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**Original article**

# Use of a variable electrical pulsing sequence in rabbit oocyte activation

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**Abstract** — Variability in oocyte activation sensitivity to electrical stimuli was shown in two types of oocytes (i.e., oocytes with a whole first polar body: w-PB1 and those with a fragmented PB1: f-PB1), of a similar post-ovulatory age. In order to initiate the normal activation display (i.e., extrusion of the second polar body), the w-PB1 oocytes required, on average, 3.6 ± 0.2 pulses and the f-PB1 oocytes 2.9 ± 0.1 pulses ($p = 0.18$). From both experimental series carried out in this work, the average haploid activation rates were 68% and 70% for w-PB1 and f-PB1 oocytes, respectively. Oocyte type did not affect the haploid embryo developmental ability observed at 24 h of culture (8-cell stage: 33-35% in Series 1 and 23–26% in Series 2), nor at 32 h of culture (16-cell stage: 77–93% and morula stage: 34–41%; Series 2). Therefore, in further experiments, the f-PB1 oocytes may also be used as potential forerunners of haploid embryos, almost up to the morula stage.

**rabbit / oocyte / electroactivation / haploid development / overripe**

**Résumé — Utilisation de différentes séquences de pulsations électriques pour l'activation des ovocytes de lapine.** La variabilité de la sensibilité de l'ovocyte à l'activation par stimulations électriques a été analysée pour deux types d'ovocytes : des ovocytes avec un premier globule polaire entier (w-PB1) ou fragmenté (f-PB1) pris au même âge post-ovulatoire. Pour accomplir la première étape d'activation (expulsion du 2$^{e}$ globule polaire), les ovocytes w-PB1 ont requis en moyenne 3,6 ± 0,2 pulses et les f-PB1 2,9 ± 0,1 ($p = 0,18$). Les résultats des deux expériences réalisées montrent que les taux d'activation haploïde ont été en moyenne de 68 et 70 % pour les w-PB1 et les f-PB1 respectivement. Aucune différence n'a été observée selon le type d'ovocyte sur le développement parthénogénétique haploïde in vitro, soit après 24 h de culture (stade de 8 cellules : 33–35 % lors de la 1$^{re}$ expérience et 23–26 % lors de la 2$^{e}$) soit après 32 h (stade de 16 cellules : 77–93 % et stade morula : 34–41 % au cours de la 2$^{e}$ expérience). Ces résultats indiquent que les ovocytes de type f-PB1 peuvent être utilisés comme des précurseurs potentiels d'embryons haploïdes au moins jusqu'au stade morula.

**lapin / ovocyte / électroactivation / développement haploïde / maturité**

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## 1. INTRODUCTION

The genetic nuclear content of the mammalian female gamete may be amplified by parthenogenetic haploid embryo development. In mice, blastomeres from haploid embryos at the 2- to 8-cell stage provide donor nuclei which, when combined with the paternal nuclear counterpart, reconstruct the normal heteroparental ploidy and develop into viable offspring [27, 29]. The study of this phenomenon and its possible future application in rabbits requires the production of haploid parthenogenetic embryos by means of an efficient oocyte activation procedure.

Under appropriate stimuli for normal rabbit oocyte activation (i.e., sperm-oocyte interaction or electroactivation), the extrusion of the second polar body (PB2) is completed within 90 min [18, 21, 23]. During this period, the number of applied pulses and their characteristics will determine the final oocyte activation rate and type [5, 23, 26]. In fact, we have recently reported that 8 pulses or more applied over 90 min overstimulate the oocytes, resulting in retention of PB2 and the production of diploid oocytes [10]. However, a reduction in the number of applied pulses to four supposes a reduction in the over-stimulation, as the improvement in normal oocyte activation rates indicate (i.e., haploid egg production). Since it has been suggested that PB2 retention is more frequent with greater pulsing frequency (number of pulses/sequence duration) [10, 23], lengthening of the sequence duration would also reduce over-stimulation. However, given that during the fertilisation process, intracytoplasmic calcium oscillations have been observed as occurring over an extended period just up to the pronuclear stage (which is estimated to start at 4–5 h post-fertilisation in rabbit zygotes [18, 21]), the total sequence should not be extended beyond this stage.

Standard activating sequences consist of a pre-determined number of electrical stimuli which are applied to all oocytes [5, 6, 10, 22, 23]. However, some factors related to the intrinsic quality of the oocyte itself, such as the strain utilised and the post-ovulatory oocyte age at the time of activation, could lead to different oocyte susceptibility to those electrical pulse stimuli [4, 5, 11, 31]. In polytocous species, such as rabbits, certain time (approximately 2–6 h) is required to complete the ovulation [3, 13, 15, 21] and so, in similarly-aged post-hCG oocytes the existence of intrinsic variability in sensitivity to stimuli would be expected. Moreover, in our experience, two types of oocytes at metaphase II were recovered from the female oviduct, at a similar post-hCG age. These types were established according to the integrity of the first polar body (PB1): oocytes with a whole first polar body (w-PB1) and those with a fragmented PB1 (f-PB1), probably as a morphological sign of over-ripeness [1, 3, 32].

The presumed variability in oocyte activation sensitivity**,** mainly determined by the post-ovulatory age factor, may be expressed by the number of electrical pulses required to induce the second polar body (PB2) extrusion.

Thus, in this work we first tested an electrical activating treatment that would reconcile the intensity and number of stimuli received with the individual oocyte sensitivity, improving both the number of oocytes that initiates the haploid activation display and early in vitro development. Since only the w-PB1 oocytes have been used in previous works [10] in order to increase the rate of haploid embryo production per doe, the activation response on the f-PB1 oocyte type was also studied here.

Further routine application of this established variable electro-activating treatment will allow us to confirm both the global haploid egg and embryo production. Haploid embryo development was conducted only up to 32 h, and the embryos produced (at the 16-cell or morula stage) were used as donors in current nuclear transfer assays.

Culture beyond this limit was not studied in this work since it had been investigated earlier [10]. In this earlier study, the limiting effect of the haploid nuclear condition upon in vitro parthenogenetic rabbit embryo development was shown to emerge at the morula stage.

## 2. MATERIALS AND METHODS

### 2.1. Oocyte recovery and preparation

Mature mixed-breed female rabbits were tested for sexual receptivity and treated with 25 I.U. of hCG (Coriogan, Lab. Ovejero, Spain). Does were not super-ovulated at all. The oocytes were recovered from oviducts at 14 h after endovenous hCG treatment by flushing in Dulbecco's phosphate-buffered saline plus 20% (v/v) homologous doe serum (s-PBS) immediately after euthanasia of the donor females. Then, oocytes were denuded by briefly placing them in $Ca^{2+}$-free DPBS supplemented with 1 $mg \cdot mL^{-1}$ bovine testes hyaluronidase (Cat. No. H4272. Type IV-S, Sigma, Spain) and then gently pipetted through a small-bore pipette. After treatment, oocytes were selected for general healthy appearance. Two types of recovered oocytes were established according to the integrity of the first polar body (PB1): oocytes with a whole first polar body (w-PB1) and those with a fragmented PB1 (f-PB1), as a morphological sign of over-ripeness [1, 2, 32]. This initial distinction was maintained throughout the activating treatment and culture. Both types of oocyte were cultured at 39 °C in Brackett's Defined Medium (DM) [3] in 7% $CO_2$ humidified atmosphere, until activation treatment.

### 2.2. Activation procedure

Oocyte activation was accomplished by electrical stimulation initiated at 15–15.5 h after ovulation induction. Handling, pulse parameters and pulsing medium characteristics have been described previously [10]. Briefly, oocytes were placed between two stainless-steel round wire electrodes approximately 0.5-mm apart and overlaid with 0.18 M mannitol containing 100 μM $CaCl_2$ and 100 μM $MgCl_2$. Square electrical D.C. pulses of 0.6 $kV \cdot cm^{-1}$ for 60 μs each were delivered with an electro-cell porator [7, 14] and monitored with an oscilloscope (TDS 320, Tektronix, Spain).

Eight pulses applied at regular time intervals over longer treatment (4.5 h) have been shown to induce oocytes to activate as haploids, although such "excessive" stimulation seems to impair subsequent development (unpublished results). Thus, we proposed this electroactivating treatment, but with a modification: before each pulse, the extrusion of the second polar body (PB2) was scored in every oocyte. The positive w- (or f-) PB1 + PB2 oocytes received no more pulses and were cultured separately in DM. In this way, at the end of the activating treatment, two groups of oocytes were distinguished: oocytes that extruded the PB2 at every pulse of the applied sequence (named PB1 + PB2); and those that, receiving the complete activation treatment (eight pulses), did not extrude the PB2 (named PB1).

### 2.3. Culture conditions

After the activation procedure, the oocytes were cultured in Ham F-10 medium plus 20% (v/v) homologous-doe serum under 7% $CO_2$ atmosphere at 39 °C for 24 h or 32 h, according to the corresponding experimental Series (see below).

### 2.4. Assessment of activation rate and type

Usually, different activation degrees were established by checking the PB2 extrusion and/or further cleavage. Three types of parthenogenetic eggs were produced [5, 10, 16, 23]. The first type had one pronucleus plus first and second polar bodies and further

cleavage (PB1 + PB2 and divided); this conduces to the haploid condition. The second type had two or more pronuclei plus the first polar body and cleaved (PB1 and divided). The third type had the first and second polar bodies but with no subsequent cleavage (PB1 + PB2 and non-divided).

### 2.5. Experimental design

Two consecutive series were performed.

In Series 1, variability in the oocyte sensitivity to electrical pulses was confirmed by the differences in the number of pulses required to induce the haploid activation display (i.e., extrusion of PB2) in both types of recovered oocytes (w-PB1 and f-PB1). At 24 h of culture, the activation rate and type were assessed, as well as the haploid embryo developmental stage reached. Culture was not extended further because all the haploid embryos at the 8-cell stage were selected for nuclear transfer assays (work in preparation). Seven replicates were performed.

In Series 2, the oocyte activation treatment was carried out over 13 sessions according to the methodology established in Series 1. The haploid embryos were cultured for 32 h in the conditions described above. At the end of the culture period (32 h) the haploid parthenogenetic embryos at either the 16-cell stage or the morula stage were checked and selected as nuclear donors in reconstructing normal heteroparental assays (work in preparation).

### 2.6. Statistical analysis

The statistical significance of the activation rate and type was determined by a Chi-square test. When a single degree of freedom was involved, the Yate's the correction for continuity was carried out.

## 3. RESULTS

After denudation, approximately half of the recovered oocytes had fragmented PB1 (f-PB1: 42%, 207/497; data not shown in tables).

In Series 1, both types of oocytes (w-PB1 and f-PB1) showed individual differences in the number of pulses required to induce the extrusion of the second polar body as an expression of initiation of the haploid activation response. In general, two to five pulses were required to extrude PB2 in both w-PB1 and f-PB1 oocytes; however, some w-PB1 oocytes (7/66: 11%, Tab. I) required more than five stimuli. The mean number of pulses required for PB2 extrusion in the w-PB1 oocyte group was slightly higher than that required in the f-PB1 group (average 3.6 ± 0.2 vs. 2.9 ± 0.1, respectively; $p = 0.18$).

After 24 h of in vitro culture, activation frequency and type were established (Series 1; Tab. II). No differences in the activation rates were observed between the pulsed oocytes with an initial whole PB1 and those with a fragmented PB1

**Table I.** Number of applied pulses to oocytes with a whole first polar body (w-PB1) or with a fragmented one (f-PB1), before PB2 extrusion (Series 1).

| Number of w-PB1 or f-PB1 oocytes with PB2 (number of pulsed) | Number of received pulses before PB2 extrusion | | | | | | | | | PB1 + PB2 [%] |
|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | mean ± s.e. | |
| w-PB1 ($n = 93$) | 0 | 16 | 20 | 15 | 8 | 3 | 3 | 1 | 3.6 ± 0.2 | 66 [71] |
| f-PB1 ($n = 34$) | 0 | 9 | 17 | 3 | 1 | 0 | 0 | 0 | 2.9 ± 0.1 | 30 [88] |

**Table II.** Effect of initial type of oocytes upon activation frequencies and types (Series 1).

| Oocyte type[a] | Number of oocytes | | | | Type of activation (%)[b] | | |
|---|---|---|---|---|---|---|---|
| | Pulsed | Lysed | Non-activated | Activated (%) | PB1 + PB2 & non-divided | PB1 + PB2 & divided | PB1 & divided |
| w-PB1 | 93 | 7 | 5 | 81 (87) | 0 (0) | 66 (81) | 15 (19) |
| f-PB1 | 34 | 2 | 0 | 32 (94) | 0 (0) | 30 (94) | 2 (6) |

[a] w-PB1: Oocytes with a whole first polar body; f-PB1: Oocytes with a fragmented PB1.
[b] PB1 + PB2 & non-divided: Oocytes with the first and second polar bodies and one pronucleus but no subsequent cleavage; PB1 + PB2 & divided: Oocytes with extruded second polar body (one pronucleus) and further cleavage (normal or haploid activation); PB1 & divided: Oocytes with suppressed extrusion of the second polar body (two or more pronuclei) and further cleavage.

(w-PB1: 87% and f-PB1: 94%, respectively; $p > 0.05$), nor in the haploid activation rates (81% vs. 94%, respectively; $p > 0.05$). All PB2-extruded eggs from the w- and f-PB1 oocytes were cleaved after 24 h of culture (Tab. II). Moreover, at this time, no differences in the percentage of embryos at the 8-cell stage were observed between both types of oocytes (w-PB1: 33% vs. f-PB1: 35%; Tab. III).

After testing the application of this electroactivating treatment on the w-PB1 oocytes in Series 1, and even on the f-PB1 oocytes, in Series 2 the recovered oocytes were routinely subjected to this variable pulsing sequence in order to obtain haploid donor nuclei. Seventy percent of pulsed w-PB1 oocytes (138/197) extruded the PB2, requiring on average 2.8 ± 0.1 pulses, which was not significantly different from f-PB1 oocyte results (3.1 ± 0.1 pulses for 127 PB2-extruded oocytes out of 173 pulsed, $p = 0.22$; data not shown in tables). The w- and f-PB1 oocytes activated and supplied haploid eggs at similar rates (83% vs. 94% and 80% vs. 70%, respectively; $p > 0.05$; Tab. IV).

**Table III.** Effect of the type of oocyte upon rates of cleaved haploid-eggs at 24 h after activation (Series 1).

| Oocyte type[a] | No. haploid embryos at the stage of (%) | | | |
|---|---|---|---|---|
| | Total | 2-cell | 4-cell | 8-cell |
| w-PB1 | 60 | 1 (2) | 39 (65) | 20 (33) |
| f -PB1 | 23 | 0 (0) | 15 (65) | 8 (35) |

[a] w-PB1: Oocytes with a whole first polar body; f-PB1: Oocytes with a fragmented PB1.

At 32 h of culture, the w- and f-PB1 + PB2-derived embryos did not show significant differences in the ability to develop to the haploid 16-cell stage: 93% vs. 77% and the haploid morula stage: 41% vs. 34% ($p > 0.05$, Tab. V).

The global efficiency in obtaining haploid embryos (Series 2) at either the 16-cell or morula stage per pulsed oocyte was 45% (165/370) and 20% (73/370) respectively (data not shown in tables).

## 4. DISCUSSION

The matured mammalian oocytes are ovulated at metaphase II and remain arrested at this stage until an activating stimulus (a fertilising spermatozoon or an artificial stimulus) drives the oocyte from this stage with the resumption of meiosis.

The fertilising sperm normally initiates multiple periodic elevation of intracellular free calcium in the oocyte over several hours, depending on the species [8, 11, 20,

**Table IV.** Effect of initial type of oocytes upon activation frequencies and types (Series 2).

| Oocyte type[a] | Number of oocytes | | | | Type of activation (%)[b] | | |
|---|---|---|---|---|---|---|---|
| | Pulsed | Lysed | Non-activated | Activated (%) | PB1 + PB2 & non-divided | PB1 + PB2 & divided | PB1 & divided |
| w-PB1 | 197 | 33 | 0 | 164 (83) | 7 (4) | 131 (80) | 26 (16) |
| f-PB1 | 173 | 10 | 0 | 163 (94) | 13 (8) | 114 (70) | 36 (22) |

[a] w-PB1: Oocytes with a whole first polar body; f-PB1: Oocytes with a fragmented PB1.
[b] See footnote on Table II.

**Table V.** Effect of the type of oocyte upon rates of cleaved haploid-eggs at 24 h and 32 h after activation (Series 2).

| Oocyte type[a] | Cultured haploid eggs[b] | Number of haploid embryos at the stage of (%) | | | | |
|---|---|---|---|---|---|---|
| | | At 24 h of culture | | | At 32 h of culture | |
| | | 2-cell | 4-cell | 8-cell | 16-cell | Advanced morula |
| w-PB1 | 102 | 1 (1) | 74 (72) | 27 (26) | 53 (52) | 42 (41) |
| f-PB1 | 91 | 3 (3) | 67 (74) | 21 (23) | 39 (43) | 31 (34) |

[a] w-PB1: Oocytes with a whole first polar body; f-PB1: Oocytes with a fragmented PB1.
[b] Only PB2-extruded eggs containing a well-defined and swelled single pronucleus were selected and cultured for 32 h.

30]. It is believed that the function of these $Ca^{2+}$ elevations is to destroy both the existing and nascent synthesised cytostatic factors (CSF), which maintain the function and the persistent high level of MPF (maturation/mitosis/meiosis promoting factor) activity [8, 11, 19, 28]. This in turn causes a decline in MPF activity, whose prolonged inactivation allows oocyte meiosis to progress and culminate in the PB2 extrusion [11, 19, 28]. A single artificial stimulus induces a single intracellular calcium elevation which can mimic the first sperm-triggered calcium peak and therefore only destroys the existing CSF [8, 11]. Since CSF is believed to be continuously synthesised in the young but not in ageing oocytes [25], multiple artificial calcium stimulations should be required to prevent re-activation of MPF in young rabbit oocytes, at least during the initial activation steps [4, 6, 10, 23, 24], whereas ageing oocytes can readily be activated by a single stimulation [16].

These dramatic differences in oocyte sensibility to artificial stimulation according to ageing have already been noted by several authors in various species [rabbit: 1, 4, 11, 32; cattle: 5, 26, 31; porcine: 17]. However, some factors more related to the oocyte itself may determine different oocyte susceptibility to those artificial stimuli [4, 5, 11, 31]. In the present work, the existence of this expected intrinsic oocyte activation variability in similarly-aged post-hCG oocytes was demonstrated. This variability may be because a certain time (around 2–6 h) is required to complete ovulation in rabbits [2, 13, 15, 21].

Such individual variability in oocyte sensitivity to artificial stimuli was observed in both types of oocyte recovered from female

oviducts at the same post-hCG age. These types, established according to the integrity of the first polar body (PB1) were those oocytes with a whole first polar body (w-PB1) and those with a fragmented PB1 (f-PB1). The PB1 fragmentation, and even its degeneration, is considered as an expression of in vivo ageing or over-ripeness [1, 2, 25, 32]. With ageing, oocytes increase their sensitivity to electrical stimuli [1, 4, 11, 22, 31]. However, after applying the established variable electrical pulsing treatment to the w-PB1 and f-PB1 oocytes, we did not detect significant differences in the mean number of pulses required to initiate the haploid activation display, nor in the normal activation rates obtained. Thus, two implications for subsequent work arise. Firstly, PB1 fragmentation should not be used to indicate greater oocyte activation sensitivity, at least in rabbits (this work). Secondly, the f-PB1 oocytes will be used, as well as the w-PB1 oocytes, as haploid embryo precursors which will contribute to improving haploid egg (or embryo) production per doe.

In earlier work, under a treatment consisting of 8 pulses applied over 90 min, high global activation and successful haploid (and diploid) embryo developmental rates were obtained, but under such treatment more abnormally activated oocytes with the suppressed PB2 were obtained, due to over-stimulation [10]. In the present work, a lower pulse frequency, derived by lengthening the sequence duration while the number of pulses remained invariable, reduced the level of applied over-stimulation and therefore improved haploid egg production per pulsed oocyte, reaching 76% (96/127) in Series 1 and 66% (245/370) in Series 2.

During fertilisation, sperm-triggered calcium oscillations persist up to the pronuclear stage which is reached at 4–5 h post-fertilisation in rabbit eggs [18, 21]. At this stage, calcium amplitude begins to decline until disappearance at the pronuclear apposition stage [12]. The multiple pulses required to complete oocyte activation display [6] should not be extended beyond 4–4.5 h from the first efficient stimulus, in order to avoid pulsing activated eggs at the pronuclear stage [6]. In fact, in some oocytes that received all eight pulses because no PB2 extrusion had been observed during the sequence, pronuclear structures could be visualised before the sequence ended (270 min), just at the time of the 6th pulse (this work). Thus, from a practical viewpoint, we suggest the application of the variable pulsing sequence proposed here, but restricted to six pulses (over 200 min total treatment). This option does not excessively penalise the haploid egg rate, since only 7 out of the 96 PB2-extruded oocytes (7%) displayed the normal activation response after receiving more than five pulses.

Under this variable sequence treatment and regardless of the initial oocyte type (w-PB1 or f-PB1 oocytes), the haploid egg-derived embryos successfully developed to the 8-cell stage (100% of cleaved eggs) and the 16-cell stage (85% of cultured haploid eggs: 165/193; Series 2). These haploid embryos were obtained from two- to five-pulsed and normally activated oocytes; however, a treatment based on four pulses over 90 min has recently been shown to not be sufficient enough to trigger and sustain subsequent haploid development [10]. Thus, the observed beneficial effects that multiple pulses exert upon parthenogenetic development in the present work may not be due so much to pulse number as to the lower pulsing frequency in this type of sequence [17, 23, this work].

In this work, the final haploid morula rate could be underestimated due to the limited in vitro culture period (32 h at maximum; Series 2). After in vitro culture of in vivo produced zygotes, a 48- to 72-h culture period is required in order to obtain the highest rates of embryos at the compacted morula stage [9]. Moreover, it must be taken into account that in the present work, the cultured embryos have a haploid nuclear condition which severely impairs their

developmental potential. To our knowledge, apart from the present and our earlier report [10], previous results on haploid embryo development in mammal species have only been reported for mice [16, 29]. In this species, the percentage of haploid parthenogenetic embryos reaching the 8- to 16-cell stage was nearly 16%, with a small number (3%) developed as morphologically normal blastocysts, a fact mainly due to the limiting effect exerted upon development by the imprinted maternal information [29].

Removal of the recently PB2-extruded oocytes from the electro-activation sequence avoids applying additional stimuli during the later activation stages which could limit subsequent parthenogenetic development, possibly by repeated handling and culture conditions [16], excessive calcium stimulation [6, 24], or membrane polarisation [5]. Whether or not this holds true could be ascertained by culturing non-extruded oocytes (having received all eight pulses); however, in the present work, this was not carried out, since our main objective was to obtain haploid embryos as donors for nuclear transfer, following Surani et al. [29].

In conclusion, we propose the flexible application of an electrical 8-pulse sequence by means of which both haploid activation and haploid parthenogenetic development, possibly up to the morula stage, are enhanced. This was achieved by adjusting the number of applied pulses to the different degrees of oocyte activation sensitivity present in a pool of similarly-aged post-ovulatory oocytes. Moreover, a common f-PB1 oocyte type has been shown to be able to activate and proceed with the haploid embryo development, as the w-PB1 oocytes do, allowing us to consider these as contributory to the haploid egg (and embryo) production efficiency.

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