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Sperm Injection

318 EFFECT OF SPERM PRETREATMENT WITH ISOBUTYLMETHYLXANTHINE AND METHYL-β-CICLODEXTRYN ON THE EFFICIENCY OF BOVINE INTRACYTOPLASMIC SPERM INJECTION

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The efficiency of intracytoplasmic sperm injection (ICSI) in bovines is lower than in other species. We propose that *in vitro* sperm capacitation could optimize the ICSI in cattle. The aim was to evaluate the effects of isobutylmethylxanthine (IBMX) and methyl-β-cyclodextrin (MβCD) on the sperm capacitation and *in vitro* development of embryos generated by ICSI. Frozen-thawed spermatozoa ($3–5 \times 10^6$ cells mL^{-1}) were pre-incubated for 2 h at 38.5°C, 5% CO_2 in defined medium (Sp-TLP/PVA) supplemented with MβCD (1 mM) or IBMX (0.4 mM) (capacitating conditions). The untreated control group (UTG; not supplemented) and vehicle group (VG) were incubated for 2 h. The non-capacitating control group (NCG) was not supplemented (neither vehicle nor IBMX or MβCD) and not incubated. The sperm viability and capacitation {intracellular calcium $[Ca^{2+}]_i$, plasma membrane fluidity (PMF), and acrosomal reaction} were evaluated by flow cytometry ($n = 3$ biological replicates). For the ICSI procedure, only motile spermatozoa were selected. After ICSI, oocytes were activated with ionomycin + cycloheximide. Culture was performed at 38.5°C, 5% CO_2, 5% O_2, 90% N_2, saturation humidity in KSOM base medium. Data were analysed by ANOVA and Scheffe's test. Pronuclear formation was evaluated by a chi-square test with Bonferroni's correction. Significance was set at $P < 0.05$. Pretreated spermatozoa showed lower ($P < 0.05$) viability (49 and 67% for IBMX and MβCD, respectively) compared with the NCG (89%), UTG (80%), and VG (78%). The $[Ca^{2+}]_i$ analysed by median fluorescence intensity (MFI) was lower ($P < 0.05$) in NCG (117 MFI) with respect to UTG (127 MFI), VG (124 MFI), IBMX (126 MFI), and MβCD (131 MFI). The PMF increased ($P < 0.05$) with IBMX (115 MFI) and MβCD (106 MFI) compared with NCG (70 MFI), UTG (89 MFI), and VG (65 MFI). Acrosome reaction improved with capacitating treatments with respect to both control groups (16, 23, 8, 4, and 3% for IBMX, MβCD, UTG, VG, and NCG, respectively). Analysis of capacitating *v.* non-capacitating conditions on ICSI efficiency revealed that the fertilization rate, assessed by pronuclear formation, was higher ($P < 0.05$) in ICSI-MβCD (76%; $n = 46$) compared with ICSI-IBMX (55%; $n = 53$) and ICSI-NCG (50%; $n = 44$). Nevertheless, there were no differences among groups in cleavage (Day 3): 85, 86, and 84% and blastocyst rates (Day 8): 19, 25, and 18% for ICSI-IBMX ($n = 8$), ICSI-MβCD ($n = 7$), and ICSI-NCG ($n = 7$), respectively. The parthenogenetic and sham injection groups yielded a lower rate of cleavage (73 and 53%, respectively) and blastocyst (13% and 10%, respectively). The results demonstrated an improvement of the fertilization rate of bovine embryos generated by ICSI using sperm capacitated by MβCD pretreatment. However, more studies are necessary to improve *in vitro* developmental potential of these embryos to the blastocyst stage.

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319 APPROACHES TO IMPROVE INTRACYTOPLASMIC SPERM INJECTION MEDIATED TRANSGENESIS AND MAXIMIZE THE USE OF SEX-SORTED SPERM IN BOVINE

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Intracytoplasmic sperm injection (ICSI) mediated transgenesis is an effective tool for transgenic animal production. However, ICSI in cattle remains inefficient. In this work, we assayed approaches to improve *egfp* expressing blastocysts production by ICSI: the sperm pretreatment with heparin and L-glutathione (Hep-GSH), the use of sex-sorted sperm (SS), the refrozen/thawing of SS sperm, and the combination of these. Quality of ICSI blastocysts was analysed by studying the expression of 4 genes, and the rates of DNA fragmentation. Cumulus-oocyte complexes from slaughtered cow ovaries were *in vitro*-matured for 21 h. Nonsorted (NS) and sex-sorted (SS) frozen straws were thawed. Some of them were incubated with 80 μM Hep-15 mM GSH for 20 h (Hep-GSH^+). The Hep-GSH-control group was not pretreated. Semen samples were co-incubated with 50 ng μL^{-1} of pCX-EGFP for 5 min before ICSI. Moreover, the SS sperm that are usually discarded after ICSI were cryopreserved and used for ICSI after a second thawing (ICSI SS refrozen). The ICSI NS, sham, and diploid parthenogenetic (Diplo PA) controls were included. Oocytes were activated with 5 μM ionomycin for 4 min, TCM-199 for 3 h (except for diploid PA), and 1.9 mM DMAP for 3 h. Cleavage and blastocyst/*egfp* expression rates were evaluated on Days 2 and 7 post-ICSI, respectively. Results are shown in Table 1. Relative expression of HMGN1, GLUT5, AQP3, and OCT4 genes from ICSI NS Hep-GSH^+ and IVF blastocysts were compared by qPCR. Data were analysed by the pair-wise fixed reallocation randomisation test. None of the 4 genes showed differences between groups. The DNA fragmented nucleus index/blastocyst cell numbers were determined by TUNEL assay, not showing differences between groups (Kruskal–Wallis test, $P \leq 0.05$). Means ± s.d. were 29 ± 17/91 ± 27 for ICSI Hep-GSH^+; 27 ± 15/63 ± 34 for ICSI Hep-GSH^-; 28 ± 17/68 ± 17 for ICSI SS, 28 ± 13/75 ± 24 for ICSI SS refrozen; and 21 ± 13/105 ± 59 for IVF SS control. The Hep-GSH pretreatment can increase blastocyst and transgene expressing blastocysts rates after TM-ICSI, except when SS semen is used. Interestingly, the use of SS sperm for ICSI can be maximized by cryopreservation and reuse of discarded sperm cells. The parameters analysed in this

SP 7017

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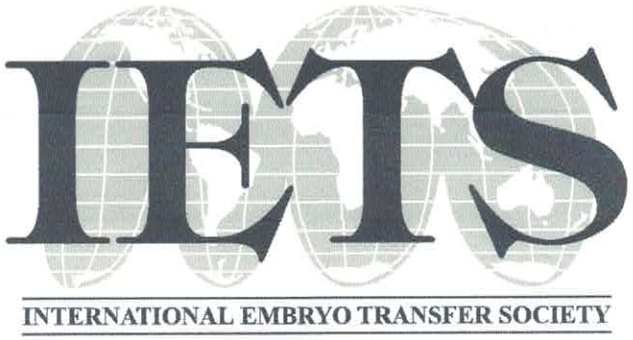

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