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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
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"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

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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

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 (Lymperi and Giwercman 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 20×10^6/ml to 15×10^6/ml (WHO 2010). In the 1940s the normal limit was considered to be 60×10^6/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).

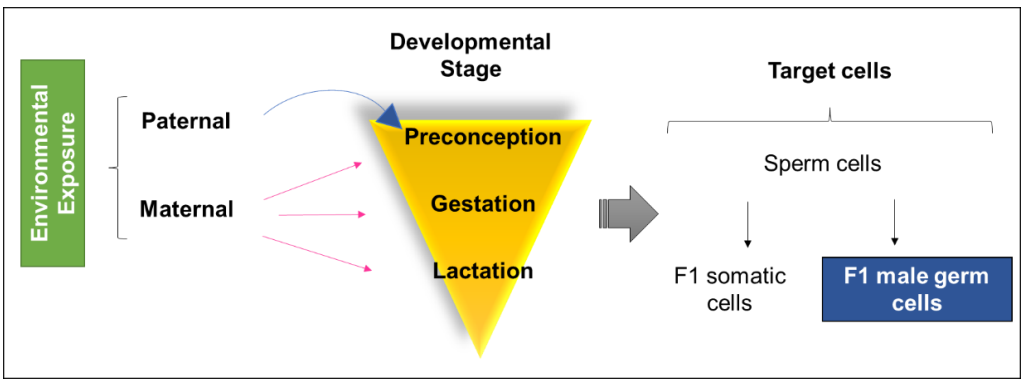


Figure 1. Developmental toxicity mechanism (Adapted from (Ong and Ozanne 2015).

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 Giwercman 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_{paired}/$A_{aligned}$ correspond to A_{pale} (Figura 2) (Singh et al. 2011).

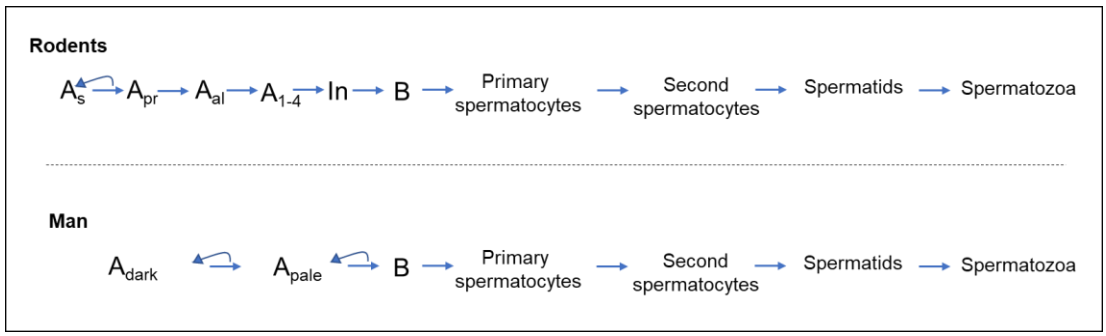


Figure 2. Schematic diagram showing stages of spermatogenesis in mouse and men (Adapted from (Singh et al. 2011).

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 α6 and β1 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 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. 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).

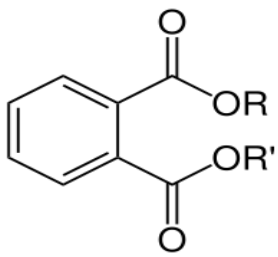
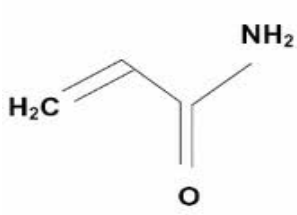
Dibutyl phthalate	Acrylamide
	

Figure 3. Chemical structures for DBP and AA.

The US Environmental Protect 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.

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