The *in vivo* and *in vitro* Developmental Toxicity and Teratogenicity of the Anti-AIDS Drugs (Human and Experimental Animal Studies)

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**ABSTRACT**

This study gives an overview about the modes of human immunodeficiency virus transmission, women and AIDS and the *in vivo* and *in vitro* developmental toxicity and teratogenicity studies of the anti-AIDS drugs (antiretrovirals), in humans and experimental animals, with especial highlights dedicated to zalcitabine effects on mice fetuses. In earlier human studies, management of AIDS positive pregnant women with antiretrovirals revealed exposure of their infants to such drugs with evidence of adverse events. However, recent publications present conflicting data about associations between antiretrovirals and adverse pregnancy outcomes. Animal embryos exposed *in vivo* to antiretrovirals exhibited significantly increased pregnancy losses, drugs incorporation into the DNA of fetal organs, external abnormalities, skeletal defects, developmental toxicity, carcinogenicity, reduced weight, anemia, deaths and significant mitochondrial damage. The *in vitro* antiretrovirals exposure of animal cells or organs resulted in cytotoxicity, growth retardation, chromosomal aberrations, mutations, sister chromatid exchange and other genotoxic effects. Zalcitabine orally (600 or 800 or 1000 mg kg⁻¹) applied to pregnant mice for five consecutive days, from day 9-13 of gestation evoked significant elevation in the number of resorption sites and dead fetuses, haematomas formation, significant reduction of body weight and length, mild to severe limb abnormalities, histopathological changes in fetal ovaries, hearts, spinal cords, brains and eyes and ultrastructural changes in ovaries. The *in vitro* exposure of mouse fibroblasts to zalcitabine revealed significant increase of abnormal metaphases and chromosomal abnormalities per metaphase. The study concluded that *in utero or in vitro* exposures to retrovirals revealed adverse outcomes in human and experimental animals.

**Key words:** Embryos and infants, women, zidovudine, zalcitabine, adverse outcomes

**INTRODUCTION**

Many types of retroviruses are known today. Undoubtedly, the best-known one is the Human Immunodeficiency Virus (HIV), the virus that causes the acquired immunodeficiency syndrome (AIDS). Unlike all other retroviruses, AIDS virus is thought to be a direct pathogen that preferentially infects and destroys OKT4 = (helper inducer) T-lymphocytes and possibly cells of the brain. The AIDS in humans is caused by two lentiviruses, HIV-1 and HIV-2, which entered the human population by transmissions from at least two different African primate species. Among infectious disease causing agents, HIV-1 is now the number one killer worldwide. Antiretroviral therapies (ARTs) have become widely prescribed in pregnancy in the absence of proof of their safety. They are widely prescribed as monotherapy and combination therapy to reduce the risk of mother-to-child HIV transmission, to treat HIV-positive pregnant women, to diminish morbidity and mortality and to reach to a point at which the benefits of therapy for both the mother and the
infant outweigh the risk. Recent publications present conflicting data about associations between ARTs and adverse pregnancy outcomes, since ART is necessary for all pregnant women. These data suggest that provision of prenatal care and ART may reduce adverse pregnancy outcomes. Given the risk-benefit ratio, these highly successful drugs will continue to be used for prevention of vertical viral transmission, however evidence of carcinogenicity and genotoxicity in mouse and monkey models and in the infants themselves would suggest that exposed children should be followed well past adolescence for early detection of potential cancer hazard. In the animal models tested, a few risks of malformations are demonstrated for some ARTs whereas others are associated with carcinogenicity, genotoxicity, chromosomal aberrations, malformations or/and developmental toxicity in rats, mice, rabbits and monkeys. Therefore, this review will first provide general description of the modes of HIV transmission and women and HIV infection. Then the impacts of the management of HIV infection in pregnant women is reported with regard to diseases and congenital abnormalities in infants exposed to ARTs in utero or during delivery. The in vivo and in vitro developmental toxicity and teratogenicity of ARTs in experimental animals were also reported with especial emphasis devoted to the developmental toxicity and teratogenicity of zalcitabine (2′,3′-dideoxycytidine, ddC).

**Modes of HIV transmission:** Bodily secretions spread HIV. Sexual intercourse, contaminated needles or blood transfusions and placental transfer from mother to fetus are the most common modes of HIV transmission (Wong-Staal and Gallo, 1985; Suligoi et al., 2010). Therefore, the virus is not naturally transmitted as a cell-free agent like other pathogenic viruses, but only congenitally, sexually, or by blood transfusion, that is, by contacts which involve exchange of infected cells (Levy et al., 1985). Transmission of HIV to infants can occur via vertical transmission through the placenta (Douglas et al., 1991; Back et al., 1992; Mattern et al., 1992; Ibboudo et al., 2009). Intrapartum transmission during delivery and postnatal transmission through breast-feeding may provide other modes of HIV infection in infants (Ziegler et al., 1985; Goedert et al., 1991; Rouzioux et al., 1995). In 1993, heterosexual contact and exceeded drug injection were the predominant mode of HIV transmission for women with AIDS (CDCP, 1993; Cohn, 2003). Heterosexual spread in the general population is the main mode of transmission in Sub-Saharan Africa, which remains the most heavily affected region, with 67% of the global burden with 35% in eight countries alone (WHO, 2008, 2009; Kilmarx, 2009). In 2007, the global prevalence of HIV-1 has stabilized at 0.8%, with 33 million people living with HIV/AIDS, 2.7 million new infections and 2.0 million AIDS deaths. Since 2001, the number of people with HIV in Eastern Europe and Central Asia increased from 650,000 to 1.5 million, heterosexual and homosexual transmission accounts for the largest proportion in these regions (Dorrucci, 2010). The HIV is the strongest risk factor for tuberculosis (TB) and in 2008, there were about 1.4 million HIV-positive TB cases, representing 15% of global TB incidence. In 2007, there were about 1.4 million HIV-positive tuberculosis cases (WHO, 2009; Granich et al., 2010).

**Women and HIV infection:** Among women of childbearing age, the rate of HIV infection is continued to increase worldwide. In the USA, of reported AIDS cases in adults, women accounted for 7% in 1985, 13% in 1993 and 23% in 1999 (CDCP, 1999). Young women are at particular risk; 41% of young adults 15 to 24 years old reported in 1999 as having AIDS were females. Davis et al. (1995) reported that approximately 6530 HIV-infected women gave birth in the USA in 1993 and an estimated 1630 of their infants were HIV infected. In addition, approximately
14,920 HIV-infected infants were born in the USA between 1988 and 1993. In 2000, an estimated 16.4 million women worldwide are living with HIV infection; 600,000 children are infected annually, most of them by mother-to-child transmission (Brown et al., 2000). In December 2002, an estimated 19.2 million women worldwide are living with HIV infection (Campsmith, 2002) most are of childbearing age. It was also estimated that 2.5 million HIV-positive women deliver infants each year, while in some countries more than 25% of pregnant women are HIV infected (Birnun et al., 2002). Among young adults 15 to 24 years old in whom AIDS was diagnosed in 1999, only 11% of males acquired HIV infection via heterosexual contact, compared with 69% of females. Women are biologically more vulnerable than men to HIV infection. Studies of CDCP (1993) reported that male to female transmission appeared to be 2-4 times more efficient than female to male transmission, in part because semen contains a far higher concentration of HIV than vaginal fluid. Women are also more likely to be recipients of blood transfusions because of haemorrhage and anemia during pregnancy and childbirth. However, young girls are particularly susceptible. Their immature cervixes and low vaginal mucus production present less of a barrier to HIV (Turmen, 2003). Because of improvements in therapy, many HIV-infected individuals are living for prolonged periods without developing clinical AIDS. In many countries of South Africa about 33% of the adult population are HIV infected and the prevalence of HIV infection in pregnant women reached 40% in some places (O’Hara et al., 2003). By the end of 2007, childbirth is an important means of spreading HIV in Sub-Saharan Africa, where 60% of all HIV patients are women (Myer et al., 2010).

Antiretroviral Therapies (ARTs): There are three classes of antiretroviral drugs (De Clercq, 2009):

- **Nucleoside Analog Reverse Transcriptase Inhibitors (NARTIs):** There are currently six NARTIs that are licensed for HIV treatment: (1) 3'-azido-2',3'-dideoxythymidine (zidovudine, AZT), (2) 2',3'-dideoxyctydine (zalcitabine, ddC), (3) 2',3'-dideoxyinosine (didanosine, ddl), (4) [(-)-b-L-2', 3'-dideoxy-3'-thiacydine] (lamivudine, 3TC), (5) 2',3'-didehydro-3'-deoxythymidine (stavudine D4T) and (6) Abacavir (Ziagen, ABC)

- **Non-Nucleoside Analog Reverse Transcriptase Inhibitors (NNARTIs):** There are three compounds that are licensed for HIV treatment: (1) Nevirapine (Viramune, NVP), (2) Efavirenz (Sustiva, EVR) and (3) Delavirdine (Recriptor, DVD)

- **Protease Inhibitors (PIs):** There are six PIs that are licensed for HIV treatment: (1) Indinavir (Crixivan, IDV), (2) Ritonavir (Norvir, RTV), (3) Nelfinavir (Viracept, NFV), (4) Lopinavir (LPV), (5) Saquinavir (Invirase, SQV) and (6) Amprenavir (Agenerase, APV)

The in vivo and in vitro developmental toxicity and teratogenicity of ARTs: In spite of the unquestionable benefits deriving from ARTs in preventing perinatal transmission of HIV, an international agreement exists on the need to continuously reevaluate the risk benefit ratio of human exposure to ARTs during embryonic development. The effectiveness of ARTs to prevent maternal-infant transmission of HIV-1 was first suggested by Connor et al. (1994).

HUMAN STUDIES

The first and the only drug that was extensively examined to reduce the risk of perinatal HIV transmission is AZT when administered according to a regimen developed by CDCP (1998). This
regimen was shown to reduce the risk for perinatal transmission by 66% in a randomized clinical trial (Sperling et al., 1996). The next two drugs developed were ddI and ddc. If combinations of ARTs are administered to the pregnant woman for treatment of her HIV infection, AZT should be included to reduce the risk for perinatal transmission. Toxic effects of chronic treatment with AZT and other NARTs were well documented both in humans and in laboratory animals. Highly active antiretroviral therapy (HAART) refers to a broad category of treatment regimens usually comprised of three or more ARTs that were expected to reduce plasma viral levels below the limits of detection (Shafer and Vuitton, 1999; Minkoff et al., 2001). Initial human studies showed that maternal use of ddc, AZT and ddI had resulted in fetal exposure to these drugs where they rapidly crossed the placenta by simple diffusion (Sandberg and Slikker, 1995). There were adverse events pre-term deliveries (PTD), biliary malformation and intracerebral haemorrhage in a small sample of 37 HIV-infected pregnant women who had received HAART and care in Switzerland (Loreni et al., 1998). Minimal hepatic and hematological abnormalities associated with short-term neonatal ARTs among African children were reported (Taha et al., 2002). Protease inhibitors were associated with the development of glucose intolerance and even diabetes mellitus. Hyperglycemia in pregnancy leads to increased risk of macrosomia, fetal distress, preeclampsia and stillbirth (Ndoye et al., 1997). Administration of any of the PIs was associated with new onset diabetes mellitus, hyperglycemia, or exacerbation of existing diabetes mellitus in HIV-infected patients (FDA Public Health Advisory, 1997). The PIs usage was associated with an increased risk of PTD (Loreni et al., 1998; Shapiro et al., 2000), increased rates of PTD and/or Low Birth Weight (LBW) were reported in women on combination NARTs with PIs (The European Collaborative Study and the Swiss Mother and Child HIV Cohort Study, 2000; Lambert et al., 2000; Kowalska et al., 2003). Elevated lactic acid levels were also found in asymptomatic ARTs exposed infants (Giaquinto et al., 2000; Alimenti et al., 2003). The use of a PI as well as EVR in a combination therapy increased risk for coronary artery disease due to elevation in the levels of triglyceride and total cholesterol (Ngondi et al., 2007). However, the changes in the atherogenicity indices showed that the regimen with NVP seems to have less risk of coronary heart disease compared to EFV (Ngoluma et al., 2010). Only LDL cholesterol was significantly higher in the cord blood of PI-exposed infants versus those not exposed to PIs in utero (Melvin et al., 2008). Among a population of HIV-1-infected women in Latin America and the Caribbean, maternal receipt of PI-containing ART regimens during pregnancy was not associated with a statistically significant increase in risk of LBW or PTD (Szyld et al., 2006) and the proportion of infants who had LBW or were born preterm declined during an era of increased maternal ART (Schulte et al., 2007). However, Cotter et al. (2006) proposed that compared with monotherapy and combination therapy without a PI, only combination therapy with a PI was associated with an increased risk of PTD. Also, Townsend et al. (2007) demonstrated an increased risk of prematurity associated with HAART and a possible association with other perinatal outcomes, including stillbirth and LBW. Use of antenatal PI-based HAART initiated before or during pregnancy was associated with a significantly increased risk of PTD at <36 weeks' gestation (Grosch-Weerner et al., 2008; Ivanovic et al., 2009; Martin and Taylor, 2009).

Although, animal studies suggest a possibility of congenital abnormalities with specific antiretrovirals, such as EVR, results from registries and cohort studies do not support an excess of congenital malformations associated with in utero ARTs exposure (Thorne and Newell, 2005). There was an association between maternal HIV-positive status and an increased risk of necrotizing enterocolitis in premature infants; abnormal umbilical artery velocity and abnormal fetal heart rate.
were significantly more frequent in fetuses that subsequently developed necrotizing enterocolitis (Desfrere et al., 2005). The ART exposure in fetal and early life had significant reducing effect on the total lymphocytes and CD8 cell counts that were prolonged until at least 8 years after birth (Bundred et al., 2005). Three deaths and additional cases of lactic acidosis and hepatic failure were described among pregnant women who began receiving D4T and ddi along with other drugs before pregnancy (Koch et al., 2003). Maternal exposures to EVR during any of the three trimesters resulted in fetal malformations and neural tube defects in children were reported (Antiretroviral Pregnancy Registry Steering Committee, 2002; Fundaro et al., 2002; De Santis et al., 2002; Saitoh et al., 2005; Jeantils et al., 2006; Bussmann et al., 2007; Watts, 2007). Exposure to both ART and folate antagonists during the first trimester was associated with an increased risk of congenital abnormalities. However, no malformations were observed in the children exposed to either ART or folate antagonists alone during the first trimester (Jungmann et al., 2001). Twins with cardiomyopathy and complete heart block were born to an HIV-infected mother treated with HAART (Lopriore et al., 2007). Conversely, there was no statistically significant increase in the prevalence of congenital abnormalities and exposure to ARTs in infants born to HIV-infected women (Townsend et al., 2006, 2009).

Symptomatic neonatal anaemia and hematologic abnormalities were increasingly reported in infants exposed to ARTs and this might worry where there is exposure to combination therapy (Silverman et al., 1998). During ART sick infants were reported with severe lactic acidosis, multi-system failure and anaemia (Scalfaro et al., 1998; Foster et al., 2001). An association between the use of combination ARTs during pregnancy and reduced neonatal hemoglobin levels was also observed, supporting the need for short- and long-term follow-up of infants exposed to ARTs during uterine life (El-Beitune and Duarte, 2006). Hematologic abnormalities and abnormal liver function were increasingly reported in infants exposed to ARTs (Myers et al., 2005; Mussi-Pinhata et al., 2007; Feiterna-Sperling et al., 2007). Conversely, no association was observed between the use of ARTs during pregnancy and adverse effects on neonatal amylase and hepatic parameters at birth (El-Beitune et al., 2007). Children who were exposed in utero to any ART did not have lower mental developmental index and psychomotor developmental index scores than unexposed children (Williams et al., 2010).

There was equivocal evidence of in utero NRTI exposure and the occurrence of Mitochondrial Dysfunction (MD) in HIV-uninfected children born of HIV-infected women (Brogly et al., 2007; Ciaranello et al., 2008; Foster and Lyall, 2008; Aldrovandi et al., 2009). The transplacental NRTI exposures induce significant similar mitochondrial DNA (mtDNA) depletion in umbilical cord endothelial cells taken from retroviral-uninfected monkey infants and from human infants born to HIV-1-infected women (Divi et al., 2007a). After 1 year, the mtDNA levels in monkeys skeletal muscles had increased but remained significantly below normal (Divi et al., 2007b). Witt et al. (2007) demonstrated that transplacental AZT exposure was genotoxic in humans and long-term monitoring of HIV-uninfected AZT-exposed infants was recommended to ensure their continued health. Zidovudine, ddi and ddC all had exerted a dose-related decrease of cell proliferation and differentiation, cytotoxic effects, metabolic disruption, increased cell mortality and mtDNA depletion on human cells in vitro (Brinkman et al., 1999; Benbrik et al., 1997; Setzer et al., 2005; Lund et al., 2007). Kunz et al. (1994) and Guimaraes et al. (2010) suggested that genetic alterations induced by NRTIs play a primary role in carcinogenesis and are also involved in secondary and subsequent steps of carcinogenesis. Taylor and Low-Beer (2001) and Volmink et al. (2007) declared that the choice of antiretroviral therapy in pregnancy may be influenced by past.
ARTs, drug resistance, effects of pregnancy on the pharmacokinetics of the drug and factors influencing tolerability and adherence. In pregnancy, tolerability may be even more important than usual, especially if therapy exacerbates common complications of pregnancy, such as vomiting and glucose intolerance.

**ANIMAL STUDIES**

*In vivo animal studies:* All the NRTIs except ddI had preclinical animal studies that indicated potential fetal risk for *in vivo* teratogenicity (Ayers, 1988; Stahlmann et al., 1988; Lindstrom et al., 1990; Toltzis et al., 1994; Mobarak, 1995; Greene et al., 1990, 1996; Minkoff and Augenbraun, 1997; Birmingham, 2000) and were classified as FDA pregnancy category C except ddI, which was classified as category B. Post-implantation embryonic AZT exposure was associated with significantly increased pregnancy losses (resorptions and intrauterine deaths) in rats (Christmas et al., 1995) and it became incorporated into the DNA of the placenta and most fetal organs during short-term intravenous infusion in pregnant rhesus monkeys (Olivero et al., 1997). In primate studies, all the NARTIs seemed to cross the placenta, but ddI and ddC apparently showed significantly less placental transfer than did AZT, D4T, ABC and 3TC (Sandberg and Slikker, 1995; Gingelmaier et al., 2006). Studies conducted in rabbits and rats indicated an increased incidence of embryonic resorptions, both early and late, after exposure to AZT (Ayers, 1988; Stahlmann et al., 1988; Greene et al., 1990). A combined *in vivo* and *in vitro* comparative studies on the prenatal toxicity of five NARTIs in rat embryos resulted in similar abnormality patterns (head, neural tube, shape) and a wide range in embryotoxic potency (Klug et al., 1991). The AZT was reported to be a strong transplacental carcinogen in mice. In mice exposed to high concentrations of AZT *in utero* and for prolonged post-natal period, vaginal tumours were observed in adult life, an increased incidence of hemangiosarcoma, mononuclear cell leukemia and hepatic carcinoma (Ayers et al., 1997; Olivero et al., 1997; Diwan et al., 1999; Walker et al., 2007). K-ras cancer gene mutations and lung carcinogenicity were found in male and female mice exposed transplacentally to NARTIs (Hong et al., 2007; Koujimani, et al., 2008). Early embryo death did not occur in rats or rabbits in teratological studies carried out by Applewhite-Black et al. (1998); however, pregnant rabbits given AZT during gestation days (GDs) 6-18 exhibited reduced weight gain, anaemia and an increase in late fetal deaths. Monkey fetuses exposed *in utero* to the combination AZT plus 3TC sustain a higher level of drug-DNA incorporation and show evidence of more telomere damage than monkey fetuses exposed to AZT alone (Olivero et al., 2002). Exposure of pregnant mice (GDs 10-19) to AZT was found to alter offspring's sensorimotor and somatic maturation by affecting body weight gain and delaying the appearance of pole-grasping reflex, the latter effect was limited to male pups (Calamandrei et al., 2002). Significant MD was found in hearts and skeletal muscles of Erythrocebus patas monkeys fetuses exposed *in utero* to doses of AZT equivalent to the normal human dose (Gerschenson et al., 2000; Gerschenson and Poirier, 2000; Ewings et al., 2000; Divi et al., 2005) and mtDNA quantity was substantially depleted (>50%) in heart, skeletal muscle, cerebellum and cerebrum from AZT plus 3TC-exposed monkey fetuses compared to unexposed controls (Gerschenson et al., 2004). The ARTs reviewed here may induce genetic damage by altering intracellular nucleotide pools. Significant levels of genetic damage were detected in erythrocytes of mouse pups treated with AZT and other nucleoside analogues *in utero* or postnataally (Von Tungeln et al., 2002; Bishop et al., 2004). AZT induced damage in nuclear DNA of mice exposed *in utero* and postnataally and mtDNA damage were observed in both human and mouse neonates following perinatal exposure to AZT and AZT/3TC in combination (Chan et al., 2007). The temporal changes in cardiomyocytes and
mitochondria at the light and electron microscopic levels, in hearts of mice exposed transplacentally to commonly used NRTIs, showed that a subset of changes in cardiac mitochondria and myofibrils persisted and progressed months after transplacental exposure of female mouse to NRTIs; with combined AZT/3TC exposure yielding additive effects compared with either drug alone (Torres et al., 2010).

In vitro animal studies: In two-cell embryos, harvested from unexposed females, exposed to low-concentration (1 μM) of AZT in vitro for 24 h, development beyond the blastocyst stage was inhibited (Toltzis et al., 1991). In contrast, drug exposure during in vitro blastocyst and postblastocyst development had resulted in little or no morphological toxicity. When embryonic or fetal mouse or human cells (from brain, limb buds, or different organ rudiments) were exposed to AZT or ddI in vitro, cytotoxicity was observed only in the micromole range, with AZT showing slightly higher cytotoxicity and brain cells appearing slightly more sensitive to both nucleosides (Sieh et al., 1992). Hitchcock (1993) showed that the in vitro activity of ddI against the HIV was generally less potent than that of AZT and ddC. However, the potency of ddI was similar to or greater than that of AZT but still less than that of ddC.

An in vitro rat whole embryo culture system to assess the embryo toxicity of various nucleoside analogues, namely, AZT, ddI and ddC and the HIV-1 protease inhibitor, indinavir, both alone and in combination was carried out by Fujinaga et al. (2000). The study concluded that AZT in combination with ddC resulted in severe growth retardation and morphologic abnormalities not seen with either agent singly. Comparison of micronucleated cell frequencies induced by AZT alone or in combination with ddI suggested that ddI potentiates AZT-induced chromosomal damage following direct exposure (Phillips et al., 1991; Witt et al., 2004). In the cell cycle of human epithelioid carcinoma (HeLa) cells exposed to AZT and 3TC alone, AZT but not 3TC causes an arrest of cells in S phase with a consistent alteration in the expression of several cell cycle genes (Olivero et al., 2005). AZT, is incorporated into cellular DNA, caused mutations and induced micronuclei, chromosomal aberrations, sister chromatid exchange, shortened telomeres and other genotoxic effects in vitro (Olivero, 2007).

The in vivo and in vitro developmental toxicity and teratogenicity of zalcitabine (ddC): Zalcitabine is extensively evaluated as monotherapy and in combination regimens for patients with HIV infection (Darbyshire et al., 2000; Sangkitporn et al., 2005; De Clercq, 2009). It is a potent antiretroviral agent that is phosphorylated to its active metabolite-2',3'-dideoxy-5'-triphosphate (ddCTP)-within both uninfected and HIV-infected cells (Jeffries, 1989; Balzarini, 1994; Devineni and Gallo, 1995). At therapeutic concentrations, ddCTP inhibited HIV replication by inhibiting the enzyme reverse transcriptase and terminating elongation of the proviral DNA chain (Adkins et al., 1997).

In the study, the data on ddC teratogenicity included, among other studies, a developmental toxicity study in C57B1/6N mice (Lindstrom et al., 1990), embryological and cytogenetical studies in NMRI mice (Mobarak, 1995), a biochemical study on the short-term cardiac side effects of ddC on rat embryos (Skuta et al., 1999) as well as in vivo studies on damaging effects of ddC on the developing heart, spinal cord of NMRI mice fetuses and damaging effect on the developing fore brains and eyes of maternally-treated CD-1 mice fetuses (Mobarak, 2001a, b, 2009).

In vivo animal studies of zalcitabine: Zalcitabine was concentrated in the fetal kidney of the rhesus monkey and was present in the brain at approximately 20% of fetal blood
concentrations (Sandberg et al., 1995). Teratological studies were performed in mice exposed to up to 2000 mg/kg/day. Doses of 400 mg/kg/day resulted in mild developmental toxicity, manifested as open eyelids and cleft palate. However, starting at 1000 mg/kg/day of ddC, an increased frequency of growth retardation, skeletal defects, micrognathia and bent long bones were observed (Lindstrom et al., 1990).

Mobarak (1995) studied the in vivo impacts of ddC on the embryonic development of NMRI strain of mice. This is a Ph.D study that was conducted from November-1990 to March-1994 in the Institute of Biology, Medical University of Lübeck, Lübeck, Germany. The experiments were designed to include three main parameters.

- The effects of the drug on maternal body weight and resorption sites
- External morphology, cartilage and bone formation on GDs 14 and 18 of mice fetuses
- Histopathological and ultrastructural changes possibly induced by the drug on the developing ovary of 14 and 18-day old mice fetuses

Two ddC doses (600 mg and 1000 mg kg⁻¹ b.wt.) were applied to pregnant mice by oral intubation for five consecutive days, from day 9-13 of gestation. Zalcitabine treatment had only reduced (statistically significant) the body weight of mothers and significantly elevated the number of resorption sites and dead fetuses of mothers given the higher dose and sacrificed at day 18 of gestation. Fetuses of mothers treated with the low dose exhibited haematomas formation on the limbs and skull, reduced body weight and length and 37.3% of fetuses exhibited External Abnormalities (EAs). On the other hand, fetuses of mothers treated with the higher dose exhibited dramatic increase in the percentage (62.9%) and severity of EAs. Head and limb abnormalities ranging from mild to severe types were encountered in such instances. Amelia, phocomelia, shortness and twisting of digits and adactylia as well as tail defects were recorded with significant incidences comparable to the controls. On GD 18, fetuses of mothers treated with either low or high doses of ddC showed reduced body weight and length and mild EAs in percentages and severity lesser than those found in 14-day-old fetuses. Also, maternally treated mice fetuses on GDs 14 and 18 fetuses had designated delay and wide variations in the processes of chondrogenesis and ossification in most of their skeletal parts.

Mobarak (1995) and Banhawy et al. (1996a, b) postulated that ovaries of the low dose (600 mg kg⁻¹ b.wt.) ddC maternally treated 14-day-old mice fetuses showed a significant increase in the mean number of degenerated germ cells, while ovaries of the high dose (1000 mg kg⁻¹ b.wt.) exhibited reduction in ovarian diameters, a marked reduction in germ cell population. On the other hand, ovaries of the same low dose ddC maternally treated, 18-day-old, fetuses showed no obvious histopathological changes except the occurrence of some cases of haemorrhages, while ovaries of the high dose treated 18-day-old mice manifested a reduction of the diameter of the ovaries accompanied with a marked significant increase of the average number of degenerated germ cells. Mild ultrastructural changes were observed in the ovaries of low dose maternally treated 14 and 18-day-old mice fetuses, while the high dose treatment had evoked severe ultrastructural changes in all ovarian components of both ages, being marked as follows: Wide intercellular gaps, autophagosomes, abundant lipid droplets and obviously deformed mitochondria, hypertrophy of Golgi complex, dilated cisternae of RER, excessive wrinkling of the nuclear envelopes and fragmentation of nuclei. In addition, various degrees of degenerative changes were observed in the germ cells, while in somatic cells all mitochondria were obviously deformed.
Fig. 1: Transverse sections of the heart of 14-day old mice fetuses (X40). (a) The heart of a control fetus shows the pulmonary trunk (2), left auricle (3) and a broad apex of ventricles. Spinal cord (1) is also seen and (b) a similar section of a fetus exposed to 1000 mg of ddC showing defected heart and spinal cord. It exhibits haemorrhage in the pericardial cavity (short arrow), an abnormal ventricular apex (long arrow) and the abdominal wall is damaged and opened. H and E stain. (Mobarak, 2001a)

Table 1: The effects of ddC on the developing heart of 14-day-old mice fetuses as compared with control

<table>
<thead>
<tr>
<th>Animal groups</th>
<th>No. of fetuses examined</th>
<th>Percentage of fetuses with abnormal heart</th>
<th>Mean diameter of ventricles ±SD (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>20</td>
<td>0.0</td>
<td>4.21±0.08</td>
</tr>
<tr>
<td>Treated (600 mg kg⁻¹)</td>
<td>20</td>
<td>10.0</td>
<td>4.29±0.1*</td>
</tr>
<tr>
<td>Treated (1000 mg kg⁻¹)</td>
<td>22</td>
<td>45.5</td>
<td>4.95±0.06*</td>
</tr>
</tbody>
</table>

*p<0.05 and *p<0.01 versus control (Student t-test)

Mobarak (2001a) had investigated the impacts of ddC on the developing heart of 14-day-old (NMRI) mice fetuses. This study was conducted from March-1996 to January-1997 in the Departement of Zoology, Faculty of Science, Suez Canal University, Ismailia, Egypt. Pregnant mice were treated with 0, 600, or 1000 mg kg⁻¹ ddC by oral gavage, from day 9-13 of gestation. The results of this study were shown in Table 1, among fetuses got the high dose treatment, 45.5% exhibited heart abnormalities in conjunction with spinal cord damage while the reverse was not true and the mean diameter (4.95±0.06 mm) of the ventricles was significantly increased (p<0.05, versus control). In the control fetuses, the heart of 14-day-old mice fetuses exhibited a completed structure of four chambers (two auricles and two ventricles), two major blood vessels (a bulbous arteriosus and a sinus venosus) and a well-developed pericardium. Figure 1a showed the heart, with a broad ventricular apex, at the level of the pulmonary trunk and left auricle, also, the interventricular septum (Fig. 2a).

In the low dose ddC-maternally-treated fetuses, the general structure of the heart was either slightly affected or did not show any change relative to the controls. However, the higher ddC dose
Fig. 2: Photomicrographs of 14-day mice fetuses showing the heart. (X40). (a) A heart section of a control fetus and (b) a similar section of a fetus exposed to 1000 mg of ddC showing thinning of interventricular septum (arrow) and ventricular wall, while the trabeculae are widely spaced. H and E stain. (Mobarak, 2001a)

Fig. 3: (a) Heart section of a 14-day fetus exposed to 1000 mg ddC kg⁻¹ displays Extrathoracic ectopia, opened body wall (1), elongated atrioventricular cushion (2) and extensive haemorrhage (H). (X40) and (b) the right atrium of Fig. 3a magnified shows nucleated fetal red blood cells in extensive haemorrhage within the heart and in pericardium. (X100). H and E stain. (Mobarak, 2001a)

resulted in ventricular defects which were the most frequent ones. These defects were represented by malformed ventricular apex (Fig. 1b), thinning out of ventricular wall, ventricular elongation, looseness of the interventricular septum and a decreased number of ventricular trabeculae (Fig. 2b). In addition, ventricular septal defect at the mesenchymal part that resulted in a direct communication between the two ventricles was observed in one fetus. Excessive haemorrhage was also found in hearts of some fetuses. Extrathoracic ectopia (a phenomenon showed a part of the heart outside the chest protruding through a defected thoracic wall). In this case, enlargement of the atrioventricular cushion, enlarged and damaged auricular and ventricular walls and excessive haemorrhage were observed (Fig. 3a, b). Among these fetuses, one fetus exhibited displacement of the heart (a condition called pericardia-diaphragmatic hernia) where the liver was seen in the left side of pleural space above the heart (Fig. 4b).
Fig. 4: (a) A cross section of a control fetus displaying the heart at the level of the atrioventricular cushion (arrow). (X40) and (b) a similar section of a fetus, exposed to 1000 mg of ddC, displays pericardiaphragmatic hernia and the liver (1) is seen above the heart. (X40). (Mobarak, 2001a)

Table 2: The effects of ddC on the developing spinal cord of 14-day old mice fetuses as compared with control

<table>
<thead>
<tr>
<th>Animal groups</th>
<th>No. of fetuses examined</th>
<th>Percentage of fetuses with abnormal spinal cord</th>
<th>Mean diameter of spinal cord±SD (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>20</td>
<td>0.0</td>
<td>2.5±0.2</td>
</tr>
<tr>
<td>Treated (600 mg kg⁻¹)</td>
<td>20</td>
<td>6.0</td>
<td>2.4±0.04*</td>
</tr>
<tr>
<td>Treated (1000 mg kg⁻¹)</td>
<td>20</td>
<td>59.1</td>
<td>2.1±0.1*</td>
</tr>
</tbody>
</table>

*p<0.05 and *p<0.05 versus control (Student t-test)

In a further study Mobarak (2001b) investigated the damaging effect of ddC on the development of the spinal cord of 14-day-old (NMR) mice fetuses. This study was conducted from March-1996 to January-1997 in the Department of Zoology, Faculty of Science, Suez Canal University, Ismailia, Egypt. Pregnant mice were treated with 0, 600, or 1000 mg kg⁻¹ ddC by oral gavage, from day 9-13 of gestation. Table 2 showed the effects of ddC on the developing spinal cord of 14-day-old mice fetuses as compared with control, generally, in the high-dose treated animals, there was a rise in the percentage (59.1%) of fetuses exhibiting spinal cord damage as well as a significant incidence (p<0.05 versus control) in the decrease of spinal cord mean diameter. Spinal cord damage in conjunction with heart abnormalities was observed in 45.8% of the above-mentioned percentage. The general structure of spinal cord in control fetuses revealed an outer marginal layer (white matter), an inner mantle layer (gray matter) and (an inverted pear-shaped) central neural canal (Fig. 5a). The spinal cord of fetuses their mothers fed the low dose did not show any change relative to the controls or slightly affected (Fig. 5b). However, the higher dose evoked severe changes in the spinal cord of the same age (14-day) fetuses. In most fetuses, the spinal cord lost its normal morphology and showed a slight elongation that gave it a more or less rectangular-shaped (Fig. 6a) or square-shape (Fig. 6b). Also, the horn like structure was absent in the majority of spinal cords due to cellular disorganization of the mantle layer. Such Malformations were seen most frequently in the lumbo-sacral and then cervical regions (Fig. 7a). The inverted pear-shaped structure of the normal neural canal was severely distorted and
Fig. 5: (a) A cross section of spinal cord of a control (14-day-old) fetus shows the Dorsal horn (Dh) Grey matter (G), White matter (W), Ependymal cells (E), Neural canal (Nc) and Ventral horn (Vh). (X 100) and (b) A similar cross section of a spinal cord from a maternally treated (600 mg ddC kg⁻¹) fetus showing elongation of the neural canal. H and E stain. (Mobarak, 2001b)

Fig. 6: (a) A rectangular shaped spinal cord from a maternally treated (1000 mg ddC kg⁻¹) fetus, has a very small neural canal. (X100) and (b) Spinal cord of another 1000 mg ddC kg⁻¹ maternally treated fetus exhibited a ± square shape, while the Neural canal (Nc) is distorted by downgrowths of the neuroepithelial cells. (X100). H and E stain. (Mobarak, 2001b)

Fig. 7: (a) Spinal cord section of a maternally treated (1000 mg ddC kg⁻¹) fetus shows absence of the neural canal, (b) higher magnification of mid dorsal part of Fig. 7a displays the Roof plate (PRp) and Ependymal cells (E) disorganized. (X400) and (c) a magnified part (in box) of Fig. 7a shows irregularity of ependymal cells, fusion of Internal Limiting Membrane (ILM), Neuroglia cells (Nr) and Neuroplasts (Np) are also seen. (X1000). H and E stain. (Mobarak, 2001b)
Fig. 8: (a) Highly damaged spinal cord of a maternally treated (1000 mg ddC kg\(^{-1}\)) fetus, exhibiting extensive hemorrhage (H) and damaged neural canal (Nc). (X100) and (b) higher magnification of mid dorsal part of Fig. 8a displays extensive Hemorrhage (H), Ependymal (E) cell death and necrosis, damaged Neural canal (Nc). (X400). H and E stain. (Mobarak, 2001b)

showed great morphological variations in all affected fetuses. In most cases, it was more or less closed (Fig. 7b, c). Enlargement of the neural canal associated with downgrowths of the neuroepithelial cells was frequently observed (Fig. 8a). Cell death and necrosis were frequently seen in the dorsal and ventral horns and roof plate cells. In some fetuses, these lesions were accompanied by extensive cystic and hemorrhagic changes (Fig. 8).

The effects of ddC on the developing fore brain and eyes of CD-1 mice fetuses were studied (Mobarak, 2009). This study was conducted from May-2006 to January- 2007 in the Departement of Zoology, Faculty of Science, Suez Canal University, Ismailia, Egypt. A single dose (800 mg kg\(^{-1}\)) of ddC was orally administered to pregnant females from day 9 to day 13 of gestation. On GDs 14 and 18, fetuses from control as well as maternally-treated groups were examined for any EAs; their heads were then processed for studying histological and anatomical changes in the fore brains and eyes. The percentages of fetuses exhibited EAs on both GDs were shown in Table 3, the percentages (58.3 and 43.4%) of EAs recorded on GD 14 and 18 were highly significant (p<0.001 and p<0.01, versus control), respectively. In both maternally-treated groups, the prevalent type of external abnormalities recorded following zalcitabine treatment was of the limb type; the four limbs showed variable degrees of mild malformations including size and length reduction, flexion and rotation. The abnormalities of the CNS were represented by microcephaly and microphthalmia. However, the severe limb abnormalities such as amelia (complete absence of limb) and adactyly previously observed by Mobarak (1995) in NMRI mice fetuses were not recorded in the present strain of mice which might represent a strain resistance to the severe teratogenic effects of ddC. On the other hand, mild to severe morphological variations and histological changes were observed in the fore brains and eyes of such fetuses in comparison with those of the control ones.
Table 3: Percentages of malformed fetuses (on GDs 14 and 18) of CD-1 mice treated with 800 mg ddC/kg from 9-13 days of gestation

<table>
<thead>
<tr>
<th>Animal groups treated (800 mg kg⁻¹)</th>
<th>No. of fetuses examined on GD 14</th>
<th>No. of fetuses examined on GD 18</th>
<th>Percentage of malformed fetuses examined on GD 14</th>
<th>Percentage of malformed fetuses examined on GD 18</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>28</td>
<td>22</td>
<td>3.5†</td>
<td>0.0</td>
</tr>
<tr>
<td>Maternally</td>
<td>24</td>
<td>18</td>
<td>58.3***</td>
<td>43.4**</td>
</tr>
</tbody>
</table>

*p<0.05 and **p<0.01, ***p<0.001 versus control (Student t-test)

Fig. 9: Photomicrographs of fore brain cross sections in control 14-days-old mice fetuses. (a) Showing normal histological features of a control fore brain. Lateral ventricles (Lv), Neopallium (Npa), Pallium (Pa), Striatum (St), Choroid plexus (Cp), Thalamus (Th), Epithalamus (Eth), Hypothalamus (Hth), the third Ventricle (3V), Cerebellum (C), a part of the forth Ventricle (4V). X40. (b) The mid-dorsal part of Fig. 9a magnified to show the neuroepithelium of the Lateral ventricles (Lv), the septum (Sp) between the ventricles, anterior Choroid Plexus (CP), Marginal plate (Mp) and the white matter of the epithalamus (arrows). X 100. (c) Another part (in rectangle) of Fig. 9a magnified to display the normal thickness of the neuroepithelium of the lateral ventricle (Lv) with Ventricular zone (Vz), Subventricular zone (SVz), Cortical plate (Cp), Marginal zone (Mz) rich with Blood vessels (Bv) and a part of the Pallium (Pa). X400 and (d) The Pallium (Pa), Choroid Plexus (CP) and striatum (St) portions of Fig. 9a magnified to show the high rate of mitosis (arrows) and a large blood vessel (arrow head) in the striatum. H and E stain. X400. (Mobarak, 2009)

The normal histological structure of the fore brain parts (lateral ventricles, cerebellum, third ventricle, thalami and anterior choroid plexus) of 14-day-old control CD-1 mice was shown in Fig. 9a-d. Zalcitabine maternally-treated fetuses exhibited mild to severe changes of the developing
Fig. 10: Photomicrographs of fore brain cross sections in Zalcitabine maternally-treated 14-days-old mice fetuses. (a) Showing quite normal fore brain parts. Lateral ventricles (Lv), Septum (Sp), Pallium (Pa), Striatum (St), Choroid plexus (Cp), Thalamus (Th), the third Ventricle (3V) and Cerebellum (C). X40. (b) The mid-dorsal part of Fig. 10a magnified to show the neuroepithelium of the lateral ventricles (Lv), the Septum (Sp) between the ventricles, anterior Choroid Plexus (CP), Marginal plate (Mp) and a slightly reduced white matter area of the epithalamus (arrows). X100. (c) A part (in rectangle) of Fig. 10b magnified to display pyknotic darkly stained neurons and gala of the Pallium (Pa) and Striatum (St), Choroid Plexus (CP) appeared abnormal. X400 and (d) A part (in rectangle) of Fig. 10a magnified to display the abnormal thickness of the neuroepithelium of the Lateral ventricle (Lv) with Ventricular zone (Vz), Subventricular zone (SVz), Cortical plate (Cp), Marginal zone (Mz) with a few blood vessels. X400. H and E stain. (Mobarak, 2009)

fore brain. In the mildly affected fetuses, brains appeared histologically to be well differentiated except the appearance of pyknotic darkly stained neurons and gala of the pallium and striatum (Fig. 10a, c). In other fetuses, the brain anatomy was quite normal while the thickness of the cerebral hemispheres was reduced. The ventricular neuroepithelial cells were also reduced in size and became less densely packed than in the control (Fig. 10d). In case of the severely damaged fore brains, of ddC maternally-treated fetuses, loss of the fore brain normal anatomy and completely deformed cerebral hemispheres and ventricles were observed (Fig. 11a). The neuroepithelial cells were less densely packed than in the control and the majority of them were darkly stained with condensed chromatin material in their nuclei, but others were fragmented and many groups of dark particles became distributed throughout the neuroepithelial layer and within the ventricular
Fig. 11: Photomicrographs of fore brain cross sections in Zalcitabine maternally-treated 14-days-old mice fetuses. (a) showing a photomicrograph of severely affected fore brain parts with completely deformed cerebral hemispheres and ventricles. X40, (b) showing a part (in square) of Fig. 11a magnified to display vacuoles (arrows), uneven thickness of the ventricular neuroepithelium (arrow head) and extravasation of blood (*). X400, (c) showing another part (in rectangle) of Fig. 11a magnified to show severely damaged lateral ventricle (Lv) with uneven neuroepithelium (arrow head) and contents of cerebral hemisphere highly damaged (Cd). X 100, (d) showing a part (in rectangle) of Fig. 11c magnified to show highly distorted neuroepithelium with numerous dense cells (arrows), abnormal cellular aggregates (large arrow heads) and irregular tissue projecting into the ventricular lumen (small arrow heads). X400, (e) showing another part (between the rectangle and arrow head) of Fig. 11c magnified to show apoptotic cells (arrows), a large cellular aggregate large arrow head) within the ventricular lumen, detached cells (small arrow heads), reduced thickness (*) and hemorrhage (arrow) in the neuroepithelium. X100 and (f) showing the part under the forth ventricle of Fig. 11a magnified to illustrate excessive hemorrhage (H). X400. H and E stain. (Mobarak, 2009)
Fig. 12: Photomicrographs of longitudinal sections in the eye of control (Fig. 12a) and Zalcitabine maternally-treated (Fig. 12b-d) 14-days-old mice fetuses. (a) Showing the control eye at the level of the Optic stalk (Os). Sclera (Sc), middle Choroid (Ch), inner Retina (R), Lens (L), Cornea (C) and Eye lids (El). X100. (b) Maternally-treated eye appeared rotated with extensive hemorrhage (arrows) in the Eye lids (El), anterior Retina (R) and Cornea (C). Detached cells (arrow head), reduced Lens (L) are also seen. X100. (c) A section showing the eye of another maternally-treated fetus with reduced Lens (L) size, Retina (R), Cornea (C) and Eye lids (El) appeared poorly developed. X100 and (d) A part (in rectangle) of the inner eye chamber and Lens (L) of Fig. 12c magnified to show detached cells and hemorrhage (arrows). X400. H and E stain. (Mobarak, 2009)

lumens (Fig. 11b,c). Also, down-growths of the neuroepithelial cells were frequently observed (Fig. 11c-e). Also, these brains exhibited extravasation of blood (hemorrhage and hematomas formation) in their outer surfaces (Fig. 11a,f).

Figure 12a showed a section in the eye of a control 14-day-old mice fetus at the level of the optic stalk, sclera, middle choroid, inner retina, lens, cornea and eye lids. The eye abnormalities of ddC maternally-treated fetuses were represented by microphthalmia, extensive hemorrhage and cellular
Fig. 13: Photographs of longitudinal hand sections of heads of control (Fig. 13a) and Zalcitabine maternally-treated (Fig. 13b-d) 18-days-old mice fetuses. (a) Showing the normal structure of the Eye (E), Olfactory epithelium (Oe), Nasal septum (Ns), Cerebellum (C) and two Cerebral hemispheres (Cb). (b) The Cerebellum (C) is abnormally elongated, the right eye (#) is completely absent and the cerebral hemispheres (Cb) are reduced in size. X20. After Mobarak (2009)

dead in the areas of the eye lids and within the eye ball (Fig. 12b-d). Both cones and rods of the sensory retina, of such maternally-treated fetuses, appeared reduced in size due to the appearance of many intercellular spaces among them.

The 18-day-old normal structure of the eye, olfactory epithelium, nasal septum, cerebellum and cerebral hemispheres were shown in a longitudinal hand section of a control head of a mouse fetus (Fig. 13a). However, in ddC-maternally treated fetuses the cerebellum was abnormally elongated, the right eye was completely absent and the cerebral hemispheres were reduced in size (Fig. 13b-d). These results could be due to the property of ddC in producing cytotoxicity and inhibition of DNA synthesis after its administration for 5 days. Also, ddC may exert its teratogenic effects by inducing fetal hypoxia, leading to vascular disruption and necrosis of existing and developing structures, after its administration to pregnant mice during a critical time in neurogenesis and circulatory system development.
In vitro animal studies of zalcitabine: Because of its effective role in Aids treatment, ddC biology was extensively characterized from a toxicological viewpoint. It had a relatively low cytotoxicity in vitro (Flint, 1994; Todtzi et al., 1994; Monte et al., 1997; Benbrik et al., 1997; Moyle, 2000; Uehio et al., 2007; Lund et al., 2007). However, ddC was designated by Mitsuya et al. (1987), Brandt et al. (1991) and Rossi et al. (1999) to be an active inhibitor of DNA synthesis that may lead to different pattern of cytotoxicity, cell death, growth inhibition associated with a pronounced increase in cell volume as well as an increased incorporation of the drug into cell DNA. A dramatic apoptosis in human glioblastoma cells exposed to ddC was reported by Ullyatt and Liang (1998). The drug was reported to be clastogenic in human peripheral lymphocytes in vitro at a concentration >7 *M mL⁻¹. Further work of Balzarmi et al. (1987), Ulman et al. (1988) and Cinatt et al. (1991) marked that ddC showed cytotoxicity and cell growth inhibition in vitro. An in vitro study carried out by Mobarak (1995) (the above mentioned Ph.D study) to evaluate the possible impacts of ddC on the chromosomes of mouse fibroblasts. Cells were treated with three concentrations of ddC (7.5, 10 and 20 *M mL⁻¹) for 12 and 24 h before arresting the culture with colcemid, respectively. Abnormal Metaphases (AM) with various types of Chromosomal Abnormalities (CA) such as fragments, minute chromosomes, simple and complex rearrangements of chromosomes, chromosomal breaks and gaps, chromatid breaks and gaps and aneuploidy were of significant incidences. The data showed that the percentage of AM and the number of CA per metaphase were highly significant than controls and were concentration and exposure time dependent. Mitochondrial DNA depletion and toxicity with impairment of several metabolic properties and oxygen production were also reported (Antonelli et al., 1997; Rossi et al., 1999; Kakuda, 2000; Moyle, 2000; Feng et al., 2001; Joseph and Levine, 2006; Bjerke et al., 2008; Kohler et al., 2009).

REFERENCES


