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Inos Deficiency Sensitizes Mouse Oocytes to Chromosome Nondisjunction in Response to Bisphenol A Exposure

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ABSTRACT

Inducible nitric oxide synthase (iNOS)-derived NO is a major paracrine mediator and physiological regulator in female reproductive processes, such as folliculogenesis, ovulation and implantation. Even though localization of the enzyme was described, the function of iNOS and its impact in response to environmental exposures on oocyte maturation remain elusive. We therefore employed mice with a targeted deletion in the iNos gene to evaluate the role of iNOS in meiotic chromosome segregation in female mice exposed to bisphenol A (BPA). BPA is an industrial chemical released into the environment that was detected in human follicular and amniotic fluid. It possesses weak affinity for estrogen receptors and is suspected to affect chromosome segregation and induce nondisjunction in mammalian oocytes. We found that there was no significant increase in aneuploid metaphase II oocytes of wild type mice (iNos+/+) orally exposed to 100 or 200µg/kg body weight BPA for 13 days. However, oocytes of the iNos-knockout mice (iNos-/-) exhibited increased error-prone chromosome segregation and precocious chromatid separation. Characterization of gene expression revealed that two components of cell cycle and spindle regulation were dramatically down regulated at the protein level, the polo-like kinase-1 (PLK1) and the RAN GTPase (RAN) in the iNos-/- oocytes. Our results therefore suggest that iNOS has a direct or indirect protective role on faithful chromosome segregation during meiosis, possibly by maintaining expression of stable levels of PLK1 and RAN in mammalian oocytes. Furthermore, our data suggest that low level BPA exposure may induce chromosomal aberrations in a iNos deficient genetic background.

KEYWORDS inducible nitric oxide synthase, bisphenol A, meiotic aneuploidy, spindle, oocyte.

Introduction Nitric oxide (NO) may serve as the second messenger in a number of processes within cells. In mammalian tissue, several isoforms of nitric oxide syntase (NOS) have been identified, neuronal NOS (nNOS or NOS-1), inducible NOS (iNOS or NOS-2), endothelial NOS (eNOS, NOS-3), and mitochondrial NOS (mtNOS). Most of the constitutive enzymes are calcium-dependent, like the calcium dependent mitochondrial NOS (mtNOS) enzyme that has the potential to regulate the steady-state concentration of NO at sub-cellular levels, regulating mitochondrial events such as activation of calcium signaling, inhibiting mitochondrial respiration, and probably regulating formation of reactive oxygen species [1, 2]. iNOS, in contrast to nNOS, eNOS and mitNOS, catalyzes the conversion of L-arginine to NO in a calcium-independent and non-constitutive manner. It is expressed in a variety of cell types and belongs to the immediate early family of genes that are rapidly up-regulated in response to multiple inflammatory stimuli [3]. Early studies have focused on the potential toxicity of the ensuing high-output NO-synthesis that may be involved in eliminating pathogens or tumor cells as well as local destruction of tissue during chronic inflammation. However, recent studies focusing on the protective facets of iNOS-derived NO challenged the dogma about NO's mainly detrimental effects [4, 5]. For instance, the expression of iNos is up-regulated with subsequent synthesis of large quantities of NO following injury of the vasculature, which has a powerful vasoprotective effect by inhibiting platelet aggregation and leukocyte chemotaxis, and promoting endothelial cell survival and growth [6]. Work with a mouse strain carrying the targeted deletion of iNos ($iNos^{-/-}$) demonstrated the protective effects of iNOS in response to stress by exposures and/or mutations, for example, protection from ozone-induced lung inflammation and injury [7]. Furthermore, a protective, anti-neoplastic role in early stages of intestinal tumorigenesis in the $ApcMin^{+/+}$ mouse (a model of familial adenomatous polyposis) [8] as well as in colitis-induced adenocarcinoma in $Il\text{-}10^{-/-}$ mice has been shown [9]. The multi-faceted effects of iNOS-derived NO suggest that a low level of iNOS expression will result in a positive host-defense response to challenges, while exaggerated or uncontrolled expression of iNOS and NO may be rather 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 folliculogenesis, ovulation, implantation, and pregnancy maintenance [10, 11]. Thus, NOS activity is necessary at a local level for normal development of mouse follicles in vitro [10]. $iNos^{-/-}$ mice do not have an overt fertility phenotype although iNOS is expressed and localized in a maturation-dependent fashion in wild type mouse oocytes [12]. So far the role of iNOS-derived NO in oocyte maturation is enigmatic. Inhibition of iNOS-derived NO by the iNOS-specific inhibitor aminoguanidine (AG) affected the phosphorylation of mitogen-activated protein (MAP) kinase in isolated oocytes [12]. We therefore used Western blotting with protein from iNos wild type and $iNos^{-/-}$ mice to assess the influence of iNOS on the MAP kinase signaling cascade, previously shown to be essential in mammalian oocyte maturation [13,14].

One hallmark of meiotic maturation in mammalian oocytes is nuclear maturation resulting in the extrusion of the first polar body following chromosome segregation at meiosis I and the alignment of the chromosomes on the spindle equator at the metaphase of meiosis II. Defects in spindle assembly, chromosome alignment (termed congression failure[15,16]; or cell cycle control [17-18] in oocytes can lead to chromosome segregation errors, which have dramatic consequences for developmental potential. In humans, it is estimated that on average 15-20%

of oocytes have numerical chromosome aberrations (aneuploidy) linked predominantly to segregation errors at meiosis I[20]. Numerical chromosomal aberrations reduce the developmental potential of the embryo and may cause physical and mental retardation of affected human individuals, for instance such suffering from aneuploidies associated with Down Syndrome, Klinefelter's syndrome or Turner's syndrome [20] .

To date, there are no data available regarding the role of iNOS in modulation of chromosome segregation although it is known that iNOS cell cycle-dependently associates with the nucleus and with chromosomes and the spindle in mammalian oocytes[12, 21]. Thus, there is an imminent need to address the physiological importance of iNOS and to elucidate the protective versus the destructive role of iNOS-derived NO under various stresses during chromosome segregation in oocytes. In the current study, we employed therefore a iNos$^{-/-}$ mouse model to evaluate the role of iNOS during meiotic chromosome segregation. First, it was tested whether knockout of iNos affects spindle formation and chromosome congression in oocytes. Furthermore, the response to an environmental stress, which potentially increases oocyte aneuploidy, was analysed by exposing iNOS-proficient and -deficient mice to bisphenol A (BPA).

BPA is a frequently used industrial chemical with aneuploidy-inducing potential[23]. BPA is a major component of polycarbonate plastics and resins [24,25] and can be released by exposure of plastics to UV light, heating or washing with highly acidic or basic detergent, or heat and exposure to microwave. Therefore, human exposure can relate to BPA release from polycarbonate plastics, lining of cans and lids of glass bottles, from baby bottles, dental sealants or even water pipes and storage tanks [26]. BPA has been detected in human follicular fluid and amniotic fluid [27,28]. Based on conservative migration values, it has recently been estimated that 6 months old infants fed both with formula from polycarbonate bottles and with canned foods and beverages may be exposed to up to 13 mg BPA/kg body weight (bw) daily in their diet whereas daily intake in adults can reach levels of 1.5 ug/kg bw per day [26]. Microgram to nanogram amounts of BPA have been detected in canned foods [29]. Concentrations of 2.0 ± 0.8 ng/ml (or 8.8 nM) and 2.4±0.8 ng/ml (or 10.5 nM) BPA were found in serum and follicular fluid in non-pregnant and premenopausal healthy women, and BPA was also detected in urine [30-32]. Although BPA is efficiently glucuronidated and excreted in urine in humans, risks by effects of chronic low dose exposure are debated [26]. There was been some tentative and unconfirmed evidence that the exposure to BPA might be associated with sub-fertility and recurrent spontaneous abortions [33,34]. BPA might effect folliculogenesis and oogenesis directly or indirectly by binding to estrogen or other steroid receptors [26,28]. Since BPA may disrupt the organization of the meiotic and mitotic spindle and induce micronuclei in somatic cells, it has to be regarded as aneugenic, at least at supra-physiological concentrations [35-40].

Previous studies suggested that inadvertent exposures of mice to low concentrations of BPA affected chromosome behavior and segregation in oocytes [41]. Since our own recent studies could not confirm these effects [39,42], we suggested that additional stressors might act synergistically to BPA to cause oocyte aneuploidy after exposure to BPA.

In search for links between BPA-induced disturbances and components in spindle assembly and chromosome congression that might be regulated by iNOS, expression of the polo-like kinase-1 (PLK1) and RAN GTPase, two enzymes in spindle assembly and function [43-46], was determined in the present study..

Materials and methods

Animals

iNOS-deficient mice (B6 129P-Nos2tm1lau, stock no. 002596^{+}) were purchased from The Jackson Laboratory (Bar Harbor, ME). In the present studies, all of the mice used were from littermate mating, and should therefore have similar genetic background (strain: 129P2/OlaHsd * C57BL/6). Animals were housed in a standard animal facility under controlled temperature (22 C) and photoperiod (12 h light, 12 h dark) with free access to water and mouse chow (Lactamin R70, soy free, Lantm?nnen AB, Sweden). All animal experiments were approved by Local Animal Ethical Committee.

Oral BPA Administration

BPA (CAS-Nr: 80-05-7, Merck) was dissolved in ethanol at a concentration of 2 mg/ml and then diluted with corn oil to 0.01mg/ml. The final concentration of ethanol in corn oil was 0.5%. Juvenile females (20- to 22-days-old) of iNos$^{-/-}$ knockout mice and the iNOS proficient wild type isogenic animals were treated once daily by oral gavages with 100 or 200ug BPA/kg bw in a volume of 10 ml/kg bw for 13 days preceding oocyte maturation, collection and analysis.

Oocyte Collection for Chromosome Analysis

To obtain ovulated MII oocytes, females at day 11 of oral BPA administration were injected with 5IU of pregnant mare serum gonadotropin (PMS, Sigma-Aldrich) in the evening followed 42-44 h later by an injection of 5IU human chorionic gonodotropin (HCG, Sigma-Aldrich). Ovulated oocytes were collected from the oviducts in the following morning, 15h after the HCG injection. The cumulus-oocyte complexes from mice in the same experimental group were pooled. Cumulus was removed by brief treatment with hyaluronidase (Sigma-Aldrich, 400-1000 units/mg) and numbers of oocytes were counted after spreading. Later, numbers of oocytes obtained on average from each animal were calculated from total number of oocytes obtained per group divided by the number of females. Therefore the number of oocytes per female in Table 1 has not been determined by counting the actual number of ovulated oocytes in each group, precluding statistical analysis, but still reflects average yield of analyzable MII oocytes obtained from individual females. Oocytes selected for Western blotting or immunofluorescence were in vitro matured overnight (see below).

Cytogenetic Analysis

Chromosome preparations of metaphase II (MII) arrested oocytes were made using a modification of the Tarkowski technique[47]. Briefly, oocytes were hypotonized in 0.3% sodium citrate, moved to a small drop of acidified water on a microscope slide, and fixed in situ with several drops of 3:1 fixative (methanol: glacial acetic acid). The slides with oocytes were denatured for 15 min with 0.2N HCl at room temperature, rinsed in distilled water and treated with preheated (37 C) 1% Ba (OH)2 for 7 min followed by a brief wash with distilled water. After that, the slides were stained with 40 ng/ml DAPI (Sigma-Aldrich) for 10 min, rinsed in phosphate-buffered saline (PBS), and then mounted with Prolong Anti-Fade (Molecular Probes) mounting medium. Chromosomes were fluorescent C-banded to enable an objective distinction between complete dyads and those chromosomes separated into two monads or oocytes containing single monads.

MII oocytes were classified as normal (euploid) if they contained 20 MII chromosomes (also termed dyads) or an equivalent number of MII chromosomes and chromatids (also termed monads; e.g. 19 dyads and 2 monads)[48,49]. The classes of abnormalities involving oocytes containing extra single chromatids and hyperploidy (n > 20) were recorded, as well as the hypoploid oocytes containing≥ 18 and <20 MII chromosomes or the respective number of MII chromosomes and chromatids (e.g. 18 MII chromosomes and 1 chromatid). Hyperploidy rate was determined from all oocytes containing ≥ 20 MII chromosomes. Predivision (precocious chromatid separation prior to anaphase II) was assessed as presence of chromatids (oocytes with single or pairs of chromatids) in all analyzed MII oocytes including hyperploids, hypoploids and euploids[49,50].

Oocyte Collection, Culture, and Fixation for Analysis of chromosome alignment For analyses of chromosome behavior and meiotic spindle formation at MII, females were injected

with 5?IU of pregnant mare serum (PMS) 42-44 h at day 11 of oral BPA administration before oocyte collection on the evening of day 13. Ovaries were removed from these females and placed in M2 medium (Sigma-Aldrich) containing 6 mg/ml BSA. Antral follicles were punctured with 26 gauge needles to obtain maturation competent oocytes at the germinal vesicle (GV) stage. GV-stage oocytes were cultured overnight for 16 h in M16 medium (Sigma-Aldrich) containing 6 mg/ml BSA at 37 C in 5% CO2 in air. Oocytes exhibiting a polar body the following morning (day 14) were embedded in a fibrin clot (bovine fibrinogen type IV, Calbiochem; bovine thrombin, Sigma-Aldrich) attached to a microscope slide as previously described (5130) and immediately fixed for 30 min at 37 C in a microtubule-stabilizing buffer containing 2% formaldehyde, 0.1% Triton-X 100, 1µM taxol, 10 U/ml aprotinin, 0.1 M PIPES, 5 mM MgCl2 and 2.5 mM EGTA and 50% deuterium oxide. Following fixation, oocytes were washed for 15 min in 0.1% normal goat serum (NGS; Gibco BRL)/phosphate buffered saline (PBS), blocked for at least 1 h at 37 C in PBS wash solution containing 10% NGS, 0.02% sodium azide and 0.1% Triton X-100, and stored at 4 C until processing.

Immunofluorescence Staining

Oocytes 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). Localisation of Polo-like kinase 1 (PLK1) 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). Chromatin was stained with DAPI for 2 minutes. Oocytes were then washed briefly in PBST (PBS with Triton X-100) and then mounted on slides with Prolong Anti-Fade (Molecular Probes) mounting medium. Slides were viewed by Leica DMRA2 and DMRXA microscopes and 63x objectives with epifluorescence. Images were captured with Hamamatsu digital charge-coupled device camera C4742-95 and OpenlabTM software version 3.1.[51].

Distribution of PLK1 was analysed in MII oocytes with aligned chromosomes displaying two spindle poles with the longitudinal axis of the spindle in the plane of view in one image.

Immunoblotting for Quantities of p-ERK1/2, PLK1 and RAN in the Oocytes 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 centrifuging at 12 000 g for 3 min, samples were frozen at –20 °C until use. The total proteins were separated by 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 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 TBST 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-phosphoERK-p42/44 mAb (1:100 in TBST) (Santa Cruz Biotechnology), or mouse anti-PLK1 antibody (1:300 in TBST) (Zymed Laboratories Inc.), or goat anti-RAN antibody (1:100 in TBST) (Santa Cruz Biotechnology) overnight at 4□C. To detect ERK1/2 and PLK1, the washed membranes were incubated with HRP-conjugated rabbit anti-mouse IgG (1:3000 in TBST) for 1 h at room temperature. For RAN, the membrane was treated with HRP-conjugated rabbit anti-goat IgG (1:2000 in TBST) 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 ß-mercaptoethanol, 1% SDS) and re-probed with anti-GAPDH (Ambion) as a loading control. Immunoblot density was determined by Image Quant 5.2 software.

Statistical Analysis of Data

All data on aneuploidy of MII oocytes was evaluated by 2 analysis. The distributions of dots of PLK1 protein were analyzed by Student t-test. Difference at $p < 0.05$ was considered significant.

Results

Ovulation of oocytes in untreated and BPA treated iNos $^{+/+}$ and iNos $^{-/-}$ mice Disruption of iNos had no effect on ovulation in several strains of mice [52,53]. In accordance, MII oocytes (Table 1) were obtained from the groups of superovulated iNos $^{+/+}$ and iNos $^{-/-}$ mice in all experimental groups of this study. In spite of the predicted essential role of iNOS in mouse oocyte maturation, similar numbers of MII oocytes were obtained from untreated wild type and iNOS deficient mice (Table 1) suggesting some functional redundancy and compensation for failure to induce NO by the iNOS pathway within the ovary. While sub-chronic oral BPA did not affect average numbers of oocytes obtained in any treated group of the iNos $^{+/+}$ mice, a lower number of oocytes/female were obtained from the iNos $^{-/-}$ mice treated with 200 mg BPA/kg bw on 13 subsequent days followed by in vivo maturation to MII overnight and in vivo ovulation before oocyte retrieval on day 14 (Table 1).

Chromosomal constitution of oocytes of BPA-exposed iNos $^{+/+}$ and iNos$^{-/-}$ mice When the juvenile female mice (20- to 22-day-old) of the iNOS expressing strain were exposed to solvent or 100 or 200 µg BPA/kg bw by daily oral doses for 13 days, respectively, (Table 2) pooled data for chromosomal constitution of oocytes revealed that hyperploidy did not increase significantly in the exposed MII oocytes of iNos $^{+/+}$ mice (from zero to 0.6% and 0.8%, respectively; Table 2). There was a small although not a significant rise in the number of oocytes showing dyads together with single chromatids or pairs of chromatids (precocious chromatid segregation, termed predivision) (Figures 1 B and C) in the oocytes of controls versus BPA exposed wild type mice from 0.7 to 1.9 and 2.1%, respectively. The significant difference in oocytes with euploid chromosome numbers (20 MII or 19 MII plus 2 chromatids) (Figures 1A and B) between wild type control and 200 µg/kg bw BPA group (98.5 versus 92.6%; $P < 0.001$; Table 2) appears mainly related to increases in the numbers of hypoploid oocytes in the treated group (from 1.5% to 7.0%, respectively) and may therefore mainly relate to spreading artifacts. In contrast to the iNos$^{+/+}$ groups, hyperploidy rate (oocytes with > 20 metaphase II chromosomes) increased much more in the oocytes of the iNos $^{-/-}$ mice of the 100 µg/ml BPA group compared to the control from 0.2% to 2.5% ($P < 0.01$), and was also elevated to 2.3% ($P < 0.05$) in the 200µg/kg bw BPA group (Table 2). Hypoploidy (only counting oocytes with 18 and 19 MII chromosomes without or with single chromatids) was not increased in the 100µg/kg bw BPA group compared to the iNOS deficient control but significantly increased from 8.4% to 23.3% in the control versus the 200µg/kg bw group although many of these oocytes might represent spreading artifacts ($P < 0.001$). Unlike in the iNOS proficient mice, hyperploidy increased significantly from 0.2% in the control to 2.5 and 2.3% in the oocytes of the Nos2 -/- mice after exposure to100 and 200 ?g BPA/kg bw for 13 days ($P< 0.01$ an 0.05, respectively; Table 2). The predivision rate also increased significantly from 1.7% in the control to 4.4% in the 100 ?g BPA/kg bw group and to 6.2% in the 200 µg BPA/kg bw group of oocytes from the iNos$^{-/-}$ mice ($P < 0.05$). Hyperploidy and predivision rate increased therefore for both types of chromosomal aberrations, hyperploidy and predivison, in the treated groups of the iNOS deficient compared to the iNOS proficient control ($P < 0.01$ or 0.001, respectively, Table 2) suggesting that the loss of iNOS activity and BPA exposure synergistically increased susceptibility to meiotic disturbances.

Chromosome alignment in oocytes of BPA-exposed iNos $^{+/+}$and iNos$^{-/-}$ mice Immunostaining with antibodies against alpha, beta and gamma-tubulin revealed that BPA exposure increased

significantly the number of oocytes with defects in the alignment of chromosomes on the meiosis II spindle (congression failure; Figure 1E-G) in the oocytes of the iNos $^{-/-}$ mice (BPA-exposed iNos $^{-/-}$ vs. iNos $^{-/-}$ control: P <0.01; BPA-exposed iNos $^{-/-}$ vs. BPA-exposed iNos $^{+/+}$: P <0.01; Table 3). Therefore, low dose BPA alone did not appear to interfere significantly with chromosome congression in healthy, young oocytes with an iNos proficient genetic background. However, the knockout of iNos apparently sensitizes oocytes to MII congression failure upon BPA exposure.

Influence of BPA on PLK1 distribution around condensed chromosomes of Nos2-/-oocytes.

MAP kinase phosphorylation, and PLK1 and RAN GTPase protein expression in iNos$^{-/-}$ and iNos$^{+/+}$ oocytes

p-ERK1/2 expression in iNos$^{-/-}$ oocytes was indistinguishable from that of the iNos$^{+/+}$ oocytes (Fig. 2, A and B), unlike expected from the previous in vitro studies with inhibitors [12]. However, it has to be noted that the previous studies compared MAP kinase phosphorylation in control MII oocytes with that in blocked germinal vesicle (GV) stage oocytes produced by the treatment of the oocytes with an iNOS inhibitor. In contrast, presently protein phosphorylation was analyzed in oocytes of iNos$^{+/+}$ as well iNos$^{-/-}$ mice that had both matured to the MII stage.

Mouse PLK1 associates with spindle poles and chromosomes, which appears critical for chromosome segregation[54-56]. Accordingly, we detected PLK1 at these two sites in metaphase II oocytes (Fig. 3). However, iImmunoblotting for quantities of PLK1 in the present study demonstrated a clear reduction in PLK1 protein expression in iNos$^{-/-}$ oocytes compared to wild type controls (Fig. 2, A and B)($p<0.01$). Since PLK1 phosphorylates RAN and RAN is also essential for bipolar spindle assembly[46]. we studied also RAN expression and noticed a down-regulation of RAN in oocytes of the iNOS deficient oocytes compared to the wild type controls (Fig. 4, A and B) ($p<0.01$).

Discussion

Ovulation in oocytes of wild type and iNos$^{-/-}$ knockout mice exposed to BPA Inhibition of NO synthesis has been shown to affect ovulation in mouse and rat [48]. In the eNos knockout mouse dysfunction in cyclicity, ovulation rate, ovarian morphology, and steroidogenesis has been detected supporting the concept that eNOS derived NO plays critical roles in ovulation and follicular development[57]. However, there was only a slight delay in E2 peak at proestrous and no obvious fertility phenotype in the iNOS deficient B6 129P-Nos2tm1lau knockout mice (http::://jaxmice.jaxorg). In accordance, numbers of MII oocytes obtained from untreated wild type and iNOS deficient mice in this study were similar. However, the reduced number of oocytes/ female obtained from the iNos $^{-/-}$ mice treated with 200 mg BPA/kg bw suggests that iNOS has a role in protecting females from adverse effects of BPA on follicle development, oocyte maturation or ovulation.

iNOS deficiency, reduced expression of PLK1, and induction of chromosome predivision, and aneuploidy. Recent research has profoundly advanced our understanding of how faithful chromosome segregation is ensured by the protein cohesin complex holding sister chromatids together until anaphase [58,59]. In meiosis, sister chromatid cohesion is released in two steps. Cohesion between the sister chromatid arms of each chromosome is lost during anaphase I of meiosis to permit chiasma resolution and homologous chromosome segregation, while centromere cohesion must persist until anaphase II when the sister chromatids finally separate. PLK1 and the activity of phosphatases like PP2A are key regulators in the sequential release of sister chromatid cohesion at anaphase of mitosis and meiosis, as well as of other processes at mitotic and meiotic divisions [60-62]. PLK1 has been implicated in chromatid arm separation in somatic cells by phosphorylating one of the cohesin proteins, SA2, which in turn causes dissociation of the cohesin complex from chromosome arms in late prometaphase of mitosis, independent of proteolysis [58,59]. In meiosis, separation of arms of sister chromatids at first anaphase involves phosphorylation of the meiotic SCC1/REC8 cohesin protein by PLK1 targeting phosphorylated REC8 protein for proteolysis by separase on chromatid arms during anaphase I[61]. Second meiotic anaphase involves SCC1/REC8 phosphorylation and proteolysis of cohesin at centromeres to induce loss of attachment of sister chromatids. Accordingly, PLK1 localises to centromeres in mitosis as well as in metaphase II oocytes [54,55]. The present study confirms this distribution and shows a speckled fluorescence by Plk-1 antibody at the MTOCs at spindle poles and around chromosomes at meiosis II.

In addition, the present report reveals for the first time that PLK1 is substantially downregulated at the protein level in the oocytes of iNos $^{-/-}$ mice. While it might be expected that this could cause delay and inhibition of chromatid segregation, all of the oocytes of the iNos$^{-/-}$ group exhibit increased rates of precocious chromatid separation (predivision) compared to the iNOS proficient oocytes. Still, this did not inevitably result in error prone meiotic division or meiotic arrest since there was no elevated hyperploidy in the untreated oocytes of the iNos$^{-/-}$ oocytes, consistent with the absence of a fertility phenotype in the knockout females. In fact, PLK1 was still found at spindle poles and chromosomes in the oocytes of the iNOS-knockout mice. However, the significant rise in predivision rate between control and BPA-exposed groups argues that the deficiency in iNOS, and, as a consequence, also deficiencies of other gene products like that of PLK1 was involved in the precocious chromatid segregation in oocytes when there was a sub-chronic exposure to low concentrations of BPA in vivo. Presence of chromatids at meiosis II can be a result of a precocious separation of sister chromatids and premature resolution of chiasmata at meiosis I associated with segregation of one or both sister chromatids to one spindle pole. It can also result from precocious loss of centromeric sister chromatid cohesion prior to anaphase II during the constitutive metaphase II arrest [48,49]. Both events may relate to the downregulation of PLK1 in the iNos$^{-/-}$ oocytes. This could increase the susceptibility to subtle disturbances by BPA, which appear inefficient to affect the 'healthy' wild type oocytes. Predivision predisposes to errors in chromosome segregation at anaphase I or II, and to oocyte aneuploidy. In accordance, hyperploidy as well as hypoploidy rate were higher in all iNOS deficient metaphase II oocytes compared to the iNOS expressing wild type oocytes although differences did not reach statistical significance in each experimental group. In addition, it must be noted that fewer oocytes were obtained from the high dose BPA group of the iNOS-deficient mice suggesting that there might have been more severe disturbances, which induced irreversible meiotic arrest and reduced ovulation in this group. Further studies have to confirm this notion. From the present study it appears that iNOS deficiency per se appears to only slightly compromise control of chromosome behavior at meiosis in healthy young oocytes but exerts a more prominent effect in conjunction with an exposure to chemicals like BPA.

Reduced RAN in Nos2 -/- oocytes and failure in chromosome congression Hanisch et al. [62] demonstrated that displacement of endogenous PLK1 in HeLa S3 cells resulted in congression failure of chromosomes. PLK1 creates the tension-sensing 3F3/2 phosphoepitope and modulates the association of proteins of the spindle assembly checkpoint (SAC) with kinetochores[63]. The SAC is a feedback mechanism and safeguards against errors in chromosome segregation. It halts cells in prometaphase when chromosomes are unattached or not under tension from spindle fibres. A number of PLK1 substrates like PICH (PLK1-interacting checkpoint "helicase") have been identified, a member of the SNF2 ATPase family, which monitors tension developing between sister kinetochores and thus helps to control the SAC [64]. Loss of cell cycle and checkpoint control might contribute to the observed sensitivity of oocytes to BPA .

Another target for PLK1 is the small GTP/GDP-binding protein RAN, a regulator of bipolar spindle assembly that is phospho-

rylated by PLK1 on serine-135 during mitosis and meiosis[46]. The present observation of congression failure support the role of RAN in control of chromosome behavior suggesting that problems in spindle and chromosome organization by reduced RAN in the iNos$^{-/-}$ oocytes may contribute to the increased sensitivity to disturbances by BPA exposure and thus predisposes to errors in chromosome segregation. Confocal laser scanning microscopy showed that RAN was concentrated at the MII spindle microtubules in mouse oocytes [65]. Manipulating levels of RAN in mouse oocytes did not inhibit assembly of a bipolar meiosis I spindle, and chromosomes still segregated normally. However, formation of the meiosis II spindle was highly aberrant with multiple ectopic asters of microtubules, and unaligned chromosomes[66]. In accordance, we found altered distribution of PLK1 around condensed chromosomes and increased congression failure in iNOS deficient oocytes, consistent with the notion that Reduced PLK1 and RAN may therefore contribute to sensitizes mouse oocytes to BPA-induced aberrations in spindle and chromosome behavior.

Deficiency in iNOS and aneugenic activity of low dose BPA
The notion of a possible negative impact of low chronic BPA exposure on meiotic maturation, chromosome congression, and faithful chromosome distribution in oocytes was initially proposed by Hunt and co-workers [41]. A repeat study with sub-chronic exposure to BPA of prepubertal mice for 7 days followed by in vitro oocyte maturation (20-100 µg/kg bw/day), did not confirm that low BPA induced aneuploidy or substantial congression failure in mouse oocytes, when healthy animals were fed on a low phytohormone diet [39]. There was also no significant BPA-induction of meiotic aneuploidy in mouse oocytes and 1-cell zygotes of sexually mature mice exposed chronically for 7 weeks through drinking water to low BPA, or sub-chronically or acute to BPA [42].

In accordance with these reports there was also no significant increase in hyperploidy rate and predivision in the oocytes of the healthy young iNOS proficient mice in the current study, when females fed on a soy-free diet were exposed to low sub-chronic BPA. In contrast, significant rises in spindle aberrations at meiosis I of in vitro maturing oocytes were noticed when mouse oocytes were exposed to high doses of BPA during in vitro maturation with and without cumulus[38-40]. Chromosome congression failure, and substantial meiotic delay or meiotic arrest [38-40] indicate that the SAC was presumably effective in blocking meiosis under these conditions and releasing only those oocytes to enter anaphase I that had well aligned and/or attached chromosomes and therefore were capable to separate chromosomes normally. In accordance, we argued that the congression failure observed at metaphase II in in vitro and in vivo exposed mouse oocytes [40] might not cause aneuploidy if the SAC was functional, explaining the failure to find aneuploid 1-cell zygotes from fertilized chronically in vivo BPA exposed oocytes[40,42]. Taken together, these observations and the current data suggest that healthy, young prepubertal and adult mice and high quality young oocytes may be capable to deal with a subtle disturbance by low BPA, either by rapidly metabolising/excreting the chemical in vivo, and/or by prolonging the SAC to protect from segregation errors. However, disturbances in oocytes leading to permissive checkpoint control, for instance, when there is reduced expression of spindle and cell cycle regulatory molecules as in aged oocytes[67-69] or in oocytes matured under sub-optimal conditions [49,70] may increase sensitivity to meiotic errors and nondisjunction. It has been repeatedly stated that more than one disturbance (more than one "hit") may be responsible for increased aneuploidy in mammalian oocytes[71]. Such a situation may arise constitutively in aged mammalian oocytes due to alterations in gene expression compromising the SAC and spindle function [16-18,69,72], especially in presence of susceptible chromosomes like those with no or single distal chiasmata. Multiple hit mechanisms could also be at the basis of the high incidence of chromosomal aberrations in the BPA-exposed iNos $^{-/-}$ mice.

Molecular bases of protective effect by iNOS-derived NO

When NO produced by iNOS at oogenesis is not available for modulating response to stress by directly or indirectly controlling gene expression in oocytes, the deficiency in PLK1 and RAN proteins may influence stringency of checkpoint sensing and control. Likewise, iNOS deficiency may also have an influence on metabolism, clearance/excretion or bioactivation of BPA and on health of mice and oocytes. BPA is effectively glucuronidated by hepatocytes in rodents in wild type animals expressing iNOS[73]. iNOS-deficient mice exhibited increased hepatotoxicity after subacute fumonisin B exposure compared to their wild type counterparts[74]. Liver regeneration was lower in iNOS$^{-/-}$ compared to that in the iNOS$^{+/+}$ mice. Deficiencies in iNOS could affect bioavailability such that higher BPA might reach follicles and oocytes. Combined genomic and non-genomic activities of BPA might then induce sub-optimal conditions at folliculogenesis and oocyte growth that indirectly affect the quality of the oocyte. This might decrease gene expression and compromise the capacity of oocytes to deal with chromosome congression failure, for instance by subtle interference with microtubule polymerisation and organization of spindle poles [23,38,39,75], repression of signalling by estrogen receptor beta and other steroid receptors[76] or by reduced stress responses involving NO signalling pathways. There is evidence for an NO-mediated anti-apoptotic pathway in ovarian follicle atresia since iNOS induced NO inhibited Fas/FasL system-induced apoptosis in rat granulosa cells by suppressing activation of the caspases [77]. Increased apoptosis of BPA-exposed iNos$^{-/-}$ granulosa cells could possibly affect follicle quality and alter gene expression in oocytes. Interestingly, addition of NO donor S-nitroso acetyl penicillamine (SNAP) protected mouse oocytes from age-related changes in microtubule dynamics, cortical granule exocytosis and zona pellucida hardening[78], and appeared to improve the integrity of the spindle apparatus. It appears now that effects of NO on abundance of proteins like PLK1 and RAN could be involved and induction of iNOS and NO could have a protective effect in cases of adverse exposures as well as aging processes affecting oocytes. A growing body of evidence demonstrates that PLK1 is a key regulator during meiotic chromosome segregation[55,56]. PLK1 substrates include early mitotic inhibitor-like 2 inhibitor (EMI2) that is involved in metaphase II arrest in mammalian oocytes and inhibits anaphase II progression until fertilization [45]. PLK1 is also essential for regulating microtubule assembly and spindle organization, partially through its phosphorylation of RAN GTPase (RAN) [46].

Currently, it is not possible to distinguish between the different direct or indirect effects of iNOS derived NO on the BPA response and further studies are required. In any case, herein we report for the first time that iNOS deficiency presents a genetically predisposing lesion and confounding factor, which increases susceptibility of mice to BPA exposure and elevates aneuploidy and predivision in oocytes in comparison to the iNOS proficient wild type controls. This is, to our knowledge, the first demonstration that iNOS deficiency has adverse influences on fertility by predisposing to errors in chromosome segregation by BPA, and possibly, also other chemicals which possess only limited aneugenic potential in a iNOS proficient genetic background. Further risk assessments for BPA exposures of the human population should therefore consider possible influences of the genetic background on the susceptibility and dose-response, apart from receptor-mediated mechanisms[79], in particular with regard to fertility and health of offspring.

Figure Legends
Figure 1. Chromosomal analysis on metaphase II (MII) oocytes from BPA-exposed iNos$^{-/-}$ females.

(A-C) Air-dried chromosome preparations from BPA-exposed iNos$^{-/-}$ females. (A) Euploid MII oocyte with 20 chromosomes. (B) MII oocyte with predivision with 19 chromosomes plus two prematurely separated sister chromatids (arrows). (C) Hyperploid oocyte with 20 chromosomes plus one prematurely separated sister chromatid (arrow). (D-G) Configurations of MII oocytes immunostained with antibodies against α +β +γ

-tublin (red) and counterstained with DAPI to visualize the chromosome (blue). (D) Normal MII configuration. (E-G) Congression failure. Bars, 5 µm.

Figure 2. Immunoblotting for quantities of p-ERK1/2.
(A) Western blot result. (B) Relative p-ERK1/2 expression quantity was determined by densitometric scans. The total amount of GAPDH present in the lower set of lanes was used to standardize the amount of p-ERK1/2 present in the upper set of lanes. The same treatment was applied to Figure 3and 4. The relative p-ERK1/2 intensity in iNos$^{-/-}$ oocytes was not significantly different from that in iNos$^{+/+}$ oocytes(P>0.05). (n = 3).

Figure 3.Immunoblotting for Quantities of Plk1.
(A) Western blot result. (B) Relative Plk1 intensity decreased in iNos$^{-/-}$ oocytes, compared to iNos$^{+/+}$ oocytes(P < 0.01).(n = 3).

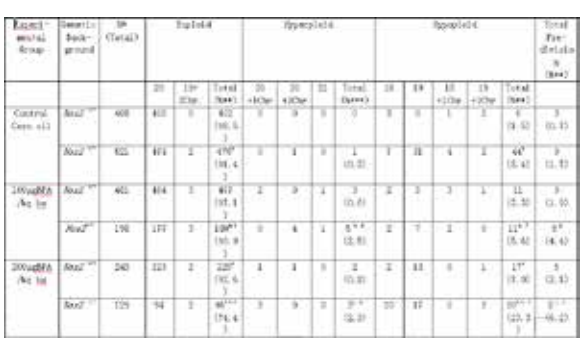
Figure 4. Immunoblotting for Quantities of Ran..
(A) Western blot result. (B) Relative Plk1 intensity decreased in iNos$^{-/-}$ oocytes, compared to iNos$^{+/+}$ oocytes(P < 0.01).(n = 3).

Table 1: Number of mice, swollen oviducts and oocytes collected from wild type (i*Nos* $^{+/+}$) and I NOS knockout (i*Nos* $^{-/-}$) mice exposed to solvent (Solvent Control; corn oil with 0.5% ethanol) or to 100 or by daily oral gavage on 13 days.

		Number of Mice	Number of Oocytes (Oocytes/Mouse)
Nos2 $^{+/+}$	Solvent Control	52	707 (13.6)
	100mgBPA /kg bw	83	917 (11.0)
	200 mg BPA/kg bw	49	724 (14.8)
Nos2 $^{-/-}$	Solvent Control	64	971 (15.2)
	100mg BPA/kg bw	43	554 (12.9)
	200mg BPA/kg bw	56	446[a] (8.0)

[a]χ2-Test: Significantly different from i*Nos*$^{-/-}$ control (P < 0.01), and i*Nos* $^{+/+}$ 200 mg BPA /kg bw (P < 0.01).

Table 2: Chromosomal constitution of metaphase II oocytes of wild type (*Nos2* $^{+/+}$) and iNOS knockout (*Nos2* $^{-/-}$) mice exposed to solvent (Solvent Control; corn oil with 0.5% ethanol) or to 100 or 200 mg BPA/kg bw by daily oral gavage on 13 days followed by *in vivo* maturation overnight and ovulation on day 14.



* Oocytes with 18 to 21 metaphase II chromosomes or the respective number of chromatids (Chr.). ** Total of all including hyperploids and hypoploids with chromatids. *** Total of all with ≥ 20 metaphase II chromosomes or the respective number of chromatids.

c2-Test -Square:
Significant difference between oocytes of *Nos2* $^{+/+}$ and *Nos2* $^{-/-}$ of same treatment group; [a]P < 0.05; [b]P < 0.001;

Significant difference between control and treatment group of same genetic background; [c]P < 0.05; [d]P < 0.01; [e]P < 0.001;

Significant difference between treated *Nos2* $^{-/-}$ and *Nos2* $^{+/+}$ control; [f] P < 0.05; [g]P < 0.01; [h]P < 0.001.

Table 3: Congression failure of chromosomes in metaphase II oocytes of wild type (*Nos2* $^{+/+}$) and iNOS knockout (*Nos2* $^{-/-}$) mice exposed to solvent (Solvent Control; corn oil with 0.5% ethanol) or to 100 mg BPA /kg bw by daily oral gavage on 13 days.

		Number of Oocytes	Oocytes with Congression Failure (%)
Nos2 $^{+/+}$	Solvent Control	539	36 (5.2)
	100mg BPA / kg bw	627	57 (9.1)
Nos2 -/-	Solvent Control	358	28 (7.8)
	100mg BPA/ kg bw	636	96[a] (15.1)

[a]χ^2-Test: Significantly different from i*Nos*$^{-/-}$ control (P < 0.01), and i*Nos* $^{+/+}$ 100 mg BPA /kg bw (P < 0.01).

Figure 1.

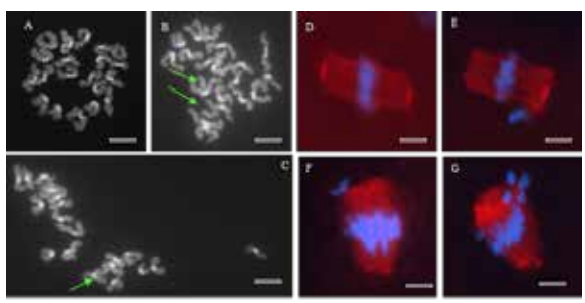
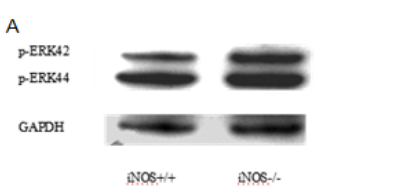


Figure 2.



B Immunoblotting for Quantities of p-ERK1/2

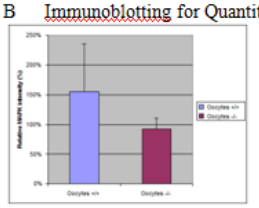
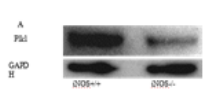
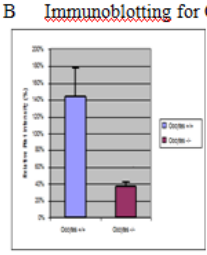
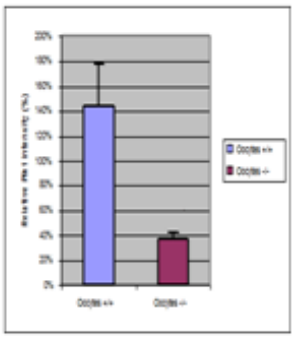


Figure 3.



B Immunoblotting for Quantities of Plk1





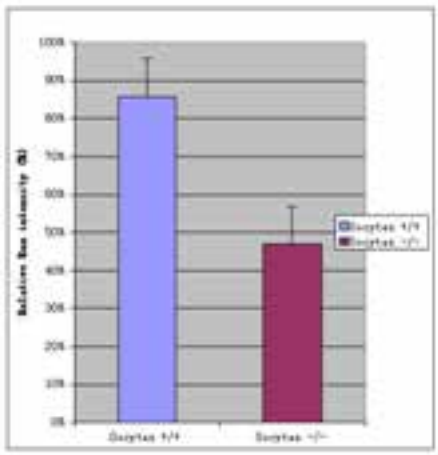
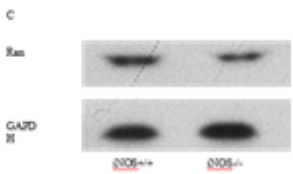


Figure 4.



B Immunoblotting for Quantities of RAN

REFERENCES

1. Giulivi C, Kato K, Cooper CE 2006 Nitric oxide regulation of mitochondrial oxygen consumption I: cellular physiology. Am J Physiol Cell Physiol 291:C1225-1231 | 2. Galkin A, Higgs A, Moncada S 2007 Nitric oxide and hypoxia. Essays Biochem 43:29-42 | 3. Stuehr DJ 1999 Mammalian nitric oxide synthases. Biochim Biophys Acta 1411:217-230 | 4. Suschek CV, Schnorr O, Kolb-Bachofen V 2004 The role of iNOS in chronic inflammatory processes in vivo: is it damage-promoting, protective, or active at all? Curr Mol Med 4:763-775 | 5. Pannu R, Singh I 2006 Pharmacological strategies for the regulation of inducible nitric oxide synthase: neurodegenerative versus neuroprotective mechanisms. Neurochem Int 49:170-182 | 6. Kibbe MR, Tzeng E 2000 Nitric oxide synthase gene therapy in vascular pathology. Semin Perinatol 24:51-54 | 7. Kenyon NJ, Last MS, Eiserich JP, Morrissey BM, Temple LM, Last JA 2006 Differentiation of the roles of NO from airway epithelium and inflammatory cells in ozone-induced lung inflammation. Toxicol Appl Pharmacol 215:250-259 | 8. Scott DJ, Hull MA, Cartwright EJ, Lam WK, Tisbury A, Poulsom R, Markham AF, Bonifer C, Coletta PL 2001 Lack of inducible nitric oxide synthase promotes intestinal tumorigenesis in the Apc(Min/+) mouse. Gastroenterology 121:889-899 | 9. Zhang R, Ma A, Urbanski SJ, McCafferty DM 2006 Induction of Inducible Nitric Oxide synthase: A protective Mechanism in Colitis-Induced Adenocarcinoma. Carcinogenesis | 10. Mitchell LM, Kennedy CR, Hartshorne GM 2004 Expression of nitric oxide synthase and effect of substrate manipulation of the nitric oxide pathway in mouse ovarian follicles. Hum Reprod 19:30-40 | 11. Lapointe J, Roy M, St-Pierre I, Kimmins S, Gauvreau D, MacLaren LA, Bilodeau JF 2006 Hormonal and spatial regulation of nitric oxide synthases (NOS) (neuronal NOS, inducible NOS, and endothelial NOS) in the oviducts. Endocrinology 147:5600-5610 | 12. Huo LJ, Liang CG, Yu LZ, Zhong ZS, Yang ZM, Fan HY, Chen DY, Sun QY 2005 Inducible nitric oxide synthase-derived nitric oxide regulates germinal vesicle breakdown and first polar body emission in the mouse oocyte. Reproduction 129:403-409 | 13. Brunet S, Maro B 2005 Cytoskeleton and cell cycle control during meiotic maturation of the mouse oocyte: integrating time and space. Reproduction 130:801-811 | 14. Inoue D, Ohe M, Kanemori Y, Nobui T, Sagata N 2007 A direct link of the Mos-MAPK pathway to Erp1/Emi2 in meiotic arrest of Xenopus laevis eggs. Nature 446(7139):1100-1104. | 15. Hodges C, Ilagan A, Jennings D, Keri R, Nilson J, Hunt P 2002 Experimental evidence that changes in oocyte growth influence meiotic chromosome segregation. Hum Reprod 17:1171-1180 | 16. Vogt E, Kirsch-Volders M, Parry J, Eichenlaub-Ritter U 2007 Spindle formation, chromosome segregation and the spindle checkpoint in mammalian oocytes and susceptibility to meiotic error. Mutat Res: doi10.10.16/jmrgentox.2007.10.015 (in press) | 17. Baker DJ, Jeganathan KB, Cameron JD, Thompson M, Juneja S, Kopecka A, Kumar R, Jenkins RB, de Groen PC, Roche P, van Deursen JM 2004 BubR1 insufficiency causes early onset of aging-associated phenotypes and infertility in mice. Nat Genet 36:744-749 | 18. Homer HA, McDougall A, Levasseur M, Yallop K, Murdoch AP, Herbert M 2005 Mad2 prevents aneuploidy and premature proteolysis of cyclin B and securin during meiosis I in mouse oocytes. Genes Dev 19:202-207 | 19. Musacchio A, Salmon ED 2007 The spindle-assembly checkpoint in space and time. Nat Rev Mol Cell Biol 8:379-393 | 20. Hassold T, Hunt P 2001 To err (meiotically) is human: the genesis of human aneuploidy. Nat Rev Genet 2:280-291 | 21. Tesfaye D, Kadanga A, Rings F, Bauch K, Jennen D, Nganvongpanit K, Holker M, Tholen E, Ponsuksili S, Wimmers K, Montag M, Gilles M, Kirfel G, Herzog V, Schellander K 2006 The effect of nitric oxide inhibition and temporal expression patterns of the mRNA and protein products of nitric oxide synthase genes during in vitro development of bovine pre-implantation embryos. Reprod Domest Anim 41:501-509 | 21. Calafat AM, Kuklenyik Z, Reidy JA, Caudill SP, Ekong J, Needham LL 2005 Urinary concentrations of bisphenol A and 4-nonylphenol in a human reference population. Environ Health Perspect 113:391-395 | 22. Pfeiffer E, Rosenberg B, Deuschel S, Metzler M 1997 Interference with microtubules and induction of micronuclei in vitro by various bisphenols. Mutat Res 390:21-31 | 23. Johnson G, Parry E 2007 Mechanistic investigations of the spindle poisons Bisphenol-A and rotenone. Mutat Res: Genet Toxico Environ Mutagen (in press) | 24. SCF 2002 Opinion of the Scientific Committee on Food on Bisphenol-A. Available on-line: http://eceuropaeu/food/fs/sc/scf/out128_enpdf | 25. EU 2003 Bisphenol-A: European Union Risk Assessment Report (CAS no. 80-05-7). Available on-line at: http://ecbjrcit/ | 26. EU 2006 Opinion of the scientific panel on food additives, flavourings , processing aids and matwrials in contact with food on request from the commission related to 2,2-bis 4-(hydroxyphenyl)propane (bisphenolA) EFD`SA Q-2005-100. The EFSA journal 428:1-75 Available at: http://www.efsa.europa.eu/en/science/afc/afc_opinions/bisphenol_a.html | 27. Ikezuki Y, Tsutsumi O, Takai Y, Kamei Y, Taketani Y 2002 Determination of bisphenol A concentrations in human biological fluids reveals significant early prenatal exposure. Hum Reprod 17:2839-2841 | 28. Tsutsumi O 2005 Assessment of human contamination of estrogenic endocrine-disrupting chemicals and their risk for human reproduction. J Steroid Biochem Mol Biol 93:325-330 | 29. Brotons JA, Olea-Serrano MF, Villalobos M, Pedraza V, Olea N 1995 Xenoestrogens released from lacquer coatings in food cans. Environ Health Perspect 103:608-612 | 30. Calafat AM, Kuklenyik Z, Reidy JA, Caudill SP, Ekong J, Needham LL 2005 Urinary concentrations of bisphenol A and 4-nonylphenol in a human reference population. Environ Health Perspect 113:391-395 | 31. Yamada H, Furuta I, Kato EH, Kataoka S, Usuki Y, Kobashi G, Sata F, Kishi R, Fujimoto S 2002 Maternal serum and amniotic fluid bisphenol A concentrations in the early second trimester. Reprod Toxicol 16:735-739 | 32. Schonfelder G, Wittfoht W, Hopp H, Talsness CE, Paul M, Chahoud I 2002 Parent bisphenol A accumulation in the human maternal-fetal-placental unit. Environ Health Perspect 110:A703-707 | 33. Sugiura-Ogasawara M, Ozaki Y, Sonta S, Makino T, Suzumori K 2005 Exposure to bisphenol A is associated with recurrent miscarriage. Hum Reprod 20:2325-2329 | 34. Berkowitz G 2006 Limitations of a case-control study on bisphenol A (BPA) serum levels and recurrent miscarriage. Hum Reprod 21:565-566; author reply 566-567 | 35. Metzler M, Pfeiffer E 1995 Effects of estrogens on microtubule polymerization in vitro: correlation with estrogenicity. Environ Health Perspect 103 Suppl 7:21-22 | 36. Ochi T 1999 Induction of multiple microtubule-organizing centers, multipolar spindles and multipolar division in cultured V79 cells exposed to diethylstilbestrol, estradiol-17beta and bisphenol A. Mutat Res 431:105-121 | 37. Parry EM, Parry JM, Corso C, Doherty A, Haddad F, Hermine TF, Johnson G, Kayani M, Quick E, Warr T, Williamson J 2002 Detection and characterization of mechanisms of action of aneugenic chemicals. Mutagenesis 17:509-521 | 38. Can A, Semiz O, Cinar O 2005 Bisphenol-A induces cell cycle delay and alters centrosome and spindle microtubular organization in oocytes during meiosis. Mol Hum Reprod 11:389-396 | 39. Eichenlaub-Ritter U, Vogt E, Cukurcam S, Sun F, Pacchierotti F, Parry J 2007 Exposure of mouse oocytes to bisphenol A causes meiotic arrest but not aneuploidy. Mutat Res: Genet Toxicol Environ Mutagen doi 10.10.16/j.mrgentox 2007.10.014 (in press) | 40. Lenie S, Cortvrindt R, Eichenlaub-Ritter U, Smitz J 2007 Continuous exposure to bisphenol A during in vitro follicular development induces meiotic abnormalities. Mutat Res: Genet Toxicol Environ Mutagen doi 10.10.16/j.mrgentox 2007 (in press) | | 41. Hunt PA, Koehler KE, Susiarjo M, Hodges CA, Ilagan A, Voigt RC, Thomas S, Thomas BF, Hassold TJ 2003 Bisphenol a exposure causes meiotic aneuploidy in the female mouse. Curr Biol 13:546-553 | 42. Pacchirotti F, Ranaldi R, Eichenlaub-Ritter U, Attia S, Adler I-D 2007 Evaluation of aneugenic effects of bisphenol A in somatic and germ cells of the mouse. Mutat Res: Genet Toxicol Environ Mutagen doi 10.10.16/j.mrgentox 2007 (in press) | 43. Warner SL, Gray PJ, Von Hoff DD 2006 Tubulin-associated drug targets: Aurora kinases, Polo-like kinases, and others. Semin Oncol 33:436-448 | 44. Rivera T, Losada A 2006 Shugoshin and PP2A, shared duties at the centromere. Bioessays 28:775-779 | 45. Hansen DV, Tung JJ, Jackson PK 2006 CaMKII and polo-like kinase 1 sequentially phosphorylate the cytostatic factor Emi2/XErp1 to trigger its destruction and meiotic exit. Proc Natl Acad Sci U S A 103:608-613 | 46. Feng Y, Yuan JH, Maloid SC, Fisher R, Copeland TD, Longo DL, Conrads TP, Veenstra TD, Ferris A, Hughes S,

Dimitrov DS, Ferris DK 2006 Polo-like kinase 1-mediated phosphorylation of the GTP-binding protein Ran is important for bipolar spindle formation. Biochem Biophys Res Commun 349:144-152 | 47.Tarkowski AK 1966 An air drying method from chromosome preparations from mouse eggs. Cytogenetics 5:394-400 | 48. Mailhes JB, Hilliard C, Fuseler JW, London SN 2003 Okadaic acid, an inhibitor of protein phosphatase 1 and 2A, induces premature separation of sister chromatids during meiosis I and aneuploidy in mouse oocytes in vitro. Chromosome Res 11:619-631 | 49. Cukurcam S, Betzendahl I, Michel G, Vogt E, Hegele-Hartung C, Lindenthal B, Eichenlaub-Ritter U 2007 Influence of follicular fluid meiosis-activating sterol on aneuploidy rate and precocious chromatid segregation in aged mouse oocytes. Hum Reprod 22:815-828 | 50. Eichenlaub-Ritter U, Winterscheidt U, Vogt E, Shen Y, Tinneberg HR, Sorensen R 2007 2-methoxyestradiol induces spindle aberrations, chromosome congression failure, and nondisjunction in mouse oocytes. Biol Reprod 76:784-793. | 51. Wang H, Hoog C 2006 Structural damage to meiotic chromosomes impairs DNA recombination and checkpoint control in mammalian oocytes. J Cell Biol 173:485-495 | 52. Jablonka-Shariff A, Basuray R, Olson LM 1999 Inhibitors of nitric oxide synthase influence oocyte maturation in rats. J Soc Gynecol Investig 6:95-101 | 53. Hefler LA, Gregg AR 2002 Inducible and endothelial nitric oxide synthase: genetic background affects ovulation in mice. Fertil Steril 77:147-151 | 54. Wianny F, Tavares A, Evans MJ, Glover DM, Zernicka-Goetz M 1998 Mouse polo-like kinase 1 associates with the acentriolar spindle poles, meiotic chromosomes and spindle midzone during oocyte maturation. Chromosoma 107:430-439 | 55. Kang YH, Park JE, Yu LR, Soung NK, Yun SM, Bang JK, Seong YS, Yu H, Garfield S, Veenstra TD, Lee KS 2006 Self-regulated Plk1 recruitment to kinetochores by the Plk1-PBIP1 interaction is critical for proper chromosome segregation. Mol Cell 24:409-422 | 56. Pouwels J, Kukkonen AM, Lan W, Daum JR, Gorbsky GJ, Stukenberg T, Kallio MJ 2007 Shugoshin 1 plays a central role in kinetochore assembly and is required for kinetochore targeting of Plk1. Cell Cycle 6:1579-1585 | 57 Mitsube K, Mikuni M, Matousek M, Br?nnstr?m M 1999 Effects of a nitric oxide donor and nitric oxide synthase inhibitors on luteinizing hormone-induced ovulation in the ex-vivo perfused rat ovary. Hum Reprod 14:2537-2543 | 58. Revenkova E, Jessberger R 2005 Keeping sister chromatids together: cohesins in meiosis. Reproduction 130:783-790 | 59. Watanabe Y 2005 Sister chromatid cohesion along arms and at centromeres. Trends Genet 21:405-412 | 60. Hauf S, Roitinger E, Koch B, Dittrich CM, Mechtler K, Peters JM 2005 Dissociation of cohesin from chromosome arms and loss of arm cohesion during early mitosis depends on phosphorylation of SA2. PLoS Biol 3:e69 | 61. Kudo NR, Wassmann K, Anger M, Schuh M, Wirth KG, Xu H, Helmhart W, Kudo H, McKay M, Maro B, Ellenberg J, de Boer P, Nasmyth K 2006 Resolution of chiasmata in oocytes requires separase-mediated proteolysis. Cell 126:135-146 | 62. Hanisch A, Wehner A, Nigg EA, Sillje HH 2006 Different Plk1 functions show distinct dependencies on Polo-Box domain-mediated targeting. Mol Biol Cell 17:448-459 | 63. Ahonen LJ, Kallio MJ, Daum JR, Bolton M, Manke IA, Yaffe MB, Stukenberg PT, Gorbsky GJ 2005 Polo-like kinase 1 creates the tension-sensing 3F3/2 phosphoepitope and modulates the association of spindle-checkpoint proteins at kinetochores. Curr Biol 15:1078-1089 | 64. Baumann C, Korner R, Hofmann K, Nigg EA 2007 PICH, a centromere-associated SNF2 family ATPase, is regulated by Plk1 and required for the spindle checkpoint. Cell 128:101-114 | 65. Cao YK, Zhong ZS, Chen DY, Zhang GX, Schatten H, Sun QY 2005 Cell cycle-dependent localization and possible roles of the small GTPase Ran in mouse oocyte maturation, fertilization and early cleavage. Reproduction 130:431-440 | 66. Dumont J, Petri S, Pellegrin F, Terret ME, Bohnsack MT, Rassinier P, Georget V, Kalab P, Gruss OJ, Verlhac MH 2007 A centriole- and RanGTP-independent spindle assembly pathway in meiosis I of vertebrate oocytes. J Cell Biol 176:295-305 | | 67. Steuerwald N, Cohen J, Herrera RJ, Sandalinas M, Brenner CA 2001 Association between spindle assembly checkpoint expression and maternal age in human oocytes. Mol Hum Reprod 7:49-55 | 68. Hamatani T, Falco G, Carter MG, Akutsu H, Stagg CA, Sharov AA, Dudekula DB, VanBuren V, Ko MS 2004 Age-associated alteration of gene expression patterns in mouse oocytes. Hum Mol Genet 13:2263-2278 | 69. Steuerwald NM, Bermudez MG, Wells D, Munne S, Cohen J 2007 Maternal age-related differential global expression profiles observed in human oocytes. Reprod Biomed Online 14:700-708 | 70. Barrett SL, Albertini DF 2007 Allocation of gamma-tubulin between oocyte cortex and meiotic spindle influences asymmetric cytokinesis in the mouse oocyte. Biol Reprod 76:949-95771 | 71. Warren WD, Gorringe KL 2006 A molecular model for sporadic human aneuploidy. Trends Genet 22:218-224 | 72. Niault T, Hached K, Sotillo R, Sorger PK, Maro B, Benezra R, Wassmann K 2007 Changing mad2 levels affects chromosome segregation and spindle assembly checkpoint control in female mouse meiosis I. PLoS ONE 2:e1165 | | 73. Kuester RK, Sipes IG 2007 Prediction of metabolic clearance of bisphenol A (4,4 '-dihydroxy-2,2-diphenylpropane) using cryopreserved human hepatocytes. Drug Metab Dispos 35:1910-1915 | 74. Suzuki H, Riley RT, Sharma RP 2007 Inducible nitric oxide has protective effect on fumonisin B1 hepatotoxicity in mice via modulation of sphingosine kinase. Toxicology 229:42-53 | 75. Lehmann L, Metzler M 2004 Bisphenol A and its methylated congeners inhibit growth and interfere with microtubules in human fibroblasts in vitro. Chem Biol Interact 147:273-285 | 76. Susiarjo M, Hassold TJ, Freeman E, Hunt PA 2007 Bisphenol A exposure in utero disrupts early oogenesis in the mouse. PLoS Genet 3:e5 | 77. Chen Q, Yano T, Matsumi H, Osuga Y, Yano N, Xu J, Wada O, Koga K, Fujiwara T, Kugu K, Taketani Y 2005 Cross-Talk between Fas/Fas ligand system and nitric oxide in the pathway subserving granulosa cell apoptosis: a possible regulatory mechanism for ovarian follicle atresia. Endocrinology 146:808-815 | 78. Goud AP, Goud PT, Diamond MP, Abu-Soud HM 2005 Nitric oxide delays oocyte aging. Biochemistry 44:11361-11368 | | 79. Pocar P, Brevini TA, Fischer B, Gandolfi F 2003 The impact of endocrine disruptors on oocyte competence. Reproduction 125(3):313-325 |