

UNIVERSIDADE ESTADUAL PAULISTA
“JÚLIO DE MESQUITA FILHO”
FACULDADE DE MEDICINA

Nathalia Pereira de Souza

Isolation and molecular characterization of testicular germ cells from male Sprague-Dawley rats exposed *in utero* and postnatally to dibutyl phthalate or acrylamide

Tese apresentada à Faculdade de Medicina, Universidade Estadual Paulista “Júlio de Mesquita Filho”, Câmpus de Botucatu, para obtenção do título de Doutor em Patologia.

Orientador: Prof. Dr. Samuel M Cohen
Co-orientadores: Prof. Dr. Joao Lauro Viana de Camargo
Dra. Merielen Garcia Nascimento e Pontes

Botucatu
2019

Nathalia Pereira de Souza

Isolation and molecular characterization of testicular germ cells from male Sprague-Dawley rats exposed *in utero* and postnatally to dibutyl phthalate or acrylamide

Tese apresentada à Faculdade de Medicina, Universidade Estadual Paulista “Júlio de Mesquita Filho”, Câmpus de Botucatu, para obtenção do título de Doutor em Patologia.

Orientador: Prof. Dr. Samuel M Cohen
Co-orientador: Prof. Dr. Joao Lauro Viana de Camargo
Dra. Merielen Garcia Nascimento e Pontes

Botucatu
2019

FICHA CATALOGRÁFICA ELABORADA PELA SEÇÃO TÉC. AQUIS. TRATAMENTO DA INFORM.
DIVISÃO TÉCNICA DE BIBLIOTECA E DOCUMENTAÇÃO - CÂMPUS DE BOTUCATU - UNESP

BIBLIOTECÁRIA RESPONSÁVEL: ROSANGELA APARECIDA LOBO-CRB 8/7500

Souza, Nathalia Pereira de.

Isolation and molecular characterization of testicular germ cells from male Sprague-Dawley rats exposed *in utero* and postnatally to dibutyl phthalate or acrylamide / Nathalia Pereira de Souza. - Botucatu, 2019

Tese (doutorado) - Universidade Estadual Paulista "Júlio de Mesquita Filho", Faculdade de Medicina de Botucatu

Orientador: Samuel M Cohen

Coorientador: Joao Lauro Viana de Camargo

Coorientador: Merielen Garcia Nascimento e Pontes

Capes: 40105008

1. Testicular diseases. 2. Germ cells. 3. Molecular biology.
4. Rat. 5. Chemicals physiological effect.

Palavras-chave: Rat; Testicular germ cells; *in utero* exposure; molecular biology.

Dedicatória

“Que os vossos esforços desafiem as impossibilidades, e lembrai-vos de que as grandes coisas do homem foram conquistadas do que parecia impossível.”

Charles Chaplin

Aos meus pais, Magda e Joel, pelo apoio incondicional e por me permitirem sonhar! Nada disso seria possível sem vocês.

*À minha irmã Andréia, pela companhia, pelas conversas, brigas (afinal, somos irmãs!), e por me ensinar o sentido da
palavra amor.*

Este trabalho é dedicado a vocês.

Agradecimientos

Agradecimentos

*À Deus, pelo dom da vida e pelas pessoas maravilhosas que
Ele coloca em meu caminho.*

*Aos familiares que torcem pelo meu sucesso e felicidade, em
especial minhas tias Mara, Marta e Mariliza. À minha vó
Ercina que está sempre presente e aos meus avós, Santo e
Lourdes, que mesmo no céu continuam cuidando de mim.*

*À Carol, Lígia, Rafa (obrigada por me abrigar!), Vivi e Ana,
por todos os anos compartilhados no TOXICAM. Vocês são
mais que colegas de trabalho, são amigas do coração. Toxicats
forever!*

*À Thania, por dividir as dificuldades enfrentadas no
laboratório. Obrigada pela amizade!*

*À professora Lillian, pela generosidade e paciência ao ensinar.
O Toxicam só tem a ganhar com sua experiência!*

*À Amanda, my BFF, por sua amizade e torcida! Sei que posso
contar sempre com você.*

*À Anelise, Raísa, Jéssica, Beatriz, Camila e Milena pela
amizade de infância que permanece até hoje. Thanks girls!*

Aos vizinhos de lab, Amanda e Gabriel, pelas junk foods,

Agradecimentos

passeios, risadas e desabafos compartilhados. Os anos de PG não seriam os mesmos sem vocês! You rock!

*À Taiane, por me ensinar a ser uma pessoa mais íntegra e correta, e por ser a melhor roomie que alguém poderia ter!
Agradeço por poder te chamar de amiga.*

À Thalitta, pela amizade que se consolidou durante esses anos! Obrigada por sempre me mostrar que devemos fazer tudo com amor e empenho.

À Jacutinga, que está comigo (mesmo distante) desde que cheguei a Botucatu. Sua amizade vale muito!

To my friend Karen Pennington, thank you for your company and for cheering me up every time my experiments failed. My days in Omaha would not be the same without you.

To the friends I won during the year in Omaha and who helped me a lot while I was there. In special Carolina, Carla, Micha, Kevin, Scott, Aggelos and Nichole. You all were essential for me to finish my goal, giving me support and making my days lighter and happier.

À Cristina, Vania e ao Paulo, pela ajuda essencial em todos os momentos da Pós-graduação.

Agradecimentos

To Chihiro and Yuri, my Japanese friends!

*To Sheri and her family, who have always been kind to me.
Thank you for the wonderful trip to South Dakota and all the
tours we had.*

*À Coordenação de aperfeiçoamento de pessoal de nível
superior, pelo suporte financeiro e pela concessão da bolsa do
programa PDSE (Processo 88881.132593/2016-01).*

*Ao Conselho Nacional de Desenvolvimento Científico e
tecnológico, CNPq, pela concessão da bolsa de mestrado
(Processo 00140333/2015-0).*

*À Universidade Estadual Paulista (UNESP), que me acolhe e
me oferece estrutura desde à graduação.*

*Ao Núcleo de Avaliação do Impacto Ambiental Sobre a Saúde
Humana, TOXICAM, pelo suporte na realização deste
trabalho.*

*A todas as pessoas que contribuíram para que este trabalho
fosse concluído, meu muito obrigada!*

*Agradecimentos
Especiais*

Agradecimentos Especiais

Ao Prof. Dr. João Lauro Viana de Camargo, o meu mais sincero agradecimento pela oportunidade, pelos conhecimentos transmitidos e pelo exemplo de dedicação à ciência. Obrigada por abrir as portas do TOXICAM e me permitir fazer parte desta família!

To Dr. Samuel Cohen, who is always sharing his knowledge and expertise. Thank you for supporting me during my 1 year in Omaha and for introducing me to your amazing family!

À Dra. Merielen Garcia Nascimento e Pontes, pela orientação na realização deste trabalho. Obrigada!

To Lora Arnold, for her encouragement even when nothing was working with my research. Thanks for your patience and friendship! I am grateful for everything you have done for me.

À Dr Maria Luíza Cotrim Sartor de Oliveira por todo apoio e amizade.

Resumo

O aumento da incidência de distúrbios testiculares e a possível influência de substâncias químicas ambientais, como o dibutilftalato (DBP) e a acrilamida (AA), exigem a identificação de modos de ação. A maioria dos estudos de toxicologia reprodutiva utiliza amostras de RNA provenientes de todo o testículo para avaliar a expressão genica; entretanto, análises de tipos celulares isolados poderiam gerar resultados mais específicos. Entre as células germinativas testiculares, as espermatogônias são importantes pois representam o início da espermatogênese. Este estudo objetivou, 1) estabelecer técnica de isolamento de espermatogônias; 2) aplicar esta técnica para verificar possíveis alterações na expressão gênica (Pou5f1, Kitlg, Mki-67, Bak1 e Spry4) em testículos de ratos pré-púberes (DPN24) e púberes (DPN45) após exposição *in utero* e pós-natal ao DBP ou à AA. A técnica foi eficiente para o isolamento das espermatogônias. A exposição ao DBP levou à redução do peso corporal da ninhada ao nascer, da distância anogenital dos filhotes machos no DPN4 e ao aumento da frequência de retenção de mamilos no DPN14. Os pesos relativos dos testículos expostos ao DBP estavam reduzidos apenas no DPN24. Animais expostos ao DBP mostraram níveis reduzidos de expressão de Pou5f1 e Mki67 no DPN24, e de Pou5f1 e Spry4 no DPN45. A exposição a AA reduziu a expressão de Pou5f1, Mki67 e Spry4, embora não significativamente. Nossos resultados sugerem que DBP atue reduzindo a proliferação celular e prejudicando a diferenciação nos testículos pré-púberes e púberes.

Abstract

The increased incidence of testicular disorders in young men and the possible influence of environmental chemicals, such as dibutyl phthalate (DBP) and acrylamide (AA), requires experimental models for identifying modes of action. Most published reproductive toxicologic studies use RNA samples from the total testis to evaluate testicular gene expression; however, analyses of isolated cell types could provide a more specific tool. Among testicular germ cells, spermatogonia are critical since they represent the onset of spermatogenesis. This study aimed, 1) to establish a technique for spermatogonia isolation; 2) to apply this isolation technique to verify possible gene expression alterations (Pou5f1, Kitlg, Mki-67, Bak1 and Spry4) in prepubertal post-natal day, (PND24) and pubertal (PND45) testes after in utero and postnatal exposure to DBP or AA. The technique was efficient for isolation of a majority of spermatogonia. In utero DBP exposure led to reduced litter body weight at birth, reduced anogenital distance of male pups on PND4, and increased frequency of male nipple retention on PND14 compared to controls. DBP-exposed relative testes weights were reduced only at PND24 compared to control but they did not differ at PND45. DBP-exposed animals showed reduced expression levels of Pou5f1 and Mki67 on PND24, and reduced expression of Pou5f1 and Spry4 on PND45. AA exposure reduced expression of Pou5f1, Mki67 and Spry4 at PND45 although not significantly. Our results suggest that DBP acts by reducing cell proliferation and impairing differentiation in prepubertal and pubertal testes.

Contents

Chapter I.....	1
Literature Review	2
Human testicular disorders	2
Prenatal programming and developmental toxicity.....	3
Male germ cell intrauterine development and spermatogenesis	6
Molecular characterization of spermatogonia.....	10
Undifferentiated spermatogonia isolation protocols.....	13
Environmental chemical exposures and testicular function impairment	16
Justification and objectives	20
References.....	22
Chapter II.....	28
Manuscript.....	29
Abstract.....	30
Introduction	31
Material and Methods.....	35
Establishment of spermatogonia isolation protocol	35
Animals	35
Testes removal	35
a. Enzymatic digestion	36
b. Cell enrichment with a discontinuous density gradient	37
c. Purifying spermatogonia.....	37
Immunohistochemistry validation	39
Environmental exposure protocol.....	40
Chemicals	40
Animals	41
Dose selection: gavage and dietary exposure	42
Experimental design	42
AGD and nipple retention.....	43
Euthanasia and sample collection	43
a. Spermatogonia isolation for PCR analysis	44
b. Testicular histology verification	44

RNA extraction, cDNA preparation and real time PCR	44
Statistical analysis.....	45
Results	47
Spermatogonia isolation and validation	47
Clinical signs (F0 dams) and morphological landmarks (F1 pups) during gestational and lactational period.....	47
Clinical parameters and testes weights (F1)	48
Histologic view and gene expression analysis	48
Discussion.....	50
Acknowledgements	56
Declaration of Conflicting Interests.....	56
Funding	57
References.....	58
Tables	62
Figures	67
Legends of figures.....	72
Conclusion.....	73
Appendix	75

Chapter I

Literature Review

Human testicular disorders

Human male reproductive disorders have increased over the past 5 decades (Xing and Bai 2018). Such disorders may occur in male newborns (cryptorchidism and hypospadias) as well as in young adult males (impaired spermatogenesis and testicular germ cell cancer [TGCT]) (Skakkebaek et al. 2001). There is experimental evidence that several adult male reproductive problems arise *in utero* and are characteristics of testicular dysgenesis syndrome (TDS) (Sharpe and Skakkebaek 2008). Although the worldwide prevalence of TDS is not well established, the majority of cases of cryptorchidism, hypospadias, TGCT, and infertility are thought to most probably be linked to TDS(Xing and Bai 2018).

Cryptorchidism and hypospadias are the two most common genital anomalies in boys (Deng et al. 2016). The incidence of both defects shows geographic variation, and increasing trends have been reported in several countries such as Denmark and Great Britain (Lymeri and Giwerzman 2018). Cryptorchidism is defined as one or both testes not present in the scrotum after birth and failure to descend in the first 6 months of life. Hypospadias occurs when the urethra opens anywhere between the ventral aspect of the glans and the perineum (Xing and Bai 2018).

Ninety-five percent of all human testicular tumors are malignant TGCT, the most frequent testicular cancer in Caucasian males. Its increased incidence (70%) in the last 20 years is probably due to a combined action of

epigenetic and microenvironmental factors (Buljubasic et al. 2018). The incidence of TGCT has doubled in the last 40 years and it is highest in countries of northern Europe, such as in Denmark and Sweden, while it is relatively low in Africa (Buljubasic et al. 2018). TGCT are clinically very important because they usually occur during the most productive age of men, between 20 and 45 years old (Buljubasic et al. 2018).

At the same time, analysis of sperm count data suggests a global downward trend but the results are inconclusive (Merzenich et al. 2010; Swan et al. 2000). The lower limit for the reference value for normal sperm concentration was reduced from $20 \times 10^6/\text{ml}$ to $15 \times 10^6/\text{ml}$ (WHO 2010). In the 1940s the normal limit was considered to be $60 \times 10^6/\text{ml}$. Whether these changes in the reference values reflect improved methodology and knowledge or an actual decline in semen quality at the population level is a matter of discussion (Sharpe and Skakkebaek 2008).

There appears to be a geographical and temporal variation in the prevalence of the four aforementioned disorders probably due to large environmental exposure variations, nutrition and lifestyles among countries (Xing and Bai 2018). It is unclear whether genetic factors might also play a role.

Prenatal programming and developmental toxicity

Epidemiological research on patterns of diseases in human populations and mechanistic studies in animals resulted in the concept of

prenatal programming (Barker 1995a, b). The findings indicate that many processes governing the regulation of our physiology are encoded during the fetal stage (Wintour et al. 2003). In addition, when pregnancy and fetal development are significantly perturbed, the normal course of maturation may then be shunted in a pathological direction, undermining the original plan for a healthy adult phenotype (Coe and Lubach 2008). For example, growth in childhood and timing of puberty were found to be tightly linked to environmental quality (Worthman and Kuzara 2005), and studies of birthweight have concluded that variation in size at birth is essentially determined by the intrauterine environment rather than the fetal genome (Barker 1995b).

During the fetal period, rapid growth and functional maturation occur and continue until after birth. The main feature of growth is cell division. Different body tissues grow during periods of rapid cell division, so-called 'critical' periods. The timing of these critical periods differs for different tissues (Barker 1995b). In this way, the same exposure at different periods would create a different spectrum of outcomes due to the divergent timing of development among the organ systems (Selevan et al. 2000). Therefore, the "programming" concept is a "setting" of physiological function by conditions operating during a sensitive developmental period resulting in long-term effects on function and thereby on health outcomes (Worthman and Kuzara 2005).

Developmental toxicity covers the entire gamut of developmental

exposures and outcomes that may result from exposure prior to conception (either parent), during prenatal development, and postnatally to the time of sexual maturation (Figure 1). The major manifestations of developmental toxicity include death of the developing organism, structural abnormality, altered growth, and functional deficiency (Selevan et al. 2000; USEPA 1991).

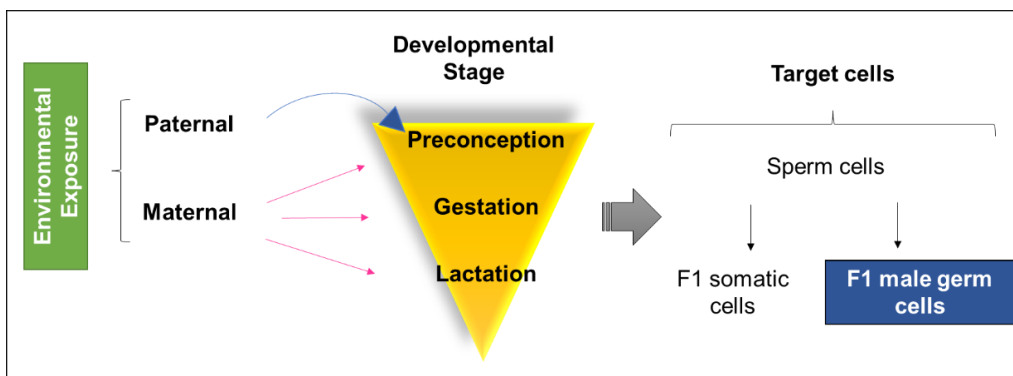


Figure 1. Developmental toxicity mechanism (Adapted from (Ong and Ozanne 2015)).

In laboratory animal studies, the early literature in experimental teratology was dominated by studies with exposures at periods of known high sensitivity for producing certain types of malformations. More contemporary studies, in particular those done for regulatory testing purposes, often include extended periods to simulate long-term human exposure. For example, in prenatal developmental toxicity studies (USEPA 1998a), dosing extends from implantation to term; dosing in two-generation reproduction studies (USEPA 1998b) is for several weeks preconception,

then prenatally and postnatally for two generations (Selevan et al. 2000).

Animal models are frequently used for hazard identification and characterization of the potential risk to human reproduction. Many of the critical steps in the formation of the male reproductive system take place during the embryogenesis process, i.e., the establishment of the primordial germ cell (PGC) lineage (the precursors of eggs and sperm) occurs early during development (Scholer 1991). Any genetic imbalance/gene mutation and/or hormonal deregulation could lead to aberrant sex differentiation, which in the mildest form, is manifested as hypospadias and/or reduced sperm production and in the most severe form as female genitalia. Furthermore, any disturbance in the development of testes in fetal or neonatal life could have a negative impact on reproductive health and function revealed in adult life (Lymperi and Giwercman 2018). Therefore, the prenatal proliferation of PGC and Sertoli cells represents sensitive windows for target toxicity, making the prenatal period a good time for exposure for evaluation in reproductive toxicology studies.

Male germ cell intrauterine development and spermatogenesis

Testicular structure is similar in humans and rodents. They are located in the scrotum, outside of the abdominal cavity, and they are composed of lobules that contain the seminiferous tubules. The seminiferous epithelium consists of Sertoli cells and germ cells. The germ cells are present at different stages of maturation (Haschek and Rousseaux

2013; Tarulli et al. 2012). In both humans and rats, Sertoli cells control germ cell maturation, and they also secrete the anti-Mullerian substance that is responsible for intrauterine testicular development (Haseltine and Ohno 1981). Leydig cells and peritubular cells are also present in the testes (Tarulli et al. 2012) and function as hormonal and structural support elements for the seminiferous tubules.

In humans, PGCs are seen in the endoderm of the allantois on gestational day (GD) 22, and they can be recognized due to the massive amount of alkaline phosphatase. PGCs migrate to the dorsal mesentery, promoting thickening of this epithelium, which becomes the gonadal ridge. Then, in the 4th week of gestation, gonadal ridge invagination occurs with subsequent development of the sexual cords. The human genital system remains undifferentiated until the 9th week of gestation (Haseltine and Ohno 1981).

In mice, PGCs are first identified on GD7 in the epiblast, close to the allantois. On GD10.5, PGCs migrate to the mesonephros promoting epithelial thickening and formation of the gonadal ridge. On GD14.5, PGCs begin a morphological differentiation into gonocytes (Zogbi et al. 2012). In rats, PGCs reach the undifferentiated gonads between GD13 and 15 (Encinas et al. 2012). Studies suggest that gonocytes appear on GD17 (Encinas et al. 2012; Zogbi et al. 2012).

After cellular migration and the establishment of the undifferentiated gonad, the embryo sex will be determined. In mammals, the activation of

SRY (sex-determining region Y) on the Y chromosome marks the onset of sexual differentiation, with testis formation (Barsoum and Yao 2006). Following SRY activation, Sertoli and Leydig cells differentiate and start secreting, respectively, anti-Müllerian hormone (AMH), which leads to the degeneration of the Müllerian duct (Merchant-Larios and Moreno-Mendoza 2001), and testosterone, which promotes Wolffian duct differentiation into the epididymis, vas deferens, seminal vesicles, and ejaculatory duct (Capel 2000; Lymperi and Giwerzman 2018). Gonocytes become quiescent at the end of embryogenesis. Gonocyte proliferation begins after birth (postnatal day [PND] 5 to 7 in rodents and 10-13 years old in humans) resulting in differentiation into spermatogonia (Fayomi and Orwig 2018; Jarvis et al. 2005; Singh et al. 2011).

In rodents, spermatogonia are divided in type A, intermediate and B. The most undifferentiated spermatogonia are called A_{single} , A_{paired} and A_{aligned} . Among the undifferentiated spermatogonia, the spermatogonial stem cells (SSCs) are the adult tissue stem cells in the testis that are at the basis of spermatogenesis and essential for male fertility (Fayomi and Orwig 2018; Phillips et al. 2010). Similar to other adult tissue stem cells, SSCs are rare, around 0.03% of the total germ cell population in mice (Fayomi and Orwig 2018). SSCs are defined by their potential for self-renewal to maintain the stem cell pool, and their ability to differentiate to maintain continuous sperm production in postpubertal males. In the postnatal rodent testis, SSC activity is widely believed to reside in the population of A_{single} spermatogonia

located on the basement membrane of the seminiferous tubules (Fayomi and Orwig 2018). These rare A_{single} cells which mitotically divide once every three days into either new A_{single} or A_{paired} spermatogonia depending on factors in the microenvironment (Singh et al. 2011). Then, A_{pr} spermatogonia produce A_{aligned} spermatogonia that are capable of differentiation into A_1 , the first generation of differentiated spermatogonia. After multiple divisions, there will be A_2 , A_3 , A_4 , intermediate, and B spermatogonia (de Rooij 2009).

The same process occurs in humans but they have only spermatogonia A and B with A_{dark} and A_{pale} being the undifferentiated spermatogonia. Rat A_{single} spermatogonia correspond to human A_{dark} , and $A_{\text{paired}}/A_{\text{aligned}}$ correspond to A_{pale} (Figura 2) (Singh et al. 2011).

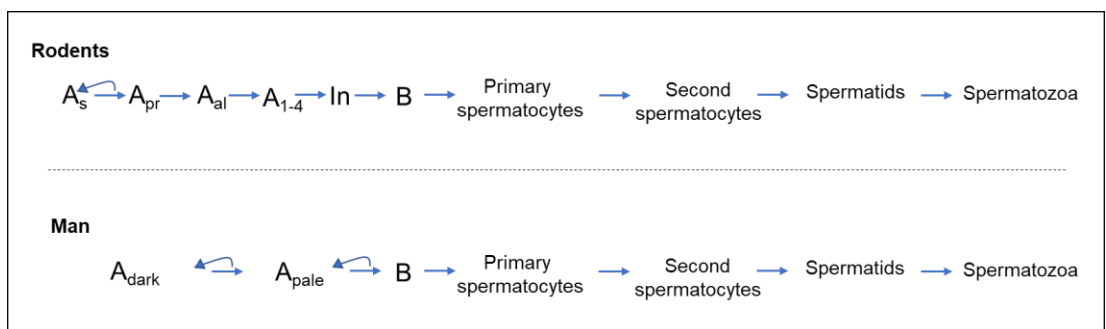


Figure 2. Schematic diagram showing stages of spermatogenesis in mouse and men (Adapted from (Singh et al. 2011).

The spermatogenesis process comprises a series of spermatogonia mitotic divisions and differentiation into spermatocytes that undergo meiosis to produce spermatids which undergo differentiation resulting in

spermatozoa production. This process is continuous and lasts 64 days in humans, 48-53 days in rats and 35 days in mice (Haschek and Rousseaux 2013; Jarvis et al. 2005).

As a clinical diagnosis, the complete depletion of testicular stem cells is defined as Sertoli cell only (SCO) syndrome meaning there are no germ cells in the testicular tubules, while a focal SCO pattern is induced by a partial depletion of testicular stem cells. Studies focusing on germ cell transplantation in mice and monkeys reveal that continuous recolonization of remaining or reintroduced testicular stem cells leads to reestablishment of spermatogenesis (Ehmcke et al. 2006). Therefore, spermatogonial stem cells play a critical role in the spermatogenesis restoration process making spermatogonia selective target cells for the preservation of the testis tissue and function (Ehmcke et al. 2006).

Molecular characterization of spermatogonia

Although spermatogenesis represents a classical stem cell model, its complexity and the lack of good *in vitro* culture systems make it difficult to study the molecular aspects underlying spermatogonial stem cell behavior (Costoya et al. 2004). In seminiferous tubule cross-sections, undifferentiated spermatogonia cannot be distinguished from each other on a morphological basis, which means it is not possible to establish whether a given cell is isolated or in a chain. However, they can be recognized by clonal arrangement in the seminiferous epithelium (single, pairs or chains) (Fayomi

and Orwig 2018; Grisanti et al. 2009).

Among the surface marker-specific antigens for undifferentiated spermatogonia, the promyelocytic leukemia zinc finger (PLZF, also known as Zbtb16) is involved in the regulation of diverse cellular processes, including cell proliferation, apoptosis, differentiation, and development (Costoya et al. 2004; Pearson et al. 2008). Plzf is a widely acknowledged biomarker of type A and B spermatogonia in zebrafish (Ozaki et al. 2011). Immunohistochemical analysis also detected Plzf expression in gonocytes and undifferentiated spermatogonia of mice (Costoya et al. 2004; Pieri et al. 2017). Mice lacking Plzf undergo a progressive loss of spermatogonia associated with an increase in apoptosis and subsequent loss of tubule structure, but without evident differentiation defects or loss of the Sertoli cells (Costoya et al. 2004).

Plzf directly represses the transcription of C-kit, a hallmark of spermatogonial differentiation. The C-kit tyrosine kinase receptor plays an important role in the postnatal stages of spermatogenesis. A point mutation in the C-kit gene blocks the initial stages of spermatogenesis and abolishes DNA synthesis in differentiating A1-A4 spermatogonia, causing infertility (Rossi et al. 2000). It is consistent that Plzf knockout mice show a significant increase of C-kit gene expression in their undifferentiated spermatogonia (Filipponi et al. 2007), suggesting that Plzf maintains the pool of spermatogonial stem cells through direct transcriptional repression of C-kit. C-kit can be re-expressed in spermatogonia following differentiation, but not

in SSCs of adult mouse testis (Fayomi and Orwig 2018). The protein Kit ligand (Kitlg) binds to the C-kit protein and turns the C-kit protein on, which activates other proteins inside the cell by adding a cluster of oxygen and phosphorus atoms at specific positions (Goddard et al. 2007).

In both humans and rodents, the Pou5f1 gene encodes the protein Oct3/4, which is critical in embryonic development and is involved in regulation of cell pluripotency. During normal perinatal maturation, Oct3/4 expression is interrupted (Ferrara et al. 2006). In rats, Oct3/4 was not expressed between GD19 and PND5. Immunohistochemical expression, although weak, was detected in 97% of the germ cells at PND8. However, expression was reduced at PND11 and PND15, where only 4% and 0.5% of cells were Oct3/4 positive, respectively (Zogbi et al. 2012). In humans, OCT3/4 positive germ cells have been used as an immunohistochemical marker of TGCT (Cools 2014).

During normal germ cell maturation, C-kit and Oct3/4 immunochemical expression decreases. Nevertheless, certain conditions/exposures can reprogram the corresponding genes increasing those protein levels in postnatal life (Cools 2014). Therefore, analysis of these genes can provide information about germ cell damage after DBP or AA exposure.

Recent genome wide association studies (GWAS) in humans identified susceptibility loci for testicular disorders such as TGCT near KITLG, SPRY4, and BAK1 (Kanetsky et al. 2009; Rapley et al. 2009). While

the role of the Kit-Kitlg pathway in survival during PGC migration is not completely understood, studies of knockout mice suggest that these genes prevent germ cell apoptosis (Runyan et al. 2006). Spry4 is also associated with the Kit-Kitlg pathway (Frolov et al. 2003) and inhibits the mitogen-activated protein (MAP) kinase pathway (Sasaki et al. 2003). The protein encoded by Bcl-2-antagonist/killer 1 (Bak1) belongs to the Bcl2 protein family and it acts as an anti- or pro-apoptotic regulator that is involved in a wide variety of cellular activities. Bak1 promotes apoptosis by binding to and antagonizing the apoptosis suppressor activity of Bcl-2 and other anti-apoptotic proteins. Expression of Bak1 in testicular germ cells is repressed by the Kit-Kitlg pathway, and interaction of Bak1 with anti-apoptotic proteins is implicated in germ cell apoptosis (Gilbert et al. 2011).

Cell proliferation is correlated with the Ki-67 labeling index. The Ki-67 antigen is known to accumulate from G1-phase to mitosis and, after mitosis, the amount of the antigen decreases to a minimal level (Kausch et al. 2003). Detailed cell cycle analysis revealed that the antigen is present in nuclei of proliferating (G1-/S-/G2-phases and mitosis) cells but not in nuclei of quiescent or resting cells (G0-phase) (Endl and Gerdes 2000). This gene can be used to detect the potential of mitosis in germ cells after chemical exposure.

Undifferentiated spermatogonia isolation protocols

Most of the current isolation protocols for undifferentiated

spermatogonia aim to characterize these cells and use them to repopulate testes with spermatogenesis impairment. Due to the small number of SSCs in the testis, *in vitro* techniques are needed to enhance the number of SSCs using biologically safe methods (Zhang et al. 2016). The most effective way to enrich germ cell populations for stem cells is to purify all forms of type A spermatogonia. In the adult mammalian testis, owing to the presence of multiple generations of germinal cells, purification of spermatogonia is more difficult than it is before puberty (Izadyar et al. 2002).

The two-step enzyme digestion method is widely used and major steps are as follows: mouse testes are usually decapsulated and digested with collagenase and EDTA-trypsin, respectively, and DNase I is employed to eliminate the intercellular adhesion (Zhang et al. 2016). There are many available modified protocols. The enzyme digestion time is the key factor in development of the isolation protocol and should be optimized by each laboratory in the first attempt at sorting, as insufficient digestion can lead to low yields, and overtreatment with enzymes is harmful to stem cells and may even destroy them (Garcia and Hofmann 2012). Compared with mechanical methods, the enzyme digestion method results in a markedly higher cell viability and purity. After digestion, the testicular cell culture is incubated overnight to remove the testicular somatic cells since the somatic cells bind tightly to the plastic culture dish when cultured in serum-containing medium, whereas germ cells do not (Hamra et al. 2008). This method primarily isolates cells from testes with a relatively low purity, as somatic cells are not

completely eliminated and a subsequent purification step is essential for enrichment of the cell type (Zhang et al. 2016).

Undifferentiated spermatogonia purification is based on differences in cell characteristics, such as wall attachment ability, size, density, and surface markers of cells. Many purification methods have been reported, however, each one has advantages and disadvantages. Researchers usually combine them to improve separation efficiency (Zhang et al. 2016). The use of a discontinuous Percoll (polyvinylpyrrolidone-coated colloidal silica particles mixed with water; non-toxic) gradient is one of the spermatogonia purification methods. The cell suspension obtained through two-enzyme digestion is loaded onto the top of a density gradient solution, followed by centrifugations with appropriate centrifugal force to separate cells with different densities. The desired cell population can be aspirated with a pipet. Because this method is roughly based on cell size and weight, all types of A spermatogonia can usually be obtained (Morena et al. 1996; Zhang et al. 2016).

Another spermatogonia enrichment technique is the use of laminin for plating selection (Hamra et al. 2008; Shinohara et al. 1999; Zhang et al. 2016). The cell adhesion molecules $\alpha 6$ and $\beta 1$ integrin have been detected on the surface of undifferentiated spermatogonia and their function is binding to laminin in the basement membrane of seminiferous tubules (Shinohara et al. 1999).

The *in vivo* exposure to environmental chemicals associated with the

spermatogonia isolation procedure may allow for analyses of spermatogonia gene expression in toxicologic studies to evaluate testicular alterations without contamination from other testicular cell types.

Environmental chemical exposures and testicular function impairment

It has been proposed (Browne et al. 2017; Lymperi and Giwerzman 2018; Picut et al. 2018) that there is an association between the exposure to endocrine disruptors (EDs) during fetal, neonatal and adult life and disturbance of normal reproductive development and function. According to the (WHO 2015), "an ED is an exogenous substance or mixture that alters functions of the endocrine system and consequently causes adverse effects in an intact organism, or its progeny". Extensive research on animal models are helping us to understand the mechanism(s) of action of EDs and confirm their toxic effects. However, human epidemiological studies have been inconclusive due to the presence of confounding factors such as the divergent biological ED actions, the continuous exposure to numerous EDs, the influence of genetic background in the manifestation of the outcome, and the lack of standardized clinical protocols for human studies (Bliatka et al. 2017). The strongest evidence in humans is that ED exposure during the prenatal period is associated with increased risk of male reproductive disorders (Bonde et al. 2016; Hauser et al. 2015).

The term EDs is used to describe a highly heterogeneous group of substances including both manufactured chemicals and natural compounds

which can disrupt the action of endogenous hormones. Industrial solvents and their by-products, such as polychlorinated biphenyls (PCBs) and polybrominated biphenyls (PBBs), plastics and plasticizers, like bisphenol A (BPA), and phthalates, like dibutyl phthalate (DBP), have all been included in this group based on animal or/and *in vitro* studies (Lymperi and Giwercman 2018).

Phthalates represent one of the most produced chemical groups in the world (450,000 tons/year) and among other uses, they confer malleability, transparency, durability and longevity to plastic products (Crinnion 2010). Phthalates are esters of phthalic acid and the common chemical structure is 1,2-benzenedicarboxylic acid diester (Figure 3). They are viscous, colorless and odorless liquids and have low solubility in water, high solubility in oil and low volatility. As they are not chemically bound, they can migrate into food and liquids or evaporate, and they have become ubiquitous environmental contaminants. The European Food Safety Authority (EFSA) established the Tolerable Daily Intakes (TDIs) for DBP as 10 µg/kg body weight/day (WHO 2005). Humans are continuously exposed to these compounds during their lifetime through oral, inhalation and dermal exposure (Perez-Albaladejo et al. 2017). DBP has been reported to cross the human placenta and reach the fetus (Wittassek et al. 2009).

Studies in humans are limited due to obvious ethical considerations and the difficulty in identifying individuals not exposed to these compounds. Male rats exposed *in utero* to 500 mg/kg of DBP often display Leydig cell

hyperplasia, multinucleated germ cells, and alterations in androgen-mediated development, as evidenced by decreased anogenital distances (AGD), supporting the hypothesis that the testis may be vulnerable to the action of phthalates during development (Swan et al. 2005). Phthalates show little or no estrogenic activity, with a growing consensus that they are antiandrogenic substances (Harris et al. 1997). However, phthalates and their metabolites do not bind to the androgen receptor (AR), indicating that they are not direct antagonists of AR (Parks et al. 2000). Moreover, exposure to DBP leads to the activation of peroxisome proliferator-activated receptors, an increase in fatty acid oxidation, and a reduction in the ability to manage increased oxidative stress (a state characterized by an imbalance between pro-oxidant molecules, including reactive oxygen species, and antioxidant defenses) that has been associated with reproductive organ malformations, reproductive defects, and decreased fertility (Mathieu-Denoncourt et al. 2015).

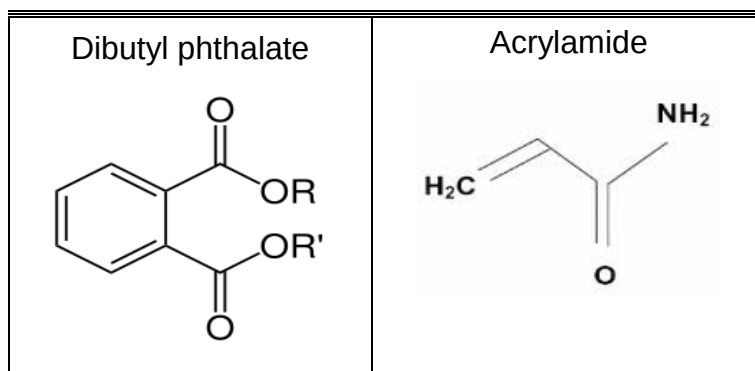


Figure 3. Chemical structures for DBP and AA.

The US Environmental Protection Agency (USEPA) selected acrylamide (Figure 3) for Tier 1 screening under the Endocrine Disruptor Screening Program (EDSP) (WHO 2015). AA exposure has become a worldwide concern because of its generation in a variety of carbohydrate rich foods (breads, potato chips, cereals, biscuits, etc) when cooked at temperatures exceeding 120°C (Friedman 2003). At these temperatures, Maillard reaction of sugars with asparagine residues produces AA (Friedman 2005). AA is also a chemical with a wide range of uses, including as a flocculant in water treatment, and it is a well-established human and rodent neurotoxin at high exposure levels (Recio et al. 2017). The 64th Joint FAO/WHO Expert Committee on Food Additives concluded that an intake of 1 µg/kg body weight/day of AA could be taken to represent the average for the general population without apparent neurotoxic or other toxic effects (Maronpot et al. 2015; WHO 2005).

AA was evaluated by the International Agency for Research on Cancer (IARC) and classified as 'probably carcinogenic to humans (IARC Group 2A)' on the basis of positive bioassay results in mice and rats and supported by evidence that AA is biotransformed in mammalian tissues to the chemically reactive genotoxic metabolite, glycidamide (WHO 1994). In rats, AA affects male reproductive performance and induces dominant lethal mutations (Tyl and Friedman 2003). Sub-chronic and short-term high-dose exposures of male rats to AA induces a variety of testicular toxicities including multinucleated giant and apoptotic cells in seminiferous tubules,

degeneration of seminiferous tubules, Sertoli and germ cell degeneration, aberrant sperm morphology, decreased sperm count, and motility (Mustafa 2012; WHO 1994)

There is little information on the effects of exposure to AA in humans, but experimental and epidemiologic studies suggest a positive association between AA exposure and probable carcinogenicity of various tissues (renal, pancreatic, gastric, breast) (Maronpot et al. 2015; Pelucchi et al. 2011).

Justification and objectives

The evidence that suggests an increased incidence of testicular disorders in young men and an influence of environmental chemical factors, led to the need to establish an experimental model for identifying risk factors in the development of these disorders and for better understanding of the mode(s) of action of DBP and AA. Most of the available reproductive toxicologic studies use RNA extracted from whole testis for gene expression analysis, instead of investigating differentially expressed genes in a specific cell type. Thus, the ability to characterize the gene expression profile of specific cells, as proposed in the present study, will allow greater understanding of testicular damage. Among the germ cells, undifferentiated spermatogonia are the most interesting since they are responsible for testicular repopulation after cell damage.

This study aimed

1. to establish an *in vitro* technique for isolation of undifferentiated spermatogonia and validate the isolation technique by Plzf immunohistochemistry (Costoya et al. 2004; Pieri et al. 2017);

2. to describe germ cell alterations induced *in vivo* by the environmental chemicals DBP and AA. After *in vivo* exposure and *in vitro* isolation of undifferentiated spermatogonia, qRT-PCR was used to determine the expressions of Pou5f1, Kitlg, Ki-67, Bak1 and Spry4 genes. The characterization of gene expressions allows comparisons between normal undifferentiated spermatogonia and those from chemically damaged testes.

References

- Barker, D.J. 1995a. Intrauterine programming of adult disease. *Mol Med Today* 1, 418-423.
- Barker, D.J. 1995b. The fetal and infant origins of disease. *Eur J Clin Invest* 25, 457-463.
- Barsoum, I. and Yao, H.H. 2006. The road to maleness: from testis to Wolffian duct. *Trends Endocrinol Metab* 17, 223-228.
- Bliatka, D., Lymperi, S., Mastorakos, G. and Goulis, D.G. 2017. Effect of endocrine disruptors on male reproduction in humans: why the evidence is still lacking? *Andrology* 5, 404-407.
- Bonde, J.P., Flachs, E.M., Rimborg, S., Glazer, C.H., Giwercman, A., Ramlau-Hansen, C.H., Hougaard, K.S., Hoyer, B.B., Haervig, K.K., Petersen, S.B., Rylander, L., Specht, I.O., Toft, G. and Brauner, E.V. 2016. The epidemiologic evidence linking prenatal and postnatal exposure to endocrine disrupting chemicals with male reproductive disorders: a systematic review and meta-analysis. *Hum Reprod Update* 23, 104-125.
- Browne, P., Noyes, P.D., Casey, W.M. and Dix, D.J. 2017. Application of Adverse Outcome Pathways to U.S. EPA's Endocrine Disruptor Screening Program. *Environ Health Perspect* 125, 096001.
- Buljubasic, R., Buljubasic, M., Bojanac, A.K., Ulamec, M., Vlahovic, M., Jezek, D., Bulic-Jakus, F. and Sincic, N. 2018. Epigenetics and testicular germ cell tumors. *Gene* 661, 22-33.
- Capel, B. 2000. The battle of the sexes. *Mech Dev* 92, 89-103.
- Coe, C.L. and Lubach, G.R. 2008. Fetal programming: Prenatal origins of health and illness. *Current Directions in Psychological Science* 17, 36-41.
- Cools, M. 2014. Germ cell cancer risk in DSD patients. *Ann Endocrinol (Paris)* 75, 67-71.
- Costoya, J.A., Hobbs, R.M., Barna, M., Cattoretti, G., Manova, K., Sukhwani, M., Orwig, K.E., Wolgemuth, D.J. and Pandolfi, P.P. 2004. Essential role of Plzf in maintenance of spermatogonial stem cells. *Nat Genet* 36, 653-659.
- Crinnion, W.J. 2010. Toxic effects of the easily avoidable phthalates and parabens. *Altern Med Rev* 15, 190-196.
- de Rooij, D.G. 2009. The spermatogonial stem cell niche. *Microsc Res Tech* 72, 580-585.
- Deng, S.L., Chen, S.R., Wang, Z.P., Zhang, Y., Tang, J.X., Li, J., Wang, X.X., Cheng, J.M., Jin, C., Li, X.Y., Zhang, B.L., Yu, K., Lian, Z.X., Liu, G.S. and Liu, Y.X. 2016. Melatonin promotes development of haploid germ cells from early developing spermatogenic cells of Suffolk sheep under in vitro condition. *J Pineal Res* 60, 435-447.
- Ehmcke, J., Hubner, K., Scholer, H.R. and Schlatt, S. 2006. Spermatogonia: origin, physiology and prospects for conservation and manipulation of the male germ line. *Reprod Fertil Dev* 18, 7-12.
- Encinas, G., Zogbi, C. and Stumpp, T. 2012. Detection of four germ cell

- markers in rats during testis morphogenesis: differences and similarities with mice. *Cells Tissues Organs* 195, 443-455.
- Endl, E. and Gerdes, J. 2000. The Ki-67 protein: fascinating forms and an unknown function. *Exp Cell Res* 257, 231-237.
- Fayomi, A.P. and Orwig, K.E. 2018. Spermatogonial stem cells and spermatogenesis in mice, monkeys and men. *Stem Cell Res* 29, 207-214.
- Ferrara, D., Hallmark, N., Scott, H., Brown, R., McKinnell, C., Mahood, I.K. and Sharpe, R.M. 2006. Acute and long-term effects of in utero exposure of rats to di(n-butyl) phthalate on testicular germ cell development and proliferation. *Endocrinology* 147, 5352-5362.
- Filipponi, D., Hobbs, R.M., Ottolenghi, S., Rossi, P., Jannini, E.A., Pandolfi, P.P. and Dolci, S. 2007. Repression of kit expression by Plzf in germ cells. *Mol Cell Biol* 27, 6770-6781.
- Friedman, M. 2003. Chemistry, biochemistry, and safety of acrylamide. A review. *J Agric Food Chem* 51, 4504-4526.
- Friedman, M. 2005. Biological effects of Maillard browning products that may affect acrylamide safety in food: biological effects of Maillard products. *Adv Exp Med Biol* 561, 135-156.
- Frolov, A., Chahwan, S., Ochs, M., Arnoletti, J.P., Pan, Z.Z., Favorova, O., Fletcher, J., von Mehren, M., Eisenberg, B. and Godwin, A.K. 2003. Response markers and the molecular mechanisms of action of Gleevec in gastrointestinal stromal tumors. *Mol Cancer Ther* 2, 699-709.
- Garcia, T. and Hofmann, M.C. 2012. Isolation of undifferentiated and early differentiating type A spermatogonia from Pou5f1-GFP reporter mice. *Methods Mol Biol* 825, 31-44.
- Gilbert, D., Rapley, E. and Shipley, J. 2011. Testicular germ cell tumours: predisposition genes and the male germ cell niche. *Nat Rev Cancer* 11, 278-288.
- Goddard, N.C., McIntyre, A., Summersgill, B., Gilbert, D., Kitazawa, S. and Shipley, J. 2007. KIT and RAS signalling pathways in testicular germ cell tumours: new data and a review of the literature. *Int J Androl* 30, 337-348; discussion 349.
- Grisanti, L., Falciatori, I., Grasso, M., Dove, L., Fera, S., Muciaccia, B., Fuso, A., Berno, V., Boitani, C., Stefanini, M. and Vicini, E. 2009. Identification of spermatogonial stem cell subsets by morphological analysis and prospective isolation. *Stem Cells* 27, 3043-3052.
- Hamra, F.K., Chapman, K.M., Wu, Z. and Garbers, D.L. 2008. Isolating highly pure rat spermatogonial stem cells in culture. *Methods Mol Biol* 450, 163-179.
- Harris, C.A., Henttu, P., Parker, M.G. and Sumpter, J.P. 1997. The estrogenic activity of phthalate esters in vitro. *Environ Health Perspect* 105, 802-811.
- Haschek, W. and Rousseaux, C. 2013. *Handbook of Toxicologic Pathology*, Academic Press.
- Haseltine, F.P. and Ohno, S. 1981. Mechanisms of gonadal differentiation.

- Science 211, 1272-1278.
- Hauser, R., Skakkebaek, N.E., Hass, U., Toppari, J., Juul, A., Andersson, A.M., Kortenamp, A., Heindel, J.J. and Trasande, L. 2015. Male reproductive disorders, diseases, and costs of exposure to endocrine-disrupting chemicals in the European Union. *J Clin Endocrinol Metab* 100, 1267-1277.
- Izadyar, F., Spierenberg, G.T., Creemers, L.B., den Ouden, K. and de Rooij, D.G. 2002. Isolation and purification of type A spermatogonia from the bovine testis. *Reproduction* 124, 85-94.
- Jarvis, S., Elliott, D.J., Morgan, D., Winston, R. and Readhead, C. 2005. Molecular markers for the assessment of postnatal male germ cell development in the mouse. *Hum Reprod* 20, 108-116.
- Kanetsky, P.A., Mitra, N., Vardhanabhuti, S., Li, M., Vaughn, D.J., Letrero, R., Ciosek, S.L., Doody, D.R., Smith, L.M., Weaver, J., Albano, A., Chen, C., Starr, J.R., Rader, D.J., Godwin, A.K., Reilly, M.P., Hakonarson, H., Schwartz, S.M. and Nathanson, K.L. 2009. Common variation in KITLG and at 5q31.3 predisposes to testicular germ cell cancer. *Nat Genet* 41, 811-815.
- Kausch, I., Lingnau, A., Endl, E., Sellmann, K., Deinert, I., Ratliff, T.L., Jocham, D., Sczakiel, G., Gerdes, J. and Bohle, A. 2003. Antisense treatment against Ki-67 mRNA inhibits proliferation and tumor growth in vitro and in vivo. *Int J Cancer* 105, 710-716.
- Lymeri, S. and Giwerzman, A. 2018. Endocrine disruptors and testicular function. *Metabolism* 86, 79-90.
- Maronpot, R.R., Thoolen, R.J. and Hansen, B. 2015. Two-year carcinogenicity study of acrylamide in Wistar Han rats with in utero exposure. *Exp Toxicol Pathol* 67, 189-195.
- Mathieu-Denoncourt, J., Wallace, S.J., de Solla, S.R. and Langlois, V.S. 2015. Plasticizer endocrine disruption: Highlighting developmental and reproductive effects in mammals and non-mammalian aquatic species. *Gen Comp Endocrinol* 219, 74-88.
- Merchant-Larios, H. and Moreno-Mendoza, N. 2001. Onset of sex differentiation: dialog between genes and cells. *Arch Med Res* 32, 553-558.
- Merzenich, H., Zeeb, H. and Blettner, M. 2010. Decreasing sperm quality: a global problem? *BMC Public Health* 10, 24.
- Morena, A.R., Boitani, C., Pesce, M., De Felici, M. and Stefanini, M. 1996. Isolation of highly purified type A spermatogonia from prepubertal rat testis. *J Androl* 17, 708-717.
- Mustafa, H.N. 2012. Effect of acrylamide on testis of albino rats. Ultrastructure and DNA cytometry study. *Saudi Med J* 33, 722-731.
- Ong, T.P. and Ozanne, S.E. 2015. Developmental programming of type 2 diabetes: early nutrition and epigenetic mechanisms. *Curr Opin Clin Nutr Metab Care* 18, 354-360.
- Ozaki, Y., Saito, K., Shinya, M., Kawasaki, T. and Sakai, N. 2011. Evaluation

- of Sycp3, Plzf and Cyclin B3 expression and suitability as spermatogonia and spermatocyte markers in zebrafish. *Gene Expr Patterns* 11, 309-315.
- Parks, L.G., Ostby, J.S., Lambright, C.R., Abbott, B.D., Klinefelter, G.R., Barlow, N.J. and Gray, L.E., Jr. 2000. The plasticizer diethylhexyl phthalate induces malformations by decreasing fetal testosterone synthesis during sexual differentiation in the male rat. *Toxicol Sci* 58, 339-349.
- Pearson, R., Fleetwood, J., Eaton, S., Crossley, M. and Bao, S. 2008. Kruppel-like transcription factors: a functional family. *Int J Biochem Cell Biol* 40, 1996-2001.
- Pelucchi, C., La Vecchia, C., Bosetti, C., Boyle, P. and Boffetta, P. 2011. Exposure to acrylamide and human cancer--a review and meta-analysis of epidemiologic studies. *Ann Oncol* 22, 1487-1499.
- Perez-Albaladejo, E., Fernandes, D., Lacorte, S. and Porte, C. 2017. Comparative toxicity, oxidative stress and endocrine disruption potential of plasticizers in JEG-3 human placental cells. *Toxicol In Vitro* 38, 41-48.
- Phillips, B.T., Gassei, K. and Orwig, K.E. 2010. Spermatogonial stem cell regulation and spermatogenesis. *Philos Trans R Soc Lond B Biol Sci* 365, 1663-1678.
- Picut, C.A., Ziejewski, M.K. and Stanislaus, D. 2018. Comparative Aspects of Pre- and Postnatal Development of the Male Reproductive System. *Birth Defects Res* 110, 190-227.
- Pieri, N., Souza, A.F., Mancanares, A., Roballo, K., Casals, J.B., Ambrosio, C.E. and Martins, D.S. 2017. Immunolocalization of proteins in the spermatogenesis process of canine. *Reprod Domest Anim* 52 Suppl 2, 170-176.
- Rapley, E.A., Turnbull, C., Al Olama, A.A., Dermitzakis, E.T., Linger, R., Huddart, R.A., Renwick, A., Hughes, D., Hines, S., Seal, S., Morrison, J., Nsengimana, J., Deloukas, P., Rahman, N., Bishop, D.T., Easton, D.F. and Stratton, M.R. 2009. A genome-wide association study of testicular germ cell tumor. *Nat Genet* 41, 807-810.
- Recio, L., Friedman, M., Marroni, D., Maynor, T. and Chepelev, N.L. 2017. Impact of Acrylamide on Calcium Signaling and Cytoskeletal Filaments in Testes From F344 Rat. *Int J Toxicol* 36, 124-132.
- Rossi, P., Sette, C., Dolci, S. and Geremia, R. 2000. Role of c-kit in mammalian spermatogenesis. *J Endocrinol Invest* 23, 609-615.
- Runyan, C., Schaible, K., Molyneaux, K., Wang, Z., Levin, L. and Wylie, C. 2006. Steel factor controls midline cell death of primordial germ cells and is essential for their normal proliferation and migration. *Development* 133, 4861-4869.
- Sasaki, A., Taketomi, T., Kato, R., Saeki, K., Nonami, A., Sasaki, M., Kuriyama, M., Saito, N., Shibuya, M. and Yoshimura, A. 2003. Mammalian Sprouty4 suppresses Ras-independent ERK activation by binding to Raf1. *Nat Cell Biol* 5, 427-432.
- Scholer, H.R. 1991. Octamania: the POU factors in murine development. *Trends Genet* 7, 323-329.

- Selevan, S.G., Kimmel, C.A. and Mendola, P. 2000. Identifying critical windows of exposure for children's health. *Environ Health Perspect* 108 Suppl 3, 451-455.
- Sharpe, R.M. and Skakkebaek, N.E. 2008. Testicular dysgenesis syndrome: mechanistic insights and potential new downstream effects. *Fertil Steril* 89, e33-38.
- Shinohara, T., Avarbock, M.R. and Brinster, R.L. 1999. beta1- and alpha6-integrin are surface markers on mouse spermatogonial stem cells. *Proc Natl Acad Sci U S A* 96, 5504-5509.
- Singh, S.R., Burnicka-Turek, O., Chauhan, C. and Hou, S.X. 2011. Spermatogonial stem cells, infertility and testicular cancer. *J Cell Mol Med* 15, 468-483.
- Skakkebaek, N.E., Rajpert-De Meyts, E. and Main, K.M. 2001. Testicular dysgenesis syndrome: an increasingly common developmental disorder with environmental aspects. *Hum Reprod* 16, 972-978.
- Swan, S.H., Elkin, E.P. and Fenster, L. 2000. The question of declining sperm density revisited: an analysis of 101 studies published 1934-1996. *Environ Health Perspect* 108, 961-966.
- Swan, S.H., Main, K.M., Liu, F., Stewart, S.L., Kruse, R.L., Calafat, A.M., Mao, C.S., Redmon, J.B., Ternand, C.L., Sullivan, S. and Teague, J.L. 2005. Decrease in anogenital distance among male infants with prenatal phthalate exposure. *Environ Health Perspect* 113, 1056-1061.
- Tarulli, G.A., Stanton, P.G. and Meachem, S.J. 2012. Is the adult Sertoli cell terminally differentiated? *Biol Reprod* 87, 13, 11-11.
- Tyl, R.W. and Friedman, M.A. 2003. Effects of acrylamide on rodent reproductive performance. *Reprod Toxicol* 17, 1-13.
- USEPA. 1991. GUIDELINES FOR DEVELOPMENTAL TOXICITY RISK ASSESSMENT. U.S. Environmental Protection Agency. In: EPA/600/FR-91/001 (Ed), Washington, DC.
- USEPA. 1998a. Prenatal Developmental Toxicity Study. Health Effects Test Guidelines. In: O. EPA and 712-C-98-207 (Eds), Washington, DC.
- USEPA. 1998b. Reproduction and Fertility Effects. Health Effects Test Guidelines. In: O. EPA and 712-C-98-208 (Eds), Washington, DC.
- WHO. 1994. IARC monographs on evaluation of carcinogenic risk to humans. International Agency for Research on Cancer, United Kingdom.
- WHO. 2005. Joint FAO/WHO Expert Committee on Food Additives Sixty-fourth meeting. pp. 7-17.
- WHO. 2010. WHO laboratory manual for the examination and processing of human semen. In: R.H.a. Research (Ed), p. 287.
- WHO. 2015. Identification of risks of endocrine disrupting-chemicals: overview of existing practices and steps ahead. p. 45.
- Wintour, E.M., Johnson, K., Koukoulas, I., Moritz, K., Tersteeg, M. and Dodic, M. 2003. Programming the cardiovascular system, kidney and the brain--a review. *Placenta* 24 Suppl A, S65-71.
- Wittassek, M., Angerer, J., Kolossa-Gehring, M., Schafer, S.D.,

- Klockenbusch, W., Dobler, L., Gunsel, A.K., Muller, A. and Wiesmuller, G.A. 2009. Fetal exposure to phthalates--a pilot study. *Int J Hyg Environ Health* 212, 492-498.
- Worthman, C.M. and Kuzara, J. 2005. Life history and the early origins of health differentials. *Am J Hum Biol* 17, 95-112.
- Xing, J.S. and Bai, Z.M. 2018. Is testicular dysgenesis syndrome a genetic, endocrine, or environmental disease, or an unexplained reproductive disorder? *Life Sci* 194, 120-129.
- Zhang, R., Sun, J. and Zou, K. 2016. Advances in Isolation Methods for Spermatogonial Stem Cells. *Stem Cell Rev* 12, 15-25.
- Zogbi, C., Tesser, R.B., Encinas, G., Miraglia, S.M. and Stumpp, T. 2012. Gonocyte development in rats: proliferation, distribution and death revisited. *Histochem Cell Biol* 138, 305-322.

Chapter II

Manuscript

Isolation and molecular characterization of spermatogonia from male Sprague-Dawley rats exposed *in utero* and postnatally to dibutyl phthalate or acrylamide

Nathália P. Souza^a (nathalips@gmail.com)

Lora L. Arnold^b (llarnold@unmc.edu)

Karen L. Pennington^b (kpenning@unmc.edu)

Merielen G. Nascimento e Pontes^a (meriennascimento@yahoo.com.br)

Helio A. Miot^a (heliomiot@gmail.com)

Samuel M. Cohen^{b, c} (scohen@unmc.edu)

João Lauro^a V. de Camargo (jdecam@uol.com.br)

^a Sao Paulo State University (UNESP), Botucatu Medical School, Botucatu Campus, Department of Pathology, Center for the Evaluation of the Environmental Impact on Human Health (TOXICAM), SP, Brazil.

^b University of Nebraska Medical Center, Department of Pathology and Microbiology, Omaha, NE, USA.

^c Havlik – Wall Professor of Oncology

Address correspondence to:

Nathalia P. Souza

UNIPLEX - bloco 5, laboratório TOXICAM

Faculdade de Medicina de Botucatu, 18618687

Botucatu, SP - Brasil

Email: nathalips@gmail.com

**Submitted to Toxicology Mechanisms and Methods*

Abstract

The increased incidence of testicular disorders in young men and the possible influence of environmental chemicals, such as dibutyl phthalate (DBP) and acrylamide (AA), requires experimental models for identifying modes of action. Most published reproductive toxicologic studies use RNA samples from the total testis to evaluate testicular gene expression; however, analyses of isolated cell types could provide a more specific tool. Among testicular germ cells, spermatogonia are critical since they represent the onset of spermatogenesis. This study aimed, 1) to establish a technique for spermatogonia isolation; 2) to apply this isolation technique to verify possible gene expression alterations (Pou5f1, Kitlg, Mki-67, Bak1 and Spry4) in prepubertal post-natal day, (PND24) and pubertal (PND45) testes after *in utero* and postnatal exposure to DBP or AA. The technique was efficient for isolation of a majority of spermatogonia. *In utero* DBP exposure led to reduced litter body weight at birth, reduced anogenital distance of male pups on PND4, and increased frequency of male nipple retention on PND14 compared to controls. DBP-exposed relative testes weights were reduced only at PND24 compared to control but they did not differ at PND45. DBP-exposed animals showed reduced expression levels of Pou5f1 and Mki67 on PND24, and reduced expression of Pou5f1 and Spry4 on PND45. AA exposure reduced expression of Pou5f1, Mki67 and Spry4 at PND45 although not significantly. Our results suggest that DBP acts by reducing cell proliferation and impairing differentiation in prepubertal and pubertal testes.

Keywords: spermatogonia isolation, dibutyl phthalate, acrylamide, molecular biology, proliferation, differentiation, rats

Introduction

There is accumulating evidence that human male reproductive disorders have increased over the past 5 decades (Xing and Bai 2018). Such disorders may occur in male newborns (cryptorchidism and hypospadias) or in young adult males (impaired spermatogenesis leading to infertility and testicular germ cell cancer [TGCT]) (Skakkebaek et al. 2001). Many processes governing the regulation of human physiology are encoded during the fetal stage (Wintour et al. 2003). Accordingly, it has been proposed (Lymperi and Giwercman 2018; Picut et al. 2018) that there is an association between the exposure to endocrine disruptors (EDs) during fetal, neonatal and adult life and disturbance of normal reproductive development and function (Bonde et al. 2016; Hauser et al. 2015).

Phthalates represent one of the most produced chemical groups in the world (450,000 tons/year) and among other uses, they confer malleability, transparency, durability and longevity to plastic products (Crinnion 2010). As they are not chemically bound, they can migrate into food and liquids or evaporate, and they have become ubiquitous environmental contaminants. Humans are continuously exposed to these compounds during their lifetime through oral, inhalation and dermal exposure particularly dibutyl phthalate (DBP), (Perez-Albaladejo et al. 2017). DBP has been reported to cross the human placenta and reach the fetus (Wittassek et al. 2009), and it is included in the ED group based on animal and *in vitro* studies (Lymperi and Giwercman 2018). Male rats

exposed to high doses *in utero* to DBP often display Leydig cell hyperplasia, multinucleated germ cells, and alterations in androgen-mediated development, as evidenced by decreased anogenital distances (AGD), a sexually dimorphic landmark which can indicate altered fetal androgen exposure (Gallavan et al. 1999), and by the presence of male nipple retention, supporting the hypothesis that the testis may be vulnerable to the action of phthalates during development (Ivell et al. 2013; Lee et al. 2004; Swan et al. 2005).

Another environmental toxicant which is also a rodent testicular toxicant is acrylamide (AA) and its exposure has become a worldwide concern because of its generation in a variety of carbohydrate rich foods (breads, potato chips, cereals, biscuits, etc) when cooked at temperatures exceeding 120°C (Friedman 2003, 2005). AA is also a chemical with a wide range of uses, including as a flocculant in water treatment, and it is a well-established human and rodent neurotoxin at high exposure levels (Recio et al. 2017). The US Environmental Protection Agency (USEPA) selected AA for Tier 1 screening under the Endocrine Disruptor Screening Program (EDSP) (USEPA 2014). In rats, AA affects male reproductive performance (Tyl and Friedman 2003). Sub-chronic and short-term high-dose exposures of male rats to AA induce a variety of testicular toxicities including multinucleated germ cells and apoptotic cells in seminiferous tubules, decreased Leydig cell viability, degeneration of seminiferous tubules, Sertoli and germ cell degeneration, aberrant sperm morphology, and decreased

sperm count and motility (Mustafa 2012).

Animal models are frequently used for hazard identification and characterization of the potential risk to human reproduction. Most published experimental reproductive toxicity studies use total testis to evaluate testicular gene expression. However, molecular analyses of an isolated cell types could provide a more specific tool for detailed exploration of testicular damage. Among testicular germ cells, spermatogonia are critical since they represent the onset of spermatogenesis. Due to the small number of spermatogonia in the testes, *in vitro* techniques are needed to enhance the number of these cells for various analyses (Izadyar et al. 2002; Morena et al. 1996; Zhang et al. 2016). Many experimental approaches have been conducted to isolate spermatogonia from testes, most are based on a two-step enzyme digestion method (Bai et al. 2008; Izadyar et al. 2002; Morena et al. 1996), but each study has utilized different protocols that differ in relation to the spermatogonia isolation medium, incubation time and enrichment process. In addition, most existing spermatogonia isolation protocols do not validate the isolation procedure by identifying the cells or they validate it only by transplanting the isolated cells into a recipient testis. Some markers, such as the promyelocytic leukemia zinc finger (Plzf, also known as Zbtb16), that are involved in the regulation of diverse cellular processes, including cell proliferation, apoptosis, differentiation, and development, can be used to characterize spermatogonias (Costoya et al. 2004; Ozaki et al. 2011; Pieri et al. 2017).

In this study, we developed and validated a spermatogonia isolation technique to assess possible gene expression alterations (Pou5f1, Kitlg, Ki-67, Bak1 and Spry4) after *in utero* and postnatal exposure to DBP or AA.

This study aimed to: 1) establish a technique for isolation of spermatogonia that was not time consuming; 2) validate the isolated cells as spermatogonia by Plzf immunohistochemistry (Costoya et al. 2004; Pieri et al. 2017); and 3) describe gene expression alterations (Pou5f1, Kitlg, Ki-67, Bak1 and Spry4) after *in utero* and postnatal exposure to DBP and AA in isolated spermatogonia.

Material and Methods

All animal procedures were conducted under protocols approved by the University of Nebraska Medical Center (UNMC) Institutional Animal Care and Use Committee (IACUC).

Establishment of spermatogonia isolation protocol

Animals

Male Sprague-Dawley rats (Charles River Breeding Laboratories, Raleigh, NC) of varying ages were used to develop a spermatogonia isolation procedure. Five additional male Sprague-Dawley rats, approximately 5-weeks old, were obtained from the same source to validate the procedure once it was developed.

Testes removal

Animals were euthanized with an overdose of Fatal Plus (Vortech Pharmaceuticals, Dearborn, Michigan), 150 mg/kg body weight. In a fume hood, the rat abdomen was sterilized with 70% ethanol and then opened. Sterile scissors and tweezers were used to manipulate and remove both testes. They were washed in saline and placed in a sterile culture dish containing 5 mL Collecting medium (from Gibco, Grand Island, NY, USA: Minimum Essential medium, 200 mM glutamine, 1M HEPES, 10 mM MEM Nonessential Amino Acids, 50 g/mL gentamicin; from Hyclone, Logan UT, USA: 10,000 IU/mL penicillin/10,000 mg/mL streptomycin). The culture dish

was transferred to a biosafety hood, the tunica albuginea was removed, and the teste were cut into small pieces.

a. Enzymatic digestion

The medium containing the testes was transferred into a sterile 50 mL tube containing 20 ml of collecting medium, 50 mg collagenase type I (2 mg/mL) (Sigma-Aldrich, St. Louis, MO, USA, #234153) and 50 mg hyaluronidase type II (2 mg/mL) (Sigma-Aldrich #H2126) solution. DNase I (5 μ g/mL; Sigma #D4513-1VL) was added to avoid viscosity. The tube was incubated for 60 minutes at 35°C for enzymatic digestion, and it was shaken every 10 minutes until incubation was complete. The solution was centrifuged at room temperature for 5 min at 30 x g (379 rpm; Sorvall Legend Mach 1.6R) and washed once with 20 mL collecting medium, and centrifuged at room temperature for 5 min at 30 x g. Approximately 3x the pellet volume of Trypsin-EDTA (0.25% Hyclone, cat no. SH30042.01) was added to lyse the cells. DNase I (5 μ g/mL) was added to neutralize the DNA release, and the solution was incubated at 35°C for 10 minutes. To stop trypsin digestion, fetal bovine serum (FBS) (10% of trypsin volume; Atlantic Biologicals, Flowery Branch, GA, USA) and collecting medium (5 ml) were added. The solution was filtered through a 70 μ m cell strainer (Sigma-Aldrich), and the filter was washed once with 2 mL collecting medium to ensure all cells were collected. Filtered cells were centrifuged at room temperature for 5 min at 30 x g (379 rpm) and 2 mL collecting medium was added.

b. Cell enrichment with a discontinuous density gradient

Isotonic 100% Percoll (GE Healthcare Bio-Sciences AB) was obtained by adding nine parts of Percoll to one part 1.5M NaCl. A discontinuous density gradient was made by diluting the Percoll suspension with collecting medium to obtain 30%, 32% and 40% Percoll (Pertoft 2000). The gradient column was prepared in a 15 ml Falcon tube by gently layering 1 ml of each of the above-mentioned solutions, starting with the 40% fraction at the bottom. Slowly, 1 mL of the cell suspension was overlaid on each tube of Percoll gradient. After centrifugation at 18°C for 30 min at 800 x g (1959 rpm), the 30% and 32% Percoll layers containing spermatogonia were carefully aspirated (Izadyar et al. 2002; Morena et al. 1996; van Pelt et al. 1996) and washed 3x in PBS (5 min at 800 x g, 18°C) to eliminate any residue of Percoll. The pellet was resuspended in 12 mL of purification medium (Minimum Essential medium, Gibco; 10% FBS, Atlantic Biologicals; 10,000 IU/mL penicillin/10,000 mg/mL streptomycin, Hyclone) and cultured overnight (approx. 12 hours) in a 100 mm culture dish at 35°C and 5% CO₂. Most somatic testicular cells remained attached to the culture plate while the spermatogonia remained suspended in the medium (Hamra et al. 2008; Izadyar et al. 2002; Morena et al. 1996; van Pelt et al. 1996).

c. Purifying spermatogonia

The day prior to the purification procedure, a 60 mm cell culture dish was coated with 4 mL of a diluted laminin solution prepared by adding of 75 µL 1mg/mL laminin (Sigma-Aldrich) to 4 mL ice-cold Collecting medium. The

dish was wrapped with parafilm and incubated overnight at 4°C. The day of the procedure, the laminin-coated dish was removed from the 4°C cooler and allowed to come to room temperature. The 100 mm cell culture dish containing the cell suspension was removed from the 35°C incubator, and the medium supernatant was collected and transferred to a 15 ml tube. The 100 mm cell culture dish was washed with 3 ml purification medium that was added to the 15 ml tube. The cell suspension was centrifuged at room temperature for 10 min at 800 x g (1959 rpm) and the pellet was resuspended in 4 mL purification medium. The laminin solution was pipetted out of the cell culture dish, 4 mL of purification medium was added to prerinse the laminin-coated dish and discarded, and 4 ml of cell suspension in purification medium was added to the dish. The culture dish was incubated for 50 min in a humidified cell culture incubator at 32°C, 5% CO₂. After incubation, the cell suspension containing the cells not bound to the laminin was removed by washing 3x with purification medium followed by addition of 4 ml -0.5% BSA in 1x PBS solution and incubated for 4 minutes at 32.5°C, 5.5% CO₂. The bound cells were detached from the laminin covered dish by pipetting the PBS-BSA solution up and down about 5 times and transferred to a 15 ml tube containing 4 ml of Purification medium. The culture dish was checked under a microscope to make sure all cells were harvested. The cell suspension was pelleted at 2000 x g for 5 minutes and the supernatant was poured off by inverting the tube and holding it upside down. The pelleted sample was stored at -80°C until all samples were ready for RNA extraction.

Immunohistochemistry validation

For the validation procedure, the method as described above was followed except that chamber slides instead of 60 mm cell culture dishes were used for purification of the spermatogonia. The wells of a chamber slide were coated with 200 μ L of the diluted laminin solution (75 μ L of 1mg/mL laminin in 4 mL ice-cold collecting medium). The chamber slide was wrapped with parafilm and incubated overnight at 4°C. The laminin-coated chamber slide was removed from the 4°C cooler and brought to room temperature. The cell culture dish containing the cell suspension was removed from the 35°C incubator, and as described above, the medium supernatant was collected and transferred to a 15 ml tube. The cell suspension was centrifuged at room temperature for 10 min at 800 x g (1959 rpm) and the pellet was resuspended in 4 mL purification medium. The laminin solution was pipetted out of each well of the chamber slide, and 200 μ L of purification medium was added to prerinse the laminin-coated wells and then discarded, and 200 μ L of the cell suspension were added to each of the laminin-coated wells. The chamber slide was incubated for 50 min in a humidified cell culture incubator at 32°C, 5% CO₂. After incubation, the cell suspension containing the cells not bound to the laminin was removed by washing 3x with purification medium. Cells were fixed by adding 200 μ L formalin to each well for 10 minutes followed by 3 washes with TBST (Tris Buffered Saline with 0.1% Tween-20) for 5 minutes each. The laminin-attached and fixed cells were processed for immunohistochemical detection

of Plzf to validate the isolation procedure. Rat testes sections were used as a positive control. Briefly, after deparaffinization of the testicular sections, both tissue and cells were exposed to 3% hydrogen peroxide (H₂O₂) for 5 min to quench endogenous peroxidase activity. Heat-induced antigen retrieval was performed only in tissue slides in 0.01 M citrate buffer, pH 6.0, using a microwave for 10 min. Avidin binding sites, endogenous biotin and biotin receptors (Avidin/Biotin Blocking kit, Vector Laboratories, Burlingame, CA) were blocked in both, tissue and cells, followed by a protein block with 1% horse serum for 30 minutes. Tissue sections and cells were immunostained overnight at 4°C with Plzf mouse monoclonal antibody (Santa Cruz Biotechnology, Inc. Dallas, Texas, United States; sc-28319), diluted 1:300. Secondary biotinylated antibody incubation was performed with anti-mouse IgG diluted 1:200 for 1 hour followed by incubation using the avidin–biotin–peroxidase complex (ABC) for 45 min (Vectastain Elite ABC kit, Vector Laboratories, Burlingame, CA). Positive reactions resulted in brown nuclear staining. Plzf was assessed as negative or positive.

Environmental exposure protocol

Chemicals

Dibutyl phthalate (DBP; CAS 84-74-2) and acrylamide (AA; CAS 79-06-1), both 99% pure) were obtained from Sigma-Aldrich). Corn oil (CAS 8001-30-7) was also obtained from Sigma-Aldrich.

Animals

Thirty female (8 weeks old) and 15 male (10 weeks old) Sprague-Dawley rats were obtained from Charles River Breeding Laboratories and underwent a 1-week acclimation period upon arrival before starting the experiment. The animal rooms were targeted at a temperature of 21°C and relative humidity of 50%. The animals were kept under a 12 hr light/dark schedule. Nylabones were added to the cages for environmental enrichment. Pelleted and autoclaved Teklad 8656 diet (Frederick, MD) was provided *ad libitum* to all animals during acclimation, breeding and gestation, and it was used as the basal diet throughout lactation and the F1 treatment phase of the study. Control animals received unsupplemented Teklad 8656. Animals exposed *in utero* to DBP or AA through gavage treatment of the dams, received Teklad 8656 diet supplemented with DBP or AA throughout lactation via the dams and after weaning. All test diets were prepared at Dyets, Inc. (Bethlehem, PA). For the purposes of diet preparation, the test materials were assumed to be 100% pure. Drinking water obtained from the Metropolitan Utilities District, Omaha, Nebraska was provided *ad libitum*. Female rats were mated overnight, two females to each male rat. Vaginal smears were collected daily and the day of sperm detection was considered to be day 0 of gestation (GD0). Pregnant rats were randomly placed in 3 experimental groups (control, DBP or AA) with 10 dams per group.

Dose selection: gavage and dietary exposure

Gavage doses were 500 mg/kg DBP in corn oil (100 g/L) or 10 mg/kg AA in deionized water (2 g/L). These doses were based on reports indicating that exposure to 500 mg/kg of DBP by gavage (embryonic days 11.5 to 20.5) was sufficient to induce rat germ cell disorders including low sperm quality and to compromise the function of fetal Leydig cells (Mahood et al. 2005; Scott et al. 2008). Exposure to 10 mg/kg AA in drinking water during 8 consecutive days caused reduction of the number of germ cells in the rat testis (Wang et al. 2010). Control dams (CTL) received corn oil by gavage according to their body weights. To treat the offspring through the diet with the same amount of chemicals provided by gavage to the dams, an estimated dose was calculated based on daily ingestion reported for an adult rat (Krizova et al. 1996) which resulted in dietary concentrations of 6000 ppm DBP and 120 ppm AA.

Experimental design

Pregnant dams were randomized into three groups and treated daily by gavage from gestational day (GD) 12 to GD 21 with plain corn oil (n=10; CTL), DBP (n = 10) or AA (n = 10). From delivery (GD 21) to postnatal day 21 (PND 21, weaning), dams were exposed to DBP or AA through the diet. Based on the literature (Fromme et al. 2011; Sorgel et al. 2002), the F1 rats were exposed to the chemicals through the placenta and through the maternal milk. After weaning, F1 male pups were allocated to their

respective groups (CTL, n=20; DBP, n=20; AA, n=20) and exposed through the diet to the respective chemicals until the end of the study on PND24 or PND45 (Figure 1). No more than two male pups from the same litter were used in the same experimental group. F1 body weights and food consumption were assessed every other week until the end of the study.

AGD and nipple retention

On PND 4, after culling, the distances between the anus and the genital tubercle (AGD) were measured in male offspring from each litter using calipers. The measurement was performed on all male pups after culling since all were exposed *in utero*, making the total number of AGD measurements greater than the total number of animals indicated in the experimental protocol. To avoid possible errors caused by differences in body size, the AGD of each animal was divided by the cube root of body weight (Gallavan et al. 1999). Litters were culled to eight pups per dam at PND 4, preferentially males. On PND 14, male pups were inspected for the presence of either areolae or nipples, with no distinction between them (Lee et al. 2004; McIntyre et al. 2002; Moore et al. 2001).

Euthanasia and sample collection

On PND 24 (n = 10/group) and PND 45 (n = 10/group), F1 animals were sacrificed by an overdose of Fatal Plus (Vortech Pharmaceuticals, Dearborn, Michigan) 150 mg/kg body weight.

a. Spermatogonia isolation for PCR analysis

At necropsy, both testes of eight animals in each group were removed, weighed and processed for spermatogonia isolation using the method described above. Following RNA isolation, the expression levels of Pou5f1, Kitlg, Mki-67, Bak1 and Spry4 in the spermatogonia were determined by qRT-PCR (Bio-Rad Laboratories, Inc., CA, USA).

b. Testicular histology verification

The testes of the remaining two animals in each group were removed, weighed and fixed in modified Davidson's fixative for 24 h (Latendresse et al. 2002). The combined testes weight for each animal was divided by 2 prior to statistical analysis. Following fixation, the tissue was processed for paraffin embedding in the Tissue Sciences Core Facility at UNMC. Approximately 4-5 micron sections were stained with hematoxylin and eosin and examined histopathologically to enable comparison of testes morphology to the PCR results.

RNA extraction, cDNA preparation and real time PCR

RNA samples from isolated spermatogonia cells (8 samples from each treatment group at PND24 and PND45 time points) were extracted following DNase I treatment using the RNeasy Micro Kit Qiagen (Qiagen, Germantown, MD, USA). Purified total RNA concentration (ng/ μ L) and RNA/protein absorbance peaks (260nm/280nm) were measured using the NanoDrop 1000 Spectrophotometer (Thermo Fischer Scientific, Waltham,

MA, USA) and RNA quality (RNA quality number- RQN) was measured using the Fragment Analyzer™ Automated CE System (Advanced Analytical, Ankeny, IA, USA). Only samples with a ratio of approximately 2 for the 260/280 absorbance and RQN > 5 were selected for cDNA preparation (Supplementary material, Table 1). The Iscript Reverse Transcription Supermix for RT-qPCR (Bio-Rad Laboratories, Inc., CA, USA) was used to obtain cDNA following the kit instructions.

To choose the best housekeeping gene to normalize gene expression levels for all treatment groups, a predesigned reference gene plate (PrimePCR Pathway Plate, 96 Well, Bio-Rad) was run and Pkg1 (Protein kinase G 1; qRnoCED0002588) gene was selected based on its stability (Supplementary material, Table 2). A singleplex reaction was performed using PrimerPCR™ SYBR® Green Assay (Bio-Rad) for the quantification of Pou5f1 (qRnoCID0002379), Kitlg (qRnoCID0006033), Bak1 (qRnoCED0008883), Mki67 (qRnoCID0001488) and Spry4 (qRnoCID0001805) gene expression (amplicon sequence and length for each primer is described in the Supplementary Material, Table 3). A Bio-Rad CFX Connect™ real-time thermal cycler was used to run all the real time PCR reactions and Bio-Rad CFX Maestro software was used to analyze data.

Statistical analysis

Since the F1 male groups had more than one pup from the same

litter, both the pups and dams were included in the statistical analyses, except where stated. Variables were analyzed by a generalized linear mixed effect model followed by a post-hoc Sidak correction (Norman and Streiner 2008). The study experimental units were the F1 male rats, adjusted by their dams. Litter body weight on PND1, the number of pups per litter on PND1, and the frequency of male/female pups on PND4 were analyzed by ANOVA, Kruskal-Wallis and Fischer's exact test, respectively. Analyses were performed with the IBM SPSS 22.0 Statistics software (Statistical Package for Social Science; SPSS Incorporation). mRNA expression analysis was performed using Bio-Rad CFX Maestro software. A p value of <0.05 was considered significant.

Results

Spermatogonia isolation and validation

To obtain an efficient release of the germ cells from the seminiferous tubules, we performed two sequential digestions of the testes with collagenase/ hyaluronidase followed by trypsin. The cell suspension obtained was composed of somatic cells and germ cells. Purification of the spermatogonial population was achieved by a two-step procedure. First, the cell suspension was fractionated on a discontinuous Percoll gradient followed by laminin incubation, resulting in adherence of spermatogonia. After isolation, immunohistochemistry showed that 95% of the isolated cells were positive for Plzf, a marker for spermatogonia (Figure 2).

Clinical signs (F0 dams) and morphological landmarks (F1 pups) during gestational and lactational period

Oral exposure in pregnant rats to DBP or AA from GD12 to GD21 had no effect on body weight (Figure 3) or food consumption (*data not shown*) compared to pregnant control rats.

On PND1, DBP *in utero* exposed litters showed reduced body weight ($74.4 \pm \text{S.D.g}$) compared to control litters ($86.1 \pm \text{S.D.g}$) (Table 2). However, the number of pups per litter did not differ among the groups.

On PND4, before culling the litter, *in utero* exposure to DBP or AA did not alter the frequency of male and female pups born compared to control pups. (Table 3). After culling the litters on PND4, both AGD and normalized

AGD were reduced in the male DBP-treated pups (3.4 and 1.6, respectively), but not in male AA treated pups compared to control (4.1 and 1.9, respectively) (Table 3). On PND14, DBP exposure resulted in increased nipple retention in male pups (78%) compared to control (3.0%) (Table 4). AA exposure did not alter this parameter (Table 4).

Clinical parameters and testes weights (F1)

Food consumption and body weight gain in animals exposed to DBP or AA were comparable to the control group for animals euthanized on both PND24 and PND45 (Figure 3). At PND24, the absolute (0.28) and relative (0.43) testes weight was decreased in the DPB group compared to controls (0.34 and 0.49, respectively), but only the decrease in relative weight was statistically significant (Table 4). The absolute and relative testes weights in the AA-treated group were similar to control. (Table 4). For PND45, absolute and relative testes weights from DBP or AA-exposed animals did not differ from controls (Table 5).

Histologic view and gene expression analysis

Two animals from each group were used for testicular histopathology at PND24 and PND45. Chemical exposure to DPB or to AA did not alter the morphology of the seminiferous tubular or overall testicular histology compared to control animals.

On PND24, AA samples were not evaluated due to the poor quality

of mRNA. Spermatogonia isolated from the testes of DBP treated rats had significantly reduced expression levels of Pou5f1 (0.19) and Mki67 (0.10) compared to control animals (1.00 and 1.00, respectively). Expression levels of Kitlg and Bak1 were comparable to the control group (Table 5). Spry4 was not analyzed due to the small amount of mRNA obtained from the isolated cells.

On PND45, spermatogonia isolated from animals exposed to DBP had reduced expression levels of Pou5f1 (0.5 ± 0.11) and Spry4 ($0.65 \pm 0.15^*$) compared to control animals (1.00 ± 0.37 and 1.00 ± 0.35), but no differences were observed in the expression levels of Kitlg, Mki67 and Bak1 genes (Table 5). In AA-exposed animals, Pou5f1, Mki67 and Spry4 expression levels in spermatogonia were reduced but the decrease was not statistically significant.

Discussion

In this study, we developed a spermatogonia isolation technique for rat testes and its efficacy was verified by immunohistochemistry for Plzf, a spermatogonia marker. Since molecular analyses of an isolated cell type can provide a more specific tool for detailed exploration of testicular damage, we applied this isolation technique to verify possible gene expression alterations (Pou5f1, Kitlg, Mki-67, Bak1 and Spry4) in prepubertal (PND24) and pubertal (PND45) testes after *in utero* and postnatal exposure to DBP or AA.

Many protocols have been published for isolation of spermatogonia but each differs in isolation medium, incubation time, enrichment process, etc. Some of them use principally cell morphology or questionable germ cells markers, such as c-kit, to confirm the success of the isolation (Bai et al. 2008; Hamra et al. 2008; Izadyar et al. 2002; Morena et al. 1996; van Pelt et al. 1996). In addition, most existing spermatogonia isolation protocols use very young animals since spermatogonia are a major component of their seminiferous epithelium, reducing the risk of contamination by other cells (Baazm et al. 2013; Hamra et al. 2008; Morena et al. 1996).

We followed previous protocols that were published in the literature, but we did not achieve successful isolation of spermatogonia. As the protocols varied, we conducted trial studies to find the best one for our experiment design. We originally did not include the laminin purification step and used a lower concentration of collagenase (1 mg/mL)/ hyaluronidase (1

mg/mL) with a shorter incubation period (15 minutes) for the first step of the enzymatic digestion, resulting in no cells at the end of the protocol. This was probably due to the short digestion period that was insufficient to open the seminiferous tubules. An increase in the concentration of the collagenase/hyaluronidase solution (2 mg/mL) and the incubation period (60 minutes) resulted in the collection of numerous cells. To refine the isolation and to have a more selected pool of spermatogonia, we investigated 2 purification steps: incubation on a lectin-coated dish (5 μ g/mL in PBS) for 60 min (somatic cells adherence) followed by incubation on a laminin-coated dish (spermatogonia adherence) for 50 min. Because the somatic cells did not bind to lectin, we did not adopt this step in the final protocol. In addition, we tried to isolate spermatogonia from rats at different ages and found that the younger the rat, the better the enriched germ cell pool. We originally attempted to use flow cytometry for surface markers of spermatogonia to validate the isolation method, however, we did not have sufficient numbers of viable cells to generate significant fluorescence. We opted for validating the isolation protocol by immunohistochemistry. We developed a new protocol that achieved efficient isolation of spermatogonia for the age of rats we used. The spermatogonia isolation procedure proposed in this study can be performed in two days, thus, it is not a time-consuming protocol. It was efficient in pubertal rats since 95% of the isolated cells were positive for Plzf in 5-week old animals, suggesting the reliability of our protocol. However, a major limitation of this isolation procedure was the small amount of extracted

mRNA that was obtained, reducing the number of samples per group.

Since the first period of exposure to the F1 males was through the dams by gavage, we monitored their clinical signs in case of toxicity. Pregnant rats orally exposed to DBP or AA during GD12 to GD21 did not show differences in their body weight or in their food consumption during the gestational period compared to pregnant control rats. Despite that, litters exposed to DBP showed reduced total body weight on PND1 compared to control litters even with the same average amount of pups per litter, suggesting that developmental exposure to DBP influences body weight changes in early life.

Androgens are responsible for normal male masculinization and development, i.e., they promote the growth of the AGD and are associated with apoptosis of nipple anlagen in male rats, causing lack of nipple development (Bowman et al. 2003; Christiansen et al. 2010). Blocking *in utero* testosterone activity may result in a shortened AGD and nipple retention in the male offspring (Hsieh et al. 2012; Li et al. 2015). In epidemiological studies, shorter AGD in males have been frequently associated with clinically relevant outcomes of reproductive health such as cryptorchidism, hypospadias, poor semen quality, infertility, small testes and low serum testosterone levels in adulthood (Foresta et al. 2018; Pasterski et al. 2015; Thankamony et al. 2016). Reduced AGD values were observed in Sprague-Dawley and Wistar rats exposed *in utero* to 300 to 900 mg/kg of DBP (Li et al. 2015; Martino-Andrade et al. 2009; Mylchreest et al. 1998).

We examined AGD at PND4 and nipple retention at PND14. DBP-exposed male pups showed reduced AGD compared to control, in addition to increased frequency of nipple retention. No significant alterations were observed in AA exposed pups. The DBP dose administered in our studies was sufficient to interfere with the endocrine system acting as an anti-androgenic substance, probably modifying the testosterone synthesis/secretion during the development of the reproductive system that could lead to testicular impairment in adulthood. However, we did not measure testosterone levels to support this hypothesis.

In animal studies, reduction in offspring body weight following *in utero* AA exposure has been consistently observed in mice and rats (Chu et al. 2017; El-Sayyad et al. 2011; Ogawa et al. 2011; Tyl and Friedman 2003). In our study, food consumption and body weight gain in DBP- or AA- exposed male rats was similar to controls in early life and at both time periods, PND21 to PND24 and PND21 to PND45. Regarding testicular toxicity, *in utero* and postnatal exposure to DBP resulted in a significant reduction in relative testes weights only at PND24; no changes were observed at PND45 compared to control. However, other laboratories have reported significant reductions in testes weights in rats exposed *in utero* to 500 mg/kg/day DBP (Fisher et al. 2003; Mylchreest et al. 1999; Mylchreest et al. 2000). The reason for a lack of such effects in rats exposed to this same dose level in our study is uncertain, but may be related to the high variability we found among the animals.

Spermatogonia express a range of genes essential for normal development, including Pou5f1, Kitlg, Mki67, Bak1, and Spry4, that can be evaluated after chemical exposure to determine possible modes of action associated with toxic effects (Marcotte et al. 2017; Poynter et al. 2012; Sisakhtnezhad 2018).

The Pou5f1 gene encodes the protein oct 3/4, which is critical in germ cell embryonic development and is involved in cell pluripotency regulation (Ferrara et al. 2006). During normal perinatal maturation, oct 3/4 expression is reduced as germ cells start to differentiate (Ferrara et al. 2006). In humans, OCT 3/4 positive germ cells have been used as an immunohistochemical marker for TGCT since the tumor is composed of germ cells that did not reach differentiation level (Cools 2014). The protein Kitlg binds to the c-kit protein in germ cells activating signaling pathways that control many important cellular processes such as cell growth and proliferation, survival, and migration (Goddard et al. 2007; Rajpert-De Meyts et al. 2004). This gene is highly expressed in fetal gonocytes but progressive cellular differentiation results in loss of expression (Singh et al. 2011). Mki67 expression is correlated with the occurrence of mitoses and it can be present independently of cellular differentiation (Kausch et al. 2003). These genes were used to investigate the pluripotency, proliferation and mitosis potential in the exposed spermatogonia. The apoptosis potential was evaluated by examining Bak1 expression that promotes apoptosis by binding to and antagonizing the apoptosis suppressor activity of Bcl-2 and other anti-

apoptotic proteins. Expression of Bak1 in testicular germ cells is repressed by the Kit-Kitlg pathway (Gilbert et al. 2011).

A common mechanism used to set up negative-feedback loops is the inducible expression of inhibitor signaling pathways through the same signaling pathways that they end up controlling. Spry4 proteins can antagonize receptor tyrosine kinase (RTK) signaling participating in the negative-feedback control. Spry4 expression is involved in diverse cellular functions, including anti-proliferative activity, inhibition of migration, and promotion of differentiation, and survival (Felfly and Klein 2013; Kim and Bar-Sagi 2004; Mason et al. 2006).

In this study, despite apparently normal seminiferous tubule histology, *in utero* and postnatal exposure to DBP (less so with AA exposure) resulted in reduced gene expression in prepubertal and pubertal spermatogonia. On PND24, only DBP-exposed animals were evaluated since the quality of the mRNA from AA samples was not good.

Most literature data show atrophy of seminiferous tubules with germ cell depletion after DBP exposure (Fisher et al. 2003; Mylchreest et al. 2000) that could be linked to alterations of the cellular proliferation or apoptosis rate. In this study, we observed reduced Pou5f1 and Mki67 expression on PND24 and reduced Pou5f1 and Spry4 expression on PND45, suggesting DBP affected the spermatogonias reducing their pluripotency and proliferation potential associated with possible impairment of cellular differentiation. As seen in DBP exposure studies, exposure to AA is related

to testicular atrophy, decreased weight of testes, and degeneration of the epithelial cells of the seminiferous tubules (Exon 2006). In this study, AA-exposed animals, despite no significantly altered testicular parameters, showed reduced Pou5f1, Mki67 and Spry4 expression at PND45, suggesting that, despite the endocrine disruption observed in DBP exposure, both DBP and AA act by altering similar intracellular signaling pathways.

The association of *in vivo* exposure followed by *in vitro* approaches to analyze reproductive toxicity may improve acquired data and facilitate analysis of modes of action for chemicals. Our results suggest that DBP acts by reducing cell proliferation in addition to impairment of differentiation in the prepubertal and pubertal testes, with DBP exposure presenting possible additional toxicity due to its potential for endocrine disruption. AA exposure showed mild effects, but not statistically significant.

Acknowledgements

The authors wish to acknowledge the support of the Fred and Pamela Buffett Cancer Center Tissue Sciences Facility Shared Resource, supported by National Cancer Institute grant P30 CA036727.

Declaration of Conflicting Interests

The authors declare no potential conflicts of interest with respect to the research, authorship, or publication of this article.

Funding

This study was supported by the Brazilian National Council for Scientific and Technological Development - CNPq (fellowship 140333/2015-0) and the Brazilian Federal Agency for Support and Evaluation of Graduate Education – CAPES (fellowship 88881.132593/2016-01). Financial support for the research was provided by funds from Dr. Cohen's endowed Havlik-Wall Professorship.

References

- Baazm, M., Abolhassani, F., Abbasi, M., Habibi Roudkenar, M., Amidi, F. and Beyer, C. 2013. An improved protocol for isolation and culturing of mouse spermatogonial stem cells. *Cell Reprogram* 15, 329-336.
- Bai, Y., Ye, Z. and Zeng, F. 2008. Isolation of subtype spermatogonia in juvenile rats. *J Huazhong Univ Sci Technolog Med Sci* 28, 435-438.
- Bonde, J.P., Flachs, E.M., Rimborg, S., Glazer, C.H., Giwercman, A., Ramlau-Hansen, C.H., Hougaard, K.S., Hoyer, B.B., Haervig, K.K., Petersen, S.B., Rylander, L., Specht, I.O., Toft, G. and Brauner, E.V. 2016. The epidemiologic evidence linking prenatal and postnatal exposure to endocrine disrupting chemicals with male reproductive disorders: a systematic review and meta-analysis. *Hum Reprod Update* 23, 104-125.
- Bowman, C.J., Barlow, N.J., Turner, K.J., Wallace, D.G. and Foster, P.M. 2003. Effects of in utero exposure to finasteride on androgen-dependent reproductive development in the male rat. *Toxicol Sci* 74, 393-406.
- Christiansen, S., Boberg, J., Axelstad, M., Dalgaard, M., Vinggaard, A.M., Metzdorff, S.B. and Hass, U. 2010. Low-dose perinatal exposure to di(2-ethylhexyl) phthalate induces anti-androgenic effects in male rats. *Reprod Toxicol* 30, 313-321.
- Chu, P.L., Lin, L.Y., Chen, P.C., Su, T.C. and Lin, C.Y. 2017. Negative association between acrylamide exposure and body composition in adults: NHANES, 2003-2004. *Nutr Diabetes* 7, e246.
- Cools, M. 2014. Germ cell cancer risk in DSD patients. *Ann Endocrinol (Paris)* 75, 67-71.
- Costoya, J.A., Hobbs, R.M., Barna, M., Cattoretti, G., Manova, K., Sukhwani, M., Orwig, K.E., Wolgemuth, D.J. and Pandolfi, P.P. 2004. Essential role of Plzf in maintenance of spermatogonial stem cells. *Nat Genet* 36, 653-659.
- Crinnion, W.J. 2010. Toxic effects of the easily avoidable phthalates and parabens. *Altern Med Rev* 15, 190-196.
- El-Sayyad, H.I., Abou-Egla, M.H., El-Sayyad, F.I., El-Ghawet, H.A., Gaur, R.L., Fernando, A., Raj, M.H. and Ouhtit, A. 2011. Effects of fried potato chip supplementation on mouse pregnancy and fetal development. *Nutrition* 27, 343-350.
- Exon, J.H. 2006. A review of the toxicology of acrylamide. *J Toxicol Environ Health B Crit Rev* 9, 397-412.
- Felfly, H. and Klein, O.D. 2013. Sprouty genes regulate proliferation and survival of human embryonic stem cells. *Sci Rep* 3, 2277.
- Ferrara, D., Hallmark, N., Scott, H., Brown, R., McKinnell, C., Mahood, I.K. and Sharpe, R.M. 2006. Acute and long-term effects of in utero exposure of rats to di(n-butyl) phthalate on testicular germ cell development and proliferation. *Endocrinology* 147, 5352-5362.
- Fisher, J.S., Macpherson, S., Marchetti, N. and Sharpe, R.M. 2003. Human 'testicular dysgenesis syndrome': a possible model using in-utero exposure of the rat to dibutyl phthalate. *Hum Reprod* 18, 1383-1394.
- Foresta, C., Valente, U., Di Nisio, A., Cacco, N., Magagna, S., Cosci, I., Presciutti, A. and Garolla, A. 2018. Anogenital distance is associated with genital measures and seminal parameters but not anthropometrics in a large cohort of young adult men. *Hum Reprod*.
- Friedman, M. 2003. Chemistry, biochemistry, and safety of acrylamide. A review. *J Agric Food Chem* 51, 4504-4526.
- Friedman, M. 2005. Biological effects of Maillard browning products that may affect acrylamide safety in food: biological effects of Maillard products. *Adv Exp Med Biol* 561, 135-156.
- Fromme, H., Gruber, L., Seckin, E., Raab, U., Zimmermann, S., Kiranoglu, M., Schlummer, M., Schwegler, U., Smolic, S. and Volkel, W. 2011. Phthalates and their metabolites in breast milk--results from the Bavarian Monitoring of Breast Milk (BAMBI). *Environ Int* 37, 715-722.

- Gallavan, R.H., Jr., Holson, J.F., Stump, D.G., Knapp, J.F. and Reynolds, V.L. 1999. Interpreting the toxicologic significance of alterations in anogenital distance: potential for confounding effects of progeny body weights. *Reprod Toxicol* 13, 383-390.
- Gilbert, D., Rapley, E. and Shipley, J. 2011. Testicular germ cell tumours: predisposition genes and the male germ cell niche. *Nat Rev Cancer* 11, 278-288.
- Goddard, N.C., McIntyre, A., Summersgill, B., Gilbert, D., Kitazawa, S. and Shipley, J. 2007. KIT and RAS signalling pathways in testicular germ cell tumours: new data and a review of the literature. *Int J Androl* 30, 337-348; discussion 349.
- Hamra, F.K., Chapman, K.M., Wu, Z. and Garbers, D.L. 2008. Isolating highly pure rat spermatogonial stem cells in culture. *Methods Mol Biol* 450, 163-179.
- Hauser, R., Skakkebaek, N.E., Hass, U., Toppari, J., Juul, A., Andersson, A.M., Kortenkamp, A., Heindel, J.J. and Trasande, L. 2015. Male reproductive disorders, diseases, and costs of exposure to endocrine-disrupting chemicals in the European Union. *J Clin Endocrinol Metab* 100, 1267-1277.
- Hsieh, M.H., Eisenberg, M.L., Hittelman, A.B., Wilson, J.M., Tasian, G.E. and Baskin, L.S. 2012. Caucasian male infants and boys with hypospadias exhibit reduced anogenital distance. *Hum Reprod* 27, 1577-1580.
- Ivell, R., Heng, K., Nicholson, H. and Anand-Ivell, R. 2013. Brief maternal exposure of rats to the xenobiotics dibutyl phthalate or diethylstilbestrol alters adult-type Leydig cell development in male offspring. *Asian J Androl* 15, 261-268.
- Izadyar, F., Spierenberg, G.T., Creemers, L.B., den Ouden, K. and de Rooij, D.G. 2002. Isolation and purification of type A spermatogonia from the bovine testis. *Reproduction* 124, 85-94.
- Kausch, I., Lingnau, A., Endl, E., Sellmann, K., Deinert, I., Ratliff, T.L., Jocham, D., Sczakiel, G., Gerdes, J. and Bohle, A. 2003. Antisense treatment against Ki-67 mRNA inhibits proliferation and tumor growth in vitro and in vivo. *Int J Cancer* 105, 710-716.
- Kim, H.J. and Bar-Sagi, D. 2004. Modulation of signalling by Sprouty: a developing story. *Nat Rev Mol Cell Biol* 5, 441-450.
- Krizova, E., Simek, V., Abelenda, M. and Puerta, M. 1996. Food intake and body weight in rats with daily food-availability restrictions. *Physiol Behav* 60, 791-794.
- Latendresse, J.R., Warbritton, A.R., Jonassen, H. and Creasy, D.M. 2002. Fixation of testes and eyes using a modified Davidson's fluid: comparison with Bouin's fluid and conventional Davidson's fluid. *Toxicol Pathol* 30, 524-533.
- Lee, K.Y., Shibutani, M., Takagi, H., Kato, N., Takigami, S., Uneyama, C. and Hirose, M. 2004. Diverse developmental toxicity of di-n-butyl phthalate in both sexes of rat offspring after maternal exposure during the period from late gestation through lactation. *Toxicology* 203, 221-238.
- Li, N., Chen, X., Zhou, X., Zhang, W., Yuan, J. and Feng, J. 2015. The mechanism underlying dibutyl phthalate induced shortened anogenital distance and hypospadias in rats. *J Pediatr Surg* 50, 2078-2083.
- Lymperi, S. and Giwercman, A. 2018. Endocrine disruptors and testicular function. *Metabolism* 86, 79-90.
- Mahood, I.K., Hallmark, N., McKinnell, C., Walker, M., Fisher, J.S. and Sharpe, R.M. 2005. Abnormal Leydig Cell aggregation in the fetal testis of rats exposed to di (n-butyl) phthalate and its possible role in testicular dysgenesis. *Endocrinology* 146, 613-623.
- Marcotte, E.L., Pankratz, N., Amatruda, J.F., Frazier, A.L., Krailo, M., Davies, S., Starr, J.R., Lau, C.C., Roesler, M., Langer, E., Hallstrom, C., Hooten, A.J. and Poynter, J.N. 2017. Variants in BAK1, SPRY4, and GAB2 are associated with pediatric germ cell tumors: A report from the children's oncology group. *Genes Chromosomes Cancer* 56, 548-558.
- Martino-Andrade, A.J., Morais, R.N., Botelho, G.G., Muller, G., Grande, S.W., Carpentieri, G.B., Leao, G.M. and Dalsenter, P.R. 2009. Coadministration of active phthalates results in disruption of foetal testicular function in rats. *Int J Androl* 32, 704-712.
- Mason, J.M., Morrison, D.J., Basson, M.A. and Licht, J.D. 2006. Sprouty proteins: multifaceted

negative-feedback regulators of receptor tyrosine kinase signaling. *Trends Cell Biol* 16, 45-54.

McIntyre, B.S., Barlow, N.J. and Foster, P.M. 2002. Male rats exposed to linuron in utero exhibit permanent changes in anogenital distance, nipple retention, and epididymal malformations that result in subsequent testicular atrophy. *Toxicol Sci* 65, 62-70.

Moore, R.W., Rudy, T.A., Lin, T.M., Ko, K. and Peterson, R.E. 2001. Abnormalities of sexual development in male rats with in utero and lactational exposure to the antiandrogenic plasticizer Di(2-ethylhexyl) phthalate. *Environ Health Perspect* 109, 229-237.

Morena, A.R., Boitani, C., Pesce, M., De Felici, M. and Stefanini, M. 1996. Isolation of highly purified type A spermatogonia from prepubertal rat testis. *J Androl* 17, 708-717.

Mustafa, H.N. 2012. Effect of acrylamide on testis of albino rats. Ultrastructure and DNA cytometry study. *Saudi Med J* 33, 722-731.

Mylchreest, E., Cattley, R.C. and Foster, P.M. 1998. Male reproductive tract malformations in rats following gestational and lactational exposure to Di(n-butyl) phthalate: an antiandrogenic mechanism? *Toxicol Sci* 43, 47-60.

Mylchreest, E., Sar, M., Cattley, R.C. and Foster, P.M. 1999. Disruption of androgen-regulated male reproductive development by di(n-butyl) phthalate during late gestation in rats is different from flutamide. *Toxicol Appl Pharmacol* 156, 81-95.

Mylchreest, E., Wallace, D.G., Cattley, R.C. and Foster, P.M. 2000. Dose-dependent alterations in androgen-regulated male reproductive development in rats exposed to Di(n-butyl) phthalate during late gestation. *Toxicol Sci* 55, 143-151.

Norman, G.R. and Streiner, D.L. 2008. *Biostatistics: the bare essentials*, PMPH-USA, 2008, Hamilton, Ontario.

Ogawa, B., Ohishi, T., Wang, L., Takahashi, M., Taniai, E., Hayashi, H., Mitsumori, K. and Shibutani, M. 2011. Disruptive neuronal development by acrylamide in the hippocampal dentate hilus after developmental exposure in rats. *Arch Toxicol* 85, 987-994.

Ozaki, Y., Saito, K., Shinya, M., Kawasaki, T. and Sakai, N. 2011. Evaluation of Sycp3, Plzf and Cyclin B3 expression and suitability as spermatogonia and spermatocyte markers in zebrafish. *Gene Expr Patterns* 11, 309-315.

Pasterski, V., Acerini, C.L., Dunger, D.B., Ong, K.K., Hughes, I.A., Thankamony, A. and Hines, M. 2015. Postnatal penile growth concurrent with mini-puberty predicts later sex-typed play behavior: Evidence for neurobehavioral effects of the postnatal androgen surge in typically developing boys. *Horm Behav* 69, 98-105.

Perez-Albaladejo, E., Fernandes, D., Lacorte, S. and Porte, C. 2017. Comparative toxicity, oxidative stress and endocrine disruption potential of plasticizers in JEG-3 human placental cells. *Toxicol In Vitro* 38, 41-48.

Pertoff, H. 2000. Fractionation of cells and subcellular particles with Percoll. *J Biochem Biophys Methods* 44, 1-30.

Picut, C.A., Ziejewski, M.K. and Stanislaus, D. 2018. Comparative Aspects of Pre- and Postnatal Development of the Male Reproductive System. *Birth Defects Res* 110, 190-227.

Pieri, N., Souza, A.F., Mancanares, A., Roballo, K., Casals, J.B., Ambrosio, C.E. and Martins, D.S. 2017. Immunolocalization of proteins in the spermatogenesis process of canine. *Reprod Domest Anim* 52 Suppl 2, 170-176.

Poynter, J.N., Hooten, A.J., Frazier, A.L. and Ross, J.A. 2012. Associations between variants in KITLG, SPRY4, BAK1, and DMRT1 and pediatric germ cell tumors. *Genes Chromosomes Cancer* 51, 266-271.

Rajpert-De Meyts, E., Hanstein, R., Jorgensen, N., Graem, N., Vogt, P.H. and Skakkebaek, N.E. 2004. Developmental expression of POU5F1 (OCT-3/4) in normal and dysgenetic human gonads. *Hum Reprod* 19, 1338-1344.

Recio, L., Friedman, M., Marroni, D., Maynor, T. and Chepelev, N.L. 2017. Impact of Acrylamide on Calcium Signaling and Cytoskeletal Filaments in Testes From F344 Rat. *Int J Toxicol* 36, 124-132.

- Scott, H.M., Hutchison, G.R., Jobling, M.S., McKinnell, C., Drake, A.J. and Sharpe, R.M. 2008. Relationship between androgen action in the "male programming window," fetal sertoli cell number, and adult testis size in the rat. *Endocrinology* 149, 5280-5287.
- Singh, S.R., Burnicka-Turek, O., Chauhan, C. and Hou, S.X. 2011. Spermatogonial stem cells, infertility and testicular cancer. *J Cell Mol Med* 15, 468-483.
- Sisakhtnezhad, S. 2018. In silico analysis of single-cell RNA sequencing data from 3 and 7 days old mouse spermatogonial stem cells to identify their differentially expressed genes and transcriptional regulators. *J Cell Biochem* 119, 7556-7569.
- Skakkebaek, N.E., Rajpert-De Meyts, E. and Main, K.M. 2001. Testicular dysgenesis syndrome: an increasingly common developmental disorder with environmental aspects. *Hum Reprod* 16, 972-978.
- Sorgel, F., Weissenbacher, R., Kinzig-Schippers, M., Hofmann, A., Illauer, M., Skott, A. and Landersdorfer, C. 2002. Acrylamide: increased concentrations in homemade food and first evidence of its variable absorption from food, variable metabolism and placental and breast milk transfer in humans. *Chemotherapy*, Copyright 2002 S. Karger AG, Basel, Switzerland, pp. 267-274.
- Swan, S.H., Main, K.M., Liu, F., Stewart, S.L., Kruse, R.L., Calafat, A.M., Mao, C.S., Redmon, J.B., Ternand, C.L., Sullivan, S. and Teague, J.L. 2005. Decrease in anogenital distance among male infants with prenatal phthalate exposure. *Environ Health Perspect* 113, 1056-1061.
- Thankamony, A., Pasterski, V., Ong, K.K., Acerini, C.L. and Hughes, I.A. 2016. Anogenital distance as a marker of androgen exposure in humans. *Andrology* 4, 616-625.
- Tyl, R.W. and Friedman, M.A. 2003. Effects of acrylamide on rodent reproductive performance. *Reprod Toxicol* 17, 1-13.
- USEPA. 2014. Final Second List of Chemicals for Tier 1 Screening for Tier 1 Screening - 1.pdf.
- van Pelt, A.M., Morena, A.R., van Dissel-Emiliani, F.M., Boitani, C., Gaemers, I.C., de Rooij, D.G. and Stefanini, M. 1996. Isolation of the synchronized A spermatogonia from adult vitamin A-deficient rat testes. *Biol Reprod* 55, 439-444.
- Wang, H., Huang, P., Lie, T., Li, J., Hutz, R.J., Li, K. and Shi, F. 2010. Reproductive toxicity of acrylamide-treated male rats. *Reprod Toxicol* 29, 225-230.
- Wintour, E.M., Johnson, K., Koukoulas, I., Moritz, K., Tersteeg, M. and Dodic, M. 2003. Programming the cardiovascular system, kidney and the brain--a review. *Placenta* 24 Suppl A, S65-71.
- Wittassek, M., Angerer, J., Kolossa-Gehring, M., Schafer, S.D., Klockenbusch, W., Dobler, L., Günsel, A.K., Muller, A. and Wiesmuller, G.A. 2009. Fetal exposure to phthalates--a pilot study. *Int J Hyg Environ Health* 212, 492-498.
- Xing, J.S. and Bai, Z.M. 2018. Is testicular dysgenesis syndrome a genetic, endocrine, or environmental disease, or an unexplained reproductive disorder? *Life Sci* 194, 120-129.
- Zhang, R., Sun, J. and Zou, K. 2016. Advances in Isolation Methods for Spermatogonial Stem Cells. *Stem Cell Rev* 12, 15-25.

Tables

Table 1. Pups per litter and litter body weight on PND1 in animals exposed *in utero* to DBP or AA.

	Number of litters	Pups per litter	Litter BW (g)
CTL	10	14 [14.0 -14.0]	86.1 ± 6.7
DBP	10	13 [12.2- 13.5]	74.4 ± 5.4*
AA	10	13 [12.0 - 14.5]	78.0 ± 13.2

Data expressed as median [Q1 – Q3] or mean ± standard deviation and statistical analysis performed by Kruskal-Wallis and ANOVA followed by Tukey post-hoc test. *Significant difference from the control group at $p < 0.05$.

Table 2. Frequency of pups born, pups' body weight and anogenital distance in animals exposed *in utero* to DBP or AA.

	Frequency of pups (PND4 before culling)			PND4 (after culling)		
	Male (Total)	Female (Total)	Male F1 (dams)	BW (g)	AGD (mm)	Normalized AGD
CTL	26% (69)	27% (73)	67 (10)	8.6 ± 1.1	4.1 ± 0.5	2.0 ± 0.2
DBP	23% (61)	24% (64)	55 (10)	8.9 ± 1.2	3.4 ± 0.3*	1.6 ± 0.1*
AA	22% (59)	25% (68)	59 (10)	9.1 ± 1.3	4.1 ± 0.4	1.9 ± 0.2

Normalized AGD = Anogenital distance/body weight ^{1/3}. Data expressed as percentages or mean ± standard deviation. Statistical analysis performed by Fischer's exact tests or by a generalized linear mixed-effect model with gamma distribution followed by Sidak correction. *Significant difference from the respective control group at p < 0.05.

Table 3. Presence of nipple retention on PND14 after DBP or AA exposure.

Male nipple retention at PND14			
	Male F1 (dams)	Presence % (N)	p value
CTL	67 (10)	3.0 (2)	
DBP	55 (10)	78.2* (43)	<0.01
AA	59 (10)	0 (0)	0.6

Data expressed as percentages. Statistical analysis performed by a generalized linear mixed-effect model with logistic distribution followed by Sidak correction. *Significant difference from the respective control group at $p < 0.05$.

Table 4. Absolute and relative testes weights on PND24 and PND45 after *in utero* and postnatal exposure to DBP or AA.

	Male F1 (dam)	Absolute testes weight (g)	Relative testes weight
PND24			
CTL	10 (5)	0.34 [0.28 - 0.36]	0.49 [0.48 - 0.57]
DBP	10 (5)	0.28 [0.25 - 0.32]	0.43 [0.42 - 0.46]*
AA	10 (5)	0.35 [0.28 - 0.37]	0.49 [0.46 - 0.53]
PND45			
CTL	10 (5)	2.15 [1.96 - 2.36]	0.87 [0.85 - 0.97]
DBP	10 (5)	2.12 [1.76 - 2.29]	0.84 [0.76 - 0.95]
AA	10 (5)	2.17 [1.96 - 2.31]	0.86 [0.83 - 0.93]

Data expressed as median [Q1 – Q3]. Statistical analysis performed by a generalized linear mixed-effect model with gamma distribution followed by Sidak correction.

*Significant difference from the respective control group at $p < 0.05$.

Table 5. mRNA expression of Pou5f1, Kitlg, Mki67, Bak1 and Spry4 genes in isolated rat spermatogonia after *in utero* and postnatally exposure to DBP or AA until PND24 or PND45.

	Pou5f1 (N)	Kitlg (N)	Mki67 (N)	Bak1 (N)	Spry4 (N)
PND24					
CTL	1.00 ± 0.23 (2)	1.00 ± 0.15 (2)	1.00 ± 0.18 (2)	1.00 ± 0.10 (2)	-
DBP	0.19 ± 0.06* (3)	0.90 ± 0.13 (3)	0.10 ± 0.02* (3)	1.08 ± 0.16 (3)	-
PND45					
CTL	1.00 ± 0.37 (3)	1.00 ± 0.47 (2)	1.00 ± 0.18 (3)	1.00 ± 0.46 (3)	1.00 ± 0.35 (2)
DBP	0.50 ± 0.11* (6)	0.88 ± 0.47 (6)	0.86 ± 0.26 (6)	1.16 ± 0.25 (6)	0.65 ± 0.15* (4)
AA	0.58 ± 0.30 (3)	0.82 ± 0.33 (3)	0.62 ± 0.21 (4)	1.16 ± 0.25 (4)	0.79 ± 0.24 (2)

N = number of samples for each gene analysis. On PND24, rat spermatogonia exposed to AA did not resulted in good quality of mRNA; only CTL and DBP groups were analyzed and there was not enough amount of mRNA to evaluate Spry4 gene. Data expressed as mean ± standard deviation. Statistical analysis performed by Bio-Rad CFX Maestro software using ANOVA followed by Tukey post-hoc test. *Significant difference from the respective control group at $p < 0.05$.

Figures

Figure 1

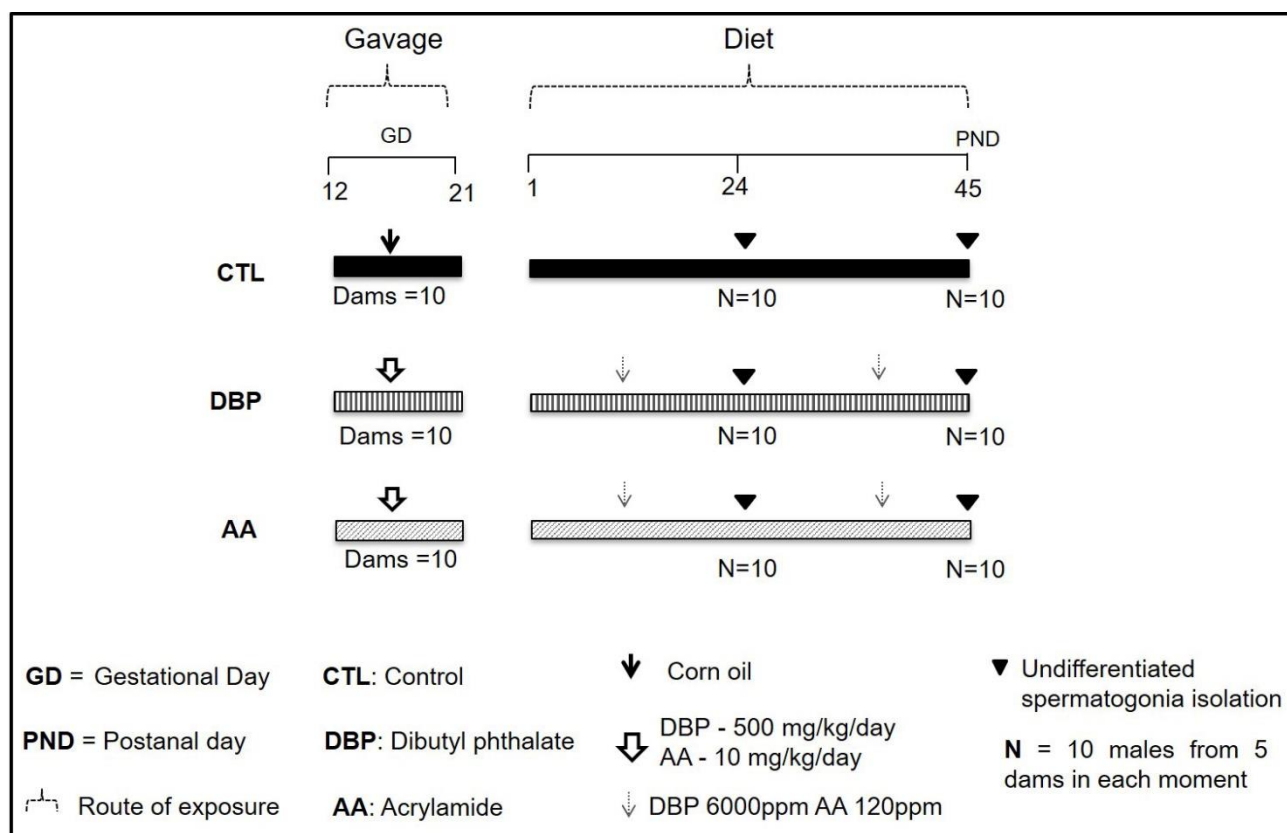


Figure 2

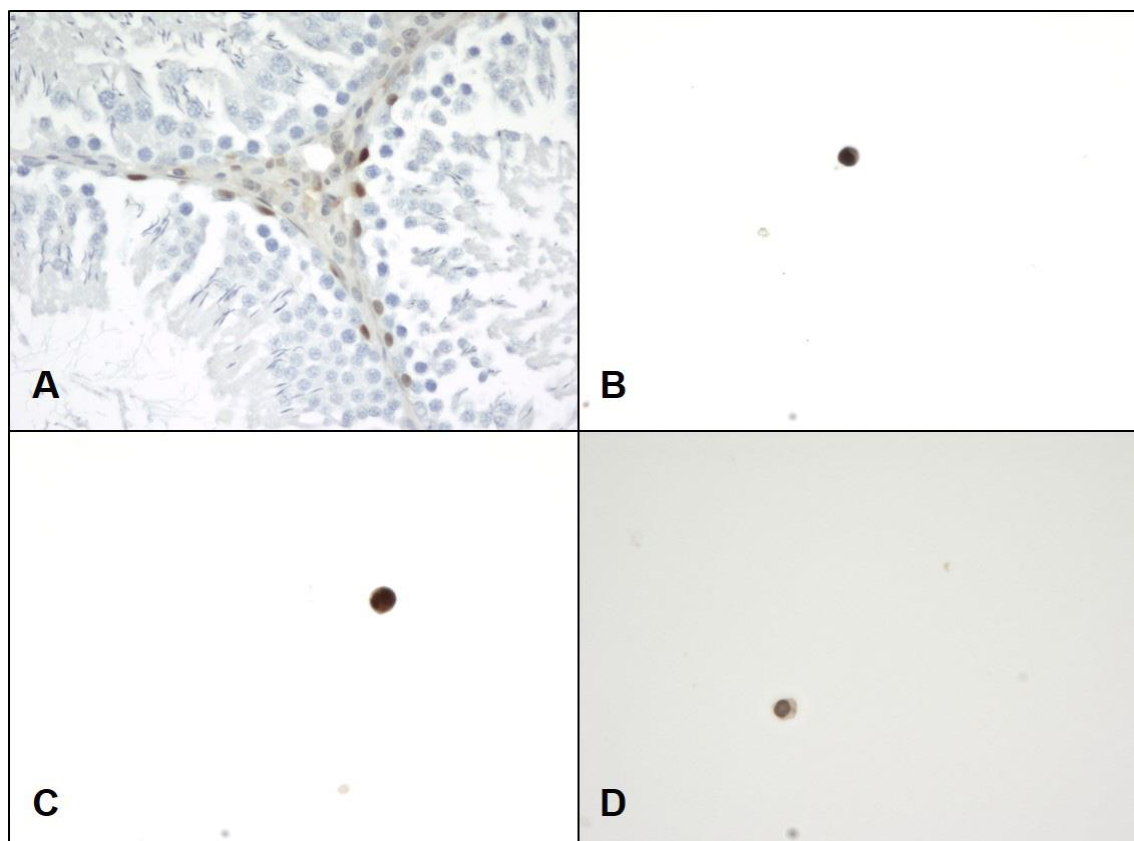


Figure 3

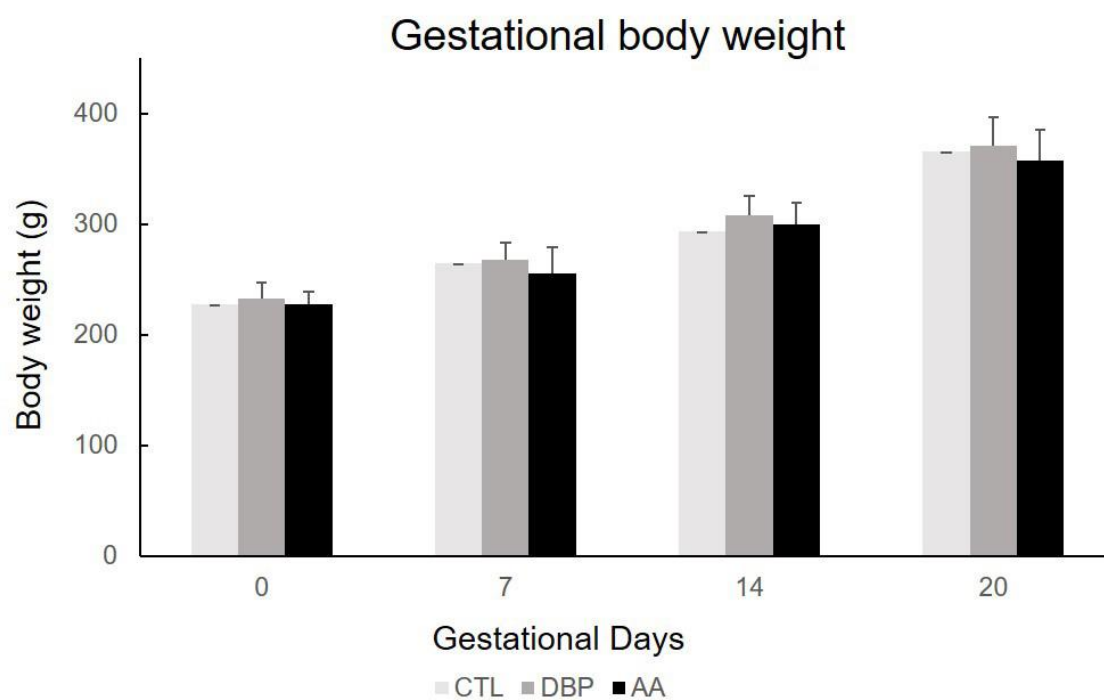


Figure 4

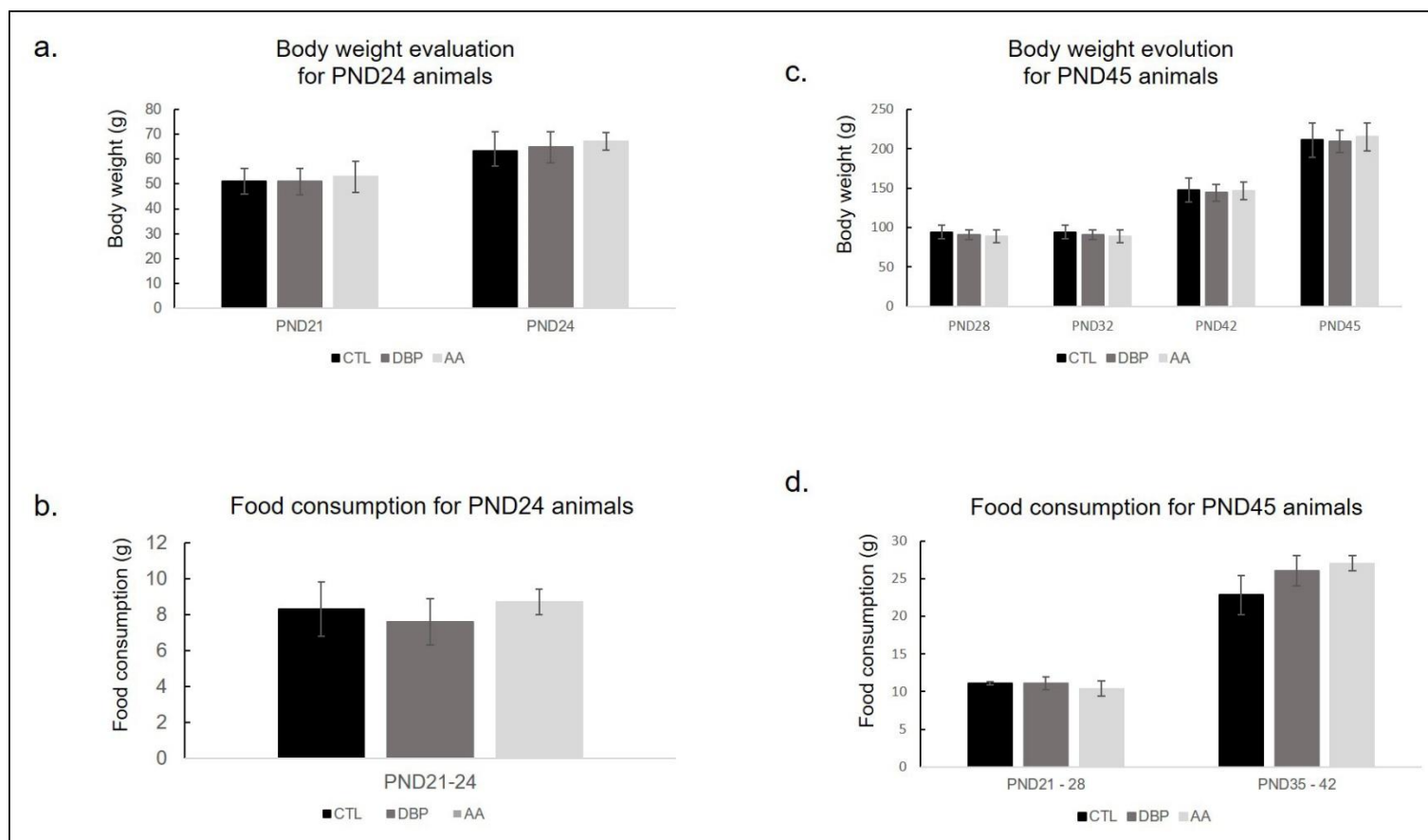
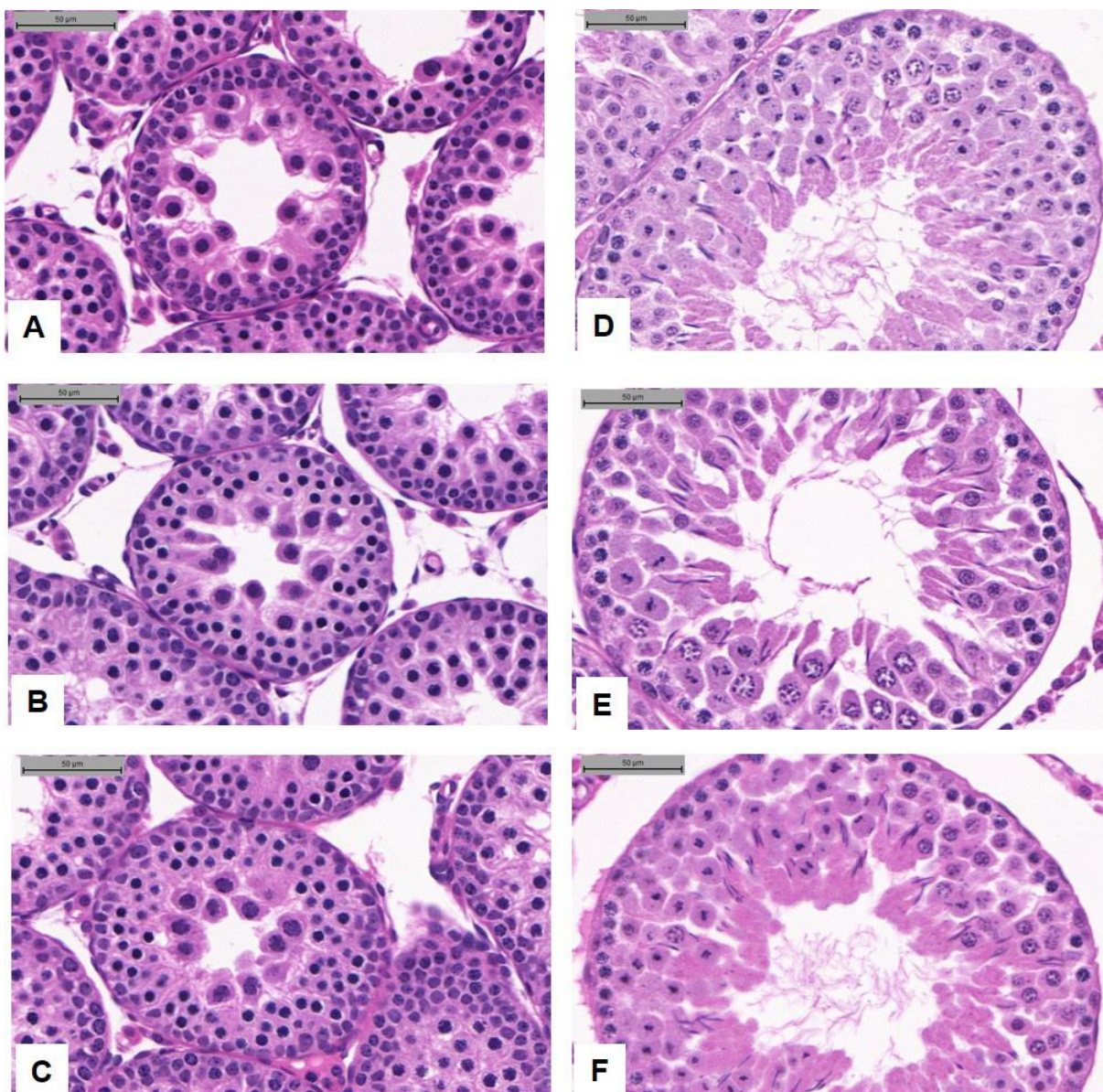


Figure 5



Legends of figures

Figure 1. Experimental design.

Figure 2. A) Rat pubertal testicular tissue used as a positive control for Plzf, an spermatogonia marker; **B-C)** Validation of the spermatogonia isolation procedure with a positive stain for Plzf.

Figure 3. Dam's body weight evolution during gestational period with oral exposure to corn oil (control), dibutyl phthalate (DBP) or acrylamide (AA) during PND12-21. Data expressed as mean $\pm$ standard deviation. Statistical analysis performed by ANOVA followed by Tukey post-hoc test. *Significant difference from the respective control group at $p < 0.05$.

Figure 4. Body weight evaluation and food consumption for animals exposed *in utero* and postnatally to DBP or AA until PND24 and PND45. Data expressed as mean $\pm$ standard deviation. Statistical analysis performed by a generalized linear mixed-effect model with gamma distribution followed by Sidak correction. *Significant difference from the respective control group at $p < 0.05$.

Figure 5. Histology of testes (H&E). **A, B, C.** Testes from control, DBP and AA animals on PND24; the germ cell epithelium was in development. **D, E, F.** Testes from control, DBP and AA animals on PND45; seminiferous tubules with complete spermatogenesis. In both moments, PND24 and PND45, seminiferous tubules exposed to DBP or AA did not differ histologically from control.

Conclusion

The spermatogonia isolation protocol developed in this study can be performed in two days, thus, it is not a time-consuming protocol; and it was efficient in pubertal rats since 95% of the isolated cells were positive for Plzf in 5-week old animals, suggesting the reliability of the protocol.

Our results suggest that DBP acts by reducing cell proliferation in addition to impairment of differentiation in the prepubertal and pubertal testes, with DBP exposure presenting possible additional toxicity due to its potential for endocrine disruption. AA exposure showed mild effects, but not statistically significant.

The association of *in vivo* exposure followed by *in vitro* approaches to analyze reproductive toxicity may improve acquired data and facilitate analysis of modes of action for chemicals.



UNESP



FACULDADE DE MEDICINA
FMB
BOTUCATU



CEUA
Ética

Comissão de Ética no Uso de Animais
Criada através da Portaria DFM nº 611 de 13/12/2012

CERTIFICADO Nº 1201/2016-CEUA

Certificamos que a proposta intitulada "Isolamento e caracterização molecular de células germinativas testiculares de ratos Sprague-Dawley expostos in utero e na vida pós-natal à acrilamida ou ao dibutilftalato", registrada com o n. 1201/2016, sob a responsabilidade da Nathália Pereira de Souza, a ser orientada pelo Prof. Dr. João Lauro Viana de Camargo, coorientado por Merielen Garcia Nascimento – que envolve a produção, manutenção ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto humanos), para fins de pesquisa científica (ou ensino) – encontra-se de acordo com os preceitos da Lei n. 11.794, de 8 de outubro de 2008, do Decreto n. 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle de Experimentação Animal (CONCEA), e foi aprovado pela Comissão de Ética no Uso de Animais da Faculdade de Medicina de Botucatu, em reunião de 24/11/16.

Finalidade	() Ensino (X) Pesquisa Científica
Vigência da autorização	01/01/2019
Espécie/Linhagem/Raça	Ratos Sprague Dawley
Nº de animais	215
Peso/Idade	até 600 gramas/16 semanas
Sexo	Macho/Fêmea
Origem	Biotério Central da UNICAMP - CEMIB

Prof. Dr. Guilherme Antônio Moreira de Barros
Presidente da CEUA

Kleber Messias Camargo
Kleber Messias de Camargo
Secretário da CEUA

Distrito Rubião Junior, s/nº - Botucatu - S.P. CEP: 18.018-687 Fone: (14) 3880-1608/3880-1609 e-mail secretaria: ceua@fmb.unesp.br



NEBRASKA'S HEALTH SCIENCE CENTER

Office of Regulatory Affairs (ORA)
Institutional Animal Care & Utilization Committee (IACUC)

May 30, 2017

Lora Arnold MS
Pathology/Microbiology
UNMC - 5900

IACUC # 17-047-05-EP

TITLE: Isolation and molecular characterization of testicular germ cells from male Sprague-Dawley rats exposed in utero and postnatally to acrylamide or dibutylphthalate

PERSONNEL:

Arnold, Lora L (Primary)
Cohen, Samuel Monroe (Contact)
Pennington, Karen L (Research Personnel)
Pereira De Souza, Nathalia (Research Personnel)

Review Date: 05/17/2017

Dear Dr. Arnold:

The Institutional Animal Care and Use Committee (IACUC) has completed its review of your protocol utilizing review criteria based on principles outlined in the Guide for the Care and Use of Laboratory Animals, Eighth Edition. As part of the final review process, a harm/benefit analysis was conducted weighing the potential adverse effects of the study against the potential benefits that are likely to accrue as a result of the research. It was determined that the benefits outweigh, or balance, the harms and the proposal is in compliance with the Public Health Service (PHS) Policy on the Human Care and Use of Laboratory Animals, USDA Regulations (where applicable), the Guide for the Care and Use of Laboratory Animals, and UNMC/UNO IACUC Animal Welfare Assurance, Guidelines and Policies. The IACUC, therefore, recommended your project for approval. This letter constitutes official notification of the approval and release of your project by the IACUC, and you are authorized to implement this project accordingly.

Please be advised that there may be additional training requirements before you are authorized to have contact with animals and/or access to the animal facilities. IACUC approval does not constitute completion of all required training nor provide authorization for contact with animals or access to animal facilities. Comparative Medicine (CM) can be reached at 559-4034 to assist you with the additional training requirements.

The Committee wishes to remind you that, under the provisions of the Animal Welfare Assurance (A3294-01) from UNMC/UNO to Animal Welfare Assurance (A3294-01) from UNMC/UNO to the Public Health Service (PHS), the principal investigator is directly responsible for keeping the IACUC informed of any proposed changes involving the care and/or use of animals. In addition, continuing review is required. Please notify the IACUC in writing if the protocol is completed during the approval period.

Best wishes for continued success in your research efforts.

Signed on: May 30, 2017

Robert G. Bennett, Ph.D., Executive Chair, IACUC
Andrew T. Dudley, Ph.D., Vice Chair, IACUC
987830 Nebraska Medical Center
Omaha, NE 68198-7830
Phone: 402-559-6046 or 3573
Fax: 402-559-3300



NEBRASKA'S HEALTH SCIENCE CENTER

Office of Regulatory Affairs (ORA)
Institutional Animal Care & Utilization Committee (IACUC)

email: iacucora@unmc.edu

Sent by:
Rebecca Bogatz
IACUC Associate
Institutional Animal Care and Use Committee (IACUC)
987830 Nebraska Medical Center
Omaha, NE 68198-7830
Phone: 402-559-3573
Fax: 402-559-3300
<mailto:iacucora@unmc.edu>
[IACUC website](http://www.unmc.edu/iacuc)