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Multiwalled carbon nanotubes vehicles for gene delivery into bovine embryos

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The *zona pellucida* (ZP) is a protective barrier of embryonic cells against chemical, physical and biological substances. For this reason, usual transfection methods are not as efficient for mammalian oocytes and embryos as they are for somatic cells. Carbon nanotubes (CNTs) have emerged as a new method for gene delivery and they can be an alternatively used for embryos transfection, but its ability to cross the PZ and mediated gene transfer is still unknown. The aim of the present study was to determine whether multiwall carbon nanotubes (MWNTs) could pass through the PZ and delivery pDNA for *in vitro*-produced bovine embryos and their effects on apoptosis in bovine blastocysts. Oocytes obtained from ovaries collected at a local slaughterhouse were *in vitro* matured with TCM 199 (Invitrogen, California, USA) supplemented with 20 µg ml-1 FSH (Sigma, St. Louis, USA) and 10% fetal calf serum (FCS; Invitrogen) in high humidity, under 5% CO2 in air at 38.5°C for 24h. Maturated oocytes were subjected to *in vitro* fertilization in 100-µl drops of Fert-TALP supplemented with heparin and 2x106 spermatozoa/ml-1 for 6h in a humidified atmosphere of 5% CO2 and 38.8 °C. Presumptive zygotes were distributed randomly in the following groups: G1 (control group - without MWNT); G2 (0.2 µg pDNA: 0.2 µg ml-1 MWNT), G3 (0.2 µg pDNA: 2 µg ml-1 MWNT) and G4 (0.2 µg pDNA: 4 µg ml-1 MWNT). Transfection was allowed to proceed in serum-free CR2aa medium for 12 h at 38.5°C in an atmosphere of 5% CO2. Subsequently, the embryos were transferred to CR2 medium containing 2.5% FCS under 5% O2, 90% N2 and 5% CO2 at 38.5°C in air and high humidity for 7 days. GFP expression in the transfected embryos at day 3 (72h post-fertilization) was observed under fluorescent microscope. Real time PCR examination (Applied Biosystems Prism 7300 Sequence Detection Systems, Foster City, EUA) was performed using the genomic DNA of the transformed embryos (n=30 per group). Blastocysts at eight day post-fertilization from G1 (n=19), G2 (n=23), G3 (n=16) and G4 (n=16) were fixed and permeabilized for TUNEL assay (DeadEndTM Florimetric TUNEL System-PROMEGA). Statistical analysis was performed by chi-square test or ANOVA ($P<0.05$). GFP expression was observed as in-situ fluorescence at 3 days post-transfection (embryos at 2 to 8-cell stage) for G2, G3 and G4. However, the expression GFP was not observed at blastocyst stage. The rate of positive-GFP embryos by PCR analysis in G4 (46.67%, n=14/30) was higher ($P<0.05$) than in G2 and G3 (3.33%, n=1/30). Apoptosis index was higher ($P<0.05$) in blastocysts from G2 (15.12±1.21), G3 (19.48±2.30) and G4 (18.20±1.90) than those in G1 (10.28±2.83). In conclusion, we showed that MWNTs are able to deliver the GFP gene into embryos. However, further studies are required to identify non-harmful exposure period and MWNT concentration.

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