Smoking women and the effect of tobacco use in the ovary

Is fertility of unborn children affected by prenatal smoke exposure?

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BACKGROUND AND INTRODUCTION

Although the adverse effect of smoking on health is renown, it is still a widely practiced habit. In the Netherlands, 37,3% of men smoke and 31.0% of women (Zatonski W. et al, 2012). Furthermore, 30% of the women in the Netherlands who are habitudinal smokers continue to smoke during pregnancy and expose their child to cigarette smoke (Goedhart G. et al, 2009). Evidence suggests that women are less likely to have success quitting smoking, as they tend to be more withdrawn by fear of weight gain than men, and social patterns have a stronger influence on their smoking behavior (Allen SS, 2014). Smoking during pregnancy increases the risk on placental abruption, premature rupture of membranes, premature delivery and restricted fetal growth (Jaddo V.W. et al. 2007). Smokers also report an increased time to pregnancy (Sarraj, Drummond, 2012) reduced in vitro fertilization success (Chra I. et al. 2001) and depleted ovarian reserves (Freour et al, 2008, Gruber I. et al, 2008). Furthermore, smoking is significantly associated with earlier natural menopause (Sun L, Tan L et al, 2012).

Evidence suggests that damage due to cigarette smoke exposure is sex-specific (Golding, J. et al, 2014). This could be explained by the fact that the germ cell pool in women is prenatally established in the ovary, and in men will be continuously renewed during the fertile period of their life. Cigarette smoking is suggested as potentially the single most toxic and preventable insult or life style factor associated with impaired fertility. As there is an increase in young women including smoking in their life style, insight in the mechanisms behind the damage is necessary for better therapeutics in quitting smoking and recovery from the damage induced by the toxic tobacco smoke.

Ovarian development in human and mice

Animal models are a useful tool in the investigation in the developmental origins of health and disease, as human embryological studies are controversial. This section elaborates on the milestones in ovarian development in man and mice.

The murine gestation period varies from 19 to 21 days. From embryonic day 6.5, primordial germ cells give rise to the oocytes derived from the extraembryonic ectoderm and visceral ectoderm. The cells become motile and migrate across the embryo to reach the developing genital ridge at day 10.5 and from the functional gonads by interacting with the surrounding somatic cells. (Chen, Zheng et al, 2013). During and after colonization of the genital ridge, the primordial germ cells (PGC) proliferate rapidly and their number increases to 25000 by day 13.5. Female germ cells initiate meiosis and arrest at the diplotene stage of prophase I during the first days after birth (see fig. 1).

In humans the gestational period lasts approximately 40 weeks. PGCs are migrating during week 3 and 5 of gestation. Shortly after the sex-specific differentiation of the gonad, PGCs start transforming into oogonia increasing steadily in number like in the mouse, until they enter meiosis to become oocytes in the newly formed ovary in gestational week 11 and 12. Multiplication is now prohibited making the store of female gametes definitive.

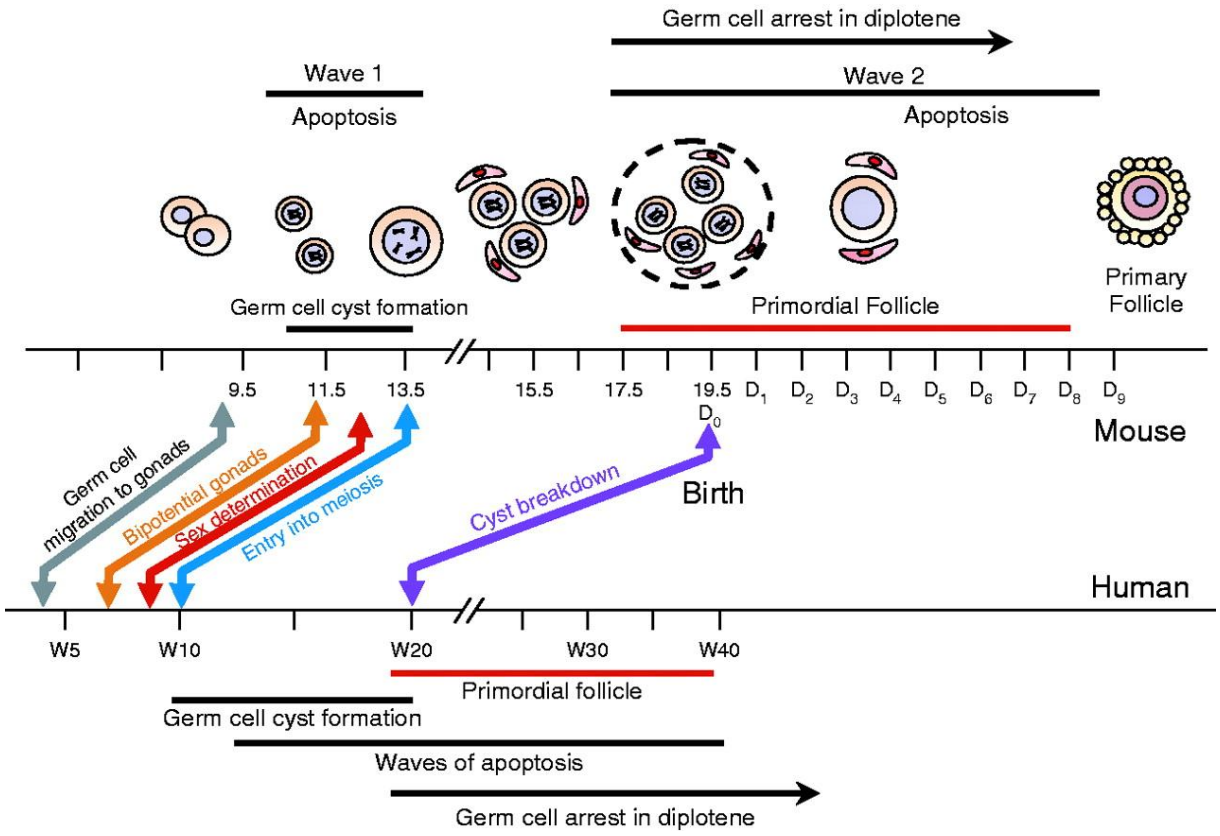


Fig. 1. *Timeline of mouse and human ovarian development, resp. in days resp. weeks post coitum. Most notorious difference is primordial follicle formation after birth in murine development and during gestational period in human (Pepling, 2006).*

Follicle development

Oocytes are surrounded by flat support cells called granulosa cells and show no biological activity. Upon recruitment into the growing follicle pool by paracrine factors, follicles become sensitive for pituitary signals. Recruited primordial follicles change shape from flat to cubical granolosa cells and both the follicle and oocyte increase in size. Stroma-like cells surround the granulosa cells and form capillary vessels to facilitate further growth. The follicle forms a fluid-filled cavity adjacent to the oocyte called the antrum (Pedersen, Hamer, 1968). The oocyte is now ready for ovulation, the last step of development. In humans this takes place in fetal life, from 20 weeks to birth whereas in mice this occurs directly after birth. Not all primordial follicles will make it to ovulation as many undergo atresia or apoptosis during development and are absorbed by the surrounding cells. It remains a mystery why so many primordial follicles are formed only to be stopped during development. Some of the suggestions are unrepaired DNA damage; defective spindle formation or insufficient pregranolosa cells (Sarray MA, Drummond, 2012). What is clear however is that primordial follicle pool is established in a balance between apoptosis, atresia and development but it remains to be shown which cell type is affected.

Tobacco smoke and ovotoxicity

Cigarette smoke consists of more than 4000 chemicals of which over 60 are known cytotoxic and carcinogenic agents (Dube M.F. et al, 1982; C.J. Smith et al., 1999). A class of toxins that is found in abundance in mainstream tobacco smoke (fig. 2) is polycyclic aromatic hydrocarbons (PAHs). PAHs are known as highly carcinogenic and mutagenic. Side-stream tobacco smoke (fig. 2) contains 10

times the content of PAHs compared to mainstream, due to incomplete combustion. Therefore, passive smoking is a considerable contributor to the damage caused by smoking.

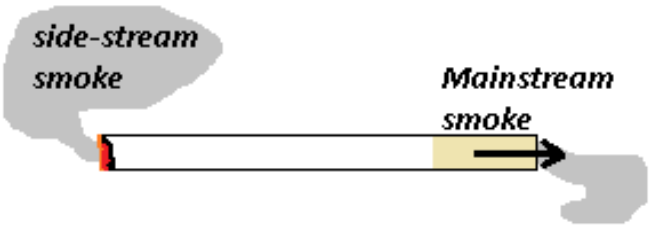


***Fig. 2**: Mainstream and side-stream cigarette smoke. Side-stream tobacco smoke contains an increased amount of PAH due to increased incomplete combustion*

Tissue in various stages of maturity reacts differently to cigarette smoke exposure. In smoke exposed mice, increased levels of primordial follicle depletion and decreased corpus lutuem formation are found, alongside with oxidative stress throughout the ovary, resulting in fewer follicles available for ovulation (Sobinoff et al. 2012). As apoptotic markers are also detected in latter stages of development alongside reduced primordial and primary follicles, a mechanism that pivotally targets the developing follicle is suggested. The apoptotic state of these follicles increases stimulation of follicular activation thereby depleting the follicle reserves. This hypothesis is supported by the fact that in human female smokers, reduced anti-Müllerian hormone levels in serum predicts a decrease of the primordial follicle reserve, indicating a similar mechanism in mice and humans (Freour et al, 2010). Early onset of menopause in women smokers is caused, at least in part, by the proapoptotic actions of tobacco smoke-derived PAHs in human oocytes found in follicular fluid.

Direct cigarette smoke exposure alters a number of parameters in mice. Increased lipid peroxidation is found, reduced glutathione contents, increased catalase activity and fewer intercellular junctions were observed in granulosa cells exposed to cadnium, a heavy metal found in cigarette smoke. Together this contributes to significant ovotoxicity (Prins, J.R., 2012).

The aromatic hydrocarbon receptor in ovotoxicity

Polycyclic aromatic hydrocarbons (PAHs) are recognized by the aryl hydrocarbon receptor pathway, shown in figure 3. By the use of Drosophila knockouts it has been shown to play a role in development, as these knockouts lack limps. Furthermore, it manifests an adaptive response by transcribing xenobiotic response enzymes, mainly found in the liver. Metabolism of Benzo(a)pyrene (BaP), a well-known potent carcinogenic substance found in cigarette smoke, is shown in figure 3. Cytochrome P450 1a1 (CYP1a1) is a phase I detoxifier and forms epoxide groups on BaP. By using glutathione as antioxidant, this normally leads to detoxification followed by biliary and urinary excretion. However, if this process fails, guanosine reacts with BaP, which activates multiple oncogenes in the p53 family.

Apart from the downstream aromatic hydrocarbon receptor (AhR)-regulated gene-transcription, crosstalk exists with other pathways. Evidence suggests interactions with the estrogen receptor, androgen receptor and thyroid hormone receptor. Furthermore, the AhR pathway may also be involved in cell cycle regulation (Callero MA1, Loaiza-Pérez AI. 2011). Other studies have found that PAHs are a cause for reactive oxygen species in the ovary and can react with DNA. PAHs have been found in covalent bonding with DNA in granulosa cells and follicle fluid of female smokers (Gruber et al., 2008). Furthermore, the clearance of the more toxic metabolic intermediates appears to be decreased. In the ovary, PAHs are potent inducers of primordial follicle depletion, by stimulating them to replace the growing follicles that are targeted for destruction (Sobinoff et al, 2012). Also

ovulated oocytes displayed evidence of oxidative stress such as increased levels of mitochondrial ROS and lipid peroxidation resulting in reduced fertilization potential (Sobinoff. A.P., 2013).

The AhR is proposed as functionally central component in follicle development. In AHR-/- mice, more follicles are found at birth suggesting a fundamental role in primordial follicle pool regulation. Furthermore, activation of the downstream target CYP1a1 has been demonstrated in smokers, suggesting that constituents of cigarette smoke reach concentrations in the circulation sufficient to induce signaling through this receptor (Hincal, F. 1986). Concentration measured in follicles shows an increase in PAH in follicles of smoking women compared to non-smokers (Baird M.W. et al, 2005, Neal M.S. et al. 2008). Also outside of the ovary AHR genes are correlated with smoke exposure. In a whole genome methylation assay of cord blood of smoking women, the most significantly altered C phosphate G (CpG) site in the DNA was in the AHR-repressor gene. In lymphoblasts and pulmonary macrophages differentially methylated CpG sites have been found in smoke exposed individuals.

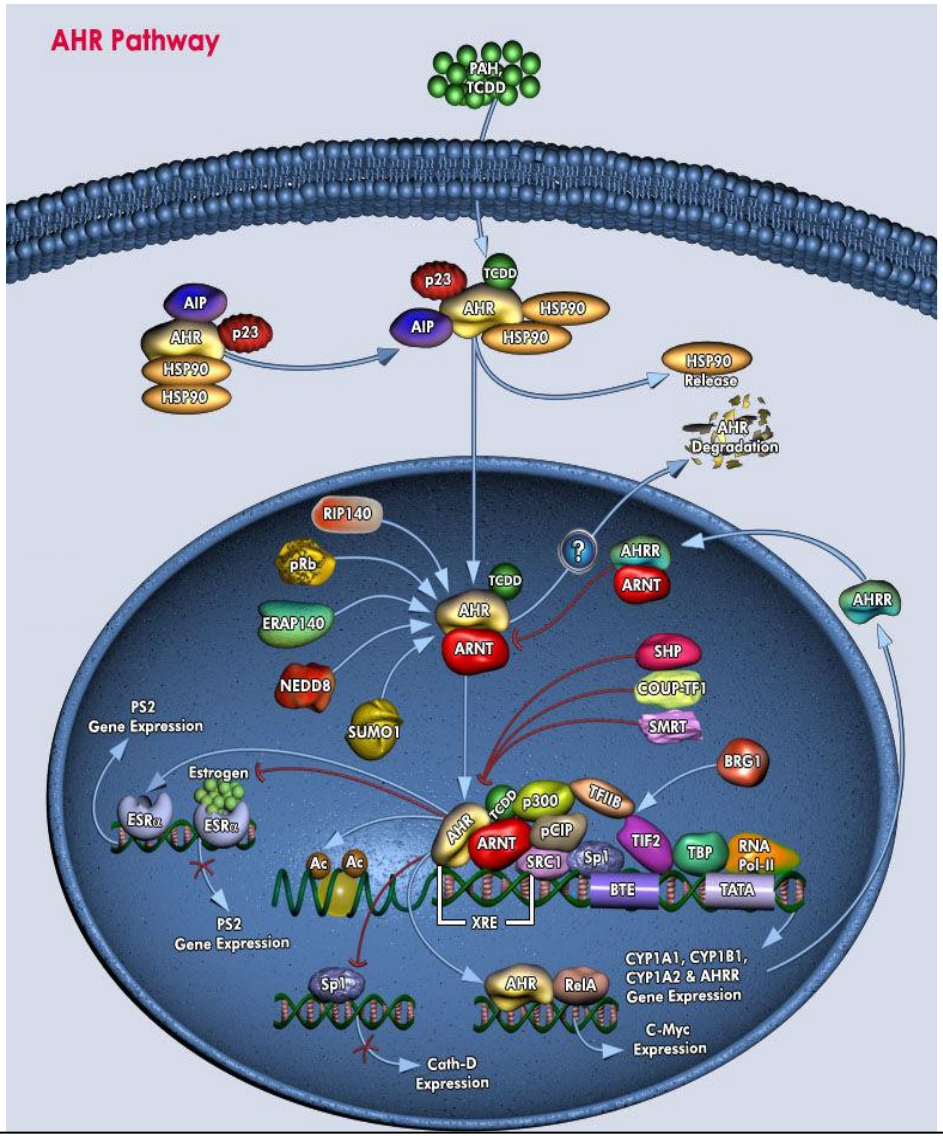


Fig. 3. *Ligand binding makes the AhR exit its dormant state and the conformational change exposes a nuclear localization signal, losing chaperone proteins as heat shock protein 90 (Hsp90). AhR transfers to the nucleus and dimers with the aryl hydrocarbon receptor nuclear translocator (ARNT). The heterodimer binds to Xenobiotic Response Element (XRE) and alters expression of genes controlled by the enhancer, which are phase I detoxifiers CYP1A1, CYP1A2, CYP1B1 and NAD(P)H-Quinone Oxireductase. Negative feedback on the AHR is mediated by the AHR-repressor to regulate the activity of the pathway.*

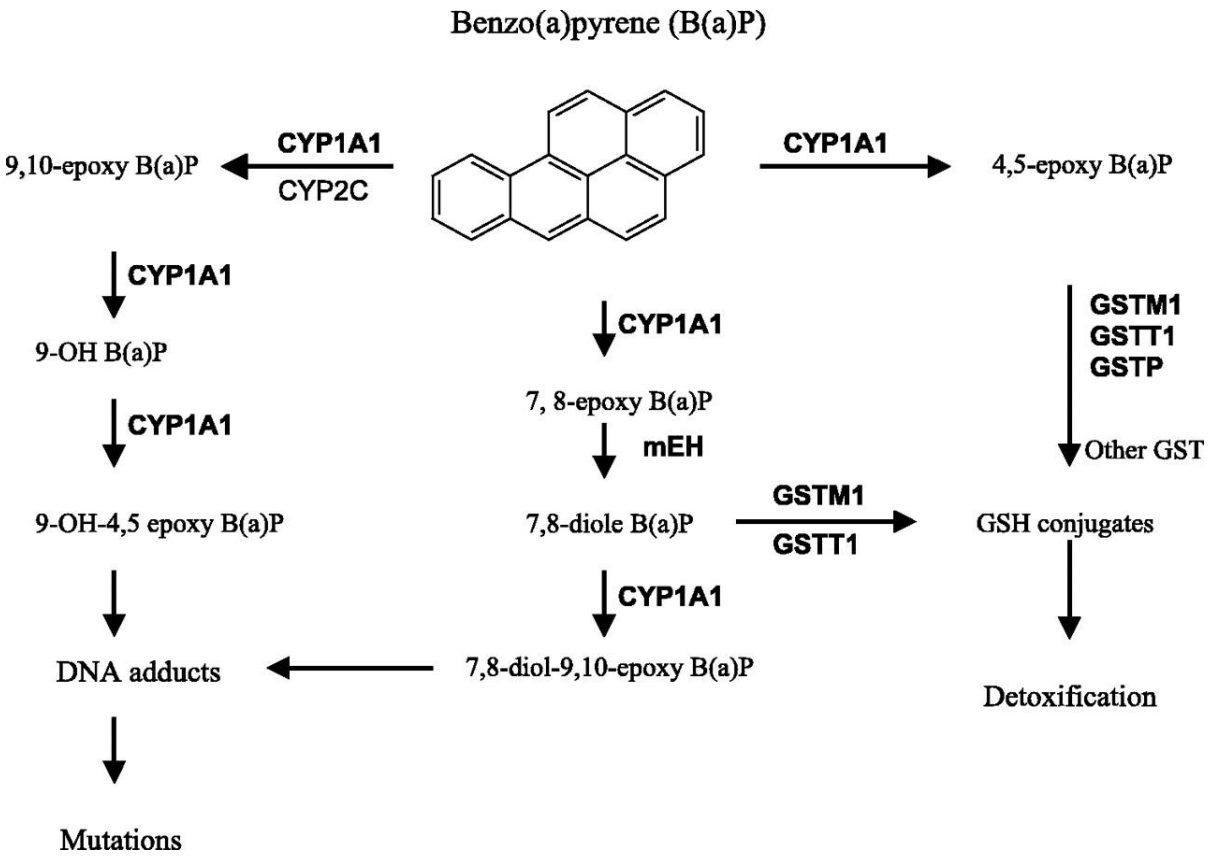


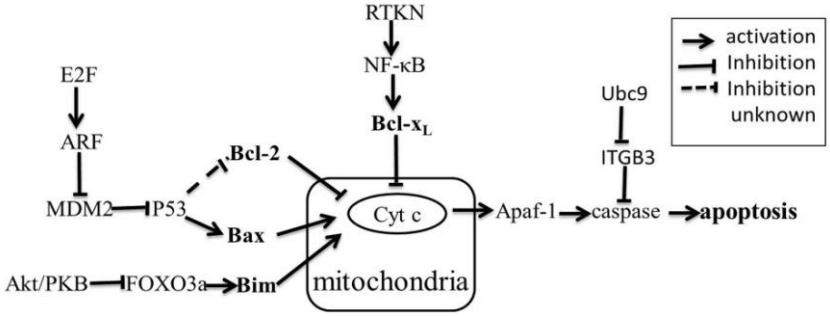
Fig. 4. *Multiple metabolism pathways of Benzo(a)pyrene, a highly carcinogenic polycyclic aromatic hydrocarbon, found in cigarette smoke. It displays normal (right) and aberrant (left) metabolism of a PAH.*

Regulation of reduced number of follicles

As smoke exposed females have a lower primordial follicle reserve, many studies have been performed on the balance between apoptosis and autophagy, the most important mechanisms leading to decrease in follicle numbers. The exact interaction of different pathways is currently under investigation. Bcl-2-associated protein X (BAX), however, has been proposed as potential regulator of PAH-associated cell death. BaP-guanosine adducts are known to activate p53, which in turn activates BAX (see fig. 3).

Autophagic pathways are also suggested to be involved in the depletion. Two important markers of autophagy, Beclin1 and microtubule associated protein light chain 3, are upregulated upon whole body side-stream smoke exposure (Sobinoff et al., 2012). In mainstream smoke exposure autophagy markers were not detected. This suggests a complex regulation of follicular depletion via multiple routes. As in this study 100% mainstream smoke is used, BAX is studied as mediator of apoptosis.

Fig. 5. Apoptosis is regulated by multiple pathways. Activation of pro-apoptotic Bcl-2 family (such as BAX) makes mitochondria release cytochrome C and eventually caspases to activate mitochondrial apoptosis. Zhong Ju Zhang, Shi Liang Ma, 2011.



Lasting the generations: epigenetics in gametes

As we reach more and more consensus on the possibility of adaptation to environmental influences via the mother to the child during pregnancy, many mechanisms of action are proposed. It is known that environmental influences can mark DNA without altering the sequence: methylation of promoter regions in genes, often where a cytosine occurs next to a guanine (CpG islands) influences gene expression, allowing for environmental adaptation. There is emerging evidence that these epigenetic marks can be transferred from parent to offspring via gametes, though the molecular nature of this inheritance remains to be elucidated. Also, therapeutic windows have been found: exposure of vinclozolin at embryonic day 13 induced a threefold increase in expressed genes, compared to embryonic day 16 (Skinner, M.K. et al, 2013). Effects of *in utero* exposure to environmental factors, such as toxic agents from cigarette smoke, have been studied and appear to have a potent effect on the development of pathologies such as cardiovascular diseases, and metabolic diseases in new-born organisms (Holloway A.C. et al, 2007). The effects of smoking on the fertility of the unborn child, however, has not been studied. The degree of stability in DNA methylation is marker-dependent and differs per tissue, therefore studies on the ovaries are awaited. In gamete production, epigenetic reprogramming is required to restore totipotency of the gamete. Studies over the past 30 years have revealed that there are two key periods of genome-wide epigenetic reprogramming, one during gametogenesis and one during embryogenesis. This raises the question how DNA methylation can inherited and is therefore studied vigorously at this moment as increased understanding may offer new therapeutic targets.

Regulatory genes of ovotoxicity

Genetic analysis correlated these insults with genes associated with detoxification, inflammation, follicular activation, immune cell mediated apoptosis and membrane organization displaying the complexity of ovotoxicity (Prins et al, 2012). Developing oocytes exposed to cigarette smoke displayed a significant upregulation in the phase I detoxifying enzyme cyp2e1, which is known to cause molecular bioactivation resulting in oxidative stress (Sobinoff. A.P., 2013). It is shown that toxic factors influence DNA methylation most strongly during gestation. As this is a period of profound development, smoke effects on oocyte reserve are expected in the second and possibly third generation. Regarding establishment of the reserve, maintenance, suppression of development and activation of growth of the ovarian follicles exists controversy (Kerr J.B., 2013). As the majority of studies have been performed in sperm, follicle investigation is awaited.

Aim of this study

This study will investigate the influence of mainstream cigarette exposure during pregnancy on numbers of ovarian follicles in a mouse model, and tries to elucidate the mechanism behind possible influences. The tissue of the mothers will be used in the pilot study to optimize all protocols for use on F1 tissue. In order to do so, a numerical analysis will be done by counting progressing classes of follicles after histological staining. Also the number and intensity of specific markers within the pathways described previously will be analyzed. As a marker of fertility furthermore pregnancy rates and litter sizes will be used.

METHODS

To investigate the effect of *in utero* cigarette smoke exposure on ovarian follicle/oocyte quantity commonly multiple parameters are used. This investigation will asses histological and immune histological staining on ovary sections. The tissue from the mothers is used as a pilot, to optimize the use of the tissue of the pups. The main investigator of this mouse model is pivotally interested in the lungs but was willing to let fellow researchers collect other organs to perform their research.

Ethics statement

Study design was set up by K. Meyer and approved for by the local ethics committee (DEC) and performed under guidance of strict international guidelines for animal care. Permission was granted to study the effect of prenatal smoke exposure on DNA methylation in epithelial cells (DEC number: 6589D, received 07-01-2013). There is hypothesized that prenatal smoke exposure affects lung development and offspring's susceptibility in later life. As this research only required use of lungs, other organs were available upon request which enabled us to make us of the ovaries.

Mice and smoke-exposure

The main investigator of this mouse model is pivotally interested in the lungs but was willing to let fellow researchers collect other organs to perform their research and so I could collect the ovaries. 48 male and 48 female C57BL/6 mice were stepwise adjusted to cigarette smoke, building towards 50 min smoke exposure (10 cigarettes) and a 3 hours 'break', followed by another 50 min exposure to the smoke of 10 cigarettes. Smoking started from the age of 6 weeks until week 11 when the males where placed with the females. To ensure high fertility rates K. Meyer needed for her experiment, the mice were hyperstimulated using two injections with pregnant mare serum gonadotrophine. 39 mice became pregnant, 3 control mice and 6 smoke exposed mice did not become pregnant (N.S.).

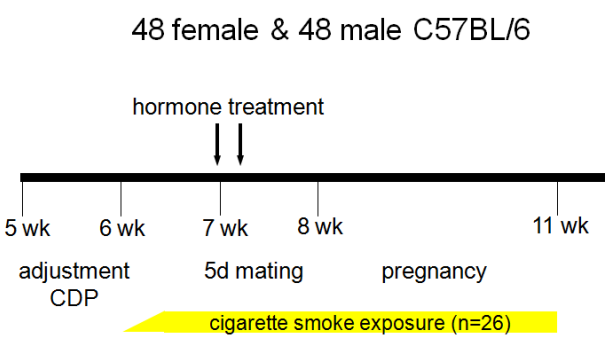


***Fig. 6**. Overview of treatment of parent mice. CDP is animal housing centre.*

Birth weight of pups was measured. Unfortunately, due to maintenance work at the animal housing centre, some of the mothers became stressed and ate their pups. 71 pups survived of which are 36 female. This strongly reduced the outcome of the pregnancies and therefore the study design had to be changed. In the new setup, two groups of mothers exist (4 smoke and 6 air exposed animals). The air exposed mother had 12 smoke exposed and 12 air exposed pups. The smoking mother had 6 smoke and 6 air exposed pups. This design enables studies regarding smoke adaptation. The pups were either smoke or air exposed for 12 weeks using the same protocol as the mothers with respect to smoke exposure.

Isolation of tissue and preparation

Ovary tissue of the mothers was collected after weaning. Left side ovaries were stored in 10% formalin, provided by the department of pathology of the UMCG. Right side tissue was snap frozen in liquid nitrogen. Tissue was obtained from sacrificed mice after lungs, kidneys and liver were harvested upon request of other researchers. To be sure no tissue was damaged, ovaries were

harvested including the surrounding tissue, which was mainly fat. Later during the research was decided that for proper processing on glass slides it is more convenient to dissect all fatty tissue from the ovary. Furthermore, not to waste any material potentially important for this or future studies, uteri and tubae were isolated from the pups and cryopreserved in using liquid nitrogen at -80°C.
After intervision with fellow research groups from Amsterdam Medical Centre and Wageningen University it was decided to store the ovaries of the pups in 4% PBS buffered paraformalin, as this is said to be more stable. However after literature study, it appears that this is of no significant effect on tissue preservation when processed within weeks after storage (Matsuda Y, Ishiwata T, 2015). From the pups ovaries, tubae and uteri were isolated separately for future research purposes, left side in 4% phosphate buffered saline paraformalin and right side frozen in liquid nitrogen.
After an incubation time of minimal 24h tissue was placed in cassettes and prepared for automated overnight paraffin embedding. Tissue was cut in sections of 4 µm thick using a Leica microtome and placed in a 50°C water bath to stretch it properly on pretreated glass slides. 1 out of 5 was taken up for histological analysis and remaining tissue was used for immune histological analysis.

Histological analysis
For histological analysis, a standard protocol for haematoxylin and eosin staining is used. After deparafinisation of the tissue in xylol (2x5') and washing in alcohol (100-70%), slides are placed in a haematoxylin bath for 10 minutes, rinsed with tap water for activation and stained with eosin for 2 minutes. Slides are dehydrated in 100% alcohol and automatically covered using the SAKURA Tissue-Tek DRS2000.
After analysis, eosin staining was changed for periodic acid – Schiff (PAS) staining upon suggestion of K. Teerds from Wageningen University. PAS stains the zona pellucida of the oocyte clearly, whereas eosin stains the cytoplasm. This change makes histological analysis easier. PAS staining was automatically applied using the SAKURA Tissue-Tek DRS2000.

Follicle classification
For an overview and digital storage means, scans were made using a Hamamatsu NanoZoomer 2.0HT and morphometrics software (Aperio ImageScope v12.1.0.5029). For detailed analysis a Leica DM2000 LED was used. Classification is based on the classification as suggested in the literature (Pedersen, Hamer, 1968). Primordial follicles are counted when a oocyte is surrounded by a single layer of flat cells. Primary follicles have a single layer of round cells around it. Secondary follicles have a layer of flat cells filled with multiple cell layers of round granulosa cells in it. Follicles are classified as antral when there is a visible fluid-filled cavity formed within the granulosa cells. To prevent double counting, only follicles with a visible nucleolus are included. After ovulation, follicles form a large corpus luteum, that were counted using printouts of the photos and labeling the visible structures in each section, as there are no nucleoli to use as a benchmark.
After use of this classification it appeared that the distinction between secondary and pre-antral is of minor significance, according to other studies. To adjust to the other studies, and make comparison easier, distinction is made in the pups between primordial, primary, pre-antral (which includes former secondary follicles and beginning antral follicles) and antral follicles (of which the antrum is at least the size of the oocyte). No changes were applied to the counting of corpora lutea.
After counting 1 out of 5 slides as mentioned before, follicle numbers are multiplied by 5 to approach absolute follicle numbers in the ovary. This was not done for corpora lutea as these numbers are already absolute per ovary.

Immune histochemistry

Immune histochemistry was performed under the supervision of M. Reijnders using a standard protocol. Four different types of antibodies are used: CYP1a1 (Santa Cruz Biotechnology, CYP1a1 G-18), AHR (Assay BioTech, (p-Ser36), AHRR (antibodies-online.com, anti-Aryl Hydrocarbon Receptor Repressor antibody) and BAX (Santa Cruz Biotechnology BCL-2 associated X protein BAX P-19 (pSER-184)). Per antibody optimal buffer solution and primary antibody concentration was decided by testing on ovary tissue of the mothers. Tested buffers are tromethamine(Tris, 10mM)/ethylenediamine tetraacetic acid (EDTA, 1mM, pH=9,0), EDTA(1mM, pH=8,0), Tris/HCl (10mM, pH=6,0) and citrate (10mM, pH=6,0). For CYP1a1 and BAX citrate buffering gave the best results, for AHR and AHRR optimal antigen retrieval buffer is Tris/HCl. All antibodies were tested at dilution 1:25; 1:50; 1:100 and 1:200. Clear staining was obtained at a dilution of 1:100 1% bovine serum albumin (BSA)/phosphate buffered saline (PBS).

Paraffin sections were deparafinized using 2x5' xylol bath, 100%-70% alcohol (stepwise washing). Citrate or Tris/HCl antigen retrieval step was performed using a microwave (heating of buffer 5'/700W, cooking of 15'/300W). Endogenous peroxidase blocking was performed in 50 ml PBS with 500µL 30% H_20_2 for 30'. Primary antibody was diluted 1:100 in 1% BSA/PBS and incubated for 1h in a humidified, dark chamber. Secondary antibodies and tertiary were 1:100 RaG-PO/GaR-PO (DAKO polyclonal Goat Anti-Rabbit or Rabbit Anti-Goat Immunoglobulins/hydrogen peroxidase, 0.250g/L) diluted in 1%BSA/PBS incubated for 30'. Hydrogen peroxidase label detection was performed using 3,3'diaminobenzidine (DAB), diluting prepackaged cups of DAB in 50mL PBS. Before application, 500µL 30% H_20_2 is added for peroxidase label activation in which tissue was incubated for 10'. Nuclear staining is performed with haematoxylin for 60 seconds, activated by rinsing with tap water. Tissue labeled using DAB was automatically sealed using SAKURA Tissue Tek Film. Positive controls were not performed as the necessary tissue was not available.

As staining of tissue from the mothers gave clear and positive staining, this protocol was applied on the material of the pups as well. However in this material it was harder to find positive staining. Therefore, new buffer tests and dilutions are performed on the material of the pups but none of the slides displayed staining as seen previously. DAB was replaced by 3-amino-9-ethylcarbazole (AEC): 2,5mL AEC was diluted in 50mL acetate buffer and filtered. Before use, 500µL 30% H_20_2 is added for peroxidase label activation in which the tissue incubated for 15'. AEC was sealed using cover slips and warm Kaizer's glycerol gelatine. Also this adjustment gave no similar results compared to the pilot material. As AEC is less sensitive but has a brighter color, a primary antibody dilution of 1:25 instead of 1:100 was used. This did not change results either.

As positive control, tissue from human kidney, kindly provided by Marjan Reijnders, was used after searching proteinatlas.com. Applying the same protocol, including testing of buffers, this gave no clear staining.

In general, antibodies give a better staining when used on frozen tissue as no antigen retrieval method is needed, frozen sections were made (4µm thick) using a cryostat. Sections were fixated on slide an acetone bath for 10'. Primary antibodies were incubated for 1h, blocking of endogenous peroxidase lasted 30' in 125µL H_2O_2 diluted in 50mL PBS. Secondary and tertiary antibodies were resp. RaG-PO and GaR-PO (DAKO polyclonal Goat Anti-Rabbit or Rabbit Anti-Goat Immunoglobulins/HRP, 0.250 g/L). Staining was performed using AEC, 15' incubation of 2,5mL AEC filtered in 50mL acetone, before use adding 50µL H_2O_2. After rinsing with demineralized water and afterstaining with heamatoxylin, slides were covered with cover slips and Kaizer's glycerol gelatine.

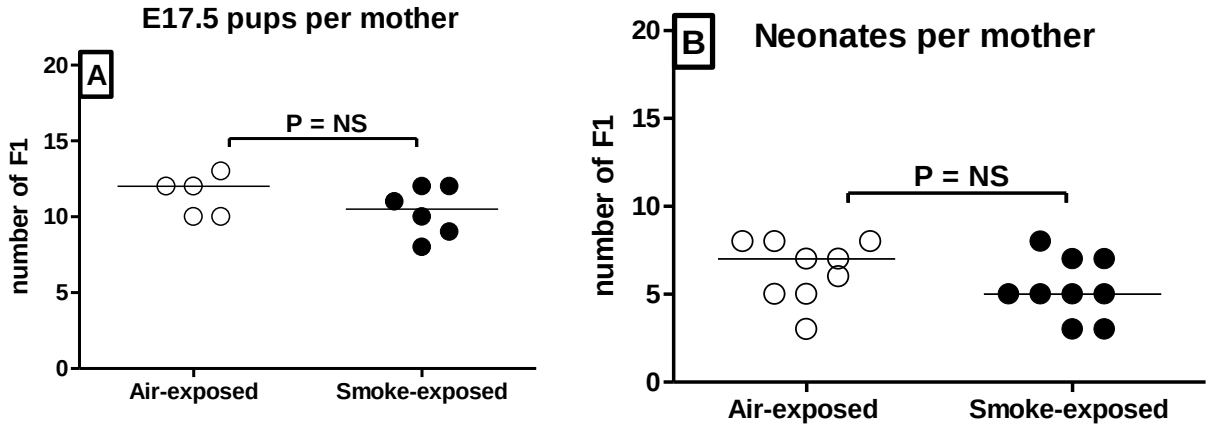
Statistical analysis

Comparison between smoke and non-smoke groups follicle count was performed using GraphPad Prism (version 5.0). One-way non-parametric T-tests were performed between groups of the same tissue but different exposure ($P>0.05$).

RESULTS

Fertility

Before the layout of the study was altered, as a result of the stressed mothers eating their pups, there were mice sacrificed at different time points to study the smoke effect over time. Therefore mice were sacrificed from both air and smoke groups on embryonic day 17,5 (graph 1a) and day 70 (graph 1b) after birth. In both groups no significant differences are found.



***Graph 1**. A: number of embryonic day 17,5 pups. Open dots display air exposed animals whereas black dots display smoke exposed animals. B: number of neonates per mother. Open dots are air exposed animals, black dots display smoking mothers.*

Counting

During the experiment, the staining of the material was changed. The difference between both staining is displayed in figure 4.

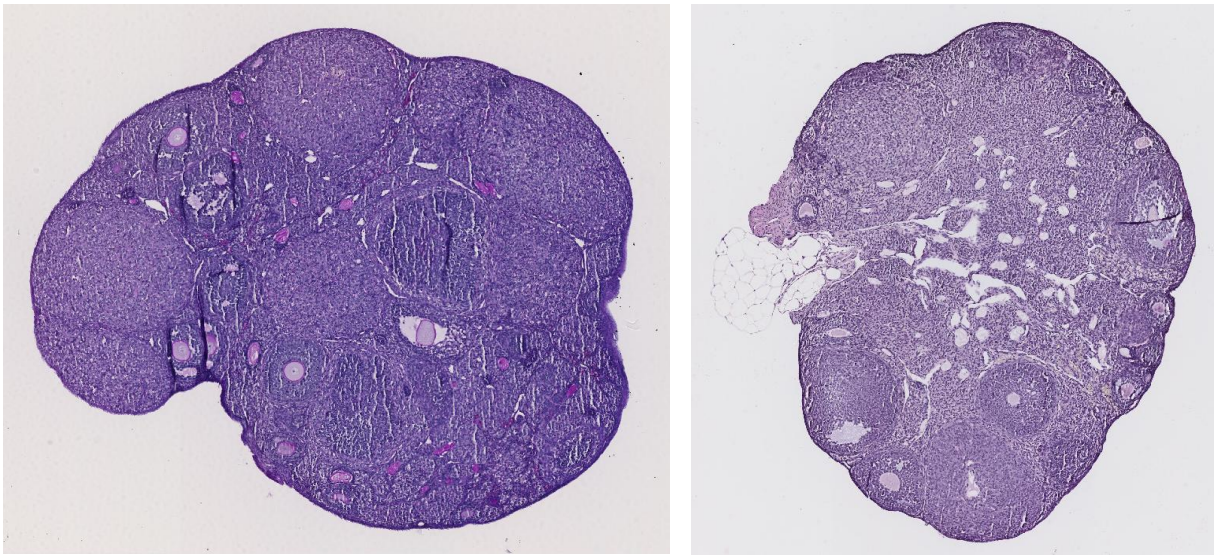


***Fig. 7**. A: HE staining: haematoxylin stains for the nuclei (purple) whereas eosin stains for the cytoplasm (pink) magnification 3,3x. B: Periodic acid-Schiff (PAS) stain. Haematoxylin stains for the nuclei (purple) whereas PAS stains polysaccharides, for example found in the zona pellucid surrounding oocytes (pink) magnification: 3.5x.*

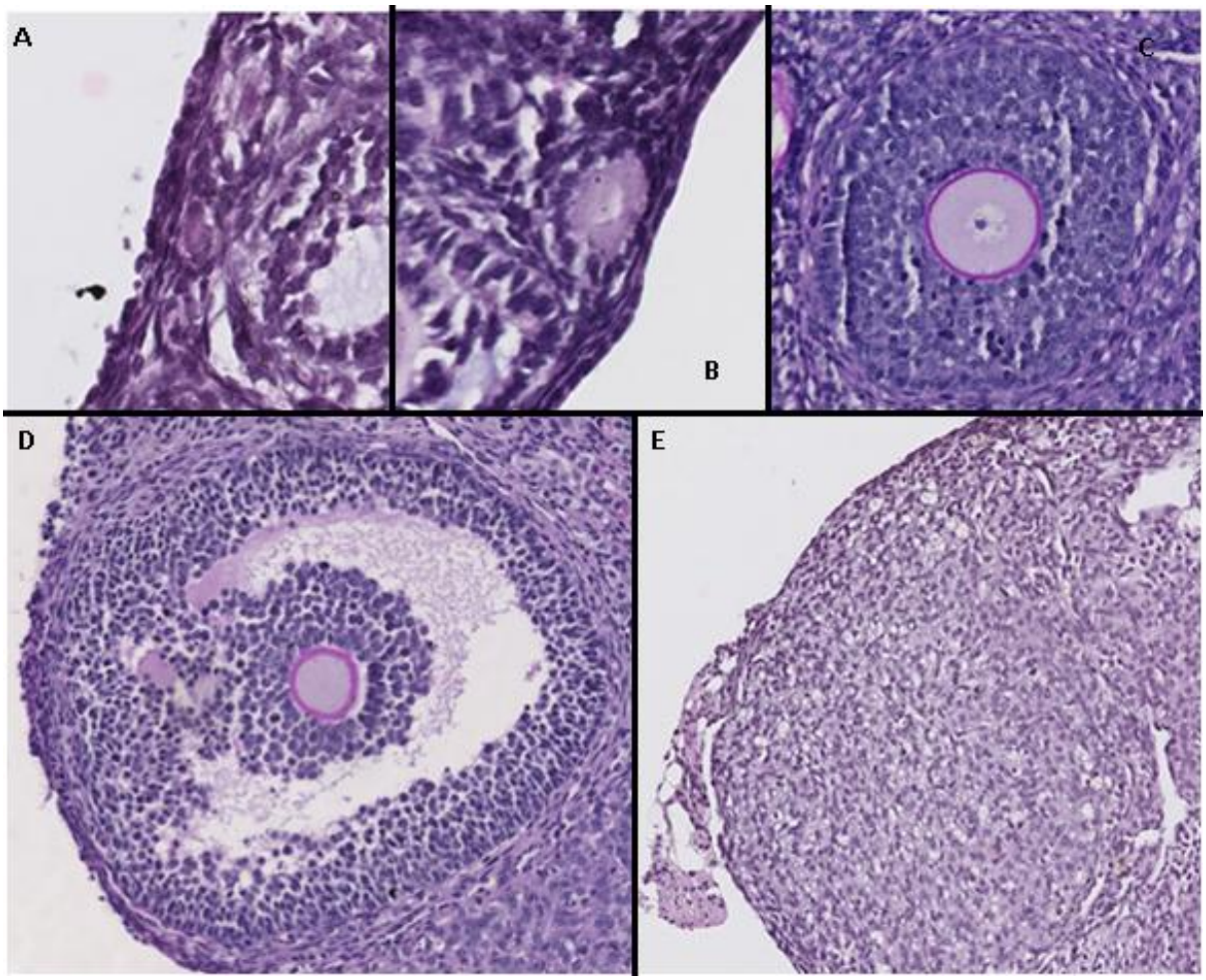
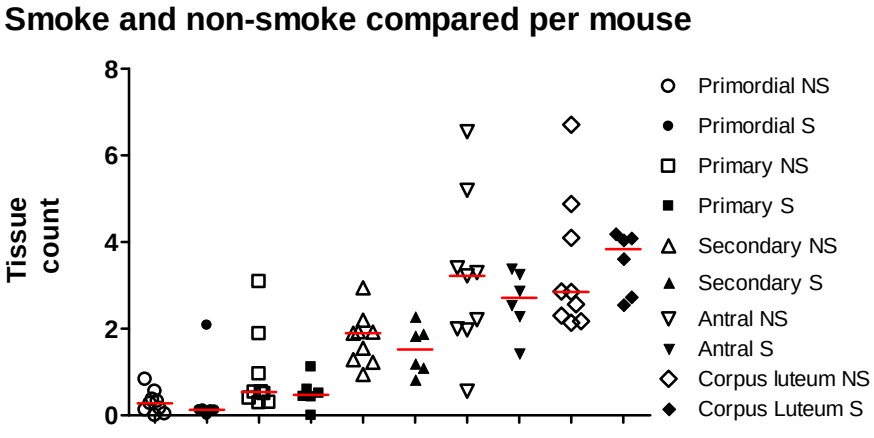


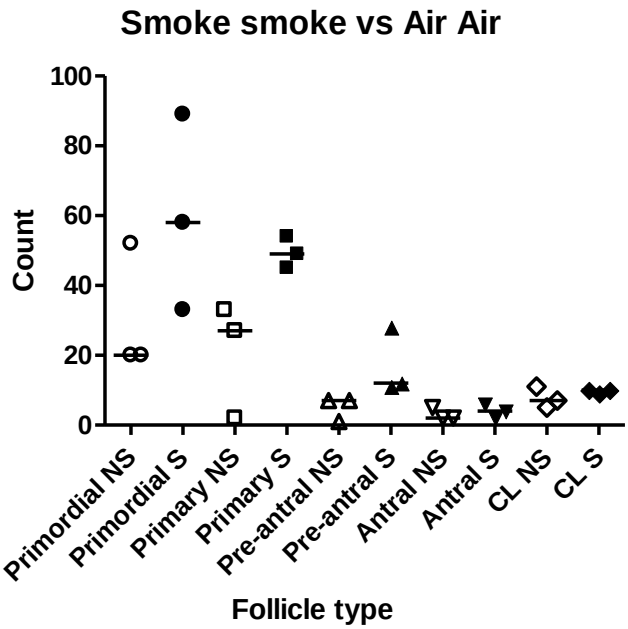
Fig. 8. *Follicles in different stages of development. A. Primordial follicle, HE staining, magn. 40x. B. Primary follicle, HE staining, magn. 40x. C. Secondary follicle with visible nucleolus, PAS staining, magn. 20x. D. Antral follicle with no visible nucleolus visible, PAS staining, magnification 16x. E. Corpus luteum, HE staining, magnification 10x.*

Classification occurred, as described earlier, into stages of development shown in figure 5. Each section was scored per follicle type. When classified into smoke and air exposed, differences between primordial, primary and corpus luteum were significant (data not shown). Taken the average number per slide per animal however showed no significant results (graph 2).



Graph 2. *Mothers exposed to air (open figures) or smoke (black figures) scored per average number of different developmental stages of follicles per slide, displayed per mouse.*

Pups also were compared with regard to number of different developmental stages of follicles. Due to time constraints, only three of the smoke exposed pups from smoke exposed mothers were compared to air exposed pups from air exposed mothers. Between these groups most profound smoke effects are expected.



Graph. 3. *Absolute count of follicles per ovary. Open dots display air-air exposed pups, closed dots display smoke-smoke exposed animals.*

Immune histochemistry

To study mechanism behind the smoke damage, ovarian tissue is stained for CYP1a1, AHR, AHRR and BAX. The tissue of the mothers is used as a pilot. Fig. 5 displays an example of an immune staining. The granulosa cells surrounding the oocyte and the oocyte itself display the strongest staining. However, after intervision with K. Teerds from Wageningen University, it appeared that the staining is not specific and is in fact an artifact from the fixation time which exceeded 48h. For the pups, this was not the case as they were processed faster. Regardless testing the antibodies, multiple optimalizations of the protocol and use of positive controls, it was difficult to obtain clear staining of ovarian tissue.

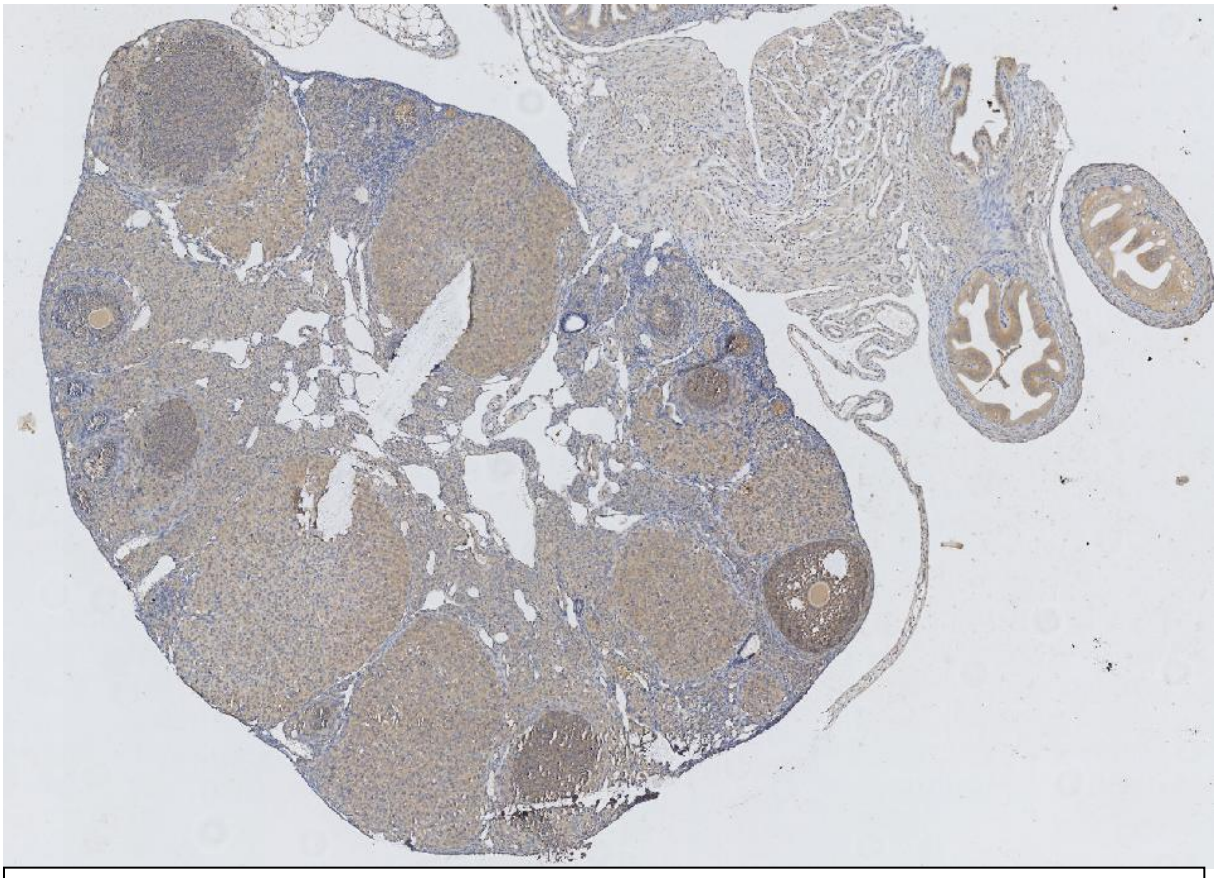


Fig. 9. *Immune staining of mother (smoke-exposed). Staining for CYP1a1 antibody (DAB-labeled), counterstained with haematoxylin. Magnification: 3.0x.*

DISCUSSION

The influence of mainstream cigarette smoke exposure during pregnancy on numbers of ovarian follicles is investigated in this study using a smoking mice model and a hypothesized mechanism behind influences is elucidated. The tissue of the mothers in the mice study is used as a pilot to ensure optimal protocols for use on the tissue of the pups. A numerical analysis is performed by classification of follicle types in different stages of maturity. The intensity of specific markers in pathways thought to be involved was studied. As a marker of fertility litter sizes and pregnancy rates have been used.

Pilot study: the tissue of the mothers

The pregnancy rates and life birth rate are an important parameter on assessing the fertility of our mice. The mothers were exposed to mainstream cigarette smoke before and during pregnancy for a total duration of 6 weeks. We did not find a significant difference with respect to the number of pups at embryonic day 17.5 nor in the number of neonates per mother. Since this study is in the first place designed to study lungs, the setup was not ideal for studying fertility, as there are two time points where the mice received hormones to ensure large numbers of offspring. Therefore the effects of smoking on natural fertility, e.g. litter size may have been masked. Our results cannot be used to support a conclusive answer to the question if fertility is altered by smoke exposure.

The sections made from maternal material all were stained using standard HE staining. Studying this helped to get familiarized with the structures and has lead to a classification based on literature supported by own observations.

The differences in follicle count in mother tissue was significant when analyzed per slide, but when displayed per mouse the differences in follicle numbers were not large enough to reach significance. A trend in decreasing numbers of primordial follicles was observed as seen in previous studies, as lower numbers of primordial follicles were expected in the smoke exposed animals.

The pups

The counting of the ovaries of the pups of two out of four groups (smoking pup from a smoke exposed mother versus air exposed pup from an air exposed mother) shows a significant difference in primordial follicle reserve, with a higher number of primordial, primary and preantral follicles in the smoke exposed animals. Due to time constraints only these groups have been counted, and only three animals per group. To make a well-founded statement on the effect of smoking on follicle count, I would recommend to count at least six animals per group. The trend that is observed is note-worthy and in an opposed direction compared to the mothers. This may be due to an adaptation effect of smoke exposure during development. In expectation of a similar exposure during adult life, the pups increase the follicle pool size to cope with the toxicity experienced during development.

New insights have optimized the protocol for counting used in this study: in the pups there was no distinction made between secondary and preantral follicles, and a different staining made counting more accurate as regressing follicles were visible. It is a challenging task to give absolute numbers of follicles within a 3D structure based on 2D images. The method used for counting the follicles in different stages of maturity is thought to give an accurate estimate of the absolute amount of follicles present in an ovary, as it is often performed and described in the literature.

Immune histochemistry of mothers and pup ovaries

The immune histological staining performed on the tissue of the mothers appeared to be successful and showed strong staining in the developing follicles as expected (Sobinoff et al., 2012). After consultation of fellow researchers however, the reliability of these staining is doubted as the staining could be a result of overfixation of the tissue. To verify the results, the staining should be repeated in the tissue from the pups, as this tissue has been fixated for a shorter period.

It should be noted that the mothers and the pups have lived under different circumstances regarding factors influencing the estrous cycle, such as hormone stimulation, pregnancy and lactation. Therefore direct comparison of tissue from mother and pup is not recommended.

When testing the antibodies on the tissue of the pups we did not manage to obtain similar results in the tissue of the pups. This could be because of the antibodies passing their expiration date, but this is unlikely as they have always been stored properly and were only 4 months old when used. The more likely explanation is that the fixation time exceeded its maximum in the mothers and therefore will not give trustworthy results.

Also cryopreserved ovaries were included in the tests for obtaining specific staining with these antibodies. As no antigen retrieval step is needed in this protocol, we expected better results in frozen sections. The morphology however is not as clearly visible as it is in paraffin embedded sections. Some staining was observed but the structure of the tissue was too obscure to conclude any tissue-specific activity within the ovary.

Future perspectives

When continuing with this research I would recommend to compare the smoke-air exposed pups and air-smoke exposed pups with the pups I already counted. This would make it possible to distinguish between an adaptive and a direct smoke effect and therefore elucidate the effect of smoke exposure during pregnancy. Also, to make well-founded statements on the effect of smoke exposure during pregnancy on the follicle pool of the pups, larger numbers of groups should be counted to have more statistical power.

In other studies mice are exposed to a mixture of mainstream and side-stream smoke which contains higher concentrations of carcinogenic polycyclic aromatic hydrocarbons. It is thought that this can alter the way of follicle loss by shifting the balance between apoptosis and autophagy (Sobinoff et. al, 2012). This balance has not been extensively studied yet and could give insight to the role of smoke toxicants in cell death. Immune histochemistral analysis should make this possible and optimaization of the protocol used in this study could be a start to understand the role of apoptosis in follicle depletion. Also DNA damage as a result of smoke exposure could be studied using a TUNEL analysis. Due to limited time this was not performed in this study but it is recommended in the future.

The study design was optimized for the use for the lung study by K. Meyer and unfortunately cannot be used to investigate fertility as litter size and time to pregnancy are altered by hormone stimulation. This experiment makes use of whole body exposure to mainstream smoke whereas in other studies investigating toxic effects of smoke exposure, smoke extract or nose-only exposure are used. Mice subjected to whole body exposure ingest smoke particles as the mice tend to clean themselves, whilst licking is prevented during nose-only exposure. However, when using the cuffs needed for nose-only exposure the mice experience increased amount of stress and animal handling as reviewed by Cheng Y.R. 2010. Consensus has not yet been reached on which method mimics human smoke exposure most accurately.

If a smoke effect is visible in the pups of smoking mothers, there must be a mechanism which enables environmental influences to be passed on to the pup. Thus far, there are only speculations on this mechanism, mainly concerning epigenetics. As the investigated pathways show up in multiple smoke related cord blood studies when analyzing differently methylated DNA regions, it would be promising to study the organ-specific effects. Using laser dissection methods, specific cell types could be analyzed and give a more detailed answer to the background of environmental influences during pregnancy. If we would fully understand the mechanism behind the influences of toxicant exposure during pregnancy, this would possibly give targets for treatment of prenatally exposed individuals.

Conclusion

The germ cell pool in females is prenatally established and can be influenced by polycyclic aromatic hydrocarbons. Further research is awaited to conclude how ovarian tissue reacts to cigarette smoke compounds. Both an increase in statistical power and a wider variety of techniques will provide insight in our observed trend that can be explained as an adaptation effect in the pups of smoke exposed mothers.

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REFERENCES

Allen S.S. (2014). Cigarette smoking among women: how can we help? Minn. Med. Mar;97(3):41-3.

Baird W.M., Hooven L.A., Mahadevan B. (2005) Carcinogenic polycyclic aromatic hydrocarbon-DNA adducts and mechanisms of action. Environ Mol Mutagen. 45(2-3):106-14.

Borman S.M., Christian P.J., Sipes, I.G., Hoyer P.B. (2000). Ovotoxicity in female Fischer rats and B6 mice induced by low-dose exposure to three polycyclic aromatic hydrocarbons: comparison through calculation of an ovotoxic index. Toxicol. Appl. Pharmacol. Sep 15; 167(3):191-8.

Callero MA., Loaiza-Pérez A. (2011) The role of aryl hydrocarbon receptor and crosstalk with estrogen receptor in response of breast cancer cells to the novel antitumor agents benzothiazoles and aminoflavone Int J of breast cancer Epub.

Chen S., Zheng Q., Zhang Y., Gao F., Liu X (2013) Disruption of genital ridge development causes aberrant primordial germ cell proliferation but does not affect their directional migration. BMC Biol. 11:22.

Dube M.F. and Green C.R. (1982). Methods of collection of tobacco smoke for analytical purposes. Recent adcances in tobacco Science 8, 42-102.

Gluckman PD, Hanson MA, Cooper C (2008), Thornburg KL. Effect of *in utero* and early-life conditions on adult health and disease. N Engl J Med;359:61-73.

Goedhart G, van der Wal MF, Cuijpers P, Bonsel GJ. (2009) Psychosocial problems and continued smoking during pregancy. Addict Behav;34:403-6.

Golding, J., Northstone K., Gergory S., Miller L.L. and Pembrey M. (2014) The anthropometry of children and adolescents may be influenced by the prenatal smoking habits of their grandmothers: a longitudinal cohort study. Am. J. Hum. Biol dec;26(6):731-9.

Gruber I, Just A, Birner M, Lasch A (2008). Effect of a woman's smoking status on oocyte, zygote, and day 3 pre-embryo quality in in vitro fertilization and embryo transfer program. Fertil. Steril. 90, 1249-1252.

Hincal, F. (1986). Effects of exposure to air pollution and smoking on the placental aryl hydrocarbon hydroxylase (AHH) acitivity. Arch. Environ. Health 41(6), 377-383.

Holloway A.C., Cuu D.Q., Morrison K.M., Gerstein H.C., Tarnopolsky M.A. (2007) Transgenerational effects of fetal and neonatal exposure to nicotine. Endocr, 2007;31:254-59.

Jaddoe V.W., Verburg B.O., de Ridder M.A., Hofman A., Mackenbach J.P, Moll H.A., Steegers E.A., Witteman J.C.M. (2007) Maternal smoking and fetal growth characteristics in different periods of pregnancy: the generation R study. Am J Epidemiol;165:1207-15.

Kerr JB, Duckett R, Myers M, Britt KL, Mladenovska TM and Findlay JK. (2006) Quantification of healthy follicels in the neonatal and adult mouse ovary: evidence for maintenance of primordial follicle supply;132:95-109.

Kerr JB, Myers M, Anderson RA. (2013) The dynamics of the primordial follicle reserve. Reproduction;146:205-15.

Matsuda Y, Ishiwata T. (2015) Fixation methods for the preservation of morphology, RNAs, and proteins in paraffin-embedded human cervical cancer cell xenografts in mice. Methods Mol. Biol. 1249:253-63.

Neal M.S., Zhu J. Foster W.G. (2008) Quantification of benzo[a]pyrene and other PAHs in the serum and follicular fluid of smokers versus non-smokers. Reprod Toxicol. Jan;25(1):100-6

Pepling, M.E. (2006) From primordial germ cell to primordial follicle: mammalian female germ cell development. Genesis Dec;44,(12):622-32.

Prins J.R., Hylkema M.N., Erwich J.J.H.M., Huitema S., Dekkema G.J., Dijkstra F.E., Faas M.M., *Melgert B.N.* (2012) Smoking during pregnancy influcences the maternal immune response in mice and humans. Am J Obstet Bynecol;207:76.e1-14

Sarraj M.A., Drummond A.E. (2012) mammalian foetal development: consequences for health and disease, 2012 143 (151-63).

Skinner M.K., Guerrero-Bosagna C., Haque M., Nilsson E., Bhandari R., McCarrey J.R. (2013) Environmentally induced trangenerational epigenetic reprogramming of primordial germ cells and the subsequent germ line; Plos one.

Smith C.J., Hansch C. (2000) The relative toxicity of compounds in mainstream cigarette smoke condensate. Food and Chemical toxicology 38; 637-646.

Sobinoff A.P., Pye V, Nixon B, Roman S.D., McLaughlin E.A. (2012). Jumping the gun: smoking constituent BaP causes premature primordial follicle activation and impairs oocyte fusibility through oxidative stress. Toxicol Appl Pharmacol. Apr 1;260(1):70-80.

Sobinoff A.P., Beckett E.L., Jarnicki A.G., Sutherland J.M., McCluskey A., Hansbro P.M., McLaughlin E.A. (2013) Scrambled and fried: cigarette smoke exposure causes antral follicle destruction and oocyte dysfunction through oxidative stress. Toxic. Appl. Pharmac. 271;156-67.

Zatonski W., Przewozniak K., Sulkowska U., West R., Wojtyla A. (2012) Tobacco smoking in countries of the European Union. Ann Agric Environ Med.