Human Reproduction, Vol.26, No.3 pp. 662–670, 2011
Advanced Access publication on January 11, 2011 doi:10.1093/humrep/deq379

human reproduction

ORIGINAL ARTICLE *Reproductive biology*

Effects of electric field on early preimplantation development *in vitro* in mice and rats

Elena Popova, Michael Bader*, and Alexander Krivokharchenko

Max-Delbrück Center for Molecular Medicine (MDC), Robert-Rössle-Str. 10, D-13125 Berlin-Buch, Germany

*Correspondence address. Fax: +49-30-9406-2110; E-mail: mbader@mdc-berlin.de

Submitted on October 6, 2010; resubmitted on November 30, 2010; accepted on December 7, 2010

BACKGROUND: Exposure of cells to electric fields is a commonly used technique for parthenogenesis, cloning and tetraploid embryo production. However, little is known about possible detrimental effects of electric fields on embryos and their development. The aim of this study was to investigate the effects of electric fields on early preimplantation development in mice and rats.

METHODS: Mouse and rat metaphase II (MII) and pre-activated oocytes, zygotes and 2-cell stage embryos were treated with electric fields with increasing voltage. Cleavage rate, morula and blastocyst formation were evaluated by *in vitro* cultivation. The effects of electric fields on embryos were investigated by measurement of reactive oxygen species (ROS) content and microtubule and microfilament distributions using fluorescence staining.

RESULTS: Pre-activated oocytes at the pronuclear stage and zygotes are more resistant to electric exposure than freshly isolated oocytes at MII stage in both studied species. Rat zygotes treated with electric fields of increasing voltage showed higher cleavage rates compared with the mouse and some of them developed beyond 4-cell stage *in vitro*. Embryos blocked at the 2-cell stage after *in vitro* cultivation of zygotes exposed to electric fields demonstrated increased level of ROS but normal distributions of microtubules and microfilaments. In both species, embryos at the 2-cell stage were more resistant to electric fields because they formed tetraploid embryos after electric field-induced blastomere fusion and these embryos could develop *in vitro* until the blastocyst stage.

CONCLUSIONS: There are stage-dependent and species-specific differences in sensitivity to electric fields in mouse and rats.

Key words: electric field / early preimplantation development / *in vitro* culture / mouse and rat embryos

Introduction

Current technologies for the creation of genetically modified mammals have been developing dramatically. All of these methodologies include large variety of different treatments and micromanipulations with preimplantation mammalian embryos.

It has recently been reported that widely used manipulations with embryos such as pronuclear microinjection, intracytoplasmic sperm injection, piezo-driven injection, cytoplasm and nuclear transfer, electrofusion procedure and *in vitro* culture techniques can induce DNA damage and epigenetic modifications in the embryos, changes of gene expression in early pre- and post-implantation embryos, apoptosis, a decreased cell number in blastocysts and lower developmental potential *in vitro* and *in vivo* (Tielens *et al.*, 2006; Yamauchi *et al.*, 2007; Yu *et al.*, 2007; Koo *et al.*, 2008; Morgan *et al.*, 2008; Zhang *et al.*, 2008a; Cheng *et al.*, 2009).

The electric field has potential as a research tool for various biotechnological experiments with mammalian gametes and embryos. Electric field-induced parthenogenetic development of oocytes has been performed in mice (Rickords and White, 1992a,b), rats (Krivokharchenko *et al.*, 2003), hamsters (Suzuki *et al.*, 2001), rabbits (Ozil, 1990), cattle (Tanaka and Kanagawa, 1997), pigs (Kure-bayashi *et al.*, 2000; Zhu *et al.*, 2002; Hirao *et al.*, 2003) and goats (Zhang *et al.*, 2008b; Guo *et al.*, 2009). Electrical activation can be used to facilitate fertilization and early embryonic development after ICSI in humans (Zhang *et al.*, 1999).

Physical and chemical treatments are well known to induce cell fusions. Membrane fusions can be achieved by Sendai virus infection, polyethylene glycol or by application of an electric field. Development of electrofusion avoids the possible toxic effect of the other methods on embryos (Zimmermann, 1986). Currently it has become most popular due to its easy use and has many applications in various embryological experiments.

Electrofusion was first applied for cloning and is currently used to fuse donor karyoplasts to enucleated cytoplasts in somatic cell nuclear transfer (SCNT) in different mammalian species such as

sheep (Wilmut *et al*., 1997), cattle (Kato *et al*., 1998; Booth *et al*., 2001; Oback *et al*., 2003), mice (Ogura *et al*., 2000), pigs (Polejaeva *et al*., 2000), horses (Galli *et al*., 2003), ferrets (Li *et al*., 2005), rats (Popova *et al*., 2009) and other mammals.

Electrofusion of blastomeres is a reliable technique for tetraploid embryo production in mice (Kubiak and Tarkowski, 1985; Nagy *et al*., 1990, 1993), rabbit (Ozil and Modlinski, 1986), rats (Krivokharchenko *et al*., 2002) and cattle (Curnow *et al*., 2000). Most importantly, tetraploid embryos obtained by electrofusion have been applied to chimera production in mice, in which either embryonic stem cells or induced pluripotent stem cells are used for tetraploid embryo complementation (Nagy *et al*., 1990, 1993; Eggan *et al*., 2002; Boland *et al*., 2009; Zhao *et al*., 2009).

Electrofusion can also be used to increase the oocyte volume for SCNT (Sayaka *et al*., 2008) and for introduction of large amounts of donor cytoplasm in mature human oocytes (Cohen *et al*., 1998). Furthermore electrofusion can be a very efficient method for novel developments in assisted reproductive technologies that could eliminate the transmission of mitochondrial diseases in affected families; these technologies include cytoplasmic, germinal vesicle and pronuclear transfer (Craven *et al*., 2010; Tachibana *et al*., 2010). The fusion technique is also used for production of human SCNT embryos (French *et al*., 2008; Li *et al*., 2009).

Efficiency of electrofusion and electroactivation depends on numerous factors, such as electrical parameters, fusion medium, type and characteristics of the cells, compositions of the cell membranes and cytoplasmic contents. In previously published data, attention has been focused on choosing the optimal parameters of electric field with the aim of improving efficiency of cell fusion or oocyte activation. However, only a few studies have investigated the possible detrimental effects on embryos. Thus, little is known about sensitivity to electric fields of mammalian embryos of different species and at various stages of early preimplantation development.

In the present study, we used mouse and rat embryos as a model to investigate the sensitivity of metaphase II (MII) oocytes, pre-activated oocytes, zygotes and 2-cell stage embryos to electric fields. We examined the efficiency of parthenogenetic activation of oocytes and tetraploid embryo production after electrical stimulation in a voltage-dependent fashion. Furthermore, we evaluated and compared the developmental ability of treated mouse and rat embryos *in vitro*.

Materials and Methods

Animals

Female C57BL/6N mice for superovulation induction (23–25 days old) were obtained from Charles River (Sulzfeld, Germany). Female Sprague–Dawley-Hannover (SD-Hann) outbred rats (26-day-old) were obtained from a commercial animal breeder (Janvier, France).

All mice and rats were kept at a temperature of 21 ± 2°C in a 12 h light/dark cycle (lights on 6.00 a.m.–6.00 p.m.) with a humidity of 65 ± 5%. All experimental protocols were performed in accordance with the guidelines for the humane use of laboratory animals by the Max-Delbrück Center for Molecular Medicine and were approved by the local Ethics Committee.

Mouse and rat embryo collection

Immature mouse and rat females were induced to superovulate by intraperitoneal injection of gonadotrophins: 5 IU for mice or 15 IU pregnant mare's serum gonadotrophin for rats (Intervet, Unterschleißheim, Germany) followed 45–50 h later by 5 IU for mice or 30 IU hCG (Intervet) for rats (Popova *et al*., 2002, 2005).

Superovulated females were sacrificed by cervical dislocation. Ovulated oocytes were collected 16–18 h after the hCG injection. To obtain zygotes, superovulated females were mated overnight with males of the same strain on the afternoon of Day 0. The criterion for mating was the presence of a vaginal plug on the following morning (Day 1 of pregnancy). Superovulated females were sacrificed at 12 p.m.–2 p.m. on Day 1 to collect zygotes. The oocytes and zygotes were recovered from the excised oviducts into M2 medium (Sigma) containing 0.1% (w/v) hyaluronidase (Sigma) to remove cumulus cells. Then, the ova were washed in M2 medium and used for manipulations. To obtain 2-cell embryos, zygotes were *in vitro* cultured overnight in M16 medium (Sigma) and used for experiments.

Spontaneous activation of oocytes

For estimation of second polar body (sPB) extrusion, mouse and rat oocytes were cultured in prewarmed and equilibrated M16 medium.

Parthenogenetic activation of oocytes

Activation by electrical stimulation

For electroactivation, the same protocol was used as described for electrofusion (Krivokharchenko *et al*., 2002). The oocytes were pre-equilibrated in fusion medium consisting of 0.3 M mannitol solution containing 0.1 mM $MgCl_2$ and 0.1 mM $CaCl_2$ (1–2 min). They were then placed between the electrodes of a fusion chamber (Pushchino, Russia) in fusion medium. An alternating current (AC) field (8 V, 500 kHz, 10 s) was used in the first step and activation was induced with the aid of a pulse generator (GI-2; Pushchino, Russia). Two direct current (DC) pulses (60 or 30 V, 20 μs) were applied with 100 ms between the pulses.

Chemical activation

Parthenogenetic activation of oocytes by strontium treatment was performed as described previously (Krivokharchenko *et al*., 2003). The oocytes were incubated for 30 min in Ca^{2+} and Mg^{2+}-free M16 medium containing 2 mM Sr^{2+} at 37°C in a CO_2 incubator.

Diploidization

To obtain diploid parthenogenetic embryos, oocytes from all experimental groups were cultured 7–8 h in the presence of 5 μg/ml cytochalasin B (Sigma).

Analysis of activation

The efficiency of pronuclear formation was analysed 10–12 h after treatment. The oocytes were observed under an inverted microscope with Hoffmann optics. Those oocytes that formed visible pronuclei were recorded as activated.

Exposure of pre-activated oocytes and zygotes to electric fields

The pre-activated oocytes and zygotes were pre-equilibrated in fusion medium then placed between the electrodes of a fusion chamber. The same electrofusion parameters which were used for induction of oocyte activation were tested to check the sensitivity of embryos to electric fields.

Electrofusion of 2-cell stage embryos

Electrofusion was performed by the method developed for the production of viable tetraploid embryos (Krivokharchenko *et al*., 2002). Additionally,

two different voltages of electric field (30 or 60 V) were applied. Most 2-cell stage embryos were fused within 30 min.

Embryo culture

For *in vitro* development of rat embryos to the blastocyst stage, 10–20 oocytes were cultured overnight in M16 medium and transferred into 700 μl mR1ECM medium (Miyoshi *et al.*, 1997) in four-well culture dishes (Nunc) and cultured for 5 days under 5% CO_2 in air at 37°C (Krivokharchenko *et al.*, 2002). Previously, the culture medium was equilibrated with the gas phase and temperature in a CO_2 incubator for 2–3 h.

Mouse parthenogenetically activated oocytes, zygotes, 2-cell stage and tetraploid embryos were cultured in M16 medium.

Measurement of reactive oxygen species contents

The level of reactive oxygen species (ROS) in each embryo was examined according to the dichlorodihydrofluorescein diacetate (DCHFDA) method described by Hashimoto *et al.* (2000). Briefly, on Day 2 of culture, 2-cell stage embryos were transferred into M2 medium containing 10 μM DCHFDA. After 30 min of culture, embryos were washed and checked using a Leica DM IRB inverted microscope under UV light with the filter at 405–435 nm for excitation. For detection of DNA, embryos were stained with Hoechst 33342 (5 μg/ml) in phosphate-buffered saline (PBS).

Staining of microtubules and microfilaments

Staining of microtubules and microfilaments was performed according to the method described by Matsumoto *et al.* (1998). Two-cell stage embryos were fixed with 4% formaldehyde in PBS at room temperature for 30 min, and permeabilized in 0.25% Tween 20 in PBS at room temperature for 5 min. For staining of microtubules, fixed embryos were incubated for 90 min at 37°C with anti-α-tubulin monoclonal antibody (Sigma) diluted 1:250 in PBS and then washed in PBS containing 0.5% Triton-X 100 and 0.5% bovine serum albumin. After this embryos were incubated in FITC-labelled goat anti-mouse antibody (Sigma) for 60 min at 37°C.

For staining of microfilaments, fixed embryos were incubated in 1 mg/ml FITC-labelled phalloidin (Sigma) for 60 min at 37°C and then washed several times. For detection of DNA, embryos of both groups were stained with Hoechst 33342 (5 μg/ml) in PBS. All samples were analysed under a Leica DM IRB inverted microscope fitted with appropriate filters.

Statistical analysis

The comparisons for multigroup and multifactorial analyses were done with a two-way analysis of variance (ANOVA) and one-way ANOVA on ranks for multiple group comparisons. Tests of significance were conducted by unpaired Student's *t*-test. A value of $P < 0.05$ was chosen as an indication of statistical significance.

Results

Spontaneous and electroactivation of mouse and rat MII oocytes

First, one group of oocytes from each mouse or rat was used for estimation of spontaneous activation (control group) and another group was used for activation by electrical stimuli. We evaluated the efficiency of pronuclei formation and cleavage rate in oocytes exposed to electric fields with increasing voltage (Table I).

After 3 h incubation, only 9.4% of mouse oocytes extruded sPB, 6.3% formed a pronucleus and none of them developed *in vitro* until the 2-cell stage. In rats, 97.4% of cultured oocytes extruded sPB and about 13% formed a pronucleus.

An electric field of 60 V, compared with 30 V, resulted in significantly greater efficiency of pronuclear formation (94.9 versus 71.8%; $P < 0.01$) in mouse oocytes but also in a lower *in vitro* cleavage rate of these embryos (5.4 versus 39.3%; $P < 0.0001$).

Efficiency of pronuclear formation (35.3%) induced by electrical stimulation in rat oocytes was only achieved using the higher voltage of 60 V while the lower voltage (30 V) did not induced any pronuclear formation at all.

Strontium activation of DC-treated mouse and rat MII oocytes

To determine the possible effect of an electric field on the efficiency of activation, mouse and rat oocytes were exposed to electric fields and then immediately activated by strontium. As a control, freshly isolated oocytes were immediately activated using the same activation protocol (Table II).

There was no significant difference in rates of pronuclei formation among all groups in both species. That means that electric fields did

Table I Electroactivation of rat and mouse oocytes.

Species	Groups	No. animals	No. of oocytes extruded sPB	Efficiency of	
				Pronuclear formation (%)	Cleavage (%)
Mouse	Spontaneous activation	12	3/32 (9.4)	2/32 (6.3)	0/2 (0)
	DC pulses 30 V, 20 ms	12		28/39 (71.8)	11/28 (39.3)
	DC pulses 60 V, 20 ms	12		37/39 (94.9)*	2/37 (5.4)**
Rat	Spontaneous activation	8	38/39 (97.4)	5/39 (12.8)	0/5 (0)
	DC pulses 30 V, 20 ms	8		0/32 (0)	0/32 (0)
	DC pulses 60 V, 20 ms	8		12/34 (35.3)$^{+}$	3/12 (25.0)$^{++}$

Values with different superscripts in the same column are significantly different.
Mouse: *Significantly different from DC 30 V ($P < 0.01$);
**Significantly different from DC 30 V ($P < 0.001$).
Rat: $^{+}$Significantly different from DC 30 V ($P < 0.001$);
$^{++}$Significantly different from DC 30 V ($P < 0.01$).

not affect the activation induced by strontium. However, the cleavage rates of electric-treated and strontium-activated mouse oocytes were significantly lower for both groups compared with the control. The higher voltage (60 V) resulted in a significantly lower cleavage rate (33%) in mouse oocytes compared with the lower voltage (30 V, 87.5%).

Unlike the mouse, the rates of development to 2-cell stage of electric-treated rat oocytes were not dependent on the voltage (51.1–53.1%). However, these parameters were significantly lower compared with control oocytes activated only by strontium (68.7%; $P < 0.05$).

Effect of electric field on *in vitro* development of MII and pre-activated oocytes

We checked the *in vitro* development of MII mouse oocytes which were first electric-treated and then activated by strontium and compared them to the group of oocytes which were first activated and then exposed to electric field after pronuclear formation. As a control of development, we used MII oocytes activated only by strontium immediately after isolation. With the aim of improving the developmental ability, oocytes of all groups were diploidized by cytochalasin B treatment.

The results presented in Table III demonstrated that the higher voltages (60 V) resulted in significantly lower cleavage rates in mouse oocytes exposed to electric field and then activated by strontium compared with all other groups. MII stage oocytes treated with 30 V and pronuclear-stage oocytes treated with 30–60 V showed similar cleavage rates compared with controls.

Oocytes at MII and pronuclear stages exposed to 60 V DC did not develop to the blastocyst stage *in vitro*. Both types of oocytes treated with 30 V developed until the blastocyst stage without any difference between experimental groups, however, at a lower rate than the controls ($P < 0.05$).

Interestingly, rat pronuclear-stage oocytes were also more resistant to electric fields of higher voltages (60 V) than were MII stage oocytes. The cleavage rates of pronuclear-stage oocytes after electric field treatments were significantly higher: 72.0% (54/75) compared with the electric-treated and activated oocytes: 51.1% (23/45) ($P < 0.05$).

Effect of electric fields on *in vitro* development of rat and mouse zygotes

We examined the effect of an electric field with 30 or 60 V on the *in vitro* development of mouse and rat zygotes. As a control we used in parallel non-manipulated zygotes (Table IV). An electric field of 60 V resulted in significantly lower cleavage rates in mice and rats (40.2 and 66.9%) compared with the control groups (90.1 and 97.8%). Mouse zygotes exposed to a DC pulse at 60 V were completely blocked at the 2-cell stage whereas a few rat zygotes could develop until morula/blastocyst stages.

Table II Strontium activation of DC-treated mouse and rat MII oocytes.

Species	Groups	No. animals	No. of oocytes used	Efficiency of	
				Pronuclear formation (%)	Cleavage (%)
Mouse	Control	12	37	33/37 (89.2)	33/33 (100)
	DC pulses 30 V, 20 msec	12	135	112/135 (82.9)	98/112 (87.5)**
	DC pulses 60 V, 20 ms	12	139	115/139 (82.7)	38/115 (33.0)*
Rat	Control	8	72	67/72 (93.1)	46/67 (68.7)
	DC pulses 30 V, 20 ms	8	52	45/52 (86.5)	23/45 (51.1)$^{++}$
	DC pulses 60 V, 20 ms	8	98	89/98 (90.8)	43/81 (53.1)$^{++}$

Control: activation only by strontium.
Mouse: *Significantly different from DC 30 V and control groups ($P < 0.001$);
**Significantly different from control group ($P < 0.05$).
Rat: $^{++}$Significantly different from control group ($P < 0.05$).

Table III Effect of electric field on *in vitro* development of MII and pre-activated mouse oocytes.

Type of manipulation	Electrofusion parameters	No. of oocytes used	*In vitro* development (%)		
			2-cells	Morula	Blastocyst
Activation of MII oocytes only by strontium		86	79/86 (91.9)	69/86 (80.2)	44/86 (51.2)
Activation of DC-treated oocytes by strontium	DC pulses 30 V, 20 ms	65	55/65 (84.6)	28/65 (43.1)	19/65 (29.2)$^{++}$
	DC pulses 60 V, 20 ms	92	30/92 (32.6)*	4/92 (4.3)*	0/92 (0)$^{+}$
Exposure of pre-activated oocytes to DC	DC pulses 30 V, 20 ms	46	43/46 (93.5)	22/46 (47.8)	15/46 (32.6)$^{++}$
	DC pulses 60 V, 20 ms	48	34/48 (89.5)	0/48 (0)*	0/48 (0)$^{+}$

Values with different superscripts in the same column are significantly different.
*Significantly different from all other groups ($P < 0.001$).
$^{+}$Significantly different from all other groups ($P < 0.001$).
$^{++}$Significantly different from control group ($P < 0.05$).

Mouse zygotes exposed to electric fields with 30 V developed until the morula and blastocyst stages but at a significantly lower rate compared with the control group ($P < 0.001$). We did not find any differences in total cell numbers in mouse blastocysts which were *in vitro* developed from zygotes exposed to an electric field of 30 V [51.6 ± 3.1 ($n = 26$)] compared with controls [48.6 ± 2.4 ($n = 37$)].

Rat zygotes were more resistant and showed no differences in developmental ability between controls and embryos exposed to an electric field of 30 V.

Electrofusion of 2-cell stage embryos and their *in vitro* development in mice and rats

The effect of electric field with voltages of 30 or 60 V on fusion rate and development of fused embryos *in vitro* until the blastocyst stage was investigated in mouse and rat (Table V). As a control, we used non-manipulated *in vitro* developed 2-cell stage embryos (Fig. 1).

The rates of fusion, cleavage and development till morula of mouse 2-cell embryos were the same in both experimental groups regardless of electrofusion parameters and were comparable with control. However, the high voltage of 60 V resulted in a significantly lower development until the blastocyst stage compared with other experimental and control groups.

Cells were counted in blastocysts developed from untreated and electrofused 2-cell mouse embryos. The number of cells in blastocysts from fused embryos was half [23.0 ± 1.6 ($n = 9$)] that of the control embryos [48.7 ± 4.9 ($n = 26$)].

In rats, the maximum rate of fusion was observed when 2-cells were exposed to a current of 60 V. At the same time, the cleavage rate of fused embryos and their *in vitro* development until morula and blastocyst stages were significantly higher for control embryos and embryos exposed to 30 V compared with those exposed to a DC of 60 V ($P < 0.05$).

Microtubules and microfilament distributions in mouse 2-cell stage embryos treated with electric field

Mouse zygotes of the experimental group were exposed to a DC pulse at 60 V then cultured overnight *in vitro* in M16 in parallel with controls (non-treated embryos) and used for staining. For comparison of microtubules and microfilament distributions, about 20 embryos from treated and control groups were analysed. Embryos of this experimental group showed microtubules and microfilament distributions similar to those in control embryos (Fig. 2).

Table IV ***In vitro*** **development of rat and mouse zygotes exposed to electric field.**

Species	Electrofusion parameters	No. of zygotes used	*In vitro* development (%)		
			2-cells	Morula	Blastocyst
Mouse	Control	91	89/91 (97.8)	80/91 (87.9)	71/91 (78.0)
	DC pulses 30 V, 20 ms	90	77/90 (85.6)*	41/90 (45.6)*	34/90 (37.9)*
	DC pulses 60 V, 20 ms	92	37/92 (40.2)**	0/92 (0)**	0/92 (0)**
Rat	Control	161	145/161 (90.1)	105/161 (65.2)	63/161 (39.1)
	DC pulses 30 V, 20 ms	113	101/113 (89.4)	55/90 (61.1)	29/90 (32.2)
	DC pulses 60 V, 20 ms	106	71/106 (66.9)$^{+}$	4/106 (3.8)$^{++}$	1/106 (0.9)$^{++}$

Values with different superscripts in the same column are significantly different.
Mouse: *Significantly different from control group ($P < 0.01$);
**Significantly different from DC 30 V and control groups ($P < 0.001$).
Rat: $^{+}$Significantly different from DC 30 V and control groups ($P < 0.001$);
$^{++}$Significantly different from DC 30 V and control groups ($P < 0.001$).

Table V ***In vitro*** **development of mouse and rat tetraploid embryos from fused 2-cells.**

Species	Electrofusion parameters	Fusion rate, (%)	*In vitro* development (%)		
			2-Cells	Morula	Blastocyst
Mouse	Control		34	32/34 (94.1)	31/34 (91.2)
	DC pulses 30 V, 20 ms	51/52 (98.1)	44/51 (86.3)	41/51 (80.4)	38/51 (74.5)
	DC pulses 60 V, 20 ms	50/50 (100)	40/50 (80.0)	34/50 (68.0)	30/50 (60.0)*
Rat	Control	–	30	25/30 (83.3)	23/30 (76.7)
	DC pulses 30 V, 20 ms	42/49 (85.7)	38/42 (90.5)	35/42 (83.3)	31/42 (73.8)
	DC pulses 60 V, 20 ms	27/27 (100)$^{+}$	19/27 (70.4) $^{++}$	15/27 (55.6)$^{++}$	12/27 (44.4)$^{++}$

Mouse: *Significantly different from control group ($P < 0.01$).
Rat: $^{+}$Significantly different from DC 30 V ($P < 0.05$);
$^{++}$Significantly different from DC 30 V and control in the same column ($P < 0.05$).

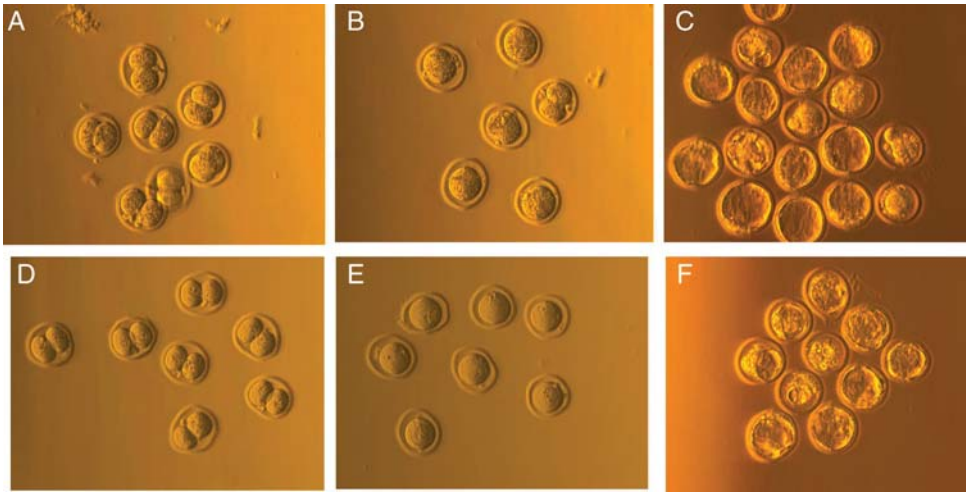

Figure 1 Electrofusion of mouse (**A**, **B**, **C**) and rat (**D**, **E**, **F**) embryos. Embryos at 2-cell stage before fusion (A and D), mouse embryos in process of fusion (B) and completely fused rat embryos (E) and blastocysts developed *in vitro* from fused 2-cell stage mouse (C) and rat embryos (F).

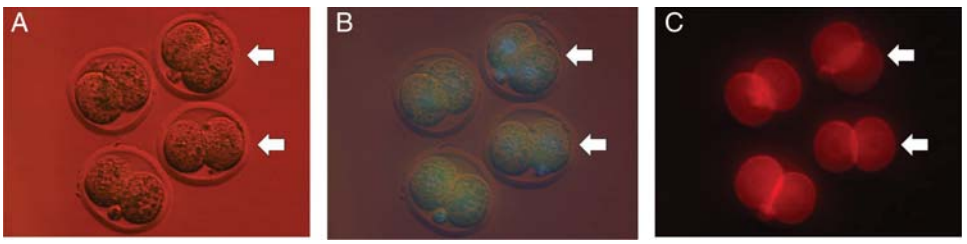

Figure 2 Cytoskeleton proteins in two control mouse 2-cell stage embryos (left) and two blocked 2-cells (indicated by white arrows) developed *in vitro* from zygotes exposed to DC pulse at 60 V. Light microscopic images (**A**), merged image of stained nuclei with Hoechst (blue) and microtubules with anti-α-tubulin antibody (green) (**B**) and stained microfilaments with FITC-labeled phalloidin (red) (**C**).

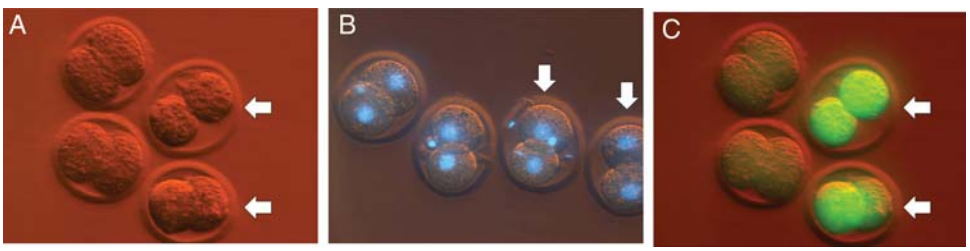

Figure 3 Generation of ROS in two control mouse 2-cell stage embryos (left) and two blocked 2-cells (indicated by white arrows) developed *in vitro* from zygotes exposed to DC pulse at 60 V. Light microscopic images (**A**), cell nuclei stained with Hoechst (**B**) and embryos stained with DCHFDA for evaluation of ROS (**C**).

The effect of electric field on ROS production in mouse 2-cells

For estimation the levels of ROS, we formed groups from two blocked 2-cell embryos developed *in vitro* from zygotes exposed to DC pulse at 60 V and two control embryos. In total, 20 treated and 20 non-treated embryos were investigated. The presence of nuclei was confirmed under UV light after Hoechst staining. The level of ROS was detected simultaneously in all embryos of each group using the DCHFDA staining. Markedly brighter fluorescence was detected in mouse 2-cell embryos developed from zygotes after electric field treatment than in control embryos (Fig. 3).

Discussion

One of the most popular techniques in different biotechnological experiments with mammalian gametes and preimplantation embryos is application of an electric field. Depending on the research purpose, early embryos at different developmental stages such as MII oocytes, pre-activated oocytes, zygotes and 2-cells can be used for various manipulations using electric fields.

However, only a few studies have been performed about the possible effects of electric fields on viability of treated gametes and embryos and the data available in the literature are quite contradictory. Simultaneously, little is known about the possible species-specific differences in sensitivity to electric stimulation of early embryos at different developmental stages.

A recent study has shown that after electrical activation of porcine oocytes, the cleavage rate was increased in the 1.5 and 2 kV/cm groups compared with 1.0 kV/cm, but blastocyst development was not significantly different among groups. A longer duration of electric pulse improved rates of both cleavage and blastocyst formation (Koo *et al*., 2008). In addition, the cleavage rate *in vitro* was also improved in cloned caprine embryos activated with 2.33 kV/cm compared with that activated with 1.67 kV/cm (Shen *et al*., 2006). At the same time other authors believe that electrofusion cannot be used for nuclear transfer experiments in mice, because the mouse oocytes are more susceptible to detrimental effects of electrical stimuli than oocytes from other species (Wu *et al*., 2007).

The results of our study showed that an electric stimulus induces activation in mouse and rat MII oocytes but the efficiency of pronuclear formation and the cleavage rate were different between species and dependent on the voltage of the electric field. Thus, the higher voltage (60 V) which was efficient for activation and cleavage rate in rats also resulted in higher pronuclear formation for mouse oocytes but dramatically decreased their further development until the 2-cell stage. However, a low DC pulse at 30 V did not induce activation in rat oocytes at all but was quite effective for mouse oocytes.

We also investigated the effect of electric fields on mouse and rat MII oocytes regarding further parthenogenetic activation by strontium, one of the most popular and effective activators. Exposure of mouse and rat oocytes to electric field before strontium activation did not affect pronuclear formation but significantly decreased the cleavage rates for both species.

Investigation of development of mouse activated diploid oocytes until the blastocyst stage *in vitro* confirmed the detrimental effect of electric field on early embryogenesis in mice. Pre-activated pronuclear-stage oocytes were more resistant than MII oocytes in mice and rats. We also demonstrated that exposure to electric fields for mouse and rat zygotes significantly decreased development *in vitro* in a dose-dependent manner and mouse embryos were more sensitive than rat embryos.

Extensive studies have been performed to find optimal parameters of electric fields at different conditions for fusion of 2-cell embryos with the aim to produce tetraploid embryos in various species such as mice (Kubiak and Tarkowski, 1985; Suo *et al*., 2009), rabbits (Ozil and Modlinski, 1986), hamsters (Suzuki *et al*., 2001), cattle (Curnow *et al*., 2000) and rats (Krivokharchenko *et al*., 2002). Furthermore recent studies define conditions for efficient electrofusion of various somatic cells with mouse, ferret, goat and bovine oocytes (Li *et al*., 2005; Gaynor *et al*., 2005; Zhang *et al*., 2008a,b).

The results of one study performed in cats showed the effects of electric field strengths on fusion and *in vitro* development of cat embryos derived by SCNT. The authors found an increase in the cleavage rate of fused embryos with an increase in the electric field strength. However, there were no differences among groups with respect to the rate of development to the morula and blastocyst stages *in vitro* (Karja *et al*., 2006). In contrast, recently, detrimental effects on cleavage rate and development to the blastocyst stage of fused 2-cell stage mouse embryos were demonstrated (Suo *et al*., 2009). Interestingly, electrofused hamster 2-cells did not even exhibit further cleavage (Suzuki *et al*., 2001).

In our experiments, we also found that a higher electric field strength decreased embryo viability. However, at optimal parameters the development of fused 2-cell embryos until the blastocyst stage was comparable with the control in both species, allowing us to conclude that these embryos are more resistant to electric fields. Probably this increased tolerance of mouse and rat 2-cell stage embryos to electric field compared with oocytes and pronuclear-stage embryos correlates with various membrane properties as was previously published for rabbit oocytes and zygotes (Arnold *et al*., 1989).

It has been shown that electrofused and blocked 2-cell stage hamster embryos demonstrated dynamic changes of the cytoskeletal distribution (Suzuki *et al*., 2001). In contrast in our experiments, we did not find any differences in microtubules and microfilament distributions in blocked 2-cell stage embryos that developed from electrotreated zygotes.

Another recent study has shown that electrical activation of oocytes induces ROS in porcine embryos. A longer duration of the electric pulse induced higher level of ROS but without detrimental effect on embryo viability (Koo *et al*., 2008). In our experiments, we found a dramatic increase of ROS in embryos after exposure to electric fields of 60 V and increasing levels of ROS were associated with decreasing of embryo viability. It is well known that ROS can induce a development block and retardation of preimplantation embryos (Guerin *et al*., 2001). Therefore, the detrimental effect of the electric field in our experiments may be mediated by ROS.

In summary, we found stage-dependent and species-specific differences in sensitivity to electric field in mouse and rats. Activation of MII oocytes in mice and rats depends on parameters of the electric field. Pronuclear stages such as pre-activated oocytes and zygotes are more stable than oocytes at the MII stage. Embryos at the 2-cell stage are more resistant to electric field in both species because the electric field induced the fusion of blastomeres and the formation of tetraploid embryos and these embryos could develop *in vitro* until the blastocyst stage. From a practical point of view, parameters of electric field chosen for one mammalian species cannot be transferred to another and should be optimized depending on the aims of experiments and stage of embryos used especially in human oocytes and embryos.

Authors' roles

A.K. and E.P. designed all experiments, collected and assembled the data. A.K., E.P. and M.B. analysed the data and wrote the manuscript.

Acknowledgements

We acknowledge Annegret Dahlke, Kerstin Voigt and Monique Bergemann for animal care.

Funding

This work was supported by a grant of the European Union (EURATRANS).

References

Arnold WM, Schmutzler RK, Al-Hasani S, Krebs D, Zimmermann U. Differences in membrane properties between unfertilised and fertilised single rabbit oocytes demonstrated by electro-rotation. Comparison with cells from early embryos. *Biochim Biophys Acta* 1989;**979**:142–146.

Boland MJ, Hazen JL, Nazor KL, Rodriguez AR, Gifford W, Martin G, Kupriyanov S, Baldwin KK. Adult mice generated from induced pluripotent stem cells. *Nature* 2009;**461**:91–94.

Booth PJ, Tan SJ, Reipurth R, Holm P, Callesen H. Simplification of bovine somatic cell nuclear transfer by application of a zona-free manipulation technique. *Cloning Stem Cells* 2001;**3**:139–150.

Cheng Y, Wang K, Kellam LD, Lee YS, Liang CG, Han Z, Mtango NR, Latham KE. Effects of ooplasm manipulation on DNA methylation and growth of progeny in mice. *Biol Reprod* 2009;**80**:464–472.

Cohen J, Scott R, Alikani M, Schimmel T, Munné S, Levron J, Wu L, Brenner C, Warner C, Willadsen S. Ooplasmic transfer in mature human oocytes. *Mol Hum Reprod* 1998;**4**:269–280.

Craven L, Tuppen HA, Greggains GD, Harbottle SJ, Murphy JL, Cree LM, Murdoch AP, Chinnery PF, Taylor RW, Lightowlers RN *et al.* Pronuclear transfer in human embryos to prevent transmission of mitochondrial DNA disease. *Nature* 2010;**465**:82–85.

Curnow EC, Gunn IM, Trounson AO. Electrofusion of two-cell bovine embryos for the production of tetraploid blastocysts *in vitro*. *Mol Reprod Dev* 2000;**56**:372–377.

Eggan K, Rode A, Jentsch I, Samuel C, Hennek T, Tintrup H, Zevnik B, Erwin J, Loring J, Jackson-Grusby L *et al.* Male and female mice derived from the same embryonic stem cell clone by tetraploid embryo complementation. *Nat Biotechnol* 2002;**20**:455–459.

French AJ, Adams CA, Anderson LS, Kitchen JR, Hughes MR, Wood SH. Development of human cloned blastocysts following somatic cell nuclear transfer with adult fibroblasts. *Stem Cells* 2008;**26**:485–493.

Galli C, Lagutina I, Crotti G, Colleoni S, Turini P, Ponderato N, Duchi R, Lazzari G. Pregnancy: a cloned horse born to its dam twin. *Nature* 2003; **424**:635.

Gaynor P, Wells DN, Oback B. Couplet alignment and improved electrofusion by dielectrophoresis for a zona-free high-throughput cloned embryo production system. *Med Biol Eng Comput* 2005; **43**:150–154.

Guerin P, El Mouatassim S, Ménézo Y. Oxidative stress and protection against reactive oxygen species in the pre-implantation embryo and its surroundings. *Hum Reprod Update* 2001;**7**:175–189.

Guo J, Liu F, Guo Z, Li Y, An Z, Li X, Li Y, Zhang Y. In vitro development of goat parthenogenetic and somatic cell nuclear transfer embryos derived from different activation protocols. *Zygote* 2009;**8**:1–9.

Hashimoto S, Minami N, Takakura R, Yamada M, Imai H, Kashima N. Low oxygen tension during *in vitro* maturation is beneficial for supporting the subsequent development of bovine cumulus–oocyte complexes. *Mol Reprod Dev* 2000;**57**:353–360.

Hirao Y, Nishimoto N, Kure-bayashi S, Takenouchi N, Yamauchi N, Masuda H, Nagai T. Influence of meiotic inhibition by butyrolactone-I during germinal vesicle stage on the ability of porcine oocytes to be activated by electric stimulation after nuclear maturation. *Zygote* 2003; **11**:191–197.

Karja NW, Otoi T, Wongsrikeao P, Shimizu R, Murakami M, Agung B, Fahrudin M, Nagai T. Effects of electric field strengths on fusion and *in vitro* development of domestic cat embryos derived by somatic cell nuclear transfer. *Theriogenology* 2006;**66**:1237–1242.

Kato Y, Tani T, Sotomaru Y, Kurokawa K, Kato J, Doguchi H, Yasue H, Tsunoda Y. Eight calves cloned from somatic cells of a single adult. *Science* 1998;**282**:2095–2098.

Koo OJ, Jang G, Kwon DK, Kang JT, Kwon OS, Park HJ, Kang SK, Lee BC. Electrical activation induces reactive oxygen species in porcine embryos. *Theriogenology* 2008;**70**:1111–1118.

Krivokharchenko A, Galat V, Ganten D, Bader M. 2002. In vitro formation of tetraploid rat blastocysts after fusion of two-cell embryos. *Mol Reprod Dev* 2002;**61**:460–465.

Krivokharchenko A, Popova E, Zaitseva I, Vil'ianovich L, Ganten D, Bader M. Development of parthenogenetic rat embryos. *Biol Reprod* 2003;**68**:829–836.

Kubiak JZ, Tarkowski AK. Electrofusion of mouse blastomeres. *Exp Cell Res* 1985;**157**:561–566.

Kure-bayashi S, Miyake M, Okada K, Kato S. Successful implantation of *in vitro*-matured, electro-activated oocytes in the pig. *Theriogenology* 2000;**53**:1105–1019.

Li Z, Sun X, Chen J, Leno GH, Engelhardt JF. Factors affecting the electrofusion of mouse and ferret oocytes with ferret somatic cells. *Mol Reprod Dev* 2005;**72**:40–47.

Li J, Liu X, Wang H, Zhang S, Liu F, Wang X, Wang Y. Human embryos derived by somatic cell nuclear transfer using an alternative enucleation approach. *Cloning Stem Cells* 2009;**11**:39–50.

Matsumoto H, Shoji N, Umezu M, Sato E. Microtubule and microfilament dynamics in rat embryos during the two-cell block *in vitro*. *J Exp Zool* 1998;**281**:149–153.

Miyoshi K, Kono T, Niwa K. Stage-dependent development of rat 1-cell embryos in a chemically defined medium after fertilization *in vivo* and *in vitro*. *Biol Reprod* 1997;**56**:180–185.

Morgan HD, Jin XL, Li A, Whitelaw E, O'Neill C. The culture of zygotes to the blastocyst stage changes the postnatal expression of an epigentically labile allele, agouti viable yellow, in mice. *Biol Reprod* 2008;**79**:618–623.

Nagy A, Gócza E, Diaz EM, Prideaux VR, Iványi E, Markkula M, Rossant J. Embryonic stem cells alone are able to support fetal development in the mouse. *Development* 1990;**110**:815–821.

Nagy A, Rossant J, Nagy R, Abramow-Newerly W, Roder JC. Derivation of completely cell culture-derived mice from early-passage embryonic stem cells. *PNAS* 1993;**90**:8424–8428.

Oback B, Wiersema AT, Gaynor P, Laible G, Tucker FC, Oliver JE, Miller AL, Troskie HE, Wilson KL, Forsyth JT *et al.* Cloned cattle derived from a novel zona-free embryo reconstruction system. *Cloning Stem Cells* 2003;**5**:3–12.

Ogura A, Inoue K, Takano K, Wakayama T, Yanagimachi R. Birth of mice after nuclear transfer by electrofusion using tail tip cells. *Mol Reprod Dev* 2000;**57**:55–59.

Ozil JP. The parthenogenetic development of rabbit oocytes after repetitive pulsatile electrical stimulation. *Development* 1990;**109**:117–127.

Ozil JP, Modlinski JA. Effects of electric field on fusion rate and survival of 2-cell rabbit embryos. *J Embryol Exp Morphol* 1986;**96**:211–228.

Polejaeva IA, Chen SH, Vaught TD, Page RL, Mullins J, Ball S, Dai Y, Boone J, Walker S, Ayares DL *et al.* Cloned pigs produced by nuclear transfer from adult somatic cells. *Nature* 2000;**407**:86–90.

Popova E, Krivokharchenko A, Ganten D, Bader M. Comparison between PMSG-and FSH-induced superovulation for the generation of transgenic rats. *Mol Reprod Dev* 2002;**63**:177–182.

Popova E, Bader M, Krivokharchenko A. Production of transgenic models in hypertension. In: Fennell JP, Baker AH (eds). *Methods Mol Med*, Vol. 108. New Jersey: Humana Press, 2005,33–50.

Popova E, Bader M, Krivokharchenko A. Efficient production of nuclear transferred rat embryos by modified methods of reconstruction. *Mol Reprod Dev* 2009;**76**:208–216.

Rickords LF, White KL. Effect of electrofusion pulse in either electrolyte or nonelectrolyte fusion medium on subsequent murine embryonic development. *Mol Reprod Dev* 1992a;**32**:259–264.

Rickords LF, White KL. Electrofusion-induced intracellular Ca2+ flux and its effect on murine oocyte activation. *Mol Reprod Dev* 1992b; **31**:152–159.

Sayaka W, Satoshi K, Van Thuan N, Hiroshi O, Takafusa H, Eiji M, Thuy BH, Masashi M, Teruhiko W. Effect of volume of oocyte cytoplasm on embryo development after parthenogenetic activation, intracytoplasmic sperm injection, or somatic cell nuclear transfer. *Zygote* 2008;**16**:211–222.

Shen PC, Lee SN, Wu JS, Huang JC, Chu FH, Chang CC, Kung JC, Lin HH, Chen LR, Shiau JW *et al*. The effect of electrical field strength on activation and development of cloned caprine embryos. *Anim Reprod Sci* 2006;**92**:310–320.

Suo L, Wang F, Zhou GB, Shi JM, Wang YB, Zeng SM, Tian JH, Zhu SE, Liu GS. Optimal concentration of calcium and electric field levels improve tetraploid embryo production by electrofusion in mice. *J Reprod Dev* 2009;**55**:383–385.

Suzuki H, Ogasawara I, Takahashi H, Imada Y, Toyokawa K. Electrofusion of blastomeres of hamster 2-cell embryos and dynamic changes of the cytoskeletal distribution. *J Reprod Dev* 2001;**47**:227–235.

Tachibana M, Sparman M, Mitalipov S. Chromosome transfer in mature oocytes. *Nat Protoc* 2010;**5**:1138–1147.

Tanaka H, Kanagawa H. Influence of combined activation treatments on the success of bovine nuclear transfer using young or aged oocytes. *Anim Reprod Sci* 1997;**49**:113–123.

Tielens S, Verhasselt B, Liu J, Dhont M, Van Der Elst J, Cornelissen M. Generation of embryonic stem cell lines from mouse blastocysts developed *in vivo* and *in vitro*: relation to Oct-4 expression. *Reproduction* 2006;**132**:59–66.

Wilmut I, Schnieke AE, McWhir J, Kind AJ, Campbell KH. Viable offspring derived from fetal and adult mammalian cells. *Nature* 1997; **385**:810–813.

Wu YG, Zhou P, Lan GC, Wang G, Luo MJ, Tan JH. The effects of delayed activation and MG132 treatment on nuclear remodeling and preimplantation development of embryos cloned by electrofusion are correlated with the age of recipient cytoplasts. *Cloning Stem Cells* 2007;**9**:417–431.

Yamauchi Y, Doe B, Ajduk A, Ward MA. Genomic DNA damage in mouse transgenesis. *Biol Reprod* 2007;**77**:803–812.

Yu Y, Ding C, Wang E, Chen X, Li X, Zhao C, Fan Y, Wang L, Beaujean N, Zhou Q *et al*. Piezo-assisted nuclear transfer affects cloning efficiency and may cause apoptosis. *Reproduction* 2007;**133**:947–954.

Zhang J, Wang CW, Blaszcyzk A, Grifo JA, Ozil J, Haberman E, Adler A, Krey LC. Electrical activation and *in vitro* development of human oocytes that fail to fertilize after intracytoplasmic sperm injection. *Fertil Steril* 1999;**72**:509–512.

Zhang G, Sun QY, Chen DY. In vitro development of mouse somatic nuclear transfer embryos: effects of donor cell passages and electrofusion. *Zygote* 2008a;**26**:1–5.

Zhang YL, Liu FJ, Sun DQ, Chen XQ, Zhang Y, Zheng YM, Zhao MT, Wang GH. Phytohemagglutinin improves efficiency of electrofusing mammary gland epithelial cells into oocytes in goats. *Theriogenology* 2008b;**69**:1165–1171.

Zhao XY, Li W, Lv Z, Liu L, Tong M, Hai T, Hao J, Guo CL, Ma QW, Wang L *et al*. iPS cells produce viable mice through tetraploid complementation. *Nature* 2009;**461**:86–90.

Zhu J, Telfer EE, Fletcher J, Springbett A, Dobrinsky JR, De Sousa PA, Wilmut I. Improvement of an electrical activation protocol for porcine oocytes. *Biol Reprod* 2002;**66**:635–641.

Zimmermann U. Electrical breakdown, electropermeabilization and electrofusion. *Rev Physiol Biochem Pharmacol* 1986;**105**:176–256.