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Parthenogenetic activation of domestic cat oocytes using ethanol, calcium ionophore, cycloheximide and a magnetic field

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Abstract

The objective of this study was to evaluate parthenogenetic activation of domestic cat oocytes after being exposed to either ethanol, magnetic field, calcium ionophore A23187, or cycloheximide and a combination of these agents. We also wished to evaluate the usefulness of the magnetic field for oocyte activation. In vitro matured oocytes subjected to artificial activation were randomly assigned into eight groups according to activating agents: (1) 10% ethanol; (2) the magnetic field (slow-changing, homogenous magnetic field with low values of induction); (3) 10% ethanol plus magnetic field; (4) 10 μM calcium ionophore A23187; (5) 10 μM calcium ionophore A23187 plus magnetic field; (6) 10% ethanol and 10 μg/mL of cycloheximide; (7) 10% ethanol and 10 μg/mL of cycloheximide plus magnetic field; (8) oocytes were not exposed to any of the activating agents. After activation oocytes were stained with Hoechst 33258 and parthenogenetic activation was defined as oocytes containing pronuclei and second polar bodies or two to four or six nuclei (embryonic cleavage). The total activation rate by using different activation treatments was 40%. The addition of the magnetic field to ethanol or calcium ionophore treatments resulted in increased parthenogenetic activation rates from 47% to 75%, and from 19% to 48%, respectively ($P < 0.001$). Instead, when the magnetic field was added to ethanol and cycloheximide treatment, activation rate decreased from 48% to 30%. Oocytes activated with magnetic field only gave the lowest activation rate (12%).

We concluded that a magnetic field can be used as an activating agent, and the combination of ethanol and magnetic field is an effective method for domestic cat oocyte activation.

Keywords: Cat; Oocyte; Activation; Parthenogenesis; Magnetic field

1. Introduction

In the process of fertilization interaction between a sperm cell and a secondary oocyte triggers off a series of morphological and biochemical transformations, known as oocyte activation. The key mechanism is a calcium signal, shared by most animal species. Several minutes after the penetration of a sperm cell into an oocyte a quick and transitory drawing occurs from intracellular reserve of calcium. This is the calcium collected in endoplasmic reticulum, although extracellular calcium can also be used to supplement the reserve, thus enabling continuation of the calcium signal [1,2]. The activation leads to meiosis resumption and extrusion of the second polar body. Consequently, pronuclei are formed, DNA synthesis begins and embryonic cleavage is initiated of artificial activation,

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caused by external stimuli. Parthenogenesis phenomenon does not occur naturally in mammals, although very rarely spontaneous can take place. Artificial activation is a necessary step in the cloning procedure, while somatic cloning can become one of the methods for saving endangered species of animals (including felids).

Although, several publications on artificial activation of feline oocytes exist, most of them are related to activation during cloning procedure. Only few studies had compared different methods of activation and their effectiveness, as measured by the number of activated oocytes and parthenogenetic embryos [2,3]. The stimulus which triggers oocyte activation, can be a physical, mechanical or chemical agent. Combination of these agents is also applied. One common, universal method has not been developed because the process is highly specific for each species. Cat oocyte activation by electric current or chemical agents (ethanol, calcium ionophore) has been reported, along with the inhibitors of protein synthesis, protein phosphorylation or histone kinases (kinases PF/MAP; cycloheximide, cytochalasine B) [6–9]. A single electrical stimulation seems to be insufficient to activate domestic cat oocytes, but their exposure to cycloheximide following electrical stimulation improved the efficacy of parthenogenetic development [3].

Domestic cat oocytes used for nuclear transfer have been activated 1–2 h post-fusion by exposing them to 7% ethanol and then culturing in cycloheximide + cytochalasin D. The results in this study demonstrated that 90% of oocytes cleaved to two to six cells and above 13% reached blastocyst stage [10]. In similar study, reconstructed cat oocytes were activated with 7% ethanol (5 min) followed by incubation in cycloheximide for 4 h. Cleavage rates (55–80%) and embryonic development to the blastocyst stage (5–8%) were similar to those observed with the presence of cytochalasin D [8].

Ethanol alone, at concentration 10% has not been used in cats to trigger parthenogenetic activation, while calcium ionophore has been mainly used in combination with other agents.

Cleavage and blastocyst development of in vivo and in vitro matured domestic cat oocytes exposed to two ionophores (A23187 or ionomycin) at two different concentrations (5 or 10 μM) for 3 or 5 min have been evaluated. In that study, higher activation rates were observed when in vivo matured oocytes were used (60% versus 47%). The cleavage on day 2 was higher for oocytes exposed to the ionophore for 5 min rather than for 3 min. Instead cleavage was not affected by the type of ionophore or its concentration [11]. Protein synthesis and protein phosphorylation inhibitors in combination with ionophores have been used for cat oocyte activation. Oocytes were exposed to calcium ionophore A23187 (5 μM) for 5 min, followed by incubation either with cycloheximide (10 μg/mL) plus cytochalasin D (2.5 μg/mL), or with 6-dimethylaminopurine (2.5 mM) for 4–5 h resulting in similar cleavage rates of 88% versus 82%, and blastocyst development 45% versus 28%, respectively [3]. It has been also demonstrated that in vitro matured cat oocytes can be activated after being exposed to ionomycin (5 μM) followed by 5 h incubation in 10 μg/mL cycloheximide. The overall rate of activation for the treated oocytes was 90% [12].

Magnetic field has not been used to induce artificial activation in oocytes of any animal species. The magnetic field can influence movement of calcium ions in the cell. Studies carried out on isolated pituitary cells had demonstrated that calcium concentration in the cells had increased almost twice from the normal concentration after being exposed to the magnetic field at frequency of 50 Hz and induction of 50 μT [13].

Most of the authors point out, however, that the results of experiments are closely connected with the parameters of magnetic fields applied and also depend on the experimental object (cellular model, animals), subjected to magnetic field. The results vary, sometimes very significantly, even for slightly different values of induction, frequency and field form. Biological effects observed may be stronger for weaker fields and inversely, some of them are proportional to the induction value or do not depend on it. Therefore, the choice of parameters is the product of many trials and the final result depends on the field values chosen.

The aim of this study was to activate in vitro matured domestic cat oocytes using ethanol, magnetic field, calcium ionophore A23187, ethanol and cycloheximide and combinations of these agents, as well as to compare their effectiveness and to evaluate magnetic field usefulness for oocyte activation.

2. Materials and methods

2.1. Oocyte recovery and maturation

Ovaries from domestic cats were obtained after ovariohysterectomy at local veterinary clinics. Oocytes selected for in vitro maturation were surrounded by compact layers of cumulus cells and dark ooplasm. Ten to fifteen cumulus–oocyte complexes (COCs) were cultured in four-well tissue culture dishes containing 700 μL of TCM-199 supplemented with 0.3% BSA, 1 UI/mL hCG, 0.5 UI/mL eCG (Intervet International

B.V., Boxmeer, Holland), 2.2 mM calcium lactate, 0.36 mM sodium pyruvate, 2 mM glutamine, 50 μg/mL gentamicin, 22 mg/mL $NaHCO_3$, 0.13 mM cysteine, 1 μL/mL 17β-estradiol (all chemicals purchased from Sigma Chemical Co., St. Louis, MO, USA unless otherwise stated) at 38 °C, for 36 h, in 5% CO_2 + air.

2.2. Oocyte activation

Magnetic field (P) as an activator was generated by Viofor JPS (Med&Life Sp. z o.o., Komorów, Poland). The equipment consists of a controller (for selecting field parameters) and an applicator. A chamber in the form of a cylinder (10.5 cm inner diameter and 13 cm high) with PVC walls was designed. The tissue culture dish containing oocytes for activation was placed in the middle of the cylinder, which is the area of homogeneous magnetic field. Two levels of field intensity were chosen on the controller: level 12, corresponding to 28.8 μT for 20 min, then level 5, corresponding to 12 μT for 10 min. The frequency of the series of impulses was 0.08–0.3 Hz.

After maturation, oocytes were washed once in HEPES buffered TCM-199 medium and exposed to the following activation treatments:

- Treatment 1: Ten percent ethanol only for 3 min (E).
- Treatment 2: Magnetic field only, slow-changing, homogenous magnetic field with low values of induction (as mentioned previously).
- Treatment 3: Magnetic field, and then exposure to 10% ethanol for 3 min.
- Treatment 4: 10 μM calcium ionophore A23187 for 7 min (J).
- Treatment 5: Ten micromolars calcium ionophore A23187 for 7 min followed by magnetic field.
- Treatment 6: Ten percent ethanol for 3 min, followed by incubation with 10 μg/mL of cycloheximide (C) for 24 h.
- Treatment 7: Ten percent ethanol for 3 min, then magnetic field, and culture in 10 μg/mL of cycloheximide for 24 h.
- Treatment 8: These oocytes were not exposed to any of the activating agents (control group), instead they were placed in HEPES buffered TCM-199 supplemented with 0.3% BSA, for the same length of time as each activating treatment.

Following activation, oocytes were cultured in TCM-199 supplemented with 0.6% BSA, 2% essential amino acids (EAA), 1% nonessential amino acids (NEAA) and 50 μg/mL gentamicin under paraffin oil for 48 h, at 38 °C, in 5% CO_2 + air.

After 48 h, oocytes were stained with Hoechst 33258, and parthenogenetic activation was defined as oocytes containing pronuclei and the second polar body, or embryonic cleavage, with the presence of two to four or six nuclei.

2.3. Statistical analysis

Data from the experiment was analyzed by using Mann–Whitney–Wilcoxon test.

3. Results

3.1. Parthenogenetic activation by using different activation treatments

A total of 1068 oocytes were activated, and 101 oocytes allocated to the control group. We evaluated one cell activated oocytes and cleaved (two, four and six cell-stage) embryos as the total number of parthenogentic activated oocytes. Table 1 shows the number and percentages of oocytes activated by each treatment.

3.2. Parthenogenetic activation by incorporating magnetic field

The efficiency of artificial activation was improved when oocytes were exposed to a combination treatment of magnetic field with ethanol (75%), or magnetic field and calcium ionophore (48%), compared to oocytes exposed only to ethanol (47%) or calcium ionophore (19%; $P < 0.001$). However, the addition of magnetic field to the combined treatment of ethanol and cycloheximide, reduced the percentage of activated oocytes (30% versus 48.4%; $P < 0.01$, respectively). The best activating effect was found when using ethanol and magnetic field (Fig. 1). The efficacy of using a magnetic field as a new activating agent was demonstrated in the present study (Fig. 2).

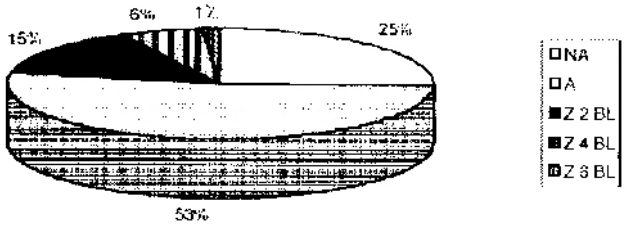

Fig. 1. Percentages of one cell activated oocytes and cleaved embryos in the group of oocytes activated by ethanol and magnetic field; NA, oocytes nonactivated; A, oocytes activated; Z 2 BL, two cell-stage embryos; Z 4 BL, four cell-stage embryos; Z 6 BL, six cell-stage embryos.

Table 1
Activation of domestic cat oocytes using different activating agents

Activating agent	No. of oocytes total	Nonactivated		Successfully activated	
		No.	%	No.	%
None (control)	101	99	98.02	2	1.98
Ethanol	153	81	52.94	72	47.06 a
Magnetic field[a]	143	126	88.11	17	11.89
Ethanol + magnetic field	154	39	25.32	115	74.68 b
Calcium ionophore	150	121	80.67	29	19.33 c
Calcium ionophore + magnetic field	148	77	52.03	71	47.97 d
Ethanol/cycloheximide	159	82	51.57	77	48.43 e
Ethanol/cycloheximide + magnetic field	155	109	70.32	46	29.68 f
Total after activation	1062	635	59.79	427	40.21

Values with different letters within columns are significantly different (a and b: $P < 0.001$; c and d: $P < 0.001$; e and f: $P < 0.01$).
[a] In the group of 149 oocytes activated by magnetic field alone, a phenomenon of cell fragmentation was observed in six cases (4.03%); these oocytes were not qualified to the group of nonactivated oocytes, nor to the group of activated oocytes and were excluded from the table.

Fig. 2 shows percentages of obtained one cell activated oocytes, cleaved embryos and their sum in particular groups. Best results were obtained in the group of oocytes activated by ethanol and magnetic field—both in the number of one cell activated oocytes and cleaved embryos. Magnetic field as the sole agent used was least effective (19%). Within all treatments, cleaved embryos constituted a small percentage. In Table 2 we compared different methods of activation, taking into account their effectiveness in the form of one cell activated oocytes and cleaved embryos. After activation with ethanol; two cell-stage embryos constituted a significant percentage in parthenogenetic embryos (53%); six cell-stage embryos had the lowest percentage (11%), while, four cell-stage embryos represented 36%. In ethanol and magnetic field treatment, 65% of two cell-stage embryos, 29% of four cell-stage embryos and 6% of six cell-stage embryos were obtained. After activation by ionophore and magnetic field the distribution of embryos was similar to the treatment with ethanol and magnetic field (64%, 29% and 7% of two, four and six cell-stage embryos, respectively). In ethanol and cycloheximide treatment, no six cell-stage embryos were obtained, while embryo cleavage for two and four cell-stage embryos were 69% and 31%, respectively.

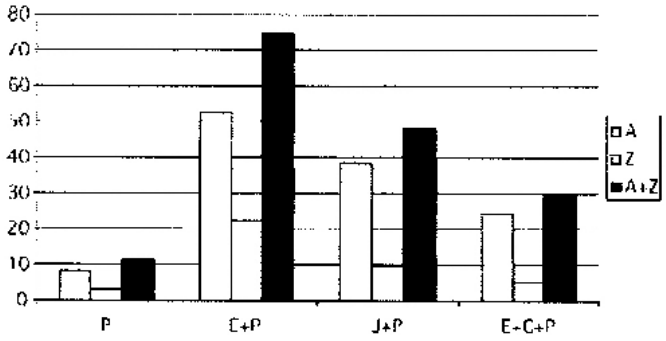

Fig. 2. Results of oocytes activation using magnetic field (P), ethanol with magnetic field – (E + P), calcium ionophore with magnetic field (J + P) and ethanol/cycloheximide with magnetic field (E + C + P); A, one cell activated oocytes; Z, cleaved embryos.

4. Discussion

Artificial activation of oocytes is one of the stages of in vitro procedures, which replace activation of an egg cell by sperm in the process of natural reproduction. Numerous comparative studies were carried out in farm animals in respect to different methods of artificial activation, and the most effective ones were selected. Different methods had not been compared for felids and several possibilities have been applied in hitherto studies, but they have not led to find a highly effective method. It was therefore purposeful to carry out a comparison of methods of artificial activation of

Table 2
Comparing the effectiveness of activation methods in obtaining one cell activated oocytes and cleaved embryos

Activating agent	One cell activated oocytes (%)	Cleaved embryos (%)
None	0	1.98
Ethanol	28.76	18.30
Magnetic field	8.39	3.50
Ethanol + magnetic field	52.6	22.08
Calcium ionophore	14.66	4.67
Calcium ionophore + magnetic field	38.51	9.46
Ethanol/cycloheximide	30.19	18.24
Ethanol/cycloheximide + magnetic field	24.52	5.16

oocytes in cats. Results obtained in this study indicated that the most effective experimental procedure was a simultaneous activation of oocytes by ethanol and magnetic field, while the lowest efficiency was observed with magnetic field alone.

In cattle, depending on the activation method, percentages of oocyte activation varies from 29% to 52% [14]. Similarly in pigs, oocyte activation measured by the presence of pronulcei varies from 22% to 74% [15], while in rabbits its lower (10% to 38%) [16]. While in the present study, the mean activation rates within different treatments was 40%.

Including the magnetic field into oocyte activation increased ethanol impact from 47% to 75% ($P < 0.001$). The same concentration of ethanol (10%) and time of application (3 min) have been used in a different study with pig oocytes, were only 15% of oocytes were activated [17]. Although in that study only activated oocytes in the pronuclei stage were considered. A high rate of oocyte activation (90%) have been observed after exposure cattle oocytes to 7% ethanol for 2 min [18]. It has to be pointed out; however, that oocyte susceptibility to artificial activating agents is species specific. While in cattle the oocyte activation efficiency was so high, in rats, activation with ethanol remains ineffective [19] or embryo development stopped at the two-cell stage [20].

Magnetic field may assist ethanol action by triggering calcium reserves, which leads to an increased concentration of the ionized calcium in the cell. A similar relationship, as for the group of oocytes activated by ethanol alone and by ethanol with magnetic field, was observed for oocytes activated by calcium ionophore alone (19%) and by calcium ionophore with magnetic field (48%). Instead, the magnetic field applied as single activator within the above mentioned parameters, is too weak, as was demonstrated with a low percentage of activation (12%). Also, the application of magnetic field to ethanol and cycloheximide treatment resulted in reducing activation effectiveness from 48% to 30% ($P < 0.01$).

In the group of oocytes activated by magnetic field alone the phenomenon of fragmentation of the activated cells was observed. The cells were divided into irregular fragments and no cell nuclei were found. On average, 4.0% of fragmented cells were observed in all oocytes activated by magnetic field alone. In cases, where magnetic field was an additional agent (to ethanol, ionophore or ethanol with cycloheximide) no fragmented cells were found. Because of lack of publications on the influence of magnetic field upon oocytes activation, the induction values used in our studies resulted from preliminary experiments. Many authors stress high dependence of the results on magnetic field parameters.

The biological model is also an important element of the experiment. Different results with the same parameters can be obtained for cells or for the whole organism. It is therefore probable, that the magnetic field parameters used in our study will not bring the same effect in other species.

Since the best activation treatment was with ethanol and magnetic field, we analyzed the data in detail in Fig. 1. From the total number of oocytes treated; 54% were activated and found at one cell-stage, 22% of treated oocytes cleaved and were distributed in two cell (14%)-, four cell (6%)- and six cell (1%)-stage embryos. The remaining numbers of oocytes were not activated (24%).

Applying magnetic field to oocyte activation is a method not previously reported, so comparing different methods with magnetic field made an important element of this study (Fig. 2). Combined application of magnetic field and ethanol proved to be the best method of oocytes activation in cats.

The highest number of cleaved embryos was obtained in the group of oocytes activated by ethanol and magnetic field (22%). However, this result did not statistically differ from the percentage of cleaved embryos observed after exposed oocytes to ethanol (18%) or ethanol and cycloheximide (18%).

A high percentage of two cell-stage embryos (70%) at 48 h after parthenogenetically activate cat oocytes with a combination of calcium ionophore and 6-DMAP has been reported [17]. Although in that study a high percentage of embryos had cleaved, we cannot compared their results with ours because we did not activated oocytes with that specific protocol. Similar protocol with calcium ionophore and 6-DMAP to activate horse oocytes had resulted in 13–45% of cleaved embryos [21]. In cattle, after application of ethanol and cycloheximide, 31% of two cell-stage embryos were found in cattle after 48 h of culture [22]. Goat oocytes, activated by ethanol and 6-DMAP divided at the level of 57.8% in respect to classic in vitro fertilization (IVF), which gave 42.7% of divided cells [23]. The results of oocytes activation prompted us to investigate whether particular methods used in our study were equally effective in respect to inducing activation and embryonic divisions.

As presented in Table 2 ethanol with magnetic field were most effective in producing both one cell activated oocytes (53%) and cleaved embryos (22%). Although the cleavage rates for oocytes exposed to the ethanol alone (18%) or ethanol with cycloheximide (18%) were

similar to those observed with the presence of ethanol with magnetic field, the combination of ethanol with cycloheximide plus magnetic field resulted in only 5% cleaved embryos. The cleavage rate of activated oocytes and their potential for further embryonic development depends on several factors, like species, source and quality of oocytes, IVM conditions, type and composition of culture media and activating agent. We suppose that in the present study the addition of magnetic field can reduce a developmental ability of oocytes activated by exposition to 10% ethanol for 3 min with prolonged (24 h) exposition to cycloheximide. This phenomenon could be species-dependent and remain to be investigated.

We concluded that a magnetic field can be used as activating agent, and the combination of ethanol and magnetic field is an effective method for domestic cat oocyte activation.

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