immature immune system. Using the cow as a model, we hypothesise that LPS found in follicular fluid can initiate an inflammatory response by TLR4 on granulosa cells, altering the follicular environment and ultimately perturb the meiotic competence of oocytes.

**Material and Methods:** Granulosa cells and cumulus-oocyte complexes (COCs) were isolated from healthy cow ovaries following aspiration of 4-8 mm follicles. Granulosa cells were challenged with a range of pathogen associated molecular patterns (PAMPs) to determine their ability to signal through various TLRs and initiate an inflammatory response. Granulosa cells were incubated in the presence of 10-fold increasing concentrations of ultrapure LPS (TLR4 ligand), lipoteichoic acid (LTA; TLR2 ligand), peptidoglycan (PGN; TLR2 ligand) or Pam3CSK4 (PAM; TLR1 and 2 ligand) ranging between 100 pg/ml and 10 μg/ml. The pro-inflammatory cytokine IL-6 and the chemokine IL-8 were measured in cell-free supernatants by ELISA. Acute responses to LPS were evaluated by qPCR for IL6 and IL8, while the phosphorylation of ERK 1/2 and p38-MAPK was assessed by immunoblot. TLR4 specific siRNA was used to demonstrate the importance of TLR4 in LPS-initiated inflammation. In vitro maturation (IVM) of intact COCs was performed using defined media in the presence of LPS. Subsequently, meiotic progression of oocytes was assessed by confocal microscopy preceded by immuno-histochemistry to label meiotic spindle structures. Oocytes which failed to reach the M-phase of meiosis II, or those which had significantly perturbed meiotic structures following IVM were deemed to have failed meiosis.

**Results:** Granulosa cells accumulated IL-6 and IL-8 in a dose dependent manner in response to LPS, LTA, PGN and PAM compared to untreated controls (P < 0.05). The accumulation of IL-6 and IL-8 was further increased following 48 h of culture for each PAMP (P < 0.05). IL6 and IL8 mRNA was significantly increased in cells following only 30 min exposure to 1 μg/ml of LPS compared to untreated controls (2.1- and 2.8-fold, respectively) and remained elevated at 180 min (2.5- and 5.3-fold, respectively; P < 0.05). Ununtreated cells showed little or no initial phosphorylation of p38-MAPK or ERK1/2, however following a 30 min treatment with 1 μg/ml of LPS, phosphorylation of both ERK1/2 and p38-MAPK was increased and maintained up to 180 min. After 24 h transfection with TLR4-siRNA granulosa cells had a 52.4% reduction in accumulation of IL-6 in response to 24 h LPS exposure compared to untreated controls (P < 0.05). LPS increased the expansion rate of COCs in the absence of FSH (0% vs 24%, P < 0.05), possibly due to increased IL-6 production compared to untreated controls (4.1-fold increase, P < 0.05). Meiotic progression of 290 COCs was assessed to ascertain the impact of LPS exposure. Untreated COCs had a meiotic failure rate of only 14.0%, however, the addition of 10μg/ml of LPS compared to untreated controls (2.1- and 2.8-fold, respectively) and remained elevated at 48 h of culture for each PAMP (P < 0.05).

**Conclusions:** For the first time these data indicate that bovine granulosa cells initiate an inflammatory response to LPS via the TLR4 pathway. Consequently, perturbations to the follicular environment adversely affect the developing oocyte and its meiotic competence. We suggest that reproductive tract infections negatively impact the developing cohort of oocytes.

**P-323 NK cells in IVF treated patients**

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**Introduction:** Decreasing fertility of endemic population represents a serious socio-economic problem in almost all developed countries and elicits an increasing effort in the diagnosis and treatment of fertility disorders. Much credit has been given for the technique of in vitro fertilization with subsequent transfer of the embryo (IVF-ET). Unfortunately, the success rate of this method is limited by low implantation rate (slightly over 30%). Consequently, the attention of investigators now switches from the technique of IVF towards the questions of embryo implantation. It follows from the very nature of implantation that the crucial factor of materno-fetal interaction is immunity. Approximately 70% of lymphocytes residing in the pregnant decidua are NK cells. These are phenotypically distinct from peripheral blood NK cells in expression of extremely high levels of CD56. Proposed functions of NK cells in the gravid (nebo pregnant?) uterus are: production of cytokines to facilitate decidualization and control of the invading trophoblast protection of fetal tissues from maternal immune attack and protection of the fetus from infectious diseases.

**Methods:** We analyzed the peripheral blood and the follicular fluid of patients from IVF center of the Institute for the Care of Mother and Child, Prague. The cohort consists of patients prepared for IVF-ET. Using FACS assay we compared successful vs unsuccessful IVF patients’ cell populations: CD56+ /CD16- (NK – cytokine producing cells) CD56+/CD16+ (NK – cytotoxic cells) and CD161/NKG-2D expression (activation of these cells) along with standard CD3/CD4/CD8 panel for T cells. We focused our attention on KIR2DL4 and CD85j (ILT2, LIR) expression, because these markers are associated with functional attenuation.

**Results and Discussion:** We observed increased surface expression (mean fluorescence intensity) of KIR2DL4 on cytotoxic NK cells (CD56+/CD16+) in follicular fluids of successfully fertilized patients. Increased KIR2DL4 expression on these cells was also observed in their activated variants (CD56+/CD16+/CD161/NKG-2D). Peripheral blood exerted increased CD85j surface expression on T helper cells (CD3+/CD4+/CD8+), bearing NK cell activation marker NKG-2D in the case of successfully fertilized patients.

**Conclusions:** These results indicate the important role of KIR2DL4 mediated attenuation of NK cell function in the scope of IVF success rate. The higher CD85j (ILT2 or LIR) expression on T helper cells may have contributed to overall immune attenuation and IVF success.

**P-324 Distinct expression pattern of Dicer1 correlates with ovari- derived steroid hormone receptor expression in human fallopian tubes**

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**Context:** Tissue-specific dicer1 knockout mice display severe, irreversible Fallopian tube damage and disruption of tubal transport. It is not known how Dicer1 affects human Fallopian tube function.

**Objective:** To investigate regulation of tubal Dicer1 expression during ovulation and the mid-secretory phase and Dicer1-associated alterations in estrogen receptor (ER) subtype and progesterone receptor (PR) expression.

**Design:** Fallopian tissue was obtained from patients at early (n = 4), late (n = 4), and postovulatory (n = 5) phases and the mid-secretory phase (n = 4). Serum was obtained immediately before surgery (sterilization or hysterectomy) to confirm ovulatory and mid-secretory phases. Localization and regulation of Dicer1, ER subtypes, and PR isoforms were determined by immunofluorescence, confocal microscopy, and quantitative RT-PCR.

**Results:** Dicer1 protein was expressed most abundantly in Fallopian epithelial cells; mRNA and protein levels peaked in the late ovulatory phase. ER subtype and PR isoform mRNA levels were not related to ovulatory stages; however, ERβ1 and ERβ2 mRNA/protein levels were highest, and PRA/B and PRB mRNA/protein levels were lowest, in the mid-secretory phase. Dicer1 mRNA expression correlated positively with ERα mRNA expression in the late ovulatory phase and negatively with ERβ2 mRNA expression in the mid-secretory phase and PRB mRNA in the early ovulatory phase.

**Conclusion:** Dicer1 expression is upregulated in cell-specific fashion in human Fallopian tubes during ovulation. The stage-dependent expression of Dicer1 affects human Fallopian tube function.

**P-325 Levonorgestrel plasma levels in patients with regular and prolonged use of the levonorgestrel-releasing intrauterine system (Mirena)**

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**Introduction:** Levonorgestrel (Mirena) is a synthetic progestogen and the active component of the copper-releasing intrauterine system (COCs) and levonorgestrel-releasing intrauterine system (LNG-IUS). The mechanism of action is likely a result of local endometrial and myometrial progestin exposure. This study was performed to assess the long-term plasma concentrations of levonorgestrel in patients with regular and prolonged use of the levonorgestrel-releasing intrauterine system.
Conclusions: The levonorgestrel-releasing intrauterine system IUS (Mirena®) is well accepted as an easy to use contraceptive with an excellent side effect profile. It contains a reservoir of 52 mg levonorgestrel (LNG) with continuous release of the steroid. Its contraceptive use is approved for five years. The aim of this study was to determine the plasma concentration of LNG and its variation with time in patients with in-dwelling Mirena.

Material and Methods: In this study we determined LNG plasma concentrations in 110 women with Mirena IUS at different time-points of use. Time from insertion of the system in the study population ranged from 20 days to 11.1 years. Quantitative LNG levels were determined using a validated liquid chromatography-tandem mass spectrometry assay.

Results: The mean ± SD LNG plasma level in all women was 147 ± 59 pg/ml. A highly significant negative correlation between LNG plasma level and Mirena time-of-use could be demonstrated. In the first year of use, LNG plasma level was as high as 191 ± 71 pg/ml, decreasing to 157 ± 68 pg/ml in the second year and 134 ± 41 pg/ml in the third year. Even after exceeding the recommended period of Mirena use, systemic LNG concentrations were detectable: 133 ± 38 pg/ml in sixth year, 133 ± 48 pg/ml in seventh year and 117 ± 45 pg/ml in eighth year. Furthermore a highly significant negative correlation between LNG plasma level and body mass index could be shown.

Conclusions: Significant systemic LNG concentrations can be found in all patients with Mirena IUS - even after 11 years of use. This data may influence recommendation for duration of Mirena use for contraception and endometrial protection in peri- and postmenopausal women. In any event, independently of duration of use the systemic LNG concentrations are much lower than in other forms of LNG application.

P-327 Pregnancy outcomes and perinatal results of unintended pregnancies following Essure® sterilization: descriptive study of 10 cases

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Introduction: The Essure® permanent birth control method was approved in 2003 for female hysteroscopic sterilization. Many studies suggest that it is a safe and effective method for female contraception. No studies have proven its safety during pregnancy, becoming important when it is used in selected cases for the treatment of hidrosalpinx prior to in vitro fertilization.

Objective: To describe the pregnancy outcomes and perinatal results of women with Essure® device in our hospital.

Material and Method: Descriptive study of pregnancies following Essure® placement in women demanding permanent sterilization. After 4500 procedures performed in our unit, 10 pregnancies in 9 women have been reported. In one patient, 2 pregnancies have been reported with four years difference (she has a unilateral placement due to a migration of one of the microinserts).

Results: 4 pregnancies finished as a first trimester abortion. From the other 6, I is a first trimester miscarriage in the 7th week of gestation. The other five pregnancies were follow-up in our High Risk Pregnancy Unit. No cases of preterm rupture membrane or preterm delivery have been reported. Four pregnancies were spontaneous etiotic delivery at 39 weeks of gestation with weights between 3040 gr till 3570 gr, appgar score 9/10 and ph up to 7.30. The other pregnancy is now on the 27th week with no complications. Neither ectopic pregnancies nor malformations have been diagnosed.

Conclusions: The results of our sample suggest that pregnancies with Essure® microinserts are safe with no adverse outcomes. No cases of ectopic pregnancies have been reported in any series. There is a lack of studies reporting the perinatal results following unintended pregnancies.

P-328 In vivo and in vitro endothelial effects of the oral contraceptive containing 2mg Chlormadinone acetate/0.3 mg Ethinylestradiol

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Introduction: The use of oral contraceptives (OC) is known to increase the risk of arterial and venous thrombosis, especially by promoting a pro-coagulant status. However, few data are available in the literature about the impact of OC on endothelial function. Thus, the objective of the present study was to assess the in vivo and in vitro endothelial effects of the OC containing 2 mg chlormadinone acetate (CMA) and 0.03 mg ethinylestradiol (EE) with in healthy women using this OC.

Methodology: The in vivo study was a prospective clinical investigation involving 40 healthy women, 20 of whom decided to use a non-hormonal contraceptive method (controls) and 20 who decided to use an OC (2 mg CMA/0.03 mg EE) for 12 months. At 6, and 12 months, all women were submitted to anthropometric measurements and to evaluation of systolic arterial pressure (SAP), diastolic arterial pressure (DAP) and of ultrasonographic markers of endothelial function: flow-mediated dilatation (FMD) of the brachial artery, and intima-media-thickness (IMT) and stiffness index (ß) of the common carotid artery. The in vitro study was carried out using immortalized endothelial cells of the ECV304 line. The in vitro endothelial effects were determined on the basis of nitric oxide (NO) synthesis and of protection against oxidative stress induced by hydrogen peroxide (H₂O₂). NO synthesis was determined by flow cytometry and the protective effect of estradiol (E₂) (100, 300 and 600 pg/mL), EE/CMA (20 pg/mL/400 pg/mL and 150 pg/mL/2000 pg/mL), EE (20, 40, 125 and 150 g/mL), and CMA (400 and 2000 pg/mL) against oxidative stress was measured by the 3-(4,5 dimethyl thiazole-2-yl) 2,5 diphenyl tetrazolium bromide (MTT) assay in order to determine cell viability. The concentrations of EE and CMA chosen for the in vitro study were those obtained after ingestion of the contraceptive containing these hormones (highest and lowest serum concentration reached after ingestion).
Results: At baseline, the clinical and echographic variables did not differ significantly between groups. At 6 months there was a significant reduction in DAP among OC users compared to non-users (OC: \(-3.23\pm 6.20 \text{ mmHg}\) vs. Controls: \(+2.71\pm 5.24 \text{ mmHg}\), \(p=0.01\)). This difference continued to be significant at 12 months (OC: \(-1.06\pm 6.75 \text{ mmHg}\) vs. Controls: \(+4.13\pm 8.33 \text{ mmHg}\), \(p=0.01\)). The remaining clinical and echographic variables showed no significant changes. In the in vitro study, only the endothelial cells incubated with \(\text{H}_2\text{O}_2\) in combination with \(\text{E}_6\) (300 and 600 pg/mL) and CMA (400 pg/mL) presented significantly greater viability (\(p<0.0001\)) than the control containing only \(\text{H}_2\text{O}_2\). Regarding NO synthesis, there was a significant increase in NO production by the endothelial cells stimulated with \(\text{E}_6\) (300 and 600 pg/mL), CMA (400 and 2000 pg/mL) and with the EE/CMA combination at concentrations of 20/400 and 150/2000 pg/mL compared to control (C), after 20, 40 and 60 minutes de incubation (\(p<0.001\)). EE alone did not cause any increase in NO synthesis compared to E.

Conclusions: The OC containing the 2mg CMA/0.03mg EE combination did not alter endothelial function compared to women not using hormonal methods over a period of 12 months. CMA proved to be a progestogen with the property of increasing NO release. This effect may explain the reduction in DAP detect ed in the group of OC users. In contrast to estradiol, EE alone did not increase NO synthesis and did not protect the cells from the oxidative stress provoked by hydrogen peroxide.

P-329 Cryopreservation and in vitro development ability of IVM oocytes from gender identity disorder (GID) patients and cancer patients

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Introduction: Recent development of IVM, as well as with oocyte and ovar ian tissue vitrification technologies, afford the possibility of preserving fertility for cancer patients or women who need to expand their reproductive lifespan. The purpose of this study was to learn the prospect for normal development of immature oocytes from the ovary. We endeavored to measure the number of normal immature (GV stage) oocytes, the maturation rate of the oocytes after IVM, the cryopreservability of the IVM oocytes, and the fertilization and in vitro developmental ability of the in vitro matured vitrified oocytes after intra-cytoplasmic sperm injection (ICSI) and in vitro culture (IVC).

Material and Methods: Oocytes were aspirated from discarded ovaries of pa tients with reproductive cancer (RC) and gender identity disorder (GID) with informed consent and IRB approval. The number and percent of total and nor mal oocytes from RC and GID patients were analyzed. IVM was performed by culture of oocytes using m-199 medium containing 20% SSS for 24 hrs at 37°C. IVM oocytes were vitrified by the Cryotop method (Kuwayama, 2005) and post-thaw viability was assessed. After warming, vitrified oocytes were then inseminated by conventional ICSI method. Fertilized oocytes (2PN) were cultured first in cleavage and then in blastocyst media for 5 days. In vitro maturation, fertilization, cleavage (4-8 cells) and blastocyst rates of all groups were examined and compared.

Results: A total of 607 oocytes were obtained from 25 RC and 25 GID patients. Post-thaw survival rate of IVM oocytes after vitrification was 85%. In vitro maturation, fertilization, cleavage (4-8 cells) and blastocyst formation rates of vitrified IVM oocytes from RC and GID patients were 54%, 50%, and 80%, respectively.

Conclusions: Transferable blastocysts were obtained from GV oocytes of ovari ies of RC and GID patients using our present IVM-ICSI-IVC system after cryopreservation by vitrification. The favorable results indicate the possibility of preserving fertility for reproductive cancer patients who lose their ovaries during cancer treatment by using cryopreserved oocytes from ovarian tissue, and also indicates the possible use of oocytes from otherwise discarded ova ries of GID women, as precious and useful as experimental material for study ing IVM. With ovarian tissue vitrification and IVM, even cancer patients with metastatic ovarian disease could have safe and effective preservation of their fertility.

P-330 Cryo aliquoting by bead vitrification

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Introduction: The presence of hypospermia at times severe is a compounding factor of male infertility that often results in a small amount of cryopreserved specimen in a single aliquot. The availability of ICSI allows a small volume of semen sample to adequately inseminate an oocyte cohort. Often shaving a layer off the frozen specimen is carried out to avoid wastage of the entire sample in severe hypospermia, pre-cancer treatment, deceased individuals, or rare donors where only one vial is available. Moreover, surgically retrieved specimens are cryopreserved in several aliquots but with inconsistent quality. Here the same procedure is carried out in order to identify the vial of higher specimen quality. This approach is carried out on the surface of the frozen sample can provide misleading information while representing a risk of partial thawing of the entire specimen.

In this study, we attempted to vitrify sperm in small CP beads and stored in a closed system.

Material and Methods: Specimens (n = 16) with normal parameters were donated from consenting men. Each sample was equally distributed among three categories: semen suspension mixed with v/v dilution of CP (eCP) or higher proportion of CP (hCP). Each sample was either cryopreserved in vapor at -80°C (30min) before plunging into LN2, or vitrified by dropping 10μl of specimen on a LN2 pre-cooled glass slide. Sperm beads were transferred to a pre-cooled vial and plunged into LN2. Control fractions were thawed by exposing vials to room temperature. Warming of cryobeads was carried out by picking up an individual bead with a pair of pre-cooled tweezers and placing it on a pre-warmed (37°C) Makler.

Results: From December 2003 to October 2010, 1,683 semen specimens with an average density of 52.7 ± 38 ± 10^6/ml, motility 52.3 ± 12%, and 3.7 ± 3% morphology were cryopreserved in standard fashion. An average post-thaw motility of 26.1 ± 15% was obtained representing a loss of about 50% loss of motile cells.

The study samples had a mean density of 55.2 ± 37 ± 10^6/ml, motility 52.2 ± 9%, and 5.2 ± 3% normal morphology. Beads cryopreserved in standard fashion yielded a post-thaw motility with eCP of 30.0 ± 14%, while with hCP 32.0 ± 13%. When cryobeads were warmed after vitrification, the post-motility with eCP was 28.4 ± 7% and with hCP 25.0 ± 10%. The decrease in progressive motility was also comparable among the two cryobead methods and with the control (45.6% vs 52.1% for eCP and hCP, respectively).

Conclusions: Bead vitrification was unrelated to the CP concentration and retained motility characteristics similar to the control. Vitrification in a closed system remarkably shortened cryopreservation time, allow multiple usage of the same vial without risk of partial thawing. Single bead assessment allowed to pick the best cryo aliquot among the same specimen.