Results: Here, we report that cultured spermatogonia respond to extracellular ATP (1 − 100 μM). ATP−induced currents show fast activation and moderate desensitization. The current−voltage relationship reveals strong inward rectification. Current potentiation by ivermectin and inhibition by an acidic extracellular pH (6.3) and extracellular copper (100μM) indicate a functional role of P2X4 receptors. Accordingly, knockdown of P2X4R expression by RNA interference significantly reduced currents activated by ATP concentrations ≤ 300 μM. Interestingly, an increased ATP concentration (>300 μM) activated an additional current with different kinetics. A similar current could be activated by 300 μM 3'−O−(4−Benzoyl)benzoyl ATP (BzATP). Knockdown of P2X7R expression decreased the current activated by higher ATP concentrations (>300 μM). Combined with molecular evidence, our results indicate that at least two different of P2X receptor subunits (P2X7R and P2X4R) are functionally expressed in spermatogonia of young prepubescent mice. Downstream of P2X receptor activation, we found a slowly activating calcium−dependent potassium current functionally antagonizing the depolarizing P2XR−mediated current.
Conclusion: To confirm these results in situ, we established a new experimental approach. Using acute tissue slices of prepubescent mouse testis we electrophysiologically analyzed spermatogonia and found ATP−induced currents with similar characteristics. Together, these data represent a first important step towards a deeper understanding of cellular purinergic communication during spermatogenesis.

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FURTHER CONFIRMATION OF SEVERAL IMPORTANT TARGETS OF SUMOYLATION IN TESTICULAR CELLS

Yuxuan Xiao, PhD[1], Daniel Pollack, BsC[1], Avi Levy[1], Miriam Andrusier[1] and Margarita Vigodner, PhD[2]
[1]Yeshiva University; [2]Yeshiva University and AECOM, New York.
(Presented By: Margarita Vigodner, PhD)

Introduction: Sumoylation (a covalent modification by Small Ubiquitin−like Modifiers or SUMO proteins) has emerged as a critical regulatory event in cell function and has been implicated in various diseases; however, its role in reproduction is largely unknown.
Methods: In a previous study in our laboratory, using the STAPUT separation technique based on a gravity sedimentation followed by immunoprecipitation with SUMO antibody and mass spectrometry analysis, multiple SUMO targets were identified in meiotic spermatocytes and round spermatids. The identified targets of sumoylation included proteins involved in regulation of transcription, metabolism and stress response. Several specific targets with an important role in germ cells were chosen for further characterization.
Results: Co−Immunoprecipitation analysis confirmed sumoylation of CDC2 and CDC5l, the large subunit of RNA Polymerase II, Piwil2, MDC1 and several other proteins with an important role in regulating spermatogenesis.
Conclusion: Bioinformatic analysis revealed the presence of one or several consensus sequences for sumoylation in the majority of the studied targets.

Monday, April 7, 2014
11:00 a.m. - 12:30 p.m.

Poster Session II*
**Not CME Accredited*
Location: Venetian

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THE TRANSCRIPTION FACTOR SOX9 IS A NOVEL REGULATOR OF STEROIDOGENIC GENES EXPRESSION IN MA−10 LEYDIG CELLS

David Landry, BSc and Luc J. Martin, PhD
Université de Moncton
(Presented By: David Landry, BSc)

Introduction and Objectives: Sox genes encode a family of transcription factors characterized by a HMG box, which can bind and bend DNA through the consensus sequence (A/T)(A/T)CAA(A/T)G. Two members, Sry and Sox9, play important roles in male sex determination and differentiation in mammals. Leydig cells are essential for testosterone production in the testis. In these cells, the StAR protein allows cholesterol to enter the mitochondria and be converted to pregnenolone by the first steroidogenic enzyme Cyp11a1. Of the 20 Sox family members identified in vertebrates, several are expressed in gonads, including adult Leydig cells. Sox9 is expressed in steroidogenic cell lines, including MA−10 and R2C Leydig and Y1 adrenal cells. Interestingly, potential DNA regulatory elements for Sox members are present in promoter regions of steroidogenic genes, supporting that Sox9 might be involved in the regulation of steroidogenesis in Leydig cells. Our objective was to determine whether Sox9 regulates StAR and Cyp11a1 in Leydig cells and to better define its mechanism of action.
Methods: Mouse MA−10 Leydig cells were used in transfection and were harvested for total protein and total mRNA extractions. Protein quantifications were done by Western blot, whereas mRNA levels were determined by qPCR. Characterizations of Sox−dependent promoter activities of steroidogenic genes were done by transient transfections of MA−10 cells with StAR or Cyp11a1 promoter constructs and electrophoretic mobility shift assays (EMSA).
Results: Multiple potential Sox−dependent regulatory elements have been found in −1kb promoter regions for StAR and Cyp11a1, and these promoter constructs were activated 3 and 14 folds, respectively, by Sox9. Interestingly, PKA−dependent phosphorylation of Sox9 consistently reduced its transcriptional activity, as shown using transfection of a constitutively active PKA expression plasmid or 8Bromo−cAMP stimulations. Using 5' progressive deletion constructs for StAR (−843, −680, −515, −355, −72 bp) and Cyp11a1 (−888, −633, −427, −262 bp) promoters, regions important for Sox9−dependent activations were located between −680 and −515 bp for StAR and −88 and −633 bp for Cyp11a1.
Conclusion: Thus, our data identify Sox9 as a new regulator of steroidogenic genes expressions in Leydig cells. Future work will focus on post−translational modifications and protein−protein interactions involved in modulation of the transcriptional activity of Sox9 in Leydig cells.

ABSTRACTS

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EFFECTS OF METHOXYCHLOR AND ITS METABOLITE 2,2−BIS(P−HYDROXYPHENYL)−1,1,1−TRICHLOROETHANE ON HUMAN AND RAT 17A−HYDROXYLASE/17,20−LYASE ACTIVITY

Leping Ye, MD[1], Ren−Shan Ge, PhD[1] and Hui Li, MD, PhD[2]
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(Presented By: Leping Ye, MD)

Introduction: Exposure to methoxychlor, an agricultural pesticide, has been associated with reduced testicular androgen secretion. However, methoxychlor is converted to 2,2−bis−(p−hydroxyphenyl)−1,1,1−trichloroethane (HPTE) in the liver, which then acts as its biologically active metabolite. Both methoxychlor and HPTE have been credited with estrogenic properties and have a weak anti−androgenic activity. However, the exact mechanisms of steroidogenic enzyme inhibition remain to be clarified.
Methods: In the present study, human and rat testis microsomes were employed to investigate the inhibitory activities of methoxychlor and HPTE on 17α−hydroxylase/17,20−lyase (CYP17A1) activity. The CYP17A1 enzyme is critical for androgen biosynthesis and catalyzes conversion of progesterone into androstenedione.
Results: The results demonstrated that HPTE directly inhibited human and rat CYP17A1 activity, while methoxychlor had no effects on enzyme activity even at a concentration of 100 μM. The IC50 values of CYP17A1 for HPTE inhibition were 1.13 ï‚± 0.10 μM (human) and 6.87 ï‚± 0.13 μM (rat), respectively. When HPTE was incubated with intact rat immature Leydig cells, it also inhibited CYP17A1 activity with an IC50 value of 6.29 ï‚± 0.1 μM. Results of enzyme inhibition studies were supported by the observation that HPTE inhibited luteinizing hormone−stimulated 5α−androstane−3α, 17β−diol and testosterone secretion by immature Leydig cells with IC50 values of 6.61 ï‚± 0.03 and 3.78 ï‚± 0.003 μM, respectively.
Conclusion: The mode of action of HPTE on CYP17A1 activity was determined to be uncompetitive with the substrate progesterone. The reported suppression of androgen secretion by methoxychlor is presumably associated with inhibition of steroidogenic enzyme activity and has implications for endocrine function of the testis.

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EXPRESSIONS OF SOX5 AND SOX13 TRANSCRIPTION FACTORS ARE INCREASED IN TESTICULAR LEYDIG CELLS OF RODENTS DURING POST−NATAL DEVELOPMENT

Mikella A. Daigle, BSc and Luc J. Martin, PhD
Université de Moncton
(Presented By: Mikella A. Daigle, BSc)

Introduction and Objectives: Members of the SRY−related HMG box (Sox) transcription factor family are proteins that have been conserved during the evolution of vertebrates. Sox members are expressed in numerous tissues and regulate a variety of developmental stages. Indeed, Sry upregulates Sox9 during sex determination and testes differentiation of the embryo. In post−natal testes, members of the Sox family, such as Sox5, Sox6, Sox8, Sox9 and Sox17, have been characterized. However, expressions of members of this family of transcription factors have never specifically been shown in adult Leydig cells. These cells supply testosterone necessary for the onset and maintenance of spermatogenesis. The objectives of this research are to locate and determine the expression profiles of two SoxD members, Sox5 and Sox13, in post−natal mice testes at different developmental stages, as well as to identify their expression in rodent Leydig cell cultures.
Methods: mRNA and protein quantifications of Sox5 and Sox13 from whole mice testes at three different ages (33 days, 8 weeks and 7 months) as well as in MA−10, R2C and primary cell cultures stimulated with 8Bromo−cAMP were done using quantitative qPCR and Western Blots, respectively. Immunohistochemistry was used to locate Sox5 and Sox13 protein expressions from whole mice testes at the same three developmental stages.
Results: Sox5 and Sox13 mRNAs and proteins have been characterized in MA−10, R2C and primary cell cultures, as well as from whole testes from 33 days, 8 weeks and 7 months old mice. Their expressions were independent of 8Bromo−cAMP stimulation. Using immunohistochemistry of mice testes, Sox5 and Sox13 expressions were confirmed to be located and to increase according to post−natal development of Leydig cells.
Conclusion: To our knowledge, this is the first study showing the presence of Sox5 and Sox13 transcription factors in adult Leydig cells. These proteins may regulate multiple functions of these cells, such as steroidogenesis important for puberty and spermatogenesis. However, their role and mechanisms of actions in post−natal testes remain to be investigated.

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DEHYDROEPIANDROSTERONE ANTAGONIZES SURGERY STRESS−INDUCED SUPPRESSION OF TESTOSTERONE PRODUCTION IN MALE RATS

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(Presented By: Han Lin, PhD, MD)

Introduction: Leydig cells secrete the steroid hormone testosterone, which is essential for male fertility and reproductive health. Stress increases the secretion of glucocorticoid (corticosterone, CORT, in rats) that decreases circulating testosterone levels in part through a direct action on receptors (GR) in Leydig cells. Intratesticular CORT level is dependent on oxidative inactivation of glucocorticoid by 11β−hydroxysteroid dehydrogenase 1 (11β−HSD1) in Leydig cells.
Methods: In the present study, we investigated the time−course changes of steroidogenic gene expression levels after acute immobilization stress in rats and the possible mechanism of dehydroepiandrosterone (DHEA) that antagonizes it.
Results: The serum CORT levels were significantly increased after 1, 3 and 6 hours of surgery−induced stress, while the serum testosterone levels were significantly decreased starting at 3 and 6 hours after stress. The 3−hour−surgery stress also decreased Star, Hsd3b1 and Cyp17a1 expression levels. Doses of 5 and 10 mg/kg DHEA were administered orally to adult Sprague Dawley rats 1 minute before surgery stress, and it antagonized surgery−mediated reduced testosterone level and the expression of Star, Hsd3b1 and Cyp17a1. DHEA was found to modulate 11β−HSD1 activities by increasing its oxidative activity and decreasing its reductive activity thus decreasing the intracellular CORT levels in the Leydig cells.
Conclusion: In conclusion, DHEA protects Leydig cell function from stress via modulating 11β−HSD1 activity.

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IMPROVING THE FERTILITY OF DOG

Gamal El−Amrawi, Professor
Alexandria University
(Presented By: Gamal El−Amrawi, Professor)

Methods: Semen quality of 22 German shepherd dogs was examined for 6 weeks before and after L−Carnitine supplementation (2 gm/dog for 2 weeks). Two ejaculates were collected by digital manipulation from each dog. The first, second and third fractions of the ejaculate were collected into 3 separate plastic test tubes via glass funnels and semen quality were assessed in the second fraction. The dogs were rested for 45 to 75 minutes (63.4±2.5 minutes) before semen collection (second ejaculate) and the same evaluation was repeated. Blood samples were collected from all dogs before semen collection for assessment of LH and testosterone concentrations.
Results: The results revealed that the second ejaculate had significantly lower values than the first one in both occasions. Semen characteristics in both the first and second ejaculates increased after L−carnitine supplementation, although values of the second ejaculates were still lower than the first one. LH and testosterone concentrations increased in the L−carnitine supplemented dogs.
Conclusion: It could be concluded that L−carnitine supplementation increased the libido and the quality of semen ejaculates in dogs.

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PREVALENCE OF BONE DENSITY DEFICIENCIES IN MEN PRESENTING FOR HYPOGONADISM TREATMENT: DO WE NEED TO WORRY?

Igor Sorokin, MD[1], Paul Feustel, PhD[2] and Andrew McCullough, MD[3]
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(Presented By: Igor Sorokin, MD)

Introduction and Objective: Hypogonadism is a known risk factor in men with osteoporosis. The prevalence of hip osteoporosis in men with total testosterone deficiency (<300ng/dL) is 4.3%. Therefore, it is recommended that baseline bone mineral density (BMD) studies be obtained in this population. The urologist is referred a unique population of men with varying durations of hypogonadism in various age groups with extremes of sex hormones. Our objective was to identify the rate of osteopenia and osteoporosis and the predictive risk factors associated with low BMD scans in the selective population that is referred to a Urologist.
Methods: A retrospective review of 95 consecutive patients with clinical hypogonadism (both symptoms and biochemical testosterone deficiency <300ng/dL) had BMD scans performed on a single Dual−energy X−ray Absorptiometry DEXA machine (Hologic 4500). Osteopenia was defined as a femoral neck, total hip, or total spine BMD T−score between −1 and −2.5. Osteoporosis was defined as a BMD T−score of −2.5 or less. Duration of hypogonadism was defined as time from 1st laboratory value noting low testosterone to BMD scan. Median testosterone and estradiol values were obtained from diagnosis of hypogonadism to BMD scans. Univariate and multivariate analysis were performed to determine the predictive risk factors of an abnormal BMD scan.
Results: The mean ± SD age of our cohort was 49.9 ±13.5 years. Median duration of hypogonadism was 10 months. The median initial testosterone at diagnosis and last testosterone before BMD scan was 179 ng/DL and 208 ng/DL, respectively. We found normal BMD in 51/95 patients (54%), osteopenia in 36/95 (38%), and osteoporosis in 8/95 (8%). On univariate analysis, age (OR 1.04, 95% CI 1.01−1.07, p=0.018) and smoking history (OR 5.2, 95% CI 2.107−12.5, p<0.001) were the only 2 significant factors associated with abnormal BMD scans. Sex hormones, Body mass index (BMI), hypertension, diabetes, or duration of hypogonadism were not predictive of abnormal BMD scans.
Conclusion: There is a very high rate of osteopenia and osteoporosis in male patients with hypogonadism referred to a urologist. We found no single testosterone value <300 ng/dL that would be predictive of an abnormal bone scan. This study reiterates the importance of obtaining BMD scans on all male patients with clinical hypogonadism.

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CHRONIC CYCLOPHOSPHAMIDE TREATMENT AFFECTS GENE EXPRESSION IN PACHYTENE SPERMATOCYTES AND ROUND SPERMATIDS

Anne Marie Downey, Barbara Hales, PhD and Bernard Robaire, PhD
McGill University
(Presente By: Anne Marie Downey)

Introduction and Objective: As the numbers of men of reproductive age who survive cancer and wish to father children increase, it is becoming increasingly important to understand the effects of chemotherapy on male germ cells and reproductive outcome. Previous studies from our laboratory have shown that paternal exposure to cyclophosphamide, a chemotherapeutic agent and immunosuppressant, has detrimental effects on sperm quality and progeny outcome. How cyclophosphamide affects the developing germ cells and how they respond to this insult remain unresolved. The purpose of this study is to test the hypothesis that cyclophosphamide affects gene expression in pachytene spermatocytes and round spermatids.
Methods: Adult Sprague−Dawley male rats were gavaged with cyclophosphamide (6 mg/kg) or saline, 6 days/week for 4 weeks. Pachytene spermatocytes (n=5) and round spermatids (n=6) were collected by unit gravity sedimentation using the STA−PUT method. Total RNA was isolated and mRNA expression was profiled using whole genome gene expression microarrays. Data was analyzed with Genespring 12.0 and Pathway Studios software.
Results: In pachytene spermatocytes 252 transcripts were significantly changed by more than 1.5 fold: 97 were up− and 155 down−regulated, compared to controls. In round spermatids, 230 transcripts were significantly changed by more than 1.5 fold: 124 were up− and 106 down−regulated, compared to control. Differential expression of transcripts coding for genes involved in the DNA damage response and the regulation of cell death was observed in both cell types. In pachytene spermatocytes, the expression of 3 genes involved in base and nucleotide excision repair pathways was altered, while in round spermatids, the expression of genes involved in base excision, homology directed and DNA alkylation repair was altered. In pachytene spermatocytes, the expression of many transcripts coding for genes involved in the tumor necrosis factor receptor 1 (TNFR1) pathway was altered. In contrast, transcripts coding for genes involved in the TNFR1 pathway were not affected by drug treatment in the round spermatids.
Conclusion: These results suggest that chronic cyclophosphamide treatment results in different DNA damage and survival responses in pachytene spermatocytes and round spermatids. The altered ability of these cells to respond to DNA damage and survive may lead to damaged mature spermatozoa.
These studies are supported by CIHR.

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ACTION OF RESVERATROL ON THE REPRODUCTIVE PARAMETERS OF LATE PUBERTAL RATS TREATED WITH ANTI−CANCER AGENTS (BEP PROTOCOL MODIFIED), FROM PERIPUBERTY

Flavia Macedo de Oliveira Neves, PhD Student, Vanessa Vendramini Vilela, Collaborator and Sandra Maria Miraglia, Advisor
Federal University of Sao Paulo − UNIFESP − Brazil
(Presented By: Flavia Macedo de Oliveira Neves, PhD Student)

Introduction and Objective: The incidence of testicular cancer in 15 to 35−years old men has increased over the last 50 years. Protocols including the co−administration of bleomycin, etoposide and cisplatin (BEP) have been effective, rising patient survival. However, deleterious effects on the reproductive health of patients have been reported. Resveratrol (R) is an antioxidant fitoalexin that shows anti−apoptotic properties. Our aim is to study the potential protection of resveratrol against the side effects on reproduction caused by the BEP administration from peripuberty.
Methods: From the 36th day post partum (dpp) rats were resveratrol−treated (gavage) with a daily single dose of 300mg/kg per 5 days and subsequently (from 41st dpp on) submitted to co−administration of R and BEP (R−BEP group) applied for three consecutive weeks: etoposide (3.50mg/kg) and cisplatin (0.70mg/kg) for 5 consecutive days/week and bleomycin (0.35mg/kg; every 2nd day of each week); all drugs were injected by intraperitoneal (ip) route. Three other groups were solely treated with: 1− BEP (BEP group), 2− resveratrol (R group), and 3− carboxi−methyl−cellulose (vehicle of resveratrol by gavage) plus saline 0.9% (ip route; SC− Sham Control group). Testis and epididymis biometric parameters, histopathological analysis, morphometric and stereological testicular parameters, spermatic evaluation and sperm mitochondrial activity were investigated. HTM−IVOS motility analyser was utilized.
Results: Rats of BEP and R−BEP groups showed reduction of: body weight, epididymal and testis weights, testis morphometric parameters and germ cell depletion. A higher frequency of sperm anomalous forms in epididymis cauda was observed in the BEP and R−BEP groups. The BEP group presented a higher frequency of TUNEL−positive germ cells and a lower mitochondrial activity when compared to the R−BEP group. Although the sperm motility characteristics were altered in BEP and R−BEP groups, the parameter reflecting sperm flagellar beating was only altered in BEP group. Sex hormone dosage, testis oxidative stress and sperm DNA fragmentation are being investigated. The reduction of apoptosis in germ cells, the improvement of sperm mitochondrial activity and of sperm flagellar beating in R−BEP group point out to a reduction of the reproductive damage caused by BEP treatment.
Conclusion: Additional studies are being performed to better clarify the potential protective action of R against the deleterious effect of this treatment.

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FETAL CYCLOPHOSPHAMIDE EXPOSURE INDUCES TESTICULAR CANCER AND REDUCES SPERMATOGENESIS IN MICE

Gunapala Shetty, PhD, Ana Luiza Drumond, PhD, Paul Comish, MS, Angabin Matin, PhD and Marvin Meistrich, PhD
University of Texas M.D. Anderson Cancer Center
(Presented By: Gunapala Shetty, PhD)

Introduction and Objectives: There has been a 3−fold increase in the incidence of testicular germ cell tumors (TGCTs) and a 50% decline in sperm counts over the past 60 years. Both these adverse outcomes have been suggested to be the results of prenatal exposure to environmental agents. Previously we showed that fetal exposure to radiation induced testicular germ cell tumors (TGCT) in 129.MOLF−congenic−L1 (L1) mice, which are genetically susceptible to testis cancer, and also reduced spermatogenic function in the testes that did not develop cancer.

Methods: Here we tested whether fetal exposure to a gonadotoxic and carcinogenic chemical could also have the same effects in L1 mice and whether it could also induce tumors in standard strains of 129 mice. We chose cyclophosphamide (CP), an alkylating agent, because pregnant women currently being treated for breast cancer are exposed to it. CP was given to pregnant L1 and 129 mice at 7.5 mg/kg on embryonic days 10.5 and 11.5.
Results: The treatments dramatically increased the TGCT incidence to 80% in the male offspring of L1 mice (control value 33%) and to 28% in the offspring of 129 mice (control value, 2%). The weights of testes with tumors in CP−treated L1 mice were higher than those in controls, indicating that treatment induced multiple foci of initiation sites in each testis. Furthermore, in utero CP exposure produced a loss of germ cells as testes weights of both 129 and L1 offspring were significantly reduced to ~70% of the respective controls and atrophic tubules were observed in about 30% of the testes. All the results obtained with CP treatment in both lines of mice are similar to those observed after irradiation.
Conclusions: The results obtained here suggest that i) DNA damage seems to be a common mechanism leading to induction of testicular cancer; ii) the susceptibility to induction of testis cancer by external agents in individuals of different genetic susceptibility is proportional to the spontaneous incidence; and iii) the male fetus of women exposed to DNA damaging chemotherapeutic agents during pregnancy might have reduced spermatogenesis and an increased risk of developing testis cancer.

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EFFECTS OF EUCOMMIAE CORTEX (EC) ON SPERM COUNT AND MOTILITY PARAMETERS IN MALE MICE

Ji Eun Lee, MS, Eun Bit Ko, MS, Jin Soo Kim, PhD, Do Rim Kim, PhD, Ha Young Kim, MS, Byung Chun Park, MS, Bong Jae Choi, PhD, Seong Kyu Park, PhD and Mun Seog Chang, PhD
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(Presented By: Mun Seog Chang, PhD)

Introduction and Objective: The process of sperm cell development is usually represented the spermatogenesis. This process explained as undergoing mature mitotic and meiototic divisions and a metamorphic change (spermatozoa). The cyclic AMP response element modulator (CREM) is a crucial role of the differentiation of round spermatids into mature spermatozoa and the expressions of many important post−meiotic genes. Eucommiae Cortex (EC), a medicinal herb, was widely used to treatment for impotence, male infertility in traditional medicine. The purpose of this study was to investigate the effects of EC on the male reproductive system and the CREM expressions in cyclophosphamide (CP)−induced mouse.
Methods: We performed Real time−PCR and western blot analysis for CREM expression and examined sperm parameters.
Results: CREM mRNA level was analyzed by real time PCR in this study in which100 mg/kg of CP and 500, 1000 mg/kg of EC treated group were significantly down regulated than CP treated group. Also, the protein levels of CP with 100 and 1000 mg/kg of EC treated groups were increased than CP treated group as well, but there was no significance.
Conclusion: Following the result data, this study suggest that Eucommiae Cortex treatment reduce the reproductive toxicity in male reproductive system by increasing CREM gene expression and protein biosynthesis in mouse testis.

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LATE REPRODUCTIVE ANALYSIS OF RAT MALE OFFSPRING EXPOSED TO NICOTINE DURING PREGNANCY AND LACTATION – FINAL PART

Mayra Miranda−Rodrigues, Masters Student, Camila C. Paccola, Collaborator, Samara U. Oliva, Collaborator and Sandra M. Miraglia, Advisor
Federal University of Sao Paulo − UNIFESP − Brazil
(Presented By: Mayra Miranda−Rodrigues, Masters Student)

Introduction and Objectives: Around 1/3 of the world population smokes and 10.4% of pregnant women report smoking during pregnancy in United States. Nicotine (Ni), present in cigarettes, reaches the maternal milk and cross the placental membrane. It inhibits steroidogenesis, suppresses testosterone secretion in adult male rats and causes erection dysfunction, testicular atrophy and infertility. Previously, we observed that Ni, when injected in rats during whole pregnancy and lactation periods, provokes, in the progeny, late morphofunctional alterations of Leydig cell, body weight raise in adulthood (90 days postpartum−dpp) as well as an evident seminiferous epithelium injury. Aiming to investigate whether the spermatogenic damage previously observed in 90dpp progenies from pregnant and lactating Ni−exposed rat dams are maintained or whether it is worsen in older rats, we analyzed the morphological testicular alterations after up to two complete periods of spermatogenesis (53 days each), spermatic parameters and sperm DNA fragmentation.
Methods: Pregnant and lactating rats were Ni−exposed (2mg/Kg/day) through an osmotic minipump implanted at the first day of pregnancy and replaced after birth. Absolute Control (no minipump implanted) and Sham Control (minipump implanted without Ni) groups were established. The offspring was killed at 143dpp and 196dpp.
Results: Significant alterations of morphometric and stereological testicular parameters were not observed in Ni−exposed rats. The testicular histopathological analysis showed small intraepithelial vacuolization and germ cell desquamation in Ni−exposed rats. Testicular concentration of step 19 spermatids, daily sperm production, concentrations and transit time of the sperm in the head/body and cauda of the epididymis did not also show significant changes. The plasmatic and intratesticular levels of cholesterol and testosterone were not significantly changed among the groups in both ages studied. However, the offspring from Ni−exposed dams exhibited a higher frequency of morphologically abnormal sperms as well as lower sperm motility in comparison with both control groups. In addition, the Ni−exposed groups showed a significant reduction of the sperm mitochondrial activity and an increased sperm DNA fragmentation (Comet Assay).
Conclusion: These results indicate a late reproductive damage in the male progenies provoked by maternal Ni−exposure, related to the decrease of the sperm quality.

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MOLECULAR ALTERATIONS IN SPERM ARE SENSITIVE INDICATORS OF TESTICULAR DYSFUNCTION

Linnea Anderson, MSc[1], Edward Derem PhD[2] Susan Huse, PhD[1] and Kim Boekelheide, MD, PhD[1]
[1]Brown University; [2]Rhode Island Hospital
(Presented By: Linnea Anderson, MSc)

Introduction: Traditional endpoints used to measure male reproductive toxicity in humans, including semen and hormone analysis, are insensitive and unreliable; those used to monitor toxicity in animal studies, while sensitive, are not easily translatable to humans. It is therefore necessary to develop sensitive and reliable molecular biomarkers of testicular injury that can be used to both monitor human reproductive function and compare animal studies with human exposures.
Objectives: The aim of this research is to use exposures to model testicular toxicants to identify sperm molecular alterations in rats, and to examine these alterations in sperm from clinically fertile and subfertile men.
Methods: Adult male rats were exposed to cyclophosphamide (CPP) for 12 weeks (1.4, 3.4, or 5.1 mg/kg/day p.o.) or 12 weeks plus an additional post−exposure recovery period of 12 weeks (5.1 mg/kg/day p.o.) as a model of germ cell toxicity. Standard reproductive endpoints were examined to assess testicular injury; in particular, germ cell apoptosis and spermatid head retention were quantified as sensitive markers of damage. mRNA isolated from cauda epididymidal sperm was analyzed for toxicant−induced alterations using a genome−wide microarray, then significant and robust alterations were further examined using qRT−PCR arrays.
Results: CPP produced dose−dependent testicular injury that resolved after a 12−week recovery period. The levels of injury correlated with specific changes in transcript abundance, indicating a utility for these mRNAs as translatable biomarkers for male reproductive dysfunction.
Conclusions: We have previously identified mRNA transcripts that are sensitive to low doses of Sertoli cell toxicants, and have now identified a panel of transcripts that sensitively identifies testicular dysfunction induced by germ cell toxicants. These transcripts will be examined in additional exposure settings, as well as both fertile and subfertile men to continue to validate the relevance of these alterations.

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NETWORK ANALYSIS OF REDOX MEDIATED PROTEIN−PROTEIN INTERACTIONS IN SPERMATOZOA

Burak Özkösem
McGill University
(Presented By: Burak Özkösem)

Introduction and Objectives: Growing evidence suggests that the cellular redox status regulates sperm function and sperm quality. Defective sperm function is the major single defined cause of infertility in humans. Redox imbalance can cause positive responses such as activation and negative responses such as inhibition and deterioration in lipid membrane and DNA packaging in spermatozoa. However, post−translational modifications in proteins are the most abundant damages caused by oxidative stress in spermatozoa. Recently, proteomic studies have started to build the protein expression datasets for human sperm, however, interactions between human sperm signaling pathway proteins and redox status in spermatozoa yet to be discovered. Predicting redox mediated protein− protein interactions (PPIs) in spermatozoa is important for the transcriptionally silenced spermatozoa and it will help identify the key regulators and their interactors that can serve as drug targets to restore redox balance and can be used for prioritization of candidate male infertility related genes. Aim of this study was to perform network analyses on manually curated and experimentally supported interactomes from different repositories and databases such as The DIPTM Database, MatrixDB, BioGRID, MINT, and IntAct. UniProtKB accession numbers for each protein were used as global protein identifiers.
Methods: Four types of PPIs were categorized as physical, regulatory, genetic interactions and similarity relations. The PPI network predictions and maps were cross−referenced with STRING when possible. Redox regulated proteins including thioredoxins (Trx), peroxiredoxins (Prdx), glutathione peroxidases (Gpx) and other peroxidases, and the proteins involved in ROS metabolism were selected as focus nodes. At 0.40 reliability score, the network analyses were performed for 84 oxidative stress proteins against each dataset where possible. Mentha interactome browser was used for the network analyses.
Results: Antioxidant proteins, proteins involved in superoxide and oxidative stress response proteins were among the highest reliability scoring nodes pointing highest number of interactions to these proteins.
Conclusion: Although measurements of PPIs tend to be noisy and incomplete, predictive network analysis of redox mediated sperm interactome would be helpful guide to better understand the signaling cascades in spermatozoa and for prioritization of candidate male infertility related genes for developing non−hormonal male contraceptives.

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MAPPING THE SPERM MEMBRANE PROTEIN INTERACTOME

Matthew Marcello, PhD[1], Marina Druzhinina[2] and Andrew Singson, PhD[2]
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(Presented By: Matthew Marcello, PhD)

Introduction: The interaction and organization of proteins in the sperm membrane are important for recognition of and fusion with the egg. We have determined the interactions between all known sperm membrane proteins in a model system for reproduction, the nematode Caenorhabditis elegans. Identification of the interactions between sperm membrane proteins will improve our understanding of and ability to characterize defects in these processes.
Methods: To identify interacting proteins, we are performed pair−wise split−ubiquitin yeast two−hybrid analysis of the full−length gene products.
Results: Our analysis revealed novel interactions between sperm membrane proteins known to have roles in spermatogenesis, spermiogenesis, and fertilization. For example, we found that a protein known to play a role in sperm function during fertilization, SPE−38 (a predicted four pass transmembrane protein), interacts with proteins necessary for spermiogenesis and spermatogenesis.
Conclusion: These novel interaction pairings will provide the foundation for understanding membrane protein interactions during spermatogenesis, spermiogenesis, and sperm function during fertilization. The interactome provides a more comprehensive view of sperm membrane protein interactions and the rationale for investigating previously unrealized connections.

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COMPARATIVE ANALYSIS OF MACAQUE AND HUMAN SPERM PROTEOMES: INSIGHTS INTO SPERM COMPETITION

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(Presented By: Tao Zhou)

Introduction and Objectives: Sperm competition is defined as sperms from different males compete for the chance of fertilization in the reproductive tract of a single female. Macaques are promiscuous and humans are monogamous, thus male macaques have higher pressure of sperm competition than male humans. Sperm competition has been a selective force that shaped many male reproductive features. Previous studies have found that macaques have larger testis size and greater sperm motility compared to humans. Our objective is to explain the differences of phenotypes between macaque and human under sperm competition at the protein level.
Methods: We firstly constructed macaque and human sperm proteomes using liquid chromatography−tandem mass spectrometry. We then detected the positively selected genes specifically on the branch of macaque based on branch−site likelihood method. Bioinformatics method was used for mining the biological and medical significance of positively selected genes. We further compared the ultrastructural differences of the mid−piece between macaque and human sperms to provide evidence for our findings using transmission electron microscopy.
Results: We identified 204 positively selected sperm genes specifically on the branch of macaque. These genes are highly associated with mitochondria and axoneme which directly drive sperm motility. We further showed that macaques have more mitochondrial gyres in mid−piece of sperm than humans. Taken the 175 human sperm orthologs of macaque sperm positively selected genes as the molecular targets of relaxation in humans, we found that ciliary motility disorder is the most significant enriched human disease. Using the information of mouse phenotypes, we also showed that the relaxation of sperm competition may be associated with poor sperm motility.
Conclusions: Our results explained the differences of phenotypes between macaque and human under sperm competition at the protein level, and also provided resources for the analysis of male infertility. We found that sperm competition has impacts on genes associated with energy production and molecular motor which are directly drive sperm motility. Sperm in humans with low motility or genetic disorders may also have higher opportunity for inheritance than in macaques. Thus we speculated that the poor sperm motility of humans may be associated with the relaxed selective pressure during evolution.

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TRANSCRIPTIONAL PROFILING OF HYPOXIA PATHWAY GENE EXPRESSION IN THE RAT TESTIS FOLLOWING P. AERUGINOSA LIPOPOLYSACCHARIDE−INDUCED INFLAMMATION

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(Presented By: Genevieve Fasano)

Introduction and Objectives: Inflammation of the male reproductive tract by bacterial infections is known to suppress androgen production and can result in infertility. Research on antimicrobial properties of the testis has advanced an understanding of specific genes and proteins involved in the detection and clearance of invading microbes. We have shown that hypoxia−inducible factor−1 (HIF−1), considered the master regulator of oxygen homeostasis, increases following lipopolysaccharide (LPS)−induced inflammation suggesting roles for hypoxia regulated genes in inflammatory responses of the testis. We hypothesize that antimicrobial protection of the testis is achieved through both classic inflammatory pathways and hypoxic pathways. The goal of this work is to determine the effects of LPS−induced inflammation on gene expression pathways of the rat testis. The objective of this project was to identify hypoxia pathway genes that are up−regulated or down−regulated following LPS administration and to determine the role of these genes in response to inflammation.
Methods: Inflammation in rats was accomplished via i.p. administration of LPS from P. aeruginosa (5 mg/kg body weight) for 3 or 6 hours (n = 6−7 animals/time point). RNA was isolated from testes and cDNA synthesized for analysis by qPCR. The RT2 Profiler™ PCR Array Rat Hypoxia Signaling Pathway (Qiagen) was used to evaluate expression of 91 genes involved in hypoxia pathways.

Results: Array results demonstrated that 9 genes (Adm, Angptl4, Egr1, Fos, Ier3, Nfkb1, Pgf, Serpine1, Slc2a1) were up−regulated after 3 hours of LPS−induced inflammation and expression of 3 genes (Angptl4, Egr1, Serpine1) remained elevated after 6 hours. In silico analysis of LPS−stimulated genes indicates that these transcripts are predominantly expressed in supporting cells of the testis (Sertoli, myoid, and Leydig cells) and not in developing germ cells. Egr1, Fos, Ier3, and Pai1 are known target genes for the transcription factor NF kappa B.
Conclusions: A subset of hypoxia−sensitive genes were up−regulated following LPS treatment and are expressed in supporting cells of the testis. While some genes are known targets of NF kappa B, their functions will be studied to propose molecular mechanisms of antimicrobial responses in the testis. Future experiments will investigate gene expression in the inflammatory pathway following LPS treatment.
Funding: Independent College Fund of NJ–Merck Undergraduate Science Endeavors Scholarship.

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OLIGOZOOSPERMIA TRANSCRIPTOME PROFILE AND GENE CANDIDATE DISCOVERY IN SEMEN FROM INFERTILE MALES

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(Presented By: Alexander N. Yatsenko, MD, PhD)

Introduction: Male infertility is a common and complex health condition. About 20% of infertile men suffer from reduced sperm count, or oligozoospermia. In many cases, a genetic factor may contribute to their infertility, though the clinical ability of detecting these abnormalities is limited. Sperm RNA could be powerful tool in determining abnormal gene expression and the viability of germ cells in fertility clinics.
Methods: We isolated high quality RNA from severe oligozoospermic (OZ) and normozoospermic (NZ) patients semen. RNAs from 6 patients with highly uniform semen parameters were pooled into 4 groups. Two experimental and 2 control groups of samples were used for analysis: Exp 1, severe OZ 2−6 $x10^6$ sperm/ml; Exp 2 mild OZ 10−12 $x10^6$ sperm/ml; Control 1, NZ 64−77 $x10^6$ sperm/ml; NZ 115−155 $x10^6$ sperm/ml. In this study, we performed semen RNA−sequencing (RNA−seq) to determine the transcriptome profiles of both OZ and NZ.
Results: After rRNA reduction and libriray construction by random primer cDNA synthesis, we obtained an average of 52 million paired 75 bp sequence reads with ~63x coverage. Sequence analysis, by Super−Trascript level coverage, revealed 17,309 total transcripts in NZ samples and 21,098 in OZ patients uniquely mapped to reference genome. Gene expression data analysis of differential transcript quantities between samples revealed 214 transcripts with reduced amount in OZ and 216 with increased amount in the OZ. Among the down regulated transcripts in OZ, 9% (20/214) were previously implicated in gene knockout mouse models displaying male factor infertility. An additional 48% (102/214) of transcripts with reduced abundance in OZ, shown high testis expression, suggesting a role in male reproduction. Pathway analysis revealed downregulation of transcription, RNA binding, cell division, energy metabolism, apoptosis, and early embryonic maintenance. Based on these results we identified 64 candidate genes for male infertility.
Conclusion: We propose that these RNAs, in the unique transcriptome profile of oligozoospermic semen, could be of high clinical utility as a powerful diagnostic tool in assessing idiopathic male infertility.

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WHEREAS ALL CASES OF FAILED FERTILIZATION WITH CONVENTIONAL OOCYTE INSEMINATION WITH NORMAL SPERM ACHIEVE GOOD FERTILIZATION RATES WITH ICSI ONLY HALF WITH NORMAL BINDING HAVE GOOD FERTILIZATION RATES

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(Presented By: Jerome Check, MD, PhD)

Introduction and Objective: Failure to fertilize any oocytes despite conventional oocyte insemination with sperm that appear normal can be related to failure of the sperm to bind to the zona pellucida or failure to induce post−binding events leading to oocyte activation. The problem can be related to a sperm defect despite the normal appearance by standard semen parameters (including absence of anti−sperm antibodies) or an oocyte defect. One objective of this study was to determine what percentage of the time is failed fertilization related to failure of sperm to attach to the zona pellucida. A second objective was to compare the relative efficacy of ICSI to overcome fertilization failure according to cause of failed fertilization, i.e., failure of sperm binding or post−binding events.
Methods: Retrospective review of all IVF cycles evaluating all IVF cycles where there was failed fertilization following conventional insemination with normal appearing sperm was performed. A minimum of 3 oocytes retrieved was required. ICSI was offered in a succeeding IVF cycle. Fertilization rates with ICSI were then compared according to reason for failed fertilization−sperm binding or failure to activate the oocyte.
Results: 12 cases of failed fertilization were identified over a 13 year period in 12,448 IVF cycles. 6 of 12 were related to very few or no sperm attached to the zona pellucida. 2 cases with zona binding defects who failed to fertilize any of 16 inseminated oocytes shared a pool of oocytes with 2 other couples. The 2 male partners of these other couples fertilized 11/15 (73.3%) of the oocytes with conventional stimulation suggesting sperm receptor defect for zona protein (ZP) 3 or ZP4 rather than mutated ZP3 or ZP4 in the oocyte. ICSI negated the sperm binding defects with all 6 couples showing >50% fertilization with a total percentage of 73% (60/82). ICSI was not as effective with failed fertilization with normal sperm binding with 2 couples out of 5 (one did not try IVF again) showing failed fertilization (0/7) or poor fertilization (12.5%, 1/8). The other 3 had very good fertilization rates of 88.8% (16/18).
Conclusions: Failed fertilization following conventional oocyte insemination with sperm with normal semen parameters is uncommon. Failure of sperm binding accounts for 50% of the cases and is corrected by ICSI. ICSI by attaining a rapid calcium influx overcomes phase I but not phase II oocyte activation defects.

ABSTRACTS

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SPERM WITH LOW HYPO−OSMOTIC SWELLING (HOS) TEST SCORES MAY BE A RARE CAUSE OF RECURRENT MISCARRIAGE

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Introduction and Objective: For over 30 years our group demonstrated that males with consistently low HOS test scores <50% do not achieve live pregnancies by either intercourse or conventional IUI. IVF with conventional oocyte insemination leads to normal fertilization of normal morphologic embryos but they do not implant and thus do not result in clinical pregnancies. However, ICSI or pre−treating the sperm with chymotrypsin−galactose prior to IUI allow normal pregnancy rates. There has been one study that suggested that low HOS can be a cause of miscarriage (Buckett et al., Fertil Steril, 1997). A prospective observational study was initiated to either confirm or refute the aforementioned study.
Methods: Our staff was alerted to report any women whose husband had a low HOS test and seemed to achieve a pregnancy without IVF with ICSI or IUI with chymotrypsin treated sperm.
Results: 5 years from Buckett's article a case of a woman who achieved a pregnancy despite a low HOS test was found but it was an ectopic. 11 years later we found a case of low HOS test and miscarriage. One couple had a live birth when the female partner was age 35 and the male partner was 51. Subsequently she had a miscarriage 6 and 10 months after delivery and then another miscarriage 3 years after delivery followed by an ectopic pregnancy 5 months later. She consulted us for recurrent miscarriage. A semen analysis with HOS test was suggested but he procrastinated. She conceived naturally again following taking letrozole for a follicular maturation defect with vaginal progesterone in the luteal phase. Unfortunately she had another miscarriage. Three months after the last miscarriage the male partner produced a semen specimen. It had low volume of 0.7mL with a concentration of 175x106/mL but only 15% motility and an HOS test of only 36%. Two subsequent semen analyses 1 week and 6 months later continued to show low % motility (6% and 8%) and low HOS test scores (37 and 30% respectively).
Conclusions: The last pregnancy and miscarriage was very likely achieved by a sperm specimen with a low HOS score. Possibly the previous ectopic or other miscarriages could have been related to the low HOS test scores. The implantation defect related to oocytes fertilized by sperm with low HOS scores rarely leads to a pregnancy, but if one occurs, it is likely to end in miscarriage or an ectopic.

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THE ROLE OF JUSTICIA GENDARUSSA BURM.F., AS MALE CONTRACEPTION, ON BLOOD LIPID PROFILE

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(Presented By: Sri Musta'ina, Master)

Introduction and Objectives: Contraceptives used to control population must consider the aspect of safety, security and the effectiveness (trusted efficacy and its use can be interfere with the need) comfort (easy to use, does not interfere husband−wife relationship, can be received by the spouse), the nature of reversibility and avoid surgery (Albar, 1991; Lissner, 1994; Liu, 1998). Gendarusin A is a major component of Justicia gendarussa Burm. f. leaves that was reported to have antifertility effect by degrading activity of hialuronidase enzyme. One of the security aspects to note is its influence on the blood lipid profile, blood lipid profile is given one of the risk factors for the occurrence of disturbed heart function. This study is to investigate the effect of 70% etanol extract of Justicia gendarussa Burm.f. in blood profile lipid.
Methods: 21 healthy men according to preliminary laboratory examine is randomized and controlled by clinical trial to consume Justicia gendarussa Burm.f. (each capsule contain 450 mg 70% ethanol extract of Justicia gendarussa leaves that equal with 2,9 mg gendarusin A) once a day after breakfast for 30 days. Blood sample were obtained at day 0, day 15, day 30 and day 60 along drug and recovery period (30 day after stopping drug treatment. Blood Serum was analyzed using Roche Modular analytics SWA system treatment. Cholesterol screening is done by the method of Cholesterol Oxidase CHOD−POD. Determination of the levels of triglycerides is done by the Enzymatic method of Glycerol Blanking, Determination of LDL−cholesterol levels is by a method of Enzymatic end point (Homogenous direct Method). Data analysis with GLM Repeated Measure Anova method.
Results: The results obtained by comparing data from laboratory examines on day 0, day 15. day 30 and day 60. Its results showed that the mean value of total cholesterol ratio, triglyceride, HDL−cholesterol, and LDL−cholesterol between day 0, day 15, day 30 and day 60 showed no significance difference to the value of $\alpha = 0.05$.
Conclusion: 70% etanol extract of Justicia gendarussa Burm.f. in capsule did not alter blood lipid profile).

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INVESTIGATING THE SPERMICIDAL PROPERTIES OF NOVEL COMPOUNDS

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(Presented By: Ashley Robertson)

Introduction and Objectives: The number of unintended pregnancies in the United States is a concern, as there are a number of associated negative economic and health related consequences. A goal in the Family Planning topic of Healthy People 2020 is to prevent unintended pregnancies. One potential mechanism to reduce unintended pregnancies is through the development of novel spermicides. Spermicides currently available are effective; however, there have been recent concerns with their safety. The most common over−the−counter spermicide ingredient is nonoxynol−9 (N9). N9 is a nonionic surfactant with spermicidal properties. Other consumer products such as shaving cream, cleaning products, poison ivy ointment, and sports cream contain N9 as well. Although N9 is effective at killing sperm, studies have shown it has detrimental side effects. The United States Food and Drug Administration issued a new rule effective June 2008 which required warning labels to be placed on over−the−counter vaginal contraceptive products containing N9. The goal of this research was to investigate the spermicidal properties of novel compounds as potential spermicides.

Methods: Human semen samples were collected according to an IRB approved Human Subjects protocol. Whole semen was initially analyzed to determine volume of ejaculate, cell concentration, pH, cell morphology, and percent motility. Spermicidal assays were conducted using whole semen. Briefly, whole semen was incubated with various concentrations of N9 or novel compound at 37°C for a minimum of 5 minutes. Following incubation, the sample was gently mixed and a small aliquot was removed to microscopically determine percent motility, cell morphology, and cell concentration.
Results: Whole semen incubated with 1% N9 for 5 minutes showed 0% motility following treatment. A slight reduction in motility was noted with treatment of whole semen with 0.001% N9 for 5 minutes. Treatment of whole semen with novel compounds A13TEG and A7TEG showed a reduction in sperm motility.
Conclusion: N9 showed spermicidal properties below over−the−counter product concentrations. Treatment of whole semen with our novel compounds resulted in reductions in sperm motility. Previous studies from our lab have shown that these novel compounds are less toxic to HeLa cells grown in culture. Taken together, these experiments support the notion that our novel compounds show potential as useful spermicides.

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A MODEL FOR STUDYING PROTECTION FROM INFERTILITY AFTER CHEMOTHERAPY: CYCLOPHOSPHAMIDE (CYP) DECREASES METASTATIC LUNG MELANOMA FORMATION AND INCREASES GERM CELL APOPTOSIS IN MICE

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(Presented By: YanHe Lue, MD)

Introduction: Preservation of fertility in young patients with cancer after chemotherapy is important for their quality of life. We have demonstrated that humanin (HN), a mitochondria derived 24 amino acid peptide, could attenuate male germ cell apoptosis after CYP treatment in rodents. This leads to the question of whether synthetic HN, while protecting germ cells from apoptosis, might also inhibit cancer cell apoptosis after CYP treatment. \
Methods: To examine whether CYP treatment was able to simultaneously suppress metastatic lung tumor formation and induce germ cell apoptosis, we studied 16 young adult male mice. Four mice were used as control. Twelve mice were challenged intravenously with B16 murine melanoma cells (200,000 cells/mouse) expressing the firefly luciferase gene (B16−Fluc). Among these 12 tumor−bearing mice, 4 mice received no treatment, and 2 groups of 4 mice were treated with a single CYP i.p. injection (200mg/kg BW) either at 1 or 2wks after B16−Fluc injection. Mice were imaged by IVIS bioluminescent imaging at 1, 2 and 3wks after 16−Fluc cell injection to detect lung metastases. Once tumor burden was determined, all mice were sacrificed at the end of 3wks. The numbers of tumors in the lungs were counted under stereomicroscopy. Germ cell apoptosis was detected by TUNEL assay and quantified as the number of apoptotic seminiferous tubules per 100 tubules expressed as Apoptosis Index (AI).
Results: CYP treatment diminished tumor burden in the lungs compared to non−treated mice. Without changes in body weight, CYP treatment decreased the number of lung tumors (NT) significantly ($p<0.001$) at 1wk (NT: 4.01±2.58) and more dramatically at 2wks (NT: 1.25±1.26) compared to non−treated tumor−bearing mice (NT: 10.33±1.16). While decreasing lung tumor formation, CYP treatment significantly ($p<0.001$) decreased testis weight at both 1 (79.75±9.64mg) and 2wks (60.25±3.95mg) as compared to control (102.10±9.64mg) and non−treated tumor−bearing (105.32±3.65mg) mice. CYP treatment for 2wks significantly ($p<0.001$) increased germ cell apoptosis (AI: 37.41±2.33) in comparison with control (AI: 15.67±2.02) and non−treated tumor−bearing (AI: 16.41±1.17) mice.
Conclusion: We conclude that 1) CYP significantly decreases metastatic lung tumor formation and increases germ cell apoptosis in mice; 2) the Metastatic Lung Melanoma (MLM) mouse model can be utilized to study oncofertility; and 3) the MLM mouse model will allow studies of humanin's fertility protective action using the CYP model of chemotherapy.

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DEVELOPMENT OF MALE NON−HORMONAL CONTRACEPTIVES BY TARGETING LATE SPERMIOGENESIS

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(Presented By: Wei Yan, MD, PhD)

Introduction: Overpopulation and high unintended pregnancy rate highlight a critical need for next−generation contraceptives, which should be safer, more convenient, effective and affordable, and can fit the needs of both women and men at different stages of their reproductive lives, with different ethnic, cultural and religious backgrounds, and different economic status worldwide. However, no male non−hormonal pills are currently available. Based on the fact that functional disruptions of late spermiogenesis (after the onset of spermatid elongation) can lead to the production of deformed and/or non−functional sperm and thus male infertility, but rarely cause testis shrinkage, we specifically proposed in 2009 that late spermiogenesis−specific gene products are ideal targets for male non−hormonal contraceptive drugs/pills. SPEM1 is a protein exclusively expressed in elongated spermatids, and inactivation of Spem1 gene leads to male infertility in mice, which is due to deformed sperm characterized by heads bent back and wrapped by residual cytoplasm, and that Spem1−null sperm cannot develop vigorous and long−lasting progressive motility. To find a compound that can cause sperm deformation similar to that seen in Spem1−null sperm, we embarked on an extensive search for known drug candidates documented to cause sperm deformation as a side or toxic effect during preclinical testing or clinical trials.
Methods: After testing numerous such compounds, we found a natural compound purified from a Chinese herb can cause sperm head−bent−back deformation in a way almost identical to that seen in Spem1−null mice. We, therefore, named the compound spermatodeformin 1 (SD−1).

Results: Our mouse efficacy testing showed that oral administration of SD−1 at 0.8−1.6mg/kg B.W. caused male infertility due to sperm deformation and lack of progressive motility in ~4 weeks. Continuous SD−1 treatment maintained the male infertility indefinitely, and male fertility was regained in ~4 weeks after the cessation of SD−1 treatment. During SD−1 treatment, no significant decrease in either testis weight or sperm counts was observed despite that close to 100% of epididymal sperm were deformed and display minimal or no progressive motility. Moreover, no any discernable side effects were observed and pups fathered by males recovered from SD−1−induced infertility were developmentally normal, healthy and fertile.
Conclusion: Our data suggest that SD−1 is a very promising oral male contraceptive agent.

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HIDE AND SEEK WITH SPERMS:MICROTESE AN OPTION IN NON OBSTRUCTIVE AZOOSPERMIA

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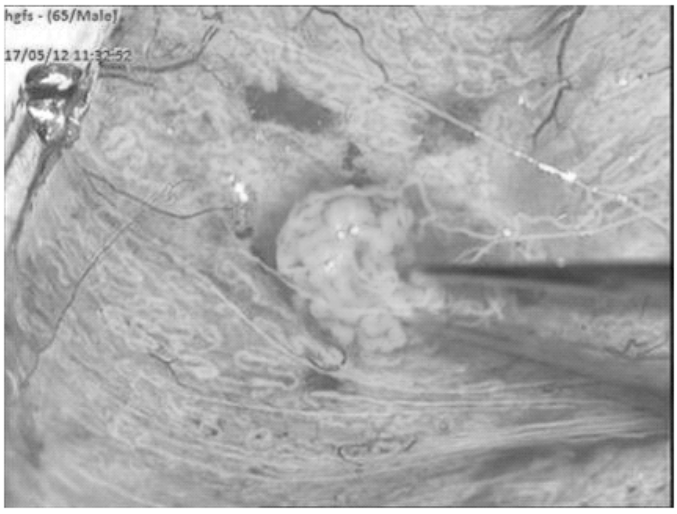
(Presented By: Dharmaraj Palanisamy, DNB (UROLOGY))

Introduction and Objective: Nonobstructive azoospermia(NOA) is an unfavourable prognostic condition for male infertility since spermatogenesis is disrupted at various levels. Sperm retrieval (SR) coupled with intracytoplasmic sperm injection (ICSI) is the only option for men with NOA seeking infertility treatment. Among the SR techniques, microdissection testicular sperm extraction (micro−TESE) has been applied with encouraging results. We present micro−TESE experiwnce in 150 patients with NOA and poor prognosis for SR.
Methods: Case series of men (n− 150) with NOA treated in a tertiary health care center Assisted reproductive technology (ART) facility was setup to perform SR using microsurgery. 150 men with NOA and prior failed retrievals or unfavourable histological results underwnt micro−TESE while their female partners underwent ovarian stimulation with oocyte retrieval(OCR). Micro− TESE was performed a day prior to OCR and testicular sperms were used for sperm injection. We assesed the retrieval rate and ICSI outcome. Outcomes of SR and ICSI were analysed descriptively. Mann Whitney and Fisher exact test were used to compare characteristics of men with successful and failed SR.
Results: The success of M−TESE is 50% in retrieving sperms with no major complications. A clear microscopic distinction between enlarged and collapsed tubules was seen in 33% of cases and sperms were retrieved in all except few. Patient with successsful and failed retrieval does not differ with respect to baseline characteristics, use of medical therapy, teticular biopsy. Sperm injection resulted in normal fertilisation and embryo cleavage of 64% and 76%. A total 50 embryo transfers with an average of 1.5 embryos resulted in cumulative clinical pregnancy rate per ICSI cycle of 30% with implantation rate of 34%.
Conclusion: We are successful in integrating the M−TESE procedures to the IVF laboratory. Our experience with micro−TESE applied to most difficult case of azoospermia is reassuring.



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ABSORBABLE CYANOACRYLATE FOR USE IN MICROSURGICAL VASOVASOTOMY:A NOVEL METHOD TO REINFORCE THE ANASTOMOSIS

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(Presented By: Tariq S. Hakky, MS)

Introduction: The absorbable cyanoacrylate surgical sealant (Ethicon OMNEX Surgical Sealant; Closure Medical Corporation, Raleigh, NC) has been applied to vascular surgery to seal and strength the anastomsis. This sealant adheres to the tissue or synthetic material, creating a flexible seal that prevents leakage of fluid in the presence of air, tissue and blood in within 1–3 minutes. We applied this applied this sealant during microsurgical vasovasotomy to seal and strength the anastomosis, decrease operative times, decrease risk of leak from the anastomosis site.
Methods: After an IRB was obtained, we performed a simple vasovastomy on four patients who requested vasectomy reversal. Once the vassal ends were cut we used four 9−0 prolene sutures. These sutured were placed at the 12, 3, 6, and 9 o'clock positions. Once the sutures were tied down the sealant was applied to a dry surgical field. We then allowed it to set for 120 seconds prior to releasing the two vassal ends. Patients had scheduled semen analysis at 3 months and 6 months. The primary endpoint was decreasing operative times from traditional one or two layered microsurgical anastomosis. The secondary endpoint included positive semen analysis post reversal. Inclusion criteria included any man who had undergone a vasectomy within the last 10 years and requested reversal. Patients were excluded if they had any prior scrotal surgeries other than vasectomy, if they did not wish to participate in the study, if their vasectomy was performed more than 10 years ago and if the patient required vasoepididymostomy.
Results: Four patients underwent microsurgical vasovastomy the mean time from vasectomy was 6.3 years. Three patients have semen analysis demonstrating the presence of sperm at the 3−6month follow up period. The fourth patient was lost to follow up. Mean operative times of (10−14) two−layered and (6−8 suture) one layered microsurgical simple vasovastomy in our institution is 320 and 155 minutes. Single layer closure with the use of absorbable cyanoacrylate is 63 minutes (50−90 minutes).

Conclusions: The cyanoacrylate surgical sealant was found to be safe and effective in the setting of microsurgical vasovastomy. It was associated with significantly decreased operatives times and did not interfere with semen passage through the anastomosis.

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ABSENCE OF NHERF−2 IN EPIDIDYMIS INCREASES LUMINAL SIZE THROUGH DYSREGULATION OF V−ATPASE LOCALIZATION.

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(Presented By: Wayland Hsiao, MD)

Introduction: Research on epididymal cell cross talk has shown that this process depends on the maintenance of sperm quiescence; specifically, the production of an acidic epididymal luminal environment through the proton pump V−ATPase. One membrane channel associated with the maintenance of low pH has been CFTR and apical expression in principal cells has been suggested as a regulator of V−ATPase. CFTR function in the nephron is regulated by Na+/H+ exchanger regulatory factors, NHERF−1 and NHERF−2. We hypothesize that NHERF−1 and NHERF−2 are involved in apical localization of CFTR leading to apical localization of V−ATPase.
Methods: We examined the morphology of epididymal tubules in NHERF−2 KO mice and found that the dimensions from the body and tail of KO mice were significantly larger when compared to age matched, wild type mice. Immunohistochemistry demonstrated that NHERF−2 expression can be seen in proximal portion of body of control mice, while V−ATPase and NHERF−1 expression begin the body and increase distally. NHERF−2 KO mice have a reduction in apical V−ATPase expression despite an elevated expression of total protein.
Results: Results suggest a lack of NHERF−2 leads to dysregulation of V−ATPase expression through upstream alterations in luminal environment. The increased size of the NHERF−2 KO epididymis body and tail may reflect the storage of increased immotile sperm due to increased pH.
Conclusion: We believe that this identification of a unique regulator of V−ATPase localization can elucidate the physiological mechanism of sperm maturation, leading to a potential treatment for male infertility and a pharmacological target for male contraception.

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INCIDENCE OF MALE AND FEMALE STERILIZATION FOLLOWING A RECENT LIVE BIRTH:
ESTIMATES FROM THE PREGNANCY RISK ASSESSMENT MONITORING SYSTEM (PRAMS), 2007−2010

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(Presented By: Lee Warner, PhD)

Introduction and Objective: Although contraceptive use is recommended postpartum, little is known about use of non−reversible contraception during this period.

Methods: We analyzed data from women from 15 states and New York City who participated in the 2007−2010 Pregnancy Risk Assessment Monitoring System (PRAMS). PRAMS is an ongoing, population−based surveillance system of women surveyed 2−6 months following delivery of a live birth. Use of non−reversible contraception after a recent live birth was assessed. Among women using contraception following delivery, we used polytomous logistic regression to separately assess predictors of tubal ligation and partner vasectomy compared with reversible contraception.
Results: Among 48,519 women who recently delivered a live birth, 11.1% (95% CI: 10.6−11.5%) reported having a tubal ligation (ranging from 6.6% in Utah to 20.8% in Mississippi) while 3.4%(95% CI: 3.2−3.7%) reported their partner had a vasectomy (ranging from 1.2% in New York City to 4.9% in Missouri). The ratio of tubal ligation to vasectomy use significantly exceeded 1 in all reporting areas, ranging from 1.9 in Utah [tubal ligation: 6.6%; vasectomy: 3.5%] to 10.4 in Mississippi [tubal ligation: 20.8%; vasectomy: 2.0%]. Multivariable modeling revealed that, compared with reversible methods, vasectomy following recent live birth was associated with being married [aOR=2.1 (95% CI=1.4−3.1)], having >=1 prior birth [eg, 4th birth vs 1st, aOR=19.2(12.8−28.9)], increased maternal age [>35 vs 20−24, aOR=2.7(1.8−4.0)], and increased maternal/paternal education [>=high school vs <high school, aOR=1.3(1.0−1.8) and aOR=1.3 (1.0−1.7), respectively]. Tubal ligation was associated with having >=1 prior birth [aOR=33.0(24.8−44.0)] and increased maternal age [>35 vs 20−24, aOR=4.2(3.4−5.2)], but inversely associated with being married [aOR=0.8 (0.7−0.9)] and maternal/paternal education [aOR=0.7(0.6−0.8) and aOR=0.6(1.0−1.7), respectively]
Conclusions: Although use of female sterilization was more common than male sterilization following delivery of a live birth, one in four women using non−reversible contraception reported their partners had a vasectomy. PRAMS data suggest significant variation by state in use of female versus male sterilization as well differences in education and marital status for users of these methods.

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PIOGLITAZONE INCREASES CYCLIC GMP CONCENTRATIONS IN A RAT MODEL OF POST−PROSTATECTOMY ERECTILE DYSFUNCTION

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(Presented By: Louis Aliperti)

Introduction: Erectile dysfunction (ED) is a common complication of radical prostatectomy. Pioglitazone (PIO) is a thiazolinedione derivative used in the treatment of diabetes mellitus. Given its known vasculoprotective and antifibrotic properties, we evaluated pioglitazone in a rat nerve−crush model of ED.

Methods: 15 Sprague−Dawley rats were stratified into three groups: 1−sham, 2−nerve crush (NC), 3−PIO treatment. Sham rats underwent an abdominal incision. Groups 2 and 3 underwent bilateral cavernosal NC. All rats subsequently underwent oral gavage(sham and NC with phosphate buffered saline, PIO treatment with PIO 0.65 mg/kg). Following a 1−day washout period, all rats underwent cavernosal nerve stimulation at 7.5V. Intracavernosal pressure to arterial pressure (ICP/MAP) was assessed as a measure of erectile function. Corporal tissue was snap frozen and analyzed for cGMP by ELISA (Cayman Chemicals Inc.). Statistics were performed using Student's t−test, with $p<0.05$ as significant.
Results: A significant decrease in ICP/MAP was observed in NC rats compared to sham animals at all voltages. However, PIO−treated animals showed voltage−dependent increases in ICP/MAP values compared to NC controls of 0.62±0.05 vs 0.42±0.05, p=0.0229, respectively. Increases in cGMP concentration were observed in PIO treated rats compared to control animals. cGMP levels in sham were 35 ± 3.5; in NC 30.4 ± 3.1; in PIO 45 ± 13.9 pmol/g (p=0.22).
Conclusion: PIO administration improves erectile function in a post−prostatectomy ED model via a cGMP−dependent pathway.

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THE PENILE DOPPLER PARAMETERS AND CLINICAL RISK FACTORS IN MEN WITH ERECTION HARDNESS SCORE 3−4 AFTER INTRACAVERNOUS INJECTION

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(Presented By: Qiang Li)

Introduction and Objectives: The Erection Hardness Score (EHS) is a simple, valid, reliable instrument to measure erection outcome and the ability for penetration. The objective of this study was to determine the best penile Doppler (PD) parameters and clinical risk factors for predicting an EHS 3−4 after intracavernous injection.
Methods: Among a total of 462 patients who underwent a PD ultrasound after intracavanous injection between July 2008 and February 2013, 221 (48%) patients achieved EHS 0−2 and 241 (52%) patients achieved EHS 3−4. The PD parameters were compared between the two groups using the Student's t test and the distribution of erectile dysfunction (ED) risk factors was determined using Chi−square test. The odds ratios (OR) of EHS 3−4 associated with PD parameters or ED risk factors were determined using a multivariable logistic regression model.
Results: Compared to patients with EHS 0−2, patients with EHS 3−4 were more likely to be younger (54 years vs 59 years, $P<0.001$) and showed significantly larger artery diameter (0.8 mm vs 0.6 mm, $P<0.001$), higher peak systolic velocity (PSV) (45.5 cm/s vs 28.5 cm/s, $P<0.001$), and lower end diastolic velocity (EDV) (0.4 cm/s vs 1.6 cm/s, $P<0.001$). EHS 3−4 was significantly associated with the presence of Peyronie's disease (p=0.01), and the absence of hypertension (p=0.001) or prostate cancer (all treatment modalities) (p=0.007). Multivariable analysis showed artery diameter (OR=14, $p<0.001$) and PSV (OR=1.03, $p<0.001$), but not EDV or resistive index, were independently associated with EHS 3−4. Patients with a history of hypertension or prostate cancer were half as likely to have an EHS 3−4 compared to patients without a history of hypertension or prostate cancer. (OR=0.5, 95%CI 0.3−0.8 p=0.005; OR=0.5, 95%CI 0.3−0.9 p=0.03, respectively).
Conclusions: The artery diameter and PSV are the strongest predictors of EHS 3−4, and hypertension and prostate cancer negatively affects EHS after intracavernous injection. Penile Doppler continues to be an indispensable tool to evaluate men with ED.

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SEXUAL FUNCTION IN MALE PARTNERS OF WOMEN PARTICIPATING IN A SURROGATE MOTHERHOOD PROGRAM

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(Presented By: Ioannis Giakoumakis, MD)

Introduction: We evaluated the sexual function of male partners of women participating in a surrogate motherhood program (SMP).
Methods: The international index of erectile function−5 (IIEF−5) outcome was calculated in 18 healthy sperm donors (group A), in 13 male partners (group B) of women participating in an in vitro fertilization (IVF) program, and in 16 male partners (group C) of women participating in an SMP. There were no significant differences in the mean value of age and peripheral serum testosterone among groups A, B and C.
Results: The mean IIEF−5 score was significantly smaller (P smaller than 0.05; Wilcoxon test) in group C (equal to 15) than in group A (equal to 22) and in group B(equal to 20). In contrast there were no statistically significant differences (P larger than 0.05) in the mean IIEF−5 score between groups A and B.
Conclusion: The significantly lower values of IIEF−5 outcome in group C compared with groups A and B may be attributed to an enhanced stress that experience the couples that participate in a SMP. Male partners of women who participate in an SMP have the hope and a strong desire one day to father their own children. However an SMP is a 20 to 30 times more expensive than an IVF program. In addition the biological parents have to participate in a legal recourse to confirm that the pregnant surrogate mother will give the child to the biological mother immediately after delivery. This results in an additional amount of stress for the biological parents. Furthermore stressful discussions concerning the financial reimbursement of the surrogate mother are necessary between the biological parents and the surrogate mother.

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THE EFFECT OF ANTIOXIDANT TREATMENT ON SEMINAL VESICLES AND VAS DEFERENS FUNCTION IN THE DIABETES MELLITUS RAT MODEL.

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ABSTRACTS

Introduction and Objectives: Diabetes Mellitus (DM) is one of the high growing diseases threatening the human health. The incidence of DM is increasing rapidly usually affecting neurological, endocrinological and reproductive functions. Previous studies reported that DM also affects the sexual function of humans, or animal models, and also cause ejaculatory disorders. In this study we investigated the effects of DM in the seminal vesicles (SV) and vas deferens (VD) functions by employing in vitro organ bath studies. We also investigated if DM−induced dysfunction of SV or VD can be reversed by antioxidant treatment.
Methods: Control group was consisted of 10 rats (Control). Diabetes was induced in 40 rats by a single dose of STZ (50 mg/kg) i.p. Diabetic rats were divided in: non−treated DM rats (20 rats; group DM), DM rats treated with edaravone 10 mg/kg i.p. daily (10 rats; group DM+E), and DM rats treated with taurine 500 mg/kg i.p. daily (10 rats; group DM+T). The treatment lasted four weeks. After the completion of the treatment both SVs and both VDs were collected from all animals. SVs and VDs functions were evaluated by in vitro organ bath studies. Contractions were induced by norepinephrine (NE) or carbachol (Crb) for SVs, and for VD contractions were induced by NE. The serum testosterone profiles were measured.
Results: The organ weights for both SVs and VDs were significantly lower in the DM group compared to the Control. Treatment with both edaravone and taurine significantly increased the SV weights compared to the DM group, while only taurine significantly increased the VD weights compared to DM group. The in vitro organ bath studies revealed a significant hypercontraction of the seminal vesicles as induced by NE or Crb in the DM group compared to the Control group. Treatment with taurine or edaravone did not significantly alter the NE−induced hypercontractions observed in the DM group, while the Crb−induced contractions were significantly normalized by both treatments with taurine or edaravone compared to DM group. The VD from DM group demonstrated significant hypercontractions compared with Control group. Both taurine and edaravone treatments significantly normalized this abnormality observed at the DM group. Testosterone levels were significantly lower in all diabetic animals compared to the Control.
Conclusion: Although antioxidant treatments did not manage to increase testosterone levels, they significantly corrected the SV and VD functions.

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ENDOTHELIAL−ERECTILE DYSFUNCTION AND CARDIOVASCULAR RISK FACTORS RELATIONSHIP

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(Presented By: Libor Zamecnik, MD, PhD)

Objective: Endothelial monolayer plays a crucial role in the vasodilatation and hemodynamic events that leads to a normal erection. Endothelial dysfunction have been well established as one of the risk factors for developing both cardiovascular disease and erectile dysfunction (ED). This might explain the association between ED and coronary artery disease (CAD), as many men diagnosed with ED are at risk for a possible subsequent atherosclerotic CV event. Our aim is to determine the proportion of men with the diagnosed of ED that suffer from endothelial dysfunction, and its association with the CVD risk factors.
Methods: We evaluated endothelial function on 50 consecutive men with the diagnosis of ED who presented to our clinic. Endothelial function was determined using ENDO−PAT 2000 (Itamar Medical, Israel) by measuring. Post−occlusive reactive hyperemia index (RHI) on peripheral arterial tonometry. Endothelial dysfunction was ruled out when RHI values are above 2.07, and diagnosed when RHI is below 1.67. In between these two values lies a gray area, which represents a zone for possible risk of developing future endothelial dysfunction. Descriptive statistical analysis was performed. The relation with CV risk factors were also evaluated.
Results: Median age was 52 years (range 32 − 82). 16 (32%) patient were confirmed to have endothelial dysfunction based on RHI, 16 pts (32 %) were in the "gray zone", and 18 pts (36 %) were in a normal RHI range. The Cohort overall CVD risk factors: hypertension 31 pts (62 %), diabetes mellitus 12 pts (24 %), dyslipidemia 35 pts (70 %), obesity 30 pts (60 %), smokers 43 %,low−HDL cholesterol in 14 pts (20 %), testosteron defficiency11 pts (22 %), and waist circumference >102cm was observed in 37 pts (74 %). Only 5 pts (10 %) did not exhibit any risk factors and they all fell in the normal RHI group. Statistical significant correlation was observed when the relationship between endothelial dysfunction patients and diabetes (p 0,4865), 2 or more comorbidities (p 0,00368), and level of triglycerides was observed (p 0,4917).
Conclusion: In our Cohort 68% of the patients with ED were diagnosed endothelial dysfunction or at risk of developing endothelial dysfunction. Endothelia dysfunction in ED patient is associated with CV risk factors. ENDO−PAT 2000 might be a useful tool to determining endothelial dysfunction in ED patient.
Study was supported by grant: PRVOUK – P25/LF1/2

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EVALUATION OF SPERM DNA DAMAGE AND ANEUPLOIDY IN MALE SURVIVORS OF PEDIATRIC HODGKIN'S LYMPHOMA

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(Presented By: Sergey Moskovtsev, MD, PhD)

Introduction: Hodgkin's lymphoma is common in adolescents/young adults and survival rates exceed 90% on contemporary multi−modality protocols. However survivors are at significant risk of impaired future fertility. Alkylating antineoplastic agents are considered the primary cause of gonadal dysfunction. The objective of this study was to assess the impact of chemotherapy on sperm quality, DNA damage and aneuploidy in survivors of pediatric Hodgkin's lymphoma.
Methods: This is a retrospective cross−sectional study of male Hodgkin's lymphoma survivors treated at a single pediatric institution from 1985−2007. Eligible males were recruited from survivors attending an aftercare clinic who were aged ≥18 years and > 3 years from completion of therapy. Study participants completed a questionnaire, underwent urological examination and an evaluation of sexual hormones and semen analysis. In consenting non−azospermic participants assessment of sperm DNA damage and sperm aneuploidy was performed. Cumulative doses of alkylator agents were expressed as tertriles and as cyclophosphamide equivalent doses.
Results: Of the 38/49 (76%) eligible male Hodgkins' lymphoma survivors contacted; 15 enrolled and completed the study. Age of participants ranged from 21−35 years (mean 26 years) with a median time to assessment of 12 years (range 6−20 years) from diagnosis. The majority (10/15; 67%) had stage I/II disease. All were treated on alkylator containing regimens. On semen analysis 47% (n=7) were normozoospermic, 20% (n=3) oligozoospermic and 33% (n=5) azoospermic. The mean cumulative alkylator score was lower in normospermic survivors (2.4 vs 3.3 and 3.4 for oligospermic and azoospermic respectively). Sperm DNA fragmentation index was normal (<15%) in the normozoospermic survivors (n=6) and borderline (16%) in the oligozoospermic survivor tested. Aneuploidy (chromosomes 13, 18,21, X/Y) was slightly elevated at 3.46% ± 0.97 in four normozoospermic participants and significantly higher at 11% in the survivor with severe oligozoospermia.
Conclusion: Infertility remains a concern for male Hodgkin's lymphoma survivors. Of those who retain spermatogenic capacity, there appears to be no long−term risk of increased sperm DNA damage, but the observed increase in the aneuploidy rates requires further evaluation in a larger cohort.

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UPDATES FROM THE CENTERS FOR DISEASE CONTROL AND PREVENTION REGARDING PROGRESS IN MALE REPRODUCTIVE HEALTH

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CDC
(Presented By: Lee Warner, PhD)

Introduction: The Centers for Disease Control and Prevention (CDC) has a longstanding history of conducting scientific and programmatic activities with direct relevance to male reproductive health. Topics that have been covered across the nation's leading public health agency range from contraceptive use and effectiveness, infertility, STD and HIV prevention, testing and treatment, unintended pregnancy, and the standardization of hormonal measurements to the effects of various occupational, environmental and physical exposures on male reproductive health function. Several publicly available, population−based surveys conducted by CDC, including the National Survey of Family Growth (NSFG), National Health and Nutrition Examination Survey (NHANES), National Health Interview Survey (NHIS), National Vital Statistics System (NVSS), and Pregnancy Risk Assessment Monitoring System (PRAMS) can also be used to examined key aspects of male health. Highlights from these and other CDC surveys and surveillance systems will be discussed and progress on incorporating data elements regarding male reproductive health into these systems will be reviewed. The presenters will also discuss opportunities for collaboration with CDC and recent progress on new initiatives regarding the reproductive health of men.

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OPIOID−FREE ANALGESIA FOLLOWING ROBOT−ASSISTED LAPAROSCOPIC PROSTATECTOMY (RALP)

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(Presented By: Xiao Gu, MD, PhD)

Objective: Opioid analgesia employed for pain control following abdominal and pelvic surgery have potential adverse events and can delay return of normal bowel function. To minimize its use, we utilized scheduled intravenous (IV) acetaminophen and ketorolac for perioperative analgesia following RALP.
Methods: Prospectively collected data from hospital records of consecutive patients who underwent transperitoneal RALP using perioperative IV acetaminophen and ketorolac for pain control were reviewed. All procedures were performed under general endotracheal anesthesia utilizing a balanced technique. The balanced anesthetic was not standardized with the exception that all patients received acetaminophen 1000 mg IV over a 15 minute infusion and ketoralac 30 mg IV prior to extubation. All patients were extubated in the operating suite and transported to the post anesthesia care unit (PACU) with supplemental oxygen by facemask and pulse oximetry monitoring. Acetaminophen 1000 mg IV was administered q6 hours post−surgery, while ketorolac 30 mg IV was administered at q8 hour intervals. Patients were provided a clear liquid diet and ambulating the evening of surgery. Once passage of flatus and tolerating a regular diet were confirmed, patients were discharged home. The hospital records were reviewed to quantitate both parenteral and oral opioid consumption.
Results: 69 patients had a median age of 62 years and an American Society of Anesthesiologists (ASA) class of 3. Median operative time was 90 minutes and estimated blood loss was 75mL. Mean hospitalization and urethral catheter duration were 21.0 hours and 5.0 days, respectively. 22 (31.9%) patients received parenteral opioid medication in the PACU, but did not require opioid medication on the hospital floor; le 39 (56.5%) patients did not require administration of parenteral/oral opioid analgesia in the PACU/hospital floor. No immediate/delayed adverse events were noted.
Conclusions: Perioperative scheduled IV acetaminophen and ketorolac are effective for pain management following RALP. Use of this regimen has the potential to decrease the need for postoperative opioid analgesia for this procedure, thereby lowering the risk of opioid−associated adverse events.

ABSTRACTS

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CORRELATION OF IMMUNOBEAD AND IMMUNOSPHERES IMMUNOGLOBULIN G (IGG) TESTS ON DETECTING ANTI-SPERM ANTIBODY (ASA) ON SPERM

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(Presented By: Aniela Bollendorf, MT, HEW)

Introduction and Objective: Production of the direct immunobead test for detection of sperm laden with antisperm antibody in phasing out. One consideration is to perform the test with immunospheres. The question is how well do they correlate.
Methods: The new direct immunosphere test for IgG is performed by mixing live motile sperm with latex beads coated with antibodies that bind to human IgG antibodies. The beads are first washed with a medium containing 1−2% bovine serum albumen and can be stored up to 3 days at 4oC. Sperm is diluted to give a final concentration of 10x106/mL. Five microliters of sperm suspension is mixed with 5 microliters of anti−IgG beads. After 1−2 minutes 150 motile sperm are counted and the percentage of sperm having beads attached is determined.
Results: There were 29 samples that were split and the presence of ASA was measured by immunobead and immunosphere test. There were 11 immunobead specimens read as zero and all 11 were similarly read as zero with immunosphere. There were 14 specimens read as zero by immunosphere with 4 slightly discordant immunobead tests read as 3, 2, and 7%, respectively. There were 11 immunobead specimens read as 100% ASA with complete agreement with immunosphere in 3. 98−99% in 3, and the others showing 95%X2, 92% and 83%, and 64%. One immunosphere read as 100% and the corresponding immunobead was 97%. There were some larger discrepancies however. One sample was 87% by immunobead read as 31% immunosphere. Other samples showed 7 vs. 0, 48 vs. 42 and 97 vs. 87.
Conclusions: There appears to be a good correlation between measuring ASA by immunobead vs. the immunosphere. Some andrologists consider with immunobead ASA levels >50% and some consider >80% as clinically important. Using the 50% cut−off value for ASA there was only 1 male with positive ASA by immunobead but negative by immunosphere and only 2 if the 80% cut−off was used.

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COX−2 AND TLR−4 AS NEW PUTATIVE BIOMARKERS OF CHRONIC INFLAMMATION IN LEUKOCYTOSPERMIA

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(Presented By: Sharika Hagan)

Introduction and Objective: Leukocytospermia (LCS) is a common cause of male infertility. Most often it is due to prostatitis and genitourinary inflammation (GUI) manifested by increased number of white blood cells (WBC), inflammatory chemokines, and reactive oxygen species (ROS) in the seminal plasma leading to decreased sperm motility and functionality and high sperm DNA damage. Many times it is idiopathic and chronic. There is an urgent need to develop much sensitive biomarkers for effective treatment of LCS before it gets to chronic stage. The current study explores newer inflammatory biomarkers such as toll−like receptor−4 (TLR−4); cyclooxygenase−2 (COX−2); and oxidative stress regulating antioxidant transcription protein, NF−E2−related factor 2 (Nrf−2) that counteracts the effects of ROS.
Method: Semen samples (n=60) collected from infertile patients (25 from non−LCS and 35 from age−matched LCS) were evaluated for sperm counts; motility/progression; morphology; and total WBC count. A differential expression profile of 60 inflammatory cytokines was determined by a commercial human cytokine antibody array (Ray Biotech; C−Series). Newer markers (TLR−4, COX−2, and Nrf−2) were evaluated by quantitative immunofluorescence microscopy (IFM).
Results: Semen samples from LCS patients showed significant decrease in sperm motility ($p<0.045$), progression ($p<0.005$), morphology ($p<0.05$) along with significant increase in WBC levels ($p<0.001$) as compared to non−LCS patients. Cytokine arrays revealed up−regulation of several pro−inflammatory cytokines and chemokines (mainly GM−CSF, IFN−γ, IL−7, MCP−2) in semen of LCS patients. The IFM data showed significant 7−fold increase ($p<0.001$) in TLR−4 and 5−fold increase ($p<0.01$) in COX−2 expression, while Nrf−2 expression showed significant 10−fold decrease ($p<0.01$) in LCS samples compared to non−LCS samples. Interestingly, these biomarkers were highly expressed in the nuclei of sperm head and in tail segments but showed much lower expression in the mid−piece section of spermatozoa collected from LCS patients, when compared to non−LCS samples.
Conclusions: These unique findings suggest that both TLR−4 and COX−2 can serve as novel biomarkers of leukocytospermia during chronic inflammation. Also, their differential localization in spermatozoa especially during GUI needs further exploration to understand their diagnostic and physiological role in male infertility practice.

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SPERM PROCESSING BY SINGLE DENSITY GRADIENT CENTRIFUGATION SELECTS FOR NORMAL HEAD, MID-PIECE AND TAIL MORPHOLOGY.

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(Presented By: Amin Herati, MD)

ABSTRACTS

Introduction: Semen analysis remains the cornerstone in the evaluation of male infertility. Motility and morphology are important parameters of the semen analysis and critical indicators of semen quality and fertility potential. Semen processing techniques have made it possible to enrich a sample for motile sperm, however, data are lacking on the morphologic profiles of these sperm. The objective of our study is to compare the morphologic profiles of an unprocessed semen specimen (unprocessed) and a semen specimen centrifuged over a colloidal silica separating media (processed) and analyzed using computer−assisted semen analysis (CASA).

Methods: Semen was prospectively collected from 18 men after at least two days of abstinence. Raw sperm semen parameters are determined with morphologies by CASA (SCA® Microptic SL, Barcelona, Spain). A 0.5 cc aliquot of raw sperm was centrifuged at 300 g over a 0.5 cc of 90% colloidal silica separating media (ISolate®, Irvine Scientific, Santa Ana, CA, USA). Computer−assisted semen analysis was then performed to determine the morphologic profiles of the processed sperm. Paired t−tests were performed for all data with 2−tailed Wilcoxon signed rank t−test where appropriate for data with nonparametric distribution.

Results: The mean volume (±SEM) for the whole cohort was 2.9 ml (±1.3), and the median days of abstinence was 3 days (range, 2−5 days). Using Kruger's strict criteria for morphology, the mean percent of normal forms in the processed sperm was significantly higher than in the raw specimen (14.7±1.9 vs 11.5±1.4; p=0.05). Similarly, the percentage of sperm with normal midpiece morphology was higher in the processed specimen (71.0±3.5 vs 61.3 ±2.7, p <0.005). Sperm isolated with processing had a statistically significant lower percentage of macroheads, piriform shaped heads, abnormal midpiece size, abnormal midpiece insertion angles and short tails.

Conclusion: Processed sperm demonstrated a higher percentage of normal head, midpiece and tail morphology. Specifically, specimens after processing have less macroheads, piriform shaped heads, abnormal midpiece sizes, abnormal insertion angles and short tails than raw sperm. Our data suggest that processing enriches the specimen for normal morphology. Additional studies are needed to determine if the processed sperm morphology more accurately reflects sperm function than the current standard.

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THE EFFECT OF CIGARETTE SMOKING QUANTITY ON SEMINAL LEUKOCYTES AND SEMEN PARAMETERS

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(Presented By: Erik Yao, BSc)

Introduction: Pyospermia can result from inflammation, infection, trauma, or other genitourinary insults. Both pyospermia and cigarette smoking have been associated with impaired semen parameters. We sought to explore the association between smoking, pyospermia, and semen parameters.

Methods: Men presenting for a fertility evaluation from 2008−2012 reporting smoking cigarettes were identified in a prospectively collected database. Patients were divided based on quantity smoked: non−smokers, < 1 packs per day (PPD), 1 PPD, 2 PPD or more. Data were analyzed for lifestyle confounders (marijuana and alcohol use) and semen parameters.

Results: Of 2787 total men, 861 (30.9%) men reported that they were current smokers. Of smokers, 695 (80.7%) smoked < 1 PPD, 116 (13.5%) 1 PPD, and 50 (5.8%) 2 PPD or more. Men without semen analyses were excluded from analysis. Marijuana was more commonly used among heavy cigarette smokers: 86.9% among those using ≥ 2 PPD compared to 20.63% in those smoking 1 PPD, 24.4% in those smoking < 1 PPD. The level of alcohol consumption did not vary between smokers and non−smokers. The proportion of men with pyospermia (defined as > 1 x10^6/ml WBC in semen) was as follows: For non−smokers 2.70% (50/1855), for those using < 1 PPD: 4.71% (13/276: p=0.065 compared with non−smokers), those smoking 1 PPD: 13.89% (5/36: p<0.001 compared with non−smokers), and 2 PPD or more: 0 (0/11: p=0.581). Semen parameters within the groups are listed in Table 1. The total motile sperm count (TMC) was not different for smokers versus non−smokers, or within smoking groups: Non−smokers: 30.8 ± 54.9 x10^6, < 1 PPD (27.5 ± 55.9 x10^6), 1 PPD (16.2 ± 21.9 x10^6), 2 PPD or more: (54.8 ± 119.1 x10^6). The total sperm count (TSC) for non−smokers: 88.0 ± 131.2 x10^6 was significantly different than of men that smoked 1 PPD, 48.7 ± 98.3 x10^6 (p=0.018). Vitality was not different between groups.

Conclusions: Approximately ¼ of infertile men are smokers. As the quantity of smoking increases, so does the proportion of men with pyospermia. TSC was lower in the 1 PPD group as compared with non−smokers, however the TMC was not different among groups. This study is limited by a small sample size in the ≥ 2 PPD smokers category.

Table 1: Demographic data and semen parameters for infertile men, divided by amount of cigarettes smoked.

Smoking Quantity (PPD)	Sample Size	Age (years)	Volume (ml)	Concentration (10^6/ml)	Motility (%)	Morphology (%)	TSC (10^6)	TMC (10^6)	Vitality (%)	% of PBMs >1 x 10^6/ml
0	n=2202	37.1 ± 6.2	2.8 ± 1.6	34.5 ± 60.1	23.9 ± 14.5	13.6 ± 10.1	88.0 ± 131.2	30.8 ± 54.9	67.1 ± 15.3	2.70
<1	n=480	36.3 ± 6.4	3.2 ± 1.6	26.3 ± 43.9	23.4 ± 14.7	12.3 ± 9.7	67.5 ± 120.6	27.5 ± 55.9	66.1 ± 17.2	4.71
1	n=63	37.0 ± 6.7	2.5 ± 1.4	22.6 ± 44.8	23.2 ± 14.1	15.3 ± 9.4	48.7 ± 98.3*	16.2 ± 21.9	67.7 ± 12.5	13.89*
≥2	n=23	36.0 ± 5.8	3.3 ± 1.9	30.7 ± 70.7	27.0 ± 21.1	16.2 ± 13.2	84.7 ± 119.1	54.8 ± 119.1	67.2 ± 8.4	0.00

* = significant at the p<0.05 level

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LOCALIZATION OF SYNAPSIN I IN HUMAN SPERM CELLS

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(Presented By: Jennifer Venditti, PhD, MS, BS)

Introduction and Objectives: Cell to cell signaling is a widespread process within organisms, and this signaling must be carefully regulated by numerous proteins. Fertilization is a carefully orchestrated cascade of events that requires communication between both the sperm and oocyte. Certain proteins known to have functions in neurons and other types of secretory cells have recently been shown to be present in human sperm. One such group of proteins, the synapsins, has been very well characterized in neurons, but very little is known about synapsin function in other types of cells. The goal of this project was to investigate the localization and distribution of synapsin in human sperm cells using immunocytochemical and protein blotting techniques.

Methods: Human semen samples were washed to remove unwanted cellular debris and seminal plasma components. Sperm cells were fixed onto a microscope slide with methanol. For immunolocalization, slides were incubated with a blocking solution for a minimum of 60 minutes, followed by primary anti−synapsin I antibodies for 60 minutes at room temperature in a moisture box. Following PBS rinses, slides were incubated with a fluorophore−conjugated secondary antibody for 30 minutes at room temperature in a moisture box. Slides were again rinsed with PBS and mounted with fluorescent mounting medium and a No. 1 coverslip. Results from immunolocalization experiments were documented using epifluorescence microscopy. Protein extracts were prepared from human sperm, mouse brain, and mouse testis/epididymis samples. The protein extracts were evaluated for the presence of synapsin I using dot−blot and Western blot techniques.
Results: Immunolocalization experiments showed positive staining for synapsin I in human sperm cells. Synapsin I localized to the plasma membrane of the sperm head, and in some cases showed an enrichment in the equatorial segment. The presence of synapsin I in human sperm was confirmed by both dot−blot and Western blot techniques.
Conclusion: The localization of synapsin I to human sperm cells is a novel finding, as this group of proteins has been thought to primarily be present in neurons. This research will enhance our understanding of regulatory synaptic proteins, and the possible role of such proteins in fertilization and reproduction.

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ELEVATED NITRIC OXIDE LEVELS MEDIATE MOTILITY DEFECTS IN JAM−A AND PMCA4 NULLS: PMCA4 IS A NEGATIVE REGULATOR OF ENDOTHELIAL NITRIC OXIDE SYNTHASE IN MURINE SPERM

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(Presented By: Patricia Martin−DeLeon, PhD)

Introduction and Objective: Reduced sperm motility (asthenospermia, AS) is a primary cause of male infertility and a large proportion of the cases are idiopathic. In mice, AS leading to infertility results from deletion of the gene encoding the highly conserved Plasma Membrane Calcium ATPase 4 (PMCA4), the major Ca2+ efflux pump in sperm. We have reported AS in mice lacking Jam−A (Junctional Adhesion Molecule A), and have shown that it results from decreased activity of PMCA4. How the absence of PMCA4, or its reduced activity, leads to AS is unknown. Our goal was to determine the mechanism by which deletion of Pmca4 and Jam−A exert its effects on motility and ultimately leads to infertility.
Methods: Since nitric oxide (NO) plays a crucial role in motility and PMCA4 (in addition to its Ca2+ efflux role) is known to modulate nitric oxide (NO) signaling by negatively regulating NO production, via nitric oxide synthases (NOSs), we used immunofluorescence to localize PMCA4, endothelial (eNOS) and neuronal (nNOS) NOS in sperm. Co−immunoprecipitaion (Co−IP) was used to study the association of eNOS and PMCA4 in uncapacitated (UNCAP) and capacitated (CAP) sperm. Intracellular NOS activity and peroxynitrite (OONO−) levels were measured in UNCAP and CAP Jam−A and Pmca4 null sperm and compared to WT, using flow cytometry.
Results: eNOS and PMCA4 were co−localized on the proximal principal piece (PPP) and over acrosome. Co−IP assays revealed an association of PMCA4 and eNOS in CAP, but not UNCAP sperm. NOS activity was significantly elevated in CAP compared to UNCAP Jam−A and Pmca4 null sperm. Similarly the levels of OONO−, a highly reactive primary effector of NO were markedly increased in Pmca4 nulls.
Conclusions: Our results show that in sperm eNOS interacts with PMCA4 which negatively regulates it. They support our hypothesis that AS in Pmca4 and Jam−A null sperm results from elevated levels of NO and its reactive byproduct (OONO−) which causes lipid peroxidation of sperm membrane, a key factor in motility loss. Our data suggest that PMCA4 mutations may be involved in AS in humans and thus may be relevant for AS diagnosis

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JAM−A−CASK COMPLEX INTERACTS WITH CD9 TETRASPANIN AND AVB3 INTEGRIN TO MEDIATE CA2+ SIGNALING IN CAPACITATION AND THE ACROSOME REACTION IN MURINE SPERM

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(Presented By: Amal Al−Dossary, MS)

Introduction and Objectives: We have shown that Junctional adhesion molecule A (JAM−A) is essential for sperm motility and is involved in the maintenance of Ca2+ homeostasis via its PDZ−ligand interaction with calcium/calmodulin−dependent serine kinase (CASK). Our objectives were to determine if JAM−A−CASK complex in sperm is a component of a larger signaling complex seen in endothelial cells and if JAM−A becomes phosphorylated which is a requirement for its signaling activity.
Methods: Sperm were capacitated and induced to acrosome−react using Ca2+ionophore (A23187) and proteins extracted for co−immunoprecipitation assays. Immunofluorescence was used for colocalization assays.
Results: We identified tetraspanin CD9 as a novel interacting partner of JAM−A and CASK in sperm and have localized it on the midpiece, the proximal principal piece (PPP), and the over the acrosome where αvβ3 resides and where we have previously localized JAM−A. CASK, a membrane−associated scaffold protein, was shown to assemble a quaternary JAM−A−CASK−CD9−αvβ3 signaling−inactive complex in uncapacitated sperm. Upon capacitation a JAM−A−CASK binary complex dissociates from the quaternary complex and JAM−A is Ser285−phosphorylated. Ser285−phosphorylated JAM−A (pJAM−A) shows a dynamic spatial and temporal tail−to−head distribution in sperm. The level of pJAM−A decreased gradually from acrosome−reacted (AR) to capacitated (2−fold lower), to uncapacitated (2−fold lower) sperm. Further in AR sperm CASK, which in uncapacitated sperm is located in the PPP only, extends to the midpiece where its partners CD9, JAM−A,and αv reside.
Conclusion: The data suggest that JAM−A is phosphorylated by its interaction with CASK. As phosphorylated JAM−A is engaged in the activation of MAPK/ERK signaling and ERK signaling is involved in sperm function, our study identifies JAM−A, CASK, and CD9 as upstream components of the ERK pathway controlling motility, capacitation, and the acrosome reaction induced by Ca2+ ionophore.
Funded by NIH−RO3 HD073523 and NIH−5P20RR015588 to PAM−D.

ABSTRACTS

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SPERM MORPHOLOGY USING A NOVEL DICHOTOMOUS KEY ALGORITHM IMPROVES ANALYSIS STABILITY, REPRODUCIBILITY AND TEACHABILITY

Anna−Marie Bort, Susan A. Rothmann, PhD, John R. Quigley, BS and Robin L. Pillow, BS
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(Presented By: Anna−Marie Bort)

Introduction and Objectives: Most Strict morphology results show few normal sperm even in fertile men. In many centers, morphology no longer has predictive value for ART. Proficiency test data show variation exceeding acceptable and useful limits. The WHO 5th edition Semen Analysis Manual adopted the Strict criteria, but their reference limits are much higher than many labs upper values. Lack of a standardized method for applying sperm classification criteria results in many different subjective interpretations of normal and makes it difficult to teach. Our objective was to develop a rational, repeatable method to apply classification criteria that would be easy to learn.
Methods: Surveyed 99 international experts on classification of 155 sperm and analyzed entropy (agreement). Reviewed photos and definitions of normal, borderline and abnormal sperm from atlases and publications. Based on these and established methods of pathology and taxonomy classification, we developed a dichotomous key algorithm with 12 queries of sperm features. Borderline normal forms were classified as a separate category using definitions from Menkveld 1990. 782 archived semen smears were analyzed for % normal with the algorithm and compared to original subjective method values.
Results: Strict % normal median with the algorithm was 18%, compared to original 4% (WHO 5th reference medians for unscreened men 14%, fertile fathers 15%). The distribution of values of 782 smears was comparable to the WHO 5th reference ranges with less than 10% of the values in the 5th centile (<4% normal Strict morphology) and a median of 20%. Regression analysis of 180 samples showed excellent inter−observer correlation with a correlation coefficient of 0.9. The method was stable over 8 months of analysis with a trend line slope of 0. An unexpected benefit was a 50% reduction in analysis time. Because borderline sperm are classified independently, the algorithm can be used to determine % normal for Traditional and Strict morphology schemes simultaneously. The algorithm was taught at two American Society of Andrology Lab Workshop where participant surveys stated the method was easy to use and adopt.
Conclusions: This novel morphology algorithm provided repeatable and stable results, with values and distributions similar to WHO 5th reference ranges. The method reduces ambiguity, decreases analytic time and reduces subjectivity.
Funding: NIH Grant R43 HD044383−01 and NIH Life Study

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COMPARISON OF SPERM CONCENTRATION AND ANALYSIS TIME USING SPERMOCYTOMETER® AND IMPROVED NEUBAUER HEMACYTOMETER COUNTING CHAMBERS

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(Presented By: Robin Pillow, BS)

Introduction and Objective: Sperm counting is an essential component of semen analysis. The improved Neubauer hemacytometer is a counting chamber intended for use with blood, but often is used for semen analysis. Its 100 micron depth allows sperm to be found in multiple focal planes and it requires dilutions, both sources of significant error. It is reusable and must be cleaned, disinfected and examined for contaminants. The Spermocytometer® (Leja Netherlands) is a disposable counting chamber specifically designed for sperm counting without dilution. Its 20 micron depth keeps sperm in a single plane. Our objective is to compare sperm concentration obtained with Spermocytometer® and hemacytometer.
Methods: Sperm counts were obtained with both chambers from 40 discarded clinical semen samples and sperm quality control reagents from 11 different lots for a total of 95 data points. For the hemacytometer, concentration was determined from two dilutions analyzed in duplicate using the method described by WHO 4th Edition Manual on the Examination of Human Semen, 1992. For the Spermocytometer®, the samples were loaded directly into the chamber without diluting. Sperm were counted with the aid of a 10 X 10 eyepiece reticle grid with 100 squares total, 1mm x 1mm each (Klarmann Rulings, KR−406B). All sperm in one grid were counted, then the stage was moved to acquire five grid counts total. Concentration was calculated from the average number of sperm per square multiplied by the measured scaling factor of the reticle. The concentrations were compared and correlation coefficient computed.
Results: The differences between concentration from Spermocytometer® and hemacytometer were not significant. The correlation coefficient value was 0.99685.
Conclusion: The Spermocytometer® consistently produced the same answer as the hemacytometer for each sample across a wide range oßf sperm counts observed in routine lab practice. This suggests that the Segre−Silberberg effect reported for similar chambers was negligible and undetectable. The hemacytometer requires cleaning and disinfection (2 min), checking for contaminants (2 min), making and counting duplicate dilutions (3−4 min) and time for the specimen to settle (5 min). The Spermocytometer® requires a 2−3 minute wait before analyzing to allow time for the sperm to stop drifting. Using the Spermocytometer® saved approximately 8 to 10 minutes per analysis.
Funding: Fertility Solutions Inc.

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CONTENT OF TESTIS−SPECIFIC ISOFORM OF NA/K−ATPASE IS INCREASED AND RAFT− AND NON−RAFT POOLS OF THIS PROTEIN ACTIVATE SPECIFIC SIGNALING PATHWAYS DURING BOVINE SPERM CAPACITATION

Gayathri Devi Rajamanickam, John Kastelic, DVM, PhD, Dip ACT[1] and Jacob Thundathil, DVM, MVSc, PhD[2]
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(Presented By: Gayathri Devi Rajamanickam)

Introduction: Capacitation comprises a series of structural and functional modifications in sperm, enabling fertilizing ability. In our previous studies, we demonstrated that incubation of bovine sperm with ouabain (a specific ligand for Na/K−ATPase) induced capacitation through a mechanism involving kinases and redistribution of ATP1A4, the testis−specific α4 isoform of Na/K−ATPase. The aim of this study was to investigate the mechanisms by which multiple signaling pathways are activated during ouabain−induced capacitation. Previous studies in somatic cells demonstrated that Na/K−ATPase interacts with lipid rafts during cell signaling. Furthermore, lipid rafts are present in bovine sperm. Therefore, we hypothesized that lipid rafts serve as a signaling hub for ATP1A4, facilitating activation of multiple signaling molecules during ouabain−induced sperm capacitation.
Methods: Raft and non−raft membrane fractions were prepared from fresh sperm collected from mature Holstein bulls using a non−detergent−based approach. Both fractions were characterised for their total content of protein and cholesterol, as well as morphology, fatty acid profile, and the presence of raft and non−raft markers.
Results: Under capacitating conditions (incubation of sperm with ouabain), content of ATP1A4 increased in the raft and non−raft fractions compared to uncapacitated sperm; these capacitation−associated increases in ATP1A4 were confirmed by immunoblotting (soluble sperm proteins) and flow cytometry−based approaches. That content of ATP1A4 remained similar in detergent−insoluble sperm protein fractions from capacitated and uncapacitated sperm excluded the translocation of this protein from subcellular compartments to the sperm membrane. Raft and non−raft membrane fractions differed in the relative content of phosphorylated signaling molecules. Whereas raft fractions predominantly contained phosphorylated caveolin−1 and Src, non−rafts fractions contained phosphorylated forms of ERK1/2 and EGFR.
Conclusion: In conclusion, we inferred that content of ATP1A4 increased during bovine sperm capacitation and that raft and non−raft pools of ATP1A4 may regulate distinct downstream signaling events leading to sperm capacitation. This study received funding from NSERC.

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ALOE VERA SP. IS AN ACCEPTABLE ALTERNATIVE TO EGG YOLK FOR PRESERVING CANINE SEMEN AT 5ºC − PRELIMINARY RESULTS

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(Presented By: Erika Oliveira, PhD)

Introduction: Diluents containing egg yolk are the most practical for preserving semen at low temperatures. However, due to the recent requirements for disease control and security with biological products, it has been suggested that animal products be eliminated from diluents used for semen conservation. Few studies have been performed on the effect of Aloe vera sp. in ram and goat semen. To date, in vitro evaluation of dog semen after cooling with use of Aloe vera sp. has not been studied.
Methods: Therefore, this study assessed the effect of 5 % (wt/vol) Aloe vera sp. in a Tris extender (T1) or in a coconut water powder extender (ACP−101) (T2) for preservation of dog semen at 5°C. The control group received Tris 20 % (vol/vol) egg yolk extender. For this, 3 ejaculates from 5 male dogs (1 ejaculate/week/dog) were used. Ejaculates were stored at 5°C. Kinetic parameters (curvilinear velocity – VCL; linear velocity – VSL; mean velocity – VAP, and linear coefficient – LIN), total motility (TM), and sperm membrane integrity (SMI) evaluated by fluorescent probes (CFDA/PI) were assessed at 0, 24, 48 and 72h after cooling.
Results: Before cooling, TM (%) for control, T1 and T2 was 67.9±19.9, 53.9±18.3 and 48.6±18.2, respectively, and control had the best average values from this time (P=0.019) to the end of the study. Treatments with Aloe vera sp. did not differ between each other through the study. Regarding kinetic parameters, after 72h of storage, it was observed that Control had the best values for VAP, when compared to the other treatments (P<0.05), and was similar to T1 for VCL and to T2 for VSL and LIN. These parameters are important for the progression of spermatozoa into cervical mucus and the penetration of zona pellucida of oocytes. Control also revealed best values (53.2±1%) for membrane integrity when compared to T1 (43.4±1%) and T2 (46.5%±1) during the 72h of storage (P=0.0001). To our knowledge, this is the first report regarding the use of Aloe vera sp. as a substitution for egg yolk in Tris and ACP−101® for preserving chilled dog semen.
Conclusion: According to our results, egg yolk still has the best characteristics for preserving the viability of chilled semen but the results observed with Aloe vera sp. are within the normal range for fertility in this species, we suggest that it can be used as a substitute for egg yolk for preserving dog semen for 72h at 5ºC.

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CALCIUM KINETIC IN BOVINE SPERMATOZOA ALTERED BY INHIBITION OF PHOSPHODIESTERASE

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(Presented By: Anthony Laroche, BScA Agr)

Introduction and Objective: Cyclic adenosine monophosphate (cAMP) is a second messenger having high physiological relevance in sperm functions such as motility, capacitation and acrosome reaction. Phosphodiesterases (PDE) are the enzymes involved in cyclic nucleotides degradation. So, we hypothesized that PDE are actively involved in sperm physiologic response. Eleven PDE families are found in mammals with different affinities for cyclic nucleotides and PDE inhibitors. However, still not much is known in term of regulation and contribution of PDE in bovine sperm physiological functions. The objective of this research project is to study the effect of a specific PDE10 inhibitor on capacitation in bovine sperm.
Methods: Freshly ejaculated bovine sperms were provided by the CIAQ (Centre d'insémination artificielle du Québec). The semen has been washed twice in Tyrodes HEPES−buffered medium (spTALP−H−PVA) and incubated (5% CO2, 37°C) for 5 hours in spTalp−BSA (6mg/ml) in presence of either a specific PDE10 inhibitor (papaverine) or a non−selective PDE inhibitor (IBMX, 3−Isobutyl−1−methylxanthine).

Results: The semen motility and progressive motility were determined by a Computer Assisted Semen Analysis (CASA) and no difference was observed between treatments. To assess calcium's (Ca) management into the sperm, the response to thapsigargin (TG), a non−competitive inhibitor of SERCA pumps (sarcoplasmic/endoplasmic reticulum Ca2+ATPase) was measured, causing Ca depletion of sperm's stores. The fluorescent probe INDO−1−AM was used and two different spectral intensities were measured depending its coupling to Ca. By using flow cytometer, it has been possible to measure several thousand of events of bull ejaculate's response to TG over a period of 7 minutes. To improve the analysis of TG response, we've developed a new approach of plotting Ca kinetic released in a sigmoid curve. Using this curve, it's possible to calculate different time response such as the time needed to reach the plateau of the sigmoid curve. Sperm incubated in control treatment has reached the plateau of the curve significantly latter (242±19 s) compare to papaverine treatment (174±11 s) and IBMX treatment (128±6 s).
Conclusion: In brief, the results show that PDE10's inhibition influence intracellular Ca and its kinetic released in bovine sperm. This new method of analyzing TG response opens on other avenues in the comprehension of sperm's physiology.
Funding: This project was made possible by the contributions of FQRNT, NSERC and L'Alliance Boviteq Inc.

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TWO SIMPLE METHODS THAT DECREASE VARIATION IN SEMEN ANALYSIS RESULTS: LESSONS FROM THE 2013 ANDROLOGY LABORATORY WORKSHOP (ALW) OF THE AMERICAN SOCIETY OF ANDROLOGY

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(Presented By: John R. Quigley, BS)

Introduction and Objectives: Semen analysis proficiency testing reveals variation among laboratories that would be unacceptable for many laboratory tests. Frequently used methods for determining count and motility have imprecision that reduces the value of the test result. The recent 2013 ALW on Semen Analysis Quality Control examined ways to improve precision. To test the effects that simple changes to sperm count and motility methods have on analytic variation.
Methods: Sperm Count: Photographs of a semen sample were created with a superimposed 10x10 counting grid. 18 participants counted the sperm in rows D and G and in all 10 rows. A high resolution video of donor semen was created for projection with a segment of untreated "live"sample and a segment of semen incubated at 56C for 5 minutes to immobilize sperm "immobilized". 15 participants analyzed the video using 3 methods: a) Estimation of % motile after viewing live segment; b) Natation: motile and non−motile sperm were counted while viewing live segment, % motile was calculated by dividing number motile by sum of motile and non−motile, multiplied by 100; c) Static: non−motile sperm were counted in live segment, then all sperm were counted in the immobilized segment, the % motile was calculated by subtracting number of non−motile sperm from total immobilized sperm to determine number of motile sperm, divided by the total immobilized sperm, multiplied by 100. To compare the variation between/among the methods, standard deviation (SD) coefficient of variation (CV) were calculated for each set of results.
Results: CV Count: Row D 37%, Row G 26%, average rows D, G 28%, 10 rows 13%. CV Motility: Estimation 21%, Natation 8%, Static 7%.
Conclusions: Counting all rows reduced sperm count CV by 2/3. Row selection influenced CV. Averaging 2 rows did not reduce CV. Objective methods for motility reduced CV by over 50%. Natation and Static results were not different, but most participants reported that Static was easier. The exercises demonstrated practical ways to reduce variation and improve precision.
Supported by American Society of Andrology

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TERMINALLY DIFFERENTIATED, POST−PUBERTAL RAT SERTOLI CELLS RESUMED PROLIFERATION AFTER TRANSPLANTATION.

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(Presented By: Jannette Dufour, PhD)

Introduction and Objective: The current dogma that post−pubertal Sertoli cells (SC) are terminally differentiated and do not proliferate, has recently been challenged suggesting that mature nondividing SC can be reprogrammed to proliferate. We have observed proliferation of SC isolated from post−pubertal rat testes after transplantation. The objective of the current study was to confirm and quantify this observation.
Methods: In this study, nondividing SC isolated from 23−27 days−old post−pubertal rats were transplanted underneath the kidney capsule of NOD scid gamma (NSG) mice or Lewis rats that were injected with 5−bromo−2'−deoxyuridine (BrdU; to label proliferating cells) or saline daily. After 10 days graft−bearing kidneys, testis, spleen and intestine were collected and tissue sections were double immunostained for Wilms' Tumor 1 (WT1; a SC marker) and BrdU.
Results: WT1 positive SC within the grafts were positive for BrdU. Germ cell within the testis and cells within the spleen and intestine were also positive for BrdU, while SC within the testis were negative for BrdU. Quantification of BrdU labeled SC demonstrated that 7.4% and 9.2% of the total transplanted SC within the grafts were proliferating in NSG mice and Lewis rats, respectively. Interestingly, the percentage of BrdU positive SC was lower when SC were arranged in tubules compared to SC located randomly outside of the tubules.
Conclusion: These data indicate that nondividing SC resumed proliferation after transplantation, and further validates previous findings that SC are not terminally differentiated. Transplantation of SC could provide a useful model to study the regulation of SC proliferation in vivo.

ABSTRACTS

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RNA POLYMERASE II PAUSING IS CRITICAL FOR SPERMATOGENESIS AND MALE FERTILITY

Prabhakara Reddi, PhD
(Presented By: Prabhakara Reddi, PhD)

Introduction: Successful completion of spermatogenesis relies upon precise spatiotemporal expression of distinct subsets of differentiation markers within the seminiferous epithelium. Failure to express genes at the correct time leads to arrested spermatogenesis and male infertility. The transcriptional mechanisms regulating this process, however, are not understood. Our work has established that RNA Pol II pausing is critical for maintaining the timing of gene expression during spermatogenesis. Paused RNA Pol II at the promoter ensures precise and rapid onset of gene transcription. This mechanism is particularly relevant to spermatogenesis wherein synchronous transcription of cohorts of genes is critical for morphogenesis and differentiation. We have identified the TAR DNA binding protein of 43 kDA (TDP−43) as a key player in maintaining paused pol II at a target gene promoter in germ cells. TDP−43 is evolutionarily conserved and highly expressed in mouse and human testis. Here we report that TDP−43 is essential for spermatogenesis.

Methods: Conditional knockout of TDP−43 in germ cells or Sertoli cells led to maturation arrest and male infertility. Loss of TDP−43 in spermatogonia, induced by the Stra8−iCre deleter, led to failure of entry into meiosis. AmhCre−induced loss of TDP−43 in Sertoli cells caused qualitative changes in spermatogenesis. While it is well−known that germ cells express genes in a precise spatiotemporal pattern, work from several laboratories established that Sertoli cells also express genes in accordance with the stage of the seminiferous epithelium. We are testing the hypothesis that Pol II pausing is critical for maintaining the timing of gene expression in these cells and that loss of TDP−43 disrupts this in a subset of genes poised for transcription.

Results: We report that TDP−43 binds to NELF, a critical component of pol II pausing and predict that mechanistically, TDP−43 guides the sequence−specific recruitment of the pause machinery.

Conclusion: This work explores a transcriptional mechanism that likely regulates the expression of a third of all genes expressed in the seminiferous epithelium, as it does in the embryo. Study of TDP−43 is highly relevant clinically because abnormal TDP−43 function is linked to a number of neurodegenerative disorders. Our future work will determine if male infertility also falls under TDP−43 proteinopathies.

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POTENTIAL GENETIC BIOMARKERS IN AZOOSPERMIA BY MICROASSAY STUDIES: NEW DIMENSION IN THE EVALUATION

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(Presented By: Vasan Srini, DNB, Fellowship – Andrology)

Introduction and Objectives: To identify potential biomarkers for azoospermia by establishing the expression patterns of genes. Our objective is to derive a novel set of candidate biomarkers for non−obstructive azoospermia (NOA) and determine a threshold for the 'reliability' of the score, which might help in identification of the potential markers.

Methods: Microarray experiments.

Results: Very high number of genes present in NOA, with ≥40 score and those present in normal testis, with ≥ 6 score, were reproduced by the microarray experiment. Genes differentially expressed with fold change >2 were identified (summarized in the table below):

Hybridization (conditions compared)	No. of genes	
	Up−regulated in condition 1	Down−regulated in condition 1
NOA vs. OA	541	557
NOA vs. Normal	1530	2093
OA vs. Normal	433	698

A new scoring system was followed which was efficient in determining the percentage overlap and the potential markers, wherever needed the consensus was derived from gene−lists across studies. Any block with a percentage value, greater than the expected random chance of occurrence, is considered as reliable block, from which the potential markers can be identified. We developed a new method to derive a more reliable expression pattern of genes, using the existing mass−scale data − from one tissue and condition at a time. The approach involved biocuration, development of a database, and deriving a consensus expression pattern across 'comparable' multiple studies for each gene. The new database and associated software serve as a 'gene expression prediction platform' which performed better than any other system in providing straight forward expression information for randomly selected genes.

Conclusions: A) The gene expression platform for mammalian testis with silico analysis provides highly reliable information with higher reliability, as per the database, were repeated frequently in the experimental data set for similar conditions. B) The analysis of the experimental results also indicated a threshold level for the reliability of the score. C) New sets of potential biomarkers identified are very promising as they contain many novel genes which could be useful for basic research.

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THE HISTONE H3 DEMETHYLASE, KDM1A IS ESSENTIAL FOR THE DIFFERENTIATION OF SPERMATOGONIA AND THE SURVIVAL OF SPERMATOGONIAL STEM CELLS.

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(Presented By: Romain Lambrot, PhD)

Introduction: Spermatogenesis is a highly complex cell differentiation process fueled by spermatogonial stem cells (SSCs). The progression from a spermatogonial stem cell to a differentiating cell involves gene expression changes that are under epigenetic control. Epigenetic mechanisms governing gene expression involve histones and their modifiers which add and remove permissive or repressive marks from histone tails. The histone demethylase KDM1A removes gene−activating methylation on histone H3 at lysine 4 (K4). KDM1A can be associated with other histone modifiers such as the histone deacetylase 1 (HDAC1), which removes gene activating H3 acetylation, forming a protein complex that will induce the silencing of the chromatin. We had previously observed that KDM1A was present in SSCs, hence we hypothesized that this protein serves in the epigenetic regulation of SSCs biology.

Methods: To determine the function of KDM1A in SSCs we generated mice with a conditional knockout of Kdm1a (cKO) specifically in spermatogonia.
Results: Analysis of the cKO revealed that KDM1A is essential for spermatogenesis, as adult cKO males were sterile and lacked germ cells. Testes were collected from cKOs at post−natal days (PND) corresponding to the appearance of spermatogonia (PND6) and meiotic cells (PND10). At PND6, spermatogonia were still present in the cKOs, however, at PND10 very few cells with an abnormal morphology were observed in place of preleptotene spermatocytes. Moreover from PND10 to 21 the number of spermatogonia in the cKO testes decreased dramatically with no germ cell remaining at PND21. To understand what mechanisms were behind the disappearance of spermatogonia and the almost complete absence of meiotic entry, we analyzed the global epigenetic profile of germ cells in the cKO. At PND6, the cKO spermatogonia presented higher levels of H3K4 di−methylation and H3 acetylation as determined by immunofluorescence. We then used RNA−sequencing to examine how KDM1A loss alters the gene expression profile of isolated SSCs and the analysis of this data is in progress.
Conclusion: These results suggest that without KDM1A the epigenome of the spermatogonia is altered and indicate that KDM1A is a master epigenetic regulator of SSCs required for SSCs survival and spermatogonia differentiation.

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SERTOLI CELLS ENFORCE PERITUBULAR MYOID CELL FATE AND ARE ESSENTIAL FOR DIFFERENTIATION, PROLIFERATION AND RETENTION OF ADULT LEYDIG CELLS

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(Presented By: Lee Smith, BSc, PhD)

Introduction: The ageing of western societies, and the associated increase in obesity, brings with it an increase in prevalence of disorders such as cardiovascular disease, diabetes, loss of bone density, muscle strength, libido and erectile dysfunction, which are associated with reduced androgen levels in men. As Leydig cells (LCs) are the source of androgens in men, establishing the mechanisms which control LC development and function is crucial to our understanding of ageing and male health. Sertoli cells (SC) regulate testicular fate in the differentiating gonad and are the main regulators of spermatogenesis in the adult testis; however, their role during the intervening period of testis development, and in particular during adult (A)LC differentiation and function remains largely unknown.
Methods: To determine whether SCs are involved in LC development two transgenic mouse models were generated which allowed controlled, cell−specific ablation of SCs in pre− and postnatal life.
Results: Results show that the SCs are required: (i) to enforce a myoid fate on peritubular cells (PTMC) in prepubertal life (ii) to maintain the ALC progenitor cell population in the postnatal testis (iii) for development of normal ALC numbers and (iv) for retention of normal ALC numbers in adulthood. Furthermore, our data shows that fetal LCs function independently from SC, germ cell or PTMC support in the prepubertal testis.
Conclusion: Together these findings describe a new paradigm, which encompasses SC−mediated control of ALC development and function and has significant implications for our understanding of both male reproductive disorders, and wider androgen−related conditions affecting male health.

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E2A AND HEB REGULATE SERTOLI CELL FUNCTION AND FERTILITY IN THE MOUSE

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Introduction and Objective: Spermatogenesis requires the support of Sertoli cells, which are the only somatic cell population in direct contact with developing germ cells. The Sertoli cell lineage is specified in the embryonic gonad and the population expands in number until early postnatal life in most mammalian species. In the testis of adult animals, Sertoli cell number is stable and the ratio of germ cells per Sertoli cell is fixed. Although it is well established that Sertoli cell functions and absolute number are crucial for spermatogenesis, the molecular mechanisms governing their proliferation and maturation remains unclear. E proteins (E2A, HEB and E2−2) are basic Helix−loop−helix (bHLH) factors that have important roles in cell differentiation and proliferation. Results of previous studies revealed that Sertoli cells express E2A and HEB; however, the functional role of these factors is unknown. The overall aim of this study was to determine whether E2A and HEB play an important role in function of Sertoli cells.
Methods: To achieve this, conditional knockout mouse models were generated using mice bearing E2A/HEB floxed alleles and a Sertoli cell specific Amh−Cre transgene. Neither testis weight nor fertility was altered in mice with single inactivation of E2A or HEB compared to littermate controls. However, double deletion of E2A and HEB resulted in a sub−fertility phenotype.
Results: At postnatal week 8, testis weight of E2A and HEB double knockout animals was significantly reduced compared to littermate controls and epididymal sperm count was decreased by more than 50%. Moreover, significant reductions in both Sertoli cell and spermatogonial numbers were found in the double knockout animals, which was likely the underlying cause of reduced sperm output and the sub−fertile phenotype. Lastly, examination of testes at postnatal weeks 3 to 4 revealed a significant reduction in testis weight and delayed emergence of elongate spermatids for the double knockout males. Further assessment of testis weight of control, single knockout and double knockout animals suggested that E protein dosage not identity is the important factor.
Conclusion: Collectively, these findings indicate that normal maturation of the Sertoli cell population during postnatal development is influenced by the transcription factors E2A and HEB.
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REGULATION OF THE PROLIFERATION AND DIFFERENTIATION OF ADULT LEYDIG STEM CELLS

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ABSTRACTS

Introduction: New Leydig cells appear in the adult rat testis after the pre−existing adult Leydig cells are eliminated with ethane dimethanesulfonate (EDS). PDGFRα+ cells were purified from the testes of adult Brown Norway rats after the animals received EDS. Depending upon culture conditions, these cells proliferated indefinitely or differentiated and produced testosterone, suggesting that the cells might be stem cells. In a second study, seminiferous tubules were isolated from the interstitium of Leydig cell−depleted testes. Culture of the tubules for one week resulted in a peak of cell division on the surface of the tubules, and then a return to basal division levels by week 2. With culture from weeks 2−4, 3βHSD+ cells appeared on the surface of the tubules, and testosterone was detected in the culture medium. These results suggest that there are stem cells on the surfaces of the tubules that divide and then cease dividing, and that the products of the divisions then give rise to the newly formed adult Leydig cells.
Methods: To begin to identify how Leydig stem cells are regulated, we screened 35 factors or their signaling molecule modulators for their effects on the division or differentiation of the stem cells, using the in vitro tubular culture system.
Results: Desert Hedgehog (DHH), PDGF−BB, FGF−2, activin, PDGF−AA, IL−1β, TGF−α and IGF−1 had stimulatory effects on cell proliferation. DHH, PDGF−AA, and inhibin had positive effects on cell differentiation. Wnt signaling inhibited cell differentiation while TGF−β inhibited both cell division and differentiation. Intriguingly, although both PDGF−AA and –BB had stimulatory effects on cell proliferation, they had completely opposite effects on the differentiation of the cells. PDGF−AA induced the cells to enter the Leydig lineage while PDGF−BB blocked the process. Interestingly, PDGF−BB may induce the cells to enter the myoid cell lineage.
Conclusion: These results suggest that Leydig stem cells may in fact be multi−potent cells, serving as the common stem cells of both Leydig and myoid cells. The use of the seminiferous tubule culture system has promise to be a good tool to examine Leydig stem cell niche and their functions despite the complexity of the tissue.
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IMPORTIN PROTEINS IN SPERMATOGENESIS AND SPERM

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(Presented By: Kate Loveland, PhD)

Introduction and Objective: Importin family proteins were initially identified by their classical nuclear import functions in which they ferry cargo containing a nuclear localization signal (NLS). The importin α proteins are adaptors that link a cargo protein to the importin β1 for transit through the nuclear pore into the nucleus. Importins are essential for cell viability and development, including gametogenesis (Miyamoto et al, 2012, 2013 BBA), and we documented the differential synthesis of many importin proteins throughout spermatogenesis in rodent and human testes (e.g. Whiley et al, 2012 Int J Androl). To discover functions of individual importins, we undertook to (1) measure their stoichiometry during successive stages of spermatogenesis, (2) identify specific cargoes for individual importin α proteins, and (3) determine the cellular distribution of importin proteins in mature spermatozoa.
Methods: Relative amounts of importins α2, α3, α4 and β1 were measured in spermatocytes and round spermatids isolated (n=3) from adult Sprague Dawley rats by elutriation and Percoll gradients to >90% purity. Western analysis of cell lysates run alongside known amounts of recombinant importin proteins was used to estimate importin levels.
Results: We calculated that importin α2 levels were 2−fold higher in spermatocytes than in spermatids, whilst importins α4 and β1 levels did not differ. Next, to find binding proteins for individual importins in rat spermatocyte and spermatid lysates, we used recombinant importin α2 and α4 proteins for pull−down experiments followed by a comprehensive proteomics analysis. Amongst the 100 candidates, only 42 had a strong classical NLS, while 8 nuclear proteins had none, indicating importin αs bind to non−canonical sequences in many proteins. In addition, these new and our previous data (Miyamoto et al 2013) reveal importin binding partners that are not nuclear proteins, but are instead typically found in mitochondria or vesicle−associated. We next performed indirect immunofluorescence to localize importin proteins in formalin−fixed cauda epididymal sperm from C57BL6 mice. Antibodies to IMPα2, IMPα3, IMPα4 and IMPβ1 each bound distinct functional regions in mature spermatozoa.
Conclusion: Individual importin protein levels are tightly regulated for distinct roles during spermatogenesis. We propose they deliver cargo proteins to non−nuclear compartments during assembly of mature spermatozoa.

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MITOCHONDRIAL METABOLIC ACTIVITY ASSISTS WITH REGULATION OF STEROID PRODUCTION IN MA−10 MOUSE LEYDIG CELLS

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(Presented By: Malena Rone, PhD)

Introduction: Mitochondria are home to many cellular processes, including oxidative phosphorylation, fatty acid metabolism, and in steroid synthesizing cells, cholesterol import and metabolism to pregnenolone. The formation of macromolecular protein complexes aids in the regulation and efficiency of these mitochondrial functions, though due to their dynamic nature are hard to identify.
Methods: To overcome this problem we utilized Blue−Native polyacrylamide gel electrophoresis (BN−PAGE) coupled to mass spectrometry on isolated mitochondria from control and hormonally stimulated mouse MA−10 Leydig cells.

Results: The data obtained identified the presence of a number of qualitatively similar mitochondrial protein machineries, under control and hCG−stimulated conditions. In addition, quantitative differences were observed in mitochondrial complex formation after hormone stimulation as compared to control cells. A prominent decrease of mitochondrial proteins involved in fatty acid import into the mitochondria was observed. From these results we implied that mitochondrial β−oxidation is not essential for steroidogenesis. To confirm this we inhibited fatty acid import utilizing the carnitine palmitoyltransferase Ia (CPT1a) inhibitor etoxomir, resulting in an increase in steroid production after 24 hour incubation of the cells with the drug. Moreover, etoxomir induced a decrease in oxygen consumption with an increase in extracellular acidification, confirming the inhibition of β−oxidation. A shift towards glycolysis with no observed lost ATP production was also observed.
Conclusion: These results suggest that changes in the metabolic profile of the mitochondria in steroidogenic cells can function as a potential regulator in cholesterol import and steroid production. We propose that upon hormonal stimulation, the mitochondria efficiently import cholesterol at the expense of other lipids necessary for energy production resulting in their specialization for steroid biosynthesis.

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THE ANCIENT AND EVOLUTIONARILY CONSERVED REGULATORS OF PROTEIN PHOSPHATASE PP1, PPP1R2, PPP1R7, AND PPP1R11, ARE EXPRESSED AS TESTIS−SPECIFIC ISOFORMS DURING SPERMIOGENESIS.

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(Presented By: Nilam Sinha)

Introduction and Objective: Two of the four Ser/Thr phosphatase type 1 (PP1) isoforms, PP1γ1 and PP1γ2, are alternate spliced products derived from one gene, Ppp1cc. Their amino acid sequences are identical except at the extreme C−termini. PP1γ1 is ubiquitous whereas PP1γ2 is highly abundant in testis. PP1γ2 isoform is present only in mammals. Knock out of Ppp1cc, which eliminates both PP1γ1 and PP1γ2, results in male infertility. Expression of PP1γ1 in testis, using transgenic approaches, is not as effective as PP1γ2 in restoring male fertility in Ppp1cc null mice. Thus PP1γ2 appears to have an isoform specific role in supporting normal sperm motility and male fertility. Three PP1regulators, PPP1R2, PPP1R7, and PPP1R11 have been proposed to be present or identified in sperm. Based on their roles in regulating PP1 in somatic cells they are suggested to bind to and regulate sperm PP1γ2. The purpose of this study was to characterize sperm PPP1R2, which was first described using indirect biochemical approaches.
Methods: We were able to demonstrate by western blot and mass spectroscopy that PPP1R2 is present in spermatozoa.
Results: We discovered that an alternatively spliced message for PPP1R2 coding for a unique isoform that is abundant in testis. Amino acid sequencing identified the unique C−terminus of this PPP1R2 isoform in testis extracts. The message for this PPP1R2 isoform is present at high levels during spermiogenesis and in adult testis. Surprisingly, we also found that the other two PP1 inhibitors, PPP1R7 and PPP1R11, are expressed as testis−specific isoforms. The temporal patterns of expression of these two proteins also parallel that of PPP1R2 and PP1γ2 in testis. Testis PPP1R7 has a unique C−terminus due to alternate splicing, while testis PPP1R11 has a unique N−terminus due to an alternate transcription start site.
Conclusion: High levels of the three ubiquitous inhibitors expressed as testis−specific isoforms suggest involvement of these proteins in the isoform specific role of PP1γ2 in supporting normal sperm function and male fertility in mammals.
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NON−STEROIDAL LIGANDS OF THE CHOLESTEROL RECOGNITION AMINO ACID CONSENSUS (CRAC) MOTIF OF THE 18−KDA TRANSLOCATOR (TSPO) PROTEIN AND THEIR EFFECTS ON STEROID HORMONE BIOSYNTHESIS

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(Presented By: Andrew Midzak, PhD)

Introduction: Steroid hormone biosynthesis by mammalian gonads and adrenals is dependent on translocation of cholesterol across the double membranes of the mitochondria and delivery to the cytochrome P450 enzyme CYP11A1, where it is metabolized to pregnenolone, the metabolic precursor of all steroids. The 18−kDa translocator protein (TSPO), a high−affinity drug−binding integral membrane protein, located in the outer mitochondrial membrane has been implicated in this cholesterol delivery process. In addition to its drug−binding ability, TSPO is also a high−affinity cholesterol−binding protein, through a conserved Cholesterol Recognition Amino Acid Consensus (CRAC) motif located at its C−terminus. To better understand the possible roles TSPO and its CRAC motif may play in steroidogenesis, we have previously identified and validated a novel CRAC motif ligand, 5−androsten−3β,17,19−triol, which was able to inhibit hormone− and drug−mediated steroidogenesis. However, a non−steroidal ligand targeting TSPO's CRAC motif would be of interest both in increasing our molecular understanding of this protein motif and as a lead compound in the development of novel drugs for the treatment of diseases of steroid excess.

Methods: In this study, we computationally constructed a pharmacophore model of TSPO and its CRAC motif and utilized structure−based virtual screening identify CRAC−binding structures from approximately 11 million small molecular structures available from structural databases. The biological activity of the top−scoring identified molecules was subsequently tested in the TSPO−rich, hormone−responsive MA−10 mouse tumor Leydig cell line and constitutively steroidogenic R2C rat tumor Leydig cell line.
Results: A series of compounds was identified capable of inhibiting with nanomolar potencies steroid production in both of these cells. This inhibition was localized to the delivery of cholesterol to CYP11A1 in the mitochondrial matrix, as the cells retained the ability to synthesize steroids when supplied with 22R−hydroxycholesterol, a water−soluble cholesterol analog which bypasses the mitochondrial cholesterol−transfer step.
Conclusion: These results identify a novel family of non−steroidal compounds targeting TSPO's CRAC domain and potently inhibiting steroidogenesis, a family which may serve as useful tools in the study of TSPO function and steroidogenesis, as well as prove effective lead compounds for the development of drug treatments for maladies of steroid imbalance.

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HUSP26 EXPRESSION AND RELATIONSHIP TO ANDROGEN RECEPTOR IN NORMAL HUMAN TESTIS

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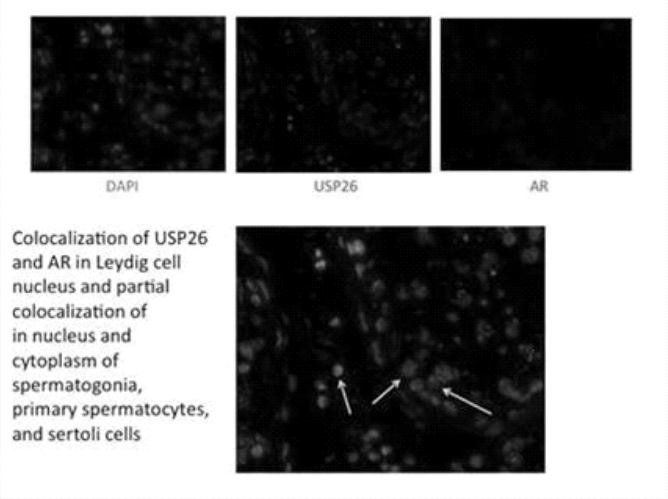
Introduction: Human ubiquitin specific protease 26 (hUSP26), an X−linked gene, is associated with male infertility and low testosterone production. hUSP26 has been recognized as a regulator of androgen receptor (AR) hormone−induced action involved in spermatogenesis and steroid production in in vitro studies. The goal of this study was to determine cellular localization of hUSP26 expression in normal human testis and its relationship to AR expression.
Methods: 3 patients with obstructive azoospermia at our institution had frozen testicular specimens available for measurement of hUSP26 and AR mRNA levels using multiplex qRT−PCR with LightCycler 480 (Roche). TATA−binding protein (TBP) was utilized for relative quantification and expression ratios were corrected with standard curves. Immunofluorescence colocalization studies were performed with paraffin−embedded and frozen tissues using primary and secondary antibodies to detect hUSP26 and AR protein expression.
Results: hUSP26 mRNA and AR mRNA is expressed in normal human testis. In normal human testis, USP26 and AR were colocalized in the Leydig cell nucleus with less in Leydig cell cytoplasm, spermatogonia, primary spermatocytes, and Sertoli cells (Figure).
Conclusions: USP26 mRNA expression and AR mRNA expression is present in human testis. USP26 colocalization with AR in Leydig and Sertoli cells, and early cells of spermatogenesis demonstrates possible interaction between these proteins in normal testis. The mechanism and implications of USP26/AR interaction in testis requires further study.



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ROLE OF ATAD3 IN THE HORMONE− INDUCED ER−MITOCHONDRIAL ORGANIZATION IN HORMONE−INDUCED LEYDIG CELL STEROIDOGENESIS

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Introduction: Leydig cell steroid formation is a multi−step process initiated in mitochondria, using cholesterol coming from intracellular stores, and finalized in the endoplasmic reticulum (ER). Cholesterol transfer from outer mitochondrial membrane (OMM) to CYP11A1 in the inner MM (IMM) is the rate−limiting step of this process and is dependent on the organization of the contact site formation. Studies on the characterization of the different proteins involved in this process, demonstrated a crucial role of the AAA+ATPase ATAD3 both in the regulation of cholesterol channeling and the integrity of contact site formation. ATAD3 is anchored in the IMM and enriched at OMM−IMM contact sites. The long isoform of ATAD3 possess an N terminus domain with 50 amino acids able to drive the insertion of the protein back into OMM. It is unclear however, whether this domain is involved in the complex making bridges between mitochondria and other cellular organelles, such ER. We hypothesized that the physical association between mitochondria and ER, named mitochondria−associated membranes (MAMs), can potentially regulate hormone−stimulated steroidogenesis.
Methods: Using the MA−10 mouse tumor Leydig cell line as a model and electron and confocal microscopy, we observed a significant increase of MAM formation upon hGC stimulation. MAMs were isolated and characterized with different specific markers such as ACSL4 and calnexin.

Results: Interestingly, we observed an enrichment of the long isoform in MAMs. Silencing ATAD3 resulted in reduced ability to form pregnenolone and progesterone in response to hCG treatment with no effect on 22−R hydroxycholesterol treatment, confirming the role of ATAD3 at the level of cholesterol delivery into mitochondria. Since progesterone is made mainly in the ER, and a profound modification of the mitochondrial inner structure was observed, we suggest that ATAD3 functions not only as a bridge between OMM−IMM but also might be involved in the organization of MAMs. MAMs could allow the transfer of the substrate cholesterol into mitochondria and steroidogenic pathway intermediates out of mitochondria. Deletion of the anchoring ATAD3 N−terminus blocked the hormone−induced steroid formation further supporting this role of ATAD3 in MAM formation.
Conclusion: Taken together, these results suggest a role of ATAD3 as a scaffold protein in the regulation of ER−mitochondria communications in Leydig cells, crucial for the optimal hormone−stimulated steroid formation.

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EFFECTS OF SILDENAFIL ON RAT SPERM DNA INTEGRITY

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Objectives: We evaluated the effects of sildenafil on rat sperm DNA integrity.
Methods: Group A included male Wistar rats (n=8, 8−week old) and served as a control group. Group B included male Wistar rats (n=8, 8−week old) that received daily an oral suspension containing 10 mg/kg of sildenafil for seven weeks. At the age of 15−week−old all rats were killed. Epididymal sperm content (ESC), the epididymal sperm motility (ESM;%), and the % epididymal caudal sperm with fully condensed chromatin (%SCC) was evaluated (Asian J Androl 2011,13:69).
Results: There were no significant differences in ESC or ESM between groups B and A (P larger than 0.05, Wilcoxon test). In contrast mean value of the %SCC was significantly larger in group A than in B (P smaller than 0.05).
Conclusions: The detrimental effect of sildenafil on sperm DNA integrity in the rat model may be attributed to inhibition of PDE5 by sildenafil that activates a nuclear cGMP−dependent protein kinase PKG with an overall detrimental effect on sperm chromatin structure. Furthermore, we may speculate that the effect of sildenafil on sperm DNA is due to the formation of hydrogen bonds between the C=O groups of the molecule of sildenafil and the NH2 group in the guanine moiety of the DNA. The latter hypothesis is very vividly supported by previous studies revealing this mechanism as the responsible mechanism for the interaction between sildenafil with salmon sperm DNA (Biosensors and Bioelectronics 22, 2007, 2471–2477).

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