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Effects of electric field on fusion rate and survival of 2-cell rabbit embryos

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SUMMARY

Electric-field-induced blastomere fusion was studied in 2-cell rabbit embryos. Field strengths (1 to 3 kV cm^{-1}) and durations (35 to 1000 μs) were chosen so as to provide the right balance between fusion rate, viability and developmental capacity of embryonic cells. Maximum plasma membrane tolerance of 2-cell rabbit embryos was observed at about 3 kV cm^{-1} for 1000 μs. All surviving 'fused' embryos were able to develop *in vitro* and most of them formed expanded blastocysts. Observation of 'fused' embryos immediately after fusion and during the whole cell cycle showed that 27·7 % of the two diploid nuclei remained separated in the hybrid cell. More than one metaphase plate was formed at the onset of mitosis causing direct cleavage into three or four 'cells'. In the remaining embryos the two diploid nuclei seemed to form a common metaphase plate and cleaved into two equal blastomeres. After transfer to recipient does, 54·4 % of these tetraploid embryos developed beyond implantation. Between day 11 and 20, ten live and morphologically fully normal embryos were recovered. Nine embryos were uniformly tetraploid and one recovered on day 18 was a diploid/tetraploid mosaic. The remaining implantation sites contained either abnormal, very retarded embryos or indefinable embryo remnants. After transfer of 'nonfused' embryos treated with 3 kV cm^{-1}, 49 % gave birth to normal live young. These results suggest that the electric field can be applied successfully in a relatively wide strength and duration range without causing any visible teratogenic effect on treated embryos. Thus, tetraploid embryos can develop normally at least until two-thirds of pregnancy, but the question whether they are able to survive till term remains open.

INTRODUCTION

Current fusion procedures applied to mammalian gametes and early embryos have certain inherent limitations and most often they are not accurately controlled (Zimmermann & Vienken, 1982). In contrast, the new electrofusion technique developed in the last few years appears to bypass some of the disadvantages by using the parameters of the vectorial system exactly defined and hence constantly repeated.

The electrofusion technique has been applied to red blood cells and lymphocytes (Zimmermann, 1982), sea-urchin eggs (Richter, Scheurich & Zimmermann, 1981) and plant protoplasts (Zimmermann, 1982), and very recently Kubiak & Tarkowksi (1985) reported successful fusion of 2-cell mouse embryos with various

Key words: rabbit embryo, fusion, electric field, tetraploid.

fusion parameters and their development beyond implantation. However, still little is known about the optimal parameters required for electrofusion of mammalian embryonic cells and about the behaviour and tolerance of such cells exposed to an electric field. Thus, it is necessary to determine the factors that provide the most advantageous conditions for the right balance between fusion rate, viability and developmental capacity of 'fused' cells.

The purpose of this study was to test the effects of physical parameters such as electric field strength and duration as well as the number of pulses applied at a wide range of values and in several combinations on the fusion, survival and developmental rate of 2-cell rabbit embryos.

Like other fusion procedures, the electrofusion technique can be used in the manipulation of ploidy and hence in the production of tetraploid embryos used in developmental and genetic research like other mammalian embryos of abnormal ploidy. Several attempts were made to induce tetraploidy in mice and a number of papers reported the effect of tetraploidy on the development of mouse eggs (Graham, 1971; Snow, 1973; Tarkowski, Witkowska & Opas, 1977; Eglitis, 1980; Spindle, 1981; Kubiak & Tarkowski, 1985). It was clearly demonstrated that mouse tetraploids can develop to blastocysts and a small proportion of them underwent postimplantation development (Snow, 1973, 1975, 1976; Tarkowski *et al.* 1977) but very seldom resulted in live-born young (Snow, 1973, 1975).

In rabbits, no attempts were made to produce tetraploid embryos, but according to some experiments (Bomsel-Helmreich, 1965, 1971) triploid embryos can develop to day 16 of pregnancy. On the other hand, Harper & Chang (1971) showed that some presumably polyploid embryos (the ploidy of embryos was not determined) obtained after cytochalasin treatment were able to develop to blastocysts and even beyond postimplantation, but failed to survive after day 17. Thus, our report concerns the production of tetraploid embryos and testing of their postimplantation development in a species other than mouse.

MATERIALS AND METHODS

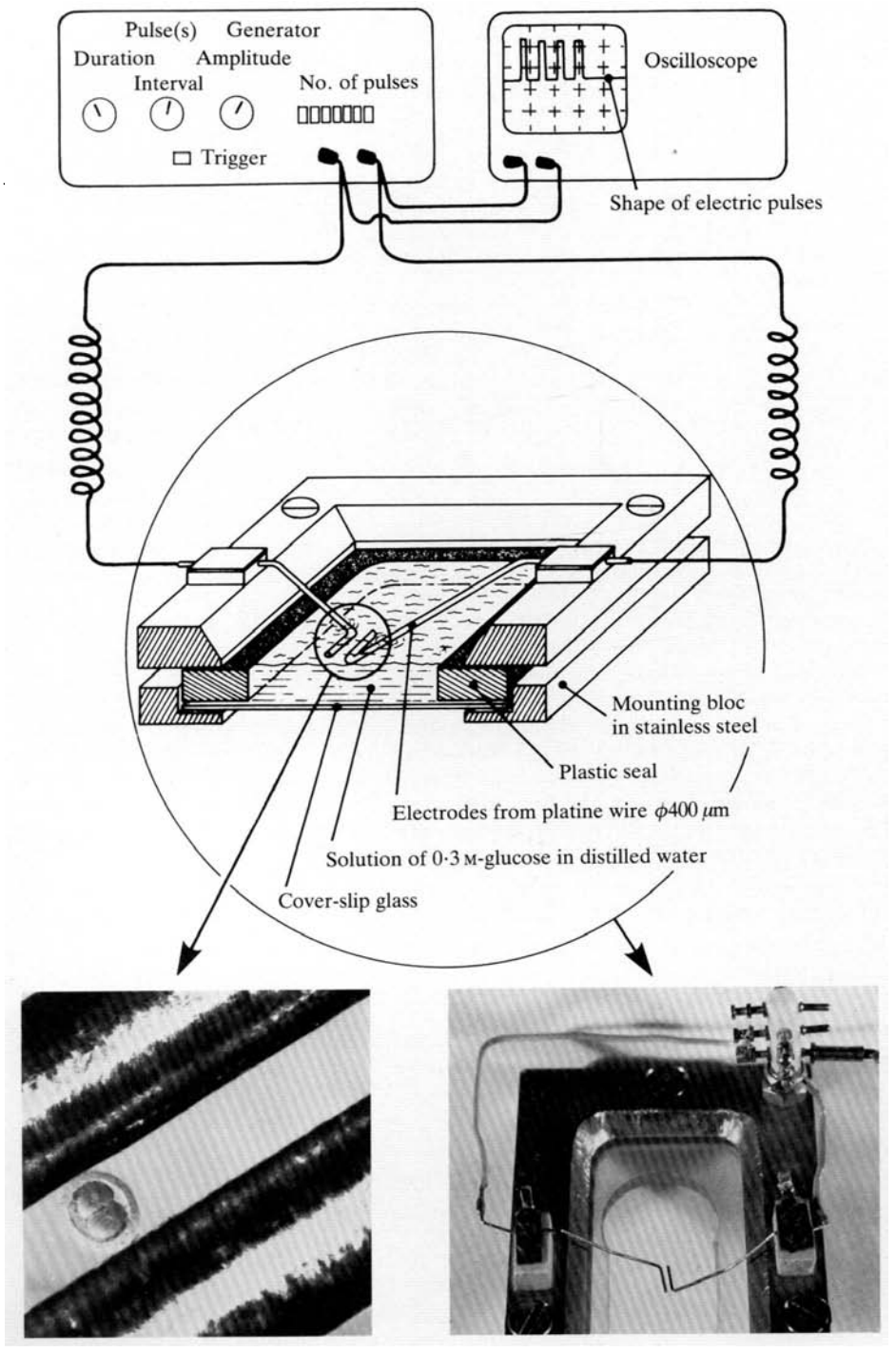
(A) *Embryo collection*

Sexually mature mixed-bred female rabbits were superovulated with follicle-stimulating hormone (FSH) followed by pituitary luteinizing hormone (LH) according to the technique described by Kennelly & Foote (1965) and modified by Thibault (personal communication). They were given 2 mg FSH in five injections at 12 h intervals: 0·250, 0·250, 0·650, 0·650 and 0·250 mg, respectively. 12 h later, just before mating, they received one injection of 0·33 mg LH.

16 to 23 h after mating, fertilized ova or 2-cell embryos were recovered from the oviducts of the does by flushing the Fallopian tubes with phosphate-buffered saline (PBS). Embryos were washed in B2 medium (Ménézo, 1976) and placed before treatment in drops of this medium under liquid paraffin. Cultures were gassed with 5 % CO_2 in air and kept at 37°C.

(B) *Experimental procedure*

The experimental procedure for electrically induced fusion was similar to that described by Zimmermann & Vienken (1982). Two cylindrical platinum wires, 400 μm in diameter, were mounted in a fusing chamber parallel and 200 μm apart (Fig. 1). The wires used as electrodes were connected to a stimulator (Anapulse Stimulator 301T WPI) equipped with a stimulus



2-cell rabbit embryo with cleavage plane parallel to the electrodes

Fig. 1. Scheme of electrical fusion procedure.

isolation unit (model 305R WPI). An oscilloscope was connected parallel to the electrodes for adjusting electric field pulse duration and strength. The experiment was carried out under a stereomicroscope at room temperature in a chamber filled with low conductive (15 to 32 kΩ) solution of 0·3 M-glucose in distilled water.

(C) *Embryo fusion, culture and transfer*

The 2-cell embryos were washed in 0·3 M-glucose solution before they were subjected to the electric field. Each embryo was placed separately between the electrodes with the cleavage plane perpendicular to the direction of the electric field vector ($\vec{E}$). Two series of experiments were carried out and the effect of the electric field, as well as of the number of pulses, was tested (groups I and II).

Group I. Effect of various electric field strengths and number of pulses

(A) 2-cell embryos were submitted to a single pulse and 35 μs of the following strengths: 1 kV cm^{-1}, 1·5 kV cm^{-1}, 2 kV cm^{-1}, 2·5 kV cm^{-1} and 3 kV cm^{-1}.

(B) Embryos were submitted to a train of four pulses of 35 μs each at 2 ms intervals, of the same strengths as in (A).

Group II. Effect of single pulse length

Embryos were exposed to one pulse of 2·5 kV cm^{-1} (subgroup A) or to 3 kV cm^{-1} (subgroup B) for 100, 500 or 1000 μs, respectively. Treated embryos were washed in B2 medium and then cultured. During the first 2–3 h embryos were observed under an inverted microscope (Diaphot, Nikon) fitted with a Nomarski interference contrast. The 'nonfused' embryos were separated from the 'fused' ones. Most eggs for all groups were cultured for at least 48 h. To test the postimplantation viability of 'fused' embryos, some of them from each group were transferred immediately after fusion to the oviduct of pseudopregnant does using the technique described by Staples (1971). Some 'fused' embryos belonging to group IB (four pulses of 3 kV cm^{-1} for 35 μs each) and to group IIB (one pulse of 3 kV cm^{-1} for 100 μs) were examined carefully after fusion till the next cleavage (see Results). Only embryos subjected to equal cleavages were transferred to pseudopregnant recipients. Autopsies of the recipients were performed between day 11 and day 20 of pregnancy and the number of implantation sites and live foetuses was determined.

(D) *Control experiments*

Three control experiments were carried out (groups I, II and III).

Control group I. Surgical transfer efficiency

Freshly collected 1- or 2-cell embryos were cultured *in vitro* for 5 h and then transferred to the pseudopregnant does. Efficiency of transfer and also the influence of short culture were determined by the implantation rate recorded on day 10 of pregnancy and by the number of live-born young.

Control group II. Embryo survival rate as affected by electric field pulse

(A) A certain number of 'nonfused' embryos treated with one or four pulses of 2 kV cm^{-1} were used as controls and transferred to recipients.

(B) 2-cell embryos placed between the electrodes with the cleavage plane parallel to $\vec{E}$ to prevent fusion were submitted either to one pulse of 3 kV cm^{-1} for 100 μs or to four pulses of 3 kV cm^{-1} for 35 μs and transferred to the oviduct of pseudopregnant does. In both subgroups the rate was determined by the number of live-born young.

Control group III. Embryo culture in vitro

Freshly collected 1- or 2-cell embryos were cultured for 96 h and the rate of development determined by the number of expanded blastocysts.

(E) *Examination of embryos*

Ploidy of cultured 'fused' preimplantation embryos was determined by chromosome preparations according to the method described by King, Linares, Gustavsson & Bane (1979). Pieces of limbs, kidney, heart and liver were treated with trypsin (0·5 mg ml^{-1} in Hank's Ca^{2+}- and Mg^{2+}-free medium). Cells were washed in MEM and then centrifuged at 3000 rev min^{-1} for 5 min. After 48 h of culture cells were treated with colcemide (0·01 mg ml^{-1}) at 37°C for 3 h whereafter the standard karyotyping methods were used. Some embryos from which only pieces of limbs or limb buds were removed to assess their ploidy were used for histological analysis. Embryos were fixed in Bouin–Holland's fluid and stained with trichrome Masson in one step. These observations will be described in detail elsewhere.

RESULTS

(A) *Effect of electric field strength and number of pulses* (group I, Table 1)

Single pulse of 35 μs

Fusion probably began at the lowest tested electric field strength (just below 1 kV cm^{-1}), since 27·3 % of treated embryos fused at that level. Efficiency of fusion increased progressively reaching a maximum at 2·5 and 3 kV cm^{-1}. Most treated embryos showed distinct signs of fusion within the first 15 min, though some fusions were only observed after 45 min. In this group no embryo lysis was seen and all fused embryos reached the early morula stage (16–32 cells) after 48 h of culture (Fig. 3F). Those in which ploidy could be assessed were diploid, tetraploid or diploid/tetraploid mosaics. Recipients with transferred 'fused' embryos were checked for implantation between day 11 and 14. Implantations were found in all recipients and their frequency increased with increasing electric field intensity. Among 15 implantations, 6 live foetuses with a normal appearance were found while 9 were retarded in their development, already dead or in various stages or resorption.

Four pulses of 35 μs

After treatment with four pulses, the fusion rate slightly increased, in comparison with the same electric field values as in group IA, reaching a maximum level of 88·4 % at 3 kV cm^{-1}. As in group IA, all embryos cultured for 48 h reached the early morula stage. Those embryos in which ploidy could be assessed were diploid or tetraploid or diploid/tetraploid mosaics. As in group IA, a certain number of 'fused' embryos at each electric field value was transferred to recipients and implantations were observed in all groups of 'fused' embryos. Among 54 implantations recorded between day 11 and 18, 9 normal live embryos were found while the remaining embryos were either abnormal and obviously retarded in their development or already resorbed. We succeeded in confirming the ploidy of three live embryos. One was diploid and two were diploid/tetraploid mosaics. In abnormal dead embryos ploidy could not be assessed.

(B) *Effect of single electric field pulse length* (group II, Table 2)

To test the effect of single pulse length, the values of electric field intensity chosen were those which in group I gave the maximum rate of fusions and also the

Table 1. *Effect of various electric field strengths and number of pulses on the fusion rate and the development of 'fused' embryos (Group 1, subgroups A and B)*

	Subgroup	No. of pulses*	Strength of electric field ($kV\,cm^{-1}$)				
			1	1·5	2	2·5	3
No. of 'fused' embryos/ no. of embryos treated	A	1	15/55 (27·3 %)	31/53 (58·5 %)	40/56 (71·4 %)	43/48 (89·6 %)	34/40 (85·0 %)
	B	4	9/15 (60·0 %)	26/40 (65·0 %)	37/48 (77·0 %)	39/47 (83·0 %)	114/129 (88·4 %)
No. of lysed embryos/ no. of embryos treated	A	1	0	0	0	0	0
	B	4	0	1 (2·5 %)	2 (4·2 %)	2 (4·3 %)	8 (6·2 %)
No. of morulae/no. of cultured 'fused' embryos†	A	1	6/6	20/20	21/21	27/27	21/21
	B	4	9/9	5/5	12/12	22/22	32/32
No. of implantations/ no. of embryos transferred	A	1	—	1/10 (10·0 %)	3/19 (15·8 %)	3/13 (23·0 %)	8/11 (72·7 %)
	B	4	—	3/15 (20·0 %)	16/23 (70·0 %)	3/13 (23·0 %)	32/76 (42·1 %)
No. of living foetuses	A	1	—	1§	0	3§	2‡
	B	4	—	0	5§	0	4‖

* Duration of the pulse(s) = 35 μs.
† Development up to 16-cell stage after 48 h of culture.
‡ Recorded at day 12.
§ Recorded at day 14.
‖ 1 recorded at day 11, 2 recorded at day 14·5, 1 recorded at day 18.

Table 2. *Effect of single electric field pulse duration on the fusion rate and the development of 'fused' embryos (group II, subgroups A and B)*

	Subgroup	Strength of electric field ($kV\,cm^{-1}$)	Duration of pulse (μs)			
			35	100	500	1000
No. of 'fused' embryos/ no. of embryos treated	A	2·5	43/48 (89·6 %)	39/45 (86·6 %)	33/42 (78·5 %)	47/65 (72·3 %)
	B	3	34/40 (85·0 %)	104/119 (87·4 %)	29/35 (82·9 %)	17/40 (42·5 %)
No. of lysed embryos (% of treated)	A	2·5	0	0	1 (2·4 %)	10 (15·4 %)
	B	3	0	1 (0·8 %)	2 (6·0 %)	21 (52·5 %)
No. of expanded blastocysts/ no. of cultured embryos*	A	2·5	—	15/18 (83·3 %)	6/9 (66·6 %)	11/16 (68·8 %)
	B	3	—	26/31 (83·9 %)	14/19 (73·7 %)	12/16 (75·0 %)
No. of implantation sites/ no. of embryos transferred	A	2·5	3/13 (23·0 %)	11/20 (55·0 %)	4/17 (23·5 %)	13/16 (81·2 %)
	B	3	8/11 (72·7 %)	42/58 (72·4 %)	—	—
No. of living foetuses	A	2·5	3‡	3§	1§	1§
	B	3	2†	6‖	—	—

* After 96 h of culture.
† Recorded at day 12.
‡ Recorded at day 14.
§ Recorded at day 16.
‖ Three recorded at day 12, two recorded at day 14·5, one recorded at day 20.

relatively highest number of implantations (i.e. 2·5 kV cm^{-1} and 3 kV cm^{-1}). At 2·5 kV cm^{-1} (subgroup IIA) the fusion rate decreased from 89·6 % at 35 μs to 86·6 % at 100 μs and from 78·5 % at 500 μs to 72·3 % at 1000 μs. Parallel to that the lysis rate increased from 0 to 15·4 %. Among the 'fused' embryos treated for 100 μs, over 83·3 % reached the expanded blastocyst stage after 96 h of culture. As the pulse duration increased, the number of embryos reaching the blastocyst stage (Fig. 3G) slightly decreased, but at least two-thirds of them were still able to complete preimplantation development *in vitro*. In all groups, implantation of 'fused' embryos and of live foetuses was recorded. For a single pulse of 3 kV cm^{-1} (subgroup IIB) the fusion rate was 85 % for 35 μs, but fell to 42·5 % for 1000 μs with a rapid increase (up to 50 %) in blastomere lysis. However, when applying the latter pulse length, 12 'fused' embryos out of 16 cultured for 96 h reached the blastocyst stage.

(C) *Behaviour of 'fused' embryos* (Table 3)

In several previous experiments cultured as well as transferred embryos were unexpectedly not uniformly tetraploids. In order to examine how a reversion of 'fused' (i.e. presumably tetraploid) embryos to a diploid state is possible, 103 embryos belonging to Group IB (treated with four pulses of 3 kV cm^{-1} for 35 μs) and 79 embryos belonging to Group IIB (treated with one pulse of 3 kV cm^{-1} and 100 μs) were carefully examined after fusion during the whole subsequent cell cycle. The typical sequence of events is shown in Fig. 2. The majority of 'fused' embryos, i.e. 61·2 and 73·4 % according to groups, followed this pattern. After fusion two diploid nuclei moved to the centre of the 'fused' cell (Fig. 2A–C) and established close contact (Fig. 2D). After that the embryos cleaved into two equal blastomeres (Fig. 2F–H). In some embryos, however, (6·8 % in embryos of group IB and 14 % in those of group IIB) small vacuoles were visible in the fusion plane

Table 3. *The behaviour of 'fused' embryos during the following cell cycle*

	Electric field treatment	
	4 pulses at 3 kV cm^{-1} for 35 μsec	1 pulse at 3 kV cm^{-1} for 100 μsec
No. of 'fused' embryos	103	79
No. of two-cell embryos after the next cleavage	63 (61·2 %)	58 (73·4 %)
No. of 'fused' embryos with visible vacuoles in the fusion plane	7 (6·8 %)	11 (14·0 %)
No. of 'fused' embryos which cleaved spontaneously in three or four cells during the next cleavage	26 (25·2 %)*	19 (24·0 %)*
No. of 'fused' embryos fragmented before the end of the cell cycle	14 (13·5 %)	2 (2·5 %)

* This number included embryos on which vacuoles were visible after fusion.

(Fig. 3A) The vacuoles swelled and migrated to the centre of the fused cell between the two nuclei (Fig. 3B). In these embryos the nuclei were not able to come into close contact and remained separated from each other and finally the cell cleaved into three or four parts (Fig. 3C–E). However, this event cannot be responsible for all atypical cleavages because in those groups the direct cleavage into three or four blastomeres (18·4 % and 10·1 %, respectively) was observed under a light microscope without any visible vacuoles on the plane of fusion.

(D) *Postimplantation development of tetraploid embryos* (Table 4)

Among 182 embryos observed during the whole cell cycle after fusion, 121 (66·5 %) embryos cleaved into two regular and equal blastomeres. These embryos were selected and transferred to recipient does. Live foetuses were recorded until day 20 of pregnancy. On day 11, three live embryos were uniformly tetraploids and no visible external abnormalities were detected. The retarded and abnormal ones showed abnormalities mainly affecting the neural tube, particularly in the brain region. On day 14·5, four live foetuses were found (Fig. 3H). All looked normal although they were slighlty smaller than the diploid embryos at the same stage. All embryos were tetraploid. The remaining implantation sites contained abnormal dead embryos with several types of abnormalities or only remnants of foetal membranes and embryos. On day 18, only one live embryo out of six implants was found. This embryo was fully developed and corresponded exactly to day 18 of pregnancy. The karyological analysis revealed diploid/tetraploid mosaicism with the distinct majority of diploid cells. On day 20, one live embryo out of 27 implants was found. This embryo was tetraploid, fully developed and its size (24×10 mm) corresponded to day 20 of pregnancy according to the table of external development of the rabbit (Edwards, 1968).

(E) *Control experiments*

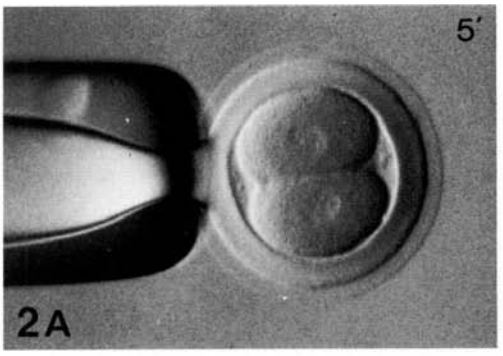
Surgical transfer

From 46 2-cell embryos transferred into recipient does, 31 normal embryos were recovered on day 10, while from 36 other transferred 2-cell embryos, 19 normal young were born (Table 5). Thus, in our experimental conditions the implantation rate after collection, culture for a few hours and surgical transfer was 67·4 % and the survival rate to term 52·7 %.

Electric field treatment (Table 5)

(a) Cleavage plane of 2-cell embryos perpendicular to $\vec{E}$. Fourteen embryos in which blastomeres did not fuse after treatment with one pulse of 2 kV cm^{-1} and 35 μs (group IA) were transferred to recipient does and gave birth to three normal live young. Ten 'nonfused' embryos treated with four pulses of 2 kV cm^{-1} and 35 μs (group IB) and transferred into pseudopregnant does gave birth to 10 normal live rabbits (Table 5).

(b) Cleavage plane of 2-cell embryos parallel to $\vec{E}$. Among 2-cell embryos treated with one pulse of 3 kV cm^{-1} for 100 μs, fusion of blastomeres occurred in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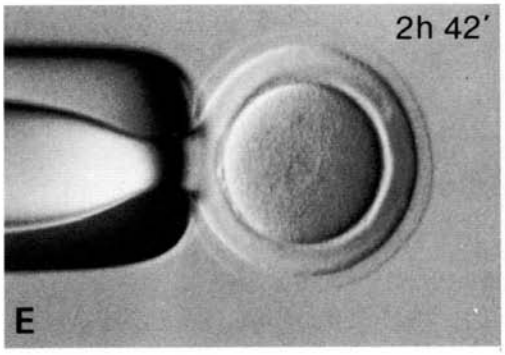
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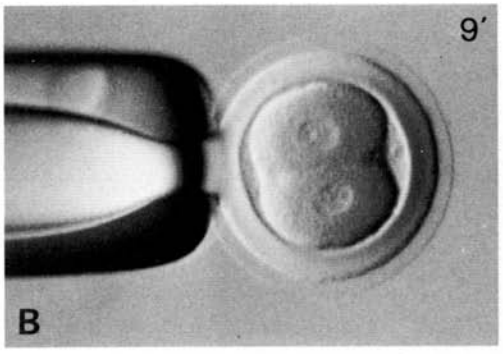
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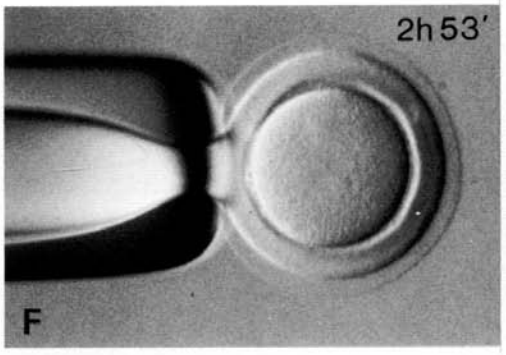
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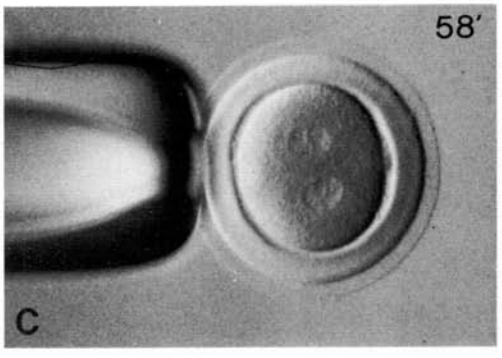
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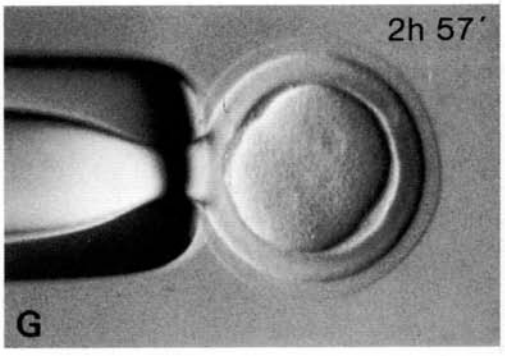
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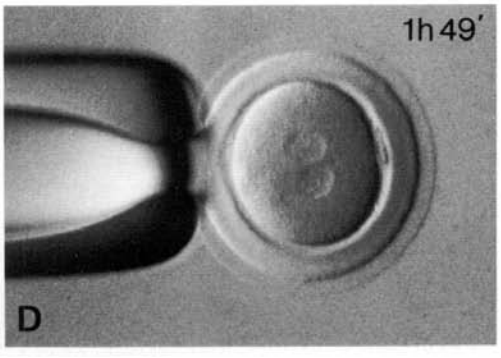
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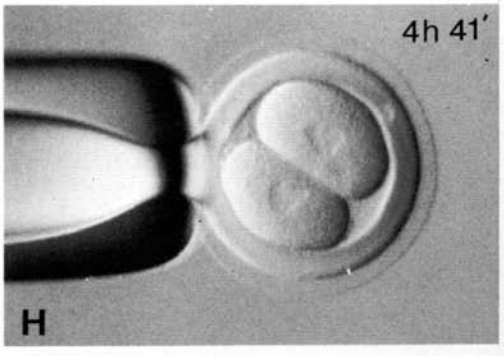
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one case and among the remaining 24 transferred embryos, 13 young live rabbits were obtained (54·2 %) (Table 5). Among 32 embryos treated with four pulses of $3\,kV\,cm^{-1}$ for 35 μs, fusion of blastomeres occurred in 3 of them while 13 live young were obtained among the remaining 29 transferred embryos (44·8 %) (Table 5).

Control of in vitro *culture* (Table 2)

Among 91 nontreated 2-cell embryos cultured for 96 h, 79 (86·8 %) reached the expanded blastocyst stage.

DISCUSSION

(A) *Fusion parameters and efficiency*

In spite of the double size of rabbit ova in comparison to mouse eggs, the minimal field strength causing fusion of blastomeres of 2-cell rabbit embryos was similar to that inducing the fusion of 2-cell mouse embryos (Kubiak & Tarkowski, 1985). It seems that the electrical breakdown of the membrane inducing the fusion of the embryonic cell is not as clearly related to cell size as in the case of other kinds of somatic cells (Zimmermann & Vienken, 1982). Electrofusion of oocytes and blastomeres taken from 2-, 4- and 8-cell embryos and small karyoplasts and cytoplasts also confirms this view (unpublished observations). The efficiency of fusion of rabbit blastomeres increases with the rise in the electric field pulse strength and duration, until these values reach a critical level. It was clearly demonstrated that the electrical breakdown of plasma membrane is dependent on pulse strength and duration (Zimmermann, 1982). However, at the lowest electric field intensity, a train of four pulses significantly increased the fusion efficiency (60 % instead of 27·2 %), most likely in relation to the development of a considerable number of pores hastening the process of fusion. At the highest field intensity (i.e. 2·5 and $3\,kV\,cm^{-1}$) the duration and number of pulses did not

Fig. 2. Typical time sequence of events of a 2-cell electric-field-treated embryo cultured in the same position at 38 °C under a microscope during the following cell cycle (×190).

(A) 2-cell rabbit embryo just after electric pulse treatment. This embryo is held with a pipette during the fusion process till the next cleavage. At 5 min (t = 5 min) it is possible to see the first sign of fusion at the cleavage furrow.

(B) The same embryo, 4 min later; the fusion of the blastomere is now clearly visible.

(C) At 58 min, this 'fused' embryo looks like a 1-cell egg, but instead of pronuclei it has two diploid nuclei.

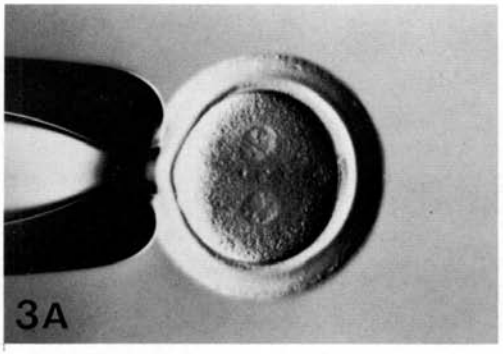
(D) 1 h 49 min after the pulse treatment the two diploid nuclei come into close contact.

(E) In the centre of the cell, it is possible to distinguish the spindle with the chromosomes in metaphase position. t = 2 h 24 min.

(F) Chromosomes of a'fused' embryo during anaphase. t = 2 h 53 min.

(G) 'Fused' embryo at the end of telophase. Formation of the tetraploid nucleus can be seen while the contractile ring begins to cleave the cell into two blastomeres. t = 2 h 57 min.

(H) 2-cell embryo but with tetraploid nuclei. t = 4 h 41 min.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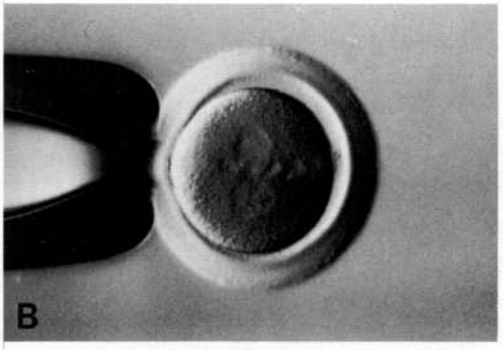
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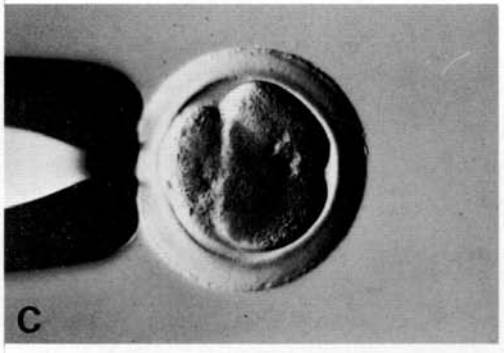
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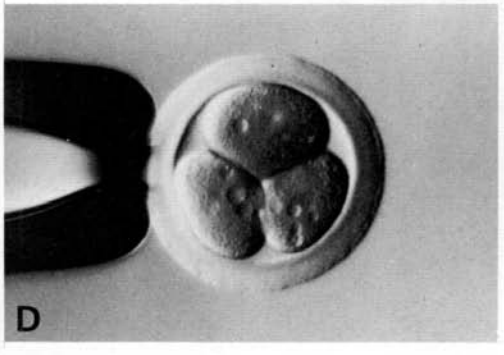
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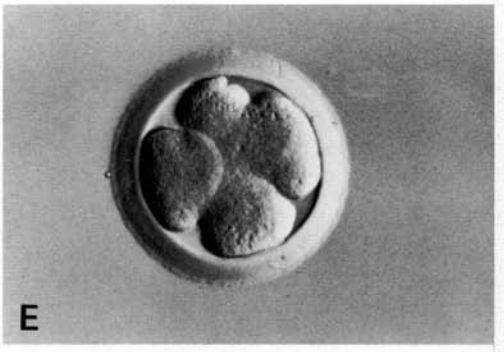
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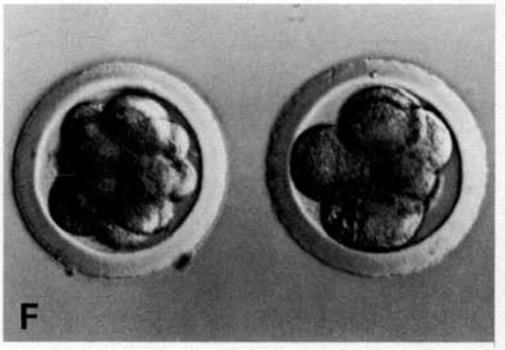
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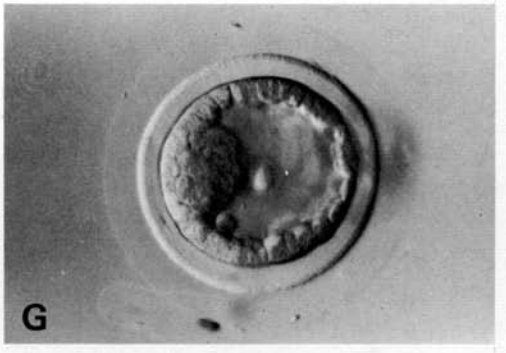
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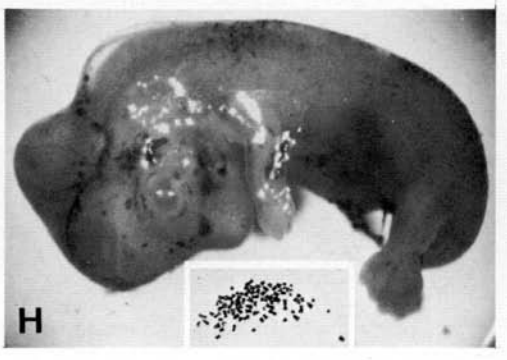
H

improve the fusion rate, which remained almost constant between 85 and 90 %. From 2·5 kV cm^{-1} the rate of lysis increased, showing at about 3 kV cm^{-1} the level of maximum tolerance for the plasma membrane. Above that critical value the effect of electric field pulse became harmful and irreversible (Benz & Zimmermann, 1980; Zimmermann, Vienken, Pilwat & Arnold, 1984). A small proportion of embryos never fused even after strong and multiple treatments, but we cannot explain this phenomenon. If the right position of embryos to be fused (i.e. cleavage plane perpendicular to $\vec{E}$) appears to be important for fusion efficiency at the lower pulse values (see also Kubiak & Tarkowski, 1985) it should not make such a significant difference at higher field intensity. When the embryos were located between electrodes with the cleavage plane parallel to $\vec{E}$ and treated with 2·5 or 3 kV cm^{-1}, around 7 % of them fused, showing that at certain field strength levels the electrical breakdown must occur on the whole surface of the cell. Similar observations were also reported by Richter *et al.* (1981). Mammalian embryos appeared to be more resistant to electric field treatment than small differentiated cells. Several types of somatic cells exhibited irreversible damage at electric field pulse values much below the critical values for embryonic cleavage stages (Zimmermann *et al.* 1984). Mouse thymocytes and follicle cells of much smaller volume (up to 40 times less) than the volume of oocyte were also more sensitive than mouse oocytes, blastomeres or even small karyoplasts (unpublished observations).

(B) *Development of 'fused' and 'nonfused' embryos*

In vitro

In all groups the development of 'fused' and 'nonfused' and treated embryos was as good as the nontreated ones. Thus, it seems that in embryonic cells an electric field pulse even relatively strong (up to 2·5 or 3 kV cm^{-1}) does not create adverse effects leading to irreversible damage of the plasma membrane and inside the cell. A high rate of development until the blastocyst stage was recorded even in groups of eggs that survived the treatment of 3 kV cm^{-1} for 1000 μs representing as

Fig. 3. A typical cleavage of 'fused' embryos and morphological aspect of tetraploid morula, blastocyst (×190) and foetus (×4).

(A) After completion of blastomere fusion, small vacuoles are visible in the fusion plane.

(B) The vacuoles have swollen and migrated to the centre of the 'fused' cell between the two nuclei.

(C) In this embryo, which cleaved into three parts, the blastomeres contain several vacuoles.

(D) Another embryo which cleaved into three parts. The blastomeres contain several vacuoles.

(E) A 'fused' embryo which cleaved spontaneously into four parts with no trace of vacuole.

(F) Two tetraploid morulae after 48 h culture. The number of cells is clearly reduced as compared to cultured diploid morulae at the same stage.

(G) A tetraploid blastocyst after 72 h culture. This blastocyst looks like a normal diploid blastocyst with a well-developed ICM.

(H) A tetraploid foetus collected on day 14·5. This foetus looks like a normal diploid foetus.

Table 4. *Postimplantation development of tetraploid embryos*

	Days *p.c.*				
	11	14·5	18	20	Total
(A) One pulse of 3 kV cm^{-1} for 100 μs					
No. of implantations/ no. of embryos transferred	10/11	5/7	—	27/40	42/58 (72·4 %)
No. of normal foetuses	3	2	—	1	6
Ploidy	3×4N	2×4N	—	1×4N	6×4N
(B) Four pulses of 3 kV cm^{-1} for 35 μs					
No. of implantations/ no. of embryos transferred	4/4	4/7	6/12	10/40	24/63 (38·1 %)
No. of normal foetuses	1	2	1	0	4
Ploidy	*	2×4N	1×2N/4N	—	2×4N

*Not determined.

previously mentioned the critical cell resistance value. However, in these experiments, current intensity never exceeded 3·7 mA; when occasionally it reached 8–10 mA due to the presence of electrolytes brought along with embryos from the culture medium, fusion occurred and the embryos survived but they never developed any further (these cases were not included in our results). Zimmermann (1982) and more recently Kubiak & Tarkowski (1985) and Blangero & Teissie (1985) showed that fusions can occur in the presence of electrolytes. Mouse embryos fused in PBS with an electric field pulse below 1 kV cm^{-1} can develop *in vitro* and complete implantation in foster mothers (Kubiak & Tarkowski, 1985). Because the relationship between potential difference (U) and current intensity (I) created by an electric field between electrodes depends on the resistance (R) of the pulsating medium (Ohm's law, U = RI), these observations indicate that fusion can be induced with a low potential difference, but a high current intensity. In PBS the resistance between electrodes is very low and for the same power applied

Table 5. *Survival to term of embryos with nonfused blastomeres after electric field treatment*

	Electric field treatment				
	1 pulse at 2 kV cm^{-1} for 35 μs	4 pulses at 2 kV cm^{-1} for 35 μs	1 pulse at 3 kV cm^{-1} for 100 μs	4 pulses at 3 kV cm^{-1} for 35 μs	Non-treated
No. of embryos transferred	14*	10*	24†	29†	36
No. of normal living young	3 (21·4 %)	10 (100 %)	13 (54·2 %)	13 (44·8 %)	19 (52·7 %)

*Embryos treated with the cleavage plane perpendicular to $\vec{E}$.
†Embryos treated with the cleavage plane parallel to $\vec{E}$.

between electrodes (Power: $P = UI$) the intensity will be greater than in non-conductive solutions. Inversely, the potential difference will be greater in a non conductive *versus* saline solution. It would be useful to consider the $U \times I$ power dissipated between the electrodes instead of only U to define the best electrical parameter values for optimum fusion and survival. Blangero & Teissie (1985) also showed that the efficiency of electric-field-induced fusion of mammalian cells (CHO) depends on the ionic content of the pulsating medium. In our conditions, current and ionic contents of the pulsating medium were not controlled, but at least in the case of rabbit embryos fusion in a nonconductive solution with a strong electric field treatment ensured a normal and undisturbed development. Although the structure of *in vitro* developed blastocysts was not accurately examined, we observed that in blastocysts originated from 'fused' embryos, ICM size was often reduced in comparison with ICM of blastocysts developed from 'nonfused' or nontreated embryos. Similar observations were reported in mouse after tetraploidization with cytochalasin B (Snow, 1975; Tarkowski *et al.* 1977). The majority of blastocysts developed from treated embryos hatched from the zona pellucida and some of them reached in the following two days a very expanded form at about 400–500 μm in diameter.

In vivo

The rate of implantation of embryos exposed to electric field pulse was similar to that of nontreated ones and confirms previous observations according to which developmental potency of 'fused' and 'nonfused' embryos is probably not altered by electric field treatment. It must be admitted that 'fused' embryos did not develop beyond two-thirds of pregnancy, but it is also difficult to relate the failure of their development to other causes than that of tetraploidy. All abnormal foetuses in which it was possible to assess ploidy were uniform tetraploids. On the other hand, several normal young rabbits were born from treated but 'nonfused' embryos. In this case the survival rate to term was similar to nontreated embryos (Table 5).

(C) *Behaviour of 'fused' embryos*

The occurrence of an atypical cleavage of 'fused' embryos was unexpected. To our knowledge such an event has not been described yet. In fact, the hybrid cell resulting from fusion is confronted with at least two problems.

The first problem is the resorption or degradation of a certain area of the plasma membrane that might reach 20 % of the original surface since the fusion between cells is accompanied by a surface reduction of constant volume (Zimmermann *et al.* 1984). A particular defective membrane resorption in the contact zone during the fusion may result in formation of vesicles located between two nuclei (see Fig. 3A,B) leading to an atypical cleavage in the next mitosis (Fig. 3C,D,F).

The second problem is the organization of a specific microtubular system, the role of which is to bring the two diploid nuclei in close contact after fusion and before the onset of mitosis, a system that appears to push the pronuclei towards

the centre of the egg. Such a system has been well described by several authors in mouse eggs (Maro, Howlett & Webb, 1985; Schatten, Simerly & Schatten, 1985). In the case of fertilized rabbit eggs we observed that at least 5% of 1-cell eggs cleave spontaneously into four parts (unpublished observation). This event resembles the one we described as 'fused' embryos and could be explained by an incorrect organization of microtubular systems causing the formation of two widely separated metaphase plates inside the cell. In such a cell, two contractile rings must operate simultaneously, cleaving the cell into four regular blastomeres (Fig. 3E). The development of diploid and diploid/tetraploid mosaic embryos could be the result of such a disfunction. In some 'fused' blastomeres we observed a transient 'apparent' fragmentation which lasted for about one hour. These cells relaxed, however, and always restored their spherical shape before the next mitosis. This event is not frequent (8·7%) (Table 3), but may suggest that application of a certain electric field pulse range may exert a reversible effect on the internal structure of the egg.

(D) *Postimplantation development of 'fused' embryos*

The obtained results clearly show that tetraploid rabbit embryos derived from 'fused' blastomeres are capable of a limited postimplantation development. Although 54·5% of tetraploids succeed in their implantation, only a few reach midpregnancy. The majority of them die between day 10 and 14. Several embryos were obviously retarded in their development, showing severe malformations mainly in the brain region. In some of them the whole central nervous system was affected. However, some tetraploids and diploid/tetraploid mosaics appeared fully normal and their morphology was consistent with gestational age (Edwards, 1968). These results are different from those obtained in mouse by Snow (1975) and by Tarkowski *et al.* (1977) who did not obtain normal 4N embryos. However, it should be mentioned that Golbus, Buchman, Witlse & Hall (1976) reported the birth of a tetraploid child which survived one year and a 2-year-old 2N/4N mosaic infant reported by Kelly & Rary (1974). A more precise analysis of post-implantation development of tetraploid rabbits is required and will be further described. Collection of a 2N/4N mosaic embryo on day 18 was surprising because in this group embryos transferred at the 2-cell stage were those selected from the 'fused' ones in which regular mitosis with common metaphase plate occurred. This observation suggests a more complex background modifying the chromosome distribution in daughter cells.

In the light of our results on rabbit embryos and those previously obtained by Kubiak & Tarkowski (1985) in mouse embryos, we may conclude that the electric field treatment of embryonic cells does not jeopardize their development and that a wide range of pulse intensities can be used to obtain a variety of embryonic cell hybrids with a sufficient efficiency so that the technique offers new perspectives in experimental embryology (for a review see Markert, 1981; Gulyas, 1985).

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