

Dawley rats. ANOVA Tukey-Kramer HSD was used for statistical analysis using a *P* value of 0.05. Each experiment contained at least four replicates with at least 100 oocytes. Experiment 1: Oocytes were collected at different times post-hCG injection (14, 18, 22 h) and cumulus cells were removed before or after 2 h of *in vitro* culture with hyaluronidase. There was no difference in SA due to oocyte aging in oviducts or hyaluronidase treatment. Experiment 2: Oocytes were cultured in Ca²⁺-containing and Ca²⁺-free KSOM for 2 h after collection at different times (14, 18, 22 h) post hCG injection. Although there were no differences among Ca²⁺-containing KSOM culture groups (91%, 82%, and 90%, respectively), Ca²⁺-free KSOM significantly decreased SA at 14, 18, and 22 h post-hCG (19%, 43%, and 51%, respectively). Experiment 3: Oocytes were treated with different doses of inhibitor of CaMKII, myr-AIP (10, 20, 50, 100 μM), and proteasome inhibitor MG132 (10, 20, 50, 100 μM) after oocyte collection. Fifty and 100 μM myr-AIP induced significantly lower SA (36%, and 17%) than 0, 10, 20 μM (91%, 65%, and 53%, respectively). Moreover, higher concentrations of MG132 (50 and 100 μM) induced significantly lower SA rates (6% and 5%) than 0, 10, 20 μM (91%, 41%, and 22%, respectively). We also investigated the effect of short exposures to myr-AIP and MG132 during the first 2 min post-recovery. The myr-AIP group produced significantly lower SA compared to control and MG132-treated groups. Experiment 4: Oocytes collected at different times (0, 10, 20, 30, 60 and 90 min) were used for CaMKII activity assay. CaMKII activity increased at 20 min and remained high for 30 min followed by decreased activity by 60 min. In conclusion, CaMKII seems to be one of the upstream signals that causes rat oocytes to spontaneously activate after recovery.

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260 REPLACEMENT OF PVA WITH FETAL BOVINE SERUM IMPROVES FORMATION AND HATCHING OF PORCINE BLASTOCYSTS PRODUCED *IN VITRO*

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Porcine embryos, derived from *in vitro* maturation and fertilization, were used to investigate the effects of timing of serum inclusion and PVA replacement in the medium for *in vitro* culture (IVC) on rates of blastocyst formation and hatching. In Experiment 1, presumptive zygotes at 20 h post-insemination (hpi) or cleaved embryos obtained by culture in porcine zygote medium (PZM-5) containing 3 mg mL⁻¹ polyvinyl alcohol (PVA) at 48 or 96 hpi were further cultured in either PZM-5 containing PVA or PZM-5 where PVA was replaced by 1%, 5%, or 10% fetal bovine serum (FBS) until Day 6 (Day 0 = the day of *in vitro* insemination). Supplementation with 1% to 10% FBS at 20 and 48 hpi reduced (*P* < 0.05; by ANOVA and Fisher's PLSD test) blastocyst rates on Days 5 (0% to 1%) and 6 (3% to 6%) compared with PVA supplementation (4% and 22%, respectively). However, addition of 10% FBS at 96 hpi increased (*P* < 0.05) blastocyst rates (30%) on Day 5 compared with PVA (11%) and 1% FBS (15%); there was no significant difference among treatments in rates of blastocyst formation on Day 6 (24% to 40%). The total number of blastomeres in Day 6 blastocysts did not differ among treatments at any timing of serum supplementation (26.5 to 48.3 cells). In Experiment 2, presumptive zygotes were cultured from 20 to 96 hpi in PVA medium, and the cleaved embryos were later transferred into PZM-5 containing PVA, or 1%, 5%, or 10% FBS for another 4 days. Hatching rates of embryos on Days 7 and 8 were significantly higher (*P* < 0.05) in PZM-5 where PVA was replaced with 10% FBS (15% and 20%, respectively) than those in PZM-5 containing PVA (1% and 5%, respectively). Moreover, the total cell number in hatching/hatched blastocysts on Day 8 were significantly greater (*P* < 0.05) in medium containing 10% FBS (135.1 cells) than that in PVA medium (77.0 cells). In Experiment 3, at 130 hpi, blastocysts derived from IVC with PZM-5 containing PVA were transferred into PZM-5 containing PVA, 3 mg mL⁻¹ bovine serum albumin (BSA) or 10% FBS for another 2 days. Hatching rates of blastocysts on Days 6, 7 and 8 were significantly higher (*P* < 0.05) in PZM-5 where PVA was replaced with 10% FBS (12%, 56%, and 64%, respectively) than those in PZM-5 containing PVA (0%, 12%, and 20%, respectively) and BSA (0%, 12%, and 20%, respectively). Moreover, the total cell number in hatching/hatched blastocysts on Day 8 were significantly greater (*P* < 0.05) in medium containing 10% FBS (138.7 cells) than that in PVA (71.7 cells) and BSA medium (70.7 cells). The results indicate that the timing of serum inclusion in the culture medium markedly affects porcine embryo development *in vitro* and that replacement of PVA with FBS in PZM-5 at 96 hpi or later improves the subsequent development of embryos to the hatching/hatched blastocyst stage.

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Male Physiology

261 FERTILITY CONTROL BY GnRH ANALOGUES IN DOGS

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GnRH plays a pivotal role in reproduction by stimulating the release of gonadotrophins. Chemical substitutions in the GnRH molecule lead to analogues possessing antagonist or agonist activity (Paramo RM *et al.* 1993 J. Reprod. Fert. Suppl. 47, 387–397). The highly potent agonist analogue, Buserelin, with up to 20 times of potency, by increasing binding affinity, desensitizing competitive receptors, and resisting metabolic degradation, shuts down rather than stimulates reproductive function (Bertschinger HJ *et al.* 2001 J. Reprod. Fert. Suppl. 57, 275–283). In man, Buserelin is employed in several gonadal hormone-dependent diseases and for prostatic cancers. We suppress gonadal function in male dogs using Buserelin. Eight intact male German sheep dogs 20 months old were divided into two groups; A, 4 subjects treated for pharmacological castration

(Buserelin acetate, 0.3 mg/each, s.c., every 8 h for 30 days) (Suprefact-Aventis Pharma, Italy); B, 4 subjects treated with placebo (NaCl, 0.9%, s.c., every 8 h for 30 days). Plasma testosterone concentrations were measured twice a week by RIA using commercial kits (Coat-A-Count, Los Angeles, USA). Clinical examination of the male genital tract was conducted by ultrasound monitoring. Before and after the pharmacological treatment, semen was collected and evaluated for macroscopic and microscopic parameters. After treatment, testicular specimens were collected by orchiectomy, fixed in Bouin's solution, and embedded in paraffin wax. Thin sections were cut and stained with hematoxylin/eosin. The presence of germ cells (spermatogonia to spermatozoa, Sertoli and Leydig cells number) were analyzed. Randomly selected fields of transverse and longitudinal sections of seminiferous tubules were observed and analyzed using a computer assisted image analyzer (MONO system, Italy). The images acquired were segmented and binarized in order to obtain the masks of the tubular profiles; the mean values of the area, major and minor axes, mean diameter, and perimeter occupied by the testicular tubules were calculated automatically. Data were analyzed by ANOVA test. After Buserelin, all dogs (group A) showed a reduction in testicular and prostatic diameters compared to group B. Azoospermia was observed in group A. Histological examination revealed a statistically significant cell reduction of the germinal line (spermatogonia and spermatocytes, $P < 0.05$; spermatids and spermatozoa, $P < 0.001$). GnRH pharmacological treatment induced a cessation of normal spermatogenesis at the spermatocyte level while no statistical difference in morphometric parameters of seminiferous tubules were observed. The basic testosterone level (3.2 ± 1.3 ng/mL) rose to 12 ± 3.7 ng/mL (21° day) and then shut down to 0.5 ± 0.3 ng/mL (30° day), giving a long-term suppression. The present study demonstrates that Buserelin is an anti-fertility agent that gives suppression of reproductive function in male dogs. The method may have a clinical application. The utilization of a Buserelin depot will be a successive step.

262 SPERM CHROMATIN STRUCTURE, OXIDATIVE STRESS AND BASIC SEMEN PARAMETERS OF MEN FROM SUBFERTILE COUPLES

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It is known that the mammalian sperm chromatin structure plays an important role in male fertility. In opposition to many other areas of biological research, the human sperm chromatin can be considered as a model for animal fertility investigations. This is due to the great number of males with high levels of chromatin abnormalities and the ease of tracking their fertility potential. The aim of the study was to find a relationship between sperm chromatin structure, level of reactive oxygen species (ROS) and the basic semen parameters: sperm concentration and motility. The semen from a total of 391 men from subfertile couples 22–51 years old was used. The sperm chromatin abnormalities were examined flow cytometrically according to the SCSA method (sperm chromatin structure assay; Evenson D.P. *Methods In Cell Biology*, vol. 33, 1990) and ROS level was examined by luminometry (Kolletis *et al.* 1999 *Fertil. Steril.*). Sperm concentration and motility were checked microscopically. Sperm concentration of the examined ejaculates ranged from 0.05×10^6 /mL to 627.5×10^6 /mL and progressive motility ranged from 0% to 70%. More than 30% of spermatozoa with abnormal chromatin (level considered as the infertility threshold) was found in 70 (17.9%) patients; 15–30% of spermatozoa with abnormal chromatin (level of decreased fertility potential) was found in 154 (39.4%) patients; and in 167 (42.7%) patients the number of abnormal spermatozoa did not exceed 15% (level of normal fertility potential; Evenson *et al.* 1999 *Hum. Reprod.*; Zini *et al.* 2001 *Fertil. Steril.*). High significant correlations were found between chromatin abnormality and: patients' age (0.1008, $P = 0.017$), sperm concentration (-0.2735 , $P < 0.001$), progressive motility (-0.4365 , $P < 0.001$), and ROS level (0.2709, $P < 0.001$). However in patients with normal sperm concentration ($>20 \times 10^6$ /mL, according to the World Health Organization), as many as 11.5% had a high level of chromatin abnormality ($>30\%$ of abnormal chromatin) and 29.7% of moderate chromatin abnormality (15–30% abnormal chromatin). Similarly, in patients with normal progressive sperm motility ($>50\%$, according to the World Health Organization) 1.7% had a high level of chromatin abnormality ($>30\%$ of abnormal chromatin), and 33.9% had a moderate level of chromatin abnormality (15–30% abnormal chromatin). Contrary to the findings of many earlier investigations, a strong relationship between sperm chromatin damages and basic semen parameters was observed in this work. The sperm chromatin structure assay should be included in standard semen examination to avoid expensive and time consuming *in vitro* procedures for spermatozoa with damaged DNA.

263 IDENTIFICATION OF A NOVEL MOPT GENE IN HUMAN AND MOUSE ADULT TESTIS

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To discover late stage germ cell-specific transcripts we prepared a cDNA library from adult testes of 35-day old mice and subtracted it with mRNA from the testes of juvenile mice. Real-time RT-PCR analysis indicated that 42 cDNA clones in the subtracted library were expressed more intensely in the adult testes than in the juvenile testes. One clone identified by subtraction is expressed preferentially in the late spermatid and is located on chromosome 17E3 in mouse and 2p22 in human. The full nucleotide and amino acid sequences of mouse and human MOPT gene are deposited in EMBL GenBank (AY367765 And AY367766). Human MOPT is spliced by 5 exons and 4 introns and encompasses 7,000 bp of genomic DNA (from bp 355 822 to 425 511) of NT-022184.13, whereas mouse MOPT is spliced by 5 exons and 4 introns and encompasses 7,382 bp of genomic DNA (from bp 6227407 to 6235588) of NT-039658.2.

Because of the limited availability of human testis samples, development-dependent expression of MOPT mRNA was conducted using its mouse homologue and semiquantitative PCR. The number of cycles completed before entering the exponential growth, recorded by amplifier PE5700 for mouse MOPT, were 1.11 ± 0.23 , 1.05 ± 0.04 , 1.5 ± 0.2 , 5.55 ± 0.65 , 19.35 ± 0.65 , 68.65 ± 2.15 , and 185.15 ± 6.15 in W/W, postnatal day 5-, 8-, 12-, 15-, 18-, 22-, and 28-day mouse tissue samples, respectively. The difference among the three times was significant ($P < 0.01$, ANOVA). These

results suggest that expression of MOPT gene increased from postnatal Day 5 to Day 28, indicating possible involvement in testicular development. The ORF encodes a protein containing 79 amino acid residues. A MORN motif, EGQFKDNMFHGLGTYTFPNG, was identified in the predicted protein sequence of MOPT; function of this motif is unknown. *In situ* hybridization of 12-week-old wild-type mouse testes using an antisense riboprobe and immuno-gold data indicated MOPT was expressed as a late spermatid and acrosome reaction.

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264 PORCINE SPERM-HEAD RECEPTOR INTERACTION WITH PROTEINS PERIPHERALLY BOUND TO THE OVIDUCTAL LUMEN

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Here we show that cell–cell interaction between boar spermatozoa and the oviductal lumen surface are mediated by specific receptor–ligand binding. We have previously demonstrated increased sperm viability following incubation of boar spermatozoa with apical plasma membrane (APM) proteins from sow oviductal epithelial cells (Fazeli A *et al.* 2003 *Reproduction* 123, 509–517). Fresh intact oviducts were internally flushed with PBS and filled with Sulfo-NHS-LC-biotin (Pierce Biotechnology, Inc., Rockford, IL, USA) in PBS (pH 8.0). Each end was clamped and incubated for 30 min at RT. Unbound biotin was quenched with 50 mM ammonium chloride for 10 min, and biotinylated soluble APM (sAPM-B) preparations were prepared. Percoll-washed boar spermatozoa ($25 \times 10^6 \text{ mL}^{-1}$) were incubated with sAPM-B ($150 \mu\text{g mL}^{-1}$) for 40 min at 39°C in 5% CO₂, and unbound sAPM-B was removed. The sperm pellet was resuspended in 0.5% SDS and incubated for 90 min at RT. Solubilized proteins were isolated by centrifugation at 14,000*g* for 5 min. The proteins were separated by SDS-PAGE alongside non-biotinylated APM and untreated “sperm-only” samples. Biotinylation was detected by NeutrAvidin/HRP. In addition, sAPM-B treated spermatozoa were smeared onto slides for the detection of biotinyl groups by anti-biotin/Alexa Fluor (Molecular Probes, Leiden, NL). NeutrAvidin/HRP Pierce detected a biotinylated sAPM band migrating to approximately 100 kDa in the sperm/sAPM-B sample. This band was not present in the “sperm-only” sample. Detection of *in situ*-labelled sAPM-B on spermatozoa showed that fluorescence was specific to the sperm head. We conclude that an oviductal protein of approximately 100 kDa is a potential viability-enhancing ligand for a sperm receptor that is mainly located over the acrosomal region.

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265 SPERM DNA FRAGMENTATION AND PREGNANCY OUTCOME

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Sperm DNA integrity is obviously important for normal embryo development and pregnancy outcome. Over the past 25 years, various methods have been developed to measure sperm DNA strand breaks *in situ*. The Sperm Chromatin Structure Assay (SCSA) treats sperm with low pH to denature DNA at the sites of DNA strand breaks, followed by acridine orange (AO) staining of green for native DNA and red for denatured DNA, as measured by flow cytometry (FCM), as well as % sperm with high DNA stainability (HDS: immature sperm with intact DNA related to decreased fertilization rates). FCM-sorted sperm from each SCSA-defined population (normal, moderate, and high DNA fragmentation and HDS sperm) show that the moderate DNA fragmentation index (DFI) population has the same image analysis characteristics as normal sperm without significant comets. Thus, an ICSI technician is not likely to differentiate between a normal and a moderate DFI sperm. The TUNEL assay uses an enzyme to add a fluorochrome-labeled base to a 3'-OH broken DNA strand. Both light microscopy and flow cytometry are used for measuring the % and extent of DNA fragmentation but cannot measure the level of HDS. For the COMET assay, sperm are suspended in an electrophoretic gel, placed on a glass microscope slide, digested with proteases and RNase, subjected to an electric field, and then stained with a DNA dye. The % of comet positive sperm is scored, but the extent of fragmentation is difficult to define and the % HDS cannot be determined. Small pieces of fragmented DNA migrate in the gel forming a “comet.” All three methods have been used for both research and clinical diagnosis and as prognosis for livestock (bulls, boars, rams, stallions) and humans. Light microscope techniques suffer from a lack of statistical soundness needed for clinical decisions as well as present a potential bias in selection of sperm for measurements. Due to the thousands of sperm randomly selected for flow cytometry measurements, the data are statistically robust. Data from all three kinds of measurements in over a hundred manuscripts clearly show that sperm DNA fragmentation has a negative impact on embryo growth and pregnancy. Infertile animals may have nearly all of the sperm with fragmented DNA. Fertility ratings in bulls and boars are clearly related to the percent and extent of DNA fragmentation. Threshold levels for fertile/sub fertile/infertile differ for different species. Likewise different methods/laboratories have suggested various threshold levels to characterize a man with a highly fertile to low/very poor potential. The range of sperm with fragmented DNA is from ~2% to 100%. The SCSA method has defined a 27–30% DFI as the point in which a man is placed into a statistical category of taking a longer time to achieve *in vivo* pregnancy, more intrauterine insemination and routine IVF cycles, or no pregnancy. Current data suggest that ICSI may help overcome the diminished pregnancy prognosis with high DFI over the other ART or natural methods.

266 ANTIOXIDANT CAPACITY OF BOAR SEMINAL PLASMA

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It has been established that antioxidants in seminal plasma play an important role in protecting the spermatozoa against oxidative stress-induced damage. This study was conducted to measure the total antioxidant capacity (TAC) of boar seminal plasma. Fifty-four ejaculates were collected from 17 mature boars of proven fertility by the gloved-hand technique. Ejaculates were collected separately in different fractions (pre-sperm, sperm-rich, and post-sperm) according to their macroscopic (color) characteristics. After centrifugation (2400g for 3 min), the sperm pellet was discarded; the supernatant was recentrifuged and filtered through a 10- μ m nylon mesh filter to remove debris or clumped spermatozoa. The seminal plasma was frozen at -20°C until further use. After thawing at room temperature, seminal plasma aliquots of 5 μL were immediately assessed for total antioxidant capacity. TAC was measured using the ABTS/ H_2O_2 /HRP decoloration method (Cano *et al.* 2000 Redox Report 5, 365–370) which allows differentiation between hydrophilic and lipophilic antioxidant activity capacity. TAC units were expressed as micromolar (μM) Trolox equivalents. Data were analyzed using ANOVA. Only the hydrophilic activity was measurable, with the lipophilic activity being undetected. The overall TAC of seminal samples (mean \pm SEM) was $1623.7 \pm 56.28 \mu\text{M}$, ranging from 674 to 2428 μM . Different TACs were observed among males ($P < 0.05$) and between ejaculates of the same male ($P < 0.05$). Ejaculate fraction had a significant effect ($P < 0.001$) on the TAC levels. The post-sperm fraction had a significantly lower TAC level ($1104.09 \pm 57.66 \mu\text{M}$) than the pre-sperm and sperm-rich fractions ($1611.95 \pm 153.68 \mu\text{M}$ and $1356.136 \pm 72.47 \mu\text{M}$, respectively, $P < 0.001$). In conclusion, hydrophilic antioxidant activity represented the main contribution to the TAC in boar seminal plasma, showing differences among males, between ejaculates of the same male, and also between the different ejaculate fractions.

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267 TESTIS TISSUE XENOGRAFTING AS A BIOASSAY FOR GERM CELL DEVELOPMENTAL POTENTIAL IN EQUINE CRYPTORCHID TESTES

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In domestic animals, spermatogenic differentiation is blocked in abnormally retained testes exposed to core body temperature. It is not known if undifferentiated germ cells are retained in long-term cryptorchid equine testes, nor is it known whether any surviving germ cells retain their ability to progress through spermatogenesis. If functional germ cells do persist in equine abdominal testes, then the possibility exists that offspring could be derived even from bilaterally cryptorchid individuals. Previously, we reported an *in vivo* model where completion of spermatogenesis with production of spermatozoa capable of fertilization occurred in fragments of testicular tissue from immature mice, domestic animals, and monkeys grafted under the skin of immunodeficient mice. Therefore, spermatogenic development in testis tissue xenografts can serve as an *in vivo* assay system for the developmental potential of germ cells. The objective of this study was to investigate if cryptorchid horse testes that had been exposed to core body temperature for 1–3 years had retained developmentally competent germ cells. Small fragments of abnormally cryptorchid testis tissue (about 1 mm^3) from three donor horses (1-, 2-, and 3-year-old Quarterhorse) were grafted under the back skin of castrated male immunodeficient mice ($n = 8, 6,$ and 3 recipient mice, respectively). At the time of grafting, donor tissue did not contain differentiated germ cells. Histological examination of the testis xenografts was performed between 5 and 45 weeks post-transplantation. Weight of the seminal vesicles in the host mouse was recorded as an indicator of bioactive testosterone produced by the xenografts. By 28 weeks after grafting, pachytene spermatocytes were observed in xenografts from all cryptorchid donor testes. While haploid gametes would be expected to be present in xenografted testis tissue from descended equine testes by 35 weeks after grafting, spermatogenesis did not progress through meiosis in the cryptorchid grafts. In all recipient animals where spermatogenic differentiation occurred, the weight of the seminal vesicles in the castrated host mice was restored to pre-castration values, indicating that xenografts were capable of releasing biologically active testosterone. These results indicate that even after 3 years of exposure to core body temperature, equine cryptorchid testes contain germ cells capable of differentiation. It remains to be investigated if supplementation of exogenous gonadotropins might support post-meiotic differentiation of germ cells in cryptorchid equine testes xenografts.

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268 GERM CELL DEVELOPMENT IN EQUINE TESTIS TISSUE XENOGRAFTED INTO MICE

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Grafting of testis tissue from immature animals under the back skin of immunodeficient mice results in complete spermatogenesis, albeit with different levels of efficiency in different species. While spermatogenesis develops comparably to that in the donor species in xenografts from pigs, sheep and goats, spermatogenic differentiation is less efficient in testis tissue from cats and bulls. Testicular maturation was significantly accelerated in rhesus monkey testis grafts whereas timing was similar to that in the donor species in cats and bulls. The objective of this study was to investigate

if grafting of immature horse testis tissue would result in spermatogenesis in a mouse host. Small fragments of testis tissue (about 1 mm³) from four sexually immature colts (2-week-old Standardbred, 5- and 8-month-old ponies, 10-month-old Warmblood) were grafted under the back skin of castrated male immunodeficient mice ($n = 5, 5, 10$ and 5 recipient mice, respectively). Histological examination of the testis xenografts was performed between 14 and 50 week post-transplantation. Weight of the seminal vesicles in the host mouse was recorded as an indicator of bioactive testosterone produced by the xenografts. At the time of grafting, the seminiferous cords of the donor testis tissue from 2-week-, 5-month- and 8-month-old colts contained only immature Sertoli cells and gonocytes. No spermatogenic differentiation occurred in xenografts from the 2-week-old colt and testosterone production was minimal. Pachytene spermatocytes were observed in testis grafts from the 5- and 8-month-old donors from 14 weeks onward. Spermatogenesis did not proceed through meiosis in grafts from the 5-month-old donor. Recipient mice carrying xenografts from the 8-month-old donor received exogenous gonadotropins (equine chorionic gonadotropin and human chorionic gonadotropin, 10 I.U./day for 2 months, beginning 14 weeks after grafting) and condensing spermatids were observed by 35 weeks after grafting. In donor tissue from the 10-month-old colt, pachytene spermatocytes were present in about 50% of tubules at the time of grafting. After 14 weeks, xenografts showed fewer differentiated germ cells than the donor tissue. However, at 35 weeks after grafting, condensing spermatids were observed, indicating that differentiated germ cells were initially lost and spermatogenesis was subsequently reinitiated. In all castrated host mice where spermatogenic differentiation occurred, the weight of the seminal vesicles was restored to pre-castration values showing that xenografts were releasing bioactive testosterone. These results indicate that horse spermatogenesis can occur in a mouse host albeit with low efficiency. Testicular maturation was not accelerated. In most cases, spermatogenesis appeared to become arrested at meiosis. The underlying mechanisms of this spermatogenic arrest require further investigation. Although equine testis xenografts produced testosterone, supplementation of exogenous gonadotropins might support post-meiotic differentiation.

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Oocyte Activation

269 INACTIVATION OF MATURATION PROMOTING FACTOR AND MITOGEN-ACTIVATED PROTEIN KINASE IN PORCINE OOCYTES BY A SINGLE ELECTRICAL PULSE

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Activities of maturation-promoting factor (MPF) and mitogen-activated protein kinase (MAPK) of mature oocytes should be decreased to begin subsequent development. In this study, activities of MPF and MAPK were investigated in porcine oocytes after artificial activation. To determine optimal electrical activation, porcine oocytes were exposed to 3 V AC pulse for 5 s followed by a single DC pulse of various electric field strengths (120, 150, 180, and 210 V/mm) and pulse durations (15, 30, 45, and 60 μ s). For chemical activation, oocytes were exposed to 5 μ M ionomycin for 5 min followed by 2 mM 6-dimethylaminopurine (6-DMAP) or 5 μ g/mL cycloheximide for 4 h or 6 h. After activation, 40 to 50 oocytes were cultured in 50- μ L drops of NCSU23 medium supplemented with 4 mg/mL BSA at 39°C, and 5% CO₂ in air. After 6 days of culture, blastocyst formation was observed and then numbers of blastocyst nuclei were counted after staining with Hoechst 33342. *In vitro* development rates and numbers of blastocyst nuclei by the field strengths were not significantly different among experimental groups ($P > 0.05$). However, development rates to the blastocyst stage of porcine oocytes exposed to 15 and 30 μ s were 27.4 and 24.4%, respectively, which were significantly higher than that (12.5%) of 60 μ s ($P < 0.05$). Mean numbers of blastocyst nuclei in 15- and 30- μ s groups (38.6 ± 13.4 and 37.9 ± 11.4 , respectively) were significantly higher than that (25.8 ± 10.5) of the 60- μ s group ($P < 0.05$). Blastocyst development after optimal electrical pulse exposure was compared with that after different chemical treatments. Electrical stimulation induced 22.9% of blastocyst formation, which was significantly higher ($P < 0.01$) than those induced by the chemical stimulators (3.4 and 2.7%). Based on these results, changes of constituent proteins (cdc2 and ERK) of MPF and MAPK after artificial activation were analyzed by immunoblotting using anti-PSTAIRE monoclonal antibody and anti-MAP kinase polyclonal antibody. Activities of both cdc2 and ERK in pig oocytes were reduced 4 h after electrical stimulus, but were maintained at optimal levels after treatment with ionomycin + 6-DMAP. Our results indicate that an optimal single electrical pulse is effective on the inactivation of MPF and MAPK in porcine oocytes, eventually resulting in activation of porcine oocytes produced *in vitro*.

270 COMBINED ELECTRICAL AND CHEMICAL ACTIVATION OF ZONA-FREE PORCINE OOCYTES

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Activation is a crucial step in mammalian somatic cell nuclear transfer (SCNT). Recently we described the application of the handmade cloning technique for porcine SCNT that uses oocytes without zona pellucida (zona-free) in a micromanipulation-independent procedure (Kragh *et al.* 2004 *Reprod. Fertil. Dev.* 16, 315–18). The purpose of the present study was to investigate the effect of a combined electrical and chemical activation of zona-free porcine oocytes. Cumulus-oocyte complexes were aspirated from ovaries of sows and matured for 41 h. Subsequently, the cumulus cells were removed by the addition of 1 mg/mL hyaluronidase in HEPES-buffered TCM-199. For zona pellucida removal, oocytes were incubated in 8 mg/mL pronase in HEPES-buffered TCM-199 supplemented with 20% cattle serum for 10 s. Three independent experiments with four treatments were conducted, and oocytes were activated by a combined electrical and chemical activation. Oocytes were washed once in activation medium (0.3 M mannitol, 0.1 mM MgSO₄, 0.1 mM CaCl₂, and 0.01% polyvinyl alcohol) and transferred to a chamber with two wires (0.5-mm