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The methylating agent streptozotocin induces persistent telomere dysfunction in mammalian cells

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ABSTRACT

We analyzed chromosomal aberrations involving telomeres in the progeny of mammalian cells exposed to the methylating agent and antineoplastic/diabetogenic drug streptozotocin (STZ), to test whether it induces long-term telomere instability (by chromosome end loss and/or telomere dysfunction). Rat cells (ADIPO-P2 cell line, derived from Sprague-Dawley rat adipose cells) were treated with a single concentration of STZ (2 mM). Chromosomal aberrations were analyzed 18 h, 10 days, and 15 days after treatment, using PNA-FISH with a pan-telomeric probe [Cy3-$(CCCTAA)_3$] to detect (TTAGGG)n repeats. Cytogenetic analysis revealed a higher frequency of chromosomal aberrations in STZ-exposed cultures vs. untreated cultures at each time point analyzed. The yield of induced aberrations was very similar at each time point. Induction of aberrations not involving telomere dysfunction was only observed 18 h and 15 days after treatment, whereas induction of telomere dysfunction-related aberrations by STZ (mainly in the form of telomere FISH signal loss and duplications, most of them chromatid-type aberrations) was observed at each time point. Our results show that STZ induces persistent telomere instability in mammalian cells, cytogenetically manifested as telomere dysfunction-related chromosomal aberrations. Neither telomere length nor telomerase activity is related to the telomere dysfunction.

1. Introduction

Telomere instability may result from chromosome end loss or, more frequently, telomere dysfunction [1–4]. Dysfunctional telomeres arise when they lose their end-capping function or become critically short, which causes chromosomal termini to behave like a double-strand break [1–4]. At the chromosomal level, this phenomenon is visualized as chromosomal aberrations directly involving terminal telomeric repeats [1–3]. These aberrations include loss or duplication of telomeric signals at one or both ends of a chromosome, association or fusion of telomeres of different chromosomes, telomere recombination at the end of a chromosome, translocation or amplification of telomeric sequences, and extrachromosomal telomere FISH signals [2,3]. Since telomeres play a fundamental role in maintaining genomic stability [1–4], the study of long-term telomere instability in cells exposed to antineoplastic drugs is of great importance to understand the genomic instability associated with chemotherapy regimens. Several studies have shown that cancer patients undergoing chemotherapy have shorter telomeres in their blood cells, even after the end of treatment [5–10]. Furthermore, a recent study showed that the chemotherapeutic agents doxorubicin and etoposide as well as γ-radiation induce short-term telomere dysfunction in normal human T lymphocytes and fibroblasts. These findings suggest that repeated exposure to chemo- and radiotherapy may cause telomere dysfunction [11]. Despite the above studies, data on the long-term effects of antineoplastic drugs on telomeres are scarce and refer to the radiomimetic compounds bleomycin (BLM) [12] and streptonigrin (SN) [13], which induce delayed telomere instability in rat cells. Both agents promote the appearance of telomere-related aberrations in treated cells, several days after exposure [12,13]. In particular, BLM induces persistent and delayed telomere instability in rat cells [12], which exhibit chromosomal abnormalities related to telomere dysfunction, such as telomere loss and duplications,

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10 days after treatment. These cells also show incomplete chromosomes (IC, a type of aberration which involves breakage at one or both chromosome ends) 10 days after exposure to BLM [12]. Moreover, SN induces persistent telomere dysfunction in the form of additional telomeric FISH signals, extra-chromosomal telomeric FISH signals, and telomere FISH signal loss and duplications in rat cells [13].

In order to gain further insight into the long-term effects of antibiotics with antineoplastic properties on mammalian telomeres, we investigated the induction of chromosomal aberrations involving telomeres in rat cells exposed to streptozotocin (STZ). This compound is an antibiotic isolated from *Streptomyces achromogenes* [14], usually used to experimentally induce diabetes mellitus in laboratory animals, and it has been considered a potential compound for the clinical treatment of some malignant diseases (see [15,16] for review). STZ is a potent alkylating agent that directly methylates DNA, giving rise to chromosome and DNA damage [15,16]. STZ exerts its clastogenic effect mainly in an S-dependent manner, inducing both chromatid- and chromosome-type aberrations [15,16]. Although the effects of STZ on telomeres from mammalian cells are partially known [17], there are no data available concerning the long-term effects of this antibiotic on telomeres. We exposed rat cells to a single pulse of STZ and determined the type and frequency of chromosomal aberrations 18 h (first mitosis after exposure), 10 days, and 15 days after treatment, by using PNA-FISH with a pan-telomeric probe. We found that STZ induces persistent telomere dysfunction in rat cells, cytogenetically detected mainly as telomere FISH signal loss and duplications. Furthermore, our results suggest that neither telomere length nor telomerase activity is related to the telomere dysfunction induced by this antibiotic.

2. Materials and methods

2.1. Cell culture, drug treatments and cell harvesting

Fibroblast-type cell line ADIPO-P2 was studied. This cell line, obtained from the Instituto Multidisciplinario de Biología Celular (IMBICE, La Plata, Argentina) cell repository, was established by Daniel Castrogiovanni (Cell Culture Section, IMBICE). ADIPO-P2 cells ($2n = 42$) are derived from dedifferentiated adipose cells of Sprague-Dawley rats. ADIPO-P2 chromosomes exhibit strong telomeric signals at their ends and do not show interstitial telomeric signals after telomere PNA-FISH [12,13]. In this way, telomere aberrations can be identified easily, with no interference from interstitial telomeric signals, i.e., all of the data concerning telomeric sequences instability can be ascribed to telomere instability. Cells were grown in D-MEM high-glucose medium (Gibco®, Grand Island, NY, USA) supplemented with 20% fetal calf serum, penicillin (100 U/mL) and streptomycin (100 μg/mL) at 37 °C and 5% CO_2 atmosphere. Cells were cultured as monolayer in TC25Corning flasks containing 1.5×10^5 cells/mL. For each experiment, three flasks were set up, two for the controls and one for the treated culture. During the log phase of growth, cells were treated with a 30 min pulse of 2 mM STZ (Sigma, CAS No. 18883-66-4). Since STZ is unstable at pH 7 [18], it was prepared immediately before use by dissolving the drug in 0.02 M sodium citrate (pH 4.4). Two negative control cultures (unexposed to STZ) were set up in parallel, i.e., one with cells treated only with the buffer sodium citrate and another one without sodium citrate. The negative control exposed to sodium citrate was included to determine if the drug solvent itself could induce cytotoxic and/or genotoxic damage in ADIPO-P2 cells. Time of exposure and concentration of STZ were chosen according to previous studies carried out in our laboratory with mammalian cells exposed to this compound [17,19–21]. At the end of the pulse treatment with STZ or sodium citrate alone, cells were washed twice with Hank's balanced salt solution and kept in culture with fresh culture medium until harvesting. Both control and STZ-treated cells were continuously maintained in culture during 7 passages or subcultures after treatment. Subcultivation was carried out whenever the cultures became confluent (approximately 4×10^5 cells/mL of culture medium). Cell cultures were analyzed at 18 h (where 100% of metaphase cells correspond to first division cells after treatment, as verified by the FPG technique) [22] and 10 and 15 days after treatment. To estimate cell viability, we applied the Trypan Blue exclusion assay. An aliquot (about 200 μL) stained with 0.4% trypan blue obtained at subcultivation steps was used to count viable cells in a Neubauer chamber. Cells were then suspended in fresh culture medium and dispensed into new culture flasks containing 1×10^5 cells/mL, to continue growing. The remainder of the cells were discarded or dispensed into another flask for cytogenetic analysis, which was performed at 18 h and 10 and 15 days after treatment. To analyze chromosomal aberrations, colchicine (0.1 μg/mL) (Sigma, CAS No. 64-86-8) was added to cell cultures during the last 3 h of culture. Chromosome preparations were made following standard procedures. After harvesting, cells were hypotonically shocked, fixed in methanol:acetic acid (3:1), spread onto glass slides and processed for PNA-FISH. Two independent experiments were carried out.

2.2. Fluorescence in situ hybridization with the PNA pan-telomeric probe (PNA-FISH)

Fluorescence in situ hybridization with the PNA pan-telomeric probe (PNA-FISH) was performed as described elsewhere [12,13], using a Cy3-conjugated PNA pan-telomeric probe [Cy3-$(CCCTAA)_3$] obtained from Panagene (Korea). Fluorescence microscopy was performed on a Nikon Eclipse 50i epifluorescence microscope equipped with an HBO 100 mercury lamp, a Nikon high-resolution digital color camera (DS-Ri-U3), and filters for DAPI and Cy3 (Chroma Technology Corp., Rockingham, VT). For more details on the PNA-FISH procedure, see the Supplementary file.

2.3. Scoring of aberrations

Chromosome analysis was performed on coded slides. For accurate observation and analysis, all images were digitalized and, when necessary, DAPI signals were enhanced for optimal contrast. This allowed the detection of even the smallest acentric fragments present in the aberrant metaphases. Centromeres were identified using the DAPI filter, whereas telomeric signals were detected using the Cy3 filter. To obtain the final, two-color FISH images, DAPI and Cy3 images were merged using the NIS-Element Imaging Software 3.22 (Nikon Corporation). The total number of centromeres and telomeric signals was counted and all unstable chromatid- and chromosome-type aberrations were scored. Since the position and number of centromeres could be easily determined with DAPI staining, all types of unstable aberrations were scored, namely: (1) dicentric chromosomes; (2) interstitial fragments, (i.e., chromosome elements without telomeric signals) which include typical acentric fragments – where chromatids lie parallel throughout their length and there is no centromeric constriction – and double minute chromosomes of variable size; (3) incomplete chromosome elements, both centric (incomplete chromosomes, lacking telomeric signals at one or both ends) or acentric (terminal fragments), (4) compound fragments (acentric fragments labeled at both ends, i.e., exhibiting four telomeric signals), and (5) mono- and isochromatid breaks. Besides the above mentioned aberrations, we scored those aberrations directly involving terminal telomeric sequences, i.e., implying telomere dysfunction, as follows: telomere fusions (giving rise to dicentric or ring chromosomes

without accompanying fragment), telomere associations, telomeric repeats translocations, additional telomeric FISH signals (which implies translocation and/or amplification of telomeric repeats), extra-chromosomal telomeric FISH signals, and telomere (FISH signal) loss or duplications [2,3]. In the case of the latter aberrations those involving one chromatid were considered chromatid type aberrations whereas those affecting both chromatids of the same chromosome were scored as chromosome type aberrations. For the discrimination and scoring of telomere fusions and associations, we used the criteria indicated in Refs. [2] and [3]. Thus, telomere associations are aberrations implying that the telomeres of two different chromosomes are very close to each other. Therefore, we scored as telomere associations those aberrations exhibiting two pairs of very close telomeric signals after PNA-FISH, each pair of signals corresponding to a different chromosome. On the contrary, telomere fusions imply the fusion of two chromosome ends, so we scored as telomere fusions those aberrations where the telomeres of adjoining chromosomes have fused into a single telomere FISH signal, one per chromatid, and the DAPI signal was continuous through the point of fusion. No multicentric or ring chromosomes were observed in any of the samples analyzed. The mitotic index (MI; expressed as the percentage of cells in mitosis) was determined by analyzing 1000 cells per sample.

2.4. Telomerase activity assay

Telomerase activity was measured in all samples with the TRAPeze™ telomerase detection kit (Millipore, Bedford, MA) and Taq Platinum (Invitrogen, Grand Island, NY) according to the manufacturer's instructions. The method used in the TRAPeze™ kit is based on an improved version of the original method described by Kim et al. [23]. Reactions were performed in triplicate. For more details on the telomerase activity assay, see the Supplementary file.

2.5. Telomere length measurement by Flow-FISH

Tel-Flow-FISH assays were performed on ADIPO-P2 cells which were fixed at 18 h, 10 days, and 15 days after treatment with STZ. Two different cell lineages with known telomere length were included as external controls: (a) the Jurkat cell line (T cell lymphoblastic lymphoma) as short telomeres control cells (11.5 kb) [24,25] and (b) spleen cells obtained from male mice (*Mus musculus*) strain C57BL/6, about one month old (telomere length equal to or greater than 20 kb [26,27]), as a control cell line for long telomeres. These control cells are easily distinguished from the test cells and provide a convenient reference point for Flow-FISH telomere fluorescence measurements. Flow cytometric determinations were carried out with a FACSVantage flow sorter (BD) using a 488 nm Innova Coherent laser tuned at 100 mW.

The signal intensity of the probe is directly proportional to the number of telomeric repeats present in the cells under study. The data obtained by Tel-Flow-FISH was analyzed according to the methodology proposed by Baerlocher et al. [28]. Calculations of telomere length from Tel-Flow-FISH data were based on the average of the telomeric length of each external control used in cytometric measurements. In each case, the difference between the mean of the fluorescent signal (corresponding to the specific hybridization of the telomeric probe) and the mean of samples processed without the telomeric probe, was calculated. The latter signal corresponds to cell autofluorescence. The data acquired represent the specific fluorescence of the telomere sequences from which telomeric length was calculated according to the methodology proposed by Baerlocher et al. [28]. The comparison between control and exposed cells was analyzed independently at each time point investigated. For further details on the Tel-Flow-FISH procedure, see the Supplementary file.

2.6. Statistical analysis

Statistical analysis was performed using GraphPad Prism version 5.00 software for Windows (GraphPad Software, San Diego, CA). Comparisons between control and exposed cultures (percentage of damaged cells) were carried out using the Chi-squared test. The significance of differences in aberration frequencies among different treatments was obtained by comparing the Z score of Poisson distributions of observed and expected values with 95% confidence intervals [29]. ANOVA with the Tukey's multiple comparison test was used to determine the statistical significance of the differences in telomere length and in the number of live cells per mL between untreated and STZ-treated samples. Differences were considered to be statistically significant at two-sided p values <0.05.

3. Results

3.1. Chromosome damage and distribution pattern of telomeric sequences in untreated cells

Previous studies from our laboratory [12,13] have demonstrated that ADIPO-P2 cells exhibit telomeric repeats localized exclusively at the terminal regions of chromosomes, some FISH signals being stronger than others (Fig. 1A). On the other hand, the high spontaneous frequency of IC and acentric fragments observed in control cells (Table 1a) cannot be linked to the results of the FISH analysis and is similar to that found in other mammalian cell lines, previously investigated in our laboratory for telomere instability [17,30–32]. Furthermore, the significant increase in the frequency of telomere FISH signal loss found in control cells 10 and 15 days after treatment (Table 1b) could be due to spontaneous extensive telomere shortening, a phenomenon usually observed in in vitro cultured cells [1–4].

3.2. Persistence of chromosome damage induced by STZ

STZ induced a significant increase in the percentage of aberrant metaphases (i.e., cells showing at least one aberration) (Fig. 2A), as well as in the frequency of total chromosomal aberrations per cell (Fig. 2B; see data on Table 1a and b) in ADIPO-P2 cells at 18 h, 10 and 15 days after treatment ($p < 0.05$), compared with control cultures. In addition, our data showed that sodium citrate alone also induced a significant increase in the percentage of cells with aberrations at 18 h and 10 days after treatment (Fig. 2A) as well as in the frequency of total chromosomal aberrations per cell (Fig. 2B; see data on Table 1a and b) in the cells at 18 h as well as 10 and 15 days after treatment ($p < 0.05$), compared with control cultures. Although sodium citrate per se seems to be non-cytotoxic (Fig. 3), it induced genotoxicity on the telomeres of these cells. Nevertheless, the fact that STZ induced about twice as many aberrations as did sodium citrate (Fig. 2B, $p < 0.05$) shows that this compound per se induces chromosome damage (i.e., a genotoxic effect). The frequency of STZ-induced aberrations in ADIPO-P2 cells remained very similar from 18 h to 15 days after treatment (Fig. 2B), which shows that STZ induces a persistent genotoxic effect in these cells. Since the drug was eliminated from the culture medium, this finding suggests that the chromosomal aberrations found at 10 and 15 days after treatment are derived aberrations scored after the first cell cycle post-treatment.

A detailed cytogenetic analysis of our data showed that significant induction of chromosomal aberrations by STZ and sodium citrate alone was mostly observed for those aberrations related

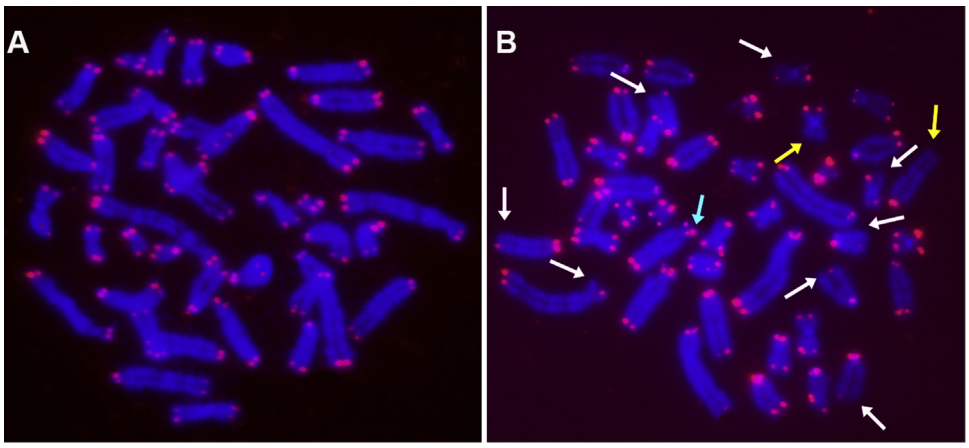

Fig. 1. Patterns of hybridization of (TTAGGG) *n* repeats in the metaphase spreads from untreated (A) and STZ-treated (B) ADIPO-P2 cells after PNA-FISH with a pantelomeric probe labeled with Cy3 (red signal). (A) Control metaphase ($2n = 42$) cell. Note the presence of terminal telomeric FISH signals and the absence of interstitial telomeric signals in ADIPO-P2 chromosomes; (B) Full metaphase of a STZ-treated culture of ADIPO-P2 cells 10 days after treatment exhibiting different types of telomere aberrations: Eight chromosomes with chromatid-type telomere loss (white arrows indicate the site where telomere loss occurred), two chromosomes with chromosome-type telomere loss (yellow arrows), and one acrocentric chromosome with additional telomeric signal (light blue arrow) at one of its chromatids.

Table 1
(a) Chromosomal aberrations not involving telomere dysfunction observed in untreated and STZ-exposed ADIPO-P2 cells after PNA-telomere FISH scoring. (b) Chromosomal aberrations implying telomere dysfunction observed in untreated and STZ-exposed ADIPO-P2 cells after PNA-telomere FISH scoring.

(a)

Treatment	Number of cells analyzed	Dic	IC[a]	Acentric fragments	Breaks		Total aberrations (frequency/cell)
					Chromatid type	Chromosome type	
Control (18 h)	181	4	18	18	7	3	50 (0.28)
Control (10 days)	199	1	22	27	5	1	56 (0.28)
Control (15 days)	143	0	8	10	1	1	20 (0.14)
SC (18 h)	147	0	13	12	2	0	27 (0.18)
SC (10 days)	176	0	13	13	1	0	27 (0.15)
SC (15 days)	136	0	5	5	0	3	13 (0.10)
STZ 2 mM (18 h)	186	3	34	34	4	8	83 (0.45)*
STZ 2 mM (10 days)	209	2	23	23	2	4	54 (0.26)
STZ 2 mM (15 days)	150	0	21	22	0	2	45 (0.30)*

(b)

Treatment	Number of cells analyzed	TF	TA	TR	ATS + ECTS	TL (chromosome/chromatid)	TD (chromosome/chromatid)	Total aberrations (frequency/cell)
Control (18 h)	181	0	4	2	2	14/5 (19)	2/5 (7)	34 (0.19)
Control (10 days)	199	1	0	0	0	18/23 (41)	2/11 (13)	55 (0.28)
Control (15 days)	143	2	0	1	4	20/26 (46)	1/1 (2)	55 (0.38)
SC (18 h)	147	0	0	3	8	70/40 (110)	2/23 (25)	146 (0.99)*
SC (10 days)	176	0	0	1	3	94/65 (159)	3/12 (15)	178 (1.01)*
SC (15 days)	136	0	0	1	0	57/49 (106)	1/6 (7)	114 (0.84)*
STZ 2 mM (18 h)	186	2	1	6	17	1116/137 (253)	17/45 (62)	341 (1.83)**
STZ 2 mM (10 days)	209	0	2	7	5	142/244 (386)	9/38 (47)	447 (2.14)**
STZ 2 mM (15 days)	150	0	0	10	2	89/102 (191)	8/37 (45)	248 (1.65)**

SC, sodium citrate; STZ, streptozotocin; Dic, dicentric chromosomes; IC, incomplete chromosomes (chromosomes lacking one end and accompanied by an acentric fragment); TF, telomere fusions; TA, telomere associations; TR, translocations of telomeric sequences; ATS, additional telomeric FISH signals; ECTS, extra-chromosomal telomeric FISH signals; TL, telomere loss; TD, telomere duplications. Telomere fusions include both dicentrics and centric rings involving fusion of their ends (these aberrations were also included in Table 1a). In the case of telomere loss and duplication, the number of chromosome- and chromatid-type telomere loss and duplications as well as the total yield of these aberrations (in brackets) are indicated.

[a] Chromosomes lacking telomere FISH signals at one or both of their ends but without accompanying acentric fragment were considered as events of chromosome-type telomere loss (included in Table 1b) instead of IC.

* $p < 0.05$ compared with the corresponding control value.

** $p < 0.05$ compared with the corresponding control value and with sodium citrate control.

to telomere dysfunction (see Fig. 1B for examples of these aberrations found in STZ-treated cells), which represents more than 80% of the aberrations induced by these compounds (see Table 1a and b for comparison). In effect, induction of aberrations not implying telomere dysfunction was only detected in metaphase cells derived from cell cultures harvested 18 h and 15 days after STZ treatment, whereas these types of aberrations were not observed in cell cultures exposed to sodium citrate alone (Table 1a).

Table 1b shows that STZ induced telomere dysfunction in ADIPO-P2 cells at 18 h, 10 and 15 days post-treatment ($p < 0.05$, Fig. 2C), cytogenetically detectable mainly as chromatid- and chromosome-type telomere FISH signal loss and duplication (Table 1b; Fig. 1B). Chromatid-type aberrations predominated over

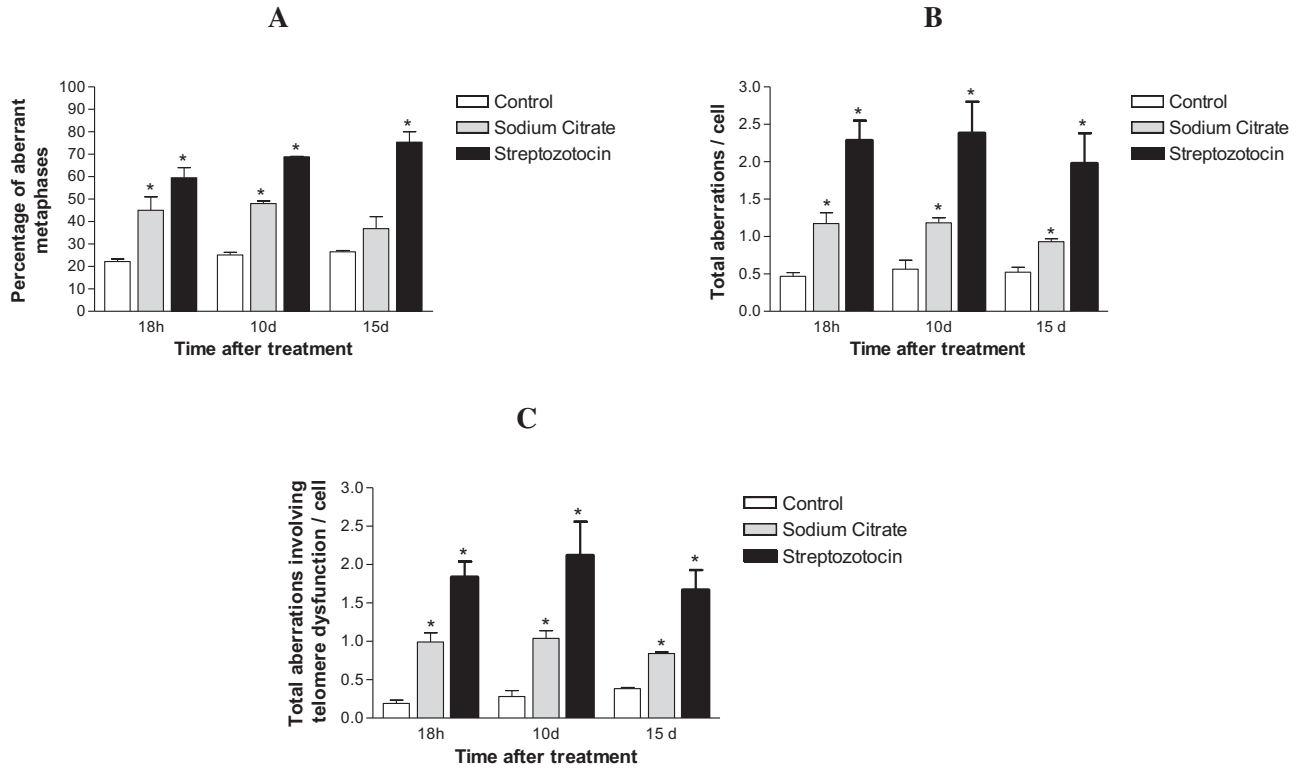

Fig. 2. (A) Percentage of aberrant metaphases (i.e., cells showing at least one chromosomal aberration) in continually subcultured (proliferating) ADIPO-P2 cells as a function of time (h = hours; d = days) after treatment with STZ (2 mM). Chi-squared test indicated significant differences (*) between control and exposed (sodium citrate or STZ) cultures at each time point analyzed ($p < 0.05$); (B) Changes in the frequency of chromosomal aberrations (all aberrations included; see data in Table 1a and b) in continually subcultured (proliferating) ADIPO-P2 cells as a function of time (h = hours; d = days) after treatment with STZ (2 mM). The *Z* score of the Poisson distribution indicated a significant increase (*) in the frequency of chromosomal aberrations induced by sodium citrate or STZ at each time point analyzed compared with untreated (control) cultures ($p < 0.05$). For this analysis, dicentrics and centric rings involving telomere fusion were counted as one aberration each; (C) Changes in the frequency of chromosomal aberrations implying telomere dysfunction (see data in Table 1b) in continually subcultured (proliferating) ADIPO-P2 cells as a function of time (h = hours; d = days) after treatment with STZ (2 mM). The *Z* score of Poisson distribution indicated significant increase (*) in the frequency of chromosomal aberrations induced by sodium citrate or STZ at each time point analyzed compared with untreated (control) cultures ($p < 0.05$). In all cases, data represent average values from two independent experiments. For each treatment, mean ± S.E. is indicated.

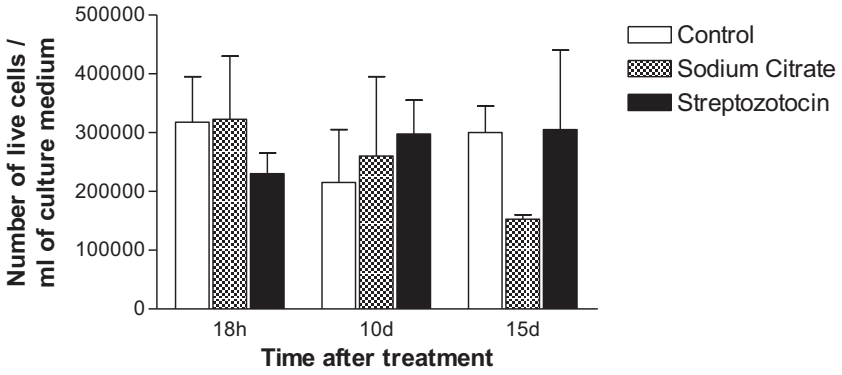

Fig. 3. Variation in the number of live cells observed in ADIPO-P2 cells with time in culture after treatment with STZ (2 mM). Data represent average values from two independent experiments. For each treatment, mean ± S.E. is indicated. No significant differences between treatments were found.

the chromosome-type ones. The yield of STZ-induced aberrations remained very similar at 18 h, 10 days, and 15 days post-treatment (Fig. 2C and Table 1b). In addition, STZ induced about twice as many aberrations as did sodium citrate alone ($p < 0.05$, Table 1b and Fig. 2C) and cell cultures exposed to the antibiotic exhibited 4–10 times as many aberrations as did control cultures (Table 1b). Moreover, sodium citrate alone induces mainly telomere FISH signal loss in ADIPO-P2 cells while the STZ induces telomere loss as well as telomere duplications ($p < 0.05$, Table 1b).

Cell viability analysis using trypan blue dye exclusion staining showed no significant differences between the negative controls (unexposed cells and sodium citrate-treated cells) and the STZ-exposed cells at each time point investigated ($p > 0.05$, Fig. 3). Thus, there was no STZ- or sodium citrate-induced cell death in ADIPO-P2 cells. Moreover, we found no significant differences in the mitotic index between different treatments (ANOVA, $p > 0.05$) (data not shown).

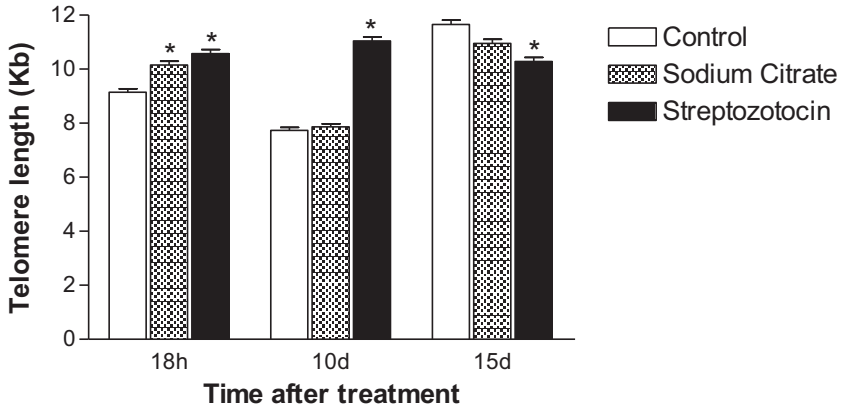

Fig. 4. Average telomere length (kb) of ADIPO-P2 cells as a function of time (h = hours; d = days) after treatment with STZ (2 mM). (*) Denotes statistically significant differences between control and sodium citrate or STZ-treated samples ($p < 0.05$).

3.3. Telomerase activity in STZ-treated and untreated cells

Analysis of the polyacrylamide gels showed that the level of telomerase activity in STZ-treated cells was very similar to that of the control (unexposed) cells with or without sodium citrate (see Supplementary Fig. 1). Thus, treatment of these cells with STZ produced no effect on telomerase activity in the long term, at least up to 15 days after the treatment.

3.4. Telomere length in STZ-treated and untreated cells

Tel-Flow FISH analysis showed that STZ treatment induced a significant increase in telomere length in ADIPO-P2 cells at 18 h and 10 days post-treatment ($p < 0.05$, Fig. 4) compared with untreated control cells. However, cell cultures exposed to sodium citrate alone also showed a significant increase in telomere length compared with untreated control cells 18 h after treatment ($p < 0.05$, Fig. 4). Therefore, the increasing effect of STZ on telomere length observed in ADIPO-P2 cells at 18 h after treatment cannot be ascribed to the antibiotic per se but to the action of sodium citrate. Moreover, 15 days after treatment, telomere lengths in sodium citrate alone and STZ-treated cells were lower than in untreated control cells (Fig. 4), the difference between untreated control and STZ-treated cultures being statistically significant ($p < 0.05$). Thus, the only significant effect on telomere length which could be ascribed to STZ in ADIPO-P2 cells was observed 10 days after treatment. Furthermore, the dramatic changes detected in telomere length in control cells could be ascribed to the loss of proliferative potential during in vitro cell culture, after a limited number of cell divisions [33]. The reduction in telomere length during the first passages could have a definitive role in the loss of chromosomal integrity among the in vitro cell culture expansion. Thus, cells cultured in vitro in the long term for several passages may present changes in the telomere length, a phenomenon usually observed in in vitro cultured cells [33]. Therefore, the comparison between control and exposed cells was analyzed independently at each time point investigated.

4. Discussion

Although there are several reports indicating that anticancer drugs affect telomeres and telomerase, most of them correspond to short-term studies. Previously, we explored the long-term effects of the chemotherapeutic agents BLM and SN on telomeres of rat cells [12,13]. In the present study, we tested whether the chemotherapeutic drug STZ (an alkylating agent) also induces telomere instability in the progeny of mammalian cells, several generations after exposure. Since STZ is widely used as a diabetogenic agent and is also employed for the clinical treatment of some malignant diseases, especially pancreatic adenocarcinomas [34,35], the study of the genomic instability induced by this compound is of great importance.

A recent study from Liu et al. [36], in a mouse spermatogonial cell line, showed that the alkylating compounds cisplatin and 4-hydroperoxycyclophosphamide (4OOH-CPA) induce telomere dysfunction in mouse cells. These authors investigated the short-term effects of the abovementioned alkylating compounds on the mouse spermatogonial cell line C18-4 and found that cisplatin and 4OOH-CPA decrease telomerase activity and shorten telomere length, thus causing telomere dysfunction [36]. However, the effects of these compounds on telomeres at the cytogenetic level have not been investigated. Thus, our study is the first one in which the effects of an alkylating compound on telomeres is analyzed at the cytogenetic level in the long term.

We found that STZ induced chromosome damage in ADIPO-P2 cells at 18 h as well as 10 and 15 days after treatment, though significant induction of chromosomal aberrations by this compound was mostly observed for those aberrations related to telomere dysfunction. Induction of aberrations not involving telomere dysfunction (including IC and terminal fragments) by STZ was observed at 18 h and 15 days after treatment. We previously found that in Chinese hamster embryo (CHE) cells STZ induces incomplete chromosome elements (i.e., chromosome end loss) at 18 h after treatment [17]. Hence, our present results confirm that STZ induces telomere instability in the form of incomplete chromosome elements, and show that this compound also induces telomere dysfunction.

Our results also indicate that sodium citrate despite inducing chromosome damage in ADIPO-P2 cells, has no cytotoxic effect on these cells in the long term. However, sodium citrate caused a certain degree of genotoxicity on telomeres of chromosomes. We suggest that this damage is related to telomere dysfunction, since this compound only induced telomere FISH signal loss and duplications. This finding has not been reported before, very likely due to the fact that this is the first work where telomere PNA-FISH is applied to mammalian cells treated with sodium citrate. However, it should be mentioned that there is at least one published study which states that sodium citrate can be cytotoxic [37].

Despite the abovementioned effect, our data showing that STZ induces twice as many aberrations than sodium citrate alone, demonstrate that STZ per se induces chromosome damage in ADIPO-P2 cells. The frequency of STZ-induced aberrations cells remained constant from 18 h to 15 days after treatment, which shows that STZ has a persistent genotoxic effect in these cells.

Our results show that STZ induces telomere dysfunction in ADIPO-P2 cells, mainly in the form of telomere FISH signal loss and duplications, most of them being chromatid-type. Our data indicate that STZ induces significantly more signal loss than duplications, telomere loss thus being the most significant effect of STZ on telomere function at the chromosome level in ADIPO-P2 cells. Telomere FISH signal loss and duplication were also observed in X-ray-surviving human fibroblasts 14 days after exposure [38], BLM-exposed ADIPO-P2 cells 10 days after treatment [12] and SN-exposed ADIPO-P2 cells 18 h and 10 and 15 days after treatment [13]. Therefore, these types of aberrations seem to be the predominant chromosome aberrations directly related to telomere dysfunction induced by ionizing radiation and anticancer drugs. Since most of the STZ-induced telomere loss and duplication events were of chromatid-type, we may assume that these aberrations mainly occur during DNA replication or in the G2 phase of the cell cycle. Similar findings were observed in BLM-treated ADIPO-P2 cells [12] and X-ray-exposed lymphoblastoid cells 14 days after exposure [38], as most of the telomere-related induced aberrations were also of the chromatid-type.

According to our data, STZ-induced telomere dysfunction is due to derived aberrations scored after the first cell cycle post-treatment, since the same telomere-related aberrations were also present in cells harvested 18 h after treatment. Similar data was previously obtained with BLM- and SN-exposed ADIPO-P2 cells [12,13]. These results differ from the findings by Ojima et al. [38] who showed that X-rays induce delayed telomere instability in the form of telomere FISH signal loss and duplication in human fibroblasts, and the observations by Tanaka et al. [39], who found delayed induction of telomere associations or fusions in human B-cell lines established from α-ray- and γ-ray-irradiated lymphocytes. Thus, while telomeres of cells exposed to ionizing radiation appear to be unstable several generations after exposure, those exposed to chemical mutagens are unstable after the first post-exposure cell division.

We found that STZ induces a significant increase in telomere length in ADIPO-P2 cells at 10 days after treatment, a delayed effect not related with telomerase activity, which remained unchanged in both treated and untreated cells. The data registered in telomerase activity in the short term agrees well with the data obtained in CHO cells, where no variations of the activity were observed 18 h post-treatment with STZ [21] in comparison to control cells. Thus, our present data indicate that telomerase activity is not directly related to telomere dysfunction induced by STZ at the chromosome in ADIPO-P2 cells, since the persistence of chromosomal aberrations related to telomere dysfunction was not accompanied by a persistent effect of this drug on telomerase activity. Moreover, our data indicate that telomere length is not directly related to telomere dysfunction in STZ-exposed cells, since the persistence of telomere aberrations was not accompanied by a persistent increase or a reduction on telomere length in these cells. Our results also show that although STZ induced damage to chromosomal DNA, no delayed cell death was induced by the antibiotic in ADIPO-P2 cells, at least up to 15 days post-treatment. In addition, no significant alteration of the mitotic index by STZ or sodium citrate was detected in these cells.

According to previous studies, the treatment or exposure to chemical agents with alkylating properties such as MNU [40], cisplatin, or cyclophosphamide [36], could induce long term telomeric loss in mammalian cells, resulting from the inhibition of the enzyme telomerase. Our present data show that the alkylating compound STZ induces telomere shortening in the long term (15 days after treatment), but this effect does not depend on the activity of telomerase. Thus, STZ mechanism of action differs from the above mentioned alkylating compounds, since STZ is not acting through telomerase but it is probably correlated to chromosomal aberrations, number of viable cells or culture conditions.

In summary, our present work shows that the antitumor antibiotic STZ induces persistent telomere instability in mammalian cells, cytogenetically manifested as telomere dysfunction-related chromosomal aberrations. Therefore, our data suggest that one of the mechanisms by which STZ causes long-term genomic instability is telomere dysfunction. Since the genotoxic action of STZ is mediated by free radicals (see [15] and [16] for review), oxidative damage to telomeres induced by the drug itself could play a significant role in the telomere dysfunction caused by STZ. This concept needs to be tested by additional experiments. Regardless of the underlying mechanism involved in the persistent telomere instability induced by STZ, our findings raise concern about the potential risks of STZ-based chemotherapy. Further studies should be aimed at determining the molecular basis of the mechanisms underlying the long-term induction of telomere instability by STZ in mammalian cells, particularly whether STZ affects telomerase RNA and/or the expression of the shelterin complex-related genes. These studies may contribute to a better understanding of the effects of alkylating antitumor antibiotics on telomeres.

Conflict of interest

The authors declare that there are no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.mrgentox.2015.09.007.

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