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Analysis of cat oocyte activation methods for the generation of feline disease models by nuclear transfer

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Abstract

Background: Somatic cell nuclear transfer in cats offers a useful tool for the generation of valuable research models. However, low birth rates after nuclear transfer hamper exploitation of the full potential of the technology. Poor embryo development after activation of the reconstructed oocytes seems to be responsible, at least in part, for the low efficiency. The objective of this study was to characterize the response of cat oocytes to various stimuli in order to fine-tune existing and possibly develop new activation methods for the generation of cat disease models by somatic cell nuclear transfer.

Methods: First, changes in the intracellular free calcium concentration $[Ca^{2+}]_i$ in the oocytes induced by a number of artificial stimuli were characterized. The stimuli included electroporation, ethanol, ionomycin, thimerosal, strontium-chloride and sodium (Na⁺)-free medium. The potential of the most promising treatments (with or without subsequent incubation in the presence of cycloheximide and cytochalasin B) to stimulate oocyte activation and support development of the resultant parthenogenetic embryos was then evaluated. Finally, the most effective methods were selected to activate oocytes reconstructed during nuclear transfer with fibroblasts from mucopolysaccharidosis I- and alpha-mannosidosis-affected cats.

Results: All treatments were able to elicit a $[Ca^{2+}]_i$ elevation in the ooplasm with various characteristics. Pronuclear formation and development up to the blastocyst stage was most efficiently triggered by electroporation (60.5 +/- 2.9 and 11.5 +/- 1.7%) and the combined thimerosal/DTT treatment (67.7 +/- 1.8 and 10.6 +/- 1.9%); incubation of the stimulated oocytes with cycloheximide and cytochalasin B had a positive effect on embryo development. When these two methods were used to activate oocytes reconstructed during nuclear transfer, up to 84.9% of the reconstructed oocytes cleaved. When the 2 to 4-cell embryos (a total of 220) were transferred into 19 recipient females, 4 animals became pregnant. All of the fetuses developed from oocytes activated by electroporation followed by cycloheximide and cytochalasin B incubation; no fetal development was detected as a result of thimerosal/DTT activation. Although heartbeats were detected in two of the cloned fetuses, no term development occurred.

Conclusion: Electroporation proved to be the most effective method for the activation of cat oocytes reconstructed by nuclear transfer. The combined thimerosal/DTT treatment followed by cycloheximide and cytochalasin B incubation triggered development effectively to the blastocyst stage; whether it is a viable option to stimulate term development of cloned cat embryos needs further investigations.

Background

Ovulated mammalian oocytes are arrested at the metaphase stage of their second meiotic division [1]. Normally, they resume meiosis and enter the first interphase at the time of fertilization when the fertilizing sperm activates the oocyte's developmental program by triggering changes in its intracellular free calcium concentration $[Ca^{2+}]_i$. Changes in the $[Ca^{2+}]_i$ can also be induced artificially and as a result parthenogenetic oocyte activation can take place. Although mammalian parthenogenetic embryos never develop to term, a great number of invertebrate and vertebrate animal species are able to reproduce via parthenogenesis [2].

During parthenogenetic activation, the increase in the $[Ca^{2+}]_i$ must be able to trigger the numerous biological events that are associated with fertilization [3]. The process is an integral part of several assisted reproductive technologies and has particular relevance in somatic cell nuclear transfer [4]. Numerous oocyte activation methods have been designed to mimic the Ca^{2+} signal induced by the sperm; however, very few of them are able to generate the oscillatory Ca^{2+} signal seen during mammalian fertilization. Thus in most cases a single $[Ca^{2+}]_i$ rise is induced to stimulate development of the reconstructed oocyte [5]. Although this was shown capable of triggering oocyte activation [6], the amplitude, frequency and duration of repetitive Ca^{2+} signals are believed to have profound effects not only on the immediate events of oocyte activation but also on peri-implantation development [7].

Activation of oocytes of many domestic species including the domestic cat has been described and used in reproductive research [8]. Cats are useful research models for a number of reasons. They are valuable for the study of hereditary diseases in humans since they can provide insight into disease etiology and pathology [9-13]. Cat models also facilitate investigation of promising treatments including gene therapies, as recently shown for the lysosomal storage diseases mucopolysaccharidosis (MPS) and α -mannosidosis (AMD); [14,15]. Reproductive research on domestic cats is also important for conserving endangered felid species [16-18]. Somatic cells isolated from nondomestic felids can be transferred into enucleated domestic cat oocytes, and it has been demonstrated that this nuclear transfer approach has the potential of generating live offspring if the two felid populations are

not too distantly related [19]. Despite the occasional successes low birth rates after nuclear transfer, just like in most other species, remain a formidable challenge. One potential reason for the low efficiency is poor embryo development following activation of the reconstructed oocytes. The number of methods known to induce parthenogenetic development of cat oocytes is rather low; the published methods are limited to electroporation, ethanol and the ionophore A23187 [8,19]. In most cases activation techniques developed for other species have been adopted for producing the cloned cat embryos; this may limit embryonic development after embryo transfer. The aims of this study were to 1) characterize $[Ca^{2+}]_i$ changes in cat oocytes induced by different stimuli; 2) develop new activation methods that could be applied after nuclear transfer to produce cleavage-stage embryos; and 3) assess the feasibility of nuclear transfer to produce cloned cats affected with the lysosomal storage diseases MPS or AMD.

Methods

All chemicals were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO, U.S.A.) unless otherwise indicated. Experiments were conducted according to guidelines approved by the Institutional Animal Care and Use Committee (IACUC) at the Cincinnati Zoo & Botanical Garden.

In vitro oocyte maturation

Cat ovaries were collected after routine ovariectomy. Cumulus-oocytes complexes (COCs) were collected by slicing in HEPES-buffered Tyrode's Lactate (TL-HEPES) medium and matured in groups of 10 in 100- μ l drops of Feline Optimized Culture Medium (FOCM; [20]) supplemented with 0.1 mM cysteamine, 0.6 mM cysteine, vitamins (1 \times MEM), ITS (1 \times), glucose (up to a total of 6 mM), 1.0 IU/ml equine chorionic gonadotropin (eCG), 2.0 IU/ml human chorionic gonadotropin (hCG), 25 ng/ml epidermal growth factor (EGF) and 4 mg/ml bovine serum albumin (BSA), for 24 h. The cumulus cells were then removed using 0.3 mg/ml hyaluronidase.

Ca²⁺ imaging and fluorescence measurements

Mature oocytes were loaded with the Ca^{2+} indicator dye fura-2 by incubation in the presence of 2 μ M fura-2 AM (acetoxymethyl ester form) and 0.02% detergent pluronic F-127 (Invitrogen; Carlsbad, CA, U.S.A.) for 45 min. The

oocytes were then rinsed and transferred to a chamber with a glass coverslip as the bottom. They were exposed to various treatments and changes in the $[Ca^{2+}]_i$ were recorded using InCyt Im2, a dual-wavelength fluorescence imaging system (Intracellular Imaging, Inc.; Cincinnati, OH, U.S.A.). Fluorescence was recorded by calculating the ratio of fura-2 fluorescence at 510 nm excited by UV light alternatively at 340 and 380 nm. The Ca^{2+} concentration was calculated by comparing the ratio of fluorescence at 340 and 380 nm against a standard curve of known Ca^{2+} concentration prepared with fura-2 potassium salt (Invitrogen). During measurements, the baseline $[Ca^{2+}]_i$ was detected first and then different stimuli were applied. For each treatment, the fluorescence measurement was performed for at least 10 min and then it was repeated several times using different oocytes.

Oocyte treatments

A number of stimuli were tested for their ability to trigger an elevation in the $[Ca^{2+}]_i$ of the oocytes. Then, based on the results of these measurements and on data published in other species, a number of treatments were identified that were deemed to have the potential to trigger parthenogenetic development of the oocytes. The oocytes were treated using the selected methods, then a portion of them were incubated in the presence of 10 μ g/ml cycloheximide (a protein synthesis blocker) and 7.5 μ g/ml cytochalasin B (an inhibitor of actin polymerization) for 4 h prior to culture; the rest of the oocytes were cultured without inhibitor supplementation. In previous studies the use of such inhibitors had a positive effect on embryo development following oocyte activation (for review see [21]). The oocytes were then cultured as described below and embryonic development induced by the different methods was monitored. The following treatments were tested.

Electric DC pulse

Individual oocytes were transferred to a chamber with two stainless steel electrodes where the bottom of the chamber had been replaced with a glass coverslip. The chamber was filled with electroporation medium (consisting of mannitol 0.3 M, $CaCl_2$ 0.1 mM, $MgSO_4$ 0.1 mM, HEPES 0.5 mM and BSA 0.01 mg/ml) and placed on the stage of an inverted microscope. After a 5-min equilibration period the steady-state Ca^{2+} levels were measured, then two DC pulses of various characteristics (1.0 kV/cm for 20 μ sec; 1.2 kV/cm for 60 μ sec; or 2 kV/cm for 60 μ sec), 1 sec apart, were applied to the oocytes [21] and changes in the $[Ca^{2+}]_i$ were monitored. To stimulate development two DC pulses of either 1.0 kV/cm for 20 μ sec or 1.2 kV/cm for 60 μ sec were applied.

Ethanol

Oocytes were placed into the Ca^{2+} measurement chamber containing TL-Hepes and ethanol (200 proof) was added

to the medium to obtain 7% (v/v) final ethanol concentration [22]. Changes in the $[Ca^{2+}]_i$ were recorded by the fluorescence imaging system. In order to trigger parthenogenetic development, oocytes were exposed to 7% (v/v) ethanol for 5 min.

Ionomycin

Each oocyte was transferred into TL-Hepes and ionomycin dissolved in dimethyl sulfoxide (DMSO) was added to the medium at a final concentration of 5 or 50 μ M [23]. The same amount of DMSO without ionomycin was added to control oocytes. For parthenogenetic development, the oocytes were incubated in TL-Hepes in the presence of 5 or 50 μ M ionomycin for 5 min.

Thimerosal

By oxidizing sulfhydryl groups on critical Ca^{2+} release proteins thimerosal is known to elevate $[Ca^{2+}]_i$ in oocytes [24]. To assess whether thimerosal has the same effect in cats, individual oocytes were transferred into TL-Hepes and thimerosal dissolved in TL-Hepes was added at a final concentration of 200 μ M [25]. Because thimerosal oxidizes sulfhydryl groups on tubulin and thus damages the meiotic spindle, the application of a reducing agent such as dithiothreitol (DTT) is necessary to regenerate the spindle [26]. For activation, mature oocytes were treated according to two different schemes with the sulfhydryl modifying agents. In the first group, the oocytes were incubated in 200 μ M thimerosal for 15 min followed by a 30-min incubation in the presence of 8 mM DTT. Oocytes in the other group were exposed to 200 μ M thimerosal for 30 min followed by a 8 mM DTT treatment for 20 min.

Strontium

The oocytes were washed in Ca^{2+}/Mg^{2+} -free TL-Hepes medium and transferred to the measurement chamber containing 500 μ l of the same medium. After recording the baseline Ca^{2+} concentration, strontium-chloride ($SrCl_2$) dissolved in TL-Hepes was added at a final concentration of 10 or 20 mM [27]. To induce development, oocytes were incubated in the presence of 10 or 20 mM $SrCl_2$ for 4 h.

Na^+ -free medium

It was shown in pig oocytes that Na^+ -free medium can induce repetitive Ca^{2+} transients via the Na^+/Ca^{2+} exchanger [28]. Based on this finding cat oocytes were placed individually into 10 μ l TL-Hepes. Following baseline detection, 2 ml Na^+ -free medium was added to the medium that resulted in an approximately 200 \times dilution of the original Na^+ concentration. The Na^+ -free medium consisted of choline chloride 114 mM, KCl 3.2 mM, KH_2PO_4 0.4 mM, $MgCl_2 \times 6H_2O$ 0.5 mM, Hepes 9.2 mM, $CaCl_2 \times 2H_2O$ 2 mM, PVA 0.1%, pH 7.0. For activation,

mature oocytes were incubated in Na⁺-free medium for 15, 30, 45 or 60 min.

Embryo culture and evaluation

Potential zygotes were cultured (in groups of 10) in 100 µl drops of FOCM IVC-1 medium (FOCM supplemented with 0.4% BSA). To evaluate the formation of pronuclei the activated oocytes were cultured for 8 h, then they were mounted on microscope slides under posted coverslips and placed in a fixative of ethanol: acetic acid (3:1) for 7 days. They were then stained with 1% (w/v) aceto-orcein and evaluated for the presence or absence of pronuclei. In order to assess development to the blastocyst stage, after 3 days of culture the groups of embryos were transferred into 100 µl drops of FOCM IVC-2 medium (FOCM supplemented with 5% fetal bovine serum) and incubated for an additional 4 days [20]. At the end of the 7-day culture period, the embryos were incubated in 5 µg/ml Hoechst 33342 for 15 min and the nuclei showing blue fluorescence were counted using an epifluorescence microscope.

Somatic cell nuclear transfer

Preparation of donor cells

The ultimate goal of these experiments was to generate cats as models for the heritable metabolic defect MPS I or AMD. The cell lines used as nuclear donors were generated by skin biopsies from affected cats. The explants were cut into small pieces and cultured in Dulbecco's Modified Eagle's medium (DMEM, Invitrogen) supplemented with 15% fetal bovine serum and 1% penicillin/streptomycin. Small aliquots of cells between passages 3 and 5 were frozen in DMEM supplemented with 10% DMSO and stored in liquid nitrogen until use.

Oocyte collection

Female cats were monitored daily for signs of behavioral estrus. Blood samples were collected from anestrous queens and the serum was assessed for progesterone to evaluate luteal status. Non-luteal queens were administered four intramuscular (i.m.) injections of porcine FSH at 24 h intervals (Day 1: 9.6 IU; Day 2: 7.6 IU; Day 3: 5.7 IU; Day 4: 5.7 IU); [29]. The last FSH injection was followed 24 h later by an i.m. injection of porcine LH (1,000 IU). Donors were anesthetized 24-27 h after the LH injection and subjected to laparoscopy and follicle aspiration. Follicles (2-5 mm in diameter) were aspirated and the COCs were collected in bicarbonate-buffered FOCM.

Nuclear transfer

Oocytes with an extruded polar body were enucleated in TL-Hepes medium supplemented with 7.5 µg/ml cytochalasin B. For enucleation, the first polar body and the adjacent cytoplasm presumably containing the metaphase-II chromosomes were removed. (Because in previous experiments this blind enucleation led to ~85%

successfully enucleated oocytes, Hoechst staining was not used during nuclear transfer in the present study). A donor cell was then transferred into the perivitelline space of each enucleated oocyte. Reconstructed oocytes were placed in an electroporation chamber filled with Ca²⁺-free electroporation medium (containing 300 mM mannitol, 0.2 mM MgSO₄, 0.5 mM Hepes, 0.1% polyvinyl alcohol) and two DC pulses (1.2 kV/cm for 60 µsec, 1 sec apart) were applied. Following fusion, part of the reconstructed oocytes were activated by electroporation (two pulses of 1.0 kV/cm for 20 µsec) and the rest by the combined method of 15 min incubation with 200 µM thimerosal followed by 30 min in 8 mM DTT. Treated oocytes were transferred to FOCM supplemented with 10 µg/ml cycloheximide and 7.5 µg/ml cytochalasin B for 4 h. Potential zygotes were then washed and cultured in FOCM/0.4% BSA until embryo transfer.

Embryo transfer

Estrual females, on Day 2 to 6 of estrus received two subcutaneous injections of gonadotropin releasing hormone (GnRH; 25 µg each, 12 h interval). The cats were anesthetized 54 to 58 h after the first GnRH injection and the ovaries were evaluated via laparoscopy. If fresh corpora lutea were present on the ovaries confirming the occurrence of recent ovulations, early cleavage stage (20-38 h after nuclear transfer) embryos (n = 8-21 per animal) were transferred through the ostium into the lumen of the left or right oviduct. All embryo recipient queens were subjected to ultrasound examination 21-23 days after embryo transfer. Pregnant females were reassessed with ultrasonography periodically to monitor fetal viability and growth.

Statistical analysis

Frequencies of pronuclear formation, cleavage and blastocyst development were compared by the chi-square test. Total cell numbers of the blastocysts were compared by the Student's t-test. Statistical comparisons were performed using either SAS or Minitab; differences were considered significant at P < 0.05.

Results

Changes in cytosolic Ca²⁺ levels

Electroporation triggered an almost immediate rise in the [Ca²⁺]_i of cat oocytes. The application of DC pulses of larger voltage or longer duration led to more robust changes in cytoplasmic Ca²⁺ levels and increasing these parameters above a certain threshold (e.g. raising voltage above ~1.8 kV/cm) caused elevated intracellular Ca²⁺ levels that did not return to baseline possibly indicating permanent membrane damage (data not shown). A typical change induced by two pulses of 1 kV/cm, 20 µsec each is shown in Figure 1A. The resting [Ca²⁺]_i in the oocytes was approximately 200 nM. The [Ca²⁺]_i elevation started 3 ±

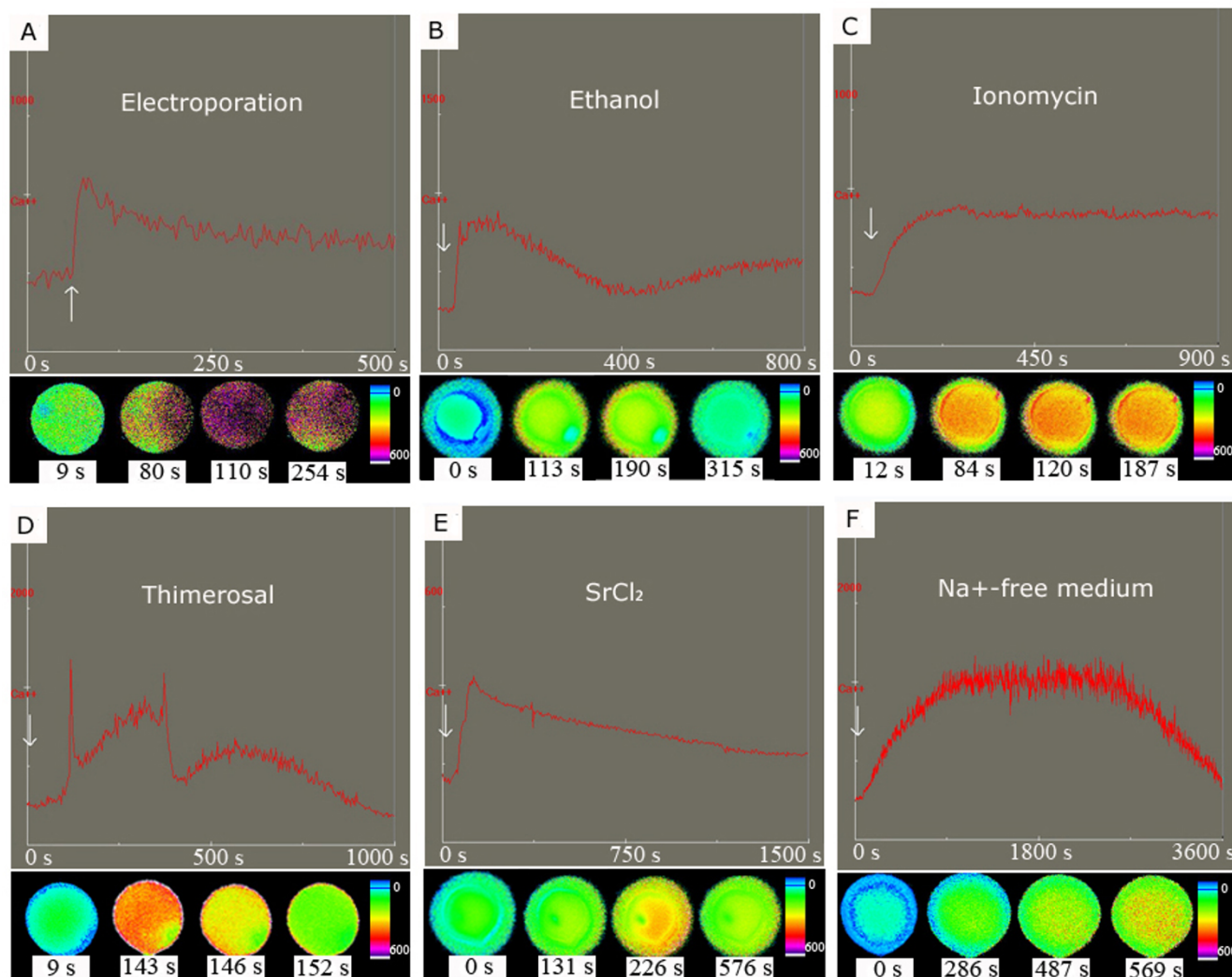


Figure 1
Changes in the $[Ca^{2+}]_i$ of cat oocytes induced by different stimuli. The upper part of each section shows cytoplasmic free Ca^{2+} levels as detected by a photometer; the arrows indicate the time when the stimulus was applied. In the lower part of each section images of $[Ca^{2+}]_i$ changes in individual cat oocytes are shown; different colors indicate different intracellular free Ca^{2+} concentrations.

0.4 sec after applying the electrical shock and peaked at a concentration of 485 ± 28 nM after which it decreased gradually. The transient had an average duration of 73 ± 15 sec and the $[Ca^{2+}]_i$ reached near baseline levels by the end of the measurements ($n = 15$). Electroporation caused a single $[Ca^{2+}]_i$ rise in all oocytes tested (Table 1).

Incubation of mature cat oocytes in 7% ethanol induced a single $[Ca^{2+}]_i$ rise in 5 out of 12 oocytes (Figure 1B). The elevation started at 110 ± 11 sec after adding the ethanol; the $[Ca^{2+}]_i$ then increased up to an average of 394 ± 87 nM and then returned to basal levels. The mean total duration

of the Ca^{2+} transient was 150 ± 41 sec. Ionomycin at both concentrations (5 and 50 μ M) could elicit a rapid Ca^{2+} response in all (a total of 10) oocytes tested. Figure 1C shows a $[Ca^{2+}]_i$ change induced by 50 μ M ionomycin. The $[Ca^{2+}]_i$ rise in these cases started within a few seconds after adding ionomycin and peaked at a concentration of 336 ± 12 nM. In all cases the $[Ca^{2+}]_i$ elevation had an extended duration that lasted as long as the oocyte was exposed to the ionophore since no $[Ca^{2+}]_i$ return to basal levels was observed during the measurement period. The Ca^{2+} rise triggered by 50 μ M ionomycin started within a shorter period of time and had higher amplitude compared to

Table 1: Characteristics of $[Ca^{2+}]_i$ changes (mean \pm SEM) induced by various artificial stimuli

Treatment	No. of oocytes (No. of responses)	Time till response (sec)	Peak $[Ca^{2+}]_i$ (nM)	Duration (sec)	Oscillation	Interval between peaks (sec)
Electroporation	15 (15)	3 \pm 0.4	485 \pm 28	73 \pm 15	No	--
7% ethanol	5 (12)	110 \pm 31	394 \pm 87	150 \pm 41	No	--
5 μ M ionomycin	5 (5)	18 \pm 2	277 \pm 27	>800	No	--
50 μ M ionomycin	5 (5)	6 \pm 1	336 \pm 12	>800	No	--
200 μ M thimerosal	15 (18)	365 \pm 51	496 \pm 75	85 \pm 8	Yes	351 \pm 30
20 mM SrCl ₂	15 (15)	85 \pm 10	431 \pm 52	335 \pm 28	No	--
Na ⁺ -free medium	15 (28)	135 \pm 24	639 \pm 79	1470 \pm 242	No	--

those stimulated by lower ionomycin concentration (data not shown). DMSO alone did not elicit changes in the $[Ca^{2+}]_i$ (data not shown).

Incubation of mature cat oocytes with 200 μ M thimerosal induced $[Ca^{2+}]_i$ elevation in 15 out of 18 oocytes; the signal took place as an oscillation in the intracytoplasmic Ca^{2+} level in 11 oocytes. On average, the initial $[Ca^{2+}]_i$ rise started 356 \pm 51 sec after the addition of thimerosal, with an average peak concentration of 496 \pm 75 nM and a mean duration of 85 \pm 8 sec. Subsequent rises in the $[Ca^{2+}]_i$ were generated at intervals of 351 \pm 30 sec; the number of Ca^{2+} spikes induced in the oocytes was between 2 and 5. In all oocytes the initial transient had the highest peak, each subsequent transient tended to have a lower average amplitude (Figure 1D). The oscillations usually lasted for 30-45 min and during this time the Ca^{2+} baseline increased gradually.

Addition of SrCl₂ caused a single long-lasting rise in the $[Ca^{2+}]_i$ in all oocytes tested (n = 30). As a result of 20 mM SrCl₂ stimulation, the baseline $[Ca^{2+}]_i$ increased to 431 \pm 52 nM within 85 \pm 10 sec. The average duration of the elevated $[Ca^{2+}]_i$ was 335 \pm 28 sec (Figure 1E). The lower (10 mM) SrCl₂ concentration elicited a similar response in the $[Ca^{2+}]_i$ (data not shown).

The profile of the $[Ca^{2+}]_i$ rise caused by Na⁺-free-medium is displayed in Figure 1F. Incubation in Na⁺-free-medium induced a gradual $[Ca^{2+}]_i$ elevation in 15 out of 28 oocytes. The rise started 135 \pm 24 sec after addition of the Na⁺-free-medium and peaked at a concentration of 639 \pm 79 nM. The average duration of the $[Ca^{2+}]_i$ rise induced by the Na⁺-free-medium was 1,470 \pm 242 sec.

Parthenogenic embryo development

The fluorescence recordings indicated that all the artificial stimuli tested were able to generate an elevation in the $[Ca^{2+}]_i$ in mature cat oocytes. Next, in preliminary experiments the different stimuli were evaluated for their ability to induce parthenogenetic development and based on the outcome of these experiments four different treatments

were finally selected and compared. The treatments selected for further evaluation were the following: 1) electroporation using two DC pulses of 1.0 kV/cm for 20 μ sec each; 2) 15 min incubation in 200 μ M thimerosal followed by a culture in 8 mM DTT for 30 min; 3) incubation in the presence of 20 mM SrCl₂ in Ca^{2+}/Mg^{2+} -free medium for 4 h, and 4) a treatment in Na⁺-free medium for 45 min. Each treatment was applied with or without subsequent incubation in the presence of 10 μ g/ml cycloheximide and 7.5 μ g/ml cytochalasin B for 4 h.

Electroporation stimulated 72 out of 119 (60.5 \pm 2.9%) oocytes to form pronuclei. Incubation of the electroporated oocytes with cycloheximide and cytochalasin B increased the percentage of oocytes having pronuclei significantly (65 out of 81, 80.2 \pm 3.1%, $P < 0.05$). The combined thimerosal/DTT treatment induced pronuclear formation in 84 out of 124 oocytes (67.7 \pm 1.8%), this frequency was significantly higher than those found after the 20 mM SrCl₂ (58/114; 50.9 \pm 4.0%) and Na⁺-free medium (62/115; 53.9 \pm 2.4%) treatments ($P < 0.05$). Oocytes in these groups tended to show higher pronuclear formation after cycloheximide and cytochalasin B incubation but the difference was not statistically significant (Figure 2).

The cleavage frequency stimulated by electroporation was 58.2 \pm 4.0% (142 out of 244 oocytes; Figure 3). The thimerosal/DTT treatment triggered 60.6 \pm 2.4% (154/254) of the oocytes to cleave, this percentage was significantly higher than that registered in the SrCl₂-treated group (128/262; 48.9 \pm 3.6%; $P < 0.05$). Of the oocytes that were activated with Na⁺-free medium, 54.9 \pm 3.4% (162/295) cleaved. We also found that incubation with cycloheximide and cytochalasin B caused a significant increase in cleavage frequency after electroporation (140/203; 69 \pm 4.2%) and SrCl₂ (118/201; 58.7 \pm 4.5%) treatments ($P < 0.05$).

In the absence of incubation with the inhibitors, blastocyst formation was the highest after electroporation (28/244; 11.5 \pm 1.7%) and the combined thimerosal/DTT treatment (27/254; 10.6 \pm 1.9%). These values were

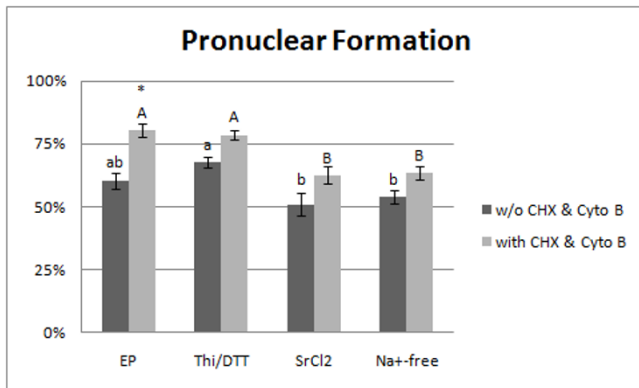


Figure 2
Pronuclear formation in cat oocytes activated by electroporation (EP), thimerosal/DDT (Thi/DDT), SrCl₂ and Na⁺-free medium (Na⁺-free) with or without 4-h incubation in cycloheximide (CHX) and cytochalasin B (Cyto B). The asterisk indicates a significant difference ($P < 0.05$) between frequencies of pronuclear formation achieved with and without CHX/Cyto B supplementation. Different lowercase letters indicate significant differences between treatments without CHX/Cyto B supplementation ($P < 0.05$); different uppercase letters indicate significant differences between treatments that were followed by CHX/Cyto B incubation ($P < 0.05$).

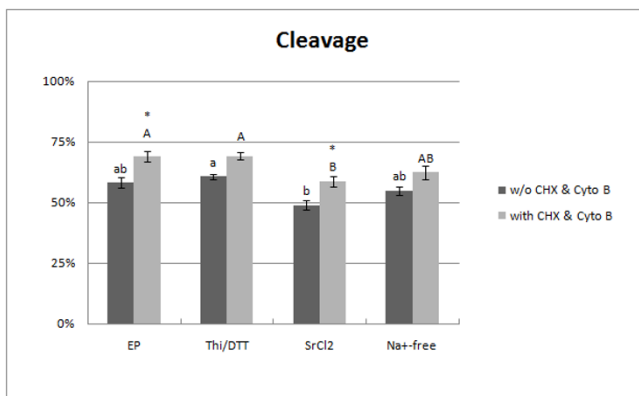


Figure 3
Cleavage development of embryos after activation by electroporation (EP), thimerosal/DDT (Thi/DDT), SrCl₂ and Na⁺-free medium (Na⁺-free) with or without a 4-h incubation in cycloheximide (CHX) and cytochalasin B (Cyto B). The asterisks indicate significant differences ($P < 0.05$) between cleavage frequencies achieved with and without CHX/Cyto B supplementation. Different lowercase letters indicate significant differences between treatments without CHX/Cyto B supplementation ($P < 0.05$); different uppercase letters indicate significant differences between treatments where the Ca²⁺ signal-inducing stimulus was followed by CHX/Cyto B incubation ($P < 0.05$).

significantly higher than those in the SrCl₂ (12/262; 4.6 ± 3.6%) and Na⁺-free medium (16/295; 5.4 ± 3.4%) - treated groups ($P < 0.05$; Figure 4). Incubation with cycloheximide and cytochalasin B significantly improved the frequency of blastocyst formation in oocytes that were activated by thimerosal/DDT (44/209; 16.7 ± 2.9%), SrCl₂ (24/201; 11.9 ± 2.8%) and Na⁺-free medium (24/245; 9.8 ± 1.4%); ($P < 0.05$).

Somatic cell nuclear transfer

A total of 157 reconstructed oocytes were produced by somatic cell nuclear transfer using MPS I-affected donor cells; these oocytes were activated by electroporation followed by incubation in cycloheximide and cytochalasin B. Following activation 114 (72.6%) reconstructed oocytes cleaved, the 2 to 4-cell embryos were transferred into the oviducts of 9 recipient females. Three of these recipients had implantations on initial palpation and ultrasonographic exam on Day 20 to 21 post-transfer. During early ultrasonography a total of 4 implantation sites and 3 developing fetuses were found; one female had only a single implantation site with no visible fetus. Heartbeats were successfully detected in two of the fetuses. However, by day 47 all 3 fetuses were absorbed (Table 2). An additional 130 reconstructed oocytes were produced by nuclear transfer using AMD-affected donor cells; 73 of which were activated by electroporation followed by cycloheximide and cytochalasin B incubation while 57 were activated by thimerosal/DDT followed by incubation with the inhibitors. In the electroporation-

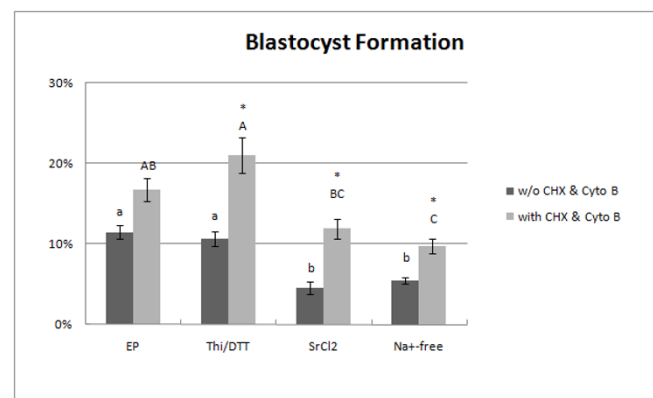


Figure 4
Blastocyst formation induced by parthenogenetic activation of cat oocytes. The asterisks indicate significant differences ($P < 0.05$) between frequencies of blastocyst formation achieved with and without CHX/Cyto B supplementation. Different lowercase letters indicate significant differences between treatments without CHX/Cyto B supplementation; different uppercase letters indicate significant differences between treatments followed by CHX/Cyto B incubation ($P < 0.05$).

Table 2: Development of reconstructed cat oocytes activated by electroporation or thimerosal/DTT.

Donor Cell type	Activation treatment	Embryos produced	Embryos cleaved and transferred (%)	Recipients	Pregnant recipients	Implantation sites (%)	No. of fetuses (%)
MPS	Electroporation	157	114 (72.6)	9	2*	4 (3.5)	3 (2.6)
AMD	Electroporation	73	62 (84.9)	6	2	4 (6.5)	2 (3.2)
AMD	Thimerosal/DTT	57	44 (77.2)	4	0	0 (0)	0 (0)

Stimulated oocytes in both cases were subsequently incubated in cycloheximide and cytochalasin B for 4 h.

*A third female had a single implantation site but with no observable fetus

activated group 62 oocytes (84.9%) cleaved; cleavage frequency in the thimerosal/DTT-treated group was 77.2% (44 out of 57 oocytes). The 62 cleaved embryos that were produced by electroporation were transferred into 6 recipients, of which 2 became pregnant. In the pregnant animals 4 implantation sites and 2 fetuses were detected on day 21. The 2 to 4-cell embryos that resulted from the thimerosal/DTT activation were transferred into 4 recipients; ultrasonography on day 21 could find neither implantation sites nor developing fetuses in these surrogates. No fetuses could be detected by ultrasonography on day 49 in any of the recipient animals.

Discussion

Following sperm-oocyte fusion, the Ca^{2+} signal triggered by the fertilizing sperm is responsible for stimulating meiotic resumption and eventually, embryonic development. In most cases, parthenogenetic activation methods also induce an elevation in the $[Ca^{2+}]_i$ to activate the oocyte's developmental program [5]. In many species numerous comparative studies have been carried out using different methods of artificial activation, and the most effective ones have been selected to activate reconstructed oocytes after nuclear transfer. In felids no such studies have been performed and the number of methods available for parthenogenetic activation of cat oocytes is rather limited. In the present study we characterized responses of cat oocytes to several artificial stimuli in order to find ways to trigger $[Ca^{2+}]_i$ changes similar to those detected during mammalian fertilization. All the methods tested could elicit a rise in the $[Ca^{2+}]_i$ of cat oocytes. Electroporation is commonly used to induce a transient elevation in the $[Ca^{2+}]_i$ of oocytes of various species; it has also been successfully used to stimulate oocyte activation during cat nuclear transfer [30]. The short, high voltage DC pulses are known to induce a significant transmembrane Ca^{2+} influx by causing a destabilization of the phospholipid bilayer. The influx is influenced by the voltage and duration of the electrical pulse as well as the ionic strength of the electroporation medium [31]. We evaluated the effect of DC pulses with various duration and magnetic field characteristics; all pulses triggered changes in the $[Ca^{2+}]_i$. To stimulate embryonic development we have selected a

set of parameters that induced a large $[Ca^{2+}]_i$ elevation without causing irreversible damage to the oocytes.

Ethanol was reported to activate oocytes of various species. In *Xenopus* it was demonstrated to stimulate the production of inositol 1,4,5-trisphosphate (IP_3) which induces the release of Ca^{2+} from the oocyte's intracellular stores [32]. Its effect on cytoplasmic Ca^{2+} levels of cat oocytes has never been characterized before. We found that 7% ethanol triggered a long-lasting Ca^{2+} signal in cat oocytes as well. However, the effects of ethanol on subsequent embryonic development have not been tested in the present study because recently it was shown by others that ethanol could activate ~50% of cat oocytes [8]. The Ca^{2+} ionophore ionomycin has also been shown to induce $[Ca^{2+}]_i$ elevations in oocytes of many species. According to the literature, the parameters of ionomycin treatment used to activate oocytes of various species vary significantly: concentrations ranging from 5 μ M to 5 mM [33] and treatment duration of 1 min to 40 min have also been reported [34]. The influence of ionomycin on cat oocytes has never been described; therefore optimizing the concentration and incubation time is critical for successful oocyte activation. As in other species, cat oocytes showed a larger elevation in the intracellular free Ca^{2+} levels after being treated with higher concentrations of ionomycin. Surprisingly, all the ionomycin concentrations that we tested for the ability to induce $[Ca^{2+}]_i$ changes caused irreversible damages and were lethal to the oocytes.

Thimerosal is known to oxidize sulfhydryl groups on intracellular Ca^{2+} release proteins [35,36], thus causing the release of stored Ca^{2+} and oscillation in the $[Ca^{2+}]_i$ levels in oocytes of a number of species [24,26,37]. Similar to those findings, thimerosal in cat oocytes was able to induce repetitive $[Ca^{2+}]_i$ changes. During incubation, the baseline $[Ca^{2+}]_i$ became increasingly elevated between transients that was probably attributable to the inhibition of Ca^{2+} -ATPases since it was reported that thimerosal not only induced Ca^{2+} release from the stores but also inhibited Ca^{2+} -ATPases and prevented the removal of excess Ca^{2+} from the cytosol [38].

Incubation in the presence of Sr^{2+} induces activation in mouse oocytes [39] but the mechanism of its action has not been completely elucidated. Because in certain cell types Sr^{2+} is handled similar to Ca^{2+} it was proposed that Sr^{2+} activates oocytes by displacing bound Ca^{2+} [40]. Recent studies showed that by using a low molecular weight heparin to antagonize the function of IP_3 receptors or treating oocytes with the phospholipase C inhibitor U73122, the Sr^{2+} -induced $[\text{Ca}^{2+}]_i$ increases in mouse oocytes were blocked and this inhibitory effect could be rescued by microinjection of IP_3 . These results indicated that Sr^{2+} triggered the release of stored Ca^{2+} through the IP_3 receptors [41]. Although in mice Sr^{2+} treatment leads to an oscillation in the oocyte $[\text{Ca}^{2+}]_i$, we found that in cat oocytes Sr^{2+} triggered a single, long-lasting Ca^{2+} elevation.

The effects of Na^+ -free medium have been described in the pig where it was reported to induce oscillatory changes in the $[\text{Ca}^{2+}]_i$ [28]. In approximately 50% of the cases, Na^+ -free medium also stimulated a $[\text{Ca}^{2+}]_i$ elevation in cat oocytes with a high amplitude and long duration. The effect of the Na^+ -free medium is probably mediated by the $\text{Na}^+/\text{Ca}^{2+}$ exchanger expressed in the plasma membrane of cat oocytes. The $\text{Na}^+/\text{Ca}^{2+}$ exchanger uses the energy of a Na^+ gradient to move Ca^{2+} [42]. However, in case of low extracellular Na^+ levels the exchanger can operate in a reverse mode, pumping Ca^{2+} into the cell. This feature of the exchanger makes Na^+ -free medium a potential candidate for stimulating oocyte activation. The exchanger was found to be present in hamster [43], mouse [44,45] and porcine [28] oocytes. Because Na^+ -free medium triggered an elevation in the $[\text{Ca}^{2+}]_i$, we decided to investigate its ability to stimulate parthenogenetic development in cat oocytes.

Based on the results of the fluorescence measurements, four different methods have been selected to activate cat oocytes. As a result of the treatments, pronuclear formation in the oocytes ranged between 50.9% and 67.7%. The combined thimerosal/DTT treatment followed by cycloheximide and cytochalasin B incubation led to blastocyst formation in 21.1% of the cases. This frequency is similar to that received after electroporation, the method most widely used for oocyte activation. Although thimerosal is able to induce Ca^{2+} oscillation in oocytes, it also oxidizes sulfhydryl groups on tubulin that prevents further development [46]. In order to eliminate its negative effects, the thimerosal treatment must be followed by an incubation in the presence of the sulfhydryl-reducing compound DTT [26]. The high percentage of blastocyst formation achieved in the present study indicates for the first time that the thimerosal/DTT activation is an effective way to stimulate parthenogenetic embryo development in cats.

Even though blastocyst formations induced by SrCl_2 or Na^+ -free medium were somewhat low, this is the first report to demonstrate that these stimuli can trigger cat oocyte activation and subsequent blastocyst development. Although Na^+ -free medium has previously been reported to induce $[\text{Ca}^{2+}]_i$ increases in porcine oocytes, no subsequent embryo development has been observed [28]. Here we found that Na^+ -free medium could induce cleavage of cat oocytes with a frequency similar to that triggered by electroporation or the combined thimerosal/DTT treatment, although the subsequent blastocyst formation was lower compared to the other treatments. It is possible that the amplitude of the Ca^{2+} signal induced by the Na^+ -free medium is insufficient to induce complete egg activation. This is in accordance with the finding that in rabbit the amplitude of the Ca^{2+} transient did not appear to affect early cleavage but it influenced the developmental competence of the produced embryo [7]. Another reason for the low blastocyst formation may be that sustained high levels of $[\text{Ca}^{2+}]_i$ induced via the $\text{Na}^+/\text{Ca}^{2+}$ exchanger caused cellular damage that negatively influenced developmental competence. Although optimization of the Na^+ -free treatment was attempted in this study, the improvement in embryonic development was not significant. Additional studies focusing on the fine-tuning of this method may be helpful to develop a method that utilizes the Ca^{2+} signal generated via the $\text{Na}^+/\text{Ca}^{2+}$ exchanger for oocyte activation.

The results obtained also showed that incubation of cat oocytes in cycloheximide and cytochalasin B after an induced Ca^{2+} signal increased the frequency of pronuclear formation, cleavage and blastocyst formation. Mature mammalian oocytes arrested at the second metaphase stage synthesize cyclin B continuously in order to maintain activity of the M-phase Promoting Factor (MPF). During activation, MPF activity has to drop in order to allow release from the metaphase II arrest [47]. Cycloheximide is a protein synthesis inhibitor that blocks the production of cyclin B and in turn decreases MPF activity. Cytochalasin B on the other hand is an inhibitor of actin polymerization frequently used to block the extrusion of second polar body. In the presence of cytochalasin B, segregation of the chromosomes occurs after parthenogenetic oocyte activation but cytokinesis does not take place which results in the formation of diploid zygotes with two pronuclei [22,48]. In our experiments, incubation of cat oocytes with cycloheximide and cytochalasin B had the tendency to improve pronuclear formation, cleavage frequency and blastocyst development after every single Ca^{2+} elevation-inducing stimulus tested and the improvement was significant in several cases. Although the incubation did not increase the total cell number of the developing blastocysts, the results indicate that these inhibitors are

beneficial to increase the efficiency of oocyte activation in the cat.

The objective of the oocyte activation experiments was to develop potential methods to be used during nuclear transfer for the generation of useful disease models. In the domestic cat, somatic cell nuclear transfer not only provides the opportunity to generate genetically identical animals for research purposes, it also has the potential to facilitate preservation of rare and valuable felid populations, including cat research models and possibly endangered felid species [30]. In addition, domestic cats are useful research models for the study of more than 200 human hereditary diseases [49]. Maintenance of cat disease models can be challenging however, because the disease state may interfere with normal breeding, gestation and/or parturition. The application of nuclear transfer technology offers an innovative approach. Cloning would eliminate the need to collect gametes from affected animals for in vitro fertilization, it would obviate the requirement to maintain populations of carrier (heterozygous) cats, and for cat models that do not survive to sexual maturity cloning would provide a direct method for their continued propagation. Based on the frequency of blastocyst formation they induced, two methods were selected to activate reconstructed cat oocytes after nuclear transfer. These methods included electroporation and the combined thimerosal/DTT treatment, each followed by incubation in the presence of cycloheximide and cytochalasin B.

For nuclear transfer, fibroblast cells from two animals affected by different metabolic defects were collected and used as nuclear donors. The defects included AMD and MPS I, two lysosomal storage diseases that have been extensively characterized in cats. They are homologous to human inborn errors, involve single gene mutations and inherited as autosomal recessive traits [49,50]. These models have been instrumental in demonstrating the value of bone marrow transplantation in alleviating these diseases and, most recently, the potential of gene therapy using an adeno-associated viral vector for treating AMD-affected cats [14,15]. Cats affected with MPS I are difficult to breed naturally due to musculoskeletal abnormalities whereas AMD-affected cats typically die before reaching puberty.

Transfer of cloned embryos in which development was stimulated with electroporation followed by cycloheximide and cytochalasin B incubation resulted in 5 females having distinct implantation sites and 4 females with established pregnancies (i.e., presence of at least one fetus). Despite a total of 8 implantation sites, 5 fetuses and detectable heartbeats in two fetuses, no term development occurred; all fetuses were eventually reabsorbed by

day 49 of pregnancy. Our previous research has demonstrated the developmental competence to term of MPS-affected and MPS-carrier IVF embryos following transfer into our primary recipient type [51]. Pregnancy loss in the current study may be attributable to deficient reprogramming of the donor nucleus in the enucleated oocyte or aberrant chromosome numbers as a result of incomplete enucleation. Furthermore, no pregnancies were detected following thimerosal/DTT-induced oocyte activation which was somewhat unexpected. Nevertheless, the thimerosal/DTT treatment stimulated cleavage divisions at a frequency comparable to that achieved by electroporation. This, in light of a recent report showing that the thimerosal/DTT activation supported term development of cloned transgenic piglets [52] implies that successful activation can be achieved by modification of sulfhydryl groups on key Ca^{2+} releasing proteins in cat oocytes.

Conclusion

This study described a number of novel methods to induce Ca^{2+} release in cat oocytes for the stimulation of embryo development. In addition, the results support previous findings that electroporation is an effective way for the activation of oocytes reconstructed by nuclear transfer. Based on in vitro data, the combined thimerosal/DTT activation may also be suitable for cat oocyte activation but this needs further verification from additional studies.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

CW carried out in vitro oocyte maturation, fluorescence measurements, parthenogenetic oocyte activation, performed statistical analysis and drafted the manuscript. WFS conceived of the study, participated in its design and performed oocyte collections and embryo transfers. JRH participated in oocyte collections and embryo transfers. KL performed nuclear transfer. ZM participated in the design and coordination of the study and helped to draft the manuscript. All authors read and approved the final manuscript.

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