RESEARCH

Assessment of basal and induced DNA damage on lymphocytes from three mouse lines by means of the alkaline comet assay

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ABSTRACT

The formation of single-strand breaks and alkali-labile sites on DNA has been extensively used for genotoxicity testing, given its involvement in degenerative disorders, cancer and oxidative stress. An alkaline variation of the single-cell electrophoresis (comet) assay used for the detection of DNA damage was developed during the eighties, providing data on this phenomenon at the level of individual cells for the first time. The present work employs the alkaline comet assay to compare three mouse lines regarding the basal and cyclophosphamide-induced frequency of single-strand breaks and the appearance of alkali-labile sites in DNA from peripheral blood lymphocytes. A total of 10 mice/sex/group of the Balb/c, OF-1 and NMRI lines were treated for 14 days, distributed into an untreated negative control group, two groups receiving excipients and a positive control group receiving intraperitoneal cyclophosphamide at 50 mg/kg. After 14 days, single cells from peripheral blood leukocytes were analyzed by alkaline electrophoresis to estimate DNA damage. It was concluded that the best choice for this type of studies is represented by the Balb/c line, due to its low basal frequency for the analyzed variables. This result indicates that Balb/c may be the best biomodel for preclinical testing of drugs, vaccines and other products.

Keywords: Comet assay, damage, DNA, mice, cyclophosphamide

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RESUMEN

Evaluación del daño basal e inducido en el ADN de linfocitos de tres líneas de ratones, mediante el ensayo cometa alcalino. Las rupturas de simple cadena y la formación de sitios lábiles al álcali en el ADN, son parámetros ampliamente utilizados para la detección de genotoxicidad. Se ha demostrado su implicación en enfermedades degenerativas, en el cáncer, y recientemente, su vínculo con el estrés oxidativo. En la década de 1980 se desarrolló la variante alcalina de la electroforesis de células individuales (ensayo cometa), para la detección de daño en el ADN. Por primera vez se proporcionaron datos en células individuales. Este artículo tuvo como objetivo la comparación de la frecuencia basal e inducida con ciclofosfamida, de las rupturas de simple cadena y la formación sitios lábiles al álcali en el ADN de leucocitos de sangre periférica, mediante el ensayo cometa alcalino, en tres líneas de ratones. Se utilizaron 10 ratones de los dos sexos, de las líneas Balb/c, OF-1 y NMRI. Se formó un grupo control negativo (al que no se no le administró ninguna sustancia), dos grupos controles, a los que se administraron sustancias vehículo, y un grupo control positivo al que se administraron 50 mg/kg de ciclofosfamida por vía intraperitoneal. Pasados 14 días, se realizó la electroforesis alcalina de células individuales en gel de leucocitos de sangre periférica, para demostrar el posible daño en el ADN. Se concluyó que la línea más adecuada para estos estudios es la Balb/c, por la baja frecuencia basal que presentan las variables analizadas. Este resultado indica que puede ser el mejor biomodelo para la evaluación preclínica de drogas, vacunas y otros productos.

Palabras clave: ensayo cometa, daño, ADN, ratones, ciclofosfamida

*I*ntroduction

The large numbers of commercially available pharmaceuticals that characterize modern life have become a double-edged sword. While, on one hand, they have undoubtedly increased our quality of life, many of them pose a significant risk due to their high toxicity [1, 2]. Current legislation requires all prospective drugs to be evaluated for safety long before their registration or marketing, so as to minimize or eliminate their use the risk/benefit ratio should be considered unacceptable to society [2].

The formation of single strand breaks and alkali-labile sites on DNA is widely used for the detection of genotoxicities. These phenomena, and DNA damage in general, have been shown to be involved in degenerative disorders, cancer and, more recently, oxidative stress [3, 4]. One of the available assays for the detection of DNA damage is the alkaline variant of single-cell electrophoresis, developed during the eighties and also known as the comet assay. This was the first DNA damage assay providing single-cell data, and since then it has become a cornerstone of the evaluation of drugs, fertilizers and pesticides [5].

In order to perform the comet assay, single cells are resuspended into low melting point agarose to form a microgel. Once immobilized in this fashion, they are lysed *in situ* to remove cellular proteins and cell DNA

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is forced to unwound [6] by breaking the hydrogen bonds holding both strands together, using alkaline/ neutral conditions. At pH higher than 13, alkali-labile sites (ALS), such as apurinic sites, quickly transform into single-strand breaks [7]; therefore, using this pH allows estimating the number of ALS by quantifying such breakages [8]. Unwound DNA is subjected to electrophoresis in an alkaline buffer [6, 9]; and staining the DNA at the end of the run produces a visual pattern much like that of a comet [10].

Cell that have sustained DNA damage exhibit increased migration in this assay [8, 11]. Control cells, with a small number of DNA breaks, have level 0 comets, following the standard classification for DNA damage. DNA migrates slowly in this case; the "tail" of the comet contains only 10% of the total DNA. In order to detect and quantify DNA damage, the gel can be stained with different agents including *e.g.* silver nitrate, although intercalating fluorescent dyes represent the most common choice. In the end, selecting one or the other depends on the specific needs of the investigation [12].

Genotoxicity evaluations are usually performed with DNA from peripheral blood leukocytes, extracted from animals treated with the compound under examination. Therefore, the availability of experimental biomodels with a low basal frequency for the appearance of single-strand breaks and ALS in their peripheral leukocyte DNA, with a high sensitivity to mutagenic and genotoxic substances, and yielding measurements with the smallest margin of error, is clearly critical.

In vivo genotoxicity studies use positive controls consisting of mutagenic substances. One of the mutagens most often used for this purpose is cyclophosphamide (CF), an alkylating agent forming mono-adducts and inter-strand crosslinks whenever a break appears due to the action of cellular DNA repair processes. Although CF is a very effective antineoplastic agent, its performance in the *in vivo* alkaline comet assay is not well described, as bleomycin is almost always used as a positive control for this technique. CF, however, is much cheaper and easier to handle, is less hazardous to the personnel and poses simpler decontamination demands [9]. Therefore, we have decided to use CF as positive control in this genotoxicity assay.

The present work compares the basal and induced frequency of single-strand breaks and ALS in DNA from peripheral leukocytes of Balb/c, OF-1 and NMRI mice of both sexes treated with CF; evaluated with the comet assay. The results will serve the identification of the best experimental biomodel for the study of other drugs or agents whose genotoxic effects need to be tested.

Materials and methods

Animals

The present study used young adults (8-9 weeks) of both sexes from the mouse lines Balb/c, OF-1 and NMRI, with a bodyweight at the end of the quarantine period of 26-30 g. They were kept at a temperature of 25 ± 2 ºC, a relative humidity of 60 ± 10%, and a 12-hour photoperiod, with water and food *ad libitum*. Their alimentation consisted on EMO1002 all-purpose autoclavable rodent chow (requiring no dietary supplementation) with batch number 1181102, supplied by the Center for the Production of Laboratory Animals (CENPALAB). The same conditions were used for all the groups of the study. The experimentation abided by established ethical guidelines for research with laboratory animals [13].

Administration and dosage

Experimental groups

Table 1 summarizes the experimental groups used in the single-cell alkaline comet assay for peripheral blood leukocytes from the three mouse lines of both sexes; as well as the administered substances, administration routes (oral or intraperitoneal) and maximum administration volume, for two replicates.

The test compounds were administered between 10:30 and 11:30 a.m., and the concentrations were adjusted weekly to match the changing bodyweight of the animals. The animals were randomly assigned to the treatment groups, using 10 mice per group, per sex, and per line. Only two replicates per experimental point were used in this study, out of economical considerations arising from the price of the reagents consumed by the assay.

Group 1 used untreated animals as negative control. These animals, however, did undergo mock gastric intubations for 14 days so that they could be directly compared to the other groups.

Group 2 used 2% Tween 65 as test compound, orally administered for 14 days and freshly prepared 2 hours before administration. This excipient is a surfactant present in most oil-based formulations [14-16].

Group 3 used 0.9% NaCl as test compound, orally administered for 14 days and freshly prepared 2 hours before administration. This solution is used to dissolve most hydrophilic drugs [17, 18].

Group 4 constituted the positive control, administered intraperitoneally with CF at 50 mg/kg bodyweight. CF (n,n-bis-(-ethyl chloride)-n'), o-esther diamide of propynel phosphoric acid ($C_{17}H_{15}C_{l2}N_2O_5P$), purchased from the Mexican manufacturer Lemri S.A., un-

Table 1. Experimental groups in the single-cell alkaline electrophoresis assay for peripheral blood leukocytes (comet assay), applied to mice of both sexes of the Balb/c, OF-1 and NMRI lines, with two replicates in each one

Experimental groups	Total of animals	Administered substance	Administration route	Maximum administered volume (mL/kg)
Negative control	20	Not treated	Oral (mock treatment)	-
Excipient 1	20	2% Tween 65	Oral	2
Excipient 2	20	0.9% NaCl	Oral	2
Positive control	20	CF[a], 50 mg/kg	i.p.[b]	15

[a] CF: Cyclophosphamide
[b] i.p.: Intraperitoneal

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der the trademark Ledoxina. It was dissolved in saline solution (0.9% NaCl) [19] and administered to the animals right after its preparation, at 48 hours, and then 24 hours before the scheduled date for euthanasia [9, 20].

Clinical observations

Two daily observations were performed; one in the morning from 8:30 to10:30 a.m. and one in the afternoon, from 3:00 to 4:30 p.m. The parameters to be monitored in each observation were the general health status of the animals, including palpation and detection of lesions, and any possible alterations of the respiratory, nervous, cardiovascular and gastrointestinal systems, as well as the appearance of coat, eyes and mucosal coloration.

Euthanasia

The animals were anesthetized under an ether atmosphere until all reflexes were lost, and were sacrificed by cervical dislocation after drawing blood samples. Groups 1, 2 and 3 were euthanized 24 hours after the last administration at day 14. Group 4, treated with CF, was euthanized 24 hours after the second administration of the mutagen, scheduled to coincide with the sacrifice date for the remaining animals.

Tests

Blood sample collection

Once all reflexes were lost, a drop of blood (equivalent to 15-20 µL) was extracted from the tail of each animal and placed into a vial containing 10 µL of sodium heparin, supplied by LIORAD Laboratories at 5000 IU/mL. The samples were handled at 4°C under dimmed light to avoid subjecting the DNA to additional damage, thus decreasing the false positive rate and minimizing the influence of experimental manipulation on the results [21].

Single-cell alkaline electrophoresis assay in peripheral blood leukocytes (alkaline comet assay)

Blood samples (15-20 µL) were resuspended in 140 µL of 0.5% low melting point agarose. Then, previously prepared agarose slides were added. They were submerged in lysis solution (2.5 M NaCl, 100 mM EDTA and 10 mM Tris, 1% Triton, 10% DMSO, pH 10) for 1.5 h at 4 ºC and denatured for 20 min in electrophoresis buffer (3% NaOH 10 N, 0.5% 200 mM EDTA, pH > 13). Electrophoresis was performed at 300 mA and 1 V/cm for 18-20 min. The slides were washed with neutralization buffer (0.4 M Tris, pH 7.5), rinsed with distilled water, and stained with 0.05% silver nitrate. The stained nucleoids were evaluated under a transmission optical microscope by three independent observers, and the obtained readings were then averaged [6, 7].

Visual analysis

Two-hundred leukocytes were analyzed per animal, quantifying one-hundred comets from the center of each gel. Each comet was classified into a DNA damage category from 0 to 4 [6, 22]. The extent of damage was expressed in arbitrary units (AU), as described by Collins in 2004 [6], using a range of values from 0 to 400 [23, 24].

The following formula was used to calculate AU:

$$UA = 0 \times TCG0 + 1 \times TCG1 + 2 \times TCG2 + 3 \times TCG3 + 4 \times TCG4$$

TCG0 = Total number of grade 0 cells (undamaged cells).
TCG1 = Total number of grade 1 cells (minimal DNA damage).
TCG2 = Total number of grade 2 cells (low DNA damage).
TCG3 = Total number of grade 3 cells (considera ble DNA damage).
TCG4 = Total number of grade 4 cells (completely damaged cells).

Statistical analysis

Mann-Whitney's U-test was used for comparisons between groups and mice lines regarding the parameters of the comet assay (AU and different damage levels). A cutoff of $\alpha = 0.05$ was chosen *a priori* for statistical signification. All statistical tests were performed with the statistical software package Statistica (Statsoft, Inc. 2003, version 6).

Results and discussion

No signs of toxicity were detected in the animals during the 14 days of the study.

Single- and double-strand DNA breaks can originate due to the formation of OH radicals. These are highly toxic species for which a cellular anti-oxidant mechanism doesn't exist. OH radicals, in addition, can generate DNA-DNA and DNA-protein crosslinks [25, 26].

There were no significant differences between the results of the negative control group and those treated with excipients 1 and 2, expressed either in arbitrary units or percentage of nucleoids, at each of the analyzed levels, for any of the studied mouse lines (Tables 2, 3 and 4). There were no significant differences between sexes either.

However, the comparison of the group treated with CF (the mutagen used as positive control in this study) with the negative control or excipients 1 and 2 did detect statistically significant differences for each of the analyzed variables. The number of AU induced by CF doubled that induced by the other substances. In addition, the main effect of this mutagen was a considerable increase in the percentage of level 3 and 4 nucleoids. These types of nucleoids are precisely those associated with the highest degrees of DNA damage and degradation.

Single-strand breaks are repaired through nucleotide and base excisions. One of the most frequent base modifications is 8-oxo-2´deoxiguanosine [23, 27]. This is a complex process that eliminates a segment of at least 29 oligonucleotides and can produce DNA migration, later corrected through the experimental calculation of AU [8, 24].

Using the AU as an indicator of cyto- and genotoxicity, the data show that the Balb/c line had a smaller interval of basal AU, from 49.51 to 57.23. Based on these criteria, the Balb/c line is therefore the best choice among the mouse lines evaluated in this study, as it exhibits the highest endogenous rate of level 0 (undamaged) nucleoids. This result agrees with pre-

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Table 2. Comet assay in Balb/c mice of both sexes, evaluating the induction of DNA damage on peripheral blood leukocytes by the two series under study[¶]

Groups (mg/kg)	Sex	Arbitrary units (mean ± SD[a])	Nucleoid percentage (mean ± SD) Level 0	Level 1	Level 2	Level 3	Level 4
Negative control 1, oral	F	49.51 ± 10.24	56.00 ± 8.01	39.29 ± 7.49	4.01 ± 4.17	0.60 ± 0.68	0.10 ± 0.05
	M	56.37 ± 7.51	51.03 ± 3.14	42.72 ± 5.68	5.10 ± 2.77	1.15 ± 1.05	0
Excipient 1, oral	F	57.23 ± 10.20	50.82 ± 9.32	42.13 ± 2.22	6.05 ± 5.28	1.00 ± 0.93	0
	M	54.25 ± 8.90	55.50 ± 9.01	36.15 ± 3.58	7.00 ± 4.66	1.30 ± 1.45	0.05 ± 0.03
Excipient 2, oral	F	52.15 ± 10.31	57.47 ± 3.76	34.27 ± 8.58	6.90 ± 4.50	1.36 ± 0.46	0
	M	56.33 ± 7.62	49.97 ± 10.03	44.92 ± 2.02	4.00 ± 5.76	1.03 ± 0.49	0.08 ± 0.02
Positive control (CF[b]), i.p.	F	118.02 ± 13.28*	23.33 ± 5.22*	53.02 ± 10.64*	10.41 ± 4.99*	8.78 ± 3.90*	4.46 ± 1.43*
	M	112.69 ± 14.11*	25.80 ± 3.41*	52.78 ± 11.98*	9.37 ± 1.90*	7.03 ± 2.01*	5.02 ± 0.91*

[¶] The values shown here differed for all groups when compared with the lines OF-1 and NMRI, except for levels 3 and 4.
[a] SD: standard deviation.
[b] CF: cyclophosphamide.
*p < 0.05 (comparison with the negative control, Mann-Whitney's U-test).

Table 3. Comet assay in OF-1 mice of both sexes for the induction of DNA damage in peripheral blood leukocytes

Groups (mg/kg)	Sex	Arbitrary Units (mean ± SD[a])	Nucleoid percentage (mean ± SD) Level 0	Level 1	Level 2	Level 3	Level 4
Negative control 1, oral	F	57.40 ± 9.64	49.10 ± 8.09	44.90 ± 3.05	5.50 ± 6.07	0.50 ± 0.86	0
	M	59.48 ± 7.51	50.00 ± 5.21	42.29 ± 2.36	6.00 ± 4.71	1.65 ± 0.02	0.06 ± 0.03
Excipient 1, oral	F	62.55 ± 10.23	48.34 ± 8.33	42.73 ± 4.72	7.06 ± 4.28	1.78 ± 0.84	0.09 ± 0.04
	M	57.68 ± 8.49	51.56 ± 7.55	40.34 ± 3.90	6.98 ± 4.48	1.10 ± 0.98	0.02 ± 0.01
Excipient 2, oral	F	56.27 ± 10.11	53.85 ± 7.12	37.29 ± 7.10	7.60 ± 4.60	1.26 ± 0.91	0
	M	59.06 ± 8.29	49.25 ± 10.01	43.84 ± 8.22	5.71 ± 3.61	1.00 ± 0.33	0.20 ± 0.10
Positive control (CF[b]), i.p.	F	123.59 ± 12.57*	22.00 ± 3.23*	51.62 ± 10.87*	12.05 ± 3.56*	9.45 ± 4.70*	4.88 ± 3.02*
	M	123.79 ± 13.16*	21.54 ± 6.40*	53.13 ± 11.58*	10.33 ± 4.34*	10.00 ± 3.08*	5.00 ± 2.17*

[a] SD: standard deviation.
[b] CF: cyclophosphamide.
*p < 0.05 (comparison with the negative control, Mann-Whitney's U-test).

vious measurements of the frequency of chromosomal aberrations, which is also lowest in Balb/c mice [28]. The AU ranges for the other lines are 56.27-62.55 in OF-1 and 61.13-71.45 AU in NMRI; agreeing with results obtained in control animals by Cancino *et al*., 2001 [19]. Taking into account the percentage of grade 0 nucleoids, the NMRI line exhibits the lowest values and the Balb/c the highest as expected, given that NMRI mice had the highest AU values.

When analyzing damage levels 1 and 2, the NMRI line presented, again, the highest values and the Balb/c line the lowest. Levels 3 and 4 tend to behave similarly on all three lines. These results also agree with previous findings by our group in a genotoxicity study performed with the micronucleus test and morphological analyses of spermatozoid heads, where the Balb/c line obtained the lowest basal results for both assays [29]; and with the findings of Rodríguez *et al*. for control animals of the Balb/c line used in a comet assay for tinidazole [30].

The highest AU and nucleoid percentages induced by CF at damage levels 1 to 4 were observed in the most susceptible line: NMRI (Tables 2, 3 and 4). Maxima of 126.21 AU were observed, with a standard deviation of 13.22. An analysis of damage level results reveals up to 55.82% of nucleoids with damage level 1 and 5.85% of nucleoids with damage level 4. Balb/c was more resistant to CF-induced damage [30] than the NMRI line [5, 26].

The high nucleoid induction percentage from levels 1 to 4 observed in these three mice lines when using CF as positive control demonstrates the usefulness of this chemical clastogen for the induction of genotoxicity, evaluated with this methodology. Its advantages, exposed above, would help to implement the utilization of this mutagen as positive control;

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Table 4. Comet assay in NMRI mice of both sexes for the induction of DNA damage in peripheral blood leukocytes

Groups (mg/kg)	Sex	Arbitrary units (mean ± SD[a])	Nucleoid percentage (mean ± SD) Level 0	Level 1	Level 2	Level 3	Level 4
Negative control 1, oral	F	63.84 ± 8.24	45.71 ± 6.09	45.99 ± 2.05	7.05 ± 6.09	1.25 ± 0.34	0
	M	61.13 ± 8.31	48.05 ± 1.30	43.86 ± 1.56	7.10 ± 4.33	0.89 ± 1.01	0.10 ± 0.06
Excipient 1, oral	F	64.54 ± 10.89	43.98 ± 2.39	49.35 ± 5.22	5.02 ± 4.28	1.45 ± 0.56	0.20 ± 0.15
	M	65.22 ± 10.56	43.57 ± 7.41	49.61 ± 3.18	5.10 ± 4.36	1.47 ± 1.20	0.25 ± 0.10
Excipient 2, oral	F	67.74 ± 10.31	44.26 ± 4.99	47.29 ± 1.40	6.90 ± 6.41	1.55 ± 0.81	0
	M	71.45 ± 9.51	41.98 ± 8.84	48.03 ± 2.02	6.83 ± 5.16	2.88 ± 2.28	0.28 ± 0.43
Positive control (CF[b]), i.p.	F	126.21 ± 13.22*	19.00 ± 3.27*	55.82 ± 10.24*	11.00 ± 2.09*	8.33 ± 4.41*	5.85 ± 4.00*
	M	123.95 ± 11.53*	20.40 ± 5.37*	54.66 ± 9.78*	10.50 ± 3.68*	9.47 ± 3.99*	4.97 ± 5.80*

[a] SD: standard deviation.
[b] CF: cyclophosphamide.
*p < 0.05 (comparison with the negative control, Mann-Whitney's U-test).

which would decrease the hazards derived from the exposure to these chemicals for the personnel performing *in vivo* genotoxicity and anti-genotoxicity assays.

The low basal levels of the Balb/c line in the micronucleus assay, in tests for chromosomal abnormalities, in morphological analyses of spermatozoid heads and now in the alkaline comet assay demonstrate that this line is genetically more stable and resistant to damage [28, 29, 31-33]. This, in turn, is coherent with the results obtained for both control and CF-treated animals by Cancino *et al*. [19], corroborating that the Balb/c mouse line is the best experimental biomodel for *in vivo* assays of potential genotoxicity and anti-genotoxicity.

Conclusions

Under our experimental conditions, Balb/c is the most suitable of the three mouse lines evaluated, independently from the sex of the experimentation animals. The low basal frequency exhibited by this line for the analyzed variables demonstrate that it is the best experimental biomodel for this type of assay. These results will, therefore, increase the efficiency of the preclinical evaluation of drugs, vaccines and other products.

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