if grafting of immature horse testis tissue would result in spermatogenesis in a mouse host. Small fragments of testis tissue (about 1 mm^3) 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 form 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 μs). For chemical activation, oocytes were exposed to 5 μM ionomycin for 5 min followed by 2 mM 6-dimethylaminopurine (6-DMAP) or 5 μg/mL cycloheximide for 4 h or 6 h. After activation, 40 to 50 oocytes were cultured in 50-μL drops of NCSU23 medium supplemented with 4 mg/mL BSA at 39°C, and 5% CO_2 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 μs were 27.4 and 24.4%, respectively, which were significantly higher than that (12.5%) of 60 μs ($P < 0.05$). Mean numbers of blastocyst nuclei in 15- and 30-μs groups (38.6 ± 13.4 and 37.9 ± 11.4, respectively) were significantly higher than that (25.8 ± 10.5) of the 60-μs 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 zonaa pellucidae (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 zonae pellucidae 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 $MgSO_4$, 0.1 mM $CaCl_2$, and 0.01% polyvinyl alcohol) and transferred to a chamber with two wires (0.5-mm

separation) covered with activation medium. After the electrical pulse, oocytes were incubated in culture medium (NCSU-37 containing 4 mg/mL BSA) supplemented with 5 μg/mL cytochalasin B and 10 μg/mL cycloheximide for 6 h. Activated oocytes were cultured in culture medium using the wells of wells system (Vajta *et al*. 2000 Mol. Reprod. Dev. 55, 256–64) in the submarine incubation system (Vajta *et al*. 1997 Theriogenology 48, 1379–85). The rate of development into blastocysts was checked on Day 7 of culture. In treatment 1, zona pellucida-intact oocytes were first activated by a single DC pulse of 1.25 kV/cm for 80 μs, and subsequently cultured in cytochalasin B and cycloheximide for 6 h. In treatments 2 and 3, oocytes without zonae pellucidae were activated by a single DC pulse of 1.25 and 0.85 kV/cm for 80 μs, respectively, and subsequently cultured in cytochalasin B and cycloheximide for 6 h. In treatment 4, oocytes without zonae pellucidae were bisected by hand under a stereomicroscope using a microblade in 5 μg/mL cytochalasin B in TCM-199 supplemented with 15 mg/mL BSA, re-fused/activated by a single DC pulse of 1.25 kV/cm for 80 μs in activation medium, and cultured in cytochalasin B and cycloheximide for 6 h. The rates of blastocyst formation from activated oocytes (mean ± SEM) in treatments 1, 2, 3, and 4 were 55 ± 4%, 40 ± 2%, 49 ± 1%, and 41 ± 8%, respectively. In conclusion, the combined electrical and chemical activation efficiently induced parthenogenetic development of zona-free oocytes. Also, a more authentic control model for activation during SCNT was established by activating and producing blasctocysts from re-fused bisected oocytes.

271 ASSESSMENT OF NUCLEAR STATUS OF ACTIVATED BOVINE OOCYTES MATURED IN DIFFERENT MATURATION CONDITIONS *IN VITRO*

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This study was carried out to assess the nuclear status after parthenogenetic activation in *in vitro* matured oocytes under different conditions. Bovine ovaries were collected from slaughtered cows at a local abattoir. Oocytes were aspirated from follicles of 3–8 mm in diameter and transferred to maturation medium: tissue culture medium (TCM)-199 supplemented with 10% (v/v) fetal calf serum, 100 mg/mL L-cysteine, 20 mg/mL sodium pyruvate, gonadotropins (each 250 IU of eCG and hCG/mL), and 10 mg/mL epidermal growth factor, with or without 5 mM hypotaurine and taurine. Oocytes were cultured at 38.9°C in 5% CO_2 in humidified air. After 24 h of culture, oocytes with polar body were selected and submitted to activation treatments. Oocytes were exposed to calcium ionomycin (5 μM for 5 min) followed by incubation with 6-DMAP (2 mM), roscovitine (50 μM), or 6-DMAP + roscovitine for 3.5 h. After activation, oocytes were cultured in mSOF medium containing 0.8% BSA at 38.9°C in 5% CO_2, 5% O_2 in humidified air for 16 h and stained with Hoechst 33342 or aceto-orcein for assessment of nuclear status. Nuclear status was recorded as follows: 1PB (polar body) + 1PN (pronucleus), 2PB + 1PN and others. Data were analyzed using chi-square test. The maturation rate of bovine oocytes cultured in maturation medium containing hypotaurine/taurine (89.3%, $n = 84$) was higher ($P < 0.05$) than those cultured without hypotaurine/taurine (72%, $n = 93$). In the oocytes matured with hypotaurine/taurine, the rates of diploid activation (1PB + 1PN) were 84% ($n = 50$) in oocytes treated with 6-DMAP + roscovitine, 78.6% ($n = 56$) with 6-DMAP, and 52% ($n = 50$) with roscovitine. In the oocytes matured without hypotaurine/taurine, the rates of diploid activation were 80% ($n = 60$) in oocytes treated with 6-DMAP + roscovitine, 72% ($n = 50$) with 6-DMAP, and 54% ($n = 50$) with roscovitine. The rates of diploid activation were not different in oocytes matured with or without hypotaurine/taurine and among activation treatments. The oocytes treated with roscovitine showed a lower rate ($P < 0.05$) of diploid activation and higher rate (39.3–40%) of second polar body extrusion (1PN + 2PB) than the other activation groups in both maturation conditions. Cleavage rates to 2-cell stage were 40–45% in all groups. Development rate of blastocysts were 7–10% in all the groups treated with 6-DMAP and 6-DMAP + roscovitine and no blastocysts were obtained from the groups treated with roscovitine alone. Hypotaurine/taurine are known to be stable and potent antioxidants, and have shown the properties of supporting oocyte maturation and further embryonic development (Guerin and Menezo 1995 Zygote 3, 333–43; Mizushima and Fukui 2001 Theriogenology 55, 1432–45). In this study, although the effectiveness of hypotaurine/taurine on promoting oocyte maturation was observed, there were no significant improvements in the rate of diploid activation in oocytes matured with hypotaurine/taurine. These results suggest that the nuclear status of activated oocytes may not have a direct relationship with the enhanced maturation condition.

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272 OOCYTE SPONTANEOUS ACTIVATION IN DIFFERENT RAT STRAINS

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Oocyte spontaneous activation (OSA) has been reported to occur during *in vitro* culture of ovulated rat eggs. Approximately 1.5 h after isolation and culture, unfertilized oocytes extrude the second polar body and enter a metaphase arrest, and by 3 h individual chromatids separate and scatter throughout the egg's cytoplasm. The objective of this study was to compare the proportion of OSA and the level of maturation promoting factor (MPF) activity in oocytes from different strains. Twelve strains were selected from two commercial sources based on litter size. Mature female rats (9 to 12 weeks old) were superovulated using 20 IU eCG s.c. followed by 30 IU eCG s.c. 5 h later. After 48 h, a dose of 50 IU hCG was administered intraperitonealy and females were mated with vasectomized males. Oocytes were collected 17 h after hCG injection and cumulus cells were removed by transfer to M2 medium containing hyaluronidase (1 mg mL^{-1}). Denuded oocytes were cultured in 50 μL drops of M16 medium under oil at 37°C and 5% CO_2 in air. In order to assess OSA, oocytes were mounted in glycerol containing Hoechst 33342 (10 μg mL^{-1}) on a glass slide after 6 h of *in vitro* culture. The proportion of activated oocytes was determined by epifluorescence microscopy. Oocytes were considered to be in metaphase II if they presented a compact group of chromosomes at the metaphase plate, and were considered activated when chromosome dispersion at different degrees was observed. Data were analyzed by ANOVA, considering each animal as an experimental unit. Significant differences were observed

between strains ($P < 0.01$, Table). In order to determine MPF activity of each strain, 10 oocytes were removed from culture at 0, 1.5, and 3 h after oocyte collection and immediately stored in kinase buffer in LN2 for posterior analysis using an ELISA based kit (MESACUP cdc2 Kinase Assay Kit, MBL, Nagoya, Japan). The log ratio of the MPF activity at 1.5 and 3 h relative to 0 h for each animal (5 per strain) was analyzed by ANOVA. There were no MPF differences between or within strains ($P > 0.3$ and $P > 0.05$, respectively). We did not observe the expected decrease in MPF activity that allows for the exit of metaphase II arrest. This could imply that OSA is not associated with a decrease in MPF, or that MPF decreased rapidly and returned to metaphase levels by 1.5 h after culture. In conclusion, different levels of OSA were observed between strains, however, no differences in MPF activity were detected at the analyzed time points.

n Strain	Rats (*n*)	Oocytes evaluated (*n*)	SOA[1] (%)
Copenhagen-CR[2]	8	107	84.9 ± 10^{a}
Wistar Han-H[3]	10	310	75.7 ± 9^{ab}
Wistar Han-CR	8	164	75.1 ± 10^{ab}
Holtzman-H	11	253	68.4 ± 9^{abc}
Sprague Dawley-H	11	255	68.1 ± 9^{abc}
Lewis-CR	9	166	66.0 ± 9^{abc}
Wistar-CR	10	267	62.2 ± 9^{abcd}
Sprague Dawley-CR	9	155	60.2 ± 9^{abcd}
Wistar Kyoto-CR	5	139	49.0 ± 13^{bcd}
Long Evans-CR	13	277	44.1 ± 8^{cd}
Wistar-H	10	255	38.4 ± 9^{d}
Long Evans-H	7	128	36.5 ± 11^{d}

[a,b,c,d] Different superscripts indicate $P < 0.05$.
[1] Mean ± SEM; [2]CR: Charles River Laboratory; [3]H: Harlan.

273 OPTIMIZATION OF PROTOCOLS FOR ACTIVATION OF GOAT OOCYTES WITH IONOMYCIN IN COMBINATION WITH 6-DIMETHYLAMINOPURINE

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The protocol of ionomycin in combination with 6-dimethylaminopurine (6-DMAP) is commonly used for activation of oocytes and reconstructed embryos of different species. Since numerous abnormalities and impaired development have been observed when oocytes are activated with 6-DMAP, this protocol needs optimization. Effects of concentration and treatment duration of both drugs on activation kinetics and parthenogenetic development of goat oocytes were examined in this study. When goat oocytes matured *in vitro* in TCM-199 were treated with 5 M ionomycin in PBS for different periods before exposure to 6-DMAP in CR1aa, the activation rate obtained with ionomycin treatment for 1 min (95.2%) was significantly ($P < 0.05$, Duncan multiple comparison test) higher than with ionomycin treatments for 3, 5, 7, or 9 min. When oocytes were treated with different concentrations of ionomycin for 1 min before exposure to 6-DMAP, activation rates obtained with 0.625, 1.25, 2.5, 5, 10, and 20 M ionomycin (87–95%) did not differ significantly but were significantly higher than that achieved with 0.3125 M ionomycin. Progressive reduction of time for 6-DMAP exposure showed that the duration of 6-DMAP treatment can be reduced to 1 h from the 2nd up to the 4th hour after ionomycin treatment, to produce activation rates greater than 85%. When oocytes were treated with different concentrations of 6-DMAP for the 3rd hour (a total of 1 h, 3 h after the exposure to ionomycin), activation rates with 4 and 2 mM 6-DMAP (>90%) were significantly higher than those with 1 and 0.5 mM. Therefore, the best protocol for goat oocyte activation would be a 1-min exposure to 2.5 M ionomycin followed by 2 mM 6-DMAP treatment for the 3rd hour. When oocytes matured *in vitro* for different times were stimulated with the best protocol, activation rates of the 27-, 30-, and 33-h oocytes (85, 85, and 91%) were significantly higher than those of the 24-, 26-, and 39-h oocytes. When activated oocytes were co-cultured in CR1aa with cumulus cell monolayers, the highest rates of cleavage (92%) and morulae/blastocysts (23%) were obtained with oocytes activated by the best protocol, and any increase in the intensity of ionomycin treatment and in the duration of 6-DMAP exposure impaired the development of the parthenotes. During anaphase II, chromosomes (the dyads) did not separate into two units in oocytes that were activated by long exposure to 6-DMAP, but they did in oocytes that were activated by short or no exposure to 6-DMAP; as a result, only one pronucleus developed in most of the former but two pronuclei were formed in most of the latter cases. Laser scanning confocal microscopy showed that microtubules also behaved differently in these two groups of activated oocytes. It is therefore concluded that to obtain better activation and development, goat oocytes matured in vitro for 27–33 h should be used, and these should be activated by a 1-min exposure to 2.5 M ionomycin followed by 2 mM 6-DMAP treatment for the 3rd hour.

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