

ABSTRACT

CISNEROS, FRANCISCO JAVIER. Epigenetic Inheritance of 5-Aza-2'-deoxycytidine (5-Aza-CdR) Induced Alterations. (Under the direction of Dr. Stacy Branch).

Methylation of DNA regulates in part genomic imprinting which plays a critical role in fetal development. Changes in methylation patterns can induce alterations of gene expression leading to abnormal growth and/or morphological defects. 5-AZA-CdR inhibits DNA methylation, thus altering gene expression. It was believed that in mammals, epigenetic defects could not be inherited because they are cleared and reset during gametogenesis and/or embryogenesis. However, recent publications sustain the existence of epigenetic inheritance. The aim of this study were: 1) to investigate if 5-AZA-CdR would induce heritable alterations in development; 2) to determine 5-AZA-CdR effects on post-natal development and reproductive capacity; 3) to confirm if these effects were due to alterations in energy metabolism or in circulating IGF-I levels; and 4) to analyze the nature of the effect on male reproductive capacity.

To determine if 5-AZA-CdR would induce heritable alterations in development, pregnant mice were administered 1 mg/kg of 5-AZA-CdR, and three subsequent generations were examined. Male and female treated offspring were mated avoiding consanguinity. In each generation, 50% of pregnant mice were killed and fetuses used for necropsy and skeletal analysis while the other 50% were allowed to give birth to produce the next generation. Leg and tail abnormalities, abnormal male mating behavior, retarded post-natal body development, cleft palate and global DNA hypermethylation were observed in exposed F1 mice. In the F2, cleft palate and increased levels of global DNA methylation were observed, while in the F3, cleft palate was evident.

Previous work determined genes whose expression is altered in developing limb buds exposed *in utero* by 5-AZA-CdR. Methylation status in abnormal palate tissue was determined to clarify if methylation of the promoter regions of developmentally related genes were affected. Statistically significant differences were not observed.

To elucidate the effects of 5-AZA-CdR on post-natal development and reproductive capacity, pregnant mice were administered 1 mg/kg of 5-AZA-CdR. Mothers gave birth and pups were weaned at 21 days of age. Number of pups per litter and weight were recorded. Body weight of males and females were collected at different ages. Lower weights in treated F1 males and females were observed every recorded time. Effects were more pronounced with age.

5-AZA-CdR F1 males and females and control mice were killed and levels of serum IGF-1, corticosterone and glucose were determined to investigate if growth retardation was a consequence of altered energy metabolism or serum IGF-1 levels induced by the treatment. No treatment effect was observed in serum corticosterone or glucose levels. However, statistically reduced levels of IGF-1 were observed in 5-AZA-CdR F1 males only.

To understand the effects on reproductive capacity, 5-AZA-CdR F1 males and females were mated with control females and males respectively. Reproductive capacity of *in utero* exposed male mice was adversely affected. However, testis histology, daily sperm production and testosterone levels were not. To clarify if the altered reproductive capacity was a behavioral phenomenon, a male sexual behavior test was conducted. A significantly decreased number of mounts and a significant increase of mount latency were observed in exposed mice. Additionally, although the treatment affected the

male:female offspring ratio, presence of Sry gene (an indicator of Y chromosome presence) in exposed male mice was not affected by the treatment.

Our data suggest that altered methylation patterns (induced initially by 5-AZA-CdR embryonic exposure) affect normal fetal and post-natal development and male sexual behavior. Induced growth retardation could be associated with lower serum IGF-1 levels. Furthermore, 5-AZA-CdR-induced alteration may be inherited by the offspring.

EPIGENETIC INHERITANCE OF 5-AZA-2'-DEOXYCYTIDINE (5-AZA-CdR)
INDUCED ALTERATIONS

by

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A dissertation submitted to the Graduate Faculty of
North Carolina State University
in partial fulfillment of the
requirements for the Degree of
Doctor of Philosophy

COMPARATIVE BIOMEDICAL SCIENCES

Raleigh

2002

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DEDICATION

To that supernatural power that I was taught to call "GOD". Who always has been showing me the path to follow. Who gave me strength in my weak moments. Who gave me understanding to learn from my mistakes and hope to continue forward to my goal.

To each and every one of my family members, who gave me their endless support, love, encouragement, and patience. To my parents, who offered me their very best always through my life. To my brothers, sisters, nephews and niece, the inspiration in those long work and study nights.

Finally to all my friends who believe in me. Who were close to me reminding me that "SI SE PUEDE" "I can do it".

BIOGRAPHY

Francisco "Javier" Cisneros was born on October 26, 1964 in Cayambe - Ecuador. He attended elementary school in his hometown. At the age of 9 years, he discovered his passion for veterinary medicine and two years later when learning about Mendel genetic experiments he realized his call for biological sciences. At the age of 12, he was sent to Quito to continue his education. At 17 years, he graduated from "Instituto Nacional Mejia" high school. At 22 years, he earned his DVM and Zoology degree at Universidad Central del Ecuador.

Three years later, while practicing veterinary medicine and working with private, government, and international agencies promoting the development of rural areas in the Ecuadorian Andes and Amazon regions, he was appointed a faculty position at the Veterinary College - Universidad Central del Ecuador.

After a period of five years of teaching, he decided to pursue further training in the USA. He completed a Teaching Internship at the Veterinary Teaching Hospital - UC Davis. After that, he moved to North Carolina, where he had the opportunity to meet and work with very successful scientists, who always encouraged him to continue with his formal education.

After working for a year at the Comparative Medicine Department of Wake Forest University School of Medicine, he decided to go back to school to pursue a Master's degree. Because of his passion for veterinary medicine and animal care, he chose the Animal Health Masters Program at North Carolina Agricultural and Technical State

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ACKNOWLEDGMENTS

I want to thank Dr. Stacy Branch, my major professor and committee chair, her kindness and considerable mentoring gave me the freedom to pursue my own research interests and successfully complete my journey at NCSU.

Special thanks go to my research advisory committee members: Drs. Robert Smart, Ida Smoak and Ross Leidy. All of them were very supportive and generous with their time, knowledge and experience. This work never would be completed without their guidance and input.

Thanks to Dr. Lee Robinette, for his positive feedback while serving as a committee member.

Thanks to Dr. Hosni Hassan for his friendship, mentoring, trust and support.

Thanks to Drs. Robert Chapin, Gary Wolf, Paul Selby, Lucy Anderson, Ralph Wilson and Greg Travlos for their interest in my project and their very valuable contribution.

Thanks to the NCSU Biological Research Facility personnel who were a key component in conducting my experiments, for all their support, kindness and professionalism.

I also would like to thank each and everyone in the Environmental and Molecular Toxicology Department for their help all these years.

Particular thanks go to Dr. Donald deBethizy, his mentoring and emotional support in those hard transition moments when starting my carrier in the USA and thereafter always will be appreciated.

Thank to Drs. Manuel Jayo, Deirdre Robinson, Linda Niedzella, for believing in me, their

words of wisdom and experience were the fuel that I needed to keep my learning machine on.

I want to extend my gratitude to my fellow graduate students, very especially to Mr. Benjamin Gersh and Dr. Gautam Ghatnekar for their honest friendship, support and assistance all these years.

Special thanks to my family for their economical, emotional and spiritual support I know that I never could make it without them on my side.

Finally, I want to thank my friend, mentor and associate, Dr. Garry Ford for being with me all the time encouraging and leading me through the Ph.D. program experience.

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INTRODUCTION

DNA methylation is a major epigenetic modification of the genome (Reik and Dean, 2001). It occurs after DNA replication and involves the transfer of a methyl group from 5-adenosyl-methionine (SAM) to the 5' position of cytosine residues, in a reaction catalyzed by the enzyme DNA methyltransferase (Dnmt 1) (Pinto and Zagonel, 1993).

In mammals, methylation of the 5' position of cytosine residues is a reversible covalent modification of DNA in the sequence 5'-CpG-3' and occasionally 5'-CpNpG-3' (Newell-Price et al., 2000). In most eukaryotic DNA, 60 to 90% of methylcytosine residues occur at CpG dinucleotide sequences (Bestor, 2000; Bird, 1986; Grippo et al., 1968; Kress et al., 2001; Robertson et al., 2000). DNA methylation occurs primarily in areas where CpG density is low. Most CpG islands, sequences 300 to 3000 bp long with high CpG density, are completely unmethylated at least in young people (Attwood et al., 2002; Issa, 2001). CpG islands often function in strong promoters and have also been proposed to function as replication origins (Delgado S, 1998). It is estimated that CpG islands are associated with about half of all mammalian genes (Antequera and Bird, 1993). Unmethylated CpG islands are associated with housekeeping genes, while the islands of many tissue-specific genes are methylated, except in the tissue where they are expressed (Yeivin and Razin, 1993). The density and location of methylated sequences are important factors (Frank et al., 1991). A weak promoter can be silenced by only a few methylated CpGs, whereas a higher density of methylation is required to repress a strong promoter (Boyes and Bird, 1992). The repression is greater if the promoter itself is methylated (Meehan et al., 1989).

However, distant methylated sequences can contribute to gene repression (Hug et al., 1996). In addition, it is sustained that interactions between proteins and DNA are changed by methylation, leading to alterations in chromatin structure and either a decrease or an increase in the rate of transcription (Jones and Takai, 2001).

A number of key biological functions have been associated with DNA methylation including genome organization, chromosome stability (Colot and Rossignol, 1999; Dean et al., 2001; Smith, 2000), gene expression, silencing some genes and activating others (Reik and Dean, 2001), cell differentiation (Jost et al., 2001), genomic imprinting (Reik et al., 2001), X chromosome inactivation (Heard et al., 1997), cancer (Baylin, 1997; Dean et al., 2001), chromatin modification (Okano et al., 1999), aging (Cooney, 1993; Holliday, 1987; Issa, 2000), and development (Jackson-Grusby et al., 2001; Kafri et al., 1993; Li et al., 1992; Martin et al., 1999; Okano et al., 1999; Reik et al., 2001; Rogers et al., 1994; Walsh and Bestor, 1999).

DNA methylation is an epigenetic mechanism that contributes to the coordination of gene regulation during vertebral development (Bulut et al., 1999; Hsieh, 2000; Kafri et al., 1992; Li et al., 1993; Li et al., 1992; Momparler and Bovenzi, 2000; Okano et al., 1999; Razin and Riggs, 1980). It is thought that the methylation pattern is established during gametogenesis (Razin and Riggs, 1980) and it changes through development in mammals (Monk et al., 1987).

During mammalian development, programmed DNA methylation and demethylation regulates the expression of genes (Riggs, 1975) controlling the interaction of regulatory

sequences with transcription factors (Holliday, 1987). Epigenetic reprogramming in germ cells and the early embryo is critical for imprinting. This imprinting has crucial roles in protecting the genome integrity (Reik et al., 2001), thus, establishing nuclear totipotency (Sutherland et al., 2000) and stem cell differentiation in animal development. Mammalian development is accompanied by two major waves of genome-wide demethylation and remethylation: one during germ-cell development and the other after fertilization (Dean et al., 2001; Howlett and Reik, 1991; Kafri et al., 1992; Monk et al., 1987; Rougier et al., 1998; Sanford et al., 1987; Surani, 1998). During embryogenesis, methylation patterns are reprogrammed genome wide, generating cells with a broad developmental potential. Passive genome-wide demethylation after fertilization (by the lack of maintenance methylation following DNA replication and cell division (Howlett and Reik, 1991; Rougier et al., 1998)) and replication-independent demethylation during early embryogenesis have been suggested (Kafri et al., 1993).

The mechanisms that establish methylation patterns during development are not completely understood (Hata et al., 2002). However, it is clear that the complete process in mammals involves maintenance methylation, de novo methylation and demethylation in which genome wide reprogramming of methylation patterns takes place (Reik et al., 2001). Although adult methylation patterns are reproduced at each round of cell division (Turker and Bestor, 1997), the precise pattern of cytosine methylation varies according to the cell type and developmental stages (Bulut et al., 1999).

The role of genetics in development and reproduction has been well substantiated (Clement et al., 1998; Gill, 1999; Hamann and Matthaei, 1996; Reseland et al., 1999). Mammalian organism size is controlled by an orchestration of gene actions participating in pathways that promote or inhibit growth through systemic or local effects (Efstratiadis, 1998; Lupu et al., 2001). Growth factors and hormones control the cellular signal pathways that in turn govern development. The insulin-like growth factors (IGF-I and IGF-II) are essential for normal growth and development in different species (Butler and LeRoith, 2001; Duclos et al., 1999; Efstratiadis, 1998; Garofalo, 2002; Le Roith et al., 2001). Genes controlling growth are members of pathways that affect cell cycle and in turn mammalian organism development (Lupu et al., 2001).

Although still in debate, many reports have indicated decreasing sperm counts and increasing reproductive problems in animals and humans as a consequence of exposure to various toxicant agents (Safe, 2000; Sinawat, 2000; Thomas K. Monsees,). Various chemical, physical and biologic conditions could affect critical events in the development of the reproductive system. These events could include early primordial germ cell determination, gonadal differentiation, gametogenesis, development of external genitalia or signaling events regulating sexual behavior (Baatrup and Junge, 2001; Gray and Ostby, 1998; Shishkina and Dygalo, 1999; Sinawat, 2000; Vandelac and Bacon, 1999). Mammalian gonadal development and sexual differentiation are complex processes that require the coordinated expression of a specific set of genes in a strict spatiotemporal manner (Viger et al., 1998). Several of these genes are more crucial than others, but all

play very important roles in maintaining these developmental pathways (Bishop et al., 2000; Page, 2002).

Male sexual behavior is mediated in part by androgens, but in several species, it is influenced by both androgens and estrogens (Cooper et al., 2000; Levis and Ford, 1989). These hormones act in the brain to influence male mating (Clancy et al., 2000; Cooper et al., 2000). In recent years, analysis of mutant animals has demonstrated that a number of imprinted genes influence brain development and behavior (Baker et al., 1996; Baum et al., 1994; Blendy et al., 1996; Isles et al., 2002; Nelson et al., 1995; Ogawa et al., 2000; Ogawa et al., 1996). Recent studies indicate that expression of genes affecting sexual behavior and reproduction such as Y chromosome specific genes (DAZ, SRY, RBMY1A, RBMY1H, RBMY1, BPY1, PRY and TSPY) (Dasari et al., 2002), IGF (Dean et al., 1998), and c-fos (Chandrasekhar and Raman, 1997) is regulated by DNA methylation.

Although genomic methylation patterns in somatic differentiated cells are generally stable and heritable (Reik et al., 2001), it was believed that acquired epigenetic modifications such as DNA methylation or stable chromatin structures were cleared and reset on passage through the germline (Rakyan et al., 2001; Roemer et al., 1997; Sutherland et al., 2000). Therefore, in mammals such epigenetic modifications could not be inherited through the germline to future generations (Landman, 1991).

Numerous recent publications have indicated that specific manipulations of early embryos, such as nuclear transplantation, can result in heritable altered patterns of gene expression and induce phenotypic alterations at later stages of development (Latham,

1994; Latham and Solter, 1991; Morgan et al., 1999; Reik et al., 1993). Roemer (1997) reported that repression and DNA methylation of genes encoding major urinary proteins, olfactory marker protein, and reduced body weight can be experimentally induced by nuclear transplantation in early embryos (Reik et al., 1993) and those acquired phenotypes are transmitted to most of the offspring of manipulated parent mice. (Roemer et al., 1997). Recently, Morgan and collaborators describe the inheritance of an epigenetic modification at the agouti locus in mice (Morgan et al., 1999). Sutherland and collaborators reported the germline inheritance of transcriptional silencing in mice and reversion to activity after as many as three generations in the silent state. These studies support the theory that silent genetic information in mammals is inherited and later reactivated, implying a mode of phenotypic inheritance less stable than Mendelian inheritance (Sutherland et al., 2000).

5-AZA-2'-deoxycytidine (5-AZA-CdR; DAC; Decitabine) is a cytidine analog with a nitrogen atom substituting the carbon in the 5' position of the heterocyclin ring (Pinto and Zagonel, 1993). 5-AZA-CdR is able to inhibit DNA methylation (Jones and Taylor, 1980; Momparler and Bovenzi, 2000) by reducing the enzymatic activity of DNA methyltransferase via the formation of a stable complex between the enzyme and 5-AZA-CdR substituted DNA (Santi et al., 1983). As a consequence, the newly synthesized DNA in exposed cells becomes significantly hypomethylated (Jones and Taylor, 1981). Antineoplastic activity has been attributed to 5-AZA-CdR. Its antileukemic activity is linked to its ability to induce the synthesis of hypomethylated DNA in target cells (Wilson et al., 1983). 5-AZA-CdR has been used both in-vivo and in-vitro to inhibit

DNA methylation (Lantry et al., 1999; Martin et al., 1999; Raman and Narayan, 1995) to induce gene expression, and cellular differentiation (Gattei et al., 1993; Jackson-Grusby et al., 1997; Juttermann et al., 1994).

Branch et al (1996) determined that 1 mg/kg of 5-AZA-CdR administered at gestation day 10 of mice causes temporally related long bone defects in the hind limbs (phocomelia, meromelia, reduced or unossified fibula, reduced pelvic bones, and reduction and curvature of the tibia). Also, presence of supernumerary ribs, tail abnormalities, and a low incidence cleft palate and vertebral defects were observed. Other than defects in locomotion due to the phocomelia, no other functional defects were apparent at this age (Branch et al., 1996). DNA methylation plays critical roles in cellular differentiation and tissue development in embryogenesis, and epigenetic inheritance has been strongly substantiated in recent years. The aims of this study were: 1) to investigate if epigenetic alterations induced by 5-AZA-CdR, expressed as altered morphological development and global DNA methylation levels, could be inherited in offspring of *in utero* exposed mice; 2) to determine the effects of 5- AZA-CdR on post-natal development and reproductive capacity of CD-1 mice since the effects of this treatment at different post-natal ages through reproductive maturity were not known; 3) to determine if these effects were due to alterations in energy metabolism or in circulating IGF-I levels of *in utero* exposed mice; and 4) to analyze the nature of the effect of 5-AZA-CdR on male mice reproductive capacity.

Findings from these studies will help the scientific community to understand how xenobiotic compounds capable of affecting gene expression during organogenesis can alter post-natal development and individual performance through the entire life of exposed individuals and possibly their offspring. These exposures could play important roles in susceptibility and disease incidence in unexposed animals when their ancestors were somehow exposed in the past. These phenomena can be explained by the existence of interactive mechanisms between genetic and epigenetic factors.

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CHAPTER I

5-AZA-2' Deoxycytidine (5-AZA-CdR): a Demethylating Agent Affecting Development and Reproductive Capacity

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ABSTRACT

The objective was to evaluate the effects of 5-AZA-2'-deoxytidine (5-AZA-CdR) on post-natal development and reproductive capacity. Pregnant mice were administered 1 mg/kg of 5-AZA-CdR at gestation day (GD) 10. The body weights of F1 control and treated (in uterine-exposed) pups were recorded. To evaluate the reproductive capacity, 5-AZA-CdR F1 males and females were mated with control mice. The presence of plugs and the number of pregnancies were recorded. 5-AZA-CdR F1 male mice were killed, and total body, testes, and epididymis weights were recorded. Spermatid head counting, histological analyses and serum testosterone levels were performed. Body weights of 5-AZA-CdR F1 mice were statistically lower than controls ($p<0.01$). Male mating capacity appeared to be more adversely affected. Mating of 5-AZA-CdR F1 males with control females resulted in a lower pregnancy rate as compared to control mating groups ($p<0.01$). Gross testicular and epididymis weights were lower in 5-AZA-CdR F1 mice ($p<0.01$). However, testicular and epididymis weight in these mice were higher than controls when correlated to body weight ($p<0.01$). In 5-AZA-CdR F1 male mice, all measured reproductive parameters including total number of spermatid heads per testis are significantly lower ($p<0.01$) than the controls except for the number of spermatid heads per mg of testis (S/mg).

Keywords: 5-aza-2'-deoxycytidine, Developmental Toxicity, Infertility, Reproductive Toxicity, Reproductive Capacity, DNA Methylation, Post-natal development, 5-AZA-CdR

INTRODUCTION

DNA methylation is an epigenetic mechanism that plays crucial roles in cellular differentiation and tissue development in embryogenesis [Bulut et al., 1999], [Kafri et al., 1992], [Li et al., 1992], [Li et al., 1993], [Okano et al., 1999]. Programmed methylation and demethylation of DNA regulate the expression of genes during mammalian development [Riggs, 1975] controlling the interaction of regulatory sequences with transcription factors [Holliday, 1987]. The possible mechanisms are 1) preventing the binding of transcription factors to their target sequences via proteins that bind preferentially to methylated promoters [Momparker and Bovenzi, 2000], 2) the methylated cytosine itself interferes with the binding of transcription factors [Kass et al., 1997], 3) alterations in chromatin structure leading to a decrease or an increase in the rate of transcription [Jones and Takai, 2001]. In most eukaryotic DNA, 60 to 90% of methylcytosine residues occur at CpG dinucleotide sequences [Bird, 1986], [Grippe et al., 1968], [Kress et al., 2001]. Just 3 to 5% of the total cytosine residues in DNA are methylated at the carbon 5' position [Cedar, 1988, Ehrlich et al., 1982], [Razin and Riggs, 1980], [Vanyushin et al., 1970].

Epigenetic modification of DNA by methylation in mammals is related to a number of key biological functions [Bird and Wolffe, 1999] including genome organization and chromosome stability [Colot and Rossignol, 1999], [Dean et al., 2001], [Smith, 2000], gene expression (silencing some genes and activating others) [Reik and Dean, 2001], cell differentiation [Jost et al., 2001], genomic imprinting [Reik et al., 2001], X chromosome

inactivation [Heard et al., 1997], cancer [Baylin, 1997], chromatin modification [Okano et al., 1999], aging [Cooney, 1993], [Holliday, 1987], [Issa, 2000] and development [Jackson-Grusby et al., 2001], [Kafri et al., 1993], [Li et al., 1992], [Martin et al., 1999], [Okano et al., 1999], [Rogers et al., 1994], [Walsh and Bestor, 1999].

Body weight and reproductive function have been correlated in some species [Ewart-Toland, Mounzih et al., 1999]. The adipocyte-specific hormone leptin, the product of the obese (ob) gene, regulates adipose-tissue mass through hypothalamic effects on satiety and energy expenditure [Clement, Vaisse et al., 1998]. Leptin plays an important role in the regulation of body weight and fat deposition [Hamann and Matthaei, 1996] and it is also important for growth, reproduction and neuroendocrine signaling [Reseland, Hollung et al., 1999]. In the mouse, the preimplantation embryonic development (Ped) locus, and a growth and reproduction complex (Grc)-like region have been reported [Gill, 1999].

5-AZA-2'-deoxycytidine (5-AZA-CdR; DAC; Decitabine) is a cytidine analog with a nitrogen atom substituting the carbon in the 5' position of the heterocyclin ring [Pinto and Zagonel, 1993]. It is able to inhibit DNA methylation [Jones and Taylor, 1980] by reducing the enzymatic activity of DNA methyltransferase via the formation of a stable complex between the enzyme and 5-AZA-CdR-substituted DNA [Santi et al., 1983]. As a consequence, the newly synthesized DNA in exposed cells becomes significantly hypomethylated [Jones and Taylor, 1981]. Antineoplastic activity has been attributed to 5-AZA-CdR. Its antileukemic activity is linked to its ability to induce the synthesis of hypomethylated DNA in target cells [Wilson et al., 1983]. 5-AZA-CdR has been used

both in-vivo and in-vitro to inhibit DNA methylation [Lantry et al., 1999], [Martin et al., 1999], [Raman and Narayan, 1995] to induce gene expression, and cellular differentiation [Gattei et al., 1993], [Jackson-Grusby et al., 1997], [Juttermann et al., 1994]. 5-AZA-2'-deoxycytidine also causes temporally related defects in the developing mouse [Branch et al., 1996].

DNA methylation is a mechanism for regulating gene expression, and alterations in methylation might significantly affect embryonic development. Branch et al (1996) determined that a 1 mg/kg dose of 5-AZA-CdR administered at gestation day 10 caused temporally related long bone defects in the hind limbs (phocomelia, meromelia, reduced or unossified fibula, reduced pelvic bones, and reduction and curvature of the tibia). Also, presence of supernumerary ribs, tail abnormalities, and a low incidence cleft palate and vertebral defects were observed. In the same study, some of the treated dams were allowed to give birth and pups evaluated for postnatal viability until post-natal day 23. Other than defects in locomotion due to the phocomelia, no other functional defects were apparent at this age [Branch et al., 1996]. Since the effects of this treatment at different post-natal ages through reproductive maturity were not known, the aim of this study was to determine the effects of 5-aza-2'-deoxycytidine on post-natal development and reproductive capacity of CD-1 mice.

MATERIALS AND METHODS

Animals

Sixteen timed pregnant CD-1 mice were purchased from Charles River Laboratories, (Raleigh, NC). Mice were allowed to acclimate for a period of 2 days. During the acclimation and experimental period animals were housed individually in polycarbonate cages with bed-o'cobs bedding in animal rooms at 24°C with a 12 h artificial light cycle. Mice were fed commercial lab chow and water *ad libitum*. Prior to tissue collection, animals were humanely killed by the approved method of cervical dislocation.

Treatment

5-AZA-2'-deoxycytidine (5-AZA-CdR) was purchased from Sigma Chemical Co. (St. Louis, MO). After weighing, animals were randomized by weight for assignment to the treatment or control groups. Equal number of animals resulted per treatment group. This randomization process allowed equal representation of all weight ranges in each treatment group. This laboratory previously determined the dose of 5-AZA-CdR (1mg/kg) treatment on gestation day (GD) 10 via i.p. injection (delivered in 0.2 mL sterile saline) [Branch et al., 1996]. Doses were calculated based on the GD 10 body weight. Controls were dosed only with 0.2 ml of sterile saline vehicle.

Data Collection and Analysis

Body Weight. At 66 and 161 days of age, body weights of F1 male and female mice were recorded. Due to observed decrease in male reproductive capacity, male body weights were recorded at two additional times (180 and 230 days of age). These time points were chosen as representations of the reproductive life span of the mice (covering early to late reproductive ages).

Reproductive Parameters. To determine the reproductive capacity in 5-AZA-CdR F1 mice, randomly selected exposed male and female offspring were mated to control females and males respectively at 3 and 5 months of age. Control groups were mated simultaneously. Mating activity was determined by presence of vaginal plug. The morning in which vaginal plug was observed, was considered GD 0. At GD 17 animals were killed and, pregnancy rates, dam body weight, uterine weight, number of pups, litter weight, number of fetal deaths, resorptions and fetus malformations were recorded.

After the second mating (5 months of age) the F1 males were killed and fresh testis and epididymis weights were collected, number of spermatid heads and serum testosterone levels was determined.

Tissue Analysis. After mating, animals were killed, and dissection of the male reproductive organs was conducted in F1 5-AZA-CdR male and control group mice. The testes with attached epididymides were removed. The epididymides were carefully

separated from the testes and each testis and epididymis were weighed separately. The right testis was frozen at -70°C for the measurement of daily sperm production (Spermatid Head Count). Counts were determined following the general procedures outlined by Blazack [Blazack et al., 1993]. The right testis was decapsulated, placed in a measured volume of physiological buffered saline (PBS) and homogenized. An aliquot of the homogenate was evaluated with a hemocytometer for the number of homogenization-resistant steps 17-19 sperm heads. The left testis and epididymis were fixed at 4°C in 4 % paraformaldehyde for 24 hours and processed to slides stained with hematoxylin and eosin and examined by light microscopy.

Serum Testosterone. As mentioned above for "tissue collection", animals were killed and blood was collected by cardiac puncture and allowed to clot at room temperature for 1 h and centrifuged at $14,000 \times g$ for 10 min. [LeBlanc and Waxman, 1988]. Serum from each animal was conserved at -20°C until assayed. Total testosterone levels in serum of treated and control males were determined by solid-phase radio-immunoassay using the Coat-A-Count Total Testosterone Kit (Diagnostic Product Corporation., Los Angeles, CA).

Data Analysis. Using StatMost Software, mean and standard error of weight values were calculated prior to Analysis of Variance (ANOVA) and Student's T, to establish differences among treatments. Spermatid head counts from treated and control mice, presence of vaginal plug, and pregnancy were analyzed.

RESULTS

No maternal toxicity (as measured by lethality or weight loss) was observed in treated dams. The expected morphological defects were observed in uterine exposed offspring. These defects included supernumerary ribs, a low incidence of cleft palate, tail defects, and hind limb long bone defects (phocomelia, meromelia, unossified and reduced fibulae) [Branch et al., 1996].

Body Weight. Body Weight (BW) of 5-AZA-CdR *in utero* exposed (F1) males and females were statistically different from those of respective controls at each of the post-natal time points examined. These differences were more pronounced with increasing age. In males, the mean BW of treated mice at 66 days of age, was 67.21 % ($P \leq 0.01$), at 161 days, 62.41% ($P \leq 0.01$), at 182 days, 54.21% ($P \leq 0.01$) while at 230 days of age, the treated group's mean BW represented just 56.59% ($P \leq 0.01$) of their control. In females, at 66 days the mean weight of the treated group was 72.48% ($P \leq 0.01$) and at 161 days it represented 48.92% ($P \leq 0.01$) of the respective control group. Male body weight gain at four different time points is presented in Table 1, while female body weight gain at two different time points is presented in Table 2.

Reproductive Parameters. As expected from preliminary data, reproductive activity in F1 5-AZA-CdR male was reduced. Measured reproductive parameters are presented in Tables 3 and 4. The presence of a vaginal plug was indicative of mating, while pregnancy was indicative of fertility. At 3 months of age, 45% of vaginal plug presence and 36 % of

pregnancy was observed in 5-AZA-CdR F1 male group, while 90% of vaginal plug presence and 81% of pregnancy was observed in 5-AZA-CdR F1 female group. At 5 months of age, 10 % of vaginal plug presence and no pregnancy resulted from mating of control females to 5-AZA-CdR F1 males, while a 100% vaginal plug presence and 75% pregnancy rate was observed in 5-AZA-CdR F1 female group. In 5-AZA-CdR F1 male mice, all measured reproductive parameters including total number of spermatid heads per testis were significantly lower ($p<0.01$) than the controls except for the number of spermatid heads per mg of testis (S/mg) (Table 5).

Pathology. Except for the organ size, there were no treatment related effects observed in the testes and epididymis of 5-AZA-CdR F1 adult mice. No abnormalities were observed histologically. Although few immature sperms were found in treated epididymis, all testes appear to have all stages of spermatogenesis evident with normal maturation and spermatid release (Figure 1). .

Serum Testosterone. No differences in total serum testosterone between treated and control males were found (Figure 2).

DISCUSSION

The mechanisms of how xenobiotic compounds affect morphological, reproductive and neurological development are not completely characterized or understood. DNA methylation regulates gene expression during mammalian development [Jackson-Grusby et al., 2001], [Kafri et al., 1992], [Li et al., 1992], [Martin et al., 1999], [Okano et al., 1999], [Reik et al., 2001], [Riggs, 1975]. It plays an important role in imprinting, and imprinted genes regulate complex mammalian traits, including growth and behavior. Therefore, alterations in methylation may significantly affect embryonic development [Rogers et al., 1994].

Evidence of the genetic role in growth and reproductive development has been described [Lyon, 1996], and influence of heredity on body size has been reported [de Castro, 1993]. In addition, maternal factors play a role in the development of the exaggerated appetite for some specific nutrients [Erkadius et al., 1996]. The family of basic helix-loop-helix (bHLH) genes comprises transcription factors involved in many aspects of growth and development. A role for *Nhlh2* in the onset of puberty and the regulation of body weight metabolism has been reported [Good et al., 1997].

It has been demonstrated that 5-AZA-CdR 1 mg/Kg at GD-10 causes axial and appendicular skeletal defects in the developing mouse without maternal toxicity [Branch et al., 1996]. Similar conditions were observed in the current study. Our findings suggest that functional defects in development can continue throughout post-natal life. In the

present study, effects on body weight are observed in males and females. *In utero* exposed mice never reached the sizes of their peer controls. This could be the cause of a delayed maturity and perhaps affects male sexual capacity.

The effects of 5-AZA-CdR include more than gross skeletal malformations. The data suggest that functional and/or homeostatic processes may be altered. These alterations are manifested in part as significantly lower body weights. Leptin, the adipocyte-specific hormone which is the product of the obese (*ob*) gene, regulates adipose tissue mass through hypothalamic alterations [Clement et al., 1998] and plays an important role in the regulation of body weight [Hamann and Matthaei, 1996], growth, reproduction and neuroendocrine signaling [Reseland et al., 1999]. A critical role for 1 alpha, 25(OH) 2D3 receptor (VDR) in growth, bone formation and female reproduction in the post-weaning stage has been established [Yoshizawa et al., 1997]. Alterations in the expression of genes involved in growth could be altered subsequent to 5-AZA-CdR treatment.

Mating activity is influenced by environmental conditions (i.e., housing specifications) and genetic factors [Bilsing et al., 1993]. Mammalian gonadal development and sexual differentiation are complex processes that require the coordinated expression of a specific set of genes in a strict spatiotemporal manner [Viger et al., 1998]. In this study, presence of vaginal plug was used as an indicator of mating. Presence of vaginal plug was very low in F1 5-AZA-CdR males in relation to their respective controls in both experimental phases. Since environment, housing, and nutrition were similar in control and treated mice, it is likely that genetic factors were altered in the treated groups. The fact that the

inheritance of specific genes promotes fertility of sterile leptin-deficient obese male mice [Ewart-Toland et al., 1999] indicates that not just mating but also fertility is controlled by genetic factors. In this study, overall number of pregnant females represents fertility. Fertility values at 3 months of age for 5-AZA-CdR F1 males was 36.36% while for 5-AZA-CdR F1 females was 81%, and at 5 months of age, 0% and 75% respectively. This suggests that treated males were more adversely affected by the reproductive insult when compared to females.

Although histological analyses of testes and epididymis did not reveal major differences, and spermatid head counts normalized by mg of testis were not statistically different between treated and control groups, the pregnancy rates were lower in 5-AZA-CdR F1 males. The absolute spermatid head counts were significantly lower in treated mice. Therefore, the lower pregnancy rates in treated males may be due in part to significantly lower numbers of spermatids available. In conclusion, 5-AZA-CdR embryonic exposure adversely affects post-natal growth in male and female CD-1 mice. Our data suggest that 5-AZA-CdR affects male reproductive capacity.

Although reduced mating activity was observed in F1 5-AZA-CdR male mice in preliminary and present studies, testis anatomy and histology and testosterone levels were not different between treated and control mice. Since many of the genes involved in growth and reproductive development are important for neuroendocrinal signaling [Ewart-Toland et al., 1999], [Gill, 1999], [Hamann and Matthaei, 1996] and

imprinted genes regulate behavior, a reproductive behavioral study is ongoing and may reveal that alterations in fertility of exposed males (F1 5-AZA-CdR) involve alterations in reproductive behavior. The results obtained from this experiment will help scientists to understand how xenobiotic compounds capable of affecting gene expression during organogenesis can alter post-natal functional parameters such as reproduction and growth.

ACKNOWLEDGEMENT

This work was supported by NIH # ES08452, National Institute of Environmental Health Sciences.

We would like to thank Dr. Robert Chapin for his assistance with the histological evaluation of the male reproductive organs and to Dr. Gary Wolf for his guidance in Spermatid head counts procedures.

Table I. 1. 5-AZA-CdR F1 Male Body Weight (gm)

LABELS	AGE (days)							
	66		161		182		230	
Group	T	C	T	C	T	C	T	C
N	22	20	19	30	19	19	4	4
Mean St. Error	29.19** ± 0.96	43.43 ± 0.89	31.04** ± 1.21	49.73 ± 1.19	31.4** ± 1.34	57.92 ± 0.99	30.45** ± 2.70	53.77 ± 1.60

Anova and Student t tests were conducted

** Statistically significant from control (p<0.01)

N = Sample number

T = Treated

C = Control

Table I. 2. 5-AZA-CdR F1 Females Body Weight (gm)

LABELS	AGE (days)			
	66		161	
Group	T	C	T	C
N	20	20	8	37
Mean St. Error	23.31** ± 1.16	32.16 ± 0.92	26.66** ± 1.27	54.49 ± 0.82

Anova and Student t tests were conducted

** Statistically significant from control (p<0.01)

N = Sample number

T = Treated

C = Control

Table I. 3. 5-AZA-CdR F1 Reproductive Parameters (3 Months of Age)

GROUP 1 5-AZA-CdR F1 MALES + NORMAL FEMALES						
Label	V. Plug♣	Pregnancy♣	D. Weight	U. Weight	Pups #	L. Weight
N	11	11	11	5	4	4
Mean St. error	5/11 (0.45)*	4/11 (0.36)**	27.84 ±0.30	17.59 ±4.35	11.2 ±2.85	13.3 ±3.36
GROUP 2 NORMAL MALES + 5-AZA-CdR F1 FEMALES						
Label	V. Plug♣	Pregnancy♣	D. Weight	U. Weight	Pups #	L. Weight
N	11	11	11	5	4	4
Mean St. error	10/11 (0.90)	9/11 (0.81)	23.26 ±1.23	12.75 ±3.85	10.50 ±1.30	11.54 ±1.31
GROUP 3 NORMAL MALES + NORMAL FEMALES						
Label	V. Plug♣	Pregnancy♣	D. Weight	U. Weight	Pups #	L. Weight
N	10	10	10	6	6	6
Mean St. error	9/10 (0.90)	9/10 (0.90)	32.17 ±1.47	20.25 ±0.54	13.5 ±0.56	14.59 ±0.41

Note: Mean refers only to Dam, Uterus, and Litter weight, and pups number.

N = sample number

♣ = Fraction of females with vaginal plug or pregnant

(corresponding number in parentheses are the decimal equivalent)

V. Plug = Vaginal Plug;

D. Weight = Dam Weight;

U. Weight = Uterus Weight;

L. Weight = Litter Weight

Anova and Student t tests were conducted

** Statistically significant from control (p<0.01)

* Statistically significant from control (p<0.05)

Table I. 4. 5-AZA-CdR F1 Reproductive Parameters (5 Months of Age)

GROUP 1 5-AZA-CdR F1 MALES + NORMAL FEMALES						
Label	V. Plug♣	Pregnancy♣	D. Weight	U. Weight	Pups #	L. Weight
N	19	19	2			
Mean St. error	2/19 (0.10) *	0/19 (0.00) *	45.5 ±4.5			
GROUP 2 NORMAL MALES + 5-AZA-CdR F1 FEMALES						
Label	V. Plug♣	Pregnancy♣	D. Weight	U. Weight	Pups #	L. Weight
N	4	4	4	3	3	3
Mean St. error	4/4 (1.00) *	3/4 (0.75) *	38.65 ±5.66	15.93 ±0.76	11.66 ±0.33	11.16 ±0.61
GROUP 3 NORMAL MALES + NORMAL FEMALES						
Label	V. Plug♣	Pregnancy♣	D. Weight	U. Weight	Pups #	L. Weight
N	11	11	5	2	2	2
Mean St. error	5/11 (0.45)	2/11 (0.18)	51.32 ±8.32	13.35 ±2.85	9 ±2.00	9.5 ±0.30

Note: Mean refers only to Dam, Uterus, and Litter weight, and pups number.

N = sample number

♣ = Fraction of females with vaginal plug or pregnant

(corresponding number in parentheses are the decimal equivalent)

V. Plug = Vaginal Plug;

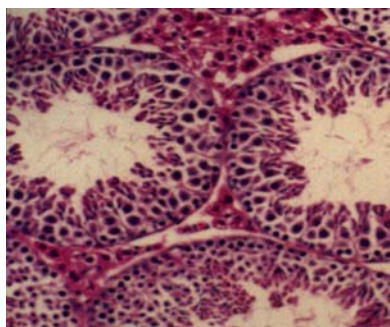
D. Weight = Dam Weight;

U. Weight = Uterus Weight;

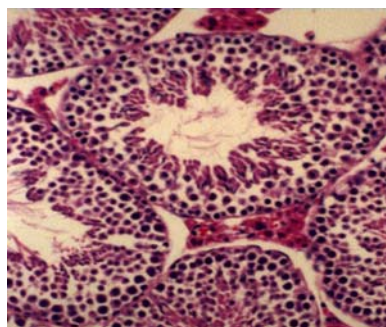
L. Weight = Litter Weight

Anova and Student t tests were conducted

* Statistically significant from control (p<0.05)



CONTROL



5-AZA-CdR F1

FIGURE I.1. TESTES OF 5-AZA-CDR F1 MICE (5 MONTHS OF AGE)

Similar structures and stages of sperm development were observed in seminiferous tubules of 5-AZA-CdR F1 and control mice, suggesting no 5-AZA-CdR induced effects on male gonadal development and spermatogenesis.

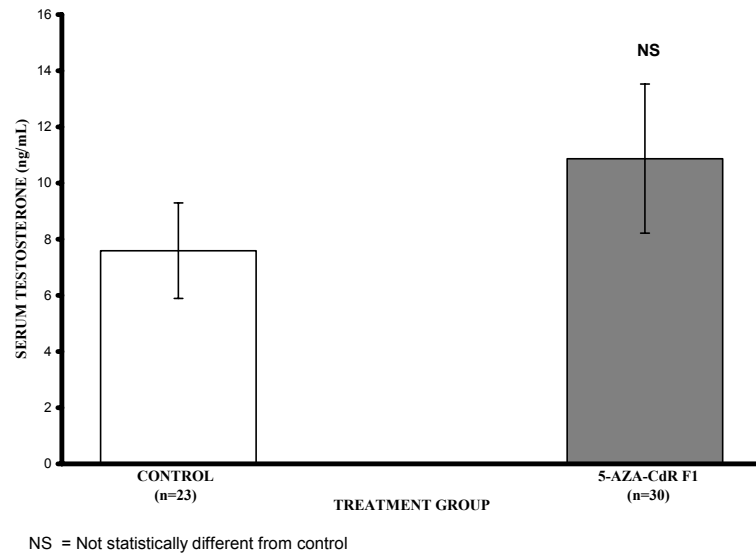


FIGURE I. 2. LEVELS OF SERUM TESTOSTERONE IN 5-AZA-CDR F1 MICE (5 MONTHS OF AGE)

Values on Y axis are means plus Standard error. Anova and Student's T test were conducted. NS= Not statistically different from control. No differences in total serum testosterone between 5-AZA-CdR F1 (n=30) and control (n=23) were observed. This observation suggests that the altered mating behavior of 5-AZA-CdR F1 male mice is not caused by altered serum testosterone levels.

Table I. 5. Testis, epididymis, and Spermatid Head Count of 5-AZA-CdR F1 (5 months of age)

Control Group (N=13)							
Label	BW (gm)	TW (mg)	EW (mg)	TWBW (%)	EWBW (%)	S/T	S/mg
Mean St. Error	56.10 ± 1.03	128.2 ± 6.46	63.8 ± 2.9	0.23 ± 0.01	0.11 ± 0.00	3613.18 ± 265.72	3.71649e+006 ± 224788
Treated Group (N= 13)							
Label	BW (gm)	TW(mg)	EW (mg)	TWBW (%)	EWBW (%)	S/T	S/mg
Mean St. Error	31.62 ± 1.65 **	101.5 ± 3.85 **	47.5 ± 2.5 **	0.33 ± 0.02 **	0.15 ± 0.00 **	2845.35 ± 203.37 **	3.48950e+006 ± 142830

Anova and Student's T test were conducted. ** =Significantly different from control (p<0.01). BW= Body Weight, TW= Testis Weight, EW= Epididymis Weight, TWBW= Testicular Weight in relation to Body Weight (%), EWBW= Epididymis Weight in relation to Body Weight (%), S/T = Absolute Spermatid heads per testis, S/mg= Spermatid heads per mg of testis tissue.

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CHAPTER II

INTRAUTERINE EXPOSURE TO 5-AZA-2' DEOXYCYTIDINE

AFFECTS SEXUAL BEHAVIOR IN MALE MICE

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ABSTRACT

We have reported that intrauterine exposure to 5-AZA-2'- deoxycytidine (5-AZA-CdR) affects growth and reproductive activity in male mice. We evaluated whether altered reproductive capacity in exposed male mice was caused by abnormal testis and epididymal morphology or sperm production. Mouse testes were collected for histological analysis and measurement of daily sperm production, both of which were unaffected. Total testosterone levels did not appear to be affected by the treatment. To elucidate if the phenomenon was a behavior alteration, CD-1 pregnant mice were administered 1.0 mg/kg i.p. of 5-AZA-CdR or left untreated. 5-AZA-CdR treatment resulted in diminished mating behavior (as measured by vaginal plug presence, mount latency and number of mounts) and reduced sexual interest of male offspring while exposed to a receptive female. Pregnant mice were allowed to give birth, then number and sex of pups were recorded. We observed that the ratio of male to female offspring was altered. Therefore, we determined the presence of Sry gene in 5-AZA-CdR F1 males. Since testes histology, sperm counts, testosterone levels and presence of Sry gene were normal, while mating behavior was abnormal, we suggest that the reproductive alterations are behaviorally related.

Keywords. 5-AZA-2'- deoxycytidine (5-AZA-CdR), daily sperm production, Sry gene, reproductive behavior.

1. INTRODUCTION

Although published data indicate that endocrine disrupters may cause alterations in female reproductive behavior and contribute to sub-fecundity, sterility, pregnancy loss, developmental retardation, and birth defects [21, 44, 51, 80, 87, 88, 92], the effects on male fertility and sexual behavior remain poorly understood [26, 79]. Male sexual behavior is mediated in part by androgens, but in several species, it is influenced by both androgens and estrogens [20, 56]. Studies have shown that androgen act in the brain to influence rat male mating [19]. Testosterone and estradiol are secreted by the testes into the blood. Testosterone is converted into dihydrotestosterone by 5 α -reductase and estradiol by aromatase [20]. Male sexual behavior is also determined by the interaction of endocrine and environmental stimuli originating from the female, yet it is unknown how and where these stimuli are integrated within the brain. [29].

Various chemical agents, heavy metals, physical factors such as radiation and global warming, as well as biologic factors such as the contamination of phyto- and xeno-estrogen in the environment could affect critical events in the development of the reproductive system. These events could include early primordial germ cell determination, gonadal differentiation, gametogenesis, external genitalia, or signaling events regulating sexual behavior [1, 34, 89, 91, 93]. In addition, many reports have indicated decreasing sperm counts and increasing reproductive problems in animals and humans as a consequence of exposure to various toxicant agents [85, 91, 92].

Halldin (1999) studied the estrogenic activity of the synthetic estrogens ethinylestradiol and diethylstilbestrol as model compounds in the Japanese quail and found that depressed male sexual behavior was the most sensitive of the studied end points. This result led to the conclusion that this ecologically relevant end point should be included in avian in vivo testing for neuroendocrine disrupters [38].

Mammalian gonadal development and sexual differentiation are complex processes that require the coordinated expression of a specific set of genes in a strict spatiotemporal manner [94]. Several of these genes are more crucial than others, but all play very important roles in maintaining these developmental pathways [7, 78]. Non Y chromosome linked genes involved in sex determination have been identified (WT1 [67], SF1 [6], DAX1 [32], and SOX9 [7, 52]). The existence of XY females (ovaries and Y chromosome are present) [67] and XX males (presence of testicular tissue in absence of Y chromosome) [7, 96] have been reported in humans and mice [33, 46, 64]. However, it is still a consensus that in humans and other mammals, a gene carried by the Y chromosome called sex-determining region, or SRY in humans and Sry in mice, is critical for male development. It triggers the entire set of molecular genetic events that leads to male sex determination [62, 65, 68, 86, 95, 97].

In recent years, analysis of mutant animals has demonstrated that a number of imprinted genes influence brain development and behavior [43]. In nNOS^{-/-}, mounting behavior continued at high rates for longer periods of time than in controls. However, these

animals presented fewer penile intromissions and ejaculations than controls when paired with estrous females. nNOS^{-/-} males displayed elevated sexual motivation when paired with neoestrous females [74]. Males with the insulin-like growth factor-1 (Igf1) gene deleted fail to display mating behavior [2]. C-fos^{-/-} males presented longer latency to mount, decreased percentages of male mounting and decreased number of mounts with or without intromissions. They also displayed significant reductions in mounting rates despite no general lack of motivation or sexual arousal [3]. Male ER α ^{-/-} are deficient in mating behavior; the number of ejaculations are reduced significantly. However, they display equivalent numbers of mounts and intromissions as controls and they appear to be feminized in mating behavior [75, 76]. Although mating behavior was normal in male and female cAMP response element modulator (CREM) ^{-/-} mice, males were infertile because CREM gene is important for spermatogenesis [9].

Given the multifarious interactions that appear to mediate the development and operation of cognitive and behavioral functions, more studies oriented to understanding these complex biological mechanisms are required [4]. Recent studies indicate that expression of genes affecting sexual behavior and reproduction such as Y chromosome specific genes (DAZ, SRY, RBMY1A, RBMY1H, RBMY2A, BPY1, PRY and TSPY) [24], IgF [25], and c-fos [17] are regulated by DNA methylation. Since DNA methylation is required for normal gastrulation and subsequent patterning of the dorsal mesoderm [63], any alteration in DNA methylation may be lethal or significantly affect normal embryonic development [84] inducing gross morphological or physiological

abnormalities that could provoke behavioral change [73]. DNA methylation is an epigenetic modification that controls in part gene expression in mammalian cells [69]. It plays critical roles in cellular differentiation and tissue development in embryogenesis [16, 49, 58, 77]. It also appears that DNA methylation plays a significant role in the differentiation of gonads into primary spermatocytes [81]. 5-AZA-CdR, an inhibitor of DNA methylation [69], has been used in vivo [70] and in vitro [10] to study gene expression [12-15]. In addition, it has been shown that a 5-AZA-CdR intrauterine insult causes temporally related defects in the developing mouse [13]. The aim of this study was to determine the nature of the effect of 5-AZA-CdR on male reproductive capacity.

2. MATERIALS AND METHODS

2.1. Animals

Timed pregnant CD-1 mice were purchased from Charles River Laboratories (Raleigh, NC). Mice were allowed to acclimate for a period of 2 days. During the acclimation and experimental period animals were housed individually in polycarbonate cages with bed-o-cobs bedding in animal rooms at 24°C with a 12 h artificial light cycle. Mice were maintained on commercial laboratory rodent diet 5015 and water *ad libitum*. Females were used to generate 5-AZA-CdR offspring in which male:female ratio, male sexual behavior, and presence of Sry gene was studied. A controls group was studied simultaneously.

2.2. 5-AZA-CdR Exposure

5-AZA-CdR was purchased from Sigma Chemical Co. (St. Louis, MO). After weighing, animals were assigned to treatment or control groups. This laboratory previously determined the dose of 5-AZA-CdR (1mg/kg) treatment on gestation day (GD) 10 via i.p. injection (delivered in 0.2 mL sterile saline). Doses were calculated based on the GD 10 body weight. Controls were dosed only with 0.2 ml of sterile saline vehicle.

2.3. Male Sexual Behavior Test

The male sexual behavior test outlined by Chubb [18] was used. This test includes:

- 1) NATURAL BREEDING.** Because sexual experience can affect the results of sexual behavior test, each male mouse to be tested was housed with an adult female for 2 weeks. During this period, vaginal plug presence was recorded every morning. Pregnancy and litter size were statistically analyzed. This information allowed us to determine the natural mating capacity in the second group of 5-AZA-CdR *in utero* exposed males.
- 2) ABSTINENCE PERIOD.** To standardize the period of male sexual abstinence, a week before the test (day 1), female mice were removed from male cages, and males were housed individually.
- 3) SEXUAL BEHAVIOR TEST.** The test was conducted from 8:00 to 11:00 AM on day 7. One observer recorded the sexual behavior of one mouse for a period of 20 minutes. With three observers present, three male mice were tested simultaneously. Each male mouse was tested four separate times, with the tests grouped into two separate

periods. Each period consisted of two consecutive days, with a two-week interval between each period. Between test periods, each tested male was housed with a new adult female mouse for one week, and individually the following week. The test started when the female mouse was placed in the male's cage. Time to mounting, number of mounts and penile intromission, and ejaculation were observed and recorded.

Prior to the test, the following important aspects were addressed:

A) Stud Mice. Among the control offspring, five males were selected at random and maintained as studs. These mice were housed alone and used repeatedly to determine the receptivity of females to be used in the mating behavior test.

B) Primed Female Mice. Sixty-day-old CD-1 female mice were used for the test. Estrus was induced by sequential i.p. injections of 2 U of pregnant mare's serum at 8:00 PM on day 4 and 3 U of human chorionic gonadotropin at 8:00 PM on day 6.

C) Evaluation of primed female mice. Female sexual receptivity can affect the outcome of male sexual behavior tests. To compensate for this effect, each treated female was monitored for sexual receptivity prior to the male sexual behavior test by pairing her with stud mice at 7:30 AM. Female mice were scored using the McGill scale (5 to 1 from more to less receptive, respectively). Females that resisted the stud (scoring less than 3) were not accepted for the test.

2.4. Sry GENE

Genomic DNA from liver of treated and control mice was isolated using DNeasy Tissue Kit (Qiagen Inc., Valencia, CA). Sry gene presence was detected by PCR. GAPDH was

used as internal PCR control. Amplification was run in duplicate. 60 ug of DNA was used for multiplex PCR in a 50 ul reaction. 0.5 ul of Taq polymerase (TaqPlus 5u/ul, Stratagene, La Jolla, CA) was used with 100 mM each dNTP (Promega Corporation, Madison, WI), 20 mM male specific Sry primers (5' -TGGGACTGGTGACAATTGTC-3' and 5' -GAGTACAGGTGTGCAGCTCT-3', synthesized by Operon) (Schiffmann et al., 1995), and 20 mM GAPDH specific primers (5' -TGTGAACGGATTTGGCCGTA-3' and 5' -TCGCTCCTGGAAGATGGTGA-3', synthesized by Genosys). A Bio-Rad Cyclor (Bio-Rad Laboratories. Hercules, CA) was used with the following program conditions: 95°C for 3 minutes, 55°C for 30 seconds and 72°C for 2 minutes followed by 30 cycles of 95°C for 30 seconds, 55°C for 30 seconds and 72°C for 2 minutes. PCR products were terminated with a final extension at 72°C for 10 minutes.

3. DATA ANALYSIS

A one -way analysis of variance (ANOVA) and Student's T using StatMost Software were calculated to establish differences among treatments. Means of measured parameters were considered to determine statistical significance at $p \leq 0.05$.

4. RESULTS

The visual morphological defects in uterine exposed offspring included tail defects, and hind limb long bone defects (phocomelia, meromalia, unossified and reduced fibulae),

while no maternal lethality or weight loss was observed in treated dams. These results corroborate previous reported studies [12].

As observed in preliminary studies, reproductive activity of *in utero* 5-AZA-CdR exposed males was reduced. The presence of a vaginal plug was indicative of mating, while pregnancy was indicative of fertility. In natural breeding of the male sexual behavioral study (Figures 1), 5-AZA-CdR exposed male produced 28 % of vaginal plug and 25 % of pregnancy. While 91% of vaginal plug presence and pregnancy was observed in the control group. There was a statistically significant ($p<0.01$) decrease in the presence of vaginal plugs and pregnancy for normal females housed with 5-AZA-CdR exposed males compared to the control mating group.

4.1. Offspring Male/Female ratio. Male to female ratio of *in utero* 5-AZA-CdR exposed mice is presented in Figure 2. A statistically significant increase in the percentage of males ($p<0.05$) was observed in offspring of 5-AZA-CdR treated females when compared with offspring of non treated females.

4.2. Male Sexual Behavior Test. Means of mount latency (the time from introduction of female to first mount) are presented in Figure 3. There was a statistically significant increase in the time to first mount in 5-AZA-CdR F1 males compared to controls ($p<0.01$). A significantly lower ($p<0.01$) mean mount number per male in the exposed group was observed as compared to controls (Figure 4). Mount and penile intromission

occurred simultaneously. Ejaculation was not observed in either control or in treated mice.

4.3. Sry Gene Amplification. A 402 bp PCR product was generated for Sry primers and a 230 bp product for GAPDH primers. Amplification product of both primers was observed in treated and control male mice, while as expected, no Sry product was observed in Control and 5-AZA-CdR *in utero* exposed females. (Figure 5).

5. DISCUSSION

Although the disruption of many metabolic pathways has been identified as a result of toxic insults, the molecular mechanisms of how these compounds affect fetal sexual differentiation, reproductive organ development and reproductive capacity are not completely characterized or understood. Furthermore, specific conditions such as sexual behavior have not been considered as indicators of toxicity [79]. DNA methylation, an epigenetic DNA modification, regulates gene expression during mammalian development [45, 49, 58, 63, 77, 82, 83]. It plays an important role in imprinting, and imprinted genes regulate complex mammalian traits, including embryonic development and sexual behavior [43]. Therefore, alterations in methylation may significantly affect embryonic development [84], sexual behavior and reproductive capacity.

Evidence of the genetic role in growth and reproductive development has been described [60]. The range of outcomes attributed to *in utero* exposure to developmental toxicants has focused on gross toxicity or teratology [42]. In this study, 5-AZA-CdR *in utero* exposed mice showed similar teratological patterns to those observed in previous studies [11, 12]. Our findings suggest that in addition to the gross skeletal malformations, 5-AZA-CdR induces defects in sexual behavior and reproductive capacity of in-utero exposed male mice. Such alterations could be caused by altered gene regulation induced by the demethylation action of 5-AZA-CdR.

The influence of genetic factors and environmental conditions (i.e., housing specifications) on mating activity are well known [5]. In this study, experimental and control groups were maintained in similar environmental conditions and fed with the same food and water. Therefore, it is likely that the observed altered mating activity could be caused by genetic changes induced by the treatment. Simultaneously, the role of genetics on fertility has been published [28]. Low fertility parameters were recorded in control females when mated to 5-AZA-CdR *in utero*-exposed males, suggesting that genes affecting fertility could be targeted by the toxic insult.

A coordinated expression of a specific set of genes in a strict spatiotemporal manner is required for normal mammalian gonad development and sexual differentiation [94]. Numerous publications emphasize the effects of toxicant intrauterine exposure on reproductive organ development. For example, administration of vinclozolin to pregnant

rats demasculinizes and feminizes male offspring due to its antiandrogenic activity [36]. The effects of Di-n-butyl phthalate (DBP) on male reproductive tract development include missing epididymis and vasa deferentia, seminal vesicle malformation, induction of hypospadias, decreased anogenital distance, retained thoracic nipples, and cryptorchism [72]. Mono (2-ethylhexyl) phthalate (MEHP) is a testicular toxicant affecting Sertoli cells in vivo. It inhibits FSH signal transduction in cultural rat Sertoli cells which could be the mechanism to decrease Sertoli cell division in vivo. [41]. Other toxic agents such as testosterone, flutamide, cyproterone acetate, tamoxifen, estradiol and diethylstilbestrol (DES) markedly alter sex differentiation of the genitalia, the accessory glands and the testis in addition to their effects on central nervous system (CNS) sex differentiation and mating behavior. These chemicals dramatically reduce testicular sperm production in the male offspring, and the most severely affected males are infertile. When a compound such as Carbendazim (MBC) and dibutyl phthalate (DBP) produced infertility through direct effects of testicular function, then testis weight, testicular histology, and testicular sperm head counts provided sensitive indicators of toxicity [35]. Data presented in the previous chapter indicated that no anatomical or histological abnormalities in testis or epididymis were induced by 5-AZA-CdR intrauterine exposure, suggesting that gonad differentiation pathways were not altered by the treatment. Therefore, the abnormal mating behavior observed in exposed male mice could be caused by the effects of treatment on the CNS.

It has been reported that the quality and quantity of human spermatozoa are facing a serious decline [91]. Exposure to environmental synthetic estrogenic chemicals and related endocrine-active compounds has been attributed as the cause for this phenomenon [85, 92]. The molecular mechanisms of how these compounds could affect spermatogenesis are far from being understood. However, the role of DNA methylation on male reproduction has been considered more closely. Recent studies have demonstrated that other enzymes that interact with Dnmt exist and affect spermatogenesis. For example, Dnmt3L, a protein required for spermatogenesis sharing homology with DNA methyltransferases, Dnmt3a and Dnmt3b, but lacking enzymatic activity, interacts with such enzymes, suggesting that Dnmt3L may regulate genomic imprinting via the Dnmt3 family enzymes [40]. In addition, a possible role of PEG1-AS, expressed in testis and in mature motile spermatozoa, in human sperm physiology and fertilization has been substantiated [59].

Since no treatment effect on spermatid head counts normalized by mg of testis was observed in 5-AZA-CdR F1 male mice (Cisneros et al., submitted), 5-AZA-CdR may not have affected genes involved in spermatogenesis. Although it has been reported that exposure to toxic agents during sex differentiation can permanently alter reproductive function and produce reproductive organ dysmorphogenesis and pseudohermaphroditism [27, 36, 37, 42, 50, 72], specific functional effects of exposure such as changes in behavioral responses or change in organ functions have not been identified and characterized [79]. Sinawat (2000) indicates that the effects of compounds and conditions

that affect male fertility can result in, not only a reduction in sperm concentration, but also alterations in sexual behavior, mood disorders and the presence of genital cancers [91].

The central nervous system (CNS)-gonadal axis and the male sexual behavior of rodents appear to be sensitive during development [87]. The role of DNA methylation in genomic imprinting is very well accepted [57]. A number of imprinted genes influencing brain development and behavior have been analyzed in mutant animals [43]. The existence of specific genes able to control arousal has been hypothesized [90]. Recently, the TRP2 gene that produces a signaling protein that regulates both aggression and sexual behavior in male mice has been identified. [22]. The regulation of protein production from genes, and, thus, genetic effects on behavior has been suggested. Research into gene regulation and neurobiology has revealed intricate interactions among genes, proteins, hormones, food, and life experiences [4]. The effects of prenatal stress on male sexual behavior and fertility at central rather than peripheral levels have been documented [23]. Studies indicating that some toxic compounds such as polychlorinated biphenyls (PCBs), fenarimol, or losulazine, alter sex differentiation and reduce fertility by affecting breeding performance alone without altering sperm and testicular measures have been published [35]. In this study, male mice included in the behavior test presented lower reproductive parameters. This abnormal condition could be caused by altered protein or hormone production (regulated by genes) at central rather than peripheral level.

In the developing male testis, testosterone is produced by fetal Leydig cells and transported to the lumen of the developing Wolffian ducts. Testosterone is the primary signal that initiates differentiation of the Wolffian ducts into the epididymides, vasa deferentia and seminal vesicles [66]. Therefore, testosterone and its metabolite dihydrotestosterone are critical determinants of the male phenotype. [48]. Recent studies suggest that aromatization of testosterone to estradiol is not necessary for normal mating behavior in some species. However, the role of androgens in male sexual behavior is imminent [20]. No statistically significant differences of total serum testosterone were observed in exposed male mice as compared to controls (Cisneros et al., submitted). These results suggested that testosterone production is not compromised by the treatment implying that 5-AZA-CdR insult may affect the sexual behavior of exposed male at the central organizational level.

Several androgen receptor antagonists produced feminization when given during the embryo-fetal period of reproductive tract differentiation [71]. Dioxin exposure has been reported to lower human secondary sex ratio, putatively through a male mediated effect. Hexachlorobenzene (HCB) exposure has been suggested as a cause of reduction of males in offspring of exposed populations [47]. The fetal male:female ratio was altered in 5-AZA-CdR *in utero*-exposed litter and manifested as an increase in the number of males as compared to females. Therefore, it is possible that 5-AZA-CdR through the demethylation capacity altered the expression of genes involved in sexual differentiation.

It has been proved that SRY in humans and Sry in mice, is the Y chromosomal gene responsible for directing the fetal gonad to begin developing testicular tissue [54]. However, exceptions (XY females and XX males) have been reported in humans and mice [46]. In this study when investigating for the presence of Sry gene as an indicator of maleness, no treatment effect was observed. This suggests that the *in utero* exposed mice were phenotypically and genotypically males. Therefore the altered reproductive capacity and male:female ratios observed in exposed male mice can not be attributed to the absence of Sry gene.

The brain-pituitary-gonads axes through the use of neurotransmitters, peptides, growth factors and hormones form the basis and control of each step and every level of reproductive physiology [61]. The role of Nhlh2 in the onset of puberty and the regulation of body weight metabolism has been supported. Loss of Nhlh2 results in a disruption of the hypothalamic-pituitary axis in mice. Male Nhlh2^{-/-} mice are microphallic, hypogonadal and infertile with alterations in circulating gonadotropins, a defect in spermatogenesis and a loss of instinctual male sexual behavior [31]. PEA3, a member of the Ets family of transcriptional regulatory proteins, is expressed in a unique spatial and temporal pattern during mouse embryogenesis. PEA3 is dispensable for embryogenesis. PEA3 deficient males failed to reproduce. PEA3 is expressed in several male sexual organs, but gross and histological analyses of the organs from PEA3 (-/-) mice revealed no abnormalities [53].

Since the hypothalamus, pituitary and gonads are essential for normal reproduction, and no treatment effects in testes, epididymis, sperm or testosterone production have been reported (Cisneros et al, submitted), gathered data suggest that a genetic alteration affecting hypothalamic and/or pituitary development could be the cause of altered sexual behavior in 5-AZA-CdR exposed males. In addition, many of the genes involved in sexual differentiation and reproductive development are important for neuroendocrinal signaling [28, 30, 39] and imprinted genes regulate brain development and behavior [43]. Findings reported in this paper may serve as the bases to understand the impact of xenobiotics and the role of altered DNA methylation on the male reproductive behavior.

In conclusion, 5-AZA-CdR embryonic exposure adversely affects male mating behavioral manifested by low reproductive parameters. Because many gene controlled factors work together in reproduction, more studies designed to clarify the toxicant-genetic relationship involved in reproductive endpoints and the related regulatory role of DNA methylation will be valuable.

ACKNOWLEDGEMENTS

This work was supported by NIH # ES08452, National Institute of Environmental Health Sciences. We would like to thank Dr. Robert Chapin for his assistance with the histological evaluation of the male reproductive organs, Dr. Gary Wolf for his guidance

in Spermatid head counts procedures, and Dr. Meredith Gooding for her assistance in Serum Testosterone determination.

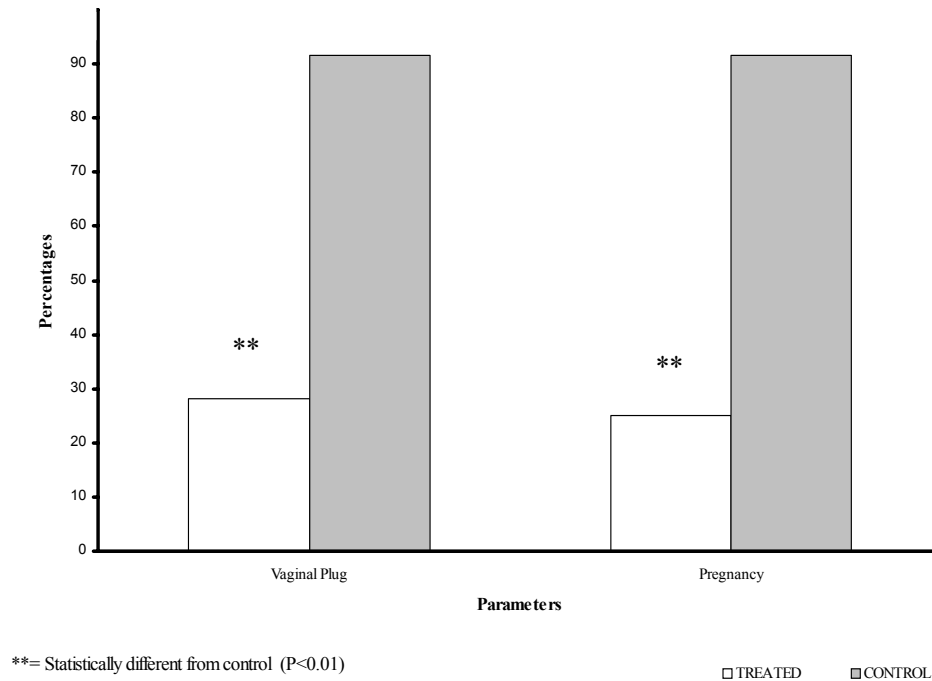


FIGURE II. 1. PERCENTAGES OF VAGINAL PLUG AND PREGNANCY IN NATURAL BREEDING OF 5-AZA-CdR *IN UTERO* EXPOSED CD-1 MICE (PHASE II).

Left bars represent group of exposed males mated with control females (n=32) while right bars represent controls (n=12). One-way analysis of variance (ANOVA) and Student's T test were calculated. **= Statistically different from control (P<0.01). The same degree of significance (P<0.01) for vaginal plug and pregnancy in the group of exposed males mated with control females when compared to their control litter mates was observed.

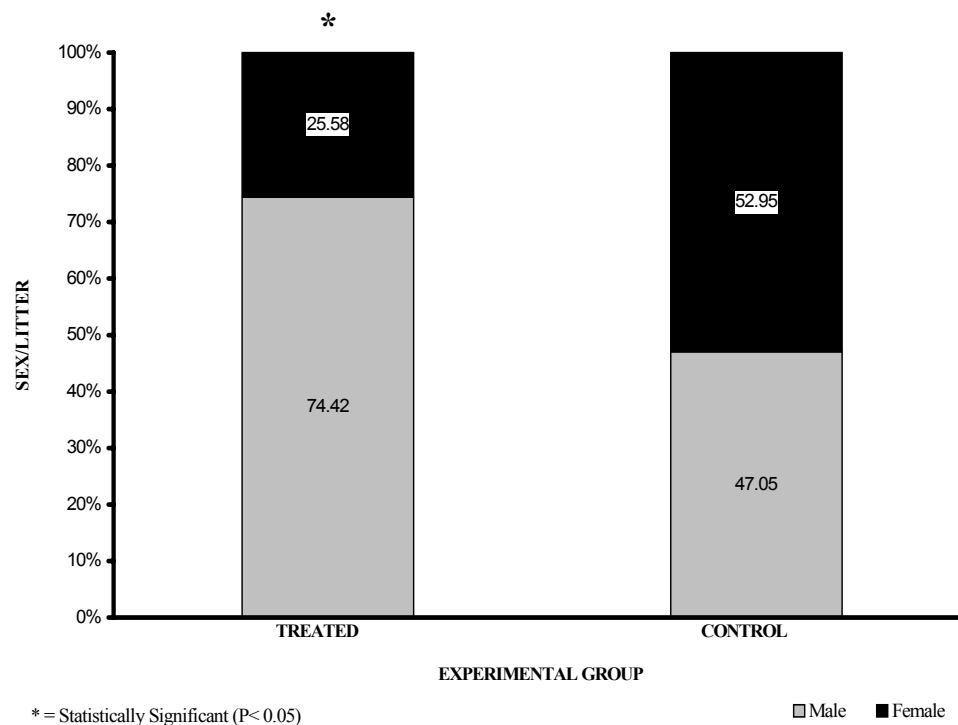
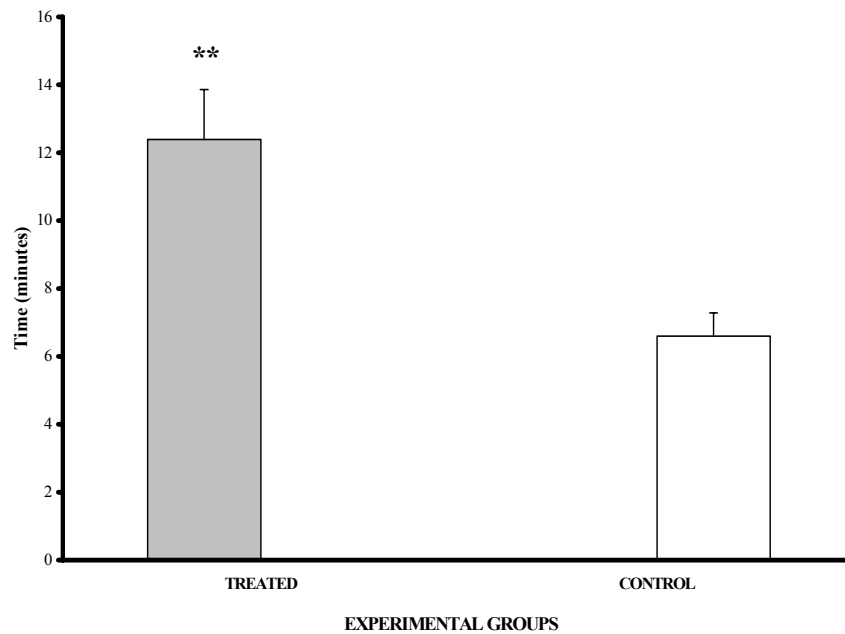


FIGURE II. 2. PERCENTAGE OF MALE/LITTER IN 5-AZA-CdR *IN UTERO* EXPOSED CD-1 MICE.

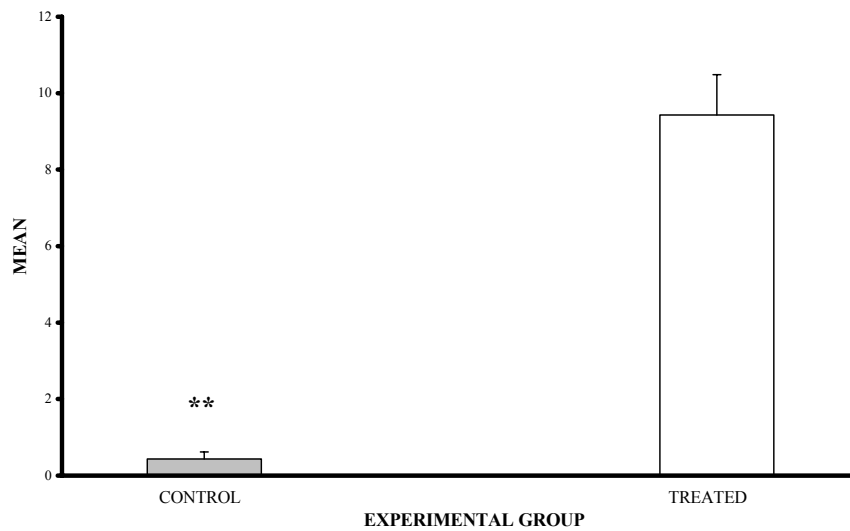
Left bar represents percentages of male and female / litter in 5-AZA-CdR *in utero* exposed offspring (n=10), while the right bar represents the control group (n=7). One-way analysis of variance (ANOVA) and Student's T test were calculated * = Statistically Significant (P< 0.05). Statistically significant difference (p<0.05) was observed on males in relation to females in treated group.



**= Statistically different from control (P<0.01)

FIGURE II. 3. MEANS OF MOUNT LATENCY IN 5-AZA-CdR *IN UTERO* EXPOSED MALE MICE.

Left bar represents the 5-AZA-CdR exposed mice (n=12), while right bar represent the control group (n=12). One-way analysis of variance (ANOVA) and Student's T test were calculated. **= Statistically different from control (P<0.01). Higher latency values were observed in exposed mice when compared to their controls.



** = Statistically different from control (P<0.01)

FIGURE II. 4. MEANS OF MOUNT NUMBER/MALE IN 5-AZA-CdR *IN UTERO* EXPOSED MALE MICE.

Left bar represents the 5-AZA-CdR exposed mice (n=12), while right bar represent the control group (n=12). One-way analysis of variance (ANOVA) and Student's T test were calculated. **= Statistically different from control (P<0.01). A statistically significant (P<0.01) lower mean of mount number was observed in exposed males when compared to the controls.

NEGATIVE
CONTROL.

The 402 bp band represent the Sry product and the 230 bp band represents the GAPDH product (PCR internal control). PCR product of both genes was observed in 5-AZA-CdR exposed and control male mice. No Sry product was observed in Control and 5-AZA-CdR *in utero* exposed females while GAPDH product was present in all the analyzed samples.

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CHAPTER III

Susceptibility to Postnatal Growth Retardation Induced by 5-Aza-2'-deoxycytidine *In Utero*: Gender Specificity and Correlation with Reduced Insulin-like Growth Factor 1

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ABSTRACT

The DNA demethylating agent 5-AZA-2'-deoxyxytidine (5-AZA-CdR) alters gene expression in mice exposed during developmental stages and causes malformations and growth suppression. The aim of this study was to determine if 5-AZA-CdR-induced growth retardation is associated with alterations in energy metabolism or in serum IGF-1 levels. Mice were exposed *in utero* to 5-AZA-CdR at gestation day 10. At postnatal day 21, exposed pups were weaned and body weights recorded. At 3 months of age, reproductive capacity was studied. At 5 months old, after body weight was recorded, mice were killed and serum was collected to determine serum glucose, corticosterone, and IGF-1 levels. The body weights of both treated males and females were reduced at weaning compared with controls, but by 5 months of age, only the male body weight was affected. Reproductive capacity of males and females was reduced with males being more affected. Levels of corticosterone and glucose were not altered. Serum IGF-1 levels were lower in males exposed *in utero* to 5-AZA-CdR when compared to controls, but not in females. Our data suggest that the decreased levels of IGF-1 associated with the treatment could partly contribute to the observed growth retardation in the *in utero*-exposed mice. A gender specific effect, where males are more affected, is evident.

Keywords: 5-AZA-deoxycytidine; *in utero* exposure; growth retardation; reproduction; gender dimorphism.

INTRODUCTION

A large body of evidence demonstrates that DNA methylation plays a role in regulating gene expression in animal cells (Attwood et al., 2002; Razin and Cedar, 1991; Santos et al., 2002) and is important for genome function during development and in adults (Reik and Dean, 2001). The demethylating agent 5-AZA-2'-deoxycytidine (5-AZA-CdR) is a potent teratogen and embryo/fetal growth suppressor in rodents (Branch et al., 1996; Branch and Henry-Sam, 2001; Cummings, 1994; Schmahl et al., 1984; Vlahovic et al., 1999). Recently, we demonstrated persistent suppressive effects of embryonic exposure to 5-AZA-CdR on body weights and reproduction in adult offspring (Cisneros et al., submitted).

Among the possible mechanisms of this permanent effect of embryonic exposure to this demethylating agent are interference with energy metabolism and reduction in circulating levels of insulin-like growth factor 1 (IGF-1), a major regulator of adult body size. IGF-1 is important for both prenatal and postnatal development (Butler and LeRoith, 2001; Liu et al., 1998), and mediates the effects of growth hormone (GH), the major regulator of postnatal development (Garcia-Aragon et al., 1992). IGF-1 knockout mice are born small in size and, if they survive, show poor postnatal development (Liu et al., 1998), suggesting that the effects of GH on postnatal growth are dependent on IGF-1 expression (Le Roith et al., 2001). There is also evidence that IGF-1 can act on growth independently of growth hormone (Zapf, 1998).

To determine if 5-AZA-CdR-induced growth retardation is associated with alterations in energy metabolism or serum IGF-1 levels, serum levels of corticosterone, glucose and IGF-1 were measured in mice exposed to 5-AZA-CdR *in utero*. While corticosterone and glucose were not significantly different after prenatal 5-AZA-CdR exposure, circulating IGF-1 levels were markedly reduced, in correlation with lower body weights of the male offspring.

MATERIALS AND METHODS

Eighteen timed pregnant CD-1 mice were purchased from Charles River Laboratories (Raleigh, NC). During the two day acclimation and experimental periods mice were housed individually in polycarbonate cages with Bed-O'cobs bedding (The Andersons, Maumee, OH) in animal rooms at 24° C with a 12 h artificial light cycle (6 a.m. to 6 p.m.). Mice were maintained on commercial laboratory rodent diet 5015 (LabDiet, Richmond, IN) and water *at libitum*.

5-AZA-CdR was purchased from Sigma Chemical Co. (St. Louis, MO) and stored at room temperature. After the period of acclimation, pregnant mice were weighed and assigned randomly to "treated" or "control" groups. To generate 5-AZA-CdR F1, 1 mg/kg of a fresh made solution of 5-AZA-CdR was administered in 0.2 mL sterile saline solution intra-peritoneally (i.p.), at 8 AM on gestation day (GD) 10. Dose calculations were based on the average GD-9 dam weight. Controls were dosed only with 0.2 mL of sterile saline vehicle.

Females were allowed to deliver. At 21 days of age, pups were weaned and body weights were recorded. Males and females were separated then housed four per cage. At 3 months of age, male and female (10 per group) 5-AZA-CdR F1 mice were weighed and mated with control females and males respectively for a 4 day period. Vaginal plug presence (indicative of mating capacity) was checked every morning, while pregnancy (indicative of fertility) was recorded at delivering time. At 5 months of age, body weight of 5-AZA-CdR F1 and control were recorded and mice were humanely killed by cervical dislocation. Blood from each animal was collected by cardiac puncture and allowed to clot at room temperature for 1 hour. Serum was obtained by centrifugation at 14,000 X g for 10 minutes (LeBlanc and Waxman, 1988) and stored at -20° C until assayed. Samples were collected alternately from treated and control mice over a period of 2 and a half hours in the morning. Serum glucose, corticosterone, and IGF-1 levels were determined with kits for glucose/hexokinase (Boehringer Mannheim, Indianapolis, IN), ¹²⁵I-corticosterone (ICN Pharmaceuticals, Costa Mesa, CA), and rat IGF-1 (Diagnostic Systems Laboratories, Houston, TX).

STATISTICAL ANALYSIS

Body weight and reproductive parameter data were analyzed using Statmost software (DataMost Corporation, Salt Lake City, UT). To determine statistical significance, Anova and Student's t test were performed. Serum glucose, corticosterone, and IGF-1 levels data were analyzed statistically using Student's t test, the t test with Welch correction, or linear regression analysis with GraphPad InStat version 3.00, GraphPad Software, San

Diego, CA. A Pearson correlation of male body weight and serum IGF-1 levels analysis was performed using Statmost software.

RESULTS

No maternal toxicity was observed. However, the expected teratogenic effects described previously (Branch et al., 1996) were observed in the offspring. At weaning, body weight means of male and female 5-AZA CdR F1 were significantly ($P < 0.05$) lower (17% for males and 11% for females) than those of age-matched controls (Figure 1). At 3 months of age, body weight means of 5-AZA CdR F1 males were significantly ($P < 0.01$) lower (22%) than those of age-matched controls. Body weight means of 5-AZA CdR F1 females were significantly ($P < 0.05$) lower (13%) than those of age-matched controls (Figure 2). By 5 months of age, a severe, statistically significant 38% reduction ($P < 0.01$) in body weight means of 5-AZA CdR F1 males was evident when compared to the respective controls. Differences in body weight means were observed in 5-AZA CdR F1 females when compared to littermate controls but were not of statistical significance (Figure 3).

At 5 months, neither average serum corticosterone nor average serum glucose was significantly different in 5-AZA CdR F1 male or female, compared with controls (Figure 4). IGF-1, by contrast, was significantly reduced in 5-AZA CdR F1 males compared with the controls ($P < 0.01$) (Figure 5).

Based on the hypothesis that IGF-1 was a major determinant of body weight linear regression was carried out. *In utero* exposed and control groups data sets were combined to show a positive relationship between serum IGF-1 and body weight ($r = 0.47$; $P = 0.0027$) (Figure 6).

To confirm the effects of *in utero* exposure to 5-AZA-CdR on reproduction, described previously (Cisneros et al., submitted), in the present experimental context, a mating test was performed at 3 month of age. Presence of vaginal plug was indicative of mating capacity while pregnancy was indicative of fertility. Pregnancy was evident in all females that presented vaginal plug. A significant, 75% reduction ($P < 0.01$) in mating capacity and fertility was observed in control females mated with males exposed to 5-AZA-CdR *in utero* ($P < 0.01$) (Figure 7). A lower effect, a 30% reduction ($P < 0.05$), was observed in females exposed to 5-AZA-CdR *in utero* mated with control males, indicating a more strongly reproductive malfunction of 5-AZA-CdR *in utero* exposed male than in female mice. These results are consistent with the more marked effects observed in the male offspring with regard to body weight and IGF-1 levels.

DISCUSSION

The data presented here confirm our recent findings (Cisneros et al., submitted). *In utero* exposure to the demethylating agent 5-AZA-CdR results not only in teratogenesis and reduced fetal size previously reported (Branch et al., 1996), but also in continued weight suppression after birth. The fact that this male weight suppression becomes more

prominent with the increasing age of the offspring indicates that it is not due simply to low birth weight. We have found that this reduced weight may have some association with a permanent (at least by 5 months of age) reduction in average serum IGF-1, and that persistence of these effects in the adult offspring is more marked in the male offspring. Effects of IGF-1 on postnatal growth have been substantiated (Dyck et al., 1999). There is a moderate positive linear relationship ($r=0.47$) between male body weight and IGF-1 protein levels. However the strength of the association (r^2) indicates that the lower IGF-1 levels do not fully explain the lower weight achievement. Therefore, the weight effect may be due to additional factors and not to lower IGF-1 protein levels alone. The significantly lower IGF-1 levels that occur in treated males when compared to control males (and the lack of this phenomenon in the females), the increase severity of the weight decrements in males and the male specific decrease in reproductive capacity suggest that the effects of the demethylating agent on the adult offspring were hormonally mediated in a gender-specific way.

The reduction in reproductive efficacy in the males in this and previous studies (Cisneros, et al., submitted) is consistent with gender-specific hormonal effects. Growth hormone is the major regulator of serum IGF-1 level (Le Roith et al., 2001), but the gender specificity and age-related nature of the observed effects suggests that sex hormones are mediators. Both male and female sex hormones control and interact with the growth hormone-IGF-1 axis (Mauras, 2001; Mauras, 1999; Mauras et al., 1996). The observed higher sensitivity of the males is consistent with the fact that the female pattern of gene expression is the default situation; the male pattern is established by testosterone-

dependent imprinting during embryonic and fetal development (McCarthy, 1994). This imprinting may be deranged by the exposure to 5-AZA-CdR, with this chemical acting as an endocrine disruptor. This is an interesting hypothesis for further experimentation.

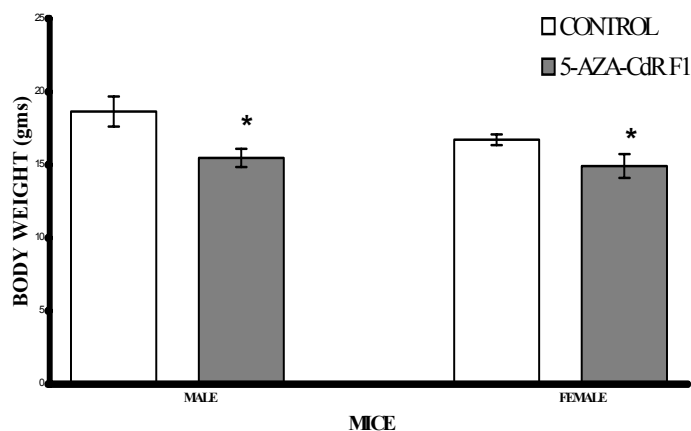
IGF-1 has been associated with reproductive activity. Males with the insuline-like growth factor-1 (IGF-1) gene deleted fail to display mating behavior (Garofalo, 2002) and has been implicated in a variety of reproductive processes (Dyck et al., 1999). Increasing evidence indicates that IGF-1 has an important role in CNS development. The role of IGF-1 in myelination during normal early development has been substantiated (Ye et al., 2002). Therefore, the lowered serum IGF-1 levels may have contributed to the lowered reproductive success by interfering with the normal brain development or altering the hypothalamic-pituitary-gonadal axis (Bartke, 1999). This suggests the possibility of a male behavior alteration induced by the toxic insult.

CONCLUSION

Findings of this study constitute the first *in vivo* evidence of postnatal retardation caused by a demethylating agent associated with serum IGF-1 levels. In addition, it presents the first evidence of gender differences in the response to an intrauterine demethylating insult. The data presented here will help in the understanding of the molecular mechanisms of intrauterine demethylating insult and how this alters postnatal development and individual performance through the entire life of exposed individuals.

ACKNOWLEDGEMENTS

This work was supported by NIH # ES08452, National Institute of Environmental Health Sciences.



* = Statistically different from control ($p < 0.05$)

FIGURE III. 1. BODY WEIGHT OF CD-1 MICE EXPOSED *IN UTERO* TO 5-AZA-CdR (21 DAYS).

One-way analysis of variance (ANOVA) and Student's T test were calculated. * Statistically different from control ($p < 0.05$). Error bars represent standard error. Body weight means of 56 control males, 49 5-AZA-CdR F1 males, 60 control females and 49 5-AZA-CdR F1 females are presented. *In utero*-exposed male and females present statistically ($p < 0.05$) lower body weight means when compared to controls.

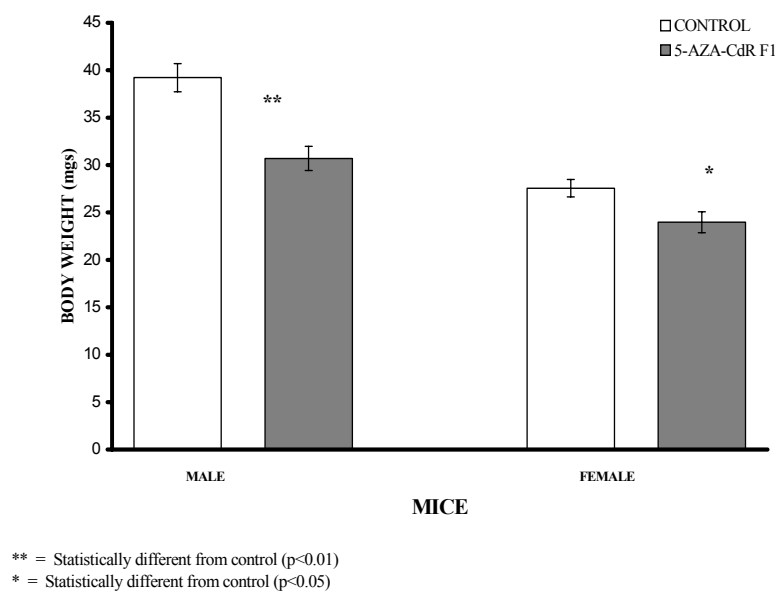


FIGURE III. 2. BODY WEIGHT OF CD-1 MICE EXPOSED *IN UTERO* TO 5-AZA-CdR (3 MONTHS).

One-way analysis of variance (ANOVA) and Student's T test were calculated. ** = Statistically different from control ($p < 0.01$). Error bars represent standard error. Body weight means of 26 control males and females and 20 5-AZA-CdR F1 males and females are presented. *In utero*-exposed male present statistically ($p < 0.05$) lower body weight when compared to controls. No body weight means difference was observed in females.

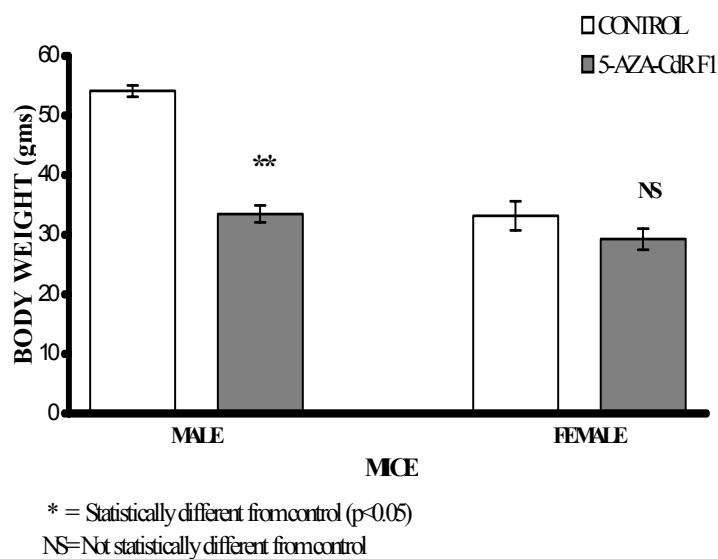


FIGURE III. 3. BODY WEIGHT OF CD-1 MICE EXPOSED *IN UTERO* TO 5-AZA-CdR (5 MONTHS).

One-way analysis of variance (ANOVA) and Student's T test were calculated. ** = Statistically different from control ($p < 0.01$). Error bars represent standard error. Body weight means of 12 control males and females, 26 5-AZA-CdR F1 males and 16 5-AZA-CdR F1 females are presented. *In utero*-exposed males present statistically ($p < 0.05$) lower body weight when compared to controls. No body weight means difference was observed in females.

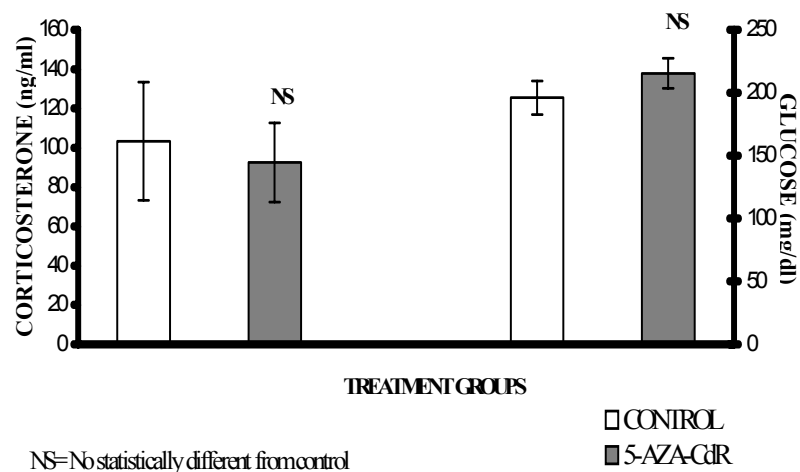


FIGURE III. 4. SERUM CORTICOSTERONE AND GLUCOSE IN CD-1 MALE MICE EXPOSED *IN UTERO* TO 5-AZA-CdR.

One-way analysis of variance (ANOVA) and Student's T test were calculated. NS = Not Statistically different from control. Error bars represent standard error. Serum corticosterone and glucose levels means of 12 control males and females, 26 5-AZA-CdR F1 males and 16 5-AZA-CdR F1 females are presented. No statistically differences in serum corticosterone and glucose levels were observed in 5-AZA-CdR F1 males or females (not shown) when compared to controls.

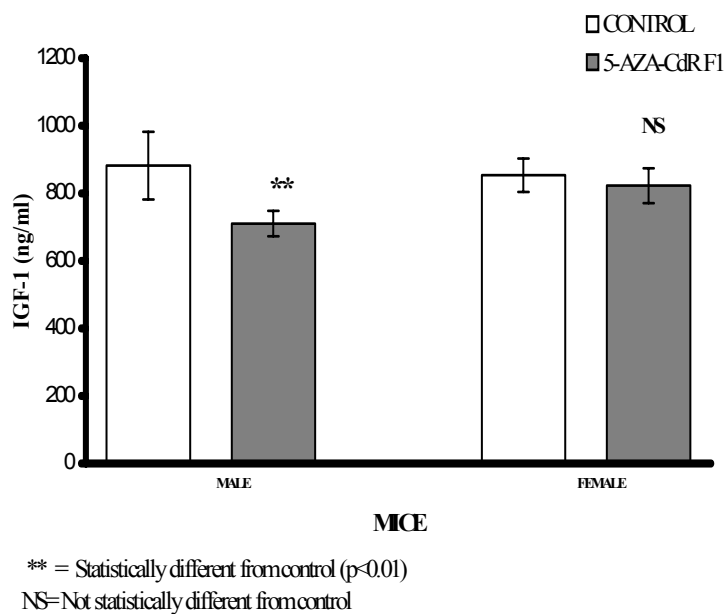


FIGURE III. 5. SERUM IGF-1 IN CD-1 MICE EXPOSED *IN UTERO* TO 5-AZA-CdR.

One-way analysis of variance (ANOVA) and Student's T test were calculated. ** = Statistically different from control (p<0.01). NS = Not Statistically different from control. Error bars represent standard error. Serum IGF-1 levels means of 12 control and 26 5-AZA-CdR F1 males are presented. *In utero*-exposed male mice present statistically (p<0.01) lower levels of serum IGF-1 when compared to controls. No difference was observed in females.

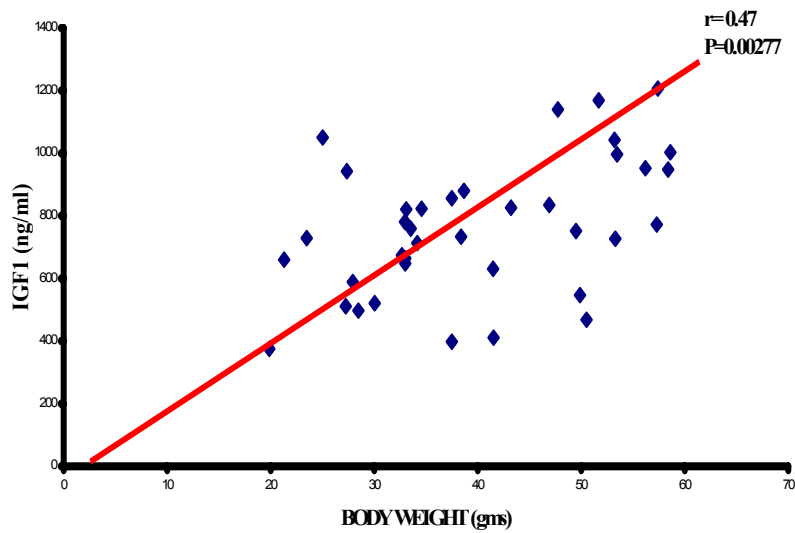


FIGURE III. 6. CORRELATION OF SERUM IGF-1 AND BODY WEIGHT IN CD-1 MALE MICE.

12 control and 26 5-AZA-CdR F1 males combined are presented. Pearson correlation analysis was conducted. 5-AZA-CdR F1 and control male combined data shows a positive correlation between serum IGF-1 and body weight ($r=0.47$; $P = 0.0027$).

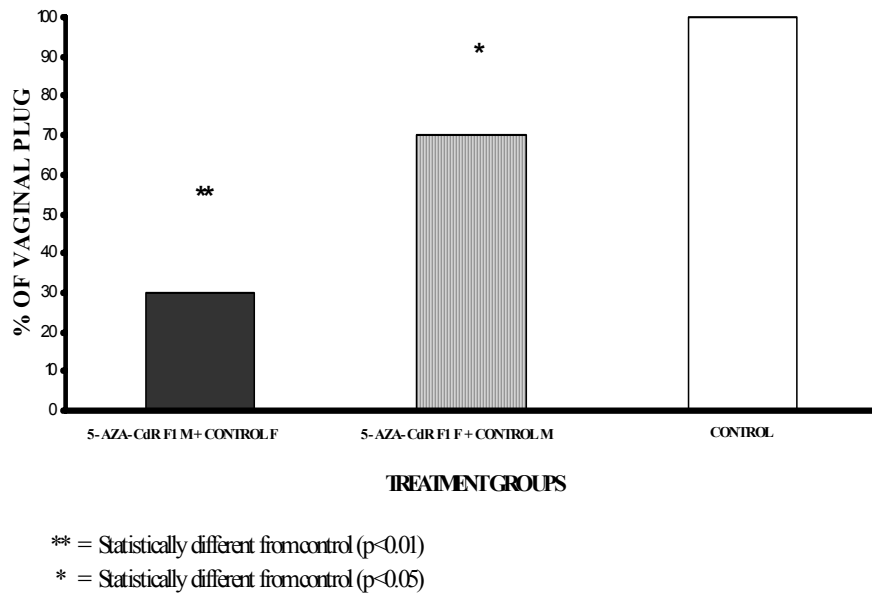


FIGURE III. 7. PERCENTAGES OF VAGINAL PLUGS IN CD-1 MICE EXPOSED *IN UTERO* TO 5-AZA-CdR.

One-way analysis of variance (ANOVA) and Student's T test were calculated. ** = Statistically different from control ($P < 0.01$), * = Statistically different from control ($P < 0.05$). Error bars represent standard error. $n=10$. Statistical differences ($P < 0.05$) and ($p < 0.01$) respectively were observed with vaginal plug when *in utero* exposed males are mated with control females. Pregnancy was observed in all females that presented vaginal plug.

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CHAPTER IV
EPIGENETIC INHERITANCE OF 5-AZA-2'-DEOXYCYTOSINE
INDUCED ALTERATIONS

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RUNNING HEAD: 5-AZA-CdR EPIGENETIC INHERITANCE

ABSTRACT

DNA methylation has been considered an epigenetic modification able to be cleared and reset in either the germ line or during embryogenesis. In recent years, evidence indicates that epigenetic inheritance exists, and it may represent a major source of phenotypic variation and sporadic diseases. The role of DNA methylation in embryo development is evident. 5-AZA-2'-deoxycytosine (5-AZA-CdR) is a potent inhibitor of DNA methylation controlling gene expression. The aim of this study was to determine if 5-AZA-CdR would induce transmissible alterations in development. A multigenerational study was designed in which three generations were examined. At gestation day (GD) 10, pregnant mice were administered 1 mg/kg of 5-AZA-CdR. Male and female offspring were mated avoiding consanguinity. In each generation, 50% of pregnant mice were killed and fetuses examined via skeletal analysis and necropsy. The remaining 50% were allowed to give birth to produce the next generation. Leg and tail abnormalities, abnormal male mating behavior, retarded post natal body development and cleft palate, as well as global DNA hypermethylation (in palate tissues of GD 17 fetus) levels were observed in 5-AZA-CdR F1 offspring. In the F2 generation, cleft palate and global DNA hypermethylation (in palate tissues of GD 17 fetus) levels were evident. In the F3 generation, an even higher incidence of cleft palate was observed. Cleft palate was observed in offspring of *in utero* exposed male and female mice. Our data suggest that altered methylation patterns (induced by 5-AZA-CdR embryonic exposure) may be inherited by the offspring of exposed parents leading to altered development.

Keywords: epigenetic inheritance, DNA methylation, germ line, embryogenesis, 5-AZA-CdR, cleft palate.

INTRODUCTION

Although genomic methylation patterns in somatic differentiated cells are generally stable and heritable [73], it was believed that acquired epigenetic modifications, such as DNA methylation or stable chromatin structures, were cleared and reset on passage through the germline [69, 78, 86]. Therefore, in mammals such epigenetic modifications could not be inherited through the germline to future generations [49]. Numerous early publications have indicated that specific manipulations of early embryos, such as nuclear transplantation, can result in heritable altered patterns of gene expression and induce phenotypic alterations at later stages of development [51, 52, 62, 74]. Roemer (1997) reported that repression and DNA methylation of genes encoding major urinary proteins, olfactory marker protein, and reduced body weight can be experimentally induced by nuclear transplantation in early embryos [74] and those acquired phenotypes are transmitted to most of the offspring of manipulated parent mice. [78]. In recent publications, Morgan and collaborators described the inheritance of an epigenetic modification at the agouti locus in mice [62]; Sutherland and collaborators reported the germline inheritance of transcriptional silencing in mice, and reversion to activity after as many as three generations in the silent state. These studies support the theory that silent genetic information in mammals are inherited and later reactivated, implying a mode of phenotypic inheritance less stable than Mendelian inheritance [86].

DNA methylation is a major epigenetic modification of the genome. It is one of the most studied epigenetic modifications of DNA in protozoa and metazoa [72]. Methylation

occurs after DNA replication and involves the transfer of a methyl group from 5-adenosyl-methionine (SAM) to the 5' position of cytosine residues, in a reaction catalyzed by the enzyme DNA methyltransferase (Dnmt 1) [67]. In most eukaryotic DNA, 60 - 90% of methylcytosine residues occur at CpG dinucleotide sequences [6, 7, 29, 48, 76]. But just 3 to 5% of the total cytosine residues in the DNA are converted to 5' methylcytosine [19, 25, 71, 75, 89].

In mammals, methylation of the 5' position of cytosine residues is a reversible covalent modification of DNA in the palindromic sequence 5'-CpG-3' and occasionally 5'-CpNpG-3' [63]. DNA methylation occurs primarily in areas where CpG density is low. It is estimated that CpG islands (areas with high CpG density) are associated with about half of all mammalian genes [2]. Unmethylated CpG islands are associated with housekeeping genes, while the islands of many tissue-specific genes are methylated, except in the tissue where they are expressed [93]. The density of methylation and the location of methylated sequences are important factors [26]. Few methylated CpGs can silence a weak promoter, whereas a higher density of methylation is required to repress a strong promoter [8]. Although the repression is greater if the promoter itself is methylated [60], distant methylated sequences can contribute to gene repression [34]. In addition, interactions between proteins and DNA are changed by methylation, leading to alterations in chromatin structure and either a decrease or an increase in the rate of transcription [39].

DNA methylation is essential to control a number of important biological mechanisms in mammals [38, 65, 72, 73, 77] including genome organization and chromosome stability

[20, 24, 84], gene expression (silencing some genes and activating others) [72], cell differentiation [43], genomic imprinting [73], X chromosome inactivation [31], cancer [4, 24], chromatin modification [64], aging [21, 32, 35] and development [36, 46, 54, 58, 64, 73, 79, 90].

Epigenetic reprogramming in germ cells and early embryos is critical for imprinting, and imprinting plays crucial roles in protecting the genome integrity [73] establishing nuclear totipotency and stem cell differentiation in animal development [86]. Mammalian development is accompanied by two major waves of genome-wide demethylation and remethylation: one during germ-cell development and the other after fertilization [24, 33, 45, 61, 80, 81, 85]. Highly methylated genes in sperm are rapidly demethylated in the zygote shortly after fertilization, before the first round of DNA replication commences, while the oocyte-derived maternal alleles either remain methylated after fertilization or become further methylated de novo. Paternally (sperm)-derived sequences are exposed to putative active demethylases in the oocyte cytoplasm while maternally (oocyte)-derived sequences are protected from this reaction [59].

During embryogenesis, methylation patterns are reprogrammed genome wide, generating cells with a broad developmental potential. Passive genome-wide demethylation after fertilization by the lack of maintenance methylation following DNA replication cell division [33, 80] and replication-independent demethylation during early embryogenesis have been suggested [46]. Active demethylation of single copy genes in the mammalian

zygote and a striking asymmetry in epigenetic methylation reprogramming has been indicated as well [66].

Although Dnmt1, a maintenance methylase with preference in vivo for hemimethylated DNA, has been characterized [5] suggesting a mechanism by which specific patterns of methylation in the genome could be maintained [28, 57]. Enzymes that can methylate DNA de novo (Dnmt3a and Dnmt3b) and some of the enzymatic related mechanisms have been identified [64, 94], and their biological purposes are being studied and interpreted [56]. The mechanisms that establish methylation patterns during development are not completely understood [30]. What is known is that the complete process in mammals involves maintenance methylation, de novo methylation and demethylation in which genome wide reprogramming of methylation patterns takes place [73]. Adult methylation patterns are reproduced at each round of cell division [88]. The precise pattern of cytosine methylation varies according to the cell type and developmental stages [16].

The effects of methylation on gene expression and specifically imprinted genes [53] and some retrotransposons [91] have been characterized. Studies in knockouts of the methylase genes result in embryo lethality or developmental defects. However, the role of methylation in the control of gene expression during normal development is still in debate [87, 90]. Therefore, the mechanisms for genome-wide alterations in methylation and the relationship to abnormal development still remains to be discovered [53, 64].

5-AZA-2'-deoxycytosine (5-AZA-CdR), a cytidine analog with a nitrogen atom substituting the carbon in the 5' position of the heterocyclin ring [67] is able to inhibit DNA methylation [40]. It reduces the enzymatic activity of Dnmt1 via the formation of a stable complex between the enzyme and 5-aza-cytosine substituted DNA [82]. Therefore, the newly synthesized DNA in exposed cells becomes significantly hypomethylated [41]. 5-AZA-CdR has been used to inhibit DNA methylation [50, 58, 70] to study gene expression and cellular differentiation [27, 37, 44]. In addition in the last few years, its teratological effects in rodents have been demonstrated [9-11, 13].

To determine if epigenetic alterations induced by 5-AZA-CdR, expressed as altered morphological development and global DNA methylation levels, could be inherited, a multigenerational study was designed in which three generations were examined. Our data show that although the degree of penetrance of malformations is low in exposed offspring, altered methylation patterns induced initially by 5-AZA-CdR embryonic exposure are not completely cleared and are able to be inherited through the germ line. This information suggests the existence of interactive mechanisms between genetics and epigenetics to produce teratological effects on *in utero* exposed animals. In addition, this type of interaction could play a role in susceptibility and disease incidence in unexposed animals that are offspring of exposed ones.

MATERIALS AND METHODS

ANIMALS

Timed pregnant CD-1 mice were purchased from Charles River Laboratories (Raleigh, NC). During the two day acclimation and experimental periods mice were housed individually in polycarbonate cages with bed-o'cobs bedding in animal rooms at 24°C with a 12 h artificial light cycle (6 a.m. to 6 p.m.). Mice were maintained on commercial laboratory rodent diet 5015 (LabDiet, Richmond, IN) and water *ad libitum*. F1 and F2 offspring of treated and control mice were weaned at 21 days of age. Males and females were separated then housed four per cage. At ten weeks of age, offspring were mated in single pairs avoiding consanguinity to produce the next generation.

5-AZA-CdR TREATMENT

5-AZA-CdR was purchased from Sigma Chemical Co. (St. Louis, MO). After the period of acclimation in our animal facilities, pregnant mice were weighed and assigned randomly to "treated" or "control" groups. To generate 5-AZA-CdR F1, on gestation day 10 (GD-10), 1 mg/kg of 5-AZA-CdR was administered in 0.2 ml sterile saline solution intra-peritoneally (i.p.). Controls were dosed with only 0.2 ml of sterile saline vehicle. Females were allowed to deliver, and pups grew up to reach sexual maturity.

GENERATION OF OFFSPRING

At 10 weeks of age, male and female 5-AZA-CdR F1 and control offspring were mated to generate F2. Four different mating groups (ten pairs per group) were established: 1) F1 males with control females; 2) F1 females with control males; 3) F1 males with F1 females avoiding consanguinity, and 4) Control males and females. Several mating incidents were required to generate 5-AZA-CdR F2 offspring since 5-AZA-CdR F1 presented poor reproductive capacity. At GD-17, pregnant females of groups 1, 2 and 4 were killed and fetuses collected. One half of them were used for skeletal analysis and the other half for fetal necropsy. Pregnant females from the group 3 were allowed to give birth the 5-AZA-CdR F2 offspring. These pups were weaned at 21 days and allowed to reach maturity. At 10 weeks of age 5-AZA-CdR F2 offspring from group 3, were mated among themselves avoiding consanguinity to generate 5-AZA-CdR F3 generation. Fifty percent of F2 mothers were allowed to give birth and the other 50% were killed and fetuses collected for analyses. Three multigenerational studies were conducted. The first study was carried to F3 generation. The following two study replicates were carried to F2 generation because enough evidence of epigenetic heritability was observed in such generation.

SKELETAL ANALYSIS

After collection at GD-17, F2 and F3 fetuses in the first study and F2 in the following study replicates were weighed and stored in 95% ethanol for 24 hours. Fetuses were

subsequently skinned and stained with Alcian Blue solution (Eastman Kodak, Rochester, NY) for cartilage, cleared with 1% KOH and then stained with Alazarin Red solution (Fisher Scientific, Fair Lawn, NJ) for bone. A detailed skeletal examination was conducted. All cranial, hyoid bones and sternebrae were examined for irregularities in size, shape, ossification degree or total absence. The axial skeleton was examined for any alteration in number and anatomical features of the vertebral bones and ribs. Pelvic and pectoral girdles as well as fore and hind limb bones were examined for any abnormality in size, shape, axis deviation, ossification degree or absence.

FETAL NECROPSY

F2 and F3 fetuses in the first study and F2 in the following study replicates were collected at GD-17. After fixation in 95% ethanol, a standard necropsy protocol was followed. A detailed examination was conducted. An external body exam was conducted in which ears, eyes, head, dorsum, tail, ventral wall, limb, digits, jaw, nares, and skin were analyzed. This was followed by an internal exam to analyze alterations in visceral anatomy.

GLOBAL DNA METHYLATION STATUS

The global DNA methylation status of 5-AZA-CdR F1, F2 and control fetuses was determined using the cytosine extension reaction described by Pogribny and collaborators [68]. Three separate experiments were conducted in duplicate. Genomic DNA from

palate of affected 5-AZA-CdR F1, F2 and control GD 17 fetuses was isolated using DNeasy Tissue Kit (Qiagen Inc., Valencia, CA). Approximately 1.00 ug of genomic DNA was digested for 12 hours with 10 units of HpaII endonuclease in a 20 uL mixture containing DNA, 10 units of HpaII (Promega, Madison, WI), 2.0 uL of 10 X Buffer for HpaII and pico pure (pp) water. Another aliquot was incubated without restriction enzyme to serve as background control.

The cytosine extension reaction was performed in a 25 ul reaction mixture containing 0.5 ug of DNA, 2.5 uL of 10 X PCR buffer without MgCl₂, 1.0 uL of 25 mM MgCl₂, 0.1 uL of Taq polymerase (TaqPlus 5u/uL. Stratagene, La Jolla, CA), 0.1 uL of 3HdCTP (43 Ci/mmol)(Perkin Elmer, Boston, MA) and pp water. The mixture was incubated at 56°C for 1 hour, then placed on ice. Duplicate 10 uL aliquots from each reaction were placed on Whatman DE-81 ion exchange filters (Fisher Scientific, Atlanta, GA) and washed three times with Na-phosphate buffer (pH 7.0) at room temperature. After drying, filters were processed for scintillation counting. Background radiolabel incorporation was subtracted from enzyme-treated samples to obtain the relative 3HdCTP incorporation /0.5 ug of DNA. The extend of 3HdCTP incorporation opposed the exposed guanine after restriction enzyme treatment is directly proportional to the number of unmethylated CpG sites.

METHYLATION STATUS OF SONIC HEDGEHOG AND HOX A-11 GENE PROMOTER REGIONS

Three experiments were conducted. One aliquot of Genomic DNA from palate of affected 5-AZA-CdR F1, F2 and control fetuses was restriction cut with HpaII and XbaI in a 20 uL mixture. The mixture contained 500 ng of DNA, 2 uL of 10X Multicore Buffer, 1 uL (10 u) of HpaII, 1 ul (10 u) of XbaI, 0.2 uL of BSA and pp water. Another aliquot of DNA was restriction cut with XbaI only, used as methylation sensitive restriction control. The reaction mixture was incubated at 37 °C for 90 minutes, followed by a 10 minute period at 70°C. The samples were then maintained at 4°C. The incubation took place in a Bio-Rad Cycler (Bio-Rad Laboratories, Hercules, CA).

Based on the known Shh promoter region sequence (NCBI Accession # AF098925 and AF019387) gene promoter primers were designed. For Hox a-11 promoter primers the known Hoxa11 gene sequence (NCBI Accession # MMU20371) was used [55]. Shh and Hox A-11 gene promoter regions presence was detected by PCR. GAPDH was used as internal PCR control. Amplification was run in duplicate. 100 ug of DNA were used in a 50 uL PCR reaction. 0.5 uL of Taq polymerase (TaqPlus 5u/uL. Stratagene, La Jolla, CA) was used with 100 mM each dNTP (Promega Corporation, Madison, WI), 20 mM Shh promoter primers (5' -TGCTGGGATAGATTGGAAGG- 3' and 5' -ACACATCGGAGTTGGGTCTC- 3', synthesized by Operon Technologies, Alameda, CA), 20 mM GAPDH specific primers (5' -TGTGAACGGATTTGGCCGTA -3' and 5' -TCGCTCCTGGAAGATGGTGA- 3', synthesized by Genosys) and 20 mM HOX A-11 promoter primers (5' -AGGAGGATCTGGAGAGTGGTTGGA -3' and

5'ATTTGTAGAAAGGCAATGGAAGGC 3', synthesized by Operon Technologies, Alameda, CA). A Bio-Rad Cyclor (Bio-Rad Laboratories, Hercules, CA) was used with the following program conditions: 95°C for 3 minutes, 55°C for 30 seconds and 72°C for 2 minutes followed by 30 cycles of 95°C for 30 seconds, 55°C for 30 seconds and 72°C for 2 minutes. PCR products were terminated with a final extension at 72°C for 10 minutes.

PCR products were analyzed in 1.5% Agarose gel and their optical densities were calculated using the Bio Image Intelligent Quantifier software (B.I. System Corporation).

DATA ANALYSIS

To determine incidence of morphological defects in 5-AZA-CdR offspring, analysis of variance (ANOVA) and Least Squares Means comparisons of measured developmental parameters using SAS 8.2 Proc GLM (SAS Institute Inc., Cary, NC) was performed. To establish differences of cytosine incorporation among experimental groups, analysis of variance (ANOVA) and Student's T test using StatMost software (DataMost Corporation, Salt Lake City, UT) were performed. To compare PCR product optical densities, analysis of Variance (ANOVA) and Student's T test using StatMost software were conducted.

RESULTS

ANIMALS

The teratogenic effects of 1 mg/Kg of 5-AZA-CdR administered at GD 10 in pregnant mice have been characterized [10]. Some of the effects include morphological alterations in tail and hind limb long bone (phocomelia, meromalia, unossified and reduced fibulae) and low percentages of cleft palate without causing toxicity (weight loss or lethality) to treated dams. In this study, no statistically significant fetal death was observed in 5-AZA-CdR exposed offspring when compared to controls. However, all dead 5-AZA-CdR F1 neonates presented cleft palate. These data corroborate results obtained in previous studies [10].

SKELETAL ANALYSIS

Supernumerary ribs, sternum defects, alterations in number of rib-sternum attachments and caudal vertebrae ossification alterations were observed in 5-AZA-CdR F2 and F3 offspring. However, there were no statistically significant differences when compared to the respective controls.

FETAL NECROPSY

Hydronephrosis, nares malformations and cleft palate were observed in 5-AZA-CdR offspring. In the first study, presence of cleft palate was not statistically significant in 5-AZA-CdR F2 offspring compared to controls. However, statistical significance ($p < 0.05$) was observed in 5-AZA-CdR F3 offspring (Fig. 1). In replicates of the study, presence of cleft palate was significantly ($p < 0.05$ when fathers are exposed and $p < 0.01$ when mothers are exposed) higher in 5-AZA-CdR F2 of exposed mice in relation to controls (Fig. 2). The degree of palate malformation is variable between animals and generations (Fig. 3).

GLOBAL DNA METHYLATION STATUS

Global methylation status of Control and 5-AZA-CdR F1 and F2 offspring are presented in Figure 4. Lower incorporation of 3HdCTP is observed in the 5-AZA-CdR F1 and F2 when compared to the control group. However, statistical significance ($p < 0.05$) was apparent just for F1. This is indicative of higher methylation levels in global DNA of F1 and F2 treated offspring groups when compared to control group.

METHYLATION STATUS OF SONIC HEDGEHOG AND HOX A-11 GENE PROMOTER REGION

Shh and Hox A-11 promoter and GAPDH PCR products from affected palate tissue of 5-AZA-CdR F1 and F2 and control GD-17 fetuses are presented in Figure 5. No statistical

differences of optical density were observed. This suggests that the methylation status of these regions was not different in the tissue of offspring from exposed lineage when compared to that of controls.

DISCUSSION

DNA methylation is one of the major epigenetic modifications of the genome [6, 7, 29, 48, 72, 76]. It is essential to control a number of important biological mechanisms in mammals [38, 65, 72, 73, 77]. The role of methylation in the control of gene expression during normal development is still in debate [87, 90]. However, gene expression [72], cell differentiation [43], genomic imprinting [73], and X chromosome inactivation [31] are some of the functions attributed to be controlled by DNA methylation. All of these functions are essential to normal embryo development; therefore, the role of DNA methylation in gene expression during normal fetal development is evident [36, 46, 54, 58, 64, 73, 79, 90]. In this and previous studies [9, 12, 14], 5-AZA-CdR, a demethylating agent, administered in developing embryonic mice was able to alter normal development inducing malformations.

The effects of methylation on gene expression and specifically imprinted genes [53] and some retrotransposons [91] have been characterized. However, it has been believed that epigenetic modifications are acquired in the developing mouse embryo and erased in the germline, to restore the totipotency of the genome in the next generation [49]. In the last years, other evidence indicated that epigenetic modifications at some mammalian alleles are not completely erased from one generation to the next, resulting in complex patterns of inheritance that do not conform to Mendelian principles [62, 69, 86]. This phenomenon has been named "Epigenetic Inheritance" which is a type of heredity based on chromatin structure rather than DNA sequence [86].

Results reported in this study suggest that altered DNA methylation patterns caused by the 5-AZA-CdR intrauterine insult were not completely erased in the germline. In the first multigenerational study that was carried to F3, presence of cleft palate (not statistically significant) was observed in F2. In F3, the incidence was higher reaching statistical significance ($p < 0.05$). In replicates of the study carried to F2, presence of cleft palate was significantly ($p < 0.05$ when fathers are exposed and $p < 0.01$ when mothers are exposed) higher in F2 of exposed mice in relation to controls. These data suggest that as in other studies [92], epigenetic information was retained during gametogenesis and development or if it was lost it was recaptured during these processes.

Global DNA methylation levels in 5-AZA-CdR F1 offspring was significantly ($p < 0.05$) higher than control group. Although not statistically significant, absolute 3H dCTP incorporation values were lower (suggesting higher methylation levels) in 5-AZA-CdR F2 than in control group. This suggests a role of the mother in maintaining the correct methylation levels in developing embryos. A possible explanation is that the maternal environment, in an attempt to compensate the DNMT enzymatic depletion caused by 5-AZA-CdR, over-produces DNMT. Then, when the demethylating agent is cleared, the excess of DNMT forces the DNA methylation machinery to incorporate more methyl groups in the novo DNA and consequently a hypermethylated DNA is produced.

These results indicate that after a first insult with 5-AZA-CdR to developing mouse embryos, methylation patterns were altered, and passed through the germ line to their offspring. This finding corroborates those of Sutherland (2000), who suggested that

epigenetic modifications can be maintained on passage through the germline. In that study, an acquired epigenetic change in the M α 7 transgene is maintained in the germline and is not erased on formation of gametes [86].

Phenotypic variations in absence of genetic differences suggesting that a number of loci that control transcription in the mouse genome could be epigenetically affected have been reported [1, 18, 86]. The inverse relationship between DNA methylation and the degree of malformation in cancer has been published [47]. In our study, the degree of cleft malformation is variable among litters and generations. This could contribute to the lack of statistical significance of global DNA methylation between 5-AZA-CdR F2 and control group. However, the biological significance is obvious because the same pattern was observed in each of the three conducted studies. A three fold decrease in absolute dCTP incorporation was observed in 5-AZA-CdR F1 offspring and a one and a half fold decrease was observed in 5-AZA-CdR F2 when compared to controls. This could be due to variations in epigenetic alterations in individual gametes or later developmental stages, especially prior to and during implantation when rapid methylation changes occur in the mouse genome [61].

Mammalian development is accompanied by two major waves of genome-wide demethylation and remethylation: one during germ-cell development and the other after fertilization [24, 33, 45, 61, 80, 81, 85]. Highly methylated genes in sperm are rapidly demethylated in the zygote shortly after fertilization, before the first round of DNA replication commences, while the oocyte-derived maternal alleles either remain

methyated after fertilization or become further methylated de novo. Paternally (sperm)-derived sequences are exposed to putative active demethylases in the oocyte cytoplasm when maternally (oocyte)-derived sequences are protected from this reaction [59]. Therefore, parent of origin specific erasure does exist suggesting the existence of epigenetic inheritance through one [62, 69, 92]. In the present study, cleft palate was observed in offspring of treated males or females; therefore, no sex specific epigenetic inheritance is evident.

Global DNA methylation status as well as the methylation status of two promoter regions of genes that have previously been shown to be altered by a 5-AZA-CdR insult [12, 14] were determined in this study. Although approximately 70% of CpG dinucleotides are methylated [3], the majority of unmethylated CpG occur in small clusters known as CpG islands. Higher levels of global DNA methylation, expressed by lower dCTP incorporation, are observed in 5-AZA-CdR F1 and F2 offspring when compared to controls. This suggests that malformations observed in exposed offspring could be caused by altered global DNA methylation patterns.

The role of Shh and Hox A-11 genes in limb patterning has been very well documented and characterized [83] [12, 15, 17, 23]. In addition, it has been suggested that Shh regulates the outgrowth and subsequent fusion of the facial primordia [95]. A 2-fold down-regulation of Hox A-11 [12] and a 2-fold up-regulation of SHH was observed in hind limb bud twelve hours after 5-AZA-CdR *in utero* exposure of fetuses at GD 10 [14]. Some of the phenotypic characteristics of 5-AZA-CdR *in utero* exposed mice include

high incidence of hind limb and tail malformations as well as low incidence of cleft palate. CpG islands are commonly found within or near promoters or first exons of housekeeping genes [7]. It is estimated that CpG islands are associated with about half of all mammalian gene promoter regions [2]. The islands of many tissue-specific genes are methylated, except in the tissue where they are expressed [93]. Density and location of methylated sequences are important factors [26] because a weak promoter can be silenced by only a few methylated CpGs, whereas a higher density of methylation is required to repress a strong promoter [8]. The methylation status of Shh and Hox A-11 promoter regions of genomic DNA extracted from palate tissue was investigated. No differences between 5-AZA-CdR F1 and F2 offspring and control groups were observed, suggesting that the methylation status of promoter regions of these two genes in palate tissue were not affected by the toxic insult. Therefore, hypermethylation observed in treated offspring did not occur in CpG islands of the promoter regions of Shh and Hox A-11 genes. Therefore, the cleft palate observed in 5-AZA-CdR F1 and F2 offspring is not associated with changes of methylation status in these promoter regions. Nevertheless, methylation changes in other genes could occur affecting the expression of genes associated with the observed malformations.

Since the precise pattern of cytosine methylation varies according to the cell type and developmental stages [16] more studies designed to elucidate the methylation status of promoter regions of these and other developmentally related genes in different tissues and developmental stages were essential.

During embryogenesis, methylation patterns are reprogrammed genome wide, generating cells with a broad developmental potential. A DNA maintenance mechanism that includes a maintenance methylase (Dnmt1) [5, 28, 57] has been identified as well as enzymes that can methylate DNA de novo (Dnmt3a and Dnmt3b) [64, 94]. To exert its effect, 5-AZA-CdR must be incorporated into the DNA [22]. Then, DNA substituted with 5-AZA-CdR forms covalent adducts with cellular DNA methyltransferase, thereby depleting the cells of enzyme activity and causing demethylation of genomic DNA as a secondary effect [42]. Opposite to the expected results, our data indicate that embryo mice 5-AZA CdR *in utero* exposed and its offspring presented higher level of global DNA methylation when compared to controls, suggesting again the mother's role in maintaining the correct methylation levels in developing embryos.

Rakyan and collaborators (2001), have postulated two models of how incomplete erasure and stochastic re-establishment of epigenetic marks could generate phenotypic diversity in the absence of genetic or environmental influences, and how phenotype can be inherited to the next generation. In these models, the allele of primordial germ cells of silent mice is initially methylated. In early gametogenesis, incomplete erasure of the mark results in some immature gametes having an allele that is still methylated. For penetrant mice, the allele is initially unmethylated, and the inability of the cell to erase the mark is inconsequential. Reestablishment of the mark is stochastic, but because some cells in the silent germline carry alleles that are already methylated, the overall proportion of mature gametes containing methylated alleles will be greater in silent mice. Genome wide epigenetic reprogramming during early embryogenesis would faithfully replicate the

original mark that was present in the mature gamete. In the second model, the original mark present in the primordial germ cell might be unchanged in epigenetic reprogramming during gametogenesis, and incomplete erasure of the mark and subsequent stochastic reestablishment would then occur in the pre-implantation embryo. For both models, the silent mice will have a higher percentage of silent offspring compared with litters from penetrant mice [69]. Data presented in this report corroborate the penetrant model described above.

It has been suggested that methylation levels in the mouse decline sharply to approximately 30% of the typical somatic levels [45, 61]. Therefore, up to 30% of methylated areas in the genome of the new zygote are not reset. When 5-AZA-CdR offspring carrying altered methylation patterns are mated they are able to transmit the altered methylation patterns to the offspring because approximately 30% of the altered methylation areas are maintained. This explains the low penetrance of observed malformations.

In conclusion, intrauterine exposure to 5-AZA-CdR alters the normal methylation patterns of the developing conceptus. This alteration elicits teratological effects that are not cleared through the germ line or during embryogenesis. Therefore inheritance of altered epigenetic modifications induced by 5-AZA-CdR occurs. Recent consideration has been given to the importance of epigenetic inheritance as a possible predisposing factor for disease. The findings reported in this study would help to understand the long term risk of individuals exposed to 5-AZA-CdR and possibly other demethylating agents

and their offspring. It may represent a major source of phenotypic variation and sporadic diseases risk if even a small proportion of human genes is subject to epigenetic inheritance.

ACKNOWLEDGMENTS

The authors want to thank Dr. Igor Pogribny and collaborators for sharing the cytosine extension assay protocol.

SUPPORTED BY NIH ES08452

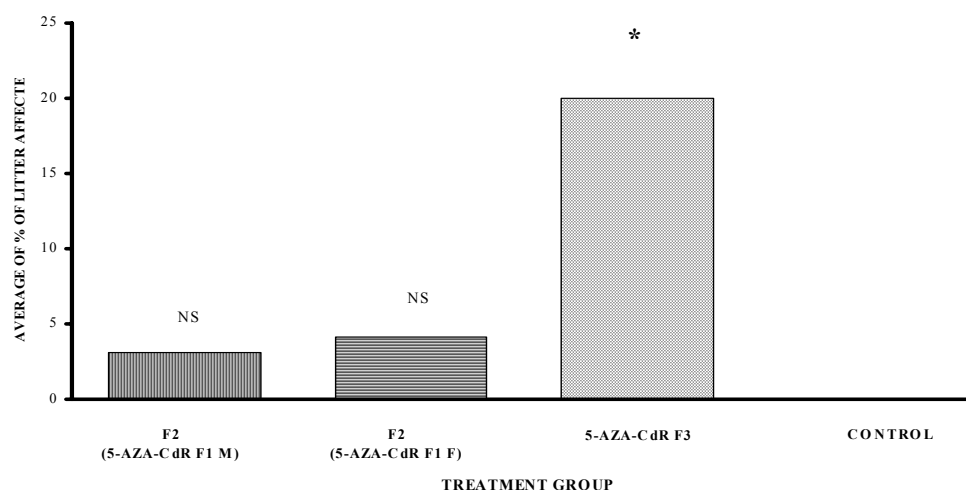


FIGURE IV. 1. INCIDENCE OF CLEFT PALATE IN 3 GENERATIONS OF 5-AZA-CdR *IN UTERO* EXPOSED MOUSE.

From left to right, the first bar represents the affected offspring of 5-AZA-CdR F1 males mated with control females (n=5). The second bar represents the affected offspring of 5-AZA-CdR F1 females mated with control males (n=9). The third bar represents the affected F3 offspring of 5-AZA-CdR F2 males mated with 5-AZA-CdR F2 females (n=10). The last bar represents the affected offspring of control mice mated among themselves. Analysis of variance (ANOVA) and Least Squares Means comparisons were performed. * = Statistically different from control (p<0.05). NS= Not statistically different from control. Error bars represent standard error. Not statistically significant cleft palate presence was observed in 5-AZA-CdR F2 when 5-AZA-CdR F1 males or females are mated to control females or males respectively. However, statistical significantly (p<0.05) higher incidence was observed in 5-AZA-CdR F3 offspring when both parents were 5-AZA-CdR *in utero* exposed mice.

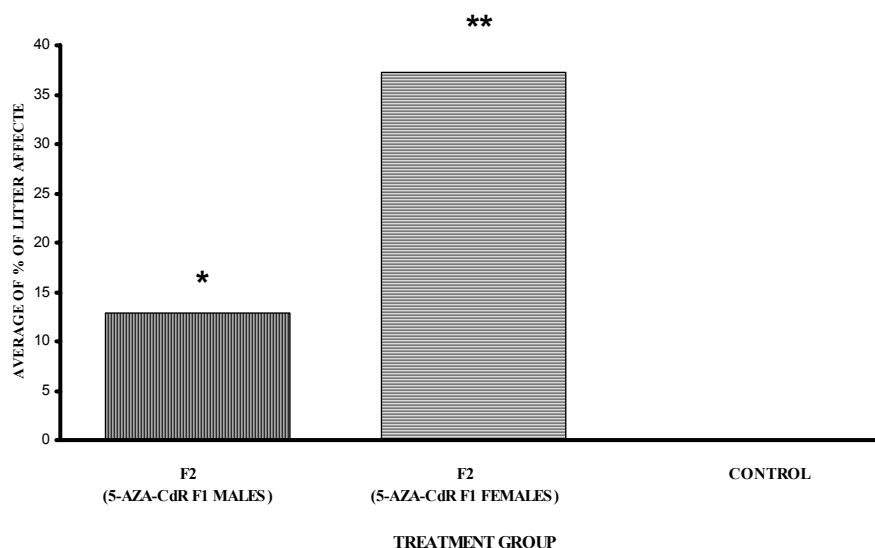


FIGURE IV. 2 . INCIDENCE OF CLEFT PALATE IN 2 GENERATIONS OF 5-AZA-CdR IN UTERO EXPOSED MOUSE (SECOND REPLICATE)

From left to right, the first bar represents the affected offspring of 5-AZA-CdR F1 males mated with control females (n=5). The second bar represents the affected offspring of 5-AZA-CdR F1 females mated with control males (n=8). The third bar represents the affected offspring of control mice mated among themselves (n=10). Analysis of variance (ANOVA) and Least Squares Means comparisons were performed. * = Statistically different from control (p<0.05). ** = Statistically different from control (p<0.01). Error bars represent standard error. Statistically significant (p<0.05) cleft palate presence was observed in 5-AZA-CdR F2 when 5-AZA-CdR F1 males were mated to control females. Statistically significant (p<0.01) higher incidence of cleft palate was observed in 5-AZA-CdR F2 when 5-AZA-CdR F1 females were mated to control males.

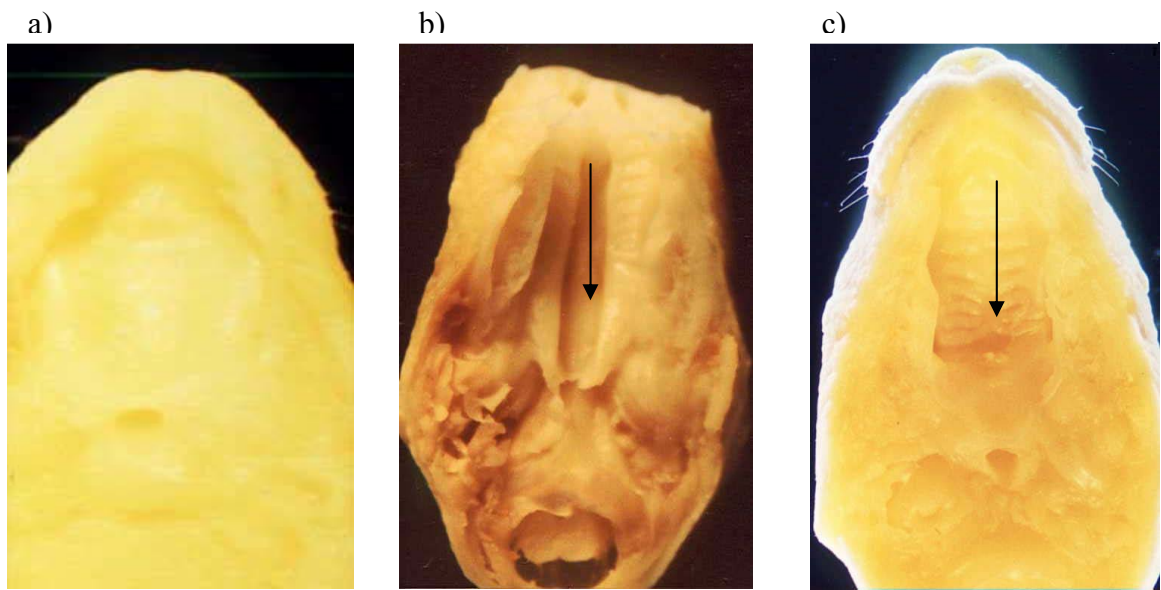


FIGURE IV. 3. ABNORMAL PALATE DEVELOPMENT IN OFFSPRING OF 5-AZA-CdR *IN UTERO* EXPOSED MICE.

a) Palate of control adult mouse. b) Palate of 5-AZA-CdR F1 offspring. A complete secondary cleft palate is evident. 5-AZA-CdR F2 offspring showed similar malformation. 3) Palate of 5-AZA-CdR F3 offspring. Cleft palate and unfused palate rugae is clear. Notice that the degree of malformation varies with and within generations.

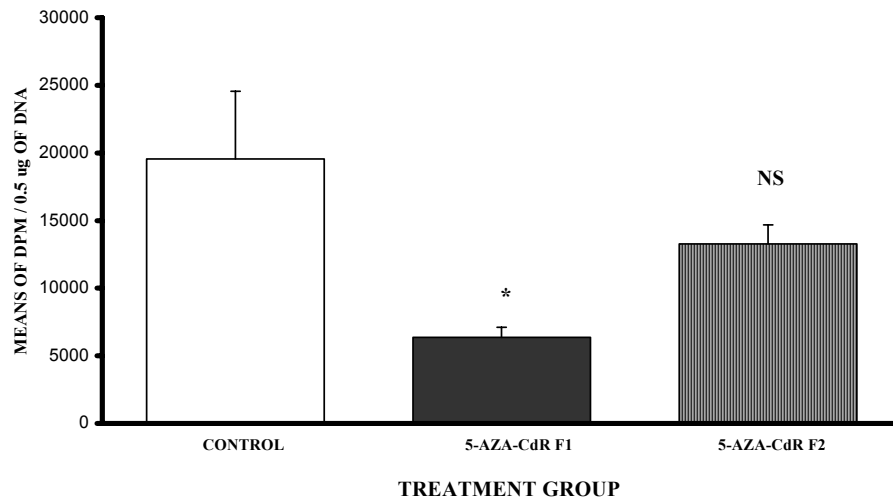


FIGURE IV. 4. LEVELS OF (3H)dCTP INCORPORATION INTO HpaII RESTRICTED GENOMIC DNA OF 5-AZA-CdR *IN UTERO* EXPOSED OFFSPRING.

From left to right, first bar represents absolute (3H)dCTP incorporation in DNA Hpa II cut from control GD-17 fetuses.(n=10) The second bar represents absolute (3H)dCTP incorporation in DNA Hpa II cut from 5-AZA-CdR F1 GD-17 fetuses (n=10). The third bar represents absolute (3H)dCTP incorporation in DNA Hpa II cut from control 5-AZA-CdR F2 GD-17 fetuses (n=10). Analysis of variance (ANOVA) and Student's T test were performed. * = Statistically different from control (p<0.05). NS= Not statistically different from control. Error bars represent standard error. A statistically significant (p<0.05) decrease in absolute (3H)dCTP incorporation was observed in 5-AZA-CdR F1 offspring when compared to controls. No statistically significant decrease of absolute (3H)dCTP incorporation was observed in 5-AZA-CdR F2 offspring when compared to

controls. However, the biological significance is evident because in the three study replicates, the same pattern was observed. Furthermore, the degree of malformation could contribute to the lack of statistical significance.

HpaII & Xba I RESTRICTION CUT DNA



XbaI RESTRICTION CUT DNA

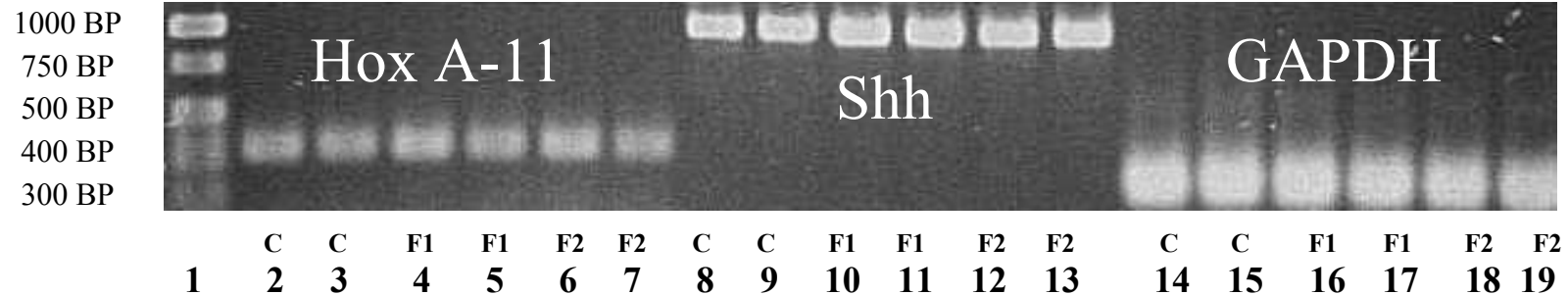


FIGURE IV. 5. PCR-BASED METHYLATION-SENSITIVE RESTRICTION ANALYSIS OF CpG REGIONS OF Hox A-11 AND Shh PROMOTERS.

Line 1= DNA ladder. Lines 2 to 7 present the Hox A-11 promoter region PCR amplification products. Lines 8 to 13 present the Shh promoter region PCR amplification products. Lines 14 to 19 present the GAPDH gene (internal control) PCR amplification products. C=Control. F1= 5-AZA-CdR F1offspring. F2= 5-AZA-CdR F2 offspring.

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SUMMARY AND CONCLUSIONS

DNA methylation is one of the major epigenetic modifications of the genome. It is a mechanism for regulating gene expression that plays crucial roles in cellular differentiation and tissue development in embryogenesis. Therefore, alterations in methylation may significantly affect embryonic and post-natal development. It has been believed that epigenetic modifications are acquired in the developing mouse embryo, and erased in the germline to restore the totipotency of the genome in the next generation. However, in recent years, evidence of the existence of epigenetic inheritance has been reported. 5-AZA-CdR, an agent able to inhibit DNA methylation has been used experimentally to induce gene expression and cellular differentiation. Its teratological effects have been studied and characterized. The aims of this study were: 1) to investigate if epigenetic alterations induced by 5-AZA-CdR, expressed as altered morphological development and global DNA methylation levels, could be inherited in offspring of *in utero* exposed mice; 2) to determine the effects of 5- AZA-CdR on post-natal development and reproductive capacity of CD-1 mice since the effects of this treatment at different post-natal ages through reproductive maturity were not known; 3) to confirm if these effects were due to alterations in energy metabolism or in circulating IGF-I levels of *in utero* exposed mice; and 4) to analyze the nature of the effect of 5-AZA-CdR on male mice reproductive capacity.

Analysis of the F3 offspring of initially *in utero* exposed parents revealed a statistically significant incidence in cleft palate. This increase in cleft palate presence was expected because 5-AZA-CdR F2 males and females were mated among themselves. Thus, in this

generation, the contribution of altered epigenetic information was twice as much than in the F2. In the study replicates, statistically significant presence of cleft palate was observed in 5-AZA-CdR F2 when 5-AZA-CdR F1 males and females were mated to control females and males, respectively, providing evidence of altered methylation patterns' heritability.

5-AZA-CdR inhibits DNA methylation by reducing the enzymatic activity of DNA methyltransferase via the formation of a stable complex between the enzyme and 5-AZA-CdR substituted DNA. As a consequence, a significantly hypomethylated newly synthesized DNA is observed in exposed cells. To determine if global DNA methylation levels were affected by the treatment, global DNA methylation analyses of affected palate tissue in 5-AZA-CdR F1 and F2 and control mice were conducted. Global DNA hypermethylation was evident in exposed 5-AZA-CdR F1 and F2. Interestingly, F2 exhibited a stronger association between treatment and increased levels of methylation when compared to controls. This suggests that malformations observed in exposed offspring could be caused by altered global DNA methylation patterns. A possible explanation to the observed phenomena, which opposes the expected results, implicates the role of the mother in maintaining the adequate methylation patterns in the conceptus. In an attempt to compensate the demethylating action of 5-AZA-CdR, more DNA methyltransferase could be produced. Then, when the demethylating agent is cleared, the over produced DNA methyltransferase forces the methylation machinery to incorporate more methyl groups in the newly synthesized DNA. This results in hypermethylated global DNA levels in *in utero* exposed mice.

Although with descending degree of severity, cleft palate is observed in 5-AZA-CdR F1, F2 and F3. Concomitantly, global DNA methylation levels are descendingly higher in 5-AZA-CdR F1 and F2 when compared to controls. This suggests that, as previously reported, the degree of methylation is proportional to the degree of severity of malformation. In addition, since the degree of cleft malformation is variable among litters and generations, being consistently less severe in F2 and F3 respectively, this could contribute to the observed lack of statistical significance of global DNA methylation between 5-AZA-CdR F2 and control group.

The same altered methylation pattern was observed in each of the three studies conducted in duplicate. A three fold decrease in absolute dCTP incorporation was observed in 5-AZA-CdR F1 offspring and a one and a half fold decrease was observed in 5-AZA-CdR F2 when compared to controls. Therefore, the biological significance of these studies is irrefutable.

Some of the phenotypic characteristics of 5-AZA-CdR *in utero* exposed mice include hind limb and tail malformations, as well as low incidence of cleft palate. A 2 fold down-regulation of Hox A-11, and a 2 fold up-regulation of SHH was observed in hind limb bud twelve hours after 5-AZA-CdR *in utero* exposure of fetuses at GD 10. DNA methylation occurs primarily in areas of low CpG dinucleotide density. CpG islands (clusters of CpG dinucleotides) are commonly associated with promoters or first exons of genes. The importance of density and location of methylation has been substantiated. To clarify if methylation of the promoter regions of Shh and Hox A-11 genes were affected

by the treatment, methylation status of promoter regions of these developmentally related genes were determined. Genomic DNA extracted from malformed palate tissue from 5-AZA-CdR F1 and F2 as well as control GD-17 fetuses was used. No differences between 5-AZA-CdR F1 and F2 offspring and control groups were observed, suggesting that the methylation status of promoter regions of these two genes in palate tissue were not affected by the toxic insult. Therefore, hypermethylation observed in treated offspring did not occur in CpG islands of the promoter regions of Shh and Hox A-11 genes. Thus, the cleft palate observed in 5-AZA-CdR F1 and F2 offspring is not associated with changes of methylation status in these two gene promoter regions. Nevertheless, methylation changes in other genes could occur affecting the expression of genes associated with the observed malformations.

Since it has been believed that methylation patterns are deleted through the germline and reset in developing embryos, studies to determine the effects of 5-AZA-CdR on germ cell methylation levels should be conducted. In addition, the precise pattern of cytosine methylation varies according to the cell type and developmental stages; therefore, more studies designed to elucidate the methylation status of promoter regions of developmentally related genes in different tissues and developmental stages would be essential.

Our data clearly show that altered methylation patterns induced by the demethylating agent 5-AZA-CdR are not completely cleared in the germline. Furthermore, they are inherited in offspring of *in utero* exposed mice. This finding corroborates previous

studies suggesting that epigenetic modifications can be maintained on passage through the germline.

In mice, evidence of inefficient erasure of epigenetic modifications has substantiated two theories explaining the existence of epigenetic inheritance. Our findings support this principle. It has been published that immediately after fertilization the zygote undergoes demethylation, in which the methylation levels decrease to 30% of the somatic cell levels. If the somatic levels are altered (in our case by the 5-AZA-CdR insult) it is clear that up to 30% of the altered methylation pattern would be passed to the next generation through the germ line. This could explain the low penetrance in the observed malformations.

Altered offspring male:female ratio and lower weights in treated F1 males and females were observed due to 5-AZA-CdR *in utero* exposure. Body weight effects were more pronounced with age. Altered offspring sex ratio induced by several toxic agents have been published. The offspring male:female ratio was altered in *in utero* 5-AZA-CdR exposed litter, and manifested as an increase in the number of males as compared to females. Therefore, it is possible that 5-AZA-CdR, through its demethylation capacity, altered the expression of genes involved in sexual differentiation. SRY in humans (Sry in mice) is the Y chromosomal gene responsible for directing the fetal gonad to begin developing testicular tissue. In this study, when investigating for the presence of Sry gene as an indicator of maleness, no treatment effect was observed. This suggests that the *in utero* exposed mice were phenotypically and genotypically males. Therefore, the altered

reproductive capacity and male:female ratios observed in *in utero* exposed male mice cannot be attributed to the absence of Sry gene.

Evidence of the genetic role in growth and reproductive development has been described and influence of heredity on body size has been reported. Our findings suggest that functional defects in development can continue throughout post-natal life. Negative treatment effects on body weight are observed in *in utero* males and females. *In utero* exposed mice never reached the sizes of their peer controls. This suggests treatment induction of altered functional and/or homeostatic processes prevalent through the entire life of *in utero* exposed mice. These findings confirm the hypothesis that in addition to the teratogenic effects, 5-AZA-CdR suppresses post-natal growth, which could be the cause of a delayed maturity, and perhaps affects male sexual capacity.

A significant 5-AZA-CdR adverse effect in mating capacity and fertility was observed in 5-AZA-CdR *in utero* exposed mice. Males were more strongly affected. The role of environmental and genetic factors in mating activity has been substantiated. In this study, experimental and control groups were maintained in similar environmental conditions and fed with the same food and water. Therefore, it is likely that altered mating activity observed and fertility could be caused by genetic changes induced by the treatment.

A coordinated expression of a specific set of genes in a strict spatiotemporal manner is required for normal mammalian gonad development and sexual differentiation. To determine if the toxic insult affected the genesis of male gonads, spermatid head counts,

analysis of testosterone levels and anatomical and histological analyses of testes and epididymides were conducted. No treatment effects were evident, suggesting that gonad differentiation pathways, testosterone production, or spermatogenesis were not altered by the treatment. However, the low fertility observed in 5-AZA-CdR F1 males could be caused by the significantly lower absolute spermatid head counts observed in these mice when compared to controls. Taken together, 5-AZA-CdR insult may affect the sexual behavior of *in utero* exposed males at the central organizational level.

Since many of the genes involved in growth and reproductive development are important for neuroendocrinal signaling and imprinted genes regulate behavior, to clarify if the altered reproductive capacity observed in male 5-AZA-CdR F1 was a behavioral phenomenon, a male sexual behavior test was conducted in AZA-CdR F1 mice. A significant decrease number of mounts and a significant increase in mount latency were observed in *in utero* exposed mice, suggesting that the observed abnormal condition could be caused by altered protein or hormone production (regulated by genes) at central rather than peripheral level.

The brain-pituitary-gonadal axes control the physiology of the reproductive system. Since no treatment effects in testes, epididymis, sperm, or testosterone production while altered sexual behavior were observed, a genetic alteration affecting hypothalamic and/or pituitary development could be the cause of altered sexual behavior in 5-AZA-CdR exposed males.

Since many of the genes involved in sexual differentiation and reproductive development are important for neuroendocrinal signaling, studies to determine the effects of 5-AZA-CdR on brain development would help to establish the mechanisms of the altered male sexual behavior observed in 5-AZA-CdR F1 males. In addition, because the male's capacity to accurately detect pheromones is a major factor to control mating activity in rodents, effects of 5-AZA-CdR on olfactory system development should be considered.

Effects of Insulin Like Growth Factor 1 (IGF-1) on postnatal growth have been substantiated. To investigate if growth retardation was a consequence of altered energy metabolism or serum IGF-1 levels induced by the treatment, 5-AZA-CdR F1 males and females as well as control mice were killed and levels of serum IGF-1, corticosterone and glucose were determined. No treatment effect was observed in serum corticosterone or glucose levels. However, significantly reduced levels of IGF-1 were observed in 5-AZA-CdR F1 males only. These results indicate that the effects of the demethylating agent on the adult offspring were hormonally mediated, in a gender-specific way. The male pattern is established by testosterone-dependent imprinting during embryonic and fetal development. This imprinting may be disturbed by the exposure to 5-AZA-CdR, with this chemical acting as an endocrine disrupter. This is a hypothesis for further experimentation.

IGF-1 has been associated with reproductive activity and has been implicated in a variety of reproductive processes. Increasing evidence indicates that IGF-1 has an important role in CNS development. The role of IGF-1 in myelination during normal early development

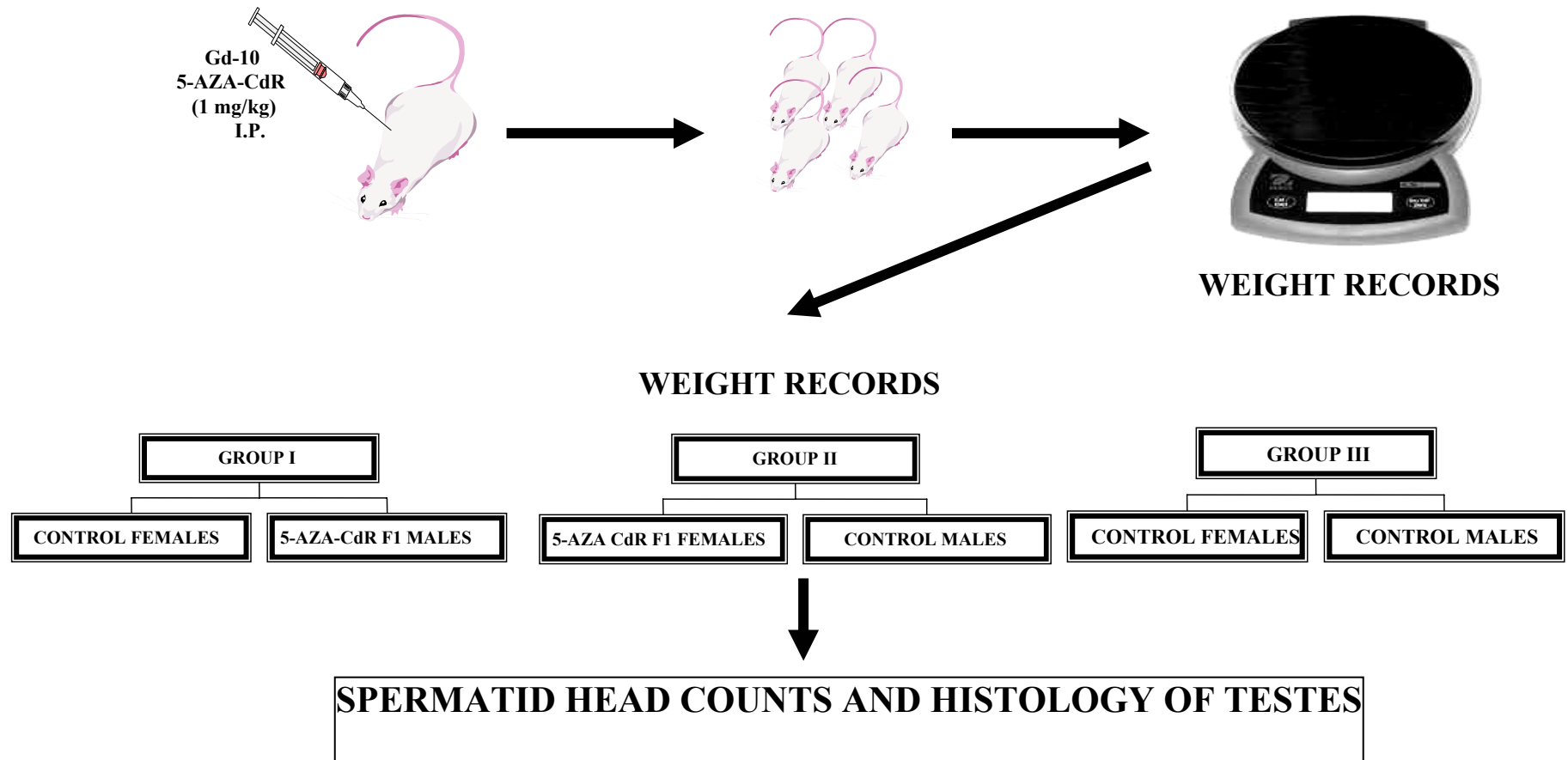
has been substantiated. Therefore, the lowered serum IGF-1 levels observed in 5-AZA-CdR F1 males may have contributed to the lowered reproductive success by interfering with the normal brain development and/or altering the hypothalamic-pituitary-gonadal axis. This corroborates findings obtained from the male sexual behavior test.

In conclusion, *in utero* exposure to 5-AZA-CdR alters the normal methylation patterns of the developing conceptus. This alteration elicits teratological effects causing malformations, postnatal retardation, and altered male sexual behavior. The observed postnatal retardation is a consequence of lower serum IGF-1 levels induced by the treatment.

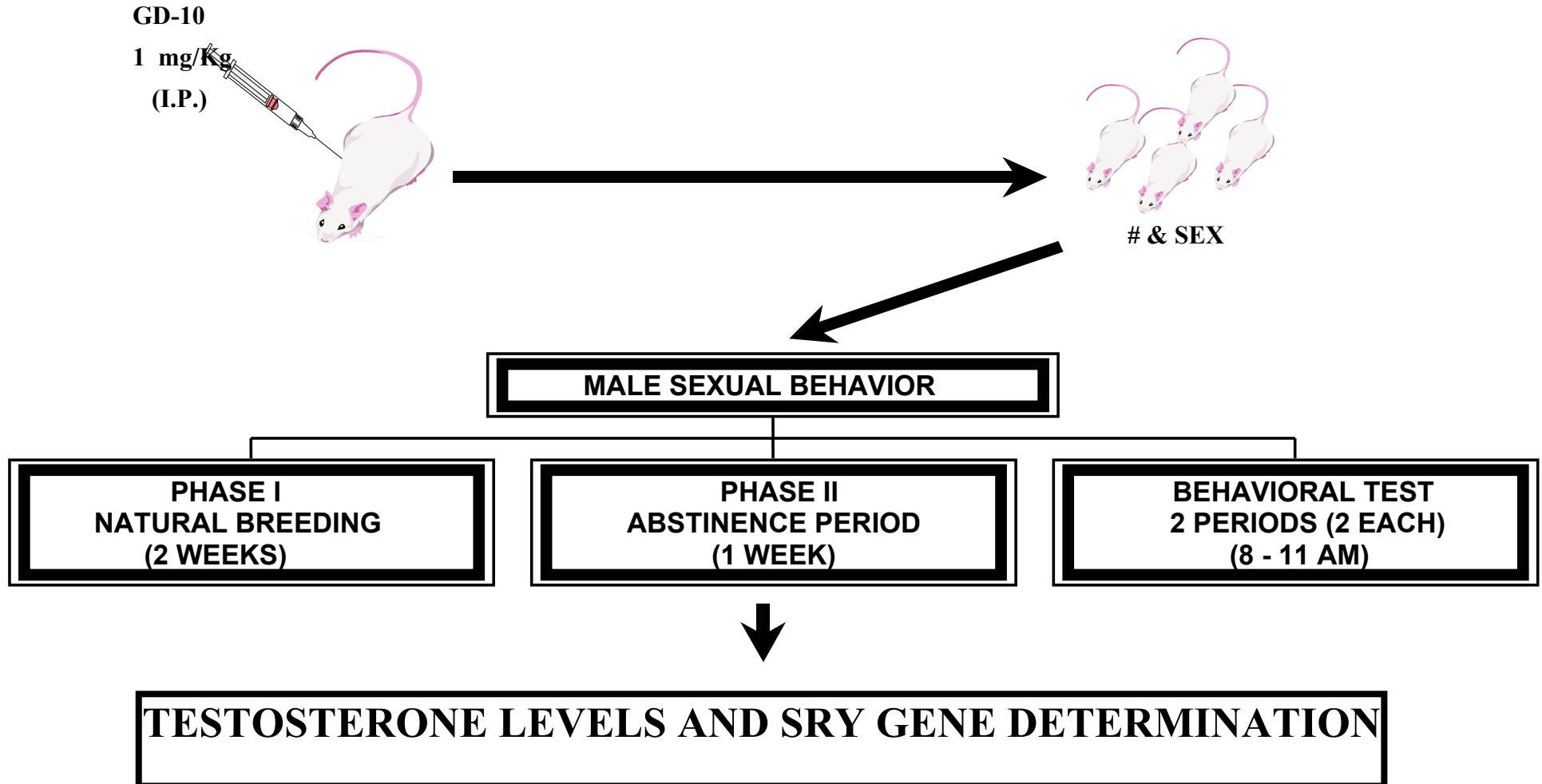
Furthermore, altered methylation patterns induced by 5-AZA-CdR insult are not cleared through the germ line or during embryogenesis. Therefore, they are inherited by offspring of *in utero* exposed mice. This publication presents the first *in vivo* evidence of gender difference in postnatal retardation caused by a demethylating agent associated with serum IGF-1 levels. Findings in this report may serve as the bases to understand the impact of xenobiotics and the role of altered DNA methylation on development through the entire life of *in utero* exposed individuals and their offspring. Epigenetic inheritance may represent a major source of phenotypic variation and sporadic diseases risk if even a small proportion of human genes is subject to epigenetic inheritance.

APPENDICES

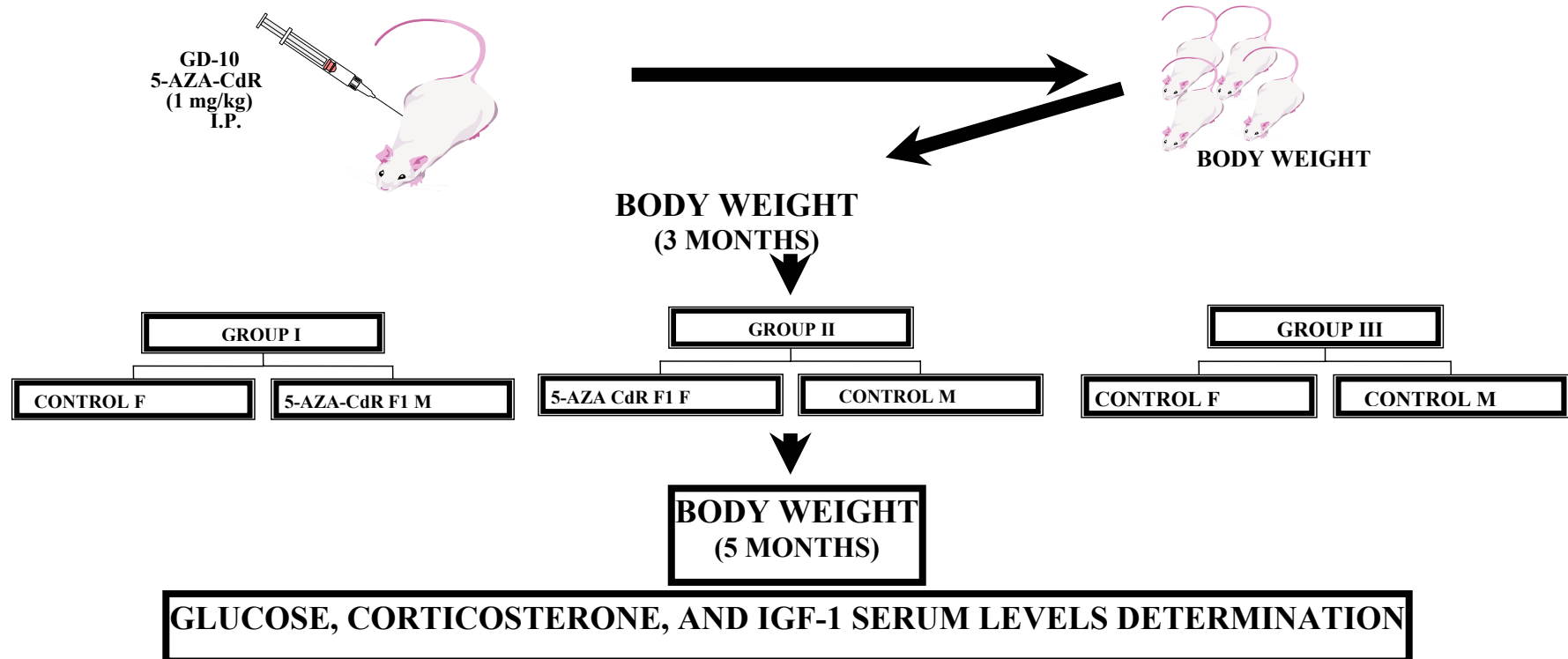
Appendix A. 5-AZA-2' Deoxycytidine (5-AZA-CdR): a Demethylating Agent Affecting Development and Reproductive Capacity: Experimental Design



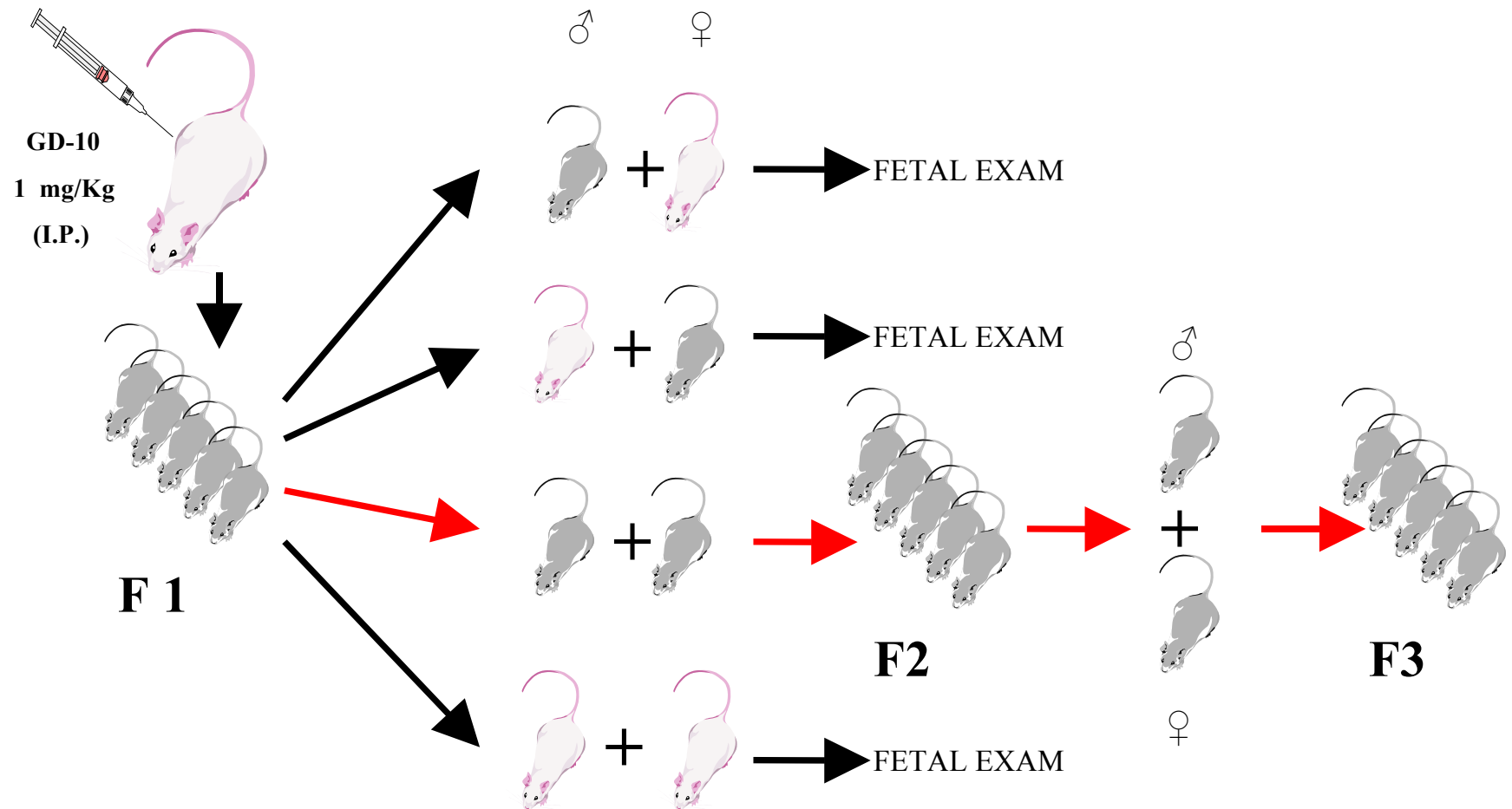
Appendix B. Intrauterine Exposure to 5-AZA-2' Deoxycytidine Affects Sexual Behavior in Male Mice: Experimental Design



**Appendix C. Susceptibility to Postnatal Growth Retardation Induced by
5-Aza-2'-deoxycytidine *In Utero*: Gender Specificity and Correlation with Reduced Insulin-like Growth
Factor 1: Experimental Design**



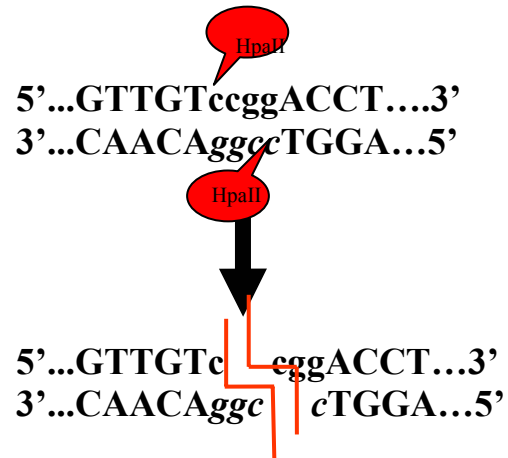
Appendix D. Epigenetic Inheritance of 5-AZA-2'-Deoxycytidine Induced Alterations: Experimental Design



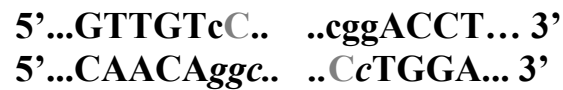
Appendix E: Cytosine Extension Reaction

**Hpa II
restriction**

Non Methylated DNA



**Cytosine
Incorporation**



Methylated DNA

