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ASSOCIATION PATTERN OF RECIPROCAL TRANSLOCATIONS INDUCED BY CHEMICALS AND IONIZING RADIATION IN MOUSE GERM CELLS: A COMPARISON BETWEEN SINGLE AND COMBINED TREATMENTS.

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ABSTRACT

The similarity degree of chemical, ionizing radiation, and combined treatments of chemical plus ionizing radiation in their capacity to induce reciprocal translocations was analyzed by means of multivariate analysis techniques on mice germ cells. The effect of three different doses of gamma rays, four doses of X-rays, and different doses of adriamycin, mitomycin C, thio-tepa and bleomycin, as well as the combined treatments of two doses of gamma rays with adriamycin, mitomycin c and thio-tepa, were studied. Our objectives were: 1) to determine the degree of similarity between the effects of chemicals and ionizing radiations in relation to the induction of reciprocal translocations in germ cells; and 2) to test the conclusions reached by previous authors using only single treatments. Data were arranged in a basic data matrix, analyzed by cluster analysis and ordination methods. The results showed that: 1) as single and combined treatments were grouped together, there was not a specific pattern of chromosomal aberration induced for physical and chemical agents; 2) the association degree between single treatments was similar to that obtained by previous authors, although in this analysis combined treatments were added; and 3) some combined treatments using 9 Gy and drugs appear in different groups, although we expected that all the combined treatments of drugs with 9 Gy were grouped together. As a working hypothesis, we propose that the variability observed when the different treatments were compared could be dose dependent.

Key words: Chromosome aberrations; multivalent configurations; chromosomal damage; multivariate analysis.

RESUMEN

Por medio de técnicas de análisis multivariado se determinó el grado de similitud de distintos agentes químicos y radiaciones ionizantes en la inducción de translocaciones recíprocas en células germinales de ratón. Se comparó el efecto de tres diferentes dosis de rayos gamma, cuatro dosis de rayos X y distintas dosis de adriamicina, mitomicina C, thio-tepa y bleomicina, así como también los tratamientos combinados con dos dosis de rayos gamma con adriamicina, mitomicina C y tio-tepa, y cuatro de rayos X. Los objetivos del presente trabajo fueron: 1) determinar el grado de similitud entre los tipos de translocaciones recíprocas inducidas por los agentes químicos y las radiaciones ionizantes; y 2) comparar estos resultados con los obtenidos previamente por otros autores al comparar los tratamientos individuales. Para la comparación de los diferentes tratamientos se elaboró una matriz de datos analizada por medio de técnicas de agrupamiento y de ordenación. Los resultados revelaron que: 1) los tratamientos simples y combinados se agruparon juntos, indicando la falta de un patrón específico de aberraciones inducido; 2) el grado de asociación entre los tratamientos simples no se vio modificado, a pesar de la incorporación de los tratamientos combinados; y 3) algunos tratamientos combinados con 9 Gy y las diferentes drogas se asociaron con otros tratamientos, en lugar de asociarse juntos como era de esperarse teniendo en cuenta los resultados obtenidos por otros autores. Como hipótesis de trabajo, proponemos que la variabilidad observada en los diferentes tratamientos podría ser dependiente de la dosis empleada.

Palabras clave: Aberraciones cromosómicas, configuraciones multivalentes, daño cromosómico, análisis multivariado.

INTRODUCTION

Genetic damage can be induced by different agents, being the DNA molecule the main target. These agents can interact with the DNA molecule in a different way through the induction of DNA double and single-strand break cross-links and base damage. In all cases the primary lesions must be amplified to be detected as chromosomal aberrations. On the other hand, ionizing radiations can interact with macromolecules or cellular structures in two ways: a), when the damage is directly induced in the macromolecules by ionized particles and b), when the damage is induced by free radicals produced by ionization of cell water. Raser and O'Shea (2005) proposed that radiation induces reactive oxygen species (ROS). This species induce genomic instability. The persistence of ROS indicates a continuous turnover of oxidative species perpetuating a source of damage over time that could account for the delayed damage observed (Morgan, 2003; Limoli et al., 2003).

In a similar way some chemicals like adriamycin (ADR), mitomycin C (MMC) and bleomycin (BLM) can interact with DNA in different ways such as intercalation, DNA adduct formation and free radical induction, respectively (Povirk and Austin, 1991); (Povirk, 1996); (Sanderson and Shield, 1996). The analysis of reciprocal translocations induced by physical and chemical agents in mammalian germ cells includes multivalent configuration types and frequencies.

The germinal epithelium is very sensitive to radiation-induced damage, with changes to spermatogonia following as little as 0.2 Gy. Testicular doses of less than 0.2 Gy had no significant effect on FSH levels or sperm counts, whereas doses between 0.2 and 0.7 Gy caused a transient dose – dependent increase in FSH and reduction in sperm concentration, with a return to normal values within 12 – 24 months. No threshold dose has been determined in cases of azoospermia standing; however, doses of 1.2 Gy and above are likely to be associated with a reduced risk of recovery of spermatogenesis. The time to recovery, if it is to occurs, is also likely to be dose dependent (Howell and Shalet, 2005). The espermatrogénesis can be affected by ionizing radiation (Hacker-Klom *et al*., 1984) to the high sensitivity of the germ cells induces both cell death as well as increase the sensibility to the induction of mutations (Van Buul *et al*., 1995). The heterogeneity in the radiosensitivity of the various cell types during spermatogenesis is well known. Despite the fact that spermatids are considered to be among the populations most susceptible to genetic damage (Meistrich, 1993), spermatogonia are the precursors of the next generations of developing and mature germ cells (Russel *et al*., 1998). The induction of translocations in diakinesis metaphase is used to evaluate the mutagenic effect of ionized radiations in mammals. This test detects the frequency of reciprocal translocations induced in spermatogonia and predicts the genetic damage in F1 (Stubbs *et al*., 1997). The knowledge of the fate of induced translocations in spermatogonia is especially important in the precise evaluation of genetic risk. Genetically impaired spermatogonia from meiosis form spermatozoa with nonbalanced, balanced or normal genome. This situation could be responsible for the appearance of dominant lethal mutations, offspring with multiple abnormalities, as well as phenotypically normal, heterozygous translocations in the progeny (Schwartz *et al*., 1986; Matsuda *et al*., 1991; Sonta, 2004).

De Luca *et al*. (2000) analyzed the similarity relationships between single treatments of chemical and physical agents, using as features different kind of multivalent configurations. These authors found that: 1) there was not a specific pattern of chromosomal damage induction for physical and chemical agents using presence/absence data; 2) the increase in the amount of reciprocal translocation observed with 9 and 10 Gy was due to an increase in the kind of multivalent configuration, and could have been dose dependent; and 3) the similarity observed in the group formed by the chemicals and the lower doses of ionizing radiation could also have been dose dependent.

In this paper we add combined treatments of drugs and/or ionizing radiations to the single treatments analyzed by (De Luca *et al*., 2000). Using the same methodology our objectives were: 1) to determine the degree of similarity or difference between the effects of chemicals and ionizing radiations in relation to the induction of reciprocal translocations in germ cells; and 2) to test the conclusions reached by these authors.

MATERIALS AND METHODS

Operational taxonomic unit (OTU) selection

Relationships between three gamma rays (GR) doses (1, 5, and 9 Gy), different doses of the chemicals ADR, MMC, BLM and thio-tepa (TT), as well as several combined treatments (*i.e.,* chemical plus ionizing radiation) were analyzed. Data were obtained from experiments carried out in male BALB/c mice (De Luca *et al.*, 1988; 1990a, b). In addition, data obtained from van Buul and Léonard (1980) about the effect of 0.25, 0.50, 0.75, 1, and 10 Gy of X-rays (XR) were included in the analysis. Table I summarizes the different treatments considered. Some of the 22 treatments induced the same types of reciprocal translocations (*i.e.*, ADR5-MMC-0.75-1 Gy; TT-BLM20-BLM40; 5 Gy-BLM60), although frequencies were different (van Buul and Léonard 1980; De Luca *et al.*, 1988; 1990a, b). Data were not clustered since this procedure did not modify the obtained results.

AGENT	DOSE	NUMBER OF ANIMALS STUDIED
ADR	5 mg/Kg	11
ADR	10 mg/Kg	4
MMC	2 mg/kg	8
BLM	20 mg/Kg	6
BLM	40 mg/Kg	6
BLM	60 mg/Kg	6
TT	0.2 mg/Kg	7
GR	1 Gy	4
GR	5 Gy	10
GR	9 GY	11
XR*	0.25 Gy	10
XR*	0.50 GY	10
XR*	0.75 Gy	10
XR*	10 GY	9
ADR + GR	5 mg/Kg + 5 Gy	7
ADR + GR	5 mg/Kg + 9 GY	6
ADR + GR	10 mg/Kg + 5 GY	4
ADR + GR	10 mg/Kg + 9 Gy	4
MMC + GR	2 m/Kg + 5 GY	10
MMC + GR	2 mg/Kg + 9 GY	8
TT + GR	0.2 mg/Kg + 5 GY	8
TT + GR	0.2 mg/Kg + 9 GY	10

Table I. Experimental protocol employed to compare eciprocal translocations types induced by the different treatment

Data accumulation

The data considered included 14 variables, *i.e.*, reciprocal translocations, induced in stem cell spermatogonia and scored as multivalent configurations in diakinesis-metaphase I. All variables were qualitative and scored as presence/absence. Table II summarizes the different configurations considered and their corresponding codifications.

MULTIVALENT CONFIGURATIONS (VARIABLES)	CODIFICATION
(A) 18 II + 1 C IV	Present (1) Absent (0)
(B) 18 II + 1 R IV	Present (1) Absent (0)
(C) 18 + 1 CIII + 1 I	Present (1) Absent (0)
(D) 17 II + 1 RVI	Present (1) Absent (0)
(E) 17 II + 1 CVI	Present (1) Absent (0)
(F) 16 II + 1 CIV + 1 RIV	Present (1) Absent (0)
(G) 16 II + 2 CIV	Present (1) Absent (0)
(H) 16 II + 2 RIV	Present (1) Absent (0)
(I) 16 II + 1 CVIII	Present (1) Absent (0)
(J) 15 II + 1 CIV + 1 CVI	Present (1) Absent (0)
(K) 15 II + 1 CIV + 1 RVI	Present (1) Absent (0)
(L) 15 II + 1 CVI + 1 RIV	Present (1) Absent (0)
(M) 15 II + 1 RVI + 1 RIV	Present (1) Absent (0)
(N) 14 II + 2 CIV + 1 RVI	Present (1) Absent (0)
TN	Total Number

Table II. Multivalent configuration codification

Data processing

Data were arranged in a basic data matrix (BDM) of 22 OTUs by 14 reciprocal translocation types using single plus combined treatments (Table III). Before accomplishing the multi-varied analysis, the MBD was analyzed to determine: (1) how many types of reciprocal translocations produced each treatment; and (2) in how many treatments each reciprocal translocation appeared. Afterwards, BDM were analyzed by means of two methods: cluster analysis and ordination. Computer analysis was done by means of PAST 2.08 (Hammer *et al.,* 2001) and NTSYSpc 2.0 (Rohlf, 1997). Details about methods and computational procedures can

	18 II + 1 C IV	18 II + 1 R IV	15 II + 1 C IV + 1 C VI	15 II + 1 C IV + 1 R IV	17 II + 1 C VI	16 II + 2 CIV	16 II + 1 C VIII	16 II + 2 R IV	18 II + 1 C III + 1 I	17 II +1 R VI	16 II + 1 C IV + 1 R VI	14 II + 2 C IV + 1 R VI	15 II + 1 CVI + 1 R IV	15 II + 1 R IV + 1 R VI
ADR5	1	1	0	0	0	0	0	0	0	0	0	0	0	0
MMC	1	1	0	0	0	0	0	0	0	0	0	0	0	0
0.75 Gy	1	1	0	0	0	0	0	0	0	0	0	0	0	0
1Gy	1	1	0	0	0	0	0	0	0	0	0	0	0	0
ADR10	1	1	0	0	1	0	0	0	0	0	0	0	0	0
5Gy	1	1	0	0	0	0	1	0	0	0	0	0	0	0
BLM60	1	1	0	0	0	0	1	0	0	0	0	0	0	0
9Gy	1	1	1	1	1	1	0	1	0	0	0	0	0	0
0.25Gy	1	1	0	0	0	0	0	0	1	0	0	0	0	0
0.50Gy	1	1	0	0	0	0	0	0	1	0	0	0	0	0
10Gy	1	1	0	0	1	0	0	1	1	1	0	0	0	0
TT	1	0	0	0	0	0	0	0	0	0	0	0	0	0
BLM20	1	0	0	0	0	0	0	0	0	0	0	0	0	0
BLM40	1	0	0	0	0	0	0	0	0	0	0	0	0	0
ADR5+5Gy	0	1	0	1	0	0	0	1	0	0	0	0	0	0
ADR5+9Gy	1	1	0	0	1	1	0	0	0	0	1	0	0	0
ADR10+9Gy	1	1	0	0	1	0	0	0	0	0	0	0	0	0
ADR10+5Gy	1	1	0	0	0	0	0	0	0	0	0	0	0	0
MMC+5Gy	1	1	0	0	1	1	0	0	0	1	0	0	0	0
MMC+9Gy	1	1	0	0	1	1	1	1	0	1	1	1	1	0
TT+5Gy	1	1	0	0	0	1	0	0	0	0	0	0	0	0
TT+9Gy	1	1	1	0	1	1	0	0	0	1	1	0	0	1

Table III. Basic matrix data (BMD) of single plus combined treatments

be found in Sneath and Sokal (1973) and Crisci and López Armengol (1983).

Cluster analysis

The BDM was transformed into a similarity association matrix (SAM) among OTUs using the Jaccard coefficient Sneath and (Sokal, 1973). SAM served as input in the calculation of a phenogram by the unweighted pair-group method using arithmetic averages (UPGMA) (Sokal and Michener, 1958). Cophenetic correlations between the phenogram and SAM input were computed using the cophenetic correlation coefficient (CCC) (Sokal and Rohlf, 1962). If more than one phenogram was obtained, a consensus tree was performed to summarize the results. A consensus tree represents the consensus topology (subset of relationship) of two or more trees, and was obtained by the majority rule consensus method (Margus and McMorris, 1981). The consensus index CIc (Rohlf, 1982) was employed as a measure of the similarity of the original trees.

Ordination

The SAM obtained in 3.1. was transformed into a scalar product form so that its eigenvalues and eigenvectors could be computed, resulting in a principal coordinate analysis (PCA) (Gower, 1966). The results of the PCA were used as an initial configuration matrix to perform a non-metric multidimensional scaling analysis (NMDS) (Kruskal, 1964a, b). This technique represents dissimilarities among any objects or variables by any point in K-dimensional space, so that the interpoint distances in the K-dimensional space corresponded as much as possible to the observed

distances between the objects. Distances were fitted in K-dimensional space to a monotone function of the original distances with a coefficient called "stress" (Kruskal, 1964a, b). This author suggests verbal evaluations for various levels of stress (S) and goodness of fit (GF):

S	GF
0.40	Poor
0.20	Fair
0.10	Good
0.05	Excellent
0.00	"Perfect"

Local distortions in the graphic representation of the NMDS were estimated by means of the Minimum Spanning Tree (MST). Consequently, MST was used as a visual aid in grouping close points. MST is the shortest possible set of connected lines connecting all points.

RESULTS

BDM Description

Taking into account the 22 treatments, the most frequent reciprocal translocations A (18 II + 1C IV) and B (18 II + 1R IV), which appear at 21 and 19 treatments, respectively, followed at distance by E (17 II + 1 CVI) and F (16 II + 2 CIV), appearing in 8 and 6 treatments, respectively (Fig. 1a). If we discriminate against single and combined treatments, it appeared that the most common translocations were still A and B, although this was more evident in the case of simple treatments (Fig. 1b). Moreover, when the other two translocations were analyzed, these remain the second largest in the combined treatments (Fig. 1c) but in the single treatments F was low, place was occupied by I (18 II + 1 CIII + 1 I) (Fig. 1c). The analysis of the BDM showed that MMC+9Gy and TT+9Gy produced the greatest amount of translocations (10 and 8 respectively), followed by 9Gy and 10Gy (with 7 and 6, respectively) (Fig. 2). It was also noted that: (a) drugs and low and medium doses of radiation produced few translocations (1 to 3) (Fig. 2); (b) in the cases of ADR5, MMC and TT, the number of translocations increased markedly by combining such drugs with high radiation dose (Fig. 2); (c) and in the case of ADR10, although there was an increase in the number of aberrations when combined with a high dose of radiation, this was much lower than the observed in the case of the other three drugs (Fig. 2).

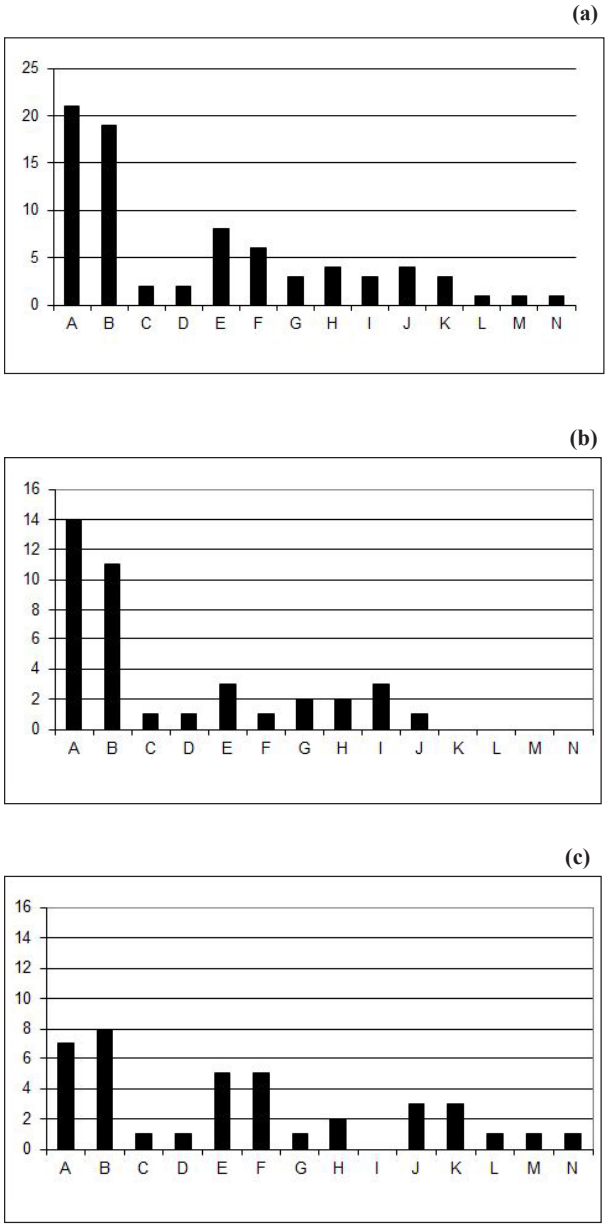

Figure 1. Number of multivalent configurations induced by the different treatments. (a) single plus combined treatments; (b) single treatments; (c) combined treatments. For references, see Table II.

Cluster analysis

We obtained eight phenograms (CCC = 0.93885) of association among OTUs. The consensus tree showed 13 groups (Fig. 3): **(a)** formed by ADR5+5Gy; **(b)** formed by ADR5, MMC, 0.75Gy, 1 Gy, ADR10+5Gy, ADR10, ADR10+9Gy, 5 Gy, BLM 60, 0.25 Gy, 0.50 Gy, TT+5Gy, TT, BLM 20, BLM 40, 9 Gy, ADR5+9 Gy, MMC+5Gy, TT+9Gy, MMC+9Gy, and 10 Gy; **(c)** formed by ADR5, MMC, 0.75Gy, 1 Gy, ADR10+5Gy, ADR10, ADR10+9Gy, 5 Gy, BLM 60, 0.25 Gy, 0.50 Gy, TT+5Gy, TT, BLM 20, and BLM 40; **(d)** formed by ADR5, MMC, 0.75Gy, 1 Gy, ADR10+5Gy, ADR10, ADR10+9Gy, 5 Gy, BLM 60, 0.25 Gy, 0.50 Gy, and TT+5Gy; **(e)** formed by TT, BLM 20, and BLM 40; **(f)** formed by ADR5, MMC, 0.75Gy, 1 Gy, and ADR10+5Gy; **(g)** formed by ADR10, and ADR10+9Gy; **(h)** formed by 5 Gy and BLM 60; **(i)** formed by 0.25 Gy and 0.50 Gy; **(j)** formed by 9 Gy, ADR5+9 Gy, MMC+5Gy, TT+9Gy, MMC+9Gy, and 10 Gy; **(k)** formed by 9 Gy, ADR5+9 Gy, MMC+5Gy, TT+9Gy, MMC+9Gy; **(l)** formed by ADR5+9 Gy, MMC+5Gy and TT+9Gy; and **(m)** formed by ADR5+9 Gy and MMC+5Gy.

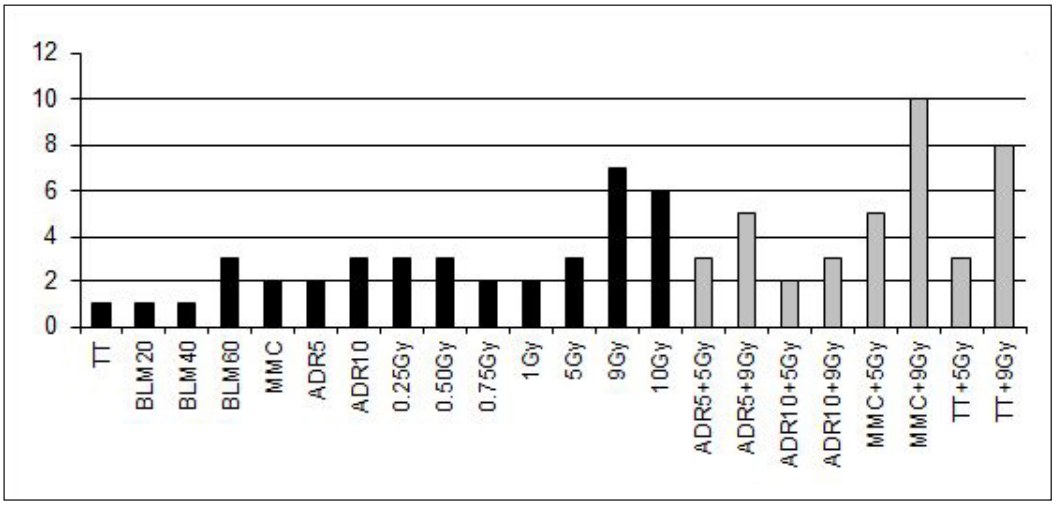

Figure 2. Total number of multivalent configurations scored for the 22 single and combined treatments. References: Black: single treatments; Grey: combined treatments.

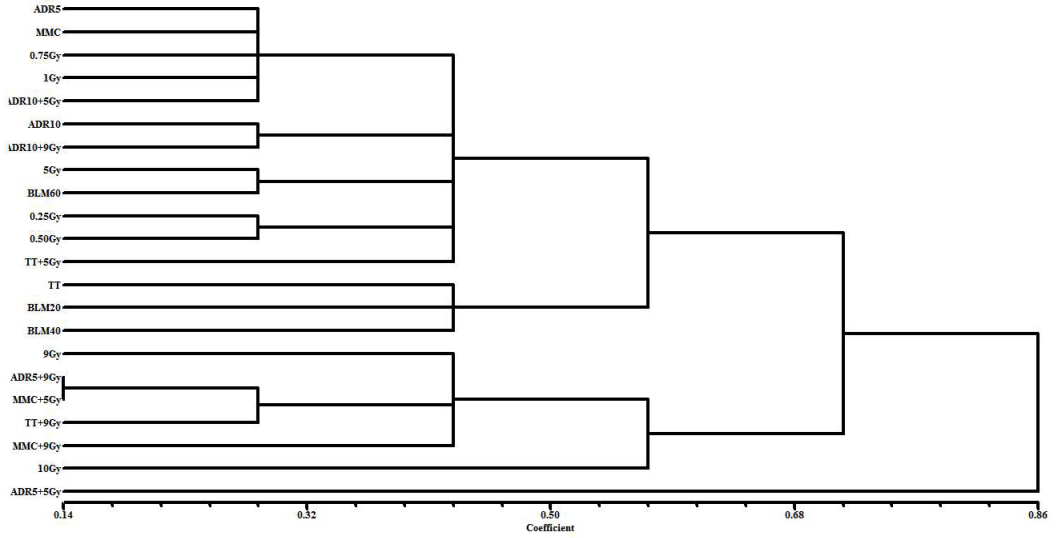

Figure 3. Consensus strict dendrogram showing the common groups among the eight phenograms obtained from the MBD of single plus combined treatments. CIc= 0.60

Ordination

In the bidimensional graphic (Fig. 4) four groups can be recognized. These groups were also found in the strict consensus dendrogram depicted in Fig. 3 [i.e., (a), (d), (e), and (j)].

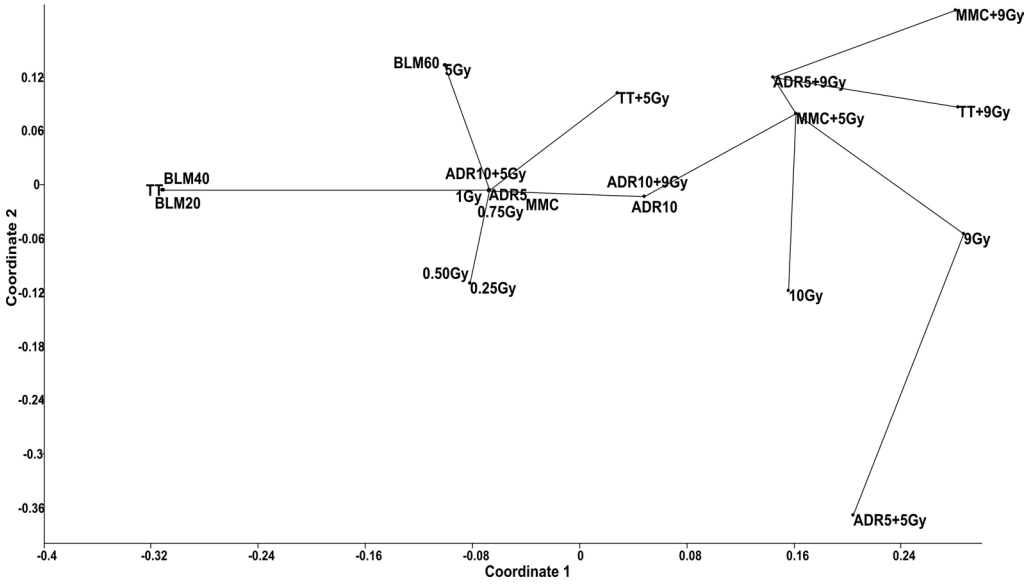

Figure 4. Multidimensional scaling plus minimum spanning tree graphic showing the 22 OTUs (single plus combined treatments) in a bidimentional graphics (axis I-II, 58.66 % of the variance). The final "stress" was 0.03893

DISCUSSION

The results obtained showed that single and combined treatments were grouped together (Figs. 3 and 4). This was an expected result, since nine of the fourteen types of reciprocal translocations were shared (Table III and Fig. 1). Consequently, there was not a specific pattern of chromosomal aberration induced for physical and chemical agents. These results were in concordance with those obtained by De Luca *et al*. (2000) when only single treatments were analyzed, although the multivalent configurations induced by the single treatments were a little less diverse than those induced by combined treatments, and produced, with the exception of 9Gy and 10Gy, a lesser type of reciprocal translocations than combined treatments (Table 3 and Figs. 1 to 5). On the other hand, the association degree between single treatments was similar to that obtained by De Luca *et al.* (2000), although combined treatments were added (compare Figs. 3 and 4 with 5 and 6). For example, the same groups observed in the consensus dendrogram obtained by De Luca *et al*. (2000) (see Fig. 3, e.g., 0.25Gy-0.50Gy-5Gy-BLM60-ADR5-MMC-0.75Gy-1Gy-ADR10; TT-BLM20-BLM40; 9 Gy-10 Gy) were recovered in the consensus dendrogram of single and combined treatments (Fig. 3).

Although, our new results showed some differences with those of De Luca *et* a*l*. (2000). On the one hand, these authors founded two main groups of reciprocal translocations one included treatments with 9 Gy of GR and 10 Gy of XR, and the other included all the chemical compounds and the remainder doses of GR and XR (Figs. 5 and 6). De Luca *et al*. (2000) concluded that 9 Gy of GR and 10 Gy of XR induced a similar kind of reciprocal translocations and a higher variability of reciprocal translocations than drugs and the remaining doses of radiation (Fig. 5). Additionally, these authors observed that these doses of radiation increased the frequency of reciprocal translocations. Taking

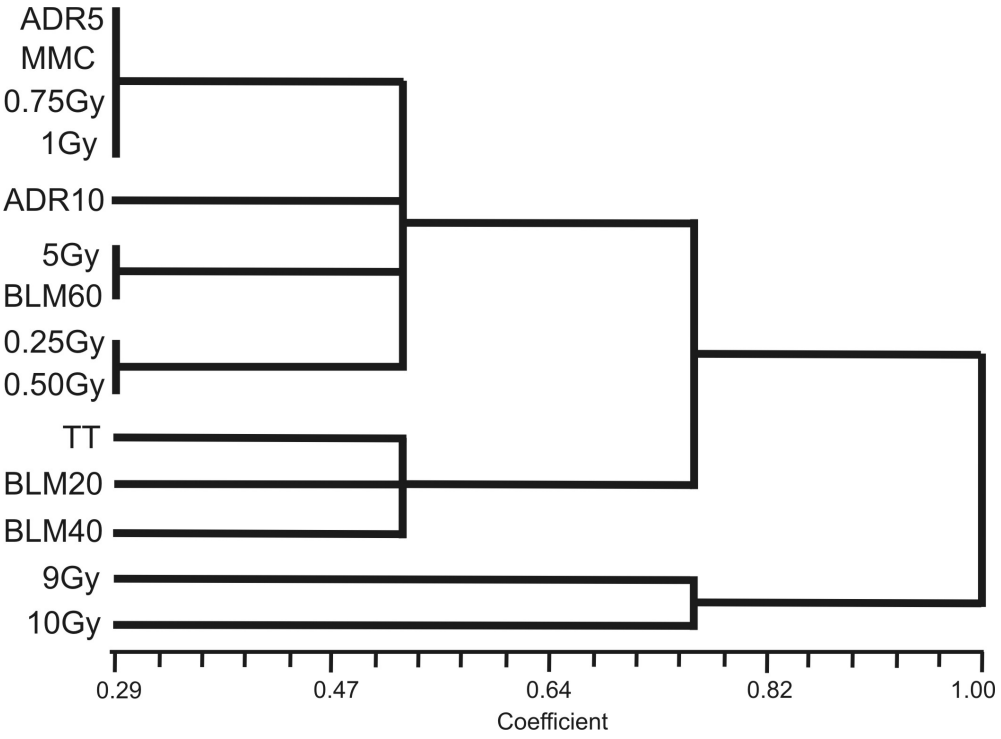

Figure 5. Consensus strict dendrogram obtained from single treatments by De Luca *et al.*, 2000 (Fig. 2).

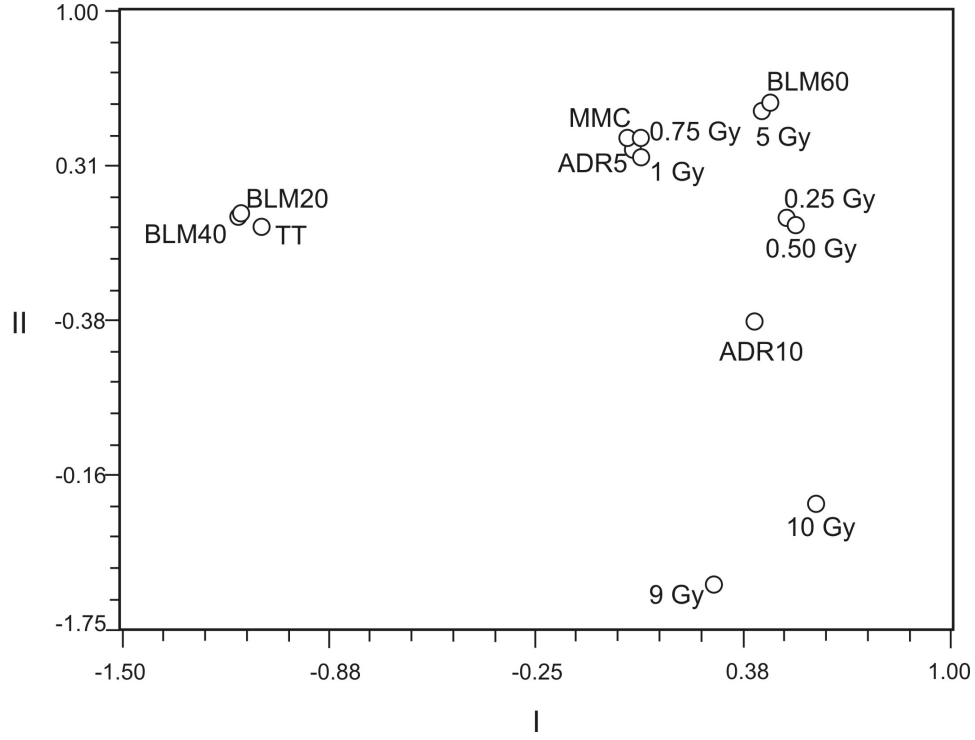

Figure 6. Multidimensional scaling graph obtained from single treatments by De Luca *et al.*, 2000 (Fig. 3)

into account these conclusions, we expected that all the combined treatments of drugs with 9 Gy were grouped together. Although, we found that some combined treatments using 9 Gy and drugs appeared in different locations. Thus, the Figs. 3 and 4 shows that: (1) ADR10+9Gy was grouped with ADR10, and both were related with ADR5, MMC, 0.75 Gy, 1 Gy, ADR10+5Gy, 5 Gy, BLM 60; and (2) MMC+5Gy was located inside the group of the highest radiation doses.

The relationship between ADR10 and ADR10+9 Gy was striking, as both treatments produced the same type of aberrations (Table III and Fig 2). In this case, the addition of 9 Gy of gamma rays did not increase the diversity of aberrations, as in most of the combination in which germ cells were irradiated with this dose of radiation (Table III and Fig 2). If we compare this behavior with ADR5 and ADR5+9 Gy, it appears that ADR5 produces only two types of translocations [the most common, i.e., A (18 II + 1 CIV) and B (18 II + 1 RIV), Table III and Fig .1 a), while ADR10 induce three [A and B plus E (17 II + 1 CVI), also produced by 9 Gy, 10 Gy and some combined treatments; see Table III and Fig 2]. However, ADR5+9 Gy produced five translocations (Table III and Fig 1b), which, two are common with ADR5 and ADR10 (A and B); one (E) is common with ADR10, 9 Gy, 10 Gy and some combined treatments; one [F (16 II + 2 CIV)] shared with 9 Gy and some combined treatments, and the remainder [K (16 II + 1 CIV + 1 RIV)] shared only with MMC+9Gy and TT+9Gy (Table III). Taking into account these results, the similarity observed between ADR10 and ADR10+9Gy could be due to a high enough dose to produce a certain of such cell damage (or death) that the later addition of 9Gy does not find the necessary substrate to produce new types of translocations. This seems to be reinforced by the fact that in the cases of the other drugs tested (i.e., TT and MMC) showed a marked increase in diversity 9Gy combination therapies (Table III and Fig 2).

Adriamycin interact with DNA through different mechanisms, mainly intercalation (ADR) (Warning, 1970) as well as free radicals production (Lambert, 1983; Gewirtz, 1999; Singal *et al*., 2000). Two different ways of free radical formation by adriamycin have been described. The first way implicates the formation of a semiquinone free radical by the action of several NADPH-dependent reductases that produce a one-electron reduction of the doxorubicin to the corresponding doxorubicin semiquinone (Olson and Mushlin, 1990; De Beer *et al*., 2001; Doroshow, J.H., 2006). In the presence of oxygen, redox cycling of adriamycin-derived quinone-semiquinone yields superoxide radicals (Singal *et al*., 2000). In the second way, adriamycin free radicals come from a non-enzymatic mechanism that involves reactions with iron. For example, Fe^{+3} reacts with adriamycin in a redox reaction after which the iron atom accepts an electron and a Fe^{+2} doxorubicin free radical complex is produced (De Beer *et al*., 2001).

Summing up, our results suggest that the induction of reciprocal translocations in mouse germ cells by single and combined treatments with ADR is dose dependent. The mice injected with 5 mg/kg of ADR and irradiated 24 hours later with 5 Gy exhibited very few multivalent configurations, and the frequency of translocations were lower than the one detected in animals irradiated only with 9 Gy of γ-rays, and equal to those recorded in animals irradiated only with 5 Gy of gamma rays (Table III and Fig. 2). However, when the dose of radiation was increased to 9 Gy of gamma rays, a dramatic increase in the type of translocations was observed. On the contrary, in mice injected with 10 mg/kg of ADR and irradiated with 5 or 9 Gy of gamma rays similar number of different translocations were found. These types were significantly lower than those detected in mice treated with 5 mg/kg of ADR and irradiated with 9 Gy of gamma rays (Table III and Fig. 2).

Leenhouts and Chadwick (1981) and van Buul and Seelen (1991) have postulated three main parameters of the stem cell spermatogonial population that affect the level of induced translocations: 1) the proportion of sensitive and resistant cells; 2) the ratio of the probabilities that a basic lesion in the DNA leads to cell killing or translocation formation; and (3) the proliferation-differentiation pattern of surviving spermatogonial stem cells after irradiation. Significant increase of intra as well as inter-individual variability is produced by high degrees of cell killing due to the increase in the effect of clonal proliferation of the relatively few surviving stem cells (Léonard and Deknudt, 1969; van Buul and Léonard, 1984). Combined treatment

results would be in concordance with the hypothesis that the resistant synchronization cells from the first treatment, is not enough to explain the frequency of reciprocal translocations induced (Cattanach and Barlow, 1984).

It has been proposed that the results observed after combined treatments could be explained assuming that depletion of any kind of spermatogonia is enough to modify the chromosomal response of stem cells (van Buul, 1984). This means that the first dose of radiation in fractionation experiments or the treatment with a chemical before irradiation induce a selective killing of proliferating spermatogonial cells, triggering the surviving stem cells to enter an active cycle to repopulate de epithelium (Cattanach *et al*., 1976; Cattanach and Crocker, 1980; Preston and Brewen, 1976; van Buul, 1983). According to this, the additive-potentiating effect observed after combined treatments with chemicals and ionizing irradiation does not depend on the amount of chromosomal damage induced by the chemical but on the ability of the compound to induce depletion of differentiating and differentiated spermatogonia. However this assumption is not enough to explain the subadditive effect observed after combined treatments with MMC and 4 Gy of X-rays (Deknudt and Leonard, 1979), MMC plus 5 Gy (De Luca *et al*., 1990a) or 5 mg/kg of ADR plus 5 Gy (De Luca *et al*., 1990b) (see below).

If the frequency of translocations recovered after combined treatments with chemicals and ionizing radiation depends only on the depletion of any kind of spermatogonia by the compound and on the amount of chromosomal damage (and cell mortality) induced by the radiation, then only two types of response can be expected, additivity and potentiating. Additivity when combined treatments induce a considerable amount of chromosomal damage without killing a great number of cells, and potentiating when combined treatments induce extensive cell death and only a few surviving cells with a high yield of chromosomal damage can be scored (see De Luca et al., 1990a,b).

On the other hand the association between all the combined treatments with 9 Gy (except the abovementioned ADR10+9Gy) could be explained because all of them showed a potentiating effect (De Luca *et al*., 1990b). Although, it is not clear why MMC+5 Gy was included in this group because it showed a subadditive effect similar to that observed in ADR 5+5 Gy in previous works (De Luca *et al*., 1990a,b). Additionally, ADR 5+5 Gy appear located into a group separated of the remaining treatments, showing a very low similarity with them (Figs. 3 and 4).

According with the BDM, MMC+5Gy produced five type of reciprocal translocations (Table III and Fig. 2), two common with most of the treatments [i.e., A (18 II + 1C IV) and B (18 II + 1 R IV)], one common with ADR10, 9 Gy, 10 Gy and some combined treatments (i.e., E (17 II + 1 CVI), one common with 9 Gy and some combined treatments [i.e., F (16 II + 2 CIV)], and one common with 10 Gy and some combined treatments [J (17 II +1 R VI)]. Among the combined treatments, MMC+5Gy was very similar to ADR5+9Gy, because they showed four reciprocal translocations and only two different (Table III).

Bioreduction of MMC leads to the formation of free radicals, which causes a cascade of reactions including lipid peroxidation, protein, DNA-damage and ultimately cell death (Kappus, 1986; Na *et al.,* 2001). Depending on the biotransformation pathway, metabolism of MMC may generate reactive oxygen species (ROS) (Gustafson *et al*., 1992). When ROS interact with cells and exceed endogenous antioxidant systems, there is indiscriminate damage to biological macromolecules such as nucleic acids, proteins, and lipids (Offord *et al*., 2000).

As was aforementioned, MMC+5Gy showed a subadditive effect in previous works (De Luca et al., 1990a,b). In order to explain the subadditivity effect, it is necessary to assume that other factors that those involved in potentiating and additivity (see above) could be involved in the mechanism of translocation induction in stem-cell spermatogonia of mice. It has been proposed that the role of the repair mechanism could be considered in this sense (De Luca *et al*., 1988; van Buul, 1984). These repair mechanisms can reduce cell mortality in such a way that higher frequency of more radio-resistant cells can survive. But with a higher dose of chemicals (e.g., ADR10) the amount chromosomal damage induced is so great that the repair mechanisms triggered are not enough to reduce cell mortality. In our study MMC facilitated the appearance of many reciprocal translocations only found in combined treatments and/or in single treatments with higher

doses of ionizing radiation (Table III). It suggests that MMC can produce a higher cell sensibility, facilitating the mutagenic action of the ionizing radiations. This effect is observed when middle doses of radiation are applied, but the effect observed is even more marked when higher doses are applied. However, this working hypothesis should be proven with appropriate experiments.

As was mentioned above, ADR5+5 Gy appeared in a monotypic group, related with the remainder treatments at low similarity values (Figs. 3 and 4). According to the BDM (Table III), this treatment produced only three kinds of reciprocal translocations: (1) B (18 II + 1 R IV) (the second more common translocation, see Table III and Fig. 1); (2) D (15 II + 1 C IV + 1 RIV), a translocation very rare, only shared with 9 Gy (Table III and Fig. 1); and (3) H (16 II + 2 RIV), a low recorded translocation, only shared with 9 Gy, 10 Gy, and MMC+9Gy (Table III and Fig. 1). Additionally, this was the only treatment in which the more frequent reciprocal translocation recorded in this study [i.e., A (18 II + 1 C IV); see Table III and Fig. 1] was absent, although we can assume that this absence simply can be due to chance. As was mentioned above, ADR5+5Gy had an subbaditive effect in previous works (De Luca et al., 1990a,b). Unlike what is observed with the MMC+5Gy, in this case an increase of sensibility to the ionizing radiations was not appreciated. In this way, the subadditive effect due to the action of the mechanisms of cell repair could explain the position of this treatment in the multivariated graphics (Figs. 3 and 4).

CONCLUSIONS

Our results show that there was not a specific pattern of chromosomal aberration induced for physical and chemical agents, because single and combined treatments were grouped together.

The association degree between single treatments was basically similar to that obtained by De Luca et al. (2000), although in our analysis combined treatments were added. Combined treatments using 9 Gy plus drugs appeared in different groups, although according to the previous work of De Luca et al (2000) we expected that all the combined treatments of drugs with 9 Gy were grouped together. We believe that the variability observed when the different treatments were compared could be dose dependent, but this hypothesis needs to be tested. Additionally, the action of potentiating, additivity, and subadditivity can help to explain the location of most of the combined treatments.

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