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Relevance of experimental models for investigation of genotoxicity induced by antiretroviral therapy during human pregnancy

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Abstract

The current incidence of HIV-1/AIDS affects around 7,000 pregnant women in the United States. When given during pregnancy, the nucleoside analog AZT significantly reduces maternal-fetal transmission. It has been previously shown that AZT is incorporated into DNA, where it causes mutations in the *HPRT* and *TK* genes. It also changes cell cycle gene expression, and induces S-phase arrest, micronuclei, chromosomal aberrations, sister chromatid exchanges, telomeric attrition, and other genotoxic effects in cultured cells. A predicted consequence of these events is genomic instability that together, with clastogenicity may contribute to the carcinogenic potency of AZT. Various aspects of genotoxicity are explored in this contribution seeking to understand the multiple effects of this antiretroviral agent in animal models and humans. This mini-review describes some of the experimental models used to elucidate the genotoxicity induced by antiretroviral therapy during human pregnancy. The use of diverse methods to detect biomarkers of exposure, such as an AZT-specific radioimmunoassay, micronuclei bearing intact chromosomes, and telomeric DNA attrition highlight the role of in-vitro models to elucidate exposure and risk. The relevance of the *in vitro* models is followed by the introduction of the role of the nucleoside analogs in transplacental carcinogenesis along with the description of a transplacental perfusion model and a transplacental carcinogenesis rodent model. In a more direct clinical application the use of AZT-DNA incorporation as a biomarker of exposure, in experiments conducted *in vivo* in *Erythrocebus patas* monkeys and in humans, addresses the possibility of elucidation of potential cancer risk in those infants exposed *in utero*.

Two relevant aspects of this contribution are the potential application of some of the models described in this mini-review, as diagnostic tools in antiretroviral-exposed populations, and the use of these models to understand the nature of the genotoxicities and minimize the undesirable side effects of the antiretroviral therapy.

Introduction

Patients infected with the human immunodeficiency virus (HIV-1) undergo extensive therapy with antiretroviral nucleoside analog drugs, among which zidovudine, (Retrovir®, 3’azido-3’-

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deoxythymidine, AZT) is used the most frequently and has become an essential component of the Highly Active Antiretroviral Therapy (HAART). The current incidence of HIV-1/AIDS affects around 7,000 pregnant women/year in the United States. When given during pregnancy, the nucleoside analog AZT significantly reduces maternal-fetal transmission. However, AZT is carcinogenic in rodents, producing 10%–22% incidences of vaginal squamous papillomas and carcinomas in adult mice [1,2]. Moreover, transplacental administration of AZT to mice results in significant increases of tumors, in liver, lung and reproductive organs of the offspring, one year after exposure [3]. It has been previously shown that AZT is incorporated into DNA, where it causes mutations in the *HPRT* and *TK* genes. It also changes cell cycle gene expression, and induces S-phase arrest, micronuclei, chromosomal aberrations, sister chromatid exchanges, telomeric attrition, and other genotoxic effects in cultured cells [4]. A predicted consequence of these events is genomic instability that, together with clastogenicity, may contribute to the carcinogenic potency of AZT. Various aspects of genotoxicity are explored in this contribution seeking to understand the multiple effects of the drug in animal models and humans.

AZT is selectively incorporated at the telomeric ends and causes telomeric shortening

Zidovudine, (Retrovir®, 3′-azido-2′,3′-dideoxythymidine; AZT), is a nucleoside reverse transcriptase inhibitor (NRTI) designed to inhibit viral replication in HIVinfected patients.

Inhibitors of the HIV-1 reverse transcriptase can also become incorporated into nuclear and mitochondrial DNA [3–6] and act as chain terminators [7] by lacking the 3′-OH of the deoxyribose sugar (Figure 1 A), then becoming unable to extend the nascent DNA chain by forming a 5′ to 3′-phosphodiester bond with the following nucleotide (Figure 1 B–C). Nucleoside reverse transcriptase inhibitors are implicated as chemical mutagens and transplacental carcinogens [3,4,8–10] and some consequences of DNA incorporation have been reported and categorized as gene mutations, chromosomal mutations, and telomere shortening [4].

In cultured cells, AZT becomes preferentially incorporated into the DNA of chromosomal ends or telomeres, causing irreversible telomeric shortening and the potential for premature senescence [3,11–13]. In experiments utilizing CHO cells exposed to 800 μM AZT for 24 hours were performed to obtain metaphase spreads that in turn were incubated with anti-AZT antibodies (Sigma, St Louis MO). A preferential localization of AZT within the telomeric region of the chromosomes was observed [14].

Furthermore, telomeric DNA attrition determined by Southern blot experiments using a telomeric probe was described in monkeys and mice exposed transplacentally to the drug [3], and in HeLa cells exposed long term to the drug [13] (Figure 2). *E.patas* monkeys were administered 40.0 mg/day AZT in food for the last half of the gestational period, and a 24 mg/day of a second nucleoside, 3TC, during the last 10 weeks of gestation. Fetuses were taken at term by C-section and analysis of the telomeric length assessed by southern blot and compared with untreated control monkeys was performed in multiple tissues after DNA extraction. Telomeric shortening was observed in brain cortex and cerebellum (Figure 2 A and B) and in heart and lung (data not shown) [15].

CD1 mice were administered AZT during the last week of a three week gestation. At the end of that period, the pups were sacrificed, the organs of the same litter pooled, DNA extracted and Southern blots performed with a specific biotinylated telomeric probe. Shortening of the telomeres was observed in lung; brain and liver of AZT exposed mice compared with untreated controls (Figure 2 B). Additionally a progressive shortening of telomeric DNA, was

demonstrated in HeLa cells exposed for 15 passages to AZT 800 μM (Figure 2 C). The shortening was not reversed after continuous growing for 25 passages in AZT free media [13].

Uncapped telomeres will confer a lack of stability to the chromosomes and allow for incorrect exchanges of chromosomal material among them in interphase. Once the mitosis occurs and the daughter cells separate during telophase, bridges of DNA could be seen as a consequence of the adhesiveness of the disrupted telomeres (Figure 3A).

Chromatin bridges and micronuclei are a consequence of telomeric attrition

Typically, the material involved in the formation of chromatin bridges will remain lost in the cytoplasm of any or both of the daughter cells as a small mass known as a micronucleus (Figure 3B). Micronuclei differ in size and most of the time a large micronucleus bears intact chromosomes inside as demonstrated by the use of kinetochore specific antibodies able to detect centromeric regions of the chromosome.

Kinetochore-specific antibodies isolated from patients with the CREST (calcinosis, Raynaud phenomenon, esophageal dysmotility, sclerodactyly, and telangiectasia) syndrome, [16] were used to incubate normal human mammary epithelial cells (NHMEC) after exposure to AZT. The mentioned cells, obtained from mammoplasty tissue were exposed to 200 μM AZT for 24 hr. After harvesting and fixation, cells were incubated with the CREST antibody and stained with Alexa 488 and DAPI. Scoring of micronuclei bearing chromosomes, identified by a CREST positive signal micronuclei, as well as those negative for the CREST antibody (Figure 3 C–D) was achieved in 500 micronucleated cells. An increment in micronuclei bearing intact chromosomes was seen in human mammary, AZT-treated cells.

AZT is a transplacental carcinogen in mice

The genomic instability described plus reports of clastogenicity and mutagenicity induced by the agent, generated interest to address the human health impact of the use of the drug in human pregnancy. A mouse transplacental model was used to address the potential of the drug to cause cancer in offspring exposed for 1 week *in utero* and allowed to grow to adulthood without further exposure. Enhanced tumorigenicity was observed in multiple organs of mice exposed transplacentally to AZT [3,17,18]. This enhanced tumorigenicity was evidenced not only by increased tumor incidences, but also by shortened tumor latency, as AZT-induced neoplasms were found in CD-1 mouse offspring at one year of age [3] in the reproductive female system, lungs for male and female and lungs of the male (Figure 4 A). Diwan *et al.*, [18] reported results from a two-year study in which they indicate finding reproductive organ tumors in mice transplacentally-exposed to AZT. Typically the strain of mice used in the study, CD-1 Swiss mice, has a very low if not absent tumor incidence in the reproductive system. Exposure to AZT, on the other hand, increased the incidence of mammary adenocarcinomas, ovarian, seminal vesicle and testicular tumors.

Investigators performing transplacental bioassays also sought to establish molecular biomarkers of carcinogenesis by analysis of the incorporation of AZT into the DNA of multiple tissues of the offspring (Figure 4 B). Incorporation of AZT into nuclear and mitochondrial DNA of multiple organs pooled from each litter was determined by an AZT-specific radioimmunoassay (RIA) and values of incorporation reported in that study ranged from undetectable to up to 100 molecules of AZT/10^6 nucleotides [3]. The transplacental carcinogenicity of AZT was subsequently confirmed in rats and another strain of mice [19]

AZT incorporates into DNA of human placentas and infant blood DNA

In an attempt to determine if the genotoxicity of AZT and other nucleoside analogs would compromise human health, experiments using placental perfusion were achieved. The model encompassed the perfusion of fresh human placentas *ex vivo* with three AZT doses on the maternal side of the organ, with perfusates recovered on the fetal side at 15 min intervals for a period of 2 hours. At that time, the maternal and fetal concentrations reached equivalent levels [20] and incorporation of AZT into DNA of the placenta was measured by AZT-RIA. A dose response was observed in the incorporation of AZT into placental DNA from tissues obtained 120 min after the initial perfusion [20] (Figure 5).

The observations in monkeys prompted a parallel study in humans to investigate the human health impact of the use of nucleoside analogs during pregnancy. For this purpose, mother-infant pairs exposed to different regimens of highly active antiretroviral therapy (HAART) were studied. Peripheral blood leukocytes from the mother and umbilical cord blood leukocytes from the fetus were isolated and DNA extracted. AZT-RIA was performed on DNA extracted from the samples obtained at birth and the results are presented in Figure 6. Each point represents the average of three separate RIAs and the amount of AZT –DNA is expressed as molecules of AZT/10^6 nucleotides. There is a lack of correlation between the time of exposure, indicated in months on the abscissa, and the amount of AZT incorporated into the DNA among these samples. Additionally, no correlation between AZT-DNA incorporation in the mother and the infant was observed. Conversely, the data suggested an inverse correlation between the amount of AZT into DNA in the mother versus the infant. One more observation seen from these data is that the high incorporation of two infants with 0.3 months of exposure at birth is not matched by any longer period of exposure. On the light of new biochemical data collected in our laboratory, it is possible to speculate that infants exposed for long time to the analog developed a resistance mechanism that is not observed in those infants exposed for a short time. *In vitro* studies in lymphoblastoid cells indicated that the expression of the enzyme TK1, responsible for phosphorylation of AZT, is decreased at earlier passages of chronic treatments to be completely abolished later on [21]. Radioisotopic labeling of the substrate of the enzyme revealed that TK1 enzymatic activity is decreased as well (unpublished data). Patients exposed for long term also present a decrease on the TK 1 enzyme activity [22,23]. Additionally, lack of expression of the active form of the enzyme has been seen in human mammary epithelial cells that do not incorporate AZT into the DNA (Olivero et al., TAAP, in press).

Combined Therapy

While perinatal AZT monotherapy decreased HIV transmission rates from 25% to 8% [24, 25], administration of combination therapy using multiple agents, known as HAART, further reduced the rate of vertical transmission to ≤2% [26]. However the increased genotoxicity associated with the combination regimens remains unclear.

The highly active antiretroviral therapy (HAART) typically includes two nucleoside reverse transcriptase inhibitors combined with either a non-nucleoside reverse transcriptase inhibitor or a protease inhibitor. HAART is recommended for HIV-1 positive mothers, to decrease vertical transmission [27]. Although there is no clear knowledge of the direct or indirect consequences of exposure to this complex regimen in the human fetus, studies in cell culture and animal models suggest increased genotoxicity is induced by the combination. *In vitro* studies revealed a synergistic effect on the frequency of *HPRT* and *TK* mutants induced by the AZT-ddI combination as compared with single drug exposures [28]. Similarly, an additive effect was observed in the incorporation of AZT into DNA of *E. patas* monkeys exposed *in utero* to AZT and 3TC during the last period of their gestation [15]. The authors indicated that the DNA damage sustained by the fetuses exposed to both drugs is double than the ones exposed

to a single compound. In that study, where monkeys were administered AZT and 3TC, incorporation of AZT into DNA of multiple tissues was achieved by a RIA, additionally, incorporation of 3TC into DNA was determined by a specific RIA. Incorporation of both nucleoside analogs was observed in the tissues, indicating a greater genotoxic potential.

With the benefits of HAART, many newborns are exposed *in utero* to multiple drugs and the use of monotherapy is rare in industrialized nations. An observation of the incorporation of AZT and 3TC into DNA of infants exposed *in utero* suggested that those infants exposed to combination therapy sustain more damage into their DNA as well [10]. Similarly to the studies *in vitro*, an enhancement in the frequency of mutations in the *HPRT* and *GPA* genes was observed in these cohorts. A preliminary study of multiplicative effects of combination therapy demonstrated an increased in the mutagenic frequency of the *HPRT* gene only in those children exposed to combination therapy when compared to AZT alone.

A third component of the HAART, protease inhibitors, have been studied as potential anticancer drugs. According to a recent report, Akt inhibition by nelfinavir, among other protease inhibitors, was the mechanism responsible of growth inhibition of non-small cell lung carcinoma xenografts and decreased viability of a panel of drug-resistant breast cancer cell lines by induction of endoplasmic reticulum stress, autophagy, and apoptosis [29].

Summary

In this mini-review a brief description of the models followed to assess genotoxicity of nucleoside analogs used in the therapy of AIDS was discussed.

The *in vitro* models have shown to be very useful tools to indicate the direction of the development of human biomarkers of exposure and, potentially, biomarkers of risk. Studies in rodents have been very revealing indicating the potential carcinogenic capacity of the agents under study. Furthermore studies in monkeys, with very similar dosage protocols than the human therapeutic regimens, gave indication of a genotoxic synergism when combination therapy was applied. Finally the possibility of elucidation of potential cancer risk in those infants exposed *in utero* is illustrated by the use of AZT-DNA incorporation as a biomarker of exposure.

Conclusion

The understanding of the genotoxicity of the drugs used to treat HIV-1 disease is paramount in the future of the AIDS therapy. The balance between the risks and the benefits provided by the drugs should be always taken into account. However, the understanding of the mechanisms by which drugs exert their detrimental effect should be studied in depth and complete knowledge of their pathways at the cellular and sub-cellular level should be characterized in order to improve therapy and drug design.

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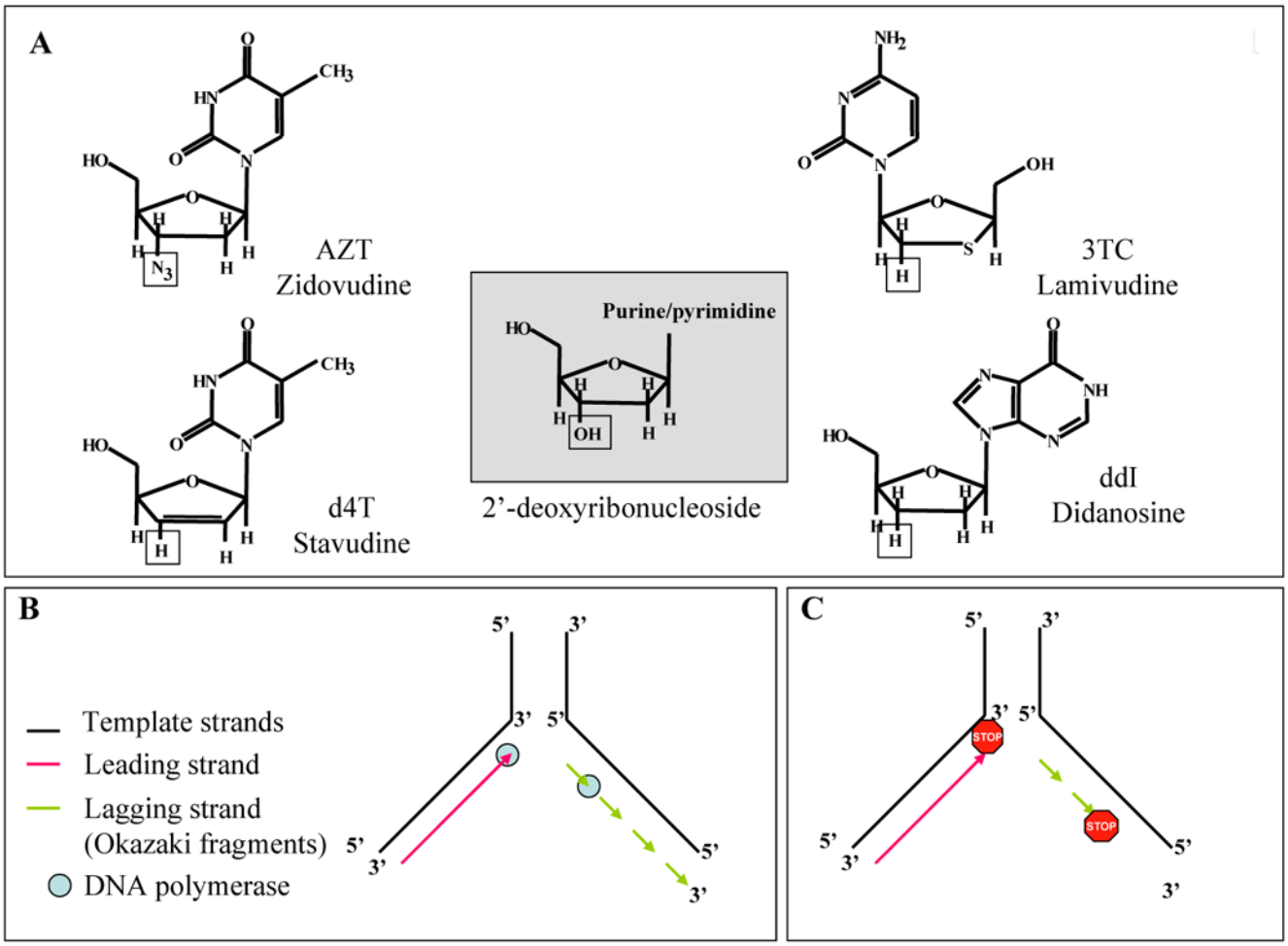

Figure 1.

A. Structures of the commonly used nucleoside analogs. The hydroxyl group at the 3' position in the 2'-deoxyribonucleoside (center box, square) is absent in the nucleoside analogs. AZT has an azido group; 3TC and ddI have hydrogen; and D4T has an unsaturated 2',3'-dideoxyribose. B. DNA replication on a normal template. C. Mechanism of inhibition of DNA replication by insertion of a nucleoside analog that lack the 3' hydroxyl, cannot form the phosphodiester link, and therefore causes chain termination. "STOP" indicates incorporated nucleoside analog.

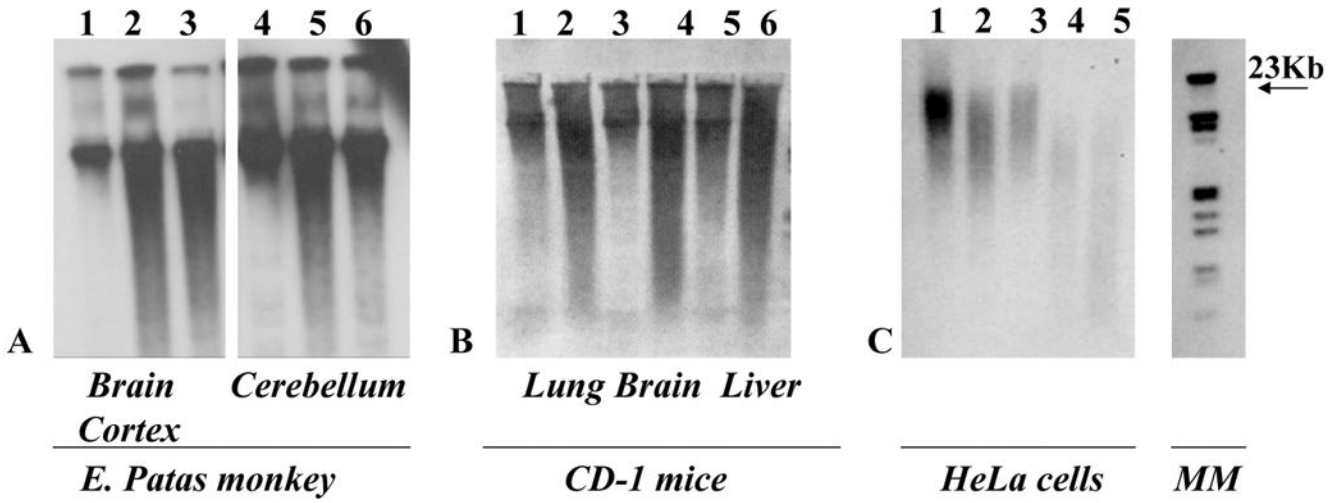

Figure 2.

Southern blot images of A. *Erythrocebus patas* monkeys brain cortex and cerebellum DNA blotted with a telomeric probe. The 1st and 4th lanes correspond to an untreated monkey while the 2nd, 3rd, 5th and 6th lanes correspond to two different newborn monkeys, exposed to AZT AND 3TC *in utero*. Lanes 1 to 3: Brain Cortex, lanes 4 to 6 Cerebellum B. lanes 1, 3 and 5 lung, brain and liver DNA of control mice; lanes 2, 4, and 6 lung, brain and liver DNA of mice exposed to AZT *in utero*. C. Control DNA of He la cells (lanes 1 to 3), long term AZT exposed DNA (lanes 4 and 5). DNA size estimated using the molecular marker (MM) on the right. Shortened telomeres are evidenced by the larger smear indicating smaller fragments of DNA. Modified from [30] and [13]

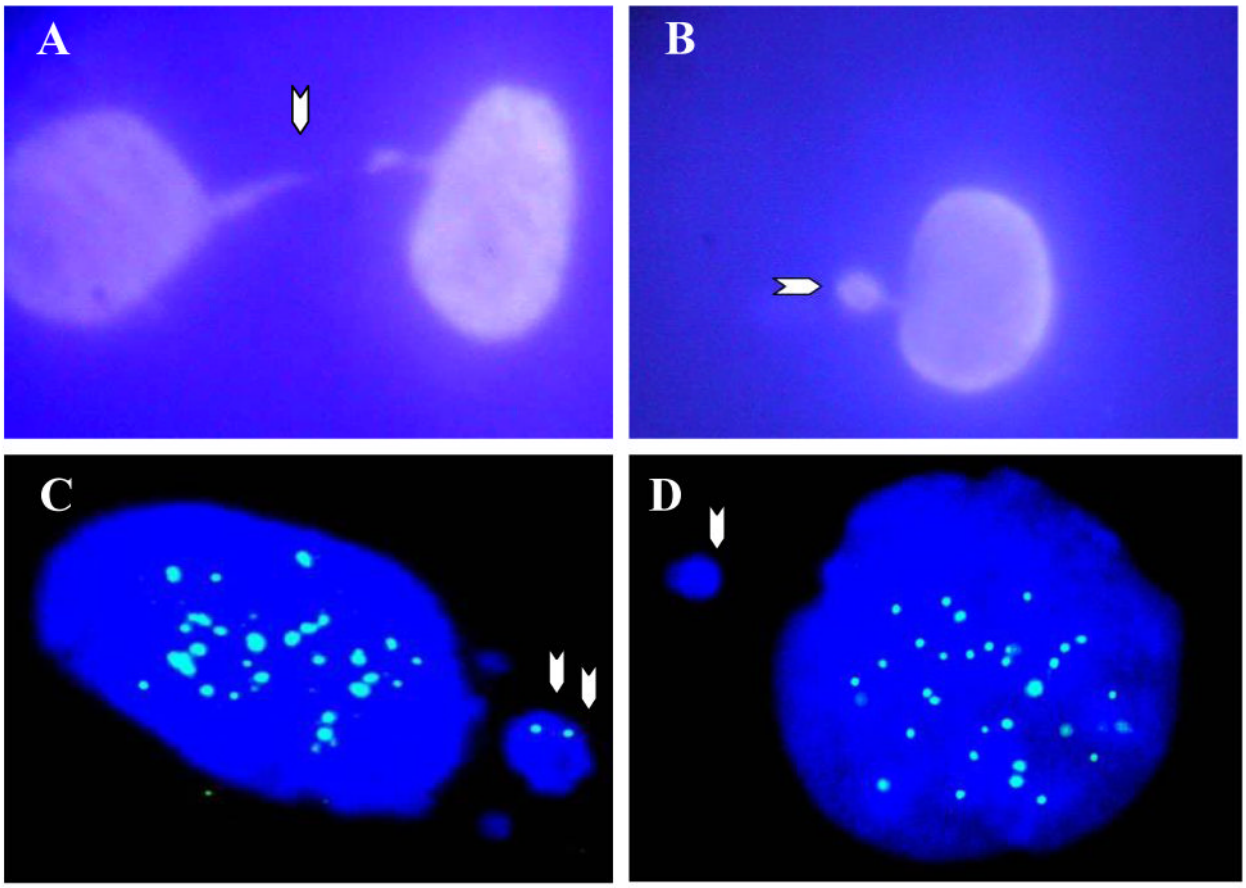

Figure 3.
DAPI staining of A. Chromatin bridge (arrow) in AZT-exposed He La cell telophase, B. micronucleus (arrow) in an AZT treated He La cell. C.CREST-positive micronucleus (blue spots in both nucleus and micronucleus, arrows) and D. CREST-negative micronucleus with positive CREST signals in the nucleus of CHO cells exposed to AZT. Adapted from [31], unpublished data.

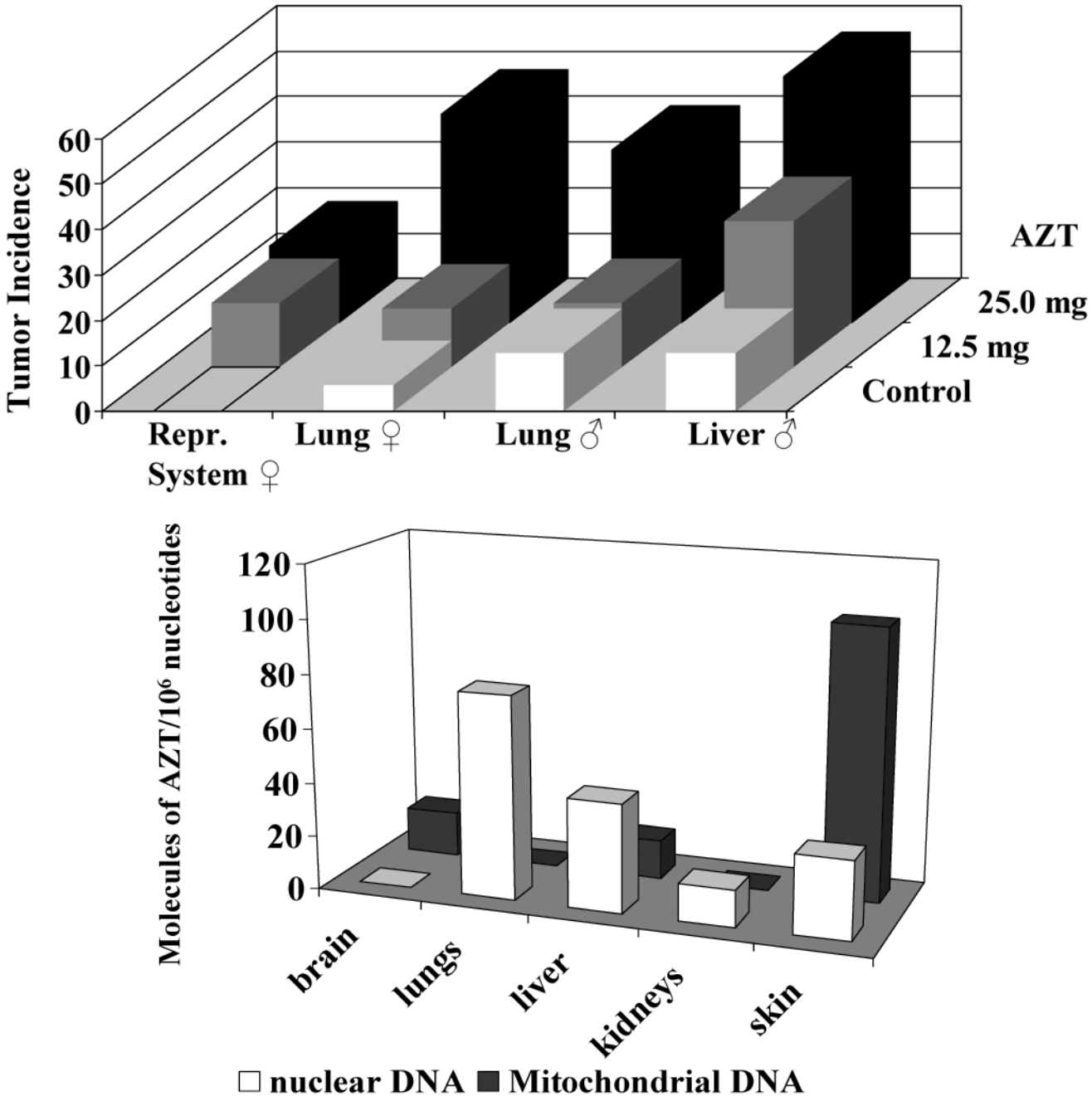

Figure 4.

A. Tumor incidence induced by 12.5 mg/day (gray bars) and 25.0 mg/day (black bars) AZT in the reproductive system, lungs and liver of CD1 mice exposed *in utero* to AZT for one week. Adapted and modified from [3]. B. Molecules of AZT/10^6 nucleotides, in nuclear (white bars) and mitochondrial (dark bars) DNA from brain, lungs, liver kidneys and skin of mice exposed to 25.0 mg/day AZT during the last week of gestation, and sacrificed at birth.

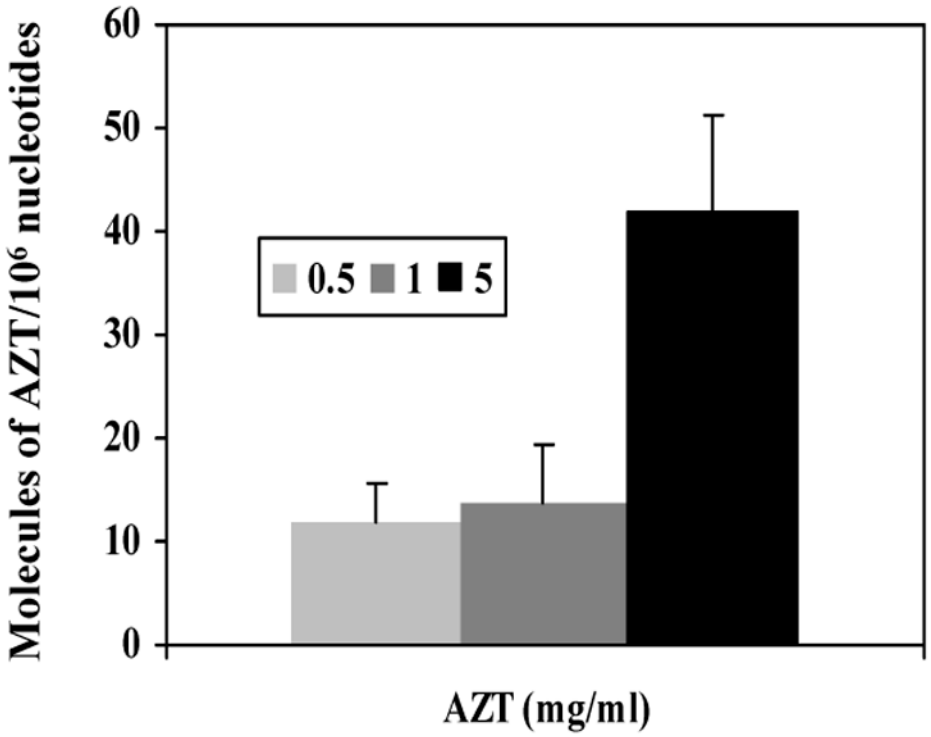

Figure 5.
AZT-DNA incorporation in human placental tissues of *ex vivo* AZT perfused placenta, 120 min after perfusion. Light bar represents 0.5 mg/ml AZT, dark gray corresponds to the 1 mg/ml AZT dose and black bar represents the 5 mg AZT dose. Adapted and modified from [20]

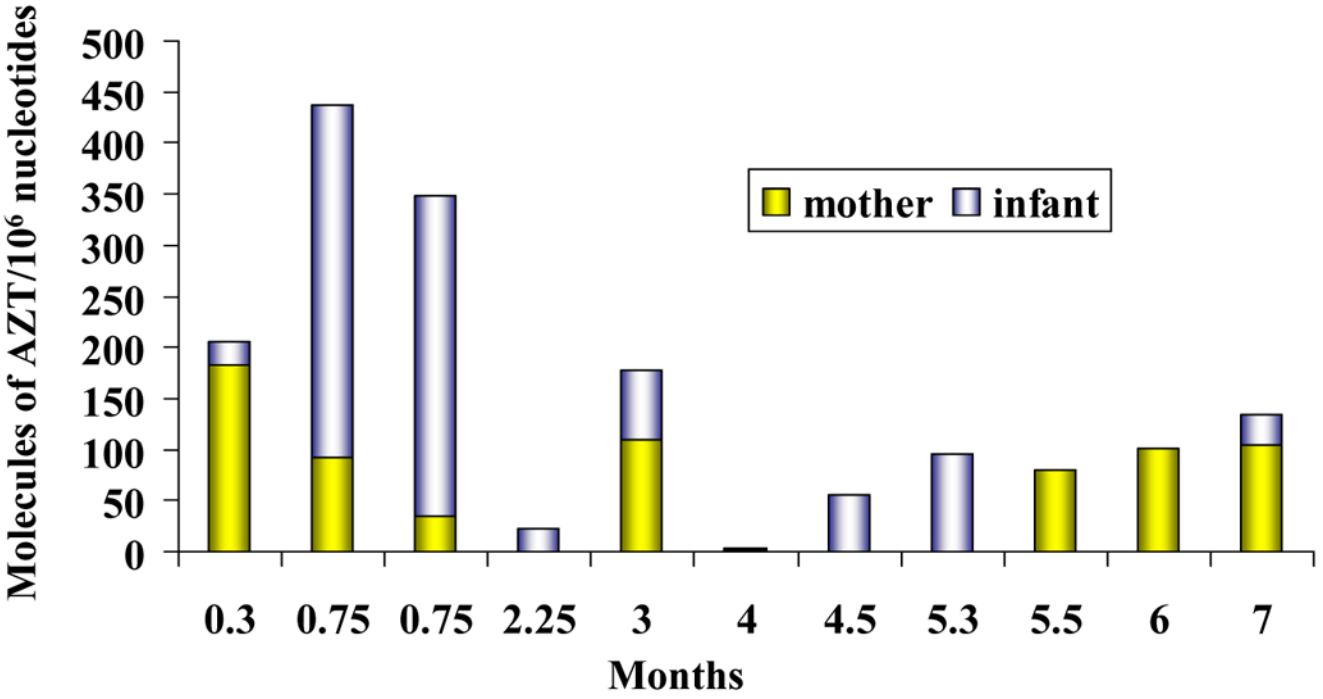

Figure 6.
AZT-DNA incorporation in mother- (yellow columns) infant (gray columns) pairs. Each bar represents the amount of AZT into DNA of peripheral blood (maternal DNA) or cord blood (infant DNA) expressed in molecules of AZT/10^6 nucleotides. The length of exposure from 0.3 to 7 months is represented in the abscissa. Adapted and modified from [5]