concentrations of resveratrol (10 and 100 mg/kg) were intraperitoneally administered 24 h before the irradiation. The ovarian follicles decreased in irradiated rats without the presence of resveratrol while the treatment with both concentration of the compound increased the number of follicles compared to non pre-treated group. Moreover, the tissue activities of antioxidant enzymes were significantly elevated in the resveratrol-treated animals. The authors suggested that this polyphenol was effective in reducing the follicle loss induced by ionizing radiation (Simsek et *al.,* 2012). One of the most recent studies (Zhang *et al.,* 2013) examined whether resveratrol can ameliorate whole-body irradiation-induced long-term bone marrow injury by inhibiting radiation-induced chronic oxidative stress and senescence in hematopoietic stem cells. Their results showed, that pre-treatment with resveratrol, effectively protected mice form radiation-induced acute bone marrow syndrome and lethality and in addition ameliorated the radiation-induced long term injury attributed to resveratrol-mediated reduction of chronic oxidative stress in hematopoietic stem cells. This reduction could be due, from their viewpoint, to resveratrol-mediated down-regulation of NADPH oxidase 4 expression and up-regulation of Sirtuin 1, SOD, and glutathione peroxidase 1 expression.

Most of the previously mentioned studies support the idea that the radiation-induced cellular damage reduction offered by resveratrol is likely mediated by the direct antioxidant properties of resveratrol or by the direct induction of antioxidants like glutathione and increases in enzymes like SOD and catalase (Carsten *et al.,* 2008).

Another approach has shown the ability of resveratrol to induce cell cycle arrest in S phase and/or at the G2/M transition. This ability has been observed by Ferry-Dumazet *et al.* (2002) in leukemia cells. It must be considered that arresting cells in any phase of the cell cycle is due to the existence of some errors during the cycle that must be resolved (Novák *et al.,* 2002). For instance, in irradiated cells the DNA becomes damaged and the cell detects the error and the cell cycle is arrested in order to repair the error (chromosomal repair). Since the ability of resveratrol to induce cell cycle arrest by itself has been demonstrated (Ferry-Dumazet *et al.,* 2002), this mechanism

will contribute to reducing the frequency of chromosome damage in irradiated cells because they will have more time to repair the damage.

The effect of resveratrol in chemically induced oxidative stress has been also observed. It was found that resveratrol was responsible for maintaining a glutathione (GSH) level in oxidation-stressed peripheral blood mononuclear cells isolated from healthy humans (Losa, 2003) and in human lymphocytes treated with hydrogen peroxide (H_2O_2) (Yen *et al.,* 2003). This is an essential action, due to the fact that GSH is a extremely important antioxidant agent preventing damage to important cellular components caused by ROS such as free radicals and peroxides (Pompella *et al.,* 2003). In addition to maintaining normal concentrations of intracellular antioxidants, resveratrol was capable, of restoring the levels of some of them such as glutathione reductase (Jang and Pezzuto, 1998) glutathione peroxidase, glutathione-S-transferase (Yen *et al.,* 2003), dismutase and catalase (Aftab and Vieira, 2010) which were reduced by chemicals. Sgambato *et al.* (2011) investigated the anti-oxidative effect of resveratrol evaluating its ability to prevent the production of ROS in normal rat fibroblasts exposed to different chemically-induced oxidative stresses. Incubation of cells with resveratrol at 30 and 90 μM for 24 h slightly decreased the levels of endogenous ROS and when cells were exposed to H_2O_2 resveratrol decreased the intracellular ROS level caused by the oxidant agent.

The results obtained in this work agreed with those obtained in other studies which support the idea of the radioprotective ability of resveratrol by different antioxidant mechanisms. Data showed that the radioprotective effect *in vitro* was around 47% corresponding to the concentration of 2.2 μM.

D.4.2. Radioprotection assessed by Premature Chromosome Condensation (PCC) technique

D.4.2.1. Radioprotection with curcumin

The effect of curcumin on the radiation-induced direct chromosome damage in G0 lymphocytes isolated from peripheral blood was investigated. For this purpose, G0 lymphocytes pre-treated with curcumin concentrations of 0.14, 1.4 and 7μM for 1 h were irradiated with γ-ray at doses of 2, 4 and 6 Gy. Two samples were considered as

controls; one only with isolated lymphocytes and the other with the addition of the compound solvent (95% ethanol). Since the lymphocytes were treated at the G0/G1 phase cell cycle, chromosomes appeared as a single chromatid. Chromosome damage was quantified scoring "chromosome fragments", as the number of chromosome fragments was in excess of 46 per cell. The damage was evaluated immediately after irradiation in the presence or absence of curcumin using cell fusion with mitotic CHO cells and PCC induction. We also tested the concentrations of 14 and 140 μM curcumin, but the cells indicated toxicity which meant that the lymphocytes could not undergo PCC. At the highest concentration (140 μM) lymphocytes suffered apoptosis and they did not fuse with the CHO cells; at 14 μM lymphocytes obtained fusion but their genetic material did not condense, appeared like the G0 phase (**Figure 48**).

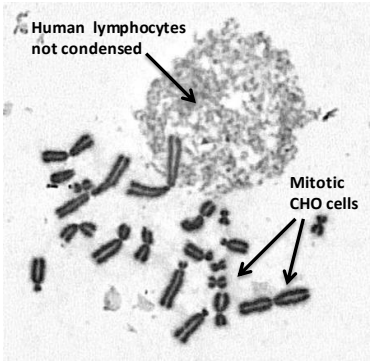

Figure 48. Incomplete fusion with human lymphocytes pre-incubated with 14 μM curcumin. The DNA molecule of lymphocytes did not condense and it appears like the G0 phase.

Because of this problem, we decreased the concentrations and finally carried out the PCC experiment with 0.14, 1.14 and 7 μM concentrations. In order to sum up the obtained results, data from the analysis of the total and the excess of fragments for each experiment are shown in **Table 17**.

Table 17. Mean average of total fragments (TF) and excess of fragments (EF) at each experiment (control, ethanol 95% and curcumin) considering that the normal human number of chromosomes without irradiation (0Gy) should be 46.

Dose (Gy)	0 Gy		2 Gy		4 Gy		6 Gy	
Fragments	**TF ± SE**	**EF**	**TF± SE**	**EF**	**TF± SE**	**EF**	**TF± SE**	**EF**
PCC	45.53±0.13	-0.47	54.54±0.48	8.54	63.43±0.24	17.43	71.69±0.22	25.69
PCC+Eth 95%	47.41±1.42*	1.45	55.33±0.55	9.33	62.60±0.66	16.60	69.96±0.65	23.96
PCC+Cur 0.14 µM	45.36±0.14	-0.63	51.17±0.35	5.17	56.13±0.45	10.13	63.1±0.64	17.07
PCC+Cur 1.4 µM	45.67±0.10	-0.33	52.03±0.35	6.03	56.90±0.49	10.90	63.87±0.90	17.87
PCC+Cur 7 µM	45.68±0.10	-0.33	51.30±0.32	5.30	57.55±0.64	11.55	63.9±0.76	17.80

Cur: curcumin: TF: total fragments; EF: excess of fragments (total – 46); *: the TF and EF values were obtained by estimation from the linear multiple regression model.

Statistical analysis was performed using a linear multiple regression model. The model considered as response variables the chromosome damage and the radiation dose, and the compound and the concentration of compound as predictive variables (**Table 18**). The best model included only the variables compound and dose (Gy).

Table 18. Multiple linear regression model used for the each PCC experiment in the presence of curcumin.

	Estimate	Std Error	p-value
Intercept	45.87	0.48	<0.001
Dose	4.33	0.11	<0.001
Ethanol	1.58	1.39	0.26
Curcumin	-0.39	0.61	0.52
Dose : ethanol	-0.49	0.32	0.13
Dose : curcumin	-1.37	0.15	<0.001

Multiple R-squared: 0.771; Adjusted R-squared: 0.770

As it can be seen in **Table 18** lymphocytes in controlled conditions had a mean total number of fragments of 45.87 with a 95% CI from 44.94 to 46.81. Regarding the data from the experiments it can be said that the γ-radiation (dose) statistically increased ($p<0.001$) the mean value of fragments in 4.33 units with a 95% CI [4.10; 4.56] which means that each Gy increased the number by 4.33 fragments. When samples were not irradiated (0Gy), neither ethanol nor curcumin statistically increased the frequency of fragments ($p=0.26$ and $p=0.52$, respectively). In the case of curcumin (Dose: curcumin), the increase in dose yielded a statistically significant ($p<0.001$)

reduction in damage (-1.37 per Gy) in comparison with the irradiated control PCC sample (45.9).

Figure 49 Shows the effect offered by curcumin in the total chromosome fragments that were radio-induced; the graphic only represents the effect of the presence of ethanol 95% and curcumin because no effect was observed when increasing the concentration of curcumin. Regarding this figure it is observed that curcumin reduced the yield of radiation induced chromosomal damage compared with the PCC control experiments. This means that curcumin may exert a radioprotective effect by reducing the direct DNA damage.

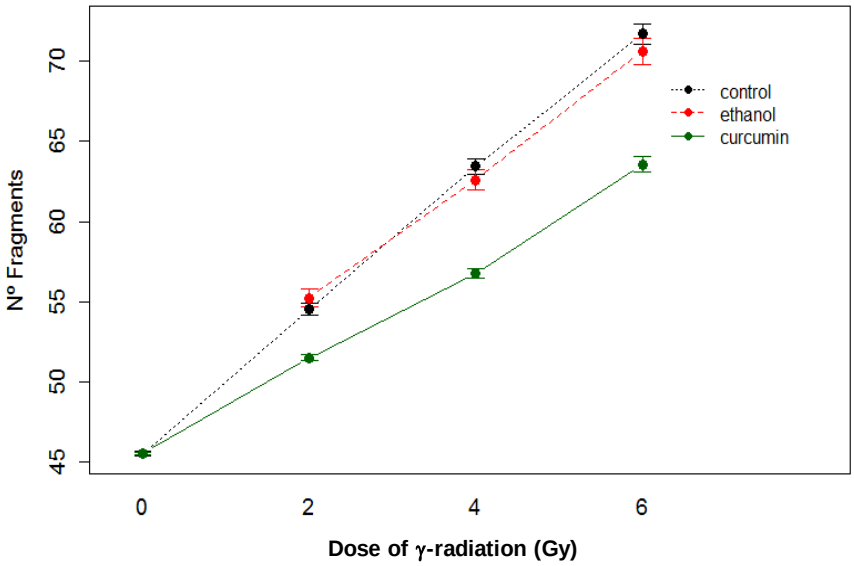

Figure 49. PCC results from control sample, lymphocytes pre-treated with ethanol 95% and with curcumin.

In order to visualize the PCC experiments, **Figure 50** shows the pictures obtained from the PCC control samples irradiated at different doses and PCC experiments with 0.14 µM curcumin (all the concentrationsare not represented because no effect was observed when increasing the concentration of curcumin).

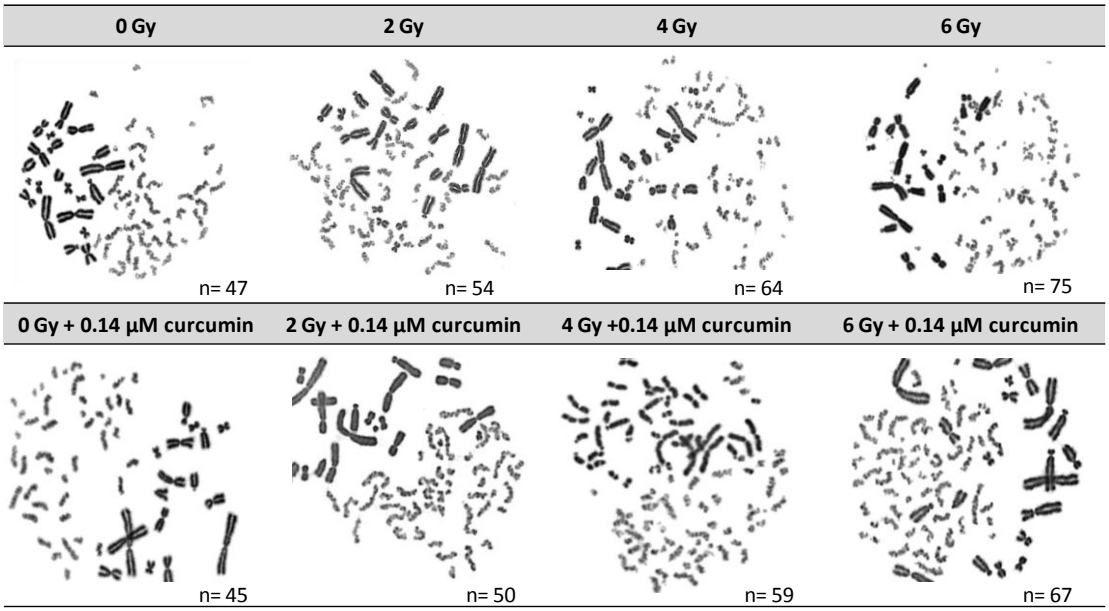

Figure 50. PCC obtained in control and 0.14 µM curcumin pre-treated PCC samples.

PCC methodology has been extensively used to assess and evaluate the direct induction and repair of chromosome damage after *in vivo* or *in vitro* exposure of human cells to ionizing radiation (Hatzi *et al.,* 2006). Given that this methodology allows visualiziation and scoring of the radiation-induced chromosome damage in G1 or G2 cells immediately after irradiation, is not necessary to go through the cell cycle to analyze the different types of CAs in order to see the radioprotective effect (Hatzi *et al.,* 2006). In the previous section (*D.4.1.1*), we observed that curcumin reduced the frequency of dicentrics compared to control samples without curcumin pre-treatment. In the dicentric assay, in addition to the radical scavenging properties of curcumin by which it can scavenge the free radicals caused by the radiation and thus diminish the chromosomal damage, other mechanisms can influence the indirect effect of radioprotection such as its ability to induce the activation of the nuclear factor NRF2 and the expression of several antioxidant enzymes (e.g., hemeoxygenase-1, glutathione peroxidase) (Aggarwal and Sung, 2009). However, with the PCC, cells not going through the cell cycle it seems reasonable therefore that the radioprotection offered by the compound must be mostly mediated by the direct free radical scavenging ability. We observed that curcumin reduced the mean value of fragments in excess of 46 when present before the irradiation.

This might be due to the fact that curcumin can manage to first cross the cell membrane, and then the nuclear membrane and be located extremely close to the DNA molecule in order to scavenge the free radicals radio-induced mainly by those water molecules present in the surroundings of the DNA double helix. In a study of Kunwar *et al.* (2008) they calculated the uptake of curcumin from spleen lymphocytes following their absorption spectrum in the cell lysate and the fluorescence spectra of cellular curcumin and further, monitoring the fluorescence by confocal microscopy and by sub-cellular fractionation in an attempt to understand its intracellular localization. The results indicated that the uptake was dose-dependent, but the amount of intracellular curcumin was extremely low (23.2 ± 4.3 pmol/10^6 cells). Analyzing the intracellular distribution, it was expected that it would be localized in the membrane since curcumin is a lipophilic molecule. However, the images of lymphocytes showed emission from the entire cells (**Figure 51**).

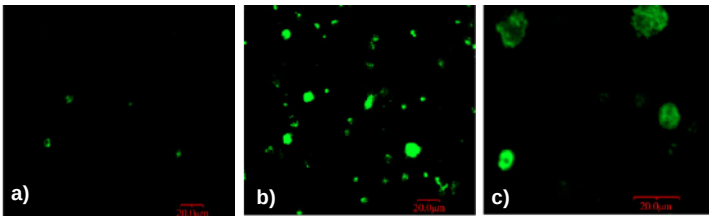

Figure 51. Confocal micrographs of spleen lymphocytes (20× objective): a) cells without curcumin; b) cells with curcumin 10 µM (2× zoom); c) cells with 10 µM (6× zoom) (Kunwar *et al.,* 2008).

Bearing in mind that in these cells, the majority of the cell volume is occupied by the nucleus, the emission could be from both the membrane and the nucleus. So, an extremely low amount of curcumin in the surroundings of the DNA molecule exists that could be responsible for the diminution of radio-induced chromosomal damage. The fact that we did not find a dose-dependent protection could be due to the fact that our concentrations are much higher than those (pmol) which can reach the nuclear region, thus, only a small number of molecules, independently of the concentration can reach the double helix of DNA and as a result, offer protection to the DNA.

D.4.2.2. Radioprotection with trans-resveratrol

The effect of *trans*-resveratrol on the initial induction of radiation-induced chromosome direct damage in G0 lymphocytes isolated from peripheral blood was investigated. G0 lymphocytes were pre-treated with *trans*-resveratrol concentrations ranging from 0-220 µM for 1h and were irradiated with doses of 2, 4 and 6 Gy. Two samples were considered as control; one only with isolated lymphocytes and the other with the addition of the compound solvent (95% ethanol). Because lymphocytes were treated at the G0/G1 phase cell cycle, chromosomes appeared as single chromatid. Chromosome damage was quantified defining "chromosome fragments", as the number of chromosome fragments in excess of 46 per cell. Data from the analysis of the total and the excess of fragments (TF and EF, respectively) for each experiment are shown in **Table 19**.

Table 19. Mean average of total fragments (TF) and excess fragments (EF) at each experiment (control, ethanol 95% and *trans*-resveratrol) considering that the normal human number of chromosomes without irradiation (0Gy) should be 46.

Dose (Gy)	0 Gy		2 Gy		4 Gy		6 Gy	
Fragments	**TF± SE**	**EF**	**TF± SE**	**EF**	**TF± SE**	**EF**	**TF± SE**	**EF**
PCC	45.53±0.13	-0.47	54.54±0.48	8.54	63.43±0.24	17.43	71.69±0.22	25.69
PCC+Eth 95%	47.41±1.42*	1.45	55.33±0.55	9.33	62.60±0.66	16.60	69.96±0.65	23.96
PCC+Res 2.2 µM	45.50±0.27	-0.50	52.45±0.20	6.45	57.42±0.05	11.42	65.13±0.51	19.13
PCC+Res 22 µM	45.66±0.10	-0.33	51.67±047	5.67	58.47±0.69	12.47	61.83±0.52	15.83
PCC+Res 220 µM	45.64±0.10	-0.33	53.52±0.18	7.52	58.92±0.28	12.92	64.67±0.45	18.67

Res: *trans*-resveratrol; TF: total fragments; EF: Excess of fragments; *: the TF and EF values were obtained by estimation from the linear multiple regression model.

Statistical analysis was performed using a linear multiple regression model. The model considered as a response variable the chromosome damage and the radiation dose, the compound and the concentration of the compound as predictive variables (**Table 20**). The best model included only the variables compound and dose.

Table 20. Linear regression model used for the each PCC experiment with the presence of *trans*-resveratrol.

	Estimate	Std Error	p-value
Intercept	45.87	0.51	<0.001
Dose	4.33	0.12	<0.001
Ethanol	1.58	1.51	0.29
***Trans*-resveratrol**	0.25	0.63	0.69
Dose : ethanol	-0.49	0.35	0.19
Dose : *trans*-resveratrol	-1.28	0.16	<0.001

Multiple R-squared: 0.771; Adjusted R-squared: 0.770

Table 20 shows that lymphocytes had a mean total number of fragments of 45.87 with a 95% CI from 44.94 to 46.81. When γ-radiation was applied (dose) the mean value of fragments was statistically increased (p<0.001) to 4.33 units with 95% CI [4.10; 4.56]. When no irradiation was applied (0Gy), neither ethanol nor *trans*-resveratrol increased the frequency of total fragments (1.58 and 0.25, respectively). In the case of *trans*-resveratrol (dose:*trans*-resveratrol), the increase in dose yielded a statistically significant (p<0.001) reduction in damage (-1.28 per Gy) in comparison with the irradiated control PCC sample.

Figure 52 shows the effect offered by *trans*-resveratrol in the total radio-induced chromosome fragments; the graphic only represents the effect of the presence of ethanol 95% and *trans*-resveratrol because no different effects have been observed by increasing the concentrations of both compounds.

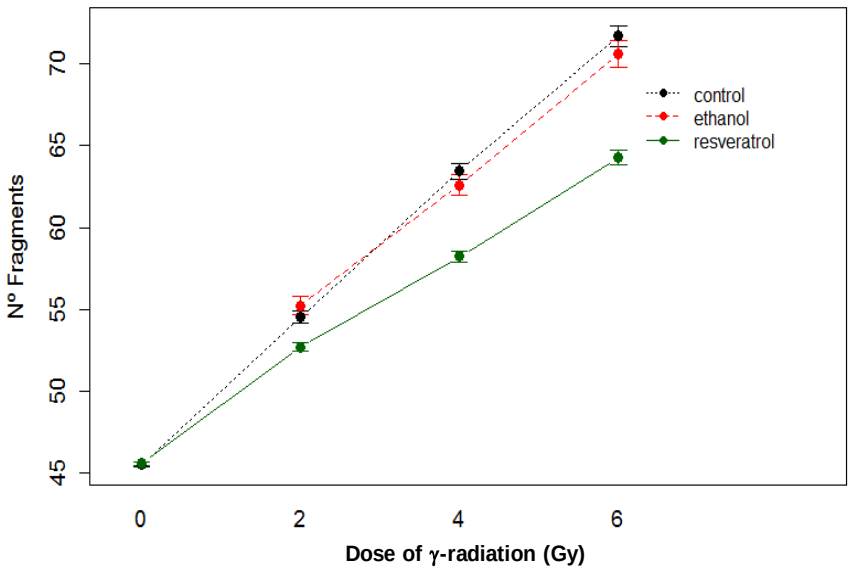

Figure 52. PCC results from control, pre-treatment with ethanol 95% and *trans*-resveratrol.

According to the data shown in **Figure 52**, *trans*-resveratrol reduced the yield of radiation induced chromosomal damage compared with the PCC control experiments without the compound. This means that *trans*-resveratrol could exert a radioprotective effect by reducing the direct radio-induced DNA damage.

In order to visualize the PCC experiments, **Figure 53** shows the pictures obtained from the PCC control experiments at different irradiation doses and PCC experiments with *trans*-resveratrol pre-treatment at 2.2 µM (it does not represent all the concentrations because no effect was observed when increasing the concentration of resveratrol).

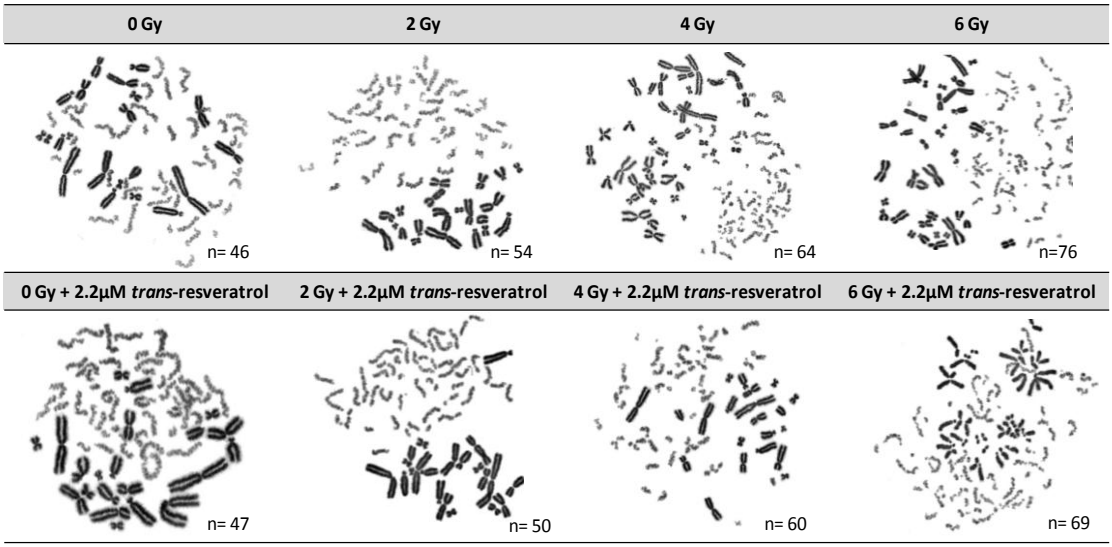

Figure 53. PCC obtained in control and *trans*-resveratrol 2.2 µM pre-treated PCC samples.

The results obtained in the present work demonstrate that 1 h pre-treatment of human peripheral blood lymphocytes with different concentrations of *trans*-resveratrol protects cells from radiation-induced chromosomal damage. Therefore, following the PCC protocol, it is possible to study the direct chromosomal damage induced by ionizing radiation and the immediate preventive effect of *trans*-resveratrol. This radioprotective effect is similar to that offered by curcumin mentioned in the previous point. The ability of *trans*-resveratrol to scavenge radiation-induced free radicals must be the most probably mechanism by which the compound can decrease an excess in the yield of fragments caused by radiation. Again, in order to exert its scavenging properties *trans*-resveratrol must cross the cellular and nuclear membranes and remain near to the DNA double helix. It has been demonstrated by

fluorescence microscopy that it is essentially present in cytoplasm and in the nucleolus region (Lancon *et al.,* 2004). Moreover, in order to accurately quantify the *trans*-resveratrol uptake, Delmas *et al.* (2011a) labeled the polyphenol with tritium in the ortho- and parapositions of the benzenic rings and they observed that the time-, dose- and temperature-dependencies of the tritiated molecule influx showed a passive diffusion (50%) and a carrier-mediated process (50%) (**Figure 54**) in several cell lines (hepatoblastoma HepG2 cells, human normal hepatocytes, in leukaemia cells and normal monocytes).

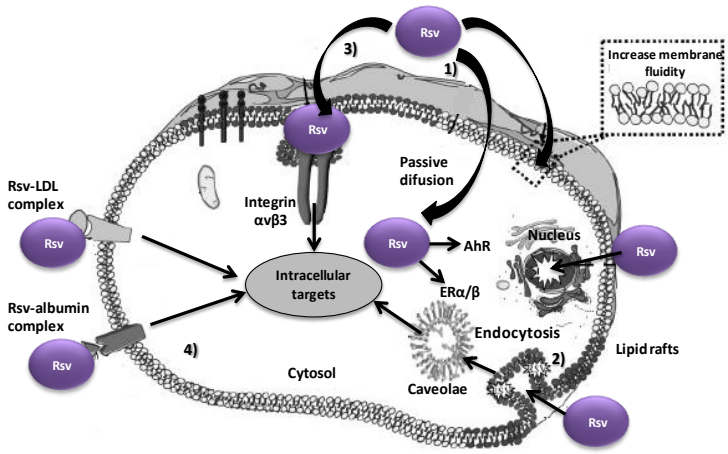

Figure 54. Resveratrol (Rsv) uptake and binding to extracellular and intracellular receptors. The uptake could be due to passive fusion (1), endocytosis via lipid rafts (2) or by binding to receptor such as integrin (3). Albumin and LDL receptors are important carriers for Rsv (4) and are very likely to play an essential role in its distribution (Delmas *et al.,* 2011b).

The results did not show a concentration-dependent increase in the radioprotective activity of *trans*-resveratrol. In the study of Delmas et al. (2011a) the value of the maximum concentration found was approximately 400 pmol/10^6 cells; it could be seen by fluorescence that resveratrol was present inside the nucleus but obviously at pmol order units. It is possible, therefore, that although the concentrations were increased, only a very small amount of molecules were able to reach the nucleus region and act as a radioprotector.

Results obtained from the PCC together with those obtained with the dicentric assay, where a radioprotective effect was also observed suggests that *trans*-resveratrol can exert a radioprotector action immediately after irradiation by being present during

the entire cell cycle, and that this action is mostly mediated by free radical scavenging properties or by regulation of different enzymes and cellular transcription pathways.

To the best of my knowledge, no literature is available concerning the use of PCC methodology to elucidate the radioprotective activity of antioxidant compounds. However, this methodology has been used to evaluate the combined effect of some chemicals and the irradiation on the DNA damage (Hazti *et al.,* 2008). For instance, some research has been developed concerning the chemical glutaraldehyde (GA) which is a high production volume compound very reactive with a wide spectrum of medical, scientific and industrial applications. GA has been extensively tested for genotoxic activity *in vitro* and *in vivo* and some authors stated that it may be considered as a suspected leukemogen. Taking into account that ionizing radiation is associated with acute myeloid leukemia; Hatzi *et al.* (2008) decided to clarify some lack of information concerning the combined effects of both GA and ionizing radiation in human cells. The authors observed pre-irradiation exposure of human peripheral blood lymphocytes to non-genotoxic doses of GA which showed a statistically significant increase in chromosomal radiosensitivity and they then used the PCC methodology to explain why this occurred. The chromosomal damage in the lymphocytes was evaluated immediately after irradiation in the presence or absence of GA and the linear dose response curves obtained show that exposure to GA affects the yield of radiation induced chromosomal damage in a statistically significant manner.

Using a similar approach to Hatzi *et al.* (2008, the PCC experiments here presented showed that both curcumin and *trans*-resveratrol provide radiation protection in the peripheral blood human lymphocytes, at least in part, by reducing the initial radiation-induced chromosomal damage.

D.5. Radiosensitivity

D.5.1. Radiosensitivity induced by curcumin and trans-resveratrol assessed by G2-assay

D.5.1.1. Radiosensitivity induced by curcumin

Results obtained from the analysis of the G2-assay with 1h pre-irradiation incubation of curcumin concentrations of 1.4 and 140 µM are presented in **Table 21**. Two samples were taken as controls; one only with blood in medium culture and the other with blood in medium culture incubated in the presence of a compound solvent (ethanol 95%).

Table 21. Results of the mean of chromatid type breaks (Mean ctb/cell ± SD) for each G2 experiment with curcumin and the G2 checkpoint efficiency.

G2 assay treatment	Mean ctb/cell ± SD		G2 checkpoint efficiency
	G2-assay	G2-assay + caffeine	
G2	3.23 ± 0.16	9.70 ± 0.61	0.67
G2 ethanol 95%	3.82 ± 0.20	9.24 ± 0.55	0.61
G2 1.4 µM Cur	4.98 ± 0.08	9.7 ± 1.04	0.49
G2 + 140 µM Cur	7.96 ± 0.17	12.25 ± 0.78	0.19

The G2 checkpoint efficiency was calculated as: (Mean ctb G2+caffeine-Mean cb G2 treatment)/Mean cbG2+caffeine. Ctb: chromatid break; Cur: curcumin; SD: Standard deviation.

From the table 21, the potent effect of caffeine in abrogating the G2 checkpoint can be deduced. As an example of this effect, it can be observed that the mean ctb/cell for the G2-assay + caffeine (9.70 ± 0.60) is almost 3-fold the value of the G2-assay without caffeine (3.23 ± 0.16). In general, it is noticeable that the presence of curcumin before the irradiation step increased the mean value in the G2-assay (4.98 for 1.4 µM and 7.96 for 140 µM) compared to the control sample (3.23). The table also shows that the G2-checkpoint efficiency was reduced in the two curcumin treatments with this reduction more than a half for 140 µM curcumin.

In order to better interpret the results, statistical analysis was performed using a linear multiple regression model. The model considered as response variable the mean value of ctb/cell and as explanatory variables the ethanol 95%, curcumin concentration and caffeine. Although caffeine has been confirmed as a G2/M checkpoint abrogation agent (Pantelias and Terzoudi, 2011) it was also taken as a variable in order to evaluate all the variables in the statistical model (**Table 22**).

Table 22. Linear regression model used for the each G2 experiment with different concentrations of curcumin.

	Estimate	Std Error	p-value
Intercept	3.14	0.26	<0.001
Ethanol	0.68	0.37	0.065
Curcumin 1.4	1.83	0.37	<0.001
Curcumin 140	4.81	0.38	<0.001
Caffeine	6.64	0.37	<0.001
Ethanol : caffeine	-1.22	0.51	0.018
Curcumin 1.4 : caffeine	-1.80	0.52	<0.001
Curcumin 140 : caffeine	-2.35	0.56	<0.001

Multiple R-squared: 0.573; Adjusted R-squared: 0.569

On the one hand, the G2-assay experiments with no caffeine added gave the following results. The mean value for ctb/cell in the G2-assay was 3.14 with a CI form 2.64 to 3.65. The pre-incubation with ethanol 95% did not statistically increase ($p=0.065$) the mean value of ctb/cell. However, concentrations of curcumin increased (1.83 for 1.4 µM and 4.81 for 140 µM) statistically ($p<0.001$) the mean value of ctb/cell. On the other hand, the G2-assay samples treated with caffeine gave the following results. As expected, the mean value for ctb/cell in the G2-assay with caffeine (6.64) was significantly increased ($p<0.001$). In case of curcumin, the increase in ctb/cell yielded a statistical reduction ($p<0.001$) in damage (-1.80 and -2.35) for both concentrations in comparison with the yield of G2 with caffeine control sample (6.64).

Figure 55 shows the graphic with the results obtained with the multiple regression statistical model. Comparing the results obtained between conventional G2 and G2 + caffeine when curcumin was present in the samples we have the following differences. On the one hand, in the treatments without caffeine, the lowest concentration of curcumin (1.4 µM) statistically increased the ctb yield (1.83 units) but this damage was masked in the G2 + caffeine which exactly reduced the ctb yield (-1.80 units) in these units. This could mean that curcumin affected somehow the G2 checkpoint in the conventional G2-assay, an effect which cannot be observed in the G2 + caffeine because caffeine totally abrogated the checkpoint. On the other hand, in the treatments with caffeine, the highest concentration of curcumin (140 µM) statistically

increased the ctb yield which was more pronounced (4.81 units) than in the lowest concentration. When caffeine was present this yield was reduced in -2.35 units; if the same situation had been present in 1.4 µM approximately the same yield in G2 assay with caffeine should have been obtained as in the conventional G2 assay without caffeine but the result was nearly double (4.81 *vs* 2.35). If we consider that caffeine totally abrogates the G2-checkpoint, the increased damage observed in the conventional G2-assay (4.81) may be due to a checkpoint alteration in addition to an initial genotoxic effect of curcumin at 140 µM which raised the yield of ctb so markedly.

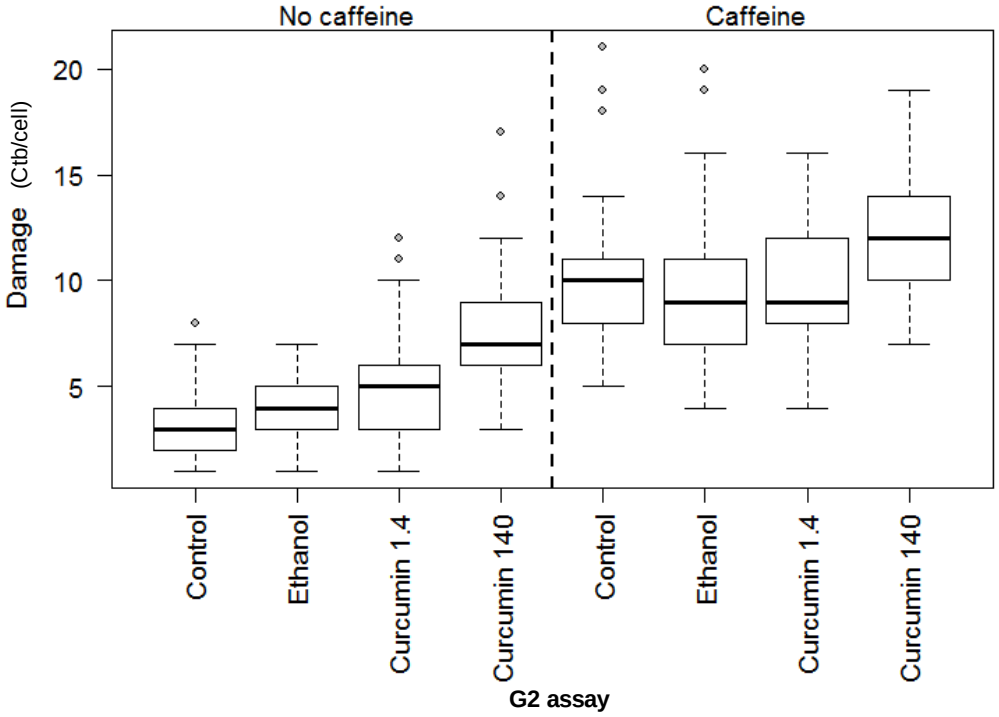

Figure 55. Different G2-assay experiments without and with caffeine (control, ethanol 95% and curcumin (µM)).

In an attempt to evaluate the effect of the curcumin in the G2 checkpoint following γ-radiation, we used conventional cytogenetics to estimate the amount of cells in metaphase, calculated with the mitotic index (MI) (**Table 23**).

Table 23. Mitotic index after G2-irradiated samples pre-incubated with ethanol 95% and curcumin.

G2-assay treatment	MI (Mean ± SD)
G2	0.008 ± 0.001
G2 + caffeine	0.030 ± 0.006
G2+ ethanol 95%	0.007 ± 0.002
G2+1.4 µM Cur	0.010 ± 0.002
G2+140 µM Cur	0.018 ± 0.004

The MI for the samples treated with curcumin following G2-irradiation was higher (0.010 for 1.4 µM and 0.018 for 140 µM) than the conventional G2-assay without compound (0.008) and became close to the G2 + caffeine (0.030) value at the highest concentration. Taking into account these results, we hypothesized that curcumin can act like caffeine in those cells which are in the G2 phase of the cell cycle (diving process), that is, inducing a less efficient G2-M checkpoint and allowing cells to go through mitosis without suffering cell repair mechanisms. This could be the reason why a higher ctb/cell yield in human peripheral blood lymphocytes pre-incubated with both concentrations of curcumin was observed. The fact that the G2 + caffeine assay at the highest concentration of curcumin (140 µM) reached a higher ctb than the G2 + caffeine control assay may be due to the genotoxic effects in some cell lines of curcumin observed at high concentrations (Cao *et al.,* 2006; Mendonça *et al.,* 2009) when joining with ionizing radiation may confer a randiosensitizing effect.

D.5.1.2. Radiosensitivity induced by trans-resveratrol

Results obtained from the analysis of G2-assay with 1h pre-irradiation incubation of *trans*-resveratrol concentrations ranging of 2.2 and 200 µM are presented in **Table 24**. One sample was taken as a compound solvent control and lymphocytes were incubated only in the presence of ethanol 95%.

Table 24. Results of the mean of chromatid type breaks (Mean ctb/cell ± SD) for each G2 experiment with *trans*-resveratrol and the G2 checkpoint efficiency. .

G2 assay treatment	Mean ctb/cell ± SD		G2 checkpoint efficiency
	G2 assay	G2 assay + caffeine	
G2	3.14 ± 0.16	9.79 ± 0.61	0.68
G2 ethanol 95%	3.82 ± 0.20	9.24 ± 0.55	0.61
G2 2.2 µM Res	4.86 ± 0.42	11.21 ± 0.10	0.50
G2 + 220 µM Res	5.29 ± 0.20	9.97 ± 0.59	0.46

The G2 checkpoint efficiency was calculated as: (Mean ctb G2+caffeine-Mean ctb G2 treatment)/Mean ctbG2+caffeine. Ctb: chromatid break; Res: *trans*-resveratrol.

Table 24 shows in general that the presence of *trans*-resveratrol before the irradiation procedure increased the mean value in the G2-assay (4.86 for 2.2 µM and 5.29 for 220 µM) compared to the control sample (3.23). The G2-checkpoint efficiency was reduced in both treatments with *trans*-resveratrol.

To better interpret the results, statistical analysis was performed using a linear multiple regression model. The model considered as a response variable the mean value of the ctb/cell and ethanol 95%, *trans*-resveratrol concentration and caffeine as explanatory variables. Although caffeine has been confirmed as a G2/M checkpoint abrogation agent it was also taken as a variable in order to evaluate all the variables in the statistical model (**Table 25**).

Table 25. Linear regression model used for the each G2 experiment with different concentrations of *trans*-resveratrol.

	Estimate	Std Error	p-value
Intercept	3.14	0.27	<0.001
Ethanol	0.68	0.38	0.08
Resveratrol 2.2	1.82	0.43	<0.001
Resveratrol 220	2.14	0.38	<0.001
Caffeine	6.64	0.38	<0.001
Ethanol : caffeine	-1.22	0.54	0.024
Resveratrol 2.2:caffeine	-0.40	0.57	0.488
Resveratrol 220 : caffeine	-1.97	0.54	<0.001

Multiple R-squared: 0.573; Adjusted R-squared: 0.569

On the one hand, the G2-assay with no caffeine addition offered the following results. The mean value for the ctb/cell in the conventional G2-assay was 3.14 with a CI from 2.61 to 3.68. The pre-incubation with ethanol 95% did not statistically increase ($p=0.08$) the yield of the ctb/cell. However, concentrations of *trans*-resveratrol statistically ($p<0.001$) increased (1.82 for 2.2 µM and 2.14 for 220 µM) the yield of the ctb/cell. On the other hand, the G2-assay samples treated with caffeine gave the following results. As expected, the mean value for the ctb/cell in the G2-assay with caffeine was significantly increased ($p<0.001$) by 6.64 units. When *trans*-resveratrol and caffeine were present, the increase in the ctb/cell yielded a statistical reduction ($p<0.001$) (-1.97) only at 220 µM in comparison with the yield of G2 with the caffeine control sample. Considering the G2 checkpoint efficiency, both ethanol and *trans*-resveratrol reduced this efficiency but not as much as curcumin did (see **Table 21**).

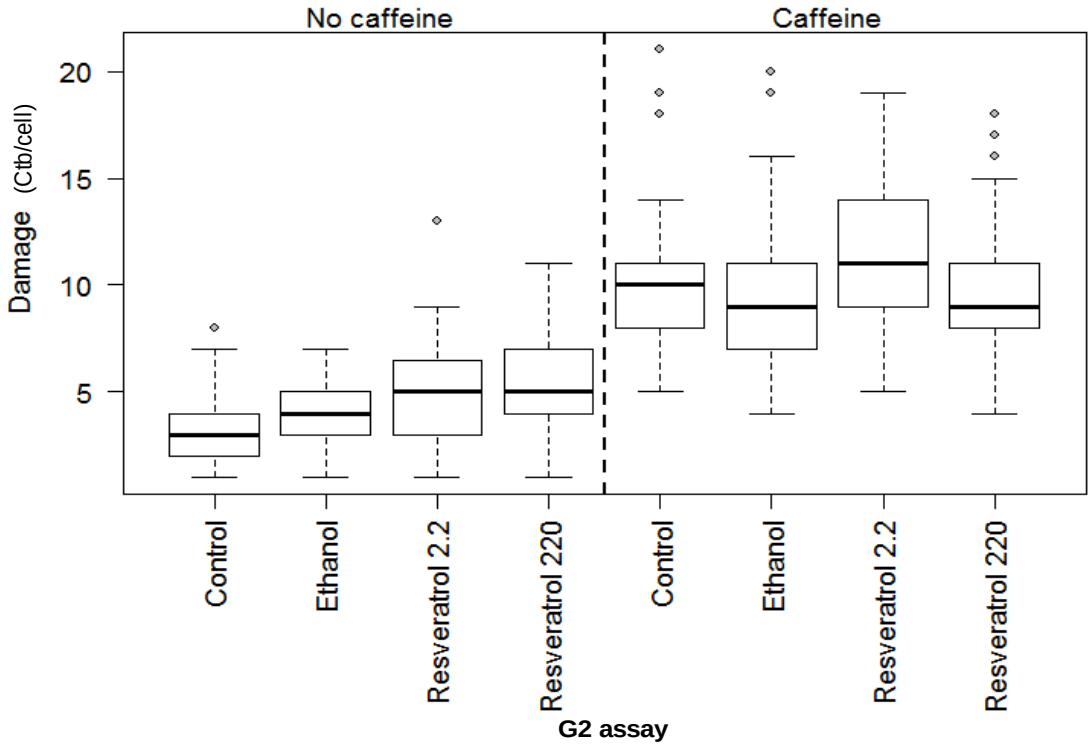

Figure 56. Different G2-assay experiments without and with caffeine (control, ethanol 95% and trans-resveratrol (µM)).

Figure 56 shows the graphic with the results obtained using the multiple regression statistical model. Comparing the results obtained between the conventional G2 and G2 + caffeine when *trans*-resveratrol was present in the samples the following

findings are shown. On the one hand, the lowest concentration (2.2 µM) statistically increased the ctb yield (1.82) in the conventional G2 assay whereas in the G2 + caffeine assay this increase was not statistically reduced (-0.40) by the presence of caffeine which is supposed to completely abrogates the checkpoint. An initial genotoxic damage caused by *trans*-resveratrol may exist which can be observed when caffeine was applied (see **Table 24**) because the ctb yield is even higher than that offered by caffeine (9.79 ± 0.61 *vs* 11.21 ± 0.10). On the other hand, at the highest concentration (220 µM) there was a statistical increase (2.14) in the ctb yield in the conventional G2-assay which is approximately counteracted by *trans*-resveratrol in the G2 + caffeine experiment (-1.97). In this case, the action of the polyphenol may reside in the G2/M checkpoint affectation.

The effect of *trans*-resveratrol at the highest concentration in the G2-checkpoint, following exposure to γ-radiation, was evaluated using conventional cytogenetics to estimate the percentage of cells in metaphase, the mitotic index (MI) (**Table 26**).

Table 26. Mitotic index after G2-irradiated samples pre-incubated with ethanol 95% and *trans*-resveratrol.

G2-assay treatment	MI (Mean ± SD)
G2	0.008 ± 0.001
G2 + caffeine	0.030 ± 0.006
G2+ ethanol 95%	0.007 ± 0.002
G2+2.2 µM Res	0.009 ± 0.004
G2+220 µM Res	0.020 ± 0.005

Bearing in mind the results for the MI, the highest concentration of *trans*-resveratrol had the closest value (0.020) to that obtained in G2 + caffeine (0.030). This means that this polyphenol might act like caffeine in those cells which are in the G2 phase of the cell cycle, inducing a less efficient G2 checkpoint and therefore, allowing cells to go through mitosis. These results match with those of Fiore *et al.* (2005) who investigated the cellular response of lymphoblastoid cells AHH-1 to treatments with X rays and *trans*-resveratrol, alone or in combination, in terms of DNA damage, cell cycle delays and induction of apoptosis. Their results showed that, as with most of the

anticancer agents, *trans*-resveratrol caused the induction of cell apoptosis, but they noticed an interesting fact, in that it prevented the G2 phase cycle arrest induced by X-rays. This observation is similar to ours given that *trans*-resveratrol pushed cells to mitosis by partially inhibiting the G2 phase arrest induced by ionizing radiation.

Some other studies have used this G2-assay to assess the chromosomal radiosensitization *in vitro* induced by specific chemical compounds. The authors (Hatzi *et al.,* 2007; Hatzi *et al.,* 2008) state that the application of this methodology can help to evaluate, *in vitro*, the potential radiosensitizing cell G2 phase of certain chemicals used in professional environments where there may be simultaneous exposure to ionizing radiation which helps to determine the potential risk of combined exposure to these chemical compounds and radiation. For instance, in the work of Hatzi *et al.* (2007) 24-h pre-irradiation exposure of peripheral blood lymphocytes to the benzene metabolite hydroquinone induced a less efficient G2-M-checkpoint and thus enhanced the G2-chromosomal radiosensitivity in a statistically significant manner.

Using the G2-assay curcumin and *trans*-resveratrol could be studied in vitro as to how they may influence the cell cycle by affecting the G2/M checkpoint. Since a deficiency in cell cycle checkpoints and an increase in G2-chromosomal radiosensitivity are linked to chromosomal instability, cancer proneness and the development of leukemia (Hatzi *et al.,* 2007), it seems important to study how certain compounds which may be combined with the treatment with irradiation can simultaneously influence the cell cycle.

D.5.2. Individual radiosensitivity. Case report.

The G2-assay for the final endpoint calculation of the individual radiosensitiviy (IRS) parameter of a patient with a radiological intervention was carried out according to Pantelias and Terzoudi (2011). In their study they investigated the induction and repair kinetics of chromatid breaks, their potential role in radiosensitivity predisposition and they proposed a standardized G2-assay in order to assess individual radiosensitivity. They blood lymphocytes analysed from healthy donors and the data obtained was used to correlate G2-checkpoint efficiency with chromatid breakage and individual radiosensitivity. Due to the significant inter-individual variation in G2

chromosomal radiosensitivity, experiments involving repair kinetics of chromatid breaks using colcemid-block and treatment with caffeine to abrogate the G2-checkpoint, generating internal controls and then, the G2-assay was standadized. They defined the individual radiosensitivity parameter as IRS = (G2/G2caf) x 100% and the ratio (G2caf-G2)/G2caf as the G2-checkpoint efficiency for preventing chromatid breakage. After the experiments, the inter-individual variation in radiosensitivity, as expressed by variations in the IRS values, was fitted by a normal distribution with a mean value of 40.1%, a standard deviation of 9.8%, and a coefficient variation of 18%. According to their proposed method, an individual can be classified as ‘‘radioresistant’’, ‘‘normal’’, ‘‘radiosensitive’’, or ‘‘highly radiosensitive’’ (**Figure 57**).

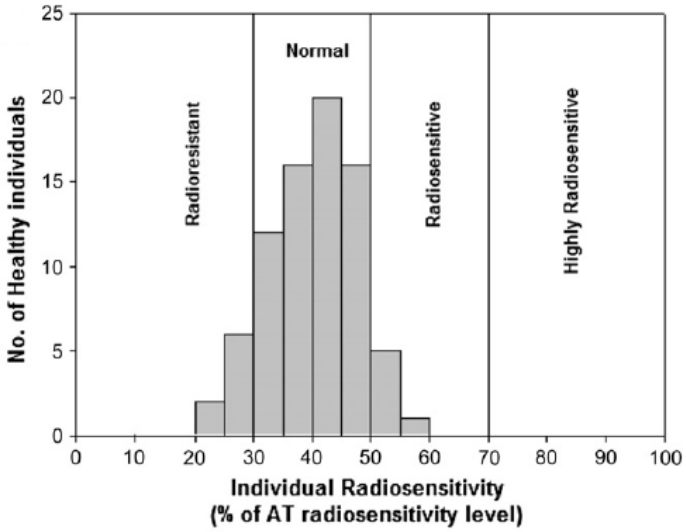

Figure 57. G2-chromosomal radiosensitivity inter-individual variation analysis obtained with the standardized G2-assay (Pantelias and Terzoudi, 2011).

In order to evaluate the individual patient radiosensitivity two distinct yields of chromatid type breaks (ctb) per individual were obtained from the analysis of the G2-assay with and without caffeine (**Table 27**).

Table 27. G2-assay yields obtained for the control donor and the patient.

G2 Assay	Ctb/cell (Yield ± SE)		IRS (%)	G2 checkpoint efficiency
	Without caffeine	With caffeine		
Control	4.25 ± 0.31	10.9 ± 0.45	38.9	0.61
Patient	5.02 ± 0.35	12.8 ± 0.56	39.2	0.61

Ctb: chromatid type breaks; SE: Standard Error; IRS: individual radiosensitivity

The mean G2 aberration yield obtained for the control donor not exposed to ionizing radiation and the patient was 4.25 ± 0.31 and 5.02 ± 0.35 ctb/cell, respectively; the mean G2 + caffeine yield for the control donor and the patient was 10.9 ± 0.45 and 12.8 ± 0.56, respectively.

The G2 checkpoint efficiency value (0.61) for preventing chromatid breakage was exactly the same in the control and the patient. Although the ctb/cell yields between the control donor and the patient seem different, the key value is the individual radiosensitivity value (IRS). According to this standardized method, the IRS values obtained from our analysis can be compared with those from the **Figure 57** and then is possible to classify the IRS parameter obtained after the G2-assay. Considering that our values are 38.9 and 39.2% for the control donor and the patient, respectively, it can be said that both subjects are classified within the "normal" individual range.

This study aimed to evaluate the possible radiosensitivity of a patient who has been exposed to ionizing radiation because of radiological diagnosis and interventions. Overall, the patient received one TAC, two panarteriographies which diagnosed three aneurisms and one interventional panarteriography for the embolization of one of the aneurysms placed on the left posterior artery. A few weeks later, the patient suffered hair loss in the treated area. According to Stecker et al. (2009) patients with a fluoroscopy time higher than 60 min should be followed up because a fluoroscopy time greater than 60 min is not itself a dose value, but is an indirect indicator of a significant radiation dose. The fluoroscopy time for the patient was 55. 47 min, and therefore due to the proximity to the established threshold it was thought appropriate to undertake a follow up.

Physic dosimetry estimated a total skin dose of 3240 mGy in the irradiated head area and an effective dose of 70 mSv (Stecker *et al.,* 2009) and biological dosimetry

estimated an absorbed dose of 112.5 mGy (confidence limits at 95%, 33.6-236). In relation to these physical and biological values, not being the same value as the whole-body dose and considering the confidence intervals of biological dosimetry, it can be said that there is a good correlation between the two results.

Data provided by Stecker et al. (2009) regarding the acute effects after a single irradiation dose on the skin stated that when the single dose is between 2 to 5 Gy it causes the depilation of the treated area after 2 to 8 weeks. According to data from physical dosimetry (3.24 Gy) the patient was in this range, so that hair loss may probably be due to irradiation rather than an individual radiosensitivity.

In this case the G2-assay helped in the clarification of the fact that the acute secondary effects of the radiation observed were not a consequence derived from the radiosensitivity of the patient. In addition this assay highlighted radiosensitive subjects which permitted improved preventive measures which could be applied in radiological procedures. The application of this assay can, therefore, be considered as a useful tool which may have a straightforward application in clinical practice by providing individual radiotherapy protocols and population screening for patients or workers in contact with ionizing radiation.

CONCLUSIONS

CONCLUSIONS

1. In the tobacco study, smoking subjects had frequencies of SCEs and HFCs significantly higher than non-smokers. Moreover, it was observed in the smoking group that the SCE distribution pattern was not random but concentrated the interchanges in a chromosome instead of being uniforminly distributed in the metaphase.

2. Subjects affected by the "Ardystil syndrome" did not present DNA damage detectable by means of SCEs or HFCs ten years after the accident. However, when the SCE distribution pattern was analyzed a statistically significant difference was found between the Ardystil group and the control group.

3. In the curcumin genotoxicity assay, the molecule was genotoxic at the highest studied concentrations inducing chromosome aberrations, mainly acentric fragments. Nevertheless, no effect on the frequency of the SCEs was observed. Curcumin also stimulated the cell division process during the cell cycle.

4. In the t*rans*-resveratrol genotoxicity assay, a genotoxic activity was observed, even at lower concentrations, inducing chromosome aberrations, characterized by a hormetic effect. In addition, this compound increased the frequency of SCEs in a dose-dependent manner. Related to the cell cycle, *trans*-resveratrol increased the cell proliferation kinetics.

5. Radioprotection studies with the dicentric assay and PCC experiments showed that curcumin can act as an *in vitro* radioprotector. The direct and indirect radioprotective mechanisms might be due to its antioxidants properties and the ability in regulating the antioxidant endogenous factors. This radioprotective activity was not concentration-dependent for both assays.

6. Radioprotection studies with dicentric assays and PCC experiments showed that *trans*-resveratrol is able to exert an *in vitro* radioprotective activity mediated by the increase of endogenous antioxidant factors and by its antioxidant properties. The radioprotective ability matched with a hormetic effect for the dicentric assay, but no concentration dependence was observed for the PCC assay.

7. Regarding the G2 radiosensitivity assay, both curcumin and *trans*-resveratrol were able to increase cellular radiosensitivity, mainly by reducing the efficiency of the checkpoint in the G2 phase of the cell cycle.

8. The study of individual radiosensitivity with the G2 assay in a case report of a patient underwent a vascular interventional process was a helpful tool, complementing the physical and biological dosimetry, to conclude that the deterministic effect of the localized hair loss induced by the intervention was not associated with patient radiosensitivity.

10. Cytogenetic techniques for analyzing biomarkers are useful, easy, not extremely expensive and are standardized methodologies for the study of human exposure to chemical and physical agents. Using cytogenetic tools it is possible to personalize and achieve more efficient medical treatments.

E. CONCLUSIONES

1. En el estudio del tabaco, se observó que los sujetos fumadores presentaban frecuencias de SCEs y HFCs significativamente más elevadas que los no fumadores. Además, en el caso de los fumadores se observó que el patrón de distribución de SCEs en los cromosomas no era al azar sino que concentraba los intercambios en un mismo cromosoma en lugar de distribuirlos uniformemente en la metafase.

2. Los sujetos afectados por el "síndrome de Ardystil" no presentaron daño en el ADN detectable por medio del análisis de SCEs o HFCs diez años después del suceso. Sin embargo, cuando se analizó el patrón de distribución de SCEs se encontraron diferencias significativas respecto al grupo control.

3. En el ensayo de genotoxicidad de la curcumina, ésta resultó genotóxica a las concentraciones más altas estudiadas induciendo aberraciones cromosómicas, principalmente fragmentos acéntricos. En cambio, no ejerció ningún efecto en la frecuencia de SCEs. En relación a su influencia en el ciclo celular, la curcumina estimuló el proceso de división de las células.

4. En el ensayo de genotoxicidad del *trans*-resveratrol se observó una acción genotóxica, incluso a concentraciones bajas, que inducía aberraciones cromosómicas, caracterizada por un efecto de hormesis. Además, este compuesto aumentó la frecuencia de SCEs de forma concentración-dependiente. Respecto al ciclo celular, el *trans*-resveratrol aumentó la cinética de proliferación de las células.

5. Los estudios de radioprotección mediante el ensayo de dicéntricos y los experimentos de PCC mostraron que la curcumina actúa como un agente radioprotector *in vitro.* Los mecanismos de radioprotección, tanto en el efecto directo como en el indirecto, podrían deberse a su capacidad antioxidante y reguladora de agentes antioxidantes endógenos. Este efecto radioprotector no fue concentración-dependiente en ninguno de los dos ensayos.

6. Los estudios de radioprotección para el *trans*-resveratrol mediante el ensayo de dicéntricos y los experimentos PCC mostraron que éste es capaz de ejercer una actividad radioprotectora, *in vitro,* debido a la regulación de la actividad de los antioxidantes endógenos y a sus propiedades antioxidantes. El efecto radioprotector

respondió a un efecto de hormesis para el ensayo de dicéntricos, no encontrándose ninguna afectación debido a la concentración en los experimentos de PCC.

7. Respecto al ensayo G2 de radiosensibilidad, tanto la curcumina como el *trans*-resveratrol fueron capaces de aumentar la radiosensibilidad celular, principalmente mediante la reducción de la eficiencia del punto de control en fase G2 del ciclo celular.

8. El estudio de radiosensibilidad individual en un caso de una paciente sometida a un proceso de intervencionismo vascular ha sido una herramienta útil, y complementaria a la dosimetría física y biológica, para concluir que el efecto determinista de la caída localizada del cabello inducido por la intervención no estuvo asociado a una radiosensibilidad propia de la paciente.

9. Las técnicas citogenéticas enfocadas al análisis de biomarcadores resultan útiles, fáciles, asequibles y reproducibles para el estudio de la exposición humana a determinados agentes químicos y físicos.

REFERENCES

F. REFERENCES

Abraham SK, Sarma L, Kesavan PC. Protective effects of chlorogenic acid, curcumin and beta-carotene against gamma-radiation-induced in vivo chromosomal damage. Mutat. Res. 1993, 303:109-12.

Aftab N, Vieira A. Antioxidant activities of curcumin and combinations of this curcuminoid with other phytochemicals. Phytother. Res. 2010, 24:500-2.

Aggarwal BB, Kumar A, Bharti AC. Anticancer potential of curcumin: preclinical and clinical studies. Anticancer Res. 2003, 23:363-98.

Aggarwal BB, Sung B. Pharmacological basis for the role of curcumin in chronic diseases: an age-old spice with modern targets. Trends Pharmacol. Sci. 2009, 30:85-94.

Ahsan H, Parveen N, Khan NU, Hadi SM. Pro-oxidant, anti-oxidant and cleavage activities on DNA of curcumin and its derivatives demethoxycurcumin and bisdemethoxycurcumin. Chem. Biol. Interact. 1999, 121:161-75.

Alaikov T, Konstantinov SM, Tzanova T, Dinev K, Topashka-Ancheva M, Berger MR. Antineoplastic and anticlastogenic properties of curcumin. Ann. N.Y. Acad. Sci. 2007, 1095:355-70.

Albertini RJ, Sram RJ, Vacek PM, Lynch J, Wright M, Nicklas JA, Boogaard PJ, Henderson RF, Swenberg JA, Tates AD, Ward JB Jr. Biomarkers for assessing occupational exposures to 1,3-butadiene. Chem. Biol. Interact. 2001, 135-136:429-53.

Alpsoy S, Aktas C, Uygur R, Topcu B, Kanter M, Erboga M, Karakaya O, Gedikbasi A. Antioxidant and anti-apoptotic effects of onion (Allium cepa) extract on doxorubicin-induced cardiotoxicity in rats. J. Appl. Toxicol. 2013, 33:202-8.

Alsner J, Andreassen CN, Overgaard J. Genetic markers for prediction of normal tissue toxicity after radiotherapy. Semin. Radiat. Oncol. 2008, 18:126-35.

Anand P, Kunnumakkara AB, Newman RA, Aggarwal BB. Bioavailability of curcumin: problems and promises. Mol. Pharm. 2007, 4:807-18.

Anderson D, Jenkinson PC, Dewdney RS, Francis AJ, Godbert P, Butterworth KR. Chromosome aberrations, mitogen-induced blastogenesis and proliferative rate index in peripheral lymphocytes from 106 control individuals of the U.K. population. Mutat. Res. 1986, 204:407-20.

Andreoli C, Gigante D, Nunziata A. A review of in vitro methods to assess the biological activity of tobacco smoke with the aim of reducing the toxicity of smoke. Toxicol. In Vitro. 2003, 17:587-94.

Antunes LM, Araújo MC, Dias FL, Takahashi CS. Modulatory effects of curcumin on the chromosomal damage induced by doxorubicin in Chinese hamster ovary cells. Teratog. Carcinog. Mutagen. 1999, 19:1-8.

Arora R, Kumar R, Sharma A, Tripathi RP. Radiomodulatory compounds of herbal origin for new frontiers in medicine, homeland security, management of radiological incidents and space applications. In: Arora R. Herbal radiomodulators. Applications in medicine, homeland defence and space. (Ed.) CAB International, Oxfordshire, UK, 2008.

Arora R. Phytodefence molecules for defence forces: utility and future prospects. In: Gupta, M, Kumar, IP, Arora, R. Herbs for Health: Application in Armed Forces. (Ed) Institute of Nuclear Medicine and Allied Sciences, Delhi, India, 2006:58–62.

Arts IC, Hollman PC. Polyphenols and disease risk in epidemiologic studies. Am. J. Clin. Nutr. 2005, 81:317-325.

Aydin S, Basaran AA, Basaran N.Modulating effects of thyme and its major ingredients on oxidative DNA damage in human lymphocytes. J. Agric. Food Chem. 2005, 53:1299-305.

Azmi AS, Bhat SH, Hanif S, Hadi SM. Plant polyphenols mobilize endogenous copper in human peripheral lymphocytes leading to oxidative DNA breakage: a putative mechanism for anticancer properties. FEBS Lett. 2006, 580:533-8.

Azzam EI, Jay-Gerin JP, Pain D. Ionizing radiation-induced metabolic oxidative stress and prolonged cell injury. Cancer Lett. 2012, 327:48-60.

Balasubramanyam M, Koteswari AA, Kumar RS, Monickaraj SF, Maheswari JU, Mohan V. Curcumin-induced inhibition of cellular reactive oxygen species generation: novel therapeutic implications. Biosci. 2003, 28:715-21.

Banerjee A, Kunwar A, Mishra B, Priyadarsini KI. Concentration dependent antioxidant/pro-oxidant activity of curcumin studies from AAPH induced hemolysis of RBCs. Chem. Biol. Interact. 2008, 174:134-9.

Barquinero JF. Master en Biología Humana. Universidad Autónoma de Barcelona. 1991.

Battershill JM, Burnett K, Bull S. Factors affecting the incidence of genotoxicity biomarkers in peripheral blood lymphocytes: impact on design of biomonitoring studies. Mutagenesis 2008, 23:423-37.

Ben Salah G, Kamoun H, Rebai A, Ben Youssef A, Ayadi H, Belghith-Mahfoudh N, Fourati A, Ayadi H, Fakhfakh F. Sister chromatid exchange (SCE) and high-frequency cells (HFCs) in peripheral blood lymphocytes of healthy Tunisian smokers. Mutat. Res. 2011, 719:1-6.

Beninson D. Biological Dosimetry: Chromosomal aberration analysis for dose assessment, IAEA Technical Reports Series, 1986, Nº 260, Viena.

Bentzen SM. Potential clinical impact of normal-tissue intrinsic radiosensitivity testing. Radiother. Oncol. 1997, 43:121-31.

Bhat SH, Azmi AS, Hadi SM. Prooxidant DNA breakage induced by caffeic acid in human peripheral lymphocytes: involvement of endogenous copper and a putative

mechanism for anticancer properties. Toxicol. Appl. Pharmacol. 2007, 218:249-55.

Bisht K, Wagner KH, Bulmer AC. Curcumin, resveratrol and flavonoids as anti-inflammatory, cyto- and DNA-protective dietary compounds. Toxicology 2010, 278:88-100.

Bonassi S, Norppa H, Ceppi M, Strömberg U, Vermeulen R, Znaor A, Cebulska-Wasilewska A, Fabianova E, Fucic A, Gundy S, Hansteen IL, Knudsen LE, Lazutka J, Rossner P, Sram RJ, Boffetta P. Chromosomal aberration frequency in lymphocytes predicts the risk of cancer: results from a pooled cohort study of 22 358 subjects in 11 countries. Carcinogenesis 2008, 29:1178-83.

Bonassi S, Ugolini D, Kirsch-Volders M, Strömberg U, Vermeulen R, Tucker JD. Human population studies with cytogenetic biomarkers: review of the literature and future prospectives. Environ. Mol. Mutagen. 2005, 45:258-70.

Borgmann K, Hoeller U, Nowack S, Bernhard M, Röper B, Brackrock S, Petersen C, Szymczak S, Ziegler A, Feyer P, Alberti W, Dikomey E. Individual radiosensitivity measured with lymphocytes may predict the risk of acute reaction after radiotherapy. Int. J. Radiat. Oncol. Biol. Phys. 2008, 71:256-64.

Brenner W, Kampen WU, Brümmer C, Von Forstner C, Zuhayra M, Muhle C, Czech N, Henze E. Myeloprotective effects of different amifostine regimens in rabbits undergoing high-dose treatment with 186rhenium-(tin)1,1- hydroxyethylidene diphosphonate (186Re-HEDP). Cancer Biother. Radiopharm. 2003, 18:887-93.

Bruneton J. Farmacognosia: fitoquímica. Plantas medicinales. In: Bruneton J. 2ª edición, 2001, Acribia, Zaragoza, Spain.

Buyukleyla M, Azirak S, Rencuzogullari E, Kocaman AY, Ila HB, Topaktas M, Darici C. The genotoxic and antigenotoxic effects of tannic acid in human lymphocytes. Drug. Chem. Toxicol. 2012, 35:11-9.

Calabrese EJ, Mattson MP, Calabrese V. Resveratrol commonly displays hormesis: occurrence and biomedical significance. Hum. Exp. Toxicol. 2010, 29:980-1015.

Cao J, Jia L, Zhou HM, Liu Y, Zhong LF. Mitochondrial and nuclear DNA damage induced by curcumin in human hepatoma G2 cells.Toxicol. Sci. 2006, 91:476-83.

Cao J, Jiang LP, Liu Y, Yang G, Yao XF, Zhong LF. Curcumin-induced genotoxicity and antigenotoxicity in HepG2 cells. Toxicon 2007, 49:1219-22.

Carballo AM, Martinez RA, Mudry MD. Nitroimidazole derivatives: non-randomness sister chromatid exchanges in human peripheral blood lymphocytes. J. Appl. Toxicol. 2009, 29:248-54.

Carrano AV, Moore DH. The rationale methodology for quantifying sister chromatid exchanges in humans, in: J.A. Heddie (Ed), New Horizons in Genetic Toxicology. 1982, 99:267–304.

Carsten RE, Bachand AM, Bailey SM, Ullrich RL. Resveratrol reduces radiation-induced chromosome aberration frequencies in mouse bone marrow cells. Radiat. Res. 2008, 169:633-8.

Catalgol B, Batirel S, Taga Y, Ozer NK. Resveratrol: French paradox revisited. Front. Pharmacol. 2012, 3:141.

Cavusoglu K, Yalcin E. Radioprotective effect of lycopene on chromosomal aberrations (CAs) induced by gamma radiation in human lymphocytes. J. Environ. Biol. 2009, 30:113-7.

Cemeli E, Baumgartner A, Anderson D. Antioxidants and the Comet assay. Mutat. Res. 2009, 681:51-67.

Chakraborty A, Gupta N, Ghosh K, Roy P. In vitro evaluation of the cytotoxic, anti-proliferative and anti-oxidant properties of pterostilbene isolated from Pterocarpus marsupium. Toxicol. In Vitro 2010, 24:1215-28.

Chen M, Wang J. Initiator caspases in apoptosis signaling pathways. Apoptosis 2002, 7:313-9.

Chendil D, Ranga RS, Meigooni D, Sathishkumar S, Ahmed MM. Curcumin confers radiosensitizing effect in prostate cancer cell line PC-3. Oncogene 2004, 23:1599-607.

Citrin D, Cotrim AP, Hyodo F, Baum BJ, Krishna MC, Mitchell JB. Radioprotectors and mitigators of radiation-induced normal tissue injury. Oncologist 2010, 15:360-71.

Clottens FL, Verbeken EK, Demedts M, Nemery B. Pulmonary toxicity of components of textile paint linked to the Ardystil syndrome: intratracheal administration in hamsters. Occup. Environ. Med. 1997, 54:376-87.

Conner MK, Rubinson HF, Ferguson JS, Stock MF, Alarie Y. Evaluation of sister chromatid exchange and cytotoxicity in murine tissues in vivo and lymphocytes in vitro following methyl isocyanate exposure. Environ. Health Perspect. 1987, 72:177-82.

Consejo de Seguridad Nucelar (CSN). Available at http://www.csn.es/index.php?option=com_content&view=article&id=129&Itemid=134&lang=es.

Cordero P, Solé A. Bronchiolitis obliterans organizing pneumonia (Ardystil syndrome) and chronic demyelinating disease of the central nervous system. Arch. Bronconeumol. 1995, 31:89-92.

Cortés R. Ardystil syndrome. A new occupational disease. An. R. Acad. Nac. Med. 1994, 111:421-50.

Cozzi R, Perticone P, Bona R, Polani S. Antimutagenic activities of naturally occurring polyamines in Chinese hamster ovary cells in vitro. Environ. Mol. Mutagen. 1991, 18:207-11.

De Salvia R, Festa F, Ricordy R, Perticone P, Cozzi R. Resveratrol affects in a different way primary versus fixed DNA damage induced by H_2O_2 in mammalian cells in vitro. Toxicol. Lett. 2002, 135:1-9.

Delmas D, Aires V, Limagne E, Dutartre P, Mazué F, Ghiringhelli F, Latruffe N. Transport, stability, and biological activity of resveratrol. Ann. N.Y. Acad. Sci. 2011, 1215:48-59.

Delmas D, Lin HY. Role of membrane dynamics processes and exogenous molecules in cellular resveratrol uptake: consequences in bioavailability and activities. Mol. Nutr. Food Res. 2011, 55:1142-53.

Devipriya N, Sudheer AR, Srinivasan M, Menon VP. Quercetin ameliorates gamma radiation-induced DNA damage and biochemical changes in human peripheral blood lymphocytes. Mutat. Res. 2008, 654:1-7.

Directiva 89/391/CEE del Consejo, de 12 de junio de 1989, relativa a la aplicación de medidas para promover la mejora de la seguridad y de la salud de los trabajadores en el trabajo. Available at: http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=CELEX:31989L0391:ES:NOT.

Dirican E, Turkez H, Toğar B. Modulatory effects of Thymbra spicata L. different extracts against the mercury induced genotoxicity in human lymphocytes *in vitro*. Cytotechnology 2012, 64:181-6.

Dunn BP, Curtis JR. Clastogenic agents in the urine of coffee drinkers and cigarette smokers. Mutat. Res. 1985, 147:179-88.

Epstein J, Docena G, MacDonald TT, Sanderson IR. Curcumin suppresses p38 mitogen-activated protein kinase activation, reduces IL-1beta and matrix metalloproteinase-3 and enhances IL-10 in the mucosa of children and adults with inflammatory bowel disease. Br. J. Nutr. 2010, 103:824-32.

Fernandes TS, Lloyd D, Amaral A. A comparison of different cytological stains for biological dosimetry. Int. J. Radiat. Biol. 2008, 84:703-11.

Ferry-Dumazet H, Garnier O, Mamani-Matsuda M, Vercauteren J, Belloc F, Billiard C, Dupouy M, Thiolat D, Kolb JP, Marit G, Reiffers J, Mossalayi MD. Resveratrol inhibits the growth and induces the apoptosis of both normal and leukemic hematopoietic cells. Carcinogenesis 2002, 23:1327-33.

Fiore M, Festa F, Cornetta T, Ricordy R, Cozzi R. Resveratrol affects X-ray induced apoptosis and cell cycle delay in human cells in vitro. Int. J. Mol. Med. 2005, 15:1005-12.

Frémont L. Biological effects of resveratrol. Life Sci. 2000 66(8):663-73.

Fucić A, Garaj-Vrhovac V, Dimitrović B, Skara M. The persistence of sister-chromatid exchange frequencies in men occupationally exposed to vinyl chloride monomer. Mutat. Res. 1992, 281:129-32.

Fujimoto A, Sakanashi Y, Matsui H, Oyama T, Nishimura Y, Masuda T, Oyama Y. Cytometric analysis of cytotoxicity of polyphenols and related phenolics to rat thymocytes: potent cytotoxicity of resveratrol to normal cells. Basic Clin. Pharmacol. Toxicol. 2009, 104:455-62.

Fukuhara K, Miyata N. Resveratrol as a new type of DNA-cleaving agent. Bioorg. Med. Chem. Lett. 1998, 8:3187-92.

Gadano AB, Gurni AA, Carballo MA. Argentine folk medicine: genotoxic effects of Chenopodiaceae family. J Ethnopharmacol 2006, 103:246-51.

Galán E. Aplicaciones del laboratorio de citogenética a la clínica. Pediatr. Integral 2002, 6:820-830.

Gil F. El papel de los biomarcadores en Toxicología Humana. Toxicología 2000, 17:19-26.

Giri AK, Das SK, Talukder G, Sharma A. Sister chromatid exchange and chromosome aberrations induced by curcumin and tartrazine on mammalian cells in vivo. Cytobios 1990, 62:111-7.

Goel A, Aggarwal BB. Curcumin, the golden spice from Indian saffron, is a chemosensitizer and radiosensitizer for tumors and chemoprotector and radioprotector for normal organs. Nutr. Cancer 2010, 62:919-30.

Goel A, Kunnumakkara AB, Aggarwal BB. Curcumin as "Curecumin": from kitchen to clinic. Biochem. Pharmacol. 2008, 75:787-809.

Gomes RN, Colquhoun A. E series prostaglandins alter the proliferative, apoptotic and migratory properties of T98G human glioma cells in vitro. Lipids Health Dis. 2012, 11:171.

Hall EJ, Giaccia AJ. Radiobiology for the radiologist. 7th edition. (Ed) Lippincott Williams & Wilkins. Philadelphia, USA, 2012.

Hall EJ, Giaccia AJ. Radiobiology for the Radiologist, 6th Edition. (Ed) Lippincott Williams & Wilkins, Philadelphia, USA, 2006.

Harvey Lodish, Arnold Berk, S Lawrence Zipursky, Paul Matsudaira, David Baltimore, and James Darnell. Molecular Cell Biology. (Ed) W.H. Freeman, New York, USA, 2000.

Hatcher H, Planalp R, Cho J, Torti FM, Torti SV. Curcumin: from ancient medicine to current clinical trials. Cell Mol. Life Sci. 2008, 65:1631-52.

Hatzi VI, Terzoudi GI, Makropoulos V, Maravelias C, Pantelias GE. Pre-irradiation exposure of peripheral blood lymphocytes to glutaraldehyde induces radiosensitization by increasing the initial yield of radiation-induced chromosomal aberrations. Mutagenesis 2008, 23:101-9.

Hatzi VI, Terzoudi GI, Makropoulos V, Pantelias GE. A cytogenetic methodology to evaluate in vitro the G2-chromosomal radiosensitization induced by chemicals at non-clastogenic doses. J. Genet. 2009, 88:349-51.

Hatzi VI, Terzoudi GI, Pantelias GE, Spiliopoulou C, Makropoulos V. The benzene metabolite hydroquinone enhances G2-chromosomal radiosensitivity by inducing

a less-efficient G2-M-checkpoint in irradiated lymphocytes. Int. J. Oncol. 2007, 31:145-52.

Hatzi VI, Terzoudi GI, Paraskevopoulou C, Makropoulos V, Matthopoulos DP, Pantelias GE. The use of premature chromosome condensation to study in interphase cells the influence of environmental factors on human genetic material. Sci. World J. 2006, 6:1174-90.

Hatzi VI, Terzoudi GI, Stavropoulou C, Malik SI, Makropoulos V, Pantelias GE. Lack of association between GSTT1 polymorphism and endogenous or benzo[a]pyrene-induced sister chromatid exchanges as analyzed in metaphase or G2-phase lymphocytes. Mol. Biol. Rep. 2011, 38:3959-66.

Hecht SM. Bleomycin: new perspectives on the mechanism of action. J. Nat. Prod. 2000, 63:158-68.

Hedner K, Högstedt B, Kolnig AM, Mark-Vendel E, Strömbeck B, Mitelman F. Sister chromatid exchanges and structural chromosome aberrations in relation to smoking in 91 individuals. Hereditas 1983, 98:77-81.

Herrera JC. La citogenética molecular y su aplicación en el estudio de los genomas vegetales. Agron. Colomb. 2007, 25:26-35.

Hoeijmakers, JHJ. Genome maintenance mechanisms for preventing cancer. Nature 2001, 411:366–374.

Hoet PH, Gilissen LP, Leyva M, Nemery B. In vitro cytotoxicity of textile paint components linked to the "Ardystil syndrome". Toxicol. Sci. 1999, 52:209-16.

Hollander DH, Tockman MS, Liang YW, Borgaonkar DS, Frost JK. Sister chromatid exchanges in the peripheral blood of cigarette smokers and in lung cancer patients; and the effect of chemotherapy. Hum. Genet. 1978, 44:165-71.

Hosseinimehr SJ. Trends in the development of radioprotective agents. Drug Discov. Today 2007, 12:794-805.

Huertas P. DNA resection in eukaryotes: deciding how to fix the break. Nature Struc. Mol. Biol. 2010, 17:11-16.

Husum B, Wulf HC, Niebuhr E. Increased sister chromatid exchange frequency in lymphocytes in healthy cigarette smokers. Hereditas 1982, 96:85-8.

Inano H, Onoda M. Radioprotective action of curcumin extracted from Curcuma longa LINN: inhibitory effect on formation of urinary 8-hydroxy-2'-deoxyguanosine, tumorigenesis, but not mortality, induced by gamma-ray irradiation. Int. J. Radiat. Onco. Biol. Phys. 2002, 53:735-43.

International Agency for Research on Cancer (IARC). Re-evaluation of some organic chemicals, hydrazine and hydrogen peroxide. IARC Monographs, 1999, Volume 71.

International Agency for Research on Cancer (IARC). Chemical agents and related occupations. A review of human carcinogens. IARC Monographs 2012, Volume 100 F.

International Agency for Research on Cancer (IARC). Tobacco Smoke and Involuntary Smoking. IARC Monographs on the Evaluation of Carcinogenic Risks to Humans 2004, Volume 83.

International Atomic Energy Agency (IAEA), Biological dosimetry: chromosomal aberration analysis for dose assessment. Technical Reports Series 260, International Atomic Energy Agency, 2001.

Itzhaki J. Department of Biochemistry. University of Oxford. Available at http://www.bioch.ox.ac.uk/aspsite/index.asp?pageid=892.

Jagetia GC, Baliga MS. Influence of the leaf extract of Mentha arvensis Linn. (mint) on the survival of mice exposed to different doses of gamma radiation. Strahlenther Onkol. 2002 178:91-8.

. Jagetia GC, Malagi KJ, Baliga MS, Venkatesh P, Veruva RR. Triphala, an ayurvedic rasayana drug, protects mice against radiation-induced lethality by free-radical scavenging. J. Altern. Complement. Med. 2004, 10:971-8.

Jagetia GC, Rajanikant GK. Role of curcumin, a naturally occurring phenolic compound of turmeric in accelerating the repair of excision wound, in mice whole-body exposed to various doses of gamma-radiation. J. Surg. Res. 2004, 120:127-38.

Jagetia GC, Venkatesha VA, Reddy TK. Naringin, a citrus flavonone, protects against radiation-induced chromosome damage in mouse bone marrow. Mutagenesis 2003, 18:337-43.

Jagetia GC. Radioprotection and radiosensitization by curcumin. Adv. Exp. Med. Biol. 2007, 595:301-20.

Jang M, Pezzuto JM. Resveratrol blocks eicosanoid production and chemically induced cellular transformation: implications for cancer chemoprevention. Pharm. Biol. 1998, 36:28–34.

Joe AK, Liu H, Suzui M, Vural ME, Xiao D, Weinstein IB. Resveratrol induces growth inhibition, S-phase arrest, apoptosis, and changes in biomarker expression in several human cancer cell lines. Clin. Cancer Res. 2002, 8:893-903.

Johnson MD, Schilz J, Djordjevic MV, Rice JR, Shields PG. Evaluation of in vitro assays for assessing the toxicity of cigarette smoke and smokeless tobacco. Cancer Epidemiol. Biomarkers Prev. 2009, 18:3263-304.

Kao-Shan CS, Fine RL, Whang-Peng J, Lee EC, Chabner BA. Increased fragile sites and sister chromatid exchanges in bone marrow and peripheral blood of young cigarette smokers. Cancer Res. 1987, 47:6278-82.

Khabour OF, Alsatari ES, Azab M, Alzoubi KH, Sadiq MF. Assessment of genotoxicity of waterpipe and cigarette smoking in lymphocytes using the sister-chromatid exchange assay: a comparative study. Environ. Mol. Mutagen. 2011, 52:224-8.

Khafif A, Hurst R, Kyker K, Fliss DM, Gil Z, Medina JE. Curcumin: a new radio-sensitizer of squamous cell carcinoma cells. Otolaryngol Head Neck Surg. 2005, 132:317-21.

Kligerman AD, Atwater AL, Bryant MF, Erexson GL, Kwanyuen P, Dearfield KL. Cytogenetic studies of ethyl acrylate using C57BL/6 mice. Mutagenesis 1991, 6:137-41.

Kligerman AD, Hu Y. Some insights into the mode of action of butadiene by examining the genotoxicity of its metabolites. Chem. Biol. Interact. 2007, 166:132-9.

Kocaman AY, Rencüzoğulları E, Topaktaş M, Istifli ES, Büyükleyla M. The effects of 4-thujanol on chromosome aberrations, sister chromatid exchanges and micronucleus in human peripheral blood lymphocytes. Cytotechnology 2011, 63:493-502.

Koide K, Osman S, AL Garner, Song F, Dixon T, Greenberger JS, Epperly MW, ACS Med The Use of 3,5,4'-Tri-O-acetylresveratrol as a Potential Pro-drug for Resveratrol Protects Mice from γ-Irradiation–Induced Death. Chem Lett. 2011, 2:270–274.

Kostoryz EL, Zhu Q, Zhao H, Glaros AG, Eick JD. Assessment of cytotoxicity and DNA damage exhibited by siloranes and oxiranes in cultured mammalian cells. Mutat. Res. 2007, 634:156-62.

Kotan E, Alpsoy L, Anar M, Aslan A, Agar G. Protective role of methanol extract of Cetraria islandica (L.) against oxidative stress and genotoxic effects of AFB_1 in human lymphocytes in vitro. Toxicol. Ind. Health 2011, 27:599-605.

Koukourakis MI, Kyrias G, Kakolyris S, Kouroussis C, Frangiadaki C, Giatromanolaki A, Retalis G, Georgoulias V. Subcutaneous administration of amifostine during fractionated radiotherapy: a randomized phase II study. J. Clin. Oncol. 2000, 18:2226-33.

Kunwar A, Barik A, Mishra B, Rathinasamy K, Pandey R, Priyadarsini KI. Quantitative cellular uptake, localization and cytotoxicity of curcumin in normal and tumor cells. Biochim. Biophys. Acta 2008, 1780:673-9.

Kunwar A, Narang H, Priyadarsini KI, Krishna M, Pandey R, Sainis KB. Delayed activation of PKCdelta and NFkappaB and higher radioprotection in splenic lymphocytes by copper (II)-Curcumin (1:1) complex as compared to curcumin. J. Cell Biochem. 2007, 102:1214-24.

Lambert B, Lindblad A, Nordenskjöld M, Werelius B. Increased frequency of sister chromatid exchanges in cigarette smokers. Hereditas 1978, 88:147-9.

Lambert JD, Hong J, Yang GY, Liao J, Yang CS. Inhibition of carcinogenesis by polyphenols: evidence from laboratory investigations. Am. J. Clin. Nutr. 2005, 81:284-291.

Lançon A, Hanet N, Jannin B, Delmas D, Heydel JM, Lizard G, Chagnon MC, Artur Y, Latruffe N. Resveratrol in human hepatoma HepG2 cells: metabolism and inducibility of detoxifying enzymes. Drug Metab. Dispos. 2007, 35:699-703.

Laugesen M, Swinburn B. New Zealand's tobacco control programme 1985-1998. Tob. Control 2000, 9:155-62.

Lazutka J, Dedonyte V, Krapavickaite D. Sister-chromatid exchanges and their distribution in human lymphocytes in relation to age, sex and smoking. Mutat. Res. 1994, 306:173-180.

Lee C, Lemyre E, Miron PM, Morton CC. Multicolor fluorescence in situ hybridization in clinical cytogenetic diagnostics. Curr. Opin. Pediatr. 2001, 13:550-5.

Lei YC, Hwang SJ, Chang CC, Kuo HW, Luo JC, Chang MJ, Cheng TJ. Effects on sister chromatid exchange frequency of polymorphisms in DNA repair gene XRCC1 in smokers. Mutat. Res. 2002, 519:93-101.

Lehnert, S.H. Biomolecular action of ionizing radiation. (Ed.) Taylor and Francis, EUA, 2008.

Leung HW. Evaluation of the genotoxic potential of alkyleneamines. Mutat. Res. 1994, 320:31-43.

Ley 31/1995, de 8 de noviembre, de prevención de Riesgos Laborales. Boletín Oficial del Estado, Número 269. Available at: http://www.boe.es/buscar/doc.php?id=BOE-A-1995-24292.

Lodish H, Berk A, Zipursky SL, Matsudaira P, Baltimore D, Darnell J. Section 13.7. Checkpoints in Cell-Cycle Regulation. Molecular Cell Biology. (Ed) Freeman, WH. New York, USA. 2000.

López AJ, Fernández, O. Signalling DNA Damage, Chapter 8 in "Protein Phosphorylation in Human Health", (Ed) Cai Huang, InTech, 2012.

Losa GA. Resveratrol modulates apoptosis and oxidation in human blood mononuclear cells. Eur. J. Clin. Invest. 2003, 33:818-23.

Marcilla A, Martínez I, Berenguer D, Gómez-Siurana A, Beltrán MI. Comparative study of the main characteristics and composition of the mainstream smoke of ten cigarette brands sold in Spain. Food Chem. Toxicol. 2012, 50:1317-33.

Marques FZ, Markus MA, Morris BJ. Resveratrol: cellular actions of a potent natural chemical that confers a diversity of health benefits. Int. J. Biochem. Cell Biol. 2009, 41:2125-8.

Martínez-Fernández ML, Sánchez-Izquierdob MD, Martínez-Frías ML. Resumen de la evolución de las técnicas de citogenética y genética molecular para la identificación de las alteraciones genéticas del desarrollo embrionario. Semergen. 2010, 36:520–525.

Masood F, Malik A. Cytotoxic and genotoxic potential of tannery waste contaminated soils. Sci. Total Environ. 2012, 444:153-160.

Mateuca RA, Carton C, Roelants M, Roesems S, Lison D, Kirsch-Volders M. Genotoxicity surveillance programme in workers dismantling World War I chemical ammunition. Int. Arch. Occup. Environ. Health 2010 83:483-95.

Matsuoka A, Furuta A, Ozaki M, Fukuhara K, Miyata N. Resveratrol, a naturally occurring polyphenol, induces sister chromatid exchanges in a Chinese hamster lung (CHL) cell line. Mutat. Res. 2001, 494:107-13.

Matsuoka A, Lundin C, Johansson F, Sahlin M, Fukuhara K, Sjöberg BM, Jenssen D, Onfelt A. Correlation of sister chromatid exchange formation through homologous recombination with ribonucleotide reductase inhibition. Mutat. Res. 2004, 547:101-7.

Matsuoka A, Takeshita K, Furuta A, Ozaki M, Fukuhara K, Miyata N. The 4'-hydroxy group is responsible for the in vitro cytogenetic activity of resveratrol. Mutat. Res. 2002, 521:29-35.

Maurya DK, Devasagayam TP, Nair CK. Some novel approaches for radioprotection and the beneficial effect of natural products. Indian J. Exp. Biol. 2006, 44:93-114.

Mayeux R. Biomarkers: potential uses and limitations. NeuroRx. 2004, 1:182-8.

Mendonça LM, Dos Santos GC, Antonucci GA, Dos Santos AC, Bianchi Mde L, Antunes LM. Evaluation of the cytotoxicity and genotoxicity of curcumin in PC12 cells. Mutat. Res. 2009, 675:29-34.

Mesa MD, Ramírez-Tortosa MC, Aguilera CM, Ramírez-Boscá A, Gil A. Efectos farmacológicos y nutricionales de los extractos de Curcuma longa L. Ars. Pharmaceutica 2000, 41:307-321.

Milić M, Kasuba V, Orescanin V, Zeljezić D, Kopjar N, Rozgaj R. Chromosome damage in workers in cigarette manufacturing industry. J. Appl. Toxicol. 2008, 28:399-404.

Montoro, A. Evaluación citogenética del efecto radioprotector del extracto etanólico de propóleos. [Tesis Doctoral]. Valencia: Departamento de Ingeniería Química y Nuclear, Universidad Politécnica de Valencia; 2006. Disponible en: http://riunet.upv.es/bitstream/handle/10251/1908/tesisUPV2369.pdf.

Montoro A, Almonacid M, Serrano J, Saiz M, Barquinero JF, Barrios L, Verdú G, Pérez J, Villaescusa JI. Assessment by cytogenetic analysis of the radioprotection properties of propolis extract. Radiat. Prot. Dosimetry 2005, 115:461-4.

Moya C, Antó JM, Taylor AJ. Outbreak of organising pneumonia in textile printing sprayers. Collaborative Group for the Study of Toxicity in Textile Aerographic Factories. Lancet 1994, 344:498-502.

Mudry MD, Martinez RA, Nieves M, Carballo MA. Biomarkers of genotoxicity and genomic instability in a non-human primate, *Cebus libidinosus* (*Cebidae, Platyrrhini*), exposed to nitroimidazole derivatives. Mutat. Res. 2011, 721:108-13.

Mukhopadhyay MJ, Saha A, Mukherjee A. Studies on the anticlastogenic effect of turmeric and curcumin on cyclophosphamide and mitomycin C in vivo. Food Chem. Toxicol. 1998, 36:73-6.

Murthy PB. Frequency of sister chromatid exchanges in cigarette smokers. Hum. Genet. 1979, 52:343-5.

National Institute for General Medical Sciences, U.S. Department of Health and Human services. Available at http://images.nigms.nih.gov/index.cfm?event=doSearch&searchTerm=&typeID=&page=8.

Norppa H, Bonassi S, Hansteen IL, Hagmar L, Strömberg U, Rössner P, Boffetta P, Lindholm C, Gundy S, Lazutka J, Cebulska-Wasilewska A, Fabiánová E, Srám RJ, Knudsen LE, Barale R, Fucic A. Chromosomal aberrations and SCEs as biomarkers of cancer risk. Mutat. Res. 2006, 600:37-45.

Norppa H. Cytogenetic biomarkers and genetic polymorphisms. Toxicol. Lett. 2004, 149:309-34.

Nota Técnica de Prevención, NTP 354. Biological monotoring of genotoxic exposures: Cytogenetic techniques. 1994. National Institute of Work Safety and Health. Spanish Ministry of Labour and Social Affairs, Spain.

Nota Técnica de Prevención, NTP 354. Genotoxic agents: biologic control. National Institute of Work Safety and Health. 1994. Spanish Ministry of Labour and Social Affairs, Spain.

Novák B, Sible J, Tyson JJ. Checkpoints in the Cell Cycle. Encyclopedia of life Sciences & Macmillan Publishers Ltd, 2002. (Ed) Nature Publishing Group.

Obe G, Pfeiffer P, Savage JR, Johannes C, Goedecke W, Jeppesen P, Natarajan AT, Martínez-López W, Folle GA,. Drets ME. Chromosomal aberrations: formation, identification and distribution Mutat. Res. 2002, 504:17-36.

Oncology Division Advisory Committee (ODAC). Fulfillment of the Accelerated Approval Requirements for the Non-Small Cell Lung Cancer Indication: "Ethyol (Amifostine) Reduces the Cumulative Renal Toxicity Associated with Repeated Administration of Cisplatin in Patients with Advanced Non-Small Cell Lung Cancer". 2003. Available at http://www.fda.gov/ohrms/dockets/ac/03/briefing/3936B1_05_MedImmune-Eythol.htm.

Ould Kadi F, Mohammed-Brahim B, Fyad A, Lellou S, Nemery B. Outbreak of pulmonary disease in textile dye sprayers in Algeria. Lancet 1994, 344:962-3.

Pantelias GE, Maillie HD. The measurement of immediate and persistent radiation-induced chromosome damage in rodent primary cells using premature chromosome condensation. Health Phys. 1985, 49:425-33.

Pantelias GE, Terzoudi GI. Radiother Oncol. A standardized G2-assay for the prediction of individual radiosensitivity. 2011, 101:28-34.

Parshad R, Sanford KK, Price FM, Steele VE, Tarone RE, Kelloff GJ, Boone CW. Protective action of plant polyphenols on radiation-induced chromatid breaks in cultured human cells. Anticancer Res. 1998, 18:3263-6.

Patt HM, Tyree EB, Straube RL, Smith DE. Cysteine Protection against X Irradiation. Science. 1949, 110:213-4.

Perkowska M, Szczygieł M, Woźniak A, Limon J. Influence of diethylenetriamine (DETA) and sodium nitroprusside (NaNP) on sister chromatid exchange frequency and cell kinetics in cultured human lymphocytes. J. Appl. Genet. 2001, 42:233-5.

Perron NR, García CR, Pinzón JR, Chaur MN, Brumaghim JL. Antioxidant and prooxidant effects of polyphenol compounds on copper-mediated DNA damage. J. Inorg. Biochem. 2011, 105:745-53.

Perry P, Evans HJ. Cytological detection of mutagen-carcinogen exposure by sister chromatid exchange. Nature. 1975, 258:121-5.

Polasa K, Naidu AN, Ravindranath I, Krishnaswamy K. Inhibition of B(a)P induced strand breaks in presence of curcumin. Mutat. Res. 2004, 557:203-13.

Pompella A, Visvikis A, Paolicchi A, De Tata V, Casini AF. The changing faces of glutathione, a cellular protagonist. Biochem. Pharmacol. 2003, 66:1499-503.

Ponzanelli I, Landi S, Bernacchi F, Barale R. The nature of high frequency sister chromatid exchange cells (HFCs). Mutagenesis 1997, 12:329-333.

Prasad NR, Mahesh T, Menon VP, Jeevanram RK, Pugalendi KV. Photoprotective effect of sesamol on UVB-radiation induced oxidative stress in human blood lymphocytes in vitro. Environ. Toxicol. Pharmacol. 2005, 20:1-5.

Prasad NR, Srinivasan M, Pugalendi KV, Menon VP. Protective effect of ferulic acid on gamma-radiation-induced micronuclei, dicentric aberration and lipid peroxidation in human lymphocytes. Mutat. Res. 2006, 603:129-34.

Reisenbichler H, Eckl PM. Genotoxic effects of selected peroxisome proliferators. Mutat Res. 1993, 286:135-44.

Rencuzogullari E, Yildiz AM, Buyukleyla M. The genotoxic and anti-genotoxic effects of Stachys petrokosmos leaf extract in human lymphocytes using microsomal fractions. Cytotechnology 2012, 64:83-94.

Rojas E, Herrera LA, Sordo M, Gonsebatt ME, Montero R, Rodríguez R, Ostrosky-Wegman P. Mitotic index and cell proliferation kinetics for identification of antineoplastic activity. Anticancer Drugs 1993, 4:637-40.

Romm H, Oestreicher U, Kulka U. Cytogenetic damage analysed by the dicentric assay. Ann. Ist. Super. Sanità. 2009, 45:251-259.

Rudiger HW, Kohl F, Mangels W, von Wichert P, Bartram CR, Wohler W, Passarge E. Benzpyrene induces sister chromatid exchanges in cultured human lymphocytes. Nature 1976, 262:290-2.

Sánchez-Campillo M, Gabaldon JA, Castillo J, Benavente-García O, Del Baño MJ, Alcaraz M, Vicente V, Alvarez N, Lozano JA. Rosmarinic acid, a photo-protective agent against UV and other ionizing radiations. Food Chem. Toxicol. 2009, 47:386-92.

Sanford KK, Parshad R, Gantt R, Tarone RE, Jones GM, Price FM. Factors affecting and significance of G2 chromatin radiosensitivity in predisposition to cancer. Int. J. Radiat. Biol. 1989, 55:963-81.

Sardaş S, Gök S, Karakaya AE. Increased frequency of sister chromatid exchanges in the peripheral lymphocytes of cigarette smokers. Toxicol. In Vitro. 1991, 5:263-5.

Secretan B, Straif K, Baan R, Grosse Y, El Ghissassi F, Bouvard V, Benbrahim-Tallaa L, Guha N, Freeman C, Galichet L, Cogliano V. WHO International Agency for Research on Cancer Monograph Working Group. A review of human carcinogens--Part E: tobacco, areca nut, alcohol, coal smoke, and salted fish. Lancet Oncol. 2009, 10:1033-4.

Scott D. Chromosomal radiosensitivity and low penetrance predisposition to cancer. Cytogenet Genome Res. 2004, 104:365-70.

Scott D, Spreadborough AR, Jones LA, Roberts SA, Moore CJ. Chromosomal radiosensitivity in G2-phase lymphocytes as an indicator of cancer predisposition. Radiat. Res. 1996, 145:3-16.

Sgambato A, Ardito R, Faraglia B, Boninsegna A, Wolf FI, Cittadini A. Resveratrol, a natural phenolic compound, inhibits cell proliferation and prevents oxidative DNA damage. Mutat. Res. 2001, 496:171-80.

Sharma RA, Gescher AJ, Steward WP. Curcumin: the story so far. Eur. J. Cancer. 2005,:1955-68.

Shcherbakova PV, Bebenek K, Kunkel TA. Functions of eukaryotic DNA polymerases. Sci. Aging Knowledge Environ. 2003, 8:RE3.

Sherr CJ, DePinho RA. Cellular senescence: mitotic clock or culture shock? Cell 2000, 102:407-10.

Shiloh Y. ATM and related protein kinases: safeguarding genome integrity. Nature Rev. Cancer 2003, 3:155-168.

Signorelli P, Ghidoni R. Resveratrol as an anticancer nutrient: molecular basis, open questions and promises. J. Nutr. Biochem. 2005, 16:449-66.

Simsek Y, Gurocak S, Turkoz Y, Akpolat N, Celik O, Ozer A, Yılmaz E, Turhan U, Ozyalin F. Ameliorative effects of resveratrol on acute ovarian toxicity induced by total body irradiation in young adult rats. J. Pediatr. Adolesc. Gynecol. 2012, 25:262-6.

Snyder RD, Arnone MR. Putative identification of functional interactions between DNA intercalating agents and topoisomerase II using the V79 in vitro micronucleus assay. Mutat. Res. 2002, 503:21-35.

Solé A, Cordero P. Ardystil syndrome. Med. Clin. 1995, 105:295-7.

Solé A, Cordero PJ, Morales P, Martínez ME, Vera F, Moya C. Epidemic outbreak of interstitial lung disease in aerographics textile workers--the "Ardystil syndrome": a first year follow up. Thorax 1996, 51:94-5.

Srinivasan M, Rajendra Prasad N, Menon VP. Protective effect of curcumin on gamma-radiation induced DNA damage and lipid peroxidation in cultured human lymphocytes. Mutat. Res. 2006, 611:96-103.

Stanimirovic Z, Stevanovic J, Jovanovic S, Andjelkovic M. Evaluation of genotoxic effects of Apitol (cymiazole hydrochloride) in vitro by measurement of sister chromatid exchange. Mutat. Res. 2005, 588:152-7.

Stecker MS, Balter S, Towbin RB, Miller DL, Vañó E, Bartal G, Angle JF, Chao CP, Cohen AM, Dixon RG, Gross K, Hartnell GG, Schueler B, Statler JD, de Baère T, Cardella JF; SIR Safety and Health Committee; CIRSE Standards of Practice Committee. Guidelines for patient radiation dose management. J. Vasc. Interv. Radiol. 2009, 20:S263-73.

Stervbo U, Vang O, Bonnesen C. A review of the content of the putative chemopreventive phytoalexin resveratrol in red wine. Food Chem. 2007, 101:449–457.

Stevenson DE, Hurst RD. Polyphenolic phytochemicals--just antioxidants or much more? Cell Mol. Life Sci. 2007, 64:2900-16.

Stone HB, Moulder JE, Coleman CN, Ang KK, Anscher MS, Barcellos-Hoff MH, Dynan WS, Fike JR, Grdina DJ, Greenberger JS, Hauer-Jensen M, Hill RP, Kolesnick RN, Macvittie TJ, Marks C, McBride WH, Metting N, Pellmar T, Purucker M, Robbins ME, Schiestl RH, Seed TM, Tomaszewski JE, Travis EL, Wallner PE, Wolpert M, Zaharevitz D. Models for evaluating agents intended for the prophylaxis, mitigation and treatment of radiation injuries. Radiat. Res. 2004, 162:711-28.

Surh YJ, Hurh YJ, Kang JY, Lee E, Kong G, Lee SJ. Resveratrol, an antioxidant present in red wine, induces apoptosis in human promyelocytic leukemia (HL-60) cells. Cancer Lett. 1999, 140:1-10.

Suspiro A, Prista J. Biomarkers of occupational exposure do anticancer agents: a minireview. Toxicol. Lett. 2011, 207:42-52.

Szende B, Tyihák E, Király-Véghely Z. Dose-dependent effect of resveratrol on proliferation and apoptosis in endothelial and tumor cell cultures. Exp. Mol. Med. 2000, 32:88-92.

Szende B, Tyihák E, Király-Véghely Z. Dose-dependent effect of resveratrol on proliferation and apoptosis in endothelial and tumor cell cultures. Exp. Mol. Med. 2000, 32:88-92.

Terzoudi GI, Hatzi VI, Barszczewska K, Manola KN, Stavropoulou C, Angelakis P, Pantelias GE. G2-checkpoint abrogation in irradiated lymphocytes: A new cytogenetic approach to assess individual radiosensitivity and predisposition to cancer. Int. J. Oncol. 2009, 35:1223-30.

Thomas SE, Malzer E, Ordonez A, Dalton LE, vant Wout EF, Liniker E, Crowther DC, Lomas DA, Marciniak SJ. p53 and translation attenuation regulate distinct cell cycle checkpoints during ER stress. J. Biol. Chem. 2013, doi:10.1074/jbc.M112.424655.

Thresiamma KC, George J, Kuttan R. Protective effect of curcumin, ellagic acid and bixin on radiation induced genotoxicity. J. Exp. Clin. Cancer Res. 1998, 17:431-4.

Tiku AB, Abraham SK, Kale RK. Eugenol as an in vivo radioprotective agent. J. Radiat. Res. 2004, 45:435-40.

Tiwari H, Rao MV. Curcumin supplementation protects from genotoxic effects of arsenic and fluoride. Food Chem. Toxicol. 2010, 48:1234-8.

Toydemir T, Kanter M, Erboga M, Oguz S, Erenoglu C. Antioxidative, antiapoptotic, and proliferative effect of curcumin on liver regeneration after partial hepatectomy in rats. Toxicol. Ind. Health 2013, doi: 10.1177/0748233712469658.

Turuguel D, Nogué S, Sanz P. The Ardystil syndrome. Med. Clin. 1996, 107:356-7.

Turuguet D, Pou R. Outbreak of organising pneumonia in textile sprayers. Lancet 1994, 344:1168.

Velioğlu-Oğünç A, Sehirli O, Toklu HZ, Ozyurt H, Mayadağli A, Ekşioğlu-Demiralp E, Erzik C, Cetinel S, Yeğen BC, Sener G. Resveratrol protects against irradiation-induced hepatic and ileal damage via its anti-oxidative activity. Free Radic. Res. 2009, 43:1060-71.

Vijayalaxmi, Reiter RJ, Meltz ML. Melatonin protects human blood lymphocytes from radiation-induced chromosome damage. Mutat. Res. 1995, 346:23-31.

Vogel W, Bauknecht T. Differential chromatid staining by in vivo treatment as a mutagenicity test system. Nature 1976, 260:448-9.

Voisin P, Roy L, Hone PA, Edwards AA, Lloyd DC, Stephan G, Romm H, Groer PG, Brame R. Criticality accident dosimetry by chromosomal analysis. Radiat. Prot. Dosimetry 2004, 110:44.

Webb MR, Ebeler SE. Comparative analysis of topoisomerase IB inhibition and DNA intercalation by flavonoids and similar compounds: structural determinates of activity. Biochem. J. 2004, 384:527-41.

Weiss JF, Landauer MR. Protection against ionizing radiation by antioxidant nutrients and phytochemicals. Toxicology 2003, 189:1-20.

Wolff S, Perry P. Differential Giemsa staining of sister chromatids and the study of chromatid exchanges without autoradiography. Chromosoma 1974, 48:341-53.

Wulf HC, Husum B, Niebuhr E. Sister chromatid exchanges in smokers of high-tar cigarettes, low-tar cigarettes, cheroots and pipe tobacco. Hereditas 1983, 98:225-8.

Yen GC, Duh PD, Lin CW. Effects of resveratrol and 4-hexylresorcinol on hydrogen peroxide-induced oxidative DNA damage in human lymphocytes. Free Radic. Res. 2003, 37:509-14.

Yoshino M, Haneda M, Naruse M, Htay HH, Tsubouchi R, Qiao SL, Li WH, Murakami K, Yokochi T. Prooxidant activity of curcumin: copper-dependent formation of 8-

hydroxy-2'-deoxyguanosine in DNA and induction of apoptotic cell death. Toxicol. In Vitro 2004, 18:783-9.

Zhang H, Zhai Z, Wang Y, Zhang J, Wu H, Wang Y, Li C, Li D, Lu L, Wang X, Chang J, Hou Q, Ju Z, Zhou D, Meng A. Resveratrol ameliorates ionizing irradiation-induced long-term hematopoietic stem cell injury in mice. Free Radic. Biol. Med. 2013, 54:40-50.

Zhao MY, Ying CJ, Shao N, Yang Y, Yang CF, Shi L, Liu WQ. The study of health effects of vinyl chloride air pollution on population. Biomed. Environ. Sci. 1994, 7:136-43.

ANNEXES

VNIVERSITAT DE VALÈNCIA
Vicerectorat d'Investigació i Política Científica

D. Fernando A. Verdú Pascual, Profesor Titular de Medicina Legal y Forense, y Secretario del Comité Ético de Investigación en Humanos de la Comisión de Ética en Investigación Experimental de la Universitat de València,

CERTIFICA:

Que el Comité Ético de Investigación en Humanos, en la reunión celebrada el día 3 de febrero de 2012, una vez estudiado el proyecto de tesis doctoral titulado:
"Marcadores citogenéticos aplicados en biomedicina",
cuya doctoranda es Dña. Natividad Sebastià Fabregat,
ha acordado informar favorablemente el mismo dado que se respetan los principios fundamentales establecidos en la Declaración de Helsinki, en el Convenio del Consejo de Europa relativo a los derechos humanos y cumple los requisitos establecidos en la legislación española en el ámbito de la investigación biomédica, la protección de datos de carácter personal y la bioética.

Y para que conste, se firma el presente certificado en Valencia, a diez de febrero de dos mil doce.

FERNANDO ALEJO|VERDU|PASCUAL
Certifico la precisión e integridad de este documento
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