

SELECTED ORAL COMMUNICATION SESSION

Session 49: Male fertility

08 July 2008

17:00–18:00

**O-186 Oral** Fetal exposure to three different classes of endocrine disruptors is associated with cryptorchidism at birth

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**Introduction:** Since in utero exposure to endocrine disruptors is deleterious to animal reproduction, it is suspected that such exposure could be harmful to human fetus as well. At the present time, there is controversy about the possible increasing incidence of congenital male genital malformations, male hypofertility and testicular cancer, and their relationship with environmental factors. Especially data are scarce on human exposure and the occurrence of cryptorchidism.

**Materials and methods:** From 2002 to 2005, we performed a prospective case-control study to assess the incidence of cryptorchidism and fetal exposure to selected endocrine disruptors (with anti-androgenic and/or estrogenic activity) in 2 maternity wards of Nice area. 6246 boys born alive at 34 weeks or more were screened at birth for cryptorchidism by senior paediatricians using standardized examination and were followed at 3 and 12 months. Each case was tightly matched with 2 controls for date and place of birth, for birth weight, and parental origin. Detailed questionnaires on parental exposure were filled after informed consent was obtained. 151 cord bloods (67 cryptorchid, 84 matched controls) and 125 colostrums (56 for cryptorchid and 69 for controls) specimen were available for xenobiotics measurements, including DDE, PCBs, and dibutylphthalate (and its metabolite monobutylphthalate–mBP). We established scores of exposure in milk and studied the possible correlations of exposure with cryptorchidism.

**Results:** 102 boys had congenital cryptorchidism (1.63%) with 83% of cases unilateral. 95 cases and 188 controls were included in this study. Incidence at 3 and 12 months was 0.8% ( $n = 50$ ). Cryptorchidism was associated to micropenis, but not hypospadias. Risk factors included prematurity (<37 weeks), paternal history of cryptorchidism, instrumental maneuver at delivery.

Median concentrations of selected chemicals in colostrums were higher though not significantly in cryptorchid vs controls, except for PCB 138 (e.g. DDE 119.4 vs 80 ng/g of fat, ?PCB 206.3 vs 166.8 ng/g of fat). Cryptorchid boys were more likely to be classified in the most contaminated groups for PCBs (57.1% vs 39.1%,  $P = 0.045$ ), DDE (53.6 vs 36.2%,  $P = 0.037$ ), and to a lesser degree mBP (58.1% vs 40%,  $P = 0.13$ ). This was also true for the composite score DDE+PCBs (30.4% vs 21.7%,  $P = 0.05$ ). Last, the odds ratio for cryptorchidism at birth was increased for the highest score of DDE: 2.03 ( $P = 0.05$ ; 95% CI 0.99–4.17), ?PCB 2.07 ( $P = 0.046$ , 95% CI 1.01–4.25, & composite score without phthalates 2.41 ( $P = 0.06$ , 95% CI 0.96–6.1) vs lowest score of those components.

**Conclusions:** our results support an association between fetal exposure to DDE, PCBs and possibly mBP, and the occurrence of congenital cryptorchidism at birth (1.6%). Higher concentrations in milk could be a marker of higher exposure or for an impaired detoxification pattern in genetically predisposed individuals.

**O-187 Oral** DNA fragmentation in morphologically normal human spermatozoa from teratozoospermic patients

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**Introduction:** The presence of a high percentage of spermatozoa with DNA damage may have a negative effect on the outcome of assisted reproductive

technologies. ICSI bypasses natural sperm selection processes, as the embryologist subjectively chooses the spermatozoon to be injected into the oocyte based on its motility and morphology appearance. However, these selection criteria will not exclude, for example, the presence of a chromosomal abnormality. The exclusion of spermatozoa with nuclear defects can thus be expected to decrease the probability of accidental injection of a DNA-damaged spermatozoon into the oocyte. The objective of this study was to evaluate the presence of DNA fragmentation in spermatozoa with normal morphology obtained from the separated fractions of highly motile sperm, as these are the cells that with high probability will be chosen by the embryologist at the time of oocyte injection for ICSI.

**Materials and methods:** Ejaculates from fertile donors ( $n = 4$ ) and infertile patients with moderate and severe teratozoospermia ( $n = 10$ ) were studied. Purified populations of highly motile sperm were obtained by swim-up. Sperm DNA fragmentation was evaluated by Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-fluorescein nick end labeling (TUNEL), using In Situ Cell Death Detection Kit, Fluorescein. Simultaneous examination of normal sperm morphology (fixed wet preparation without staining) and DNA fragmentation in the same cell were performing using phase contrast and TUNEL respectively. A total of 400 cells were evaluated in two droplets per patient. During this examination, every time a spermatozoon with normal morphology was found, the light was immediately switched to the fluorescence to determine DNA integrity. Spermatozoa were considered normal when the head had a normal shape, a symmetrical and oval head configuration, vacuoles occupying less than 20% of the head area, an acrosomal region comprising 40–70% of the head area, a symmetrical insertion of the tail, and absence of midpiece or neck defects.

**Results:** The proportion of TUNEL positive cells was  $3.9\% \pm 2.9$  (mean  $\pm$  SD) for the fertile group and  $21.2\% \pm 13.4$  for the infertile group. There was a significant difference between groups ( $P < 0.05$ ). The percentages of normal sperm morphology in the fixed wet preparations without staining examined under phase contrast were: fertile group,  $7.5\% \pm 0.6$  and infertile group  $1.0\% \pm 0.3$  ( $P < 0.05$ ). Next, the spermatozoa with normal morphology were examined with fluorescence for TUNEL analysis. No DNA fragmentation was found in spermatozoa with normal morphology in any of the samples from the fertile men. However, in all the samples from the patients in the infertile between 20% to 66.6% of sperm with normal morphology presented DNA fragmentation ( $P < 0.05$ ).

**Conclusions:** Spermatozoa from infertile men with moderate and severe teratozoospermia, with apparently normal morphology and recovered from the motile fractions after swim-up may have DNA fragmentation. Results pose a question on the use of “normal morphology” alone as a reliable attribute for the selection of sperm for ICSI in this group of patients. Ideally, an evaluation of sperm morphology and DNA fragmentation in the same cell should be performed in all patients will be undergoing ICSI, in order to increase the fertility outcome and prevent or reduce the risk of inducing genetic alteration in the offspring.

**O-188 Oral** Comparison of semen samples from normal and muscular dystrophy affected animal models by high throughput fluorescence activated cell sorting

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**Introduction:** Duchenne's muscular dystrophy (DMD) is the most common human muscular affection with high frequency in male individuals. The condition is characterized by progressive muscle degeneration, weakness and loss of motion capacity. The signs of DMD start around 3 to 6 years, by the age of 12 years almost all patients are unable to walk and the muscle deterioration becomes fatal for most of the affected individuals during their second decade of life. However, life expectancy is increasing due to technical improvements in patient care. The increase of lifespan raises new questions ranging from ethical to physiological issues never observed before. Individuals affected by DMD may manifest their interest for parenthood. In such cases, the function of the reproductive organs and fertility should be known to assist infertility practitioners to choose the most adequate treatments. Animal models are

required to address these questions. Golden Retriever's muscular dystrophy (GRMD) is a natural experimental model of DMD, since the dysfunction of the dystrophin is involved in the pathogenesis shared by the two conditions. Thus, this study aimed to compare the spermogram and the mitochondrial activity and acrosomal integrity by a high throughput fluorescence activated cell sorting (FACS) of non-affected and GRMD affected Golden Retrievers.

**Materials and methods:** Twenty three ejaculates from 4 non-affected (control) aging from 2.0 to 4.5 years old and 5 affected Golden Retrievers (GRMD) aging from 1.5 to 4.5 years old were obtained by manipulations in three different occasions. Semen samples were processed by two experienced theriogenologists and initial analysis comprised sperm concentration, percent of straightforward motility and morphology. Samples were diluted to a final concentration of  $10^6$  spermatozoa/mL prior incubation with each fluorescence probe. Acrosome integrity assessment was conducted with *Pisum sativum* lecithin conjugated to fluorescein-isothiocyanate (PSA-FITC; 100  $\mu$ g/mL) for 10 minutes and mitochondrial activity was assessed by JC-1 (50  $\mu$ g/mL) for 10 minutes. Fluorescence activated cell sorting was performed in  $10^3$  spermatozoa, from a pre-selected gate which contained only these cells. Acrosome integrity was evaluated by the geometric mean of green fluorescence of the histogram of sperm fluorescence after incubation with PSA-FITC. Similarly, mitochondrial activity was assessed by the geometric mean of green fluorescence emitted by JC-1 dimeric molecules produced during high mitochondrial activity. Data were checked for non-affected distribution and homoscedasticity by Kolgomov-Smirnov test and F test for equal variances. Comparisons between the two groups were made by Student's *t* test.

**Results:** The volume of the ejaculate (control =  $1.12 \pm 0.11$  mL and GRMD =  $1.16 \pm 0.26$  mL;  $P = 0.87$ ), straightforward motility percents (control =  $83.8 \pm 2.3\%$  and GRMD =  $83.9 \pm 1.81\%$ ;  $P = 0.98$ ), and percent of sperm defects (control =  $11.6 \pm 2.1\%$  and GRMD =  $13 \pm 1.4\%$ ;  $P = 0.58$ ) were similar between non-affected and affected dogs. However, the sperm concentration was significantly higher in dogs affected by GRMD ( $714 \pm 128 \times 10^6$  spermatozoa/mL) in comparison to non-affected dogs ( $311 \pm 116 \times 10^6$  spermatozoa/mL;  $P = 0.03$ ). FACS analysis of semen samples from non-affected and affected dogs revealed no differences in acrosomal integrity (control =  $108 \pm 391$  and GRMD =  $143 \pm 88$ ;  $P = 0.71$ ) and mitochondrial activity (control =  $67.3 \pm 21$  and GRMD =  $77.5 \pm 5.4$ ;  $P = 0.1$ ).

**Conclusions:** Muscular dystrophy is a debilitating disease, but it does not seem to affect the production and quality of dog semen. This animal model suggests that ejaculates of DMD men can be obtained by the proper stimulation, and that the conventional *in vitro* fertilization techniques combined with pre-implantation genetic diagnosis for single gene disorders may be efficient to satisfy the desire of paternity of DMD patients with the birth of non-affected children.

### O-189 Oral The fate of paternal histones in zygotes

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**Introduction:** About 15% to 30% of the DNA in human sperm is packed in nucleosomes and transmission of this fraction (containing histone variants and histone modifications) to the embryo potentially serves as a mechanism to facilitate paternal epigenetic programs during embryonic development. However, until now it has not been established whether these nucleosomes are removed like the protamines or indeed contribute to paternal zygotic chromatin.

**Materials and methods:** We evaluated the presence of histone H3 variants in *in vitro* decondensed human spermatozoa and in paternal chromatin of zygotes obtained after heterologous ICSI (injection of human sperm into mouse oocytes). Decondensation of the sperm head in the oocyte and formation of the paternal pronucleus (PN) was observed in 60 injected oocytes. Presence of histone H3 and the H3.1/H3.2 DNA replication dependent variants was evaluated with *in situ* immunofluorescence (IF).

To clarify the fate of sperm-derived nucleosomes we have used the deposition characteristics of histone H3 variants; the H3.1/H3.2 replication variants present in the paternal PN prior to S-phase are derived from sperm. After sperm entry, the maternal nucleosome forming machinery utilizes the H3.3 variant to remodel paternal chromatin. Using a monoclonal antibody (mAb) against histone H3.1/H3.2 and a polyclonal antibody Pan-H3, we evaluated the

presence of nucleosomes containing these histone variants in (a) *in vitro* decondensed sperm, (b) after sperm penetration in a heterologous mouse-human ICSI system and (c) in abnormally fertilized human zygotes.

**Results:** IF with the highly specific mAb for H3.1/H3.2 showed a positive signal in 97% of decondensed human spermatozoa. After heterologous ICSI, zygotes were incubated for different periods of time (75 to 370 min) before fixation. Using the same mAb, a positive fluorescent signal was consistently detected at all intervals, from the early decondensing sperm head (75 minutes post-injection) up to the pronuclear (PN) stage prior to the S-phase (370 minutes post-injection). In addition, staining of human multipronuclear (3 PN) zygotes revealed an identical localisation of H3.1/H3.2 as in the heterologous zygotes. An increase of the H3.1/H3.2 replication variants was observed in the paternal chromatin comparing pre-S-phase with post-S-phase human zygotes.

**Conclusions:** These findings reveal that sperm-derived nucleosomal chromatin contributes to paternal zygotic chromatin. Hence, the execution of epigenetic programs originating from transmitted paternal chromatin during subsequent embryonic development is likely a consequence of this observation.

## SELECTED ORAL COMMUNICATION SESSION

### Session 50: Endometrium - Contraception

08 July 2008

17:00–18:00

#### O-190 Oral EGF regulates the proliferative factor CYR61 via an activation of STAT3 proteins in endometrial cells

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**Introduction:** CYR61 (cysteine-rich protein 61, CCN1) is a growth factor that stimulates angiogenesis and tumorigenesis. A recent study identified murine Cyr61 in the luminal epithelium surrounding the implanting blastocyst suggesting an involvement of this protein in implantation. In the human endometrium, CYR61 expression is regulated during the menstrual cycle with a peak in the proliferative phase and during menstruation. Since the epidermal growth factor (EGF) is implicated in the proliferative phase, we aimed to investigate its possible involvement in CYR61 regulation. EGF can activate the EGFR/MAPK I-pathway or the signal transducers and activators of transcription proteins (STATs). Here, we identified the EGF mediated signalling pathways resulting in CYR61 mRNA and protein regulation in human endometrial cells.

**Materials and methods:** The spontaneously immortalized endometrial epithelial cell line HES express both estrogen receptors (ER $\alpha$  and ER $\beta$ ), the EGF receptor and CYR61. The cells were treated with EGF (20 ng/ml), the typhostins AG1478 and AG490, the MEK inhibitor PD98059 as well as with a specific STAT3 inhibitor. Knock down studies applied a transient transformation with 10nM CYR61 siRNA. The expression of CYR61 was determined by quantitative RT-PCR and western blot analysis. The mRNA and protein levels were normalised to the expression levels of the house keeping  $\beta$ -actin. Cellular proliferation was measured using a cell counter and additional by BrdU labelling and ELISA.

**Results:** Like in the uterus, EGF mediates the proliferation of the HES cells. Treatment with EGF, leading to phosphorylation and internalisation of the EGF receptor, showed significant induction of CYR61 expression already after 30 minutes. However, an inhibition of C-terminal EGFR phosphorylation by AG1478 did not reduce CYR61 levels. Next, we studied the EGF induced MAP-kinase/ERK pathway. Surprisingly, the HES-cells revealed an upregulation of CYR61 mRNA expression after inhibition of the MAPK/ERK pathway upon EGF treatment. However, simultaneous application of EGF with the inhibitors AG490 and a specific STAT3 inhibitor, respectively, revealed that the EGF signalling pathway was mediated by JAK2/STAT3. To proof, if the induction of cellular proliferation via EGF is mediated by an increase of CYR61, we performed CYR61 siRNA studies. Application of CYR61 siRNA resulted in 50% decrease of proliferation.

**Conclusions:** We identified CYR61 as a proliferative factor and downstream of STAT3 in endometrial cells. EGF mediated CYR61 upregulation in HES cells