Title
Endocannabinoids: Discovery of a unique signaling system for regulating sperm function and fertilization.

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basis. However, there was an association of fragmentation within a cohort with increased pregnancy and implantation. Increased fragmentation of the PB may reflect the length of time since PB extrusion, which may in turn be responsible for the variation in developmental potential after ICSI.

Supported by: Section of Reproductive Endocrinology and Infertility.

P-270

The recovery of full-length DAZ mRNA from mature, ejaculated human spermatozoa. D. C. Sprague, P. G. Conley, M. L. Brown, A. R. Ficht, T. J. Kuehl. Scott & White Clin and Texas A&M Univ System Health Science Ctr Coll of Medicine, College Station, TX; Texas A&M Univ System Health Science Ctr Coll of Medicine, College Station, TX; Scott & White Clin, Temple, TX; Scott & White Clin and Texas A&M Univ System Health Science Ctr Coll of Medicine, Temple, TX.

Objective: The Deleted in AZoospermia (DAZ) gene has been implicated as important in spermatogenesis. Reduced fertility in human males has been associated with spontaneous deletions of the portions of the Y-chromosome including regions that contain the DAZ gene and/or other genes of the AZFa, AZFb, AZFc, and AZFd (AZoospermia Factor) regions. Most current molecular diagnostics focus on detecting microdeletions in the genomic complement of DNA. We speculate that point mutations, like microdeletions, can frequently occur spontaneously in the DAZ gene located on the human Y-chromosome and contribute to reduced spermatogenesis. Alternatively, transcripts of the chromosome 3 copy of the DAZ gene may salvage spermatogenesis in the absence of non-expressed DAZ transcripts from the Y locus. To test this hypothesis, we explored methods to extract full-length DAZ mRNA transcripts from ejaculated sperm cells of individual males.

Design: Residues of sperm cells from semen analysis were processed using various sperm separation methods, storage conditions for stabilizing mRNA, and various methods of mRNA separation and purification away from genomic DNA.

Materials/Methods: We extracted RNA from mature ejaculated, spermatozoa separated from other cellular components of the ejaculate using a swim-up technique. RNA was extracted with a method adapted from Chirgwin using guanidinium HCl and multiple precipitation steps in a single container. Because the extraction of RNA without DNA contamination is difficult, we designed primers for our PCR reaction that would not amplify genomic DNA. RT-PCR was performed using MMLV reverse transcriptase coupled with specific PCR primers to produce DAZ-specific transcripts. The resulting cDNA was sized and sequenced.

Results: Ejaculated spermatozoa were found to contain two DAZ transcripts. One was 1062 bp and 1200 bp and in agreement with the putative Y-chromosome DAZ products while the other was 750 bp and displayed sequence homology with the putative chromosome 3 DAZ product. Methods were replicated in 2 samples from 2 different male subjects without a detectable micro-deletion in the coding sequence of Y-chromosome DAZ locus.

Conclusions: These results confirm that messages from both sources of DAZ transcripts can be found in ejaculated sperm cells from individual men. The availability of this noninvasive method will allow further analyses to identify types of mutations that are compatible with normal and reduced levels of spermatogenesis as reflected by characteristics of semen analyses.

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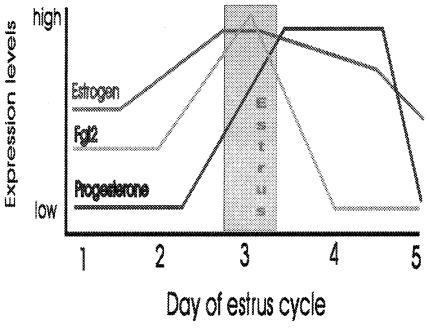
Uterine Fgl-2 expression in the Sprague-Dawley rat estrus cycle correlates with peak estrogen levels. D. F. Rychlik, E. Chien, L. Thomas, D. Wolff, M. Phillippe. Univ of Chicago, Chicago, IL.

Objective: Fgl-2, a novel tissue prothrombinase, activates the conversion of prothrombin to thrombin independent of the coagulation cascade. Fgl-2 expression in the rat uterus is low at mid-gestation, when progesterone is high and up-regulated at the end of gestation when estradiol levels are peaking. To better understand the steroid hormone relationships to Fgl-2 expression, these studies sought to determine Fgl-2 expression during the estrus cycle in the rat.

Design: Reverse transcriptase-polymerase chain reaction (RT-PCR) and protein Western blot studies of Fgl-2 were performed using rat uterine tissue obtained at the various stages of the estrus cycle.

Materials/Methods: Uterine tissues were obtained from spontaneously cycling, adult female Sprague-Dawley rats. Total cellular RNA was isolated using the acidic guanidinium thiocyanate-phenol-chloroform extraction technique. After, DNase treatment, the RNA was utilized for RT-PCR studies using primers for rat Fgl-2. Ethidium bromide stained DNA gels allowed qualitative estimates of Fgl-2 expression at the mRNA level. Proteins from crude homogenates of the uterine tissues were utilized for Western blot analysis of Fgl-2 protein expression using rabbit polyclonal antisera generated against a peptide fragment of rat Fgl-2.

Results: RT-PCR analysis demonstrated expression of Fgl-2 mRNA during all phases of the estrus cycle. Western blot analysis demonstrated that Fgl-2 protein levels underwent significant variation during the estrus cycle: the protein was apparent on the day of proestrus, peaked on estrus, and was undetectable on metestrus and diestrus.



Conclusions: These studies have demonstrated that Fgl-2 protein expression undergoes significant modulation during the estrus cycle in the Sprague-Dawley rat. Fgl-2 protein is up-regulated during estrus, a time of peak estrogen levels, and undetectable during metestrus and diestrus, periods of falling estrogen and increased progesterone in the rat. The regulation of Fgl-2 expression in uterine tissues could occur through modulation of translation and/or protein turnover. The biologic role for the upregulation of Fgl-2, a tissue prothrombinase, in the uterus at the time of ovulation is yet to be defined. We speculate that estrogen dependent uterine up-regulation of Fgl-2 may have a role in embryo implantation.

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P-272

Endocannabinoids: Discovery of a unique signaling system for regulating sperm function and fertilization. L. J. Burkman, H. Schuel, J. Lippes, A. Makriyannis, A. Giuffrida, D. Piomelli. State Univ of New York at Buffalo, Buffalo, NY; Univ of Connecticut, Storrs, CT; Univ of CA, Irvine, Irvine, CA.

Objective: There is currently no known comprehensive regulatory system which might explain such diverse events as inhibition of capacitation in semen, and suppression of sperm motility within the oviductal isthmus, and timed capacitation of sperm in the female reproductive tract. Arachidonylethanolamide (AEA), also known as anandamide, is an endogenous ligand for cannabinoid (marijuana-like) receptors. We have previously shown that AEA can block acrosome reactions (AR) and enhance sperm motility in sea urchins. AEA is also produced in the rodent testis and uterus. Is AEA present in human reproductive tract fluids? Does AEA or its analogs alter sperm function in the human?

Design: Frozen human reproductive tract fluids were analyzed for AEA and similar compounds. The effect of a potent synthetic AEA analog, AM-356, was tested in three in vitro sperm function assays, using fresh semen from research donors. THC, the active substance in marijuana, was studied for acrosomal effects. AEA alone was also evaluated for its influence on hyperactivated motion.

Materials/Methods: Frozen samples of human seminal plasma, mid-cycle oviductal fluid and follicular fluid from IVF were analyzed for AEA-like compounds by HPLC/MS. The presence of a cannabinoid receptor on

human sperm was studied via radioligand binding assays. In vitro capacitation and analysis by CASA was used to assess human sperm motility and hyperactivation (HA). The influence of AM-356 on sperm/egg binding was followed using the Hemizona Assay. The incidence of completed AR, as well as newly-described intermediate modifications over the acrosomal cap, were determined by triple stain methods.

Results: AEA is present within the three human reproductive fluids: seminal plasma (13.7 nM $\pm$ 3), oviductal fluid (10.5 nM $\pm$ 2), and follicular fluid (3.7 nM $\pm$ 1). Two other endocannabinoids, palmitylethanolamide and oleylethanolamide are also present. Human sperm contain a putative cannabinoid receptor (KD 9.7 nM for the specific analog). AM-356 inhibits ($P < 0.01$) alterations over the acrosomal cap (IC50 = 6 picomolar), without affecting the low rate of completed AR. THC (150 nM) likewise suppresses acrosomal modifications. Treatment with 1 nM AM-356 inhibits sperm/zona binding by 50% ($P < 0.001$), in theory by slowing capacitation. AM-356 elicits biphasic effects on sperm HA motility: 2.5 nM will inhibit HA while a lower concentration (0.25 nM) produces HA stimulation ($P < 0.05$). Preliminary data reveal that AEA can stimulate HA motility by 26-71%.

Conclusions: The presence of endocannabinoids in seminal plasma, oviductal and follicular fluids may imply sequential exposure of sperm to these agents while moving from the male tract and up through the female reproductive tract. A presumptive modulatory role for endocannabinoids during sperm transport, capacitation and fertilization in vivo is suggested by concentration-dependent inhibition and stimulation of HA motility as well as the ability to inhibit acrosomal modifications and sperm preparation for zona binding. Such data also support the conclusion that smoking marijuana could impact these reproductive functions in the male and female.

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P-273

CD146 expression in human preimplantation blastocyst. H. Wang, Y. Wen, S. Mooney, B. Behr, M. L. Polan. Stanford Univ Sch of Medicine, Stanford, CA.

Objective: CD146, also known as Mel-CAM, MUC18, is a membrane glycoprotein which functions as a Ca^{++}-independent cell adhesion molecule involved in heterophilic cell-cell interactions. CD146 contains the characteristic V-V-C2-C2-C2 Ig-like domain structure, suggesting that CD46 may be involved in the interactions between cells and the extracellular matrix. Interestingly, CD146 has been detected in human intermediate trophoblast cells, not in the syncytiotrophoblast and cytotrophoblast cell. The origin and the function of the intermediate trophoblast cell is not clear. Successful implantation and embryonic development depend on a series of complex, co-ordinate interactions between maternal and embryonic tissue that is facilitated by a unique cell population, the trophoblast cells. In mammals, the trophoblast cell lineage is specified before implantation into the uterus. The lineage appears at blastocyst stage as the trophoectoderm cell. The polar trophoectoderm (the subset of the trophoectoderm that is in direct contact with inner cell mass) maintains a proliferation capacity and forms the extraembryonic ectoderm, the ectoplacental cone, and the secondary giant cells of the early conceptus. The rest of the trophectoderm ceases to proliferate and becomes primary giant cells. Studies suggest that stem cells exist in extraembryonic ectoderm that contribute descendants to the ectoplacental cone and the polyploid giant cells. The goal of this investigation is to determine whether CD146 is expressed in human preimplantation blastocysts.

Design: mRNA was extracted from single preimplantation blastocysts and examined for CD146 gene expression using RT and two rounds of nested PCR.

Materials/Methods: In-vitro fertilization programs, embryos which were uni-pronucleate, tri-pronucleate, or excessively fragmented, and thus not suitable for transfer, were examined.

Results: CD146 was expressed by all human preimplantation blastocysts (25/25) examined. Respective 268-bp signals consistent with the expected sizes of CD146 was demonstrated.

Conclusions: CD146 is expressed in preimplantation human embryos, which indicated that CD146 may play an important role in the development of trophoblast stem cells and promote human embryonic implantation. More studies need to local CD146 in trophoblast stem cell.

Supported by: The Children's Health Initiative Innovations Fund.

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Single sperm testing demonstrates acrosomal loss after sperm tail breaking. N. Katz, B. Bartoov, S. Segal. I. Tur-Kaspa, Barzilai Medical Center/Ben-Gurion University; Faculty of Life Science, Bar-Ilan Univ, Ashkelon, Israel; Faculty of Life Science, Bar-Ilan Univ, Ramat-Gan, Ramat-Gan, Israel; IVF Unit, Dept of Ob/Gyn, Barzilai Medical Ctr, Ben-Gurion Univ, Ashkelon, Israel, Ashkelon, Israel.

Objective: The mechanism by which sperm tail breaking before ICSI improve fertilization rate is not clear. We studied the impact of tail breaking on sperm acrosomal status.

Design: Basic research.

Materials/Methods: Evaluation of the single sperm was performed by two methods: scanning electron microscopy (SEM) and Kruger staining. Scanning electron microscopy method was modified for observation of small number of treated spermatozoa. Number of experiments was done to ensure efficient sperm remaining (around 70%) after preparation. 70 sperm cells from five normozoospermic samples were studied by SEM. The sperm were fixed 5, 15 and 30 minutes after immobilization. Unbroken sperm cells served as control. 120 treated spermatozoa from eight normozoospermic samples were placed into diamond marked circle on poly-L-lysine coated slide and stained by standard Kruger method. Slides, prepared from appropriate samples for routine morphological analysis, served as control.

Results: Immobilized sperm demonstrated 68% of acrosome reaction (partial and complete loss) 5–7 minutes after the tail insult (13 from 19), 89% in 15 $\pm$ 2 minutes (24 from 27) and 92% in 30 $\pm$ 2 minutes (22 from 24) following sperm immobilization. Totally, 84% of the sperm cells observed by SEM were acrosomally reacted half an hour post sperm tail insult versus 27% in control. The Kruger staining demonstrated an average of 74% (53–93%) of acrosome loss after sperm insult versus 33% (17–42%) in control.

Conclusions: Sperm immobilization by tail breaking prior ICSI leads to partial or complete acrosomal loss during half an hour post insult. This phenomenon may indicate some form of capacitation, which is still important for successful ICSI outcome.

P-275

Suppressed ICSI blastocyst hatching in vitro compared with hatching of blastocysts from conventional insemination. P. Morton, R. Porter, J. Graham, M. Tucker, P. E. Browne, E. Widra. Georgia Reproductive Specialists, Atlanta, GA; Shady Grove Fertility Reproductive Science Ctr, Rockville, MD.

Objective: Implantation of ICSI embryos has either been reported to be lower than or similar to conventionally inseminated embryos. With extended in vitro culture to the blastocyst stage, observation of the behavior of embryos hatching from the zona pellucida is possible, and this allows comparison of hatching rates between different groups of embryos. We wished to study whether ICSI embryos showed blastocyst formation and hatching rates that differed in any way from conventionally inseminated embryos.

Design: Retrospective observational review of embryonic development in extended routine culture, taken from IVF laboratory records. All embryonic culture was individualized to track daily growth, which is standard practice in our laboratory.

Materials/Methods: Embryos not used for transfer or cryopreservation, due to compromised morphology or lack of patient consent, were observed for an extended period in routine culture, until development arrested as per our standard disposal protocol. Embryos, regardless of their eventual fate, were initially inseminated in HTF, and moved into individual droplets of IVC-1 [In Vitro Care, San Diego, CA] for development day-1 through day–3. Thereafter individually marked embryos were cultured in CCM [Vitrolife, Göteborg, Sweden], and were moved to fresh medium every 48 hrs. No embryos continued to develop past day-9 in regular culture.

Results: There was no significant difference in the maternal ages of the patients providing the 2 sets of embryos: 31.4 yrs (ICSI), and 33.8 yrs (conventionally inseminated group—CONV). From 64 IVF cycles (25 ICSI and 39 CONV) 489 blastocysts developed from 930 2PN embryos (overall blastocyst formation = 52.2%), 191 ICSI and 298 CONV. Average number of embryos in the ICSI group was 14, and in the CONV group it was 15 [NS]. Average number of blastocysts formed from ICSI was 7.5 (54%), and