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Cycle arrest and aneuploidy induced by zidovudine in murine embryonic stem cells

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Zidovudine (3′-azido-3′-deoxythymidine; AZT) is a nucleoside analogue widely used for the treatment of acquired immune deficiency syndrome (AIDS). Medical guidelines recommend the use of AZT by pregnant women in order to reduce risk of HIV vertical transmission. Although it is efficacious, little is known about the side effects of AZT on embryonic development. In this sense, we used murine embryonic stem (mES) cells as a model to investigate the consequences of AZT exposure for embryogenesis. Firstly, mES colonies were incubated with AZT (50 or 100 μM) and cell cycle profile was evaluated. While 27.7 ± 5.43% of untreated mES cells were in G2/M phase, this percentage raised to 45.96 ± 4.18% after AZT exposure (100 μM). To identify whether accumulation of cells in G2/M phase could be related to chromosome missegregation with consequent cell cycle arrest, aneuploidy rate was evaluated after AZT treatment. Untreated colonies presented 39.6 ± 8.4% of cells aneuploid, while after AZT 100 μM treatment, the proportion of aneuploid cells raised to 67.8 ± 3.4% with prevalence of chromosome loss. This event was accompanied by micronuclei formation as AZT 100 μM treated mES cells presented a 2-fold increase compared to untreated ones. These data suggest that AZT exerts genotoxic effects and increases chromosome instability at early stages of embryonic development.

Introduction

Acquired immune deficiency syndrome (AIDS) is described as a deregulation of immune system, manifested by opportunistic infections and unusual neoplasms due to infection by Human Immunodeficiency Virus (HIV). According to the World Health Organization, 33.3 million of people live with HIV/AIDS worldwide and it was estimated that ∼370 000 children (<15 years) were newly infected in 2009, mostly through vertical transmission.

Zidovudine (3′-azido-3′-deoxythymidine; AZT), a nucleoside reverse transcriptase inhibitor, was the first drug approved by the U.S. Food and Drug Administration for HIV treatment. During viral DNA synthesis by reverse transcription, AZT is incorporated into DNA in place of thymidine, causing early termination of DNA synthesis and inhibiting reverse transcriptase activity (1,2). In infected women known to be pregnant, medical guidelines suggest that antiviral therapy should be continued and with specific interventions, the risk of mother-to-child transmission of virus can be reduced to <10% (3). Although the well-documented efficacy of AZT treatment in preventing mother-to-child transmission of virus during pregnancy, little is known about its effect on early stages of embryogenesis.

Embryonic stem (ES) cells have the ability to differentiate into any kind of cell of adult organism as a feature of pluripotency. In view of the difficulty in studying embryogenesis *in vivo*, ES cells brought a functional *in vitro* system to investigate events occurring during early embryo development (4).

Recently, it was shown that AZT treatment induces telomere attrition, promotes centrosomal amplification and increases aneuploidy rate in mammary cells and Chinese hamster ovary (CHO) cells (5). Despite previous studies using adult cells, the effects of AZT treatment on pluripotent cells have never been explored. Here, we used murine embryonic stem (mES) cells as a model to study the consequences of AZT for early stages of embryogenesis.

Materials and methods

Culture of mES cells

mES cells line USP1 (6) was cultured onto inactivated mouse embryonic fibroblast (MEF) feeder in order to retain their pluripotency. The colonies were maintained in Dulbecco's modified Eagle's medium (Life Technologies, CA, USA) supplemented with 15% of Knockout Serum Replacement (KSR; Life Technologies), 1% of non-essential amino acids (Life Technologies), 2 mM L-glutamine (Life Technologies), 55 μM 2-mercapthoethanol (Life Technologies), 50 μg/ml gentamicin (Schering-Plough, NJ, USA) and 0.2% of conditioned medium from CHO cells carrying leukaemia inhibitor factor-encoding vector in a humidified incubator at 5% CO_2 and 37°C.

Zidovudine treatment

Colonies of mES were treated during 4 days with AZT at 50 and 100 μM concentrations, being the medium replaced on the second day. Concentrations of AZT used in this work were based on previous studies conducted *in vitro* and *in vivo* (5,7,8). The drug was obtained from Far-Manguinhos/Fundação Oswaldo Cruz, Rio de Janeiro, Brazil, diluted and prepared on the day of use. In addition, the estimated population doubling time of the mES cells line USP1 cells was ∼24 h (Figure 1B) and cells on logarithmic phase of growth were used to conduct the experiments.

Cell cycle analysis

Flow cytometric analyses of the cell cycle were performed at the last day of AZT treatment. Cells were detached from plates, washed in phosphate-buffered saline, incubated with Cycloscope solution containing propidium iodide (Cytognos, Salamanca, Spain) and passed through a Becton Dickinson FACScantoII flow cytometer. Data were acquired with the use of BD FACSDiva software (Becton Dickson, NJ, USA) and analysis performed by Infinicity software (Cytognos).

Cell viability assay

Cell viability was measured by 3(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) reduction assay. Briefly, cells were cultured and exposed to AZT

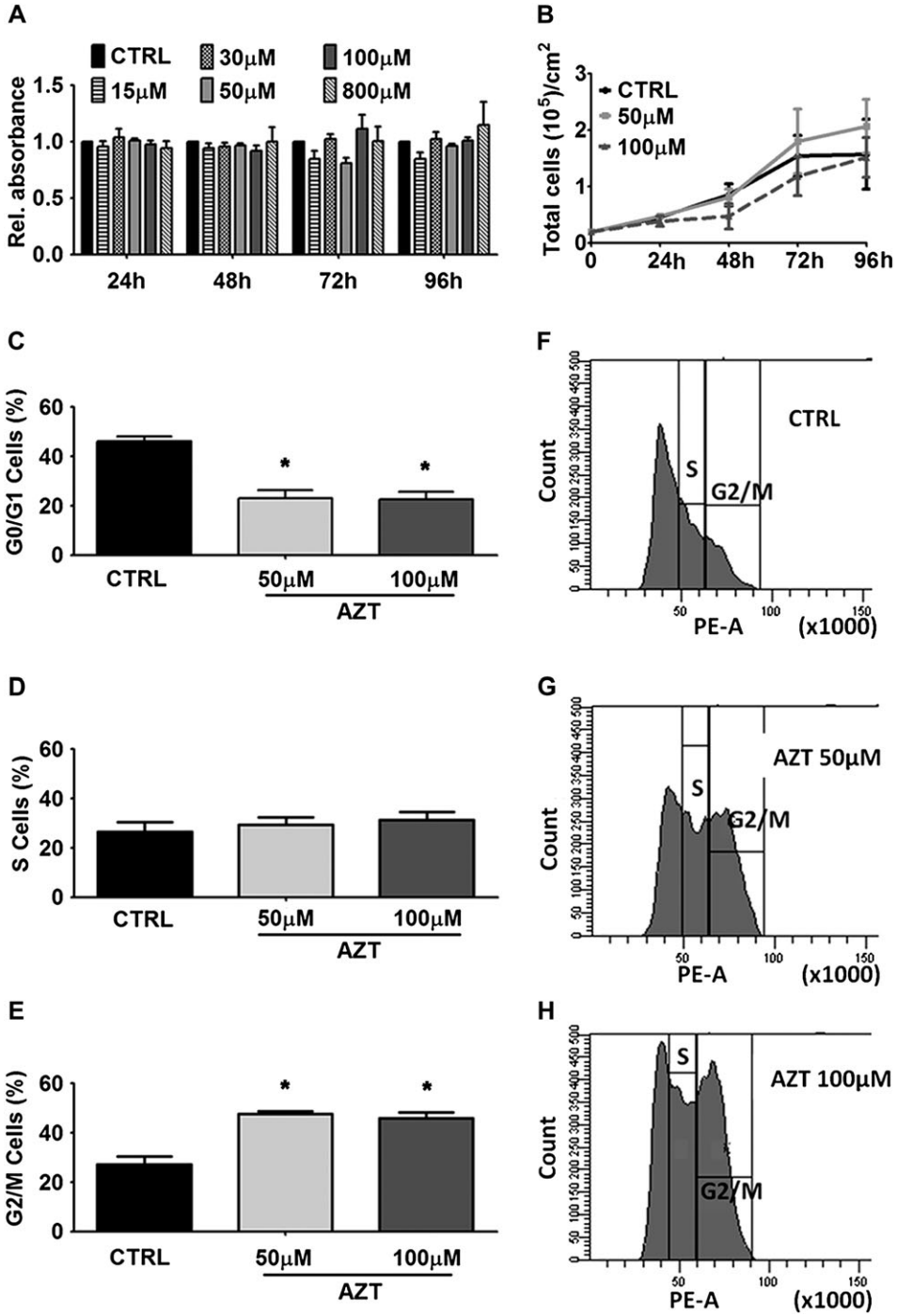

Fig. 1. Zidovudine induces G2/M arrest. MTT relative absorbance graph reveals that no significant change in the number of viable cells was detected after AZT treatment (A). No alteration in the number of total cells was observed during treatment (B). After 4 days of AZT treatment, the proportion of cells in G0/G1 phase decreased from 46.21 ± 3.45% in control to 23.04 ± 5.51% (50 μM AZT) and 22.68 ± 5.28% (100 μM AZT) (C). No alteration in the proportion of S phase cells was observed (D). In contrast, the rate of cells G2/M phase accumulated from 27.27 ± 5.43% in control to 47.63 ± 1.98% and 45.96 ± 4.18% in 50 and 100 μM AZT, respectively (E). (F–H) The histograms are related to control (F), AZT 50 μM (G) and AZT 100 μM (H) and correspond to one of three independent assays. Data represent three independent assays. Asterisk means significant difference ($P < 0.05$) compared to control group.

in triplicate wells of a 96-well plate. After 24, 48, 72 and 96 h of treatment, cells were incubated with MTT (0.5 mg/ml) (Sigma–Aldrich Corp., MO, USA) diluted in culture medium. After 3 h of incubation, an equal volume of dimethyl sulfoxide (Sigma–Aldrich Corp.) was added to dissolve the formazan salts and absorbance was measured at 490 nm by spectrophotometer (Infinite M200 Pro, i-control software; Tecan, Zürich, Switzerland).

Chromosome counting

Chromosome spreads were prepared as already described (9). For this specific experiment, cells were incubated with 0.1 μg/ml Karyo MAX colcemid (Life Technologies) for 3 h to cause mitotic arrest in G2/M phase. Next, cells were detached from dishes using trypsin/EDTA 0.05% and incubated with KCl hypotonic solution (75 mM) for 15 min at 37°C. The swollen cells were then fixed in methanol/glacial acetic acid 3:1 (v:v) solution and stored at 4°C overnight. On the next day, cell suspension was washed with fixative solution and then spread onto clean glass slides. The slides were stained with DAPI (4′-6-diamidino-2-phenylindole) and cover slipped with *n*-propyl galate. The mounted slides were examined under fluorescence microscope (Axioplan, AxioVision software; Carl Zeiss, NY, USA) (Figure 2D–F). The number of chromosomes was evaluated in five

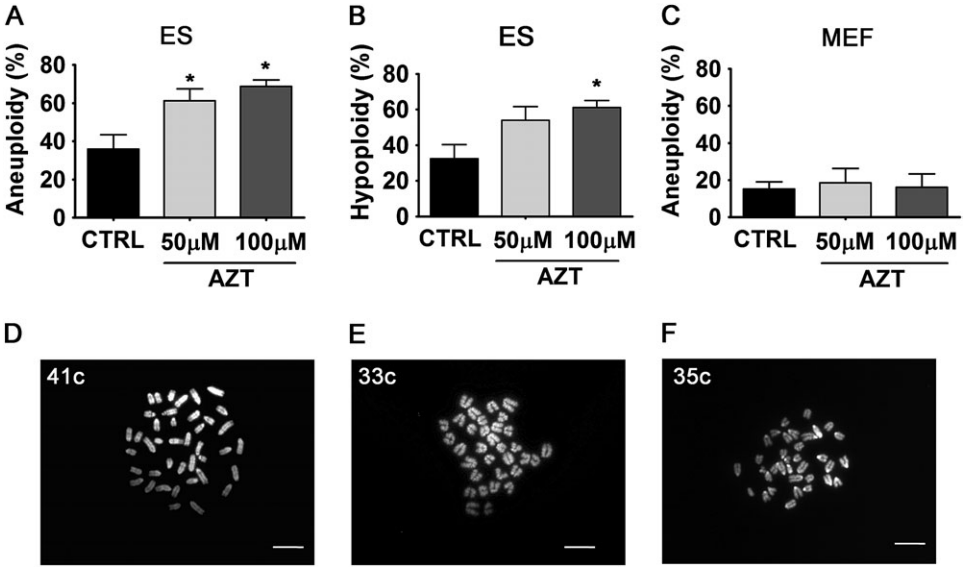

Fig. 2. AZT induces aneuploidy in mES cells. Aneuploidy rate raised from 39.6 ± 8.4% in control to 62.5 ± 6.0% (50 μM AZT) and 67.8 ± 3.4% (100 μM AZT) (A). The number of hypoploid ES cells changed from 32.4 ± 7.8% in control to 53.9 ± 7.7% (50 μM AZT) and 61.0 ± 4.0% (100 μM AZT) (B). In contrast, no alteration in ploidy rate could be observed in MEFs (C). Fluorescence images of metaphase spreads from control (D), 50 μM AZT (E) and 100 μM AZT (F) exhibiting 41, 33 and 35 chromosomes, respectively. Bars indicate mean ± SEM from three independent assays. Asterisk means significant difference ($P < 0.05$) compared to control group. Scale bar = 50 μm.

independent assays and at least 50 metaphase spreads were examined per assay.

Micronuclei analysis

Cells were incubated for 24 h with cytochalasin B (6 μg/ml; Sigma–Aldrich Corp.) in the last day of AZT treatment to cause an accumulation of binucleated cells. After 24 h of incubation, cells were fixed in methanol/glacial acetic acid (3:1). By distributing 30 μl of cell suspension onto the slides, samples were counterstained with DAPI and analysed under fluorescence microscope. At least 2000 nuclei were examined per condition.

Data analysis

Data are expressed as mean ± standard error mean (SEM). Comparisons among doses were performed by one-way analysis of variance, with multiple comparisons versus control group being conducted by Newman–Keuls multiple comparison test.

Results

AZT induces arrest in G2/M phase

It has been described that AZT is capable of inducing S and G2/M arrest of HepG2, 3T3 and Hela cells (10–12). Flow cytometry was used to identify whether AZT also interferes with mES cell cycle dynamics. Before proceeding with experiments, MTT assay was performed with five different AZT concentrations to determine the doses to be used in this work. As show in Figure 1, mES cells viability was not significantly affected by AZT treatment (Figure 1A). So 50 and 100 μM AZT were chosen in attempt to be comparable with other groups (7,8,11,13). As see in Figure 1, all experiments were conducted in mES cells on logarithmic phase and the estimated population doubling time of cells is ~24 h. In addition, no significant alteration in the number of total cells could be observed after AZT treatment (Figure 1B).

Despite treatment with AZT has not changed mES cells viability, cells treated with AZT exhibited significant decrease in G0/G1 cells with consequent increase in G2/M phase cells.

The percentage of G0/G1 cells in control was 46.21 ± 3.45%, while AZT 50 and 100 μM treated cells presented a 2-fold decrease, exhibiting 23.04 ± 5.51 and 22.68 ± 5.28% of cells in this phase, respectively (Figure 1C). AZT caused accumulation of cells in G2/M phase in which the percentage of cells raised from 27.27 ± 5.43% in control to 47.63 ± 1.98% (50 μM AZT) and 45.96 ± 4.18% (100 μM AZT) (Figure 1E). No significant alteration in S phase was observed (Figure 1D).

AZT induces aneuploidy in mES cells

Checkpoints in G2/M phase block the progression into mitosis when DNA is damaged or when chromosomes are not attached or under tension with mitotic spindle. This surveillance mechanism avoids abnormal distribution of genetic material between daughter cells, ensuring correct chromosome segregation (14). To identify whether accumulation of cells in G2/M phase could be related to chromosome missegregation with consequent cell cycle arrest, aneuploidy rate was evaluated after AZT treatment.

According to our analysis, untreated mES cells (control group) showed 47.7% of cells with 41 chromosomes instead of 40 (16.4%), the diploid number for mouse. It has been reported that depending on culture conditions, cells can acquire genomic alterations, which seems to be a common feature among ES cells lineages (15–18). Moreover, ES cells present rapid cycles of cell division with short G1 phase and absence of G1 DNA-damage checkpoint (19), postulated to be a property of 'stemness'. Thus, 40 and 41 chromosomes were considered as the euploid modal number for this cell line. Numbers <40 chromosomes were considered as hypoploidy (loss of chromosomes) and >41 were considered hyperploidy (gain of chromosomes). Analysis of chromosomal complement in mES cells colonies showed that aneuploidy increased from 39.6 ± 8.4% in control to 62.5 ± 6.0% in 50 μM AZT and 67.8 ± 3.4% in 100 μM AZT (Figure 2A). Interestingly, hypoploidy was the prevalent kind of aneuploidy in cells treated with AZT. The percentage of hypoploid cells was 32.4 ± 7.8% in control, while with AZT, the proportion of chromosome loss raised to 53.9 ± 7.7% (50 μM AZT) and 61.0 ± 4.0% (100 μM AZT) (Figure 2B). No

difference in hyperploidy rate was observed after AZT treatment (data not shown). In order to identify whether non-pluripotent cells also become aneuploid after exposure to AZT, MEFs were treated with AZT under the same conditions. No difference in ploidy rate was observed (Figure 2C).

Aneuploidy induced by AZT is associated to micronuclei formation

Finally, we investigated whether micronuclei formation could be associated with AZT-induced aneuploidy in mES cells. These structures can be composed by chromosome fragments or whole chromosomes that are excluded from daughter cells nuclei during mitosis (20). After AZT treatment, mES cells containing micronuclei raised from 1.32 ± 0.17% in control to 2.01 ± 0.24% (50 μM AZT) and 2.66 ± 0.97% (100 μM AZT) (Figure 3).

Discussion

Zidovudine is the most effective drug for preventing vertical transmission of HIV. Being a nucleoside analogue, it permeates transplacental barrier and is rapidly incorporated into DNA of placenta tissues (21). Guidelines recommend the onset of treatment with at last two trimesters of gestation. Even so, micronuclei increased incidence and mitochondrial DNA depletion have been reported in children born from AZT-treated mothers (22,23). Besides, rodent progeny exposed intra-utero to AZT, during gestation, presented high tumour incidence after 1 year of age (24). Zidovudine has been described to induce S and G2/M arrest and aneuploidy in cultured cells including CHO, HeLa and human hepatocytes; however, the consequences of AZT on ES cells have never been explored. In fact, all these cell types previously used to study AZT effects represent adult cells, in which no correlation to embryogenesis can be performed. By using ES cells, it is possible to elucidate what happens during early embryogenesis.

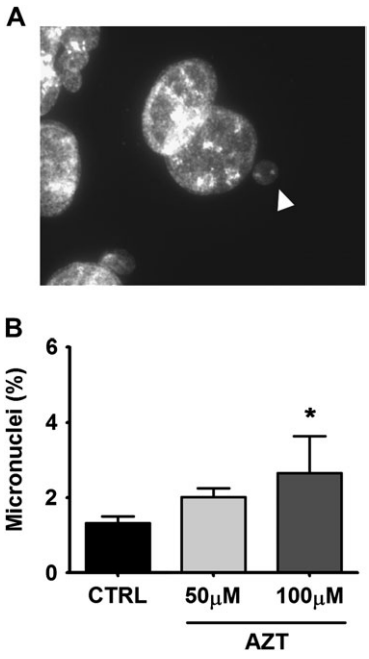

Fig. 3. Aneuploidy induced by AZT is related to micronuclei formation. Micronucleus is represented by arrow head (A). The percentage of micronuclei in ES cells raised from 1.32 ± 0.17% in control to 2.01 ± 0.24% in 50 μM AZT and 2.66 ± 0.97% in 100 μM AZT group (B). Bars indicate mean ± SEM from three independent assays. Asterisk means significant difference ($P < 0.1$) compared to control group.

Considering ES cells as a reliable model for studying nucleoside analogues effects during embryogenesis *in vitro*, we investigated consequences of AZT treatment in mES cells. We selected 50 and 100 μM of AZT doses to be in comparison with other works and also to mimic *in vivo* murine system (8). Here, we demonstrated that AZT induces cell cycle arrest followed by increased aneuploidy and micronuclei formation in mES cells.

It has been previously reported that AZT leads to reduced viability and induces cell S and G2/M arrest in hepatocytes and Hela cells (11,12). In accordance, we observed accumulation of cells in G2/M phase. Cells arrest in G2/M phase when DNA injuries are recognised and cell cycle is delayed until damage is corrected (25). Over time, doubling is prolonged and results in decreased number of viable cells. Also, when cells fail to fix damage, they activate apoptotic cell death pathways. It is interesting to note that even though significant alteration on viability and total number of cells was not detected during 4 days of AZT treatment, the extended accumulation of cells in G2/M may result in reduction of viability and consequent cell death. This event has been previously observed in stem-like cells derived from glioblastomas. After short periods of radiation, those cells were arrested in G2/M and delayed cell death (26). This phenomenon occurs as a consequence of mitotic catastrophe, characterised by aberrant mitosis associated with many events such as aneuploidy, micronucleation and also morphological and biochemical changes (27).

Our data also show that AZT causes a 2-fold increase of aneuploidy in mES cells as previously demonstrated in immortalised CHO cells (5). Once MEFs treated with AZT presented no alteration in ploidy rate, we speculate that aneuploidisation induced by AZT can be related to pluripotency and/or immortalisation. Moreover, while being a primary culture of somatic cells, MEF is expected to present robust checkpoint mechanisms and to have better response to DNA damage.

Aneuploidy is commonly associated to human diseases, spontaneous abortions and infertility (28–30). While AZT is carcinogenic in rodents, chromosomal alterations are a hallmark of many types of cancer (31). It has been reported that mice treated intra-utero with AZT, presented high incidence of lung neoplasms and liver tumours after 1 year of age (24,32). Accordingly with those data, we show that AZT is capable of promoting aneuploidy in ES cells without increasing cell death.

In order to identify a mechanism to explain the increase in aneuploidy induced by AZT, micronuclei formation was evaluated. We observed that micronuclei frequency doubled in mES cells exposed to AZT in comparison to untreated cells. These structures detach from major nuclei in most of cases because of incorrect segregation and contribute to aneuploidy generation. Many mechanisms can contribute to micronuclei formation since centrosome amplification to incorrect interactions between kinetochores and microtubules (33). As a consequence, incorrect divisions of genetic material for daughter cells can occur. In recent work, Borojerdi *et al.* demonstrated that AZT causes centrosome amplification accompanied by increased chromosome instability in CHO cells (5). Similarly, Dutra reported the occurrence of nuclear buds (considered as precursors of micronuclei) in AZT-treated cells even after 24 h (7).

We demonstrated that AZT is capable of inducing micronuclei formation associated to aneuploidy even on early stages

of development. Moreover, the prevalence of hypoploidy in mES-treated cells may result from micronuclei once these structures are compromised to chromosome elimination (34). A few studies have shown that mice and children exposed intra-utero to AZT have 10 times more micronuclei incidence compared to untreated ones (22,35).

These data emphasise the importance of studying the consequences of AZT during early stages of development, once micronuclei have been recently associated not only to tumorigenesis (36,37) but also to neurological disorders including Alzheimer and Parkinson disease in adulthood (38). Besides, people treated for HIV infection show signs of premature ageing and the event seems to be related to mitochondrial dysfunction (39). Another interesting point is that aneuploidy usually converges to senescence (40).

In conclusion, our results point to possible deleterious effects of AZT in mES cells and emphasise the importance of further studying its side effects during embryogenesis. Future studies are necessary to improve knowledge about risks and benefits of using AZT drug during pregnancy.

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