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THE MOUSE OOCYTE AS A MODEL IN REPRODUCTIVE TOXICOLOGY STUDIES

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To my beloved family

ABSTRACT

It is suspected that certain chemicals may cause alterations in female reproduction, including ovarian failure and birth defects. Sub-fecundity, infertility, pregnancy loss, fetal growth retardation, and birth defects are consequences proposed to occur. It has been estimated that about 3% of the observed birth defects could be attributed to the exposure to chemicals and other physical agents, and that as much as 25% of the observed birth defects may be due to a combination of genetic and environmental factors. Aneuploidy is a major cause of birth defects and it is mostly derived from errors in chromosome segregation during female germ cell development (oogenesis). The mechanisms of chemically induced aneuploidy in the oocyte are not well understood for any chemical.

The overall aim of the present study was therefore to provide new knowledge, which could aid in the development of a mouse experimental system for the detection and evaluation of chemicals, with aneugenic potential. More specifically, the present study was designed to 1) elucidate the underlying mechanism(s) involved in the reported aneugenic potential of bisphenol A (BPA), and to 2) evaluate the potential of different protein components, critically involved in meiotic chromosomal segregation, as markers for chemically induced aneuploidy in the mouse oocyte.

As opposed to previous reports, we could not confirm that BPA induces aneuploidy at the previously reported dose-level in C57BL 6/129 mice with mixed background. However, we were able to demonstrate that the mice lacking inducible nitric oxide synthase (iNOS) exhibited increased error-prone chromosome segregation in female meiosis after BPA exposure as compared to their wild-type counterparts. Further mechanistic characterization revealed that the protein expression of both polo-like kinase-1 (Plk1) and Ran GTPase (Ran) was dramatically reduced in iNOS-deficient oocytes.

Using female C57BL/6N wildtype mice we were able to demonstrate the specific localization of Wapl (wings apart-like) on the synaptonemal complex, a meiosis-specific structure connecting one pair of sister chromatids to the homologous pair of oocyte chromosomes.

Taken together, results obtained in the present study suggest that mouse oocytes have the potential to become a useful test-model for detection and evaluation of aneugenic chemicals. Oocytes derived from iNOS-deficient mice may aid in understanding the interactions between the fundamental biology of chromosomal segregation and the toxicological aspects of aneuploidy induction. Wapl, localized on the synaptonemal complex in female meiotic chromosomes and known to be affected by for example dioxin and 3-methylcholanthrene, has the potential to become a marker of chemicals, which interferes with the proper function of meiotic chromosomes, as well as an exposure marker of Ah-receptor ligands, which targets female germ cells.

LIST OF PUBLICATIONS

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LIST OF ABBREVIATIONS

BPA	Bisphenol A
DAPI	4', 6-diamidino-2-phenylindole
ER	Estrogen receptor
FSH	Follicle-stimulating hormone
iNOS	Inducible nitric oxide synthase
MI	Metaphase I
MII	Metaphase II
MTOCS	Microtubule organizing centers
NO	Nitric oxide
Plks	Polo-like kinases
SC	Synaptonemal complex
SMC	structural maintenance of chromosome
Wapl	Wings apart-like

BACKGROUND

1 GENERAL INTRODUCTION AND STUDY AIM

It is suspected that certain chemicals may cause alterations in female reproduction, including ovarian failure and birth defects. Sub-fecundity, infertility, pregnancy loss, fetal growth retardation, and birth defects are consequences proposed to occur (Bhatt 2000; Sharara *et al.* 1998). How reproductive toxicants can affect ovarian function is generally not well understood but their effects can be due to one or several possible mechanisms. Indirect effects on ovarian function might result from altered pituitary output of gonadotropins, due to disruption of neuroendocrine feedback systems. Alternatively, reproductive toxicants can have direct effect on steroid hormone production, affecting oocyte maturation and early maintenance of pregnancy (Gandolfi *et al.* 2002). Steroidogenesis, expansion of oocyte-cumulus complex, and meiotic maturation of the oocyte all represent intrafollicular processes taking important part in the background of successful fertilization.

It has been estimated that about 3% of the observed birth defects in humans could be attributed to the exposure to chemicals and other physical agents, and that as much as 25% of the observed birth defects may be due to a combination of genetic and environmental factors. Among a variety of the birth defects, chromosomal aneuploidy is a major cause mostly derived from errors in chromosome segregation during female germ cell development (oogenesis). For instance, in humans, aneuploidy is the most common genetic disorder, occurring in approximately 25% of all conceptions, 50% of all spontaneous abortions and 0.3% of all live births with significant physical or intellectual abnormalities such as Down Syndrome, Klinefelter's syndrome and Turner's syndrome (Hassold and Hunt 2001). Those aneuploid individuals are particularly prone to development of cancer due to gain or loss of one chromosome leading to a massive change in gene expression (Koller 1972).

Despite the high frequency of aneuploidy and its severe impact on reproduction, relatively little is known about the etiology and the molecular mechanisms of aneuploidy. Even less information is available about the impact of exogenous agents on the genesis of aneuploidy. Nine out of sixteen evaluated chemicals, including carbendazims, colchicine, and diethylstilbestrol, were classified as definitive aneugens by an European aneuploidy task force, which reviewed the role of aneuploidy in human disease and critically evaluated available data (Aardema *et al.* 1998). The task force appreciated that a variety of chemicals are capable of inducing aneuploidy and thus represents a potential hazard to the health of exposed individuals and their offspring, with a specific concern of agricultural herbicides (40% of those licensed for use in the European Union) and fungicides in use. The task force emphasised the urgent need to develop more specific, sensitive and efficient experimental systems for the detection and evaluation of aneugens, and therefore urged researchers to develop mechanism-based animal models, which could aid in the identification and understanding of environmental risk factors interfering with germ cell development.

On these grounds, the overall aim of the present study was to provide new knowledge, which could aid in the development of a mouse experimental system for the detection and evaluation of chemicals, which induce aneuploidy (i.e. aneugens). More specifically the present study was designed to 1) elucidate the underlying mechanism(s) involved in the reported aneugenic potential of bisphenol A (BPA), and to 2) evaluate the potential of

different protein components, which are known to be critically involved in meiotic chromosomal segregation, as markers for chemically induced aneuploidy in the mouse oocyte.

2 MOUSE OOGENESIS AND OOCYTE MATURATION

Oogenesis has been widely defined as the transformation of oogonia into primary oocytes, which embark on meiosis, and meiotic maturation. The production of a fertilizable ovum (egg) is a protracted process spanning the embryonic, postnatal, prepubertal and sexually mature life of the mammalian female.

In mice, oogonia undergo a final DNA replication and enter meiosis at 13.5 days post coitum (p.c.) Over the next couple of days they pass through leptotene, zygotene, and pachytene and finally reach the dictyate (prolonged resting) stage by 5 days post partum (p.p.). At birth, these resting oocytes are found in primordial follicles, surrounded by a single layer of granulosa cells. At puberty, recruitment of a pool of oocytes into a growth period in response to cyclic variations in gonadotrophins occurs. During this growth period, the primary oocyte increases in size but remains arrested in prophase of meiosis I (MI). Concomitantly, the number of granulosa cells surrounding the oocyte increases. After completion of this growth and proliferation phase to form the mature follicle, the primary oocytes resume meiosis and oocyte meiotic maturation starts. This is a process involving resumption of meiosis, progression to metaphase II (MII), and the accompanying cytoplasmic changes that prepare the oocyte for fertilization and subsequent embryonic development. In response to the preovulatory gonadotropin surge (Hyttel *et al.* 1986) the prominent nucleus (termed as germinal vesicle) disappears following chromosome condensation and dissolution of the nuclear membrane (germinal vesicle breakdown). Meiosis continues with chromosome segregation, asymmetric division, and extrusion of a polar body, and it proceeds to a second division, which is arrested in metaphase II until fertilization (Hyttel *et al.* 1989).

3 CHROMOSOMAL SEGREGATION

Recent research has profoundly advanced our understanding of how the faithful segregation of meiotic chromosomes is ensured by a protein complex called cohesin, which holds sister chromatids together, and several regulator proteins, such as the Shugoshin/MEI-S332 protein family, Plk1/Polo kinases, and the wings apart-like (Wapl) protein.

3.1 Cohesin complex and functions

The cohesin complex connects the sister chromatids from the time they are replicated. The mitotic cohesin complex is built by four core subunits: two members of the SMC (structural maintenance of chromosomes) family proteins (Hirano 2005; Jessberger 2003), i.e. Smc1 and Smc3, and two accessory subunits, i.e. Scc1 and Scc3 (Nasmyth 2001; Uhlmann 2003). Recent studies suggest that Smc1-Smc3 heterodimers, by their long stretches of coiled-coil, topologically embrace DNA strands. Scc1 interacts with the two ends of the cohesion ring, presumably with the aid of Scc3 (Haering and Nasmyth 2003), thereby closing it. The disassembly of the cohesin complex triggers sister chromatid separation, following cleavage of Rad21/Scc1 by separase. Thereby, the cohesin ring is opened to release sister chromatid cohesion, resulting in the separation of sister chromatids at the metaphase to anaphase transition (Uhlmann *et al.* 1999).

Cohesin is also present in cells undergoing meiosis. In addition to the mitotic cell components in mammals, three proteins involved specifically in meiotic chromosome cohesion have been identified: Rec8 (Rad21/Scc1 homolog), Stage3 (a Scc3 homolog) and Smc1 β (a Smc1 α isoform) (Parisi *et al.* 1999; Prieto *et al.* 2001; Revenkova *et al.* 2001). At the first meiotic division (meiosis I), homologous chromosomes, rather than sister chromatids, segregate from one another. Sister chromatids disjoin only at the second meiotic division (meiosis II) as they do in mitosis. The segregation of homologous chromosomes requires the release of chromosome arm cohesion at the onset of anaphase of meiosis I (during meiosis I), while centromere cohesion must remain between sisters until the metaphase II/anaphase II transition when the sisters finally separate (Watanabe 2005b). Therefore, the maintenance of centromere cohesion is essential to prevent chromosome missegregation in meiosis, but the molecular mechanisms underlying the control of this cohesive force have only recently begun to be understood.

3.2 *Shogushin*

It has been shown that the cohesin complex is protected at the centromere by a family of Shugoshin (Sgo) centromere protector proteins (the Shugoshin/MEI-S332 protein family), initially characterized as the *Drosophila* MEI-S332 protein (Kerrebrock *et al.* 1995) and subsequently found in yeast and vertebrates (Watanabe 2005a). MEI-S332 localizes to the centromere regions during prophase and is present until the onset of anaphase II, consistent with its role in the maintenance of centromeric sister chromatid cohesion. MEI-S332 mutations lead to precocious loss of sister chromatid cohesion beginning at the onset of anaphase I (Kerrebrock *et al.* 1995). One of the human Sgo proteins, hSgo1, has also been shown to associate with centromeres from prophase to anaphase, and to be essential to prevent premature sister chromatid separation (Tang *et al.* 2004). Segregation defects observed in HeLa cells depleted of hSgo1 by small interfering (si)RNA could be partially rescued by expressing a version of the SA2 (Scc3 homolog) cohesin subunit which can not be phosphorylated. MEI-S332/Sgo is regulated by protein kinase and phosphatase, such as Plk1/Polo kinase/phosphatase PP2A (Clarke *et al.* 2005; Rivera and Losada 2006).

3.3 *Polo-like kinase*

Polo-like kinase is a member of the polo protein family of serine/threonine kinases, a family which is conserved among organisms from yeast to mammals (Glover *et al.* 1998). Polo-like kinases (Plks) are involved in multiple stages of the cell cycle, including initiation of M phase, bipolar spindle formation, mitotic exit, cytokinesis (Glover *et al.* 1998), and DNA damage checkpoint regulation. A growing body of evidence strongly suggests that Plk1/Polo plays a critical role in mediating sister chromatid cohesion. In budding yeast, phosphorylation of Scc1 by the POLO kinase ortholog Cdc5 at serine residues adjacent to Scc1 cleavage sites strongly enhances their cleavage by separase (Alexandru *et al.* 2001). Cleavage of the meiosis-specific Rec8 protein by separase is completely dependent on Cdc5 (Clyne *et al.* 2003). More recently, it has been shown that both condensin and Cdc5 are required to phosphorylate and remove meiotic cohesin from chromosomes prior to the onset of anaphase I when homologs segregate (Yu and Koshland 2005). The release of *Drosophila* MEI-S332 from centromeres is regulated via phosphorylation by POLO kinase. In vertebrate cells, Plk1 has been implicated in chromatid arm separation through cohesin removal during early mitosis (Sumara *et al.* 2002). Moreover, displacement of endogenous Plk1 in HeLa S3 cells via overexpression of the polo-box domain of Plk1 resulted in congression failure of chromosomes, suggesting that maintenance of kinetochore-microtubule attachments depended on localized Plk1 activity (Hanisch *et al.* 2006).

In line with this multitude of proposed functions, it seems that Plk1 is associated with multiple distinct complexes/proteins, likely at discrete subcellular locations at different stages of the cell cycle. One of the newly identified proteins is the small GTP/GDP-binding protein Ran, which is a substrate of Plk1 and is phosphorylated on serine-135 during mitosis. It is now known that Ran is not only a chromatin signal stimulating spindle assembly, but is also required for chromosome congression at the metaphase plate as well as postmetaphase events, including chromosome segregation and the assembly of the microtubule midbody (Silverman-Gavrila and Wilde 2006).

The subcellular localization of mouse Plk1 during oocyte meiotic maturation displayed a punctate localisation along the broad spindle poles and on the meiotic chromosomes at metaphase II (Wianny *et al.* 1998), while Plk1 appeared at the spindle poles and then translocated to the middle region of the spindle at the early MII stage (Tong *et al.* 2002). In addition, confocal laser scanning microscopy revealed that Ran was concentrated to the MII spindle microtubules in mouse oocytes (Cao *et al.* 2005).

3.4 *Wapl, a cohesin-binding protein*

Drosophila Wapl was originally identified as a factor important for normal chromosome segregation and heterochromatin organization (Dobie *et al.* 2001; Verni *et al.* 2000). Recently two groups have demonstrated that the human ortholog of Wapl is a cohesin-binding protein that promotes sister-chromatid resolution in mitotic prophase (Gandhi *et al.* 2006; Kueng *et al.* 2006). Depletion of Wapl from HeLa cells causes transient accumulation of prometaphase-like cells with chromosomes that display poorly resolved sister chromatids. Conversely, overexpression of Wapl causes premature separation of sister chromatids. Both the mouse and human homolog of Wapl are expressed predominantly in the testes (Kuroda *et al.* 2005a). Detailed analysis by immunocytochemical staining showed that the mouse Wapl was localized on the synaptonemal complex (SC), a meiosis-specific structure connecting one pair of sister chromatids to the homologous pair in mouse pachytene spermatocytes. Intuitively, one could expect that Wapl plays a similar role in regulating chromosome segregation during female meiosis through cohesin complex.

4 ANEUPLOIDY

Aneuploidy, referring to a change in chromosome number from the normal diploid or haploid number for the species, originates from the malsegregation of chromosomes during cell division in somatic and germ cells. The underlying mechanism(s) of aneuploidy are not well understood but both genetic and environmental factors are known to contribute.

Aneuploidy in somatic cells is associated with the pathogenesis of many neoplasms (Li *et al.* 2000). It has been shown that most cancer cells, including virtually all of the over 5 000 solid human cancers that have been analyzed, are aneuploid, due to lost or gained chromosomes. In addition, an increased risk of tumour development has been observed with certain congenital aneuploidies. For example, individuals with Down's syndrome have a greater risk of developing leukaemia (Porter and Paul 1974). Increased incidence of breast tumour and gonadoblastoma are associated with Klinefelter's syndrome (Simpson and Photopoulos 1976). Evidence is now rapidly accumulating suggesting that genetic instability caused by aneuploidy is an important factor in tumor pathogenesis.

Aneuploidy in germ cells is a significant contributor to genetic abnormalities, which accounts for much human morbidity and mortality. An estimated 10-25 % of fertilized human oocytes are aneuploid, and numerical chromosome abnormalities are the leading cause of miscarriage or congenital defects (Hassold and Hunt 2001). In approximately 35% of all spontaneous abortions aneuploidy is the leading cause, and 4% of stillborn infants have a numerical chromosome abnormality. Of live-births, 0.3% are aneuploid (Hassold and Hunt 2001). The most frequent abnormality is trisomy 21 (Downs syndrome) with an incidence of 1 in 600 live births. More than 90 % of the karyotypic abnormalities observed in germ cells are due to a highly error-prone female meiotic process (Hassold and Hunt 2001). It is well known that oocyte meiosis is sensitive to endogenous and exogenous factors that could disturb the orderly sequence of oocyte maturation and dispose oocytes to faulty chromosome segregation (Eichenlaub-Ritter *et al.* 1996; Hansmann and Pabst 1992; Mailhes *et al.* 1997). Considerable efforts have been directed to identify factors that increase meiotic nondisjunction and a number of potential risk factors, including irradiation (Sperling *et al.* 1994), smoking (Yang and Ma 2001) oral contraceptives (Harlap *et al.* 1979), and chemicals (Czeizel *et al.* 1993) have been suggested.

Chemicals, which induce aneuploidy are known as aneugens. Unlike direct DNA-damaging agents, aneugens may act upon a wide variety of cellular targets involved in cell division as well as the chromosomes themselves. Consequently aneuploidy need not always be correlated with gene mutation or chromosomal aberration induction. Disruptive effects on any part of the cell division apparatus, e.g. spindle, microtubules, microtubule organizing centers (MTOCS) or kinetochores, may affect correct chromosome segregation. Direct damage to the chromosomes may also affect their orientation and attachment to the spindle apparatus (Aardema *et al.* 1998). Aneugens have been categorized as spindle poisons, meiosis inhibitors, and estrogenic chemicals as exemplified in Box 1.

5 BISPHENOL A CHEMISTRY AND TOXICOLOGY

In this project Bisphenol A (BPA, Figure 1) has been selected as the aneugenic model compound. BPA is a widely used industrial chemical, which in recent years, has received much attention due to the possibility that low, environmentally relevant doses can cause multiple effects on development and reproduction via an estrogenic mode-of-action (Box 1).

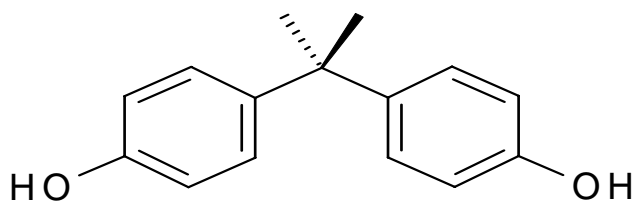


Figure 1. Structural formula of bisphenol-A

5.1 BPA chemistry, use, and stability

BPA is frequently used as a monomer to manufacture polycarbonate plastics, and as a component of epoxy resins widely used in consumer products, such as reusable food and drink containers, shatter-resistant baby bottles, electrical and electronic equipment and as lining for food and beverage cans. Furthermore, some polymers used in dental filling also contain BPA.

Heat and contact with either acidic or basic compounds accelerate hydrolysis of the ester bond linking BPA molecules in polycarbonate plastic and resins. Specially, heating of cans to sterilize food, the presence of acidic or basic food or beverage in cans or polycarbonate plastic, and repeated washing of polycarbonate products have all been shown to result in an

BOX 1: Examples of industrial chemicals and environmental pollutants with suspected aneugenic potential.

Carbendazims are spindle poisons, which affect tubulin polymerization and microtubular formation, with demonstrated reproductive toxicity. Carbendazim, inhibits microtubule assembly by binding to the B-subunit of tubulin. The actual binding to tubulin exhibits a characteristic dose-response pattern, which includes a threshold level where a critical number of target sites must be occupied before the biological effect is expressed. The damage is impacted by pharmacokinetic factors since the binding to the target sites is usually reversible and repeated exposure (particularly at low doses) may not be additive.

Estrogenic compounds can have a variety of physiological effects, especially on the reproductive system. Chemicals with estrogenic activity that are present in the environment may thus be considered potentially hazardous to reproduction and development. The potent synthetic therapeutic estrogen diethylstilbestrol (DES) and the widely used chemical bisphenol A (BPA), a structural analogue of DES, which is used in this study, belong to this category of aneugenic chemicals (*Marselos and Tomatis 1992*).

AhR ligands such as PAHs and dioxins are ubiquitous environmental contaminants acting on the reproductive system at low exposure levels. Young men exposed to air pollutants containing a higher level of PAHs exhibit aneuploid sperm, especially Y disomy, which may have implications for the risk of having an aneuploid child (*Sram et al. 1999*). PAHs also inhibit the progression of spermatocytes through meiotic division and are highly cytotoxic (*Georgellis et al. 1990*). Dioxin exposure affects gene expression in mammals. For example, the expression of the wings apart-like (*Wapl*) was induced by dioxin exposure in mouse embryonic stem cells (*Kuroda et al. 2005a*). *Wapl* protein was localized on the synaptonemal complex (SC), a meiosis-specific structure connecting one pair of sister chromatids to the homologous pair in mouse pachytene spermatocytes (*Kuroda et al. 2005a*) and oocytes (*Zhang et al. 2007*), suggesting that *Wapl* may have the potential of being a specific effect marker of chemicals during mammalian meiosis.

Acrylamide is a genetic, reproductive, and neural toxicant. The major concern for its genotoxicity centers on its clastogenic activity (*Aardema et al. 1998; Schmid et al. 1999*). This clastogenic activity has been observed in germinal tissues, which suggests the possible heritability of acrylamide-induced DNA alterations (*Dearfield et al. 1988*). In addition acrylamide causes meiotic delay that is correlated to aneuploidy induction. Acrylamide may act through a variety of biochemical mechanisms, such as effects on the motor protein kinesin that would affect meiosis/mitosis, axonal transport, and sperm flagellar activity (*Gassner and Adler 1995; Laws et al. 2000*). Chauhan et al (*Chauhan et al. 1993*) found that acrylamide affected microtubule associated proteins in rat brain, possibly inducing their loss and thereby changing the microtubule assembly kinetics.

increased rate of leaching of BPA (Brotons *et al.* 1995; Howdeshell *et al.* 2003; Kang *et al.* 2003; Kang and Kondo 2002). Ester bonds linking BPA molecules is also subject to hydrolysis, resulting in leaching of BPA monomer from new polycarbonate into water at room temperature (Howdeshell *et al.* 2003).

5.2 Human exposure to BPA

Several studies have shown that there is a broad exposure of the general population to BPA throughout all life-stages (Table 1). BPA was for example detected in 95% of the urine samples from a reference human population (Calafat *et al.* 2005). Other studies have shown that BPA is detected in mothers milk (Kuruto-Niwa *et al.* 2007; Sun *et al.* 2004), amniotic fluid and foetal plasma (Ikezuki *et al.* 2002). Milk levels were in the range 0.6-1.9 ng/ml, while serum levels varied between 0.6 and 4.4 ng/ml. It has been estimated that present human exposure levels are in the range 0.2-13 µg/kg bw/day (EFSA 2006).

Table 1. Average concentrations of BPA in human breast milk, serum, umbilical cord blood and urine.

Countries	n	BPA (total or free) (ng ml ⁻¹)	Samples	Methods	References
Japan (Shizuoka)	101	3.41 ± 0.13 (total)	Colostrum ^a	ELISA	(Kuruto-Niwa <i>et al.</i> 2007)
Japan	23	0.61 ± 0.20 (free)	Breast milk	HPLC ^b	(Sun <i>et al.</i> 2004)
USA	20	1.3 (free)	Breast milk	HPLC ^c	(Ye <i>et al.</i> 2006)
USA	20	1.9 (total)	Breast milk	HPLC ^c	(Ye <i>et al.</i> 2006)
Japan	9	0.46 ± 0.20 (free)	Maternal blood sera	HPLC ^b	(Kuroda <i>et al.</i> 2003)
Japan	9	0.62 ± 0.13 (free)	Umbilical cord blood sera	HPLC ^b	(Kuroda <i>et al.</i> 2003)
Japan	11	1.49 ± 0.11 (total)	Normal male sera	ELISA	(Takeuchi and Tsutsumi 2002)
Japan	14	0.64 ± 0.10 (total)	Normal female sera	ELISA	(Takeuchi and Tsutsumi 2002)
Germany	37	4.4 ± 3.9 (free)	Maternal blood	GC/MS	(Schonfelder <i>et al.</i> 2002)
Germany	37	2.9 ± 2.5 (free)	Umbilical cord blood	GC/MS	(Schonfelder <i>et al.</i> 2002)
USA	394	≥0.1 µg/L	Urine	GC/MS	(Calafat <i>et al.</i> 2005)

Data are shown as mean ± SD. ^a Within three days after delivery. ^b With column-switching and fluorescence detection. ^c On-line solid-phase extraction-HPLC-MS/MS.

5.3 BPA toxicology in vivo

The exposure of both female and male rats and mice to BPA has been reported to produce adverse reproductive effects such as increased prostate size, decreased epididymal weight,

increased androgen receptor binding activity (Nagel *et al.* 1997), induction of an uterotrophic response, modified estrous cycle (Kim *et al.* 2001; Laws *et al.* 2000). Exposure of pregnant mice during the last half of gestation, i.e. days 11-17, to BPA at 2 or 20 µg/kg body weight per day was associated with small increases in prostate and preputial gland weight and small decreases in daily sperm production and epididymal weight of adult male offspring at the age of 6 month (Nagel *et al.* 1997; vom Saal *et al.* 1998). However, in two separate studies (Ashby *et al.* 1999; Cagen *et al.* 1999), the adverse effects of low-dose BPA exposure were not replicated using the same strain of mice and design as in the previous studies.

Unintentional and intentional BPA exposure has, in a series of experiments in mice, been linked to a dramatic increase in aneuploidy levels as well as congression failure, which otherwise are infrequent in mice (Hunt *et al.* 2003). BPA derived from the cages as well as the plastic water bottles were identified as the causative agent and controlled treatment of juvenile female mice with BPA for 6–8 days confirmed the increased congression failure (Hunt *et al.* 2003).

5.4 *BPA toxicology in vitro*

BPA exerts estrogenic activity in a number of in vitro test systems (Kim *et al.* 2001) via binding to both estrogen receptor α and β (Gaido *et al.* 1997; Krishnan *et al.* 1993).

Low doses of BPA (1-3 nM) promote the development of 2-cell mouse embryos, while 100 µM concentrations inhibit developmental progression and reported that 1-10 µM BPA induced cell proliferation (Kim *et al.* 2001; Takai *et al.* 2000). BPA display a moderate to severe microtubule-disrupting effect at 75 µM doses (Nakagomi *et al.* 2001) and BPA disturbed centrosome and microtubular organization and disposed the formation of abnormal spindles and chromosome non-disjunction in mouse oocytes during meiosis (Can *et al.* 2005). It has also been shown that BPA induce meiotic cell cycle delay via degrading centrosomal proteins, thus perturbing the spindle microtubule organization and chromosome segregation (Can *et al.* 2005).

5.5 *Evaluation of BPA toxicology data*

Attempts to evaluate the BPA toxicology literature, including published and unpublished scientific data based on positive and negative effects on low dose BPA exposure, has been performed by the Endocrine Disruptors Low Dose Peer Review Panel comprised of experts from government, academia and industry (NIEH 2001) and the European Union Risk Assessment (EC 2003).

The NIEHS panel concluded that the low-dose effect of BPA had not been established as a general and reproducible finding on the basis of the number and power of studies that failed to show an effect (NIEH 2001). Furthermore, the mode-of-action and the biological relevance of the reported low dose effects were considered unclear. The discrepancies in the data were attributed to such factors as the intrauterine position phenomenon, dietary influences (such as background levels of phytoestrogens and caloric intake), animal strain differences (and differences developed within the same strain), caging (steel vs polycarbonate), bedding and housing (group vs individual) and seasonal variation, as well as differences among the studies in body and prostate weights of the unexposed control animals. The panel suggested that further mechanistic studies are needed to investigate the developmental effects of BPA in the

µg/kg bw dose range. Furthermore, it was suggested that differences between species and strains should be identified.

The conclusions regarding possible low-dose effects of BPA in the risk assessment from the European Union (EU 2003) support the conclusions of the NIEHS panel. A few studies reporting low-dose effects on the development of the male reproductive tract were reviewed but were disregarded in the final risk assessment. The reasons stated were that these results were not reproducible in further studies and the effects seen were of questionable biological relevance for human health. The available conflicting low-dose data did, however, raise uncertainties that this data could not be entirely dismissed. It was therefore concluded that further testing regarding developmental toxicity of BPA at low doses (in the µg-range) was needed. Furthermore, only a provisional NOAEL for developmental toxicity was set awaiting the results of further studies. The indication of differences in the sensitivity of different mice strains to the effects of estrogen was discussed as one reason for the conflicting developmental toxicity data.

A more recent opinion issued by the European Food Safety Authority (EFSA 2006) concludes that low-dose effects of BPA in rodents have still not been demonstrated in a robust and reproducible way and also expresses doubts regarding the relevance to humans of any low-dose effects observed in rodents.

In addition, a scientific review analysed 120 published reports on BPA toxicology and found that 109 of the studies demonstrated that low doses, i.e. below 50 µg/kg/day of BPA, caused significant effects in experimental animals, but that another 11 studies concluded that BPA caused no effects (vom Saal and Welshons 2006). The authors concluded that, based on examination of the published studies on low-dose effects of BPA, there is a need for a re-evaluation of the prior estimate of the acceptable level of daily human exposure to BPA.

THE PRESENT STUDY

1 AIM OF THE STUDY

The overall aim of this thesis project was to provide new knowledge, which could aid in the development of a mouse experimental system for the detection and evaluation of chemicals, which induce aneuploidy in oocytes.

More specifically the present study was designed to:

- elucidate the underlying mechanism(s) involved in the reported aneugenic potential of bisphenol A (BPA) in the mouse oocyte
- evaluate the potential of cohesion, Shugoshin, polo-like kinases (plks), and wapl, which are proteins known to be critically involved in meiotic chromosomal segregation, as markers for chemically induced aneuploidy in the mouse oocyte.

2 METHODS

2.1 *Animal models*

In the present studies, we employed mice with two different genetic background, i.e. study 1 with C57BL/129 (Jackson Laboratory, Bar Harbor, USA) and study 2 with C57BL/6N (Charles River, Germany). To increase the possibility of detecting chromosomal aberrations, we also used iNOS-deficient mice and their wild-type littermates. This strain of mice was chosen because it has been shown that the inducible nitric oxide synthase (iNOS) is involved in follicle development and iNOS was recently shown to be localized in mouse oocytes, suggesting that iNOS-derived NO acts directly on oocytes.

2.2 *Oral BPA Administration*

BPA (CAS-Nr: 80-05-7, Merck) was dissolved in ethanol (2 mg/ml) and then diluted with corn oil to 0.01 mg/ml. The final concentration of ethanol was 0.5% in corn oil. Preceding oocyte collection and analysis, juvenile females from iNOS^{+/+} and iNOS^{-/-} genotype (20- to 22-days-old) were treated with daily doses of 100 or 200 µg/kg body weight (bw) in a volume of 10 ml/kg bw for 13 days by gavage.

2.3 *"Dry-down" technique (paper 1)*

The ovaries were dissected from female embryos at 18.5 dpc in wild type mouse and spread using a "dry-down" technique (Peters *et al.* 1997). The fetal ovaries were dissected out and placed in PBS, and then transferred to a hypo-extraction buffer containing 30 mM Tris, 50 mM Sucrose; 17 mM Trisodium citrate dihydrate; 5 mM EDTA; 0.5 mM DTT; 0.1 mM PMSF pH 8.2 for 25-30 minutes. After that, each pair of ovaries was moved to a 25 µl drop of 100 mM sucrose solution and dissociated using fine needles. Then, 10 µl of suspension was dropped onto a slide dipped in a freshly made 1% PFA containing 10 mM sodium

borate pH 9.2 and 0.15% Triton-X100. The slide was immediately placed in the humid chamber to dry slowly for 2-6 hours. After drying, the slides were washed in a 0.04% solution of Kodak PhotoFlo in water and air dried. The dried slides were stored at -20°C or -80°C until analysis.

2.4 *Immunofluorescence staining (paper 1 and paper 2)*

Oocytes at pachytene and MII stages were incubated with primary and secondary antibodies for 1 h per antibody at 37°C followed by 1 h washes at 37°C in a PBS-blocking solution containing 10% NGS, 0.02% sodium azide and 0.1% Triton X-100 between incubation steps.

For labeling of microtubules and centrosomes, the oocytes were incubated in a 1:1 mixture of anti- α + β tubulin mouse monoclonal antibodies (1:100 dilution) (Sigma-Aldrich) and a 1:100 dilution of monoclonal mouse anti- γ -tubulin (Santa Cruz Biotechnology), followed by a 1:1200 dilution of an affinity-purified Cy3-conjugated donkey anti-mouse IgG (Jackson ImmunoResearch Laboratories). Plk localisation was visualised using a monoclonal mouse anti-Plk antibody (1:100 dilution) (Zymed Laboratories Inc.) and Cy3-conjugated donkey anti-mouse antibody (1:1200 dilution).

In study 2, pachytene oocytes were incubated with the two primary antibodies, polyclonal guinea pig anti- SYCP2 (1:500 dilution) (Novak *et al.* 2006) and polyclonal rabbit anti-Wapl (1:400 dilution) (Kuroda *et al.* 2005a), followed by a 1:1200 dilution of an affinity-purified Cy3-conjugated donkey anti-mouse IgG (Jackson ImmunoResearch Laboratories, PA, USA) and a 1:100 dilution of swine-anti-rabbit FITC (DakoCytomation).

Following antibody incubation, chromatin was stained with DAPI for 2 minutes. Oocytes were then washed briefly in PBST and then mounted on slides with Prolong Anti-Fade (Molecular Probes) mounting medium. Slides were viewed by Leica DMRA2 and DMRXA microscopes with epifluorescence, and images were captured with Hamamatsu digital charge-coupled device camera C4742-95 and OpenlabTM software version 3.1.4 (Wang and Hoog 2006).

2.5 *Fluorescence C-banding (paper 1)*

In conventional cytogenetic analysis, Standard Giemsa C-banding (Salamanca and Armendares 1974) is a method to distinguish and identify aneuploidy in mammalian germ cells (A'Arabi S *et al.* 1997; Mailhes *et al.* 2003; Mailhes *et al.* 1998). The shortage of the Standard Giemsa C-banding is that staining is so weak that chromosomes are difficult to visualize on slides. We therefore directly stained chromosomes using 4',6-diamidino-2-phenylindole (DAPI; 40 ng/ml) denaturalization after treatment with HCl and Ba (OH).

2.6 *The extraction of oocyte protein (paper 1)*

Ovaries were removed and placed in M2 medium (Sigma-Aldrich, USA) containing 6 mg/ml BSA. Antral follicles were punctured with 26 gauge needles to obtain immature oocytes at the germinal vesicle stage. Germinal vesicle stage oocytes were cultured for 16 h in M16 medium (Sigma-Aldrich, USA) containing 6 mg/ml BSA at 37°C in 5% CO_2 in air. Oocytes exhibiting a polar body the following morning were collected. Proteins from 50 or 100 oocytes/sample were collected in SDS sample buffer and heated to 100°C for 4 min.

After cooling on ice and centrifugation at 12 000 g for 3 min, the samples were frozen at –20°C until use.

2.7 *Immunoblotting*

Total oocyte protein was used for testing expression of Plk1, Ran and p-ERK1/2. Separation was carried out using SDS-PAGE with a 4% stacking gel and a 10% separating gel for 30 min at 90 V and 2.5 h at 120 V, respectively, and were then electrophoretically transferred onto nitrocellulose membrane for 2.5 h at 200 mA, at 4°C. Then the membrane was blocked overnight at 4°C in PBST buffer (20 mM Tris, 137 mM NaCl, 0.1% Tween 20, pH 7.4) containing 5% low-fat milk. After that, the membrane was incubated with mouse anti-p-ERK1/2 antibody (Santa Cruz Biotechnology, Inc), or mouse anti-Plk1 antibody (Zymed Laboratories Inc., South San Francisco, CA), goat anti-Ran antibody (Santa Cruz Biotechnology, Inc) overnight at 4°C. To check ERK1/2 and Plk1, the washed membranes were incubated with HRP-conjugated rabbit anti-mouse IgG for 1 h at room temperature. For Ran, the membrane was treated with HRP-conjugated rabbit anti-goat IgG for 1 h at 37°C. Finally, the membranes were washed three times in TBST and then the specific proteins were visualized using chemiluminescence detection system. The blot membranes were later stripped with stripping buffer (50 mM Tris–HCl pH 6.8, 100 mM β -mercaptoethanol, 1% SDS) and re-probed with anti-GAPDH (Ambion) as a loading control. Immunoblot density was determined by Image Quant 5.2 software.

2.8 *Statistical methods*

Chi-square (two-tailed) analysis and Fisher's exact test were used for analyzing the cytogenetic data and congression failure. Fisher's exact test was used when the numerator was less than five. Immunoblotting for quantities of p-ERK1/2, Plk1 and Ran as well as distribution of Plk1 protein were analyzed by Student t-test. Difference at $p < 0.05$ were considered significant.

3 **RESULTS AND DISCUSSION**

3.1 *Fluorescence C-banding*

Karyotype analysis of mouse oocytes with the conventional C-banding Giemsa staining gave unsatisfactory results either due to loss of chromosome morphology or to the absence of distinct staining. Paper 1 described the development of a new technique, named "Fluorescence C-banding", which revealed better results with centromeric regions in bright, white color and arms in blue under fluorescence microscope (Figure 2). This method, has the advantages of decreased appearance of dissolved chromosome arms and improved staining compared both to the "Giemsa C-binding method" and the "combined formamide DAPI method". In addition, the self-developed method is considerably simpler and less time-consuming than the conventional C-banding technique.

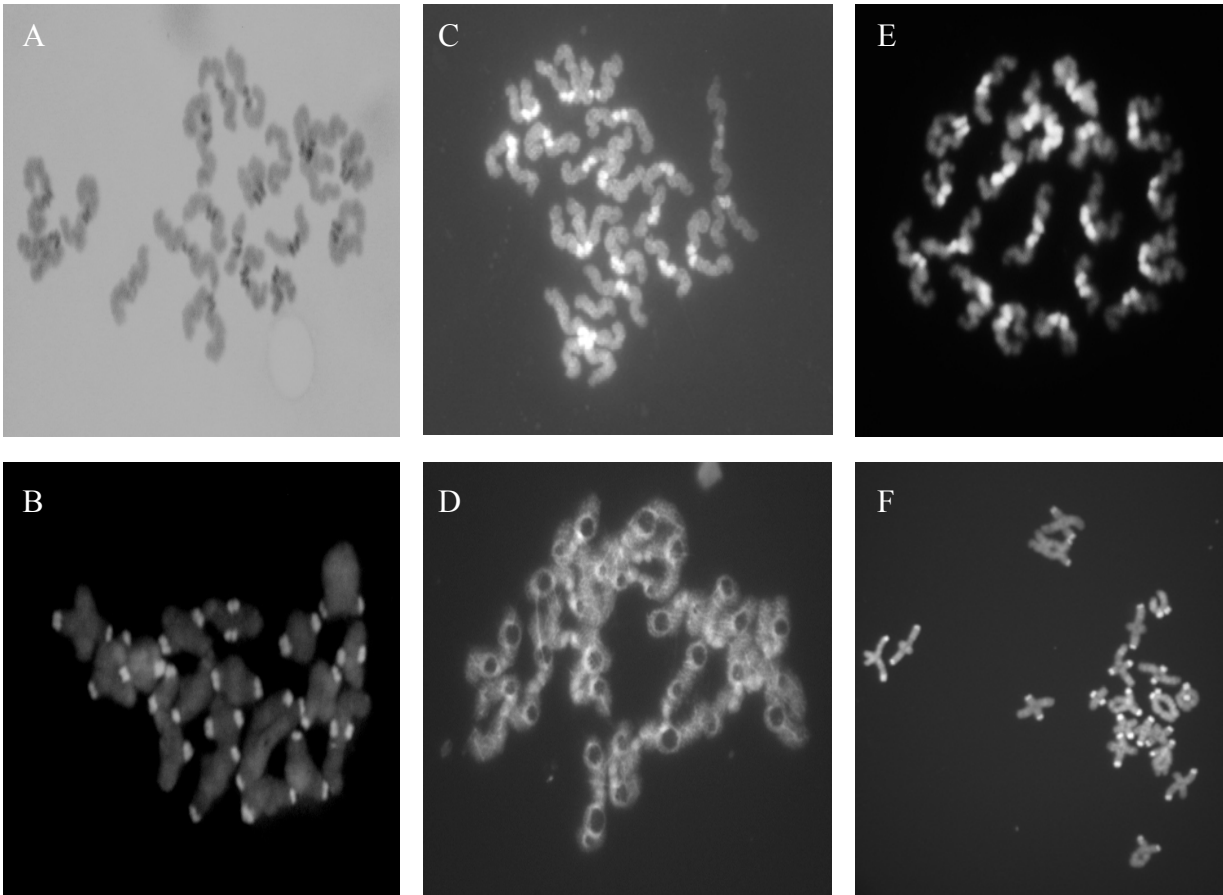


Figure 2. Cytogenetic analysis in mouse oocytes. A-B: Giemsa C-binding. A)- mouse MII oocyte, normal, $n=20$ chromosomes. B) Mouse MI oocyte, normal, $n=20$ bivalents. C-D: Formamide pretreatment and DAPI staining. C) Mouse MII oocyte, aneuploidy, 19 chromosomes plus 2 chromatids-arrowed, chromosomal arms were partly dissolved. D) Mouse MII oocyte, chromosomes were mostly dissolved. E-F: Direct DAPI staining: E) Mouse MII oocyte, normal, $n=20$ chromosomes. F) Mouse MI oocyte, normal, $n=20$ bivalents.

3.2 *iNOS deficiency sensitizes mouse oocytes to BPA-induced disturbances in chromosome congression, precocious chromatid segregation and aneuploidy*

As reported in Paper 1, we could not confirm that BPA induced meiotic aneuploidy at the previously reported dose-level in C57BL6/129 mice with mixed background. However, we did find that the frequencies of aneuploid oocytes were significantly higher in iNOS^{-/-} female mice exposed either to 100 or 200 µg/kg BPA compared to iNOS^{+/+} controls. Of note, single chromatids resulting from premature centromere separation were significantly elevated in those oocytes derived from BPA-exposed iNOS^{-/-} female mice. Immunostaining with antibodies against α + β + γ -tubulins revealed defects in the alignment of the chromosomes on the MII spindle, i.e. congression failure, which was raised in oocytes from BPA-exposed iNOS^{-/-} mice. Further mechanistic characterization revealed that the protein expression of both polo-like kinase-1 (Plk1) and Ran GTPase (Ran) was dramatically reduced in iNOS-deficient oocytes compared to iNOS^{+/+} controls.

Our finding of failure of BPA to increase aneuploidy was contradictory to Hunt's report (Hunt et al 2003), but was consistent with others (Eichenlaub-Ritter 2007; Lenie 2007; Pacchirotti 2007). Eichenlaub-Ritter et al. (2007) found that BPA did not increase hyperploidy at meiosis II at any tested concentration (50 ng/ml to 10 µg/ml) in vitro. 30 M BPA reduced granulosa cell proliferation slightly in early stages of preantral follicle culture, and at this concentration significantly fewer oocytes extruded a first polar body as compared to controls (Lenie 2007). Low oral BPA exposure in vivo during the final stages of oocyte growth subtly affected spindle morphology and oocyte maturation rate in vitro in prepubertal mice (Eichenlaub-Ritter et al. 2007). However, there was no evidence that low BPA doses increased hyperploidy at meiosis II. No significant induction of hyperploidy was observed at any treatment conditions (chronic, subchronic, or acute exposure) in adult mice in vivo (Pacchirotti 2007).

Our analyses in paper 1 showed that iNOS^{-/-} mice did not display a higher incidence of congression failure and aneuploidy as compared to wildtype. However, we observed an increased frequency of aneuploid oocytes in iNOS^{-/-} mice treated with BPA leading us to suspect that iNOS deficiency may be a genetically predisposing lesion that increases the susceptibility to BPA exposure. In line with this observation, several other studies have recently demonstrated the protective effects of iNOS in various test systems. For example, iNOS^{-/-} mice were more susceptible to ozone-induced lung inflammation and injury than their isogenic iNOS^{+/+} counterparts (Kenyon et al. 2006). The iNOS-deficient mice exhibited increased hepatotoxicity after subacute fumonisin B exposure compared to their wild type counterparts, the liver regeneration was lower in iNOS^{-/-} mice compared to that in the WT mice (Suzuki et al. 2007). It is now known that a low level of expression of iNOS will reflect a positive host-defense response to challenge, but that exaggerated or uncontrolled expression of iNOS itself becomes detrimental.

iNOS-derived NO has long been known to be a major paracrine mediator and physiological regulatory agent in various female reproductive processes, such as ovulation, implantation, and pregnancy maintenance (Lapointe et al. 2006; Mitchell et al. 2004). However, the present study for the first time demonstrated that iNOS-derived NO acted directly on meiotic oocytes during chromosome segregation. We also found a clear reduction of both Plk1 and Ran protein expression in iNOS^{-/-} oocytes and altered distribution of Plk1 around condensed chromosomes in BPA-exposed iNOS^{-/-} oocytes, compared to their corresponding controls. It has been suggested that a major function of the Plk1/Polo kinase during chromosome

segregation is being a key regulator of the release of sister chromatid cohesin (Revenkova and Jessberger 2005). For example, Polo kinase controls the release of the *Drosophila* cohesion protein MEI-S332 from centromeres through phosphorylation (Clarke *et al.* 2005). In vertebrate cells, Plk1 is required for chromatid arm separation through cohesin removal during early mitosis (Gimenez-Abian *et al.* 2004; Hauf *et al.* 2005; Sumara *et al.* 2002). Displacement of endogenous Plk1 in HeLa S3 cells through overexpression of the polo-box domain of Plk1 resulted in congression failure of chromosomes, suggesting that maintenance of kinetochore-microtubule attachments depended on localized Plk1 activity (Hanisch *et al.* 2006). A number of Plk1 substrates have been identified, of which the small GTP/GDP-binding protein Ran is phosphorylated by Plk1 on serine-135 during mitosis (Feng *et al.* 2006). It is now known that Ran is not only a chromatin signal stimulating spindle assembly, but also required for chromosome congression at the metaphase plate as well as postmetaphase events, including chromosome segregation and the assembly of the microtubule midbody (Silverman-Gavrila and Wilde 2006).

Taken together, we propose that absence of iNOS in mouse oocytes results in reduced Plk1 expression that is required for proper regulation of Ran in cells. In turn, both deregulation of Plk1 and Ran sensitize mice to BPA and lead to a significantly increased incidence of aneuploidy in mouse oocytes.

3.3 *Wapl localization on the synaptonemal complex of meiotic chromosomes in mouse oocytes*

To characterize protein components involved in meiotic chromosome segregation, we selected Wapl to examine its expression pattern at the prophase of meiosis I in mouse embryonic oocytes. With immunofluorescence assay, we found that Wapl was localized on the synaptonemal complex in the mouse oocytes as revealed by colocalization with SYCP2 (a marker for SC in mammals).

Our finding in paper 2, together with Kuroda's report (Kuroda *et al.* 2005a), strongly suggests that Wapl may play a crucial role in meiotic chromosome remodelling at early meiosis. It is known that Wapl is a cohesin-binding protein, which forms a stable subcomplex with cohesin and controls cohesin dissociation from the vertebrate chromatin (Gandhi *et al.* 2006; Kueng *et al.* 2006; Kuroda *et al.* 2005a). Aberrant Wapl caused embryonic lethality (Oikawa *et al.* 2004), cervical cancer (Oikawa *et al.* 2004), and induced aneuploidy and chromatid breaks and accelerated chromosomal instability (Ohbayashi *et al.* 2007). In addition, the expression of Wapl is affected by dioxin and 3-methylcholanthrene (3-MC), known as chemical carcinogens present in the environment (Kuroda *et al.* 2005a; Kuroda *et al.* 2005b).

Taken together, we propose that Wapl in mouse oocytes has the potential of being a specific effect marker of chemicals, which interfere with the proper function of the synaptonemal complex during meiosis, as well as an exposure marker of potent Ah-receptor ligands, which targets female germ cells.

4 CONCLUSIONS

Taken together, results obtained in the present study suggest that mouse oocytes have the potential to become a useful test-model for detection and evaluation of aneugenic chemicals.

Oocytes derived from iNOS-deficient mice may aid in the understanding of interactions between the fundamental biology of chromosomal segregation and the toxicological aspects of aneuploidy induction, due to their potential for increased susceptibility to estrogen-like chemical exposure.

Wapl, localized on the synaptonemal complex in female meiotic chromosomes and known to be affected by dioxin and 3-MC, has the potential to become a marker of chemicals, which interferes with the proper function of meiotic chromosomes, as well as an exposure marker of potent Ah-receptor ligands, which targets female germ cells.

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