$[Mg^{2+}]_o/[Ca^{2+}]_o$ determines Ca^{2+} response at fertilization: tuning of adult phenotype?

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Abstract

Alteration of the postnatal phenotype has sparked great concern about the developmental impact of culture media used at fertilization. However, the mechanisms and compounds involved are yet to be determined. Here, we used the Ca^{2+} responses from mouse eggs fertilized by ICSI as a dynamic and quantitative marker to understand the role of compounds in egg functioning and establish possible correlations with adult phenotypes. We computed 134 Ca^{2+} responses from the first to the last oscillation in media with specific formulations. Analyses demonstrate that eggs generated two times as many Ca^{2+} oscillations in KSOM as in M16 media (18.8 ± 7.0 vs 9.2 ± 2.5). Moreover, the time increment of the delay between two consecutive oscillations, named TlbO, is the most sensitive coefficient characterizing the mechanism that paces Ca^{2+} oscillations once the egg has been fertilized. Neither doubling external free Ca^{2+} nor dispermic fertilization increased significantly the total number of Ca^{2+} oscillations. In contrast, removing Mg^{2+} from the M16 boosted Ca^{2+} oscillations to 54.0 ± 35.2 . Hence, $[Mg^{2+}]_o/[Ca^{2+}]_o$ appears to determine the number, duration and frequency of the Ca^{2+} oscillations. These changes were correlated with long-term effects. The rate of female's growth was impacted with the 'KSOM' females having only half the fat deposit of 'M16' females. Moreover, adult animals issued from M16 had significantly smaller brain weight vs 'KSOM' and 'control' animals. TlbO is a new Ca^{2+} coefficient that gauges the very early functional impact of culture media. It offers the possibility of establishing correlations with postnatal consequences according to IVF medium formulation.

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Introduction

In humans and animals, several reports demonstrate that the culture media used at fertilization affects the developmental potential and may have postnatal consequences (Fernandez-Gonzalez *et al.* 2004, Watkins *et al.* 2007, Donjacour *et al.* 2014, Kleijkers *et al.* 2014, Morbeck *et al.* 2014). Given the considerable use of *in vitro* fertilization (IVF) technologies in human, the elucidation of the developmental influence of the culture media remains a critical challenge.

In mammals, fertilization triggers a series of Ca^{2+} oscillations, the number, frequency and duration of which are highly variable (Saunders *et al.* 2002, Kurokawa & Fissore 2003, Vanden Meerschaut *et al.* 2013) and which have an impact on the developmental processes (Ozil *et al.* 2006, Yu *et al.* 2008).

In the present study, we used the ultra-sensitive nature of Ca^{2+} oscillations triggered by fertilization as a quantitative marker to understand how media formulation alters egg functioning and could correlate with postnatal outcome.

The frequency of the Ca^{2+} oscillations has been extensively used to characterize the Ca^{2+} response at fertilization in mammals. This variable is however not

accurate enough to describe the egg's response because the occurrence of Ca^{2+} oscillations is neither strictly periodic nor chaotic. To assess the evolution of the time interval better, we used a linear regression method to define a new coefficient called TlbO (for time increments between oscillations) that most closely quantifies the time interval increase between two consecutive oscillations.

Considerable progress has been made in understanding how these Ca^{2+} oscillations are generated, maintained and terminated following fertilization (Miyazaki 1995, Kono *et al.* 1996, Politi *et al.* 2006, Swann & Lai 2013, Kashir *et al.* 2014, Dupont & Combettes 2016). It is well established that the Ca^{2+} response triggered by fertilization is determined by two decisive factors: firstly the dose of $PLC\zeta$ brought into play by the incoming sperm that confers calcium-induced Ca^{2+} release (CICR) excitability to the egg (Rice *et al.* 2000, Saunders *et al.* 2007, Sanusi *et al.* 2015) and secondly the transmembrane Ca^{2+} influx that supports the persistence of Ca^{2+} oscillations (Mohri *et al.* 2001, Miao *et al.* 2012, Takahashi *et al.* 2013, Wakai *et al.* 2013).

In this context, to explore the sensitivity of the Ca^{2+} responses to various media formulations, we managed to fertilize a large number of mouse eggs by intra-

cytoplasmic sperm injection (ICSI). We systematically recorded the whole Ca^{2+} response in the presence of M16 or KSOM medium, which have the same chemical compounds but in different proportions (Whittingham 1971, Biggers 1998). We then varied the concentration of a series of compounds such as $[\text{CaCl}_2]_0$, $[\text{MgSO}_4]_0$, $[\text{KH}_2\text{PO}_4]_0$ and $[\text{BSA}]_0$ to identify their impacts on the time interval between oscillations. We conducted analysis to detect if the evolution of the time interval measured by TlBO is correlated with the total number of Ca^{2+} oscillations, which provides a quantitative estimate of the work carried out by the egg (Ozil & Huneau 2001, Ducibella *et al.* 2002, Ozil *et al.* 2005, 2006, Ducibella & Fissore 2008) according to medium formulation.

Finally, we explored whether the Ca^{2+} responses set by M16 and KSOM media correlate with changes in postnatal growth and animal phenotypes.

Materials and methods

Ethics statement

Animal experiments were carried out in strict accordance with the recommendations of the Code for Methods and Welfare Considerations in Behavioral Research (Directive 86/609EC) and were approved by the Committee on the Ethics of Animal Experiments of the author's institution, permit number 12165 INRA.

Gametes production and collection

Female mice (C57BL/6 $\times$ CBA; 7–11 weeks old, Janvier, Le Genest, France) were superovulated by i.p. injection of 8 IU of PMSG (Chronogest Intervet) followed 48 h later by injection of 7.5 IU of hCG (Chorulon Intervet). Metaphase II oocytes were collected from the oviducts 15–16 h after hCG injection, and then placed in 0.1% hyaluronidase (Sigma H-3506) in Hepes buffered M2 medium (Sigma M-7167) in a Petri dish, to remove the cumulus cells. After washing and selection, oocytes were put into M16 medium under mineral oil (Sigma-M8410) and incubated under an atmosphere of 5% CO_2 in air at 37°C. When fertilized eggs were needed, females were caged overnight with F1 males. *In vivo* fertilized eggs were recovered 17 h after hCG injection and eggs that displayed two pronuclei (PNs) were subjected to experimental protocol. Spermatozoa from C57BL/6 $\times$ CBA males (8–12 weeks old, Janvier) were collected from the caudae epididymides and suspended in 2 mL of M16 (Kuretake *et al.* 1996).

Culture media

M16 and KSOM media were made without EDTA, antibiotics or amino acid (Table 1). ICSI media was made up of 134.0 mM KCl, 2.6 mM NaCl, BSA (4 g/L) and 0.6 μM cytochalasin D (Sigma C-8273). All inorganic and organic components were purchased from Sigma Chemical Company. When needed, NaCl was substituted for MgSO_4 or KH_2PO_4 in the M16 formulation (Table 1).

Intra-cytoplasmic sperm injection

Sperm heads were separated from their tails by applying ultrasonic pulse with the digital sonicator (Model DS 250 Ultrasonic Corporation, USA) (Kuretake *et al.* 1996). Before ICSI, MII oocytes were maintained for 15 min in M16 with cytochalasin D to make the plasma membrane more malleable during ICSI intervention. Oocytes and sperm heads were placed together into ICSI media deprived of Ca^{2+} ions under the stage of a NIKON TMD at room temperature. The micropipette containing one or two sperm head(s) were advanced through the oocyte plasma membrane and a puncture was made by using a piezo-pulse (Piezodrill Burleigh, USA) (Kimura & Yanagimachi 1995). After release of the sperm head(s) into the cytosol, just as the pipette was being pulled back out of the zona, the part of the plasma membrane, made fluent by cytochalasin, was pulled into the pipette and squeezed against the zona with the tip of the pipette to mechanically seal the plasma membrane in the absence of external Ca^{2+} (Fig. 1). Eggs were replaced in culture medium at 37°C in less than 3 min following ICSI. When Ca^{2+} recording was carried out, sperm injections were given in ICSI medium supplemented with 500 μM of Fura 2 Dextran (F3029 Molecular Probes Fisher Technology Lot No. 983882 with 1.6 dye/mol of Dextran).

Level of [free Ca^{2+}] in culture media

The free Ca^{2+} concentrations were measured with a perfect ION Ca^{2+} ion-selective electrode connected to the Ph/Ionomètre S500 device from Mettler Toledo SAS France. Results are expressed as mean $\pm$ s.d. of at least 9 measurements of different aliquots of the same medium formulation.

Ca^{2+} signal recording

Two eggs in turn, held opposite one another in the microfluidic chamber by suction pipettes (Ozil & Swann 1995), were subjected to ICSI and then immediately washed by a super flux of M16 medium (10 $\mu\text{L/s}$) to

Table 1 M16 and KSOM culture media formulation in mM.

	Compounds									
	NaCl	CaCl_2	NaHCO_3	KCl	MgSO_4	KH_2PO_4	NaLac	NaPyr	Glucose	BSA (g/L)
M16	95	1.7	25	4.8	1.2	1.2	23	0.33	5.6	4
KSOM	95	1.7	25	2.5	0.2	0.35	10	0.2	0.2	1
Difference	0	0	0	2.3	1	0.85	13	0.13	5.4	3

M16 and KSOM have close formulations, but in M16, some components have higher concentration than that in KSOM. These might be responsible for the differences in Ca^{2+} responses between the two media. In bold, the differences in the concentration of media tested.

Part 1: Analysis of Ca²⁺ responses following ICSI and culture in various media

Part 2: Developmental responses following ICSI and culture in M16 or KSOM

Figure 1 Schematic representation of the experimental design. (Part 1) Oocytes were fertilized by ICSI. Ca²⁺ responses were recorded inside a microfluidic device until the cessation of Ca²⁺ oscillations. Eggs were checked for the presence of 2 or 3 PN and then discarded. Ca²⁺ records were automatically analyzed. The photo shows details of the ICSI procedure. After sperm deposition, the plasma membrane, made fluent by cytochalasin, was squeezed against the zona in order to close the plasma membrane onto itself by mechanical pressure and thus avoid any ions influx into the cytosol during the membrane healing period. After ICSI, eggs are immediately placed in the culture medium at 37°C. (Part 2) Oocytes were fertilized by ICSI and cultured in the incubator in the presence of M16 or KSOM for 4 h. Eggs with 2 PN were cultured before being transferred into female recipients at the 1-cell at D₀ or 2-cell stage at D₁ according to the availability of recipients. Control eggs were fertilized *in vivo* and collected at the time of PN formation i.e. 3–4 h after fertilization. They were cultured in KSOM and transferred into female recipients as described for experimental eggs. The rate of animal growth was recorded up to the 8th week of age.

remove the ICSI medium. The chamber was positioned in the viewing field of a Nikon TE2000 inverted microscope fitted with a Fluor 40× oil immersion objective (NIKON). The optical field was illuminated by a 75-W Xenon arc lamp and wavelengths were selected at 340 ± 5 nm and 380 ± 5 nm by using a Cairn OptoScan Monochromator. Light emitted from the Fura 2 Dextran loaded eggs was transmitted to an EMCCD Photometric camera (EVOLVE Roper Scientific, USA). Intracellular $[Ca^{2+}]$ levels were recorded in terms of a ratio of fluorescence (F340/F380) at 0.5 Hz from the minute following their deposition in the microfluidic chamber until the cessation of Ca^{2+} oscillations. The intensity of UV light was highly attenuated by neutral dense (ND) filters (×32) to avoid cell heating and perturbing the permeation of Ca^{2+} ions through the plasma membrane that manifests itself by a progressive acceleration of the Ca^{2+} oscillation during the time course of recording (personal observation). The permanent flow of culture media in the microfluidic chamber, 1.4 μ L/s at $37^\circ\text{C} \pm 0.1$, ensured constant refreshment of O_2 , CO_2 , pH, salt composition and carbohydrates under the zona pellucida. This makes it possible to keep eggs spiking throughout the period of egg activation with the $[Ca^{2+}]_i$ resting line remaining horizontal and flat between two successive oscillations. This is a mandatory condition for automatic detection of the first rising data point at the root of every $[Ca^{2+}]_i$ rise before Ca^{2+} release takes place. Eggs were sometimes observed for PN formation before cessation of Ca^{2+} oscillations and always after cessation. The number of independent replicates is half the total number of Ca^{2+} records, i.e. 67.

Automated Ca^{2+} signal analysis: coefficients and parameters definition

An exploratory data analysis of the Ca^{2+} response (EDA, NIST/SEMATECH e-Handbook of Statistical Methods <http://www.itl.nist.gov/div898/handbook/eda/eda.htm>) was conducted to find out what Ca^{2+} parameter was the most sensitive to changes in media formulation.

Every Ca^{2+} record was automatically analyzed using SigmaPlot 13.0 homemade macro scripts. Two parameters and one coefficient were used to characterize the whole Ca^{2+} response:

1. The total number of Ca^{2+} oscillations was counted by automatic peaks detection (Fig. 2A).
2. The duration of the Ca^{2+} response was computed by number of the data points between the initial rise of the first oscillation and the peak of the last oscillation divided by the acquisition frequency (0.5 Hz; Fig. 2A).
3. The linear regression coefficient 'a' between the row order of Ca^{2+} oscillations and the time interval between n and $n + 1$ oscillation as detailed in Fig. 2B ($y = ax + b$, with y = time interval between oscillations; x = order). The regression coefficient, named TlbO, most closely estimates the step of time that is added after every Ca^{2+} oscillation before the next oscillation pops up (Fig. 2B). The details of the algorithm will be published elsewhere.

Figure 2 Automated analysis of the Ca^{2+} response. (A) Example of the decomposition of a Ca^{2+} response following ICSI in a series of critical points. Every Ca^{2+} peak represents a single oscillation and is automatically labeled by a red star and counted (here 9 oscillations). The time interval between two consecutive oscillations corresponds to the number of data points counted between two consecutive minima identified by the macro script (red downward triangle) divided by the frequency of acquisition (0.5 Hz) (e.g. see the 6th time period). The total duration of the Ca^{2+} response is defined as the time elapsed between the lowest data point at the root of the first Ca^{2+} oscillation (first red downward triangle) and the last maximum (last red star). (B) Linear regression line ($y = ax + b$) between the rank order of the Ca^{2+} oscillations (x) and their respective time interval (y) shown by a red circle symbol. The regression coefficient 'a' is the coefficient that best reflects the increments of the time interval between two consecutive oscillations. It is called TlbO. The y-intercept b corresponds to the initial time interval. Given its linearity, TlbO and y-intercept can be estimated on a short time window encompassing a few number of oscillations. It can be used to predict the total number of oscillations ($NofO_p$), the total duration (TD_p) and the predicted frequency (F_p). For example, with $TlbO = 187.533$ s; y-intercept = 262.778; $NofO_p = f(TlbO) = 9.029$ (computed with the parameters of the empirical function Fig. 7).

$$\text{Then, } TD_p = \sum_{x=1}^{f(TlbO)} TlbO * x + b = 10,803 \text{ s} = 3 \text{ h } 0 \text{ min}$$

with $F_p = (NofO_p / TD_p) * 3600 = 3.0$ oscillations/h. The accuracy of the prediction relies on the R value of the linear regression and the quality of the Ca^{2+} recording (frequency of data point acquisition).

Culture media and embryo treatment

Monospermic eggs fertilized by ICSI were immediately put into 40- μ L drops of either M16 or KSOM and cultured for 4 h (Fig. 1). After 4 h, eggs with 2 PNs were cultured further in M16 until their transfer into pseudo-pregnant recipients either the day of the experiment at (D_0) or the next day at the 2-cell stage (D_1) depending on the number of recipients available at D_0 and D_1 . *In vivo*-fertilized eggs used as control were cultured in KSOM until their transfer to a recipient at D_0 or D_1 .

Embryo transfer

Female mice (SWISS, CD1, 8–12 weeks old, Janvier) were used as recipients. They were mated to vasectomized males and the morning of the experimental day or the next day, the recipients were checked for the presence of a vaginal plug. Eggs showing 2 PNs were transferred in groups of 8 to the left oviduct of female recipients either at the one-cell stage (D₀) or at the two-cell stage (D₁).

Postnatal phenotype

Offspring were weighed weekly beginning the first week after the day of birth. The pups were weaned at the 4th week and males and females were separated and placed in groups at a maximum of 5 in independent cages. At the 8th week of age, animals were killed by cervical dislocation. The livers, kidneys, hearts, lungs, brains and fat were dissected out and weighed.

Statistical analysis

The data were analyzed using SigmaPlot automation analysis procedures (SigmaPlot 13.0 software). The sizes of the groups in Tables 2 and 3 are fairly small (less than 30). Approximate normality of observations was assumed for the purposes of this exploratory analysis. For data Table 2, we applied unpaired *t*-test to test the differences between means without any correction for multiple comparisons in order to let the data speak for themselves and reveal their structure. For Table 3, we applied chi-square (χ^2) test for analysis of contingency tables and Z-test analysis to compare the proportion of males vs females as indicated in the footnote. To detect whether two factors such as the culture media (three levels: M16, KSOM and control) and the day of transfer (two levels: D₀ and D₁) affect the animal growth until the 8th week of age, we used two-way

ANOVA with all pairwise comparisons with Bonferroni *t*-test correction for every week and female and male animals separately (Table 4). The difference in mean of organ weights and organ:bodyweight ratios according to the medium formulations were analyzed by one-way ANOVA (three levels: M16, KSOM and control) with pairwise multiple comparison procedures and Bonferroni *t*-test correction for female and male animals separately (Table 5). Since the determinism of the long-term impact is not known, the results and the signification of the statistical tests in Tables 4 and 5 should be regarded as a trend that merits further researches peering into the programming role of the fertilization process.

Results

Impact of the culture media formulation on the Ca²⁺ response

The Ca²⁺ responses in M16 and KSOM culture media were different

Two typical Ca²⁺ responses following monospermic fertilization by ICSI in M16 and KSOM are shown on Fig. 3A and B. The first oscillation always showed a series of very rapid dampened sinusoidal oscillations after the peak (Fig. 3).

The mean number of Ca²⁺ oscillations was twice as high in KSOM as in M16, i.e. 18.8 ± 7.0 vs 9.2 ± 2.5 respectively ($P < 0.001$; Table 2). The mean durations of the Ca²⁺ response were not significantly different (3 h 35 ± 36 min vs 3 h 19 ± 36 min respectively, Fig. 4A) but the mean frequencies were significantly different (5.1 ± 1.5 vs 2.7 ± 0.4 ; $P < 0.001$, Table 2).

Regarding the evolution of the time interval between oscillations, Figs. 3A and B show that the time intervals

Table 2 Calcium response parameters following fertilization by ICSI (monospermic or dispermic) in presence of M16, KSOM and various declinations of these two culture media formulations.

Culture media [†]	[Ca ²⁺] _{free} (mM)	<i>n</i> of eggs	Mean <i>n</i> of oscillations	Mean duration	Mean frequency (spike/h)	Mean T1bO
M16 (1)	$1.16 \pm 0.10^{a,c}$	17	$9.2 \pm 2.5^{d,i,w}$	3 h 19 ± 36 min ^j	$2.7 \pm 0.4^{e,h,k}$	3 min 18 ± 2 min 38 ^l
KSOM (1)	$1.39 \pm 0.11^{a,b}$	18	$18.8 \pm 7.0^{d,p,m,u}$	3 h 35 ± 36 min	$5.1 \pm 1.5^{e,g,n,q}$	46 s ± 32 s ^{f,o,r,v}
M16 2 × [Ca ²⁺] ₀ (1)	$2.44 \pm 0.23^{b,c}$	10	11.3 ± 4.2^u	3 h 25 ± 54 min	3.6 ± 2^g	2 min 47 ± 2 min 11 ^v
M16 (2)	1.16 ± 0.10	9	10.3 ± 3.0	2 h 49 ± 33 min	3.6 ± 0.5^h	2 min 18 ± 1 min 24
KSOM (2)	1.39 ± 0.11	14	20.3 ± 7.4	3 h 32 ± 46 min	5.6 ± 1.6	56 s ± 47 s
M16-MgSO ₄ (1)	1.12 ± 0.117	10	54.0 ± 35.2^i	4 h 14 ± 31 min ^j	12.3 ± 7^k	12 s ± 9 s ^l
M16-KH ₂ PO ₄ (1)	1.13 ± 0.113	9	9.1 ± 2.1	3 h 0 ± 30 min	3.0 ± 0.8	3 min 43 ± 2 min 39
KSOM _{X-M16} (1)	1.27 ± 0.159	14	10.6 ± 3.8^m	3 h 8 ± 46 min	3.4 ± 1.0^n	3 min 20 ± 2 min 14 ^o
M16 _{X-KSOM} (1)	1.15 ± 0.142	14	$12.3 \pm 3.5^{p,w}$	3 h 50 ± 35 min	3.2 ± 0.7^q	2 min 11 ± 1 min 10 ^r
M16 _{0-0.4} (1)	1.14 ± 0.127	10	57.8 ± 22.2^s	3 h 59 ± 45 min	15.2 ± 7.7	8.2 s ± 3.8 s ^t
M16 _{0-0.1} (1)	1.25 ± 0.132	9	83.2 ± 28.2^s	4 h 16 ± 42 min	19.3 ± 5.7	4 s ± 1.9 s ^t

[†](1): monospermic, (2): dispermic; M16_{X-KSOM} corresponds to a M16 formulation with the [MgSO₄] and [KH₂PO₄] from the KSOM. KSOM_{X-M16} corresponds to a KSOM formulation made with the [MgSO₄] and [KH₂PO₄] from the M16. M16_{0-0.4} corresponds to a M16 formulation without ([MgSO₄] and [KH₂PO₄]) and 4 g/L BSA. M16_{0-0.1} corresponds to a M16 formulation without ([MgSO₄] and [KH₂PO₄]) and 1 g/L BSA. The same letter is used to denote statistical difference. ^a(*** $P < 0.001$); ^b(*** $P < 0.001$); ^c(*** $P < 0.001$); ^d(*** $P < 0.001$); ^e(*** $P < 0.001$); ^f(*** $P < 0.001$); ^g(** $P = 0.031$); ^h(*** $P < 0.001$); ⁱ(*** $P < 0.001$); ^j(*** $P < 0.001$); ^k(*** $P < 0.001$); ^l(*** $P < 0.001$); ^m(*** $P < 0.001$); ⁿ(*** $P < 0.001$); ^o(*** $P < 0.001$); ^p(** $P = 0.004$); ^q(*** $P < 0.001$); ^r(*** $P < 0.001$); ^s(** $P = 0.042$); ^t(** $P = 0.009$); ^u(** $P = 0.005$); ^v(*** $P < 0.001$); ^w(** $P < 0.008$). Diagrams Fig 4 and 6 reveal the enslavement of the parameters upon themselves.

Figure 3 Typical mono and dispermic Ca^{2+} responses in M16 and KSOM. Time zero corresponds to the start of Ca^{2+} recording in the microfluidic chamber occurring in less than 3 min following ICSI. The oscillations usually started in less than 1 min after the egg was held in the microfluidic chamber by pipette. The first oscillation is shown at higher temporal magnification on the left side of every record. A series of dampened sinusoidal super-oscillations can always be seen on the top of the first oscillation after an initial shoulder. We used the pattern of this first oscillation as a quality check of ICSI (see text). (A) Monospermic egg response in M16; (B) in KSOM; (C) dispermic egg response in M16; (D) dispermic egg response in KSOM. After a few hours, oscillations stop abruptly, sometimes with a slight increase of $[\text{Ca}^{2+}]$, that appears insufficient to trigger the $n + 1$ oscillation. This is called abortion of the last oscillation on the record. The number of oscillations, TlbO parameter with R and the total duration are shown for every single record.

between two consecutive oscillations were regularly incremented by a rather constant value. The mean value of TlbO coefficient (described above) most closely estimates this constant value, which corresponds to the mean step of time that regularly increments the

time interval between two successive oscillations (TlbO value is shown for every record Fig. 3). The mean values of the TlbO coefficient were significantly different between the Ca^{2+} responses in KSOM and in M16 ($46 \text{ s} \pm 32 \text{ s}$ vs $3 \text{ min } 18 \pm 2 \text{ min } 38$; $P < 0.001$;

Figure 4 Functional linkage between the mono and dispermic Ca^{2+} response parameters in M16 and KSOM (exploratory data analysis of the Ca^{2+} responses): (A) KSOM vs M16 responses. (B) M16 $2 \times [\text{Ca}^{2+}]$ vs M16 and KSOM. (C) Dispermic and monospermic responses in M16. (D) Dispermic and monospermic responses in KSOM. The axes of the Radar Plot are in arbitrary units for each of the four parameters. The color lines represent the average values while the color areas represent the standard deviations. Stars represent the significant differences; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; ns $P > 0.05$. The key figures are detailed in Table 2. Note that the duration is the less variable parameter.

Figure 5 Six typical Ca²⁺ signal responses following monospermic ICSI in various media formulations. (A) Upon replacement of M16 by M16-MgSO₄, oscillations frequency starts rising with a concomitant decrease in Ca²⁺ peak amplitude, before slowing progressively with a coincident increase in Ca²⁺ peak amplitude and finally ending with a drop in Ca²⁺ peak amplitude. (B) Ca²⁺ response in M16-KH₂PO₄. The response was not different from the response in standard M16 (Table 2). (C) Ca²⁺ response in M16-X-KSOM. The number of Ca²⁺ oscillations was slightly increased (Table 2). (D) Ca²⁺ response in KSOM-X-M16, the number of Ca²⁺ oscillations was significantly reduced (Table 2). (E) Ca²⁺ response in M16_{0-0.4}. (F) Ca²⁺ response in M16_{0-0.1}. Note that in the absence of Mg²⁺ responses, (A, E and F) show higher number of oscillations and similar typical M-shape formats.

Table 2). So, in average, the mean time interval between two consecutive oscillations was increased by a time step that is 4 times less in KSOM than in M16. The

relative functional dependence of the parameters upon themselves according to the culture media is shown on the diagram (Fig. 4A).

Figure 6 Functional linkage between the Ca²⁺ response parameters in various media formulations (exploratory data analysis of the Ca²⁺ responses): (A) M16 vs M16 without Mg²⁺. (B) M16 vs M16 without KH₂PO₄. (C) M16-X-KSOM vs M16 and KSOM. (D) KSOM-X-M16 vs KSOM and M16. Stars represent the significant differences; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; ns $P > 0.05$. The key figures are detailed in Table 2.

Doubling external $[Ca^{2+}]_o$ did not significantly change the Ca^{2+} response in M16

Doubling extracellular $[Ca^{2+}]_o$ to 3.4 mM in M16 (Table 1) essentially doubled the free Ca^{2+} concentration (2.44 ± 0.23 mM vs 1.16 ± 0.10 mM; $P < 0.001$, Table 2). However, the mean number of Ca^{2+} oscillations was not significantly increased (11.3 ± 4.2 vs 9.2 ± 2.5 , Fig. 4B), nor was the mean total duration of the responses (3 h 25 ± 54 min vs 3 h 19 ± 36 min, Fig. 4B) or the mean frequency that increased to 3.6 ± 2 from 2.7 ± 0.4 but still remaining significantly below the 5.1 ± 1.5 mean frequency in KSOM ($P = 0.031$; Fig. 4A and B).

Regarding the evolution of the time interval between oscillations, the means of TlBO were not significantly different (2 min 47 ± 2 min 11 vs 3 min 18 ± 2 min 38, Fig. 4B). The higher free Ca^{2+} concentration in KSOM was therefore not responsible for the difference in mean number of Ca^{2+} oscillations between KSOM and M16.

Dispermic fertilization had limited effect on the Ca^{2+} response in M16 or KSOM

With dispermic eggs, the mean number of Ca^{2+} oscillations and the mean duration of the Ca^{2+} responses (Fig. 3C and D) were not statistically different from their respective monospermic counterparts in M16 or KSOM (Fig. 4C, D and Table 2). However, in M16, dispermic fertilization increased significantly the mean frequency (3.6 ± 0.5 vs 2.7 ± 0.4 ; Fig. 4C), which is associated with a slight reduction of the mean duration (2 h 49 ± 33 min vs 3 h 19 ± 36 min; Fig. 4C). In both cases, the means of TlBO were not significantly different from their respective monospermic counterparts. Therefore, M16 and KSOM media both appear capable of cushioning the effect of dispermic ICSI but with higher intensity in M16 than in KSOM (Fig. 4C and D).

Effects of deprivation of Mg^{2+} or $H_2PO_4^-$ on the Ca^{2+} response are different

In M16 deprived of $MgSO_4$ (M16- $MgSO_4$), the mean number of Ca^{2+} oscillations increased dramatically from 9.2 ± 2.5 to 54.0 ± 35.2 ($P < 0.001$; Figs 5A and 6A) along with an increase in mean duration from 3 h 19 ± 36 min to 4 h 14 min ± 31 min ($P < 0.001$; Fig. 6A) and a significant increase in mean frequency from 2.7 ± 0.4 to 12.3 ± 7.1 ($P < 0.001$; Fig. 6A). The TlBO was decreased from 3 min 18 ± 2 min 38 to 12 s ± 9 s ($P < 0.001$, Fig. 6A). Therefore, the deprivation of Mg^{2+} in M16 increased by almost 5.7 times the number of Ca^{2+} oscillations, 1.27 times the total duration and decreased by 16 times the mean TlBO coefficient (Fig. 6A and Table 2).

Moreover, we can see that the pattern of the time courses of the peak amplitude of Ca^{2+} oscillations

(Fig. 5A) took an M-shape with three phases: (i) the peak amplitude decreased while the frequency was rising; (ii) the peak amplitude re-increased while the frequency slowed down; (iii) the peak amplitude decreased again while the frequency continued to slow down before stopping. This pattern appeared typical of media deprived of Mg^{2+} (Fig. 5A, E and F), and in this case, PN's were observed before oscillations stopped (data not shown).

In contrast, deprivation of $[KH_2PO_4]_o$ in M16 (M16- KH_2PO_4) made no difference (Fig. 6B).

Reciprocal changes of $[MgSO_4]_o$ and $[KH_2PO_4]_o$ between M16 and KSOM had different effects

To reveal any complex interactions between external Mg^{2+} and $H_2PO_4^-$ with some others components, we switched their concentrations between M16 and KSOM (Table 1). A new M16 was made with the $[MgSO_4]$ and $[KH_2PO_4]$ from the KSOM that is called M16 $_{X-KSOM}$. Conjointly a new KSOM was made with the $[MgSO_4]$ and $[KH_2PO_4]$ from the M16, that is called KSOM $_{X-M16}$.

In KSOM $_{X-M16}$, the means of the number of Ca^{2+} oscillations, frequency, duration and TlBO were not significantly different from those obtained in M16 (Fig. 6C and Table 2) but were significantly different from those obtained in KSOM (the mean number of Ca^{2+} oscillations decreased from 18.8 ± 7.0 to 10.6 ± 3.8 ; ($P < 0.001$); the mean frequency decreased from 5.1 ± 1.5 to 3.4 ± 1.0 ($P < 0.001$) and the mean of TlBO increased from 46 s ± 32 s to 3 min 20 ± 2 min 14 ($P < 0.001$); Fig. 6C). Hence, the combined increase of $MgSO_4$ and KH_2PO_4 in KSOM to their M16 levels was sufficient to restore almost the typical M16 Ca^{2+} response (Fig. 6C).

In contrast with M16 $_{X-KSOM}$, the mean number of Ca^{2+} oscillations remained significantly lower than that in KSOM (12.3 ± 3.5 vs 18.8 ± 7.0) ($P = 0.004$, Fig. 6D) and significantly higher than that in M16 (12.3 ± 3.5 vs 9.2 ± 2.5) ($P = 0.008$, Fig. 6D). The mean duration was not significantly different (Fig. 6D), which made the frequency significantly lower than that in KSOM (3.2 ± 0.7 vs 5.1 ± 1.5 ; $P < 0.001$) but not significantly different from the frequency obtained in M16 (3.2 ± 0.7 vs 2.7 ± 0.4 ; $P = 0.054$). The mean TlBO remained significantly higher than the mean TlBO in KSOM (2 min 11 ± 1 min 10 vs 46 s ± 32 s ($P < 0.001$) but not significantly different from the TlBO obtained in M16 (2 min 11 ± 1 min 10 vs 3 min 18 s ± 2 min 38 s, Fig. 6D). Hence, the combined decrease of $MgSO_4$ and KH_2PO_4 in M16 to their KSOM levels was insufficient to restore the typical KSOM Ca^{2+} response (Fig. 6D).

Thus, the reciprocal changes in $[MgSO_4]$ and $[KH_2PO_4]$ between M16 and KSOM had different effects and suggest that other components might impact the Ca^{2+} response.

Change of [BSA]₀ impacted the Ca²⁺ response

To test the possible impact of BSA, the concentration of which is 4 times higher in M16 than that in KSOM (Table 1), we firstly removed both MgSO₄ and KH₂PO₄ from the M16 in order to exacerbate the Ca²⁺ response and then we decreased the [BSA]₀ from 4 g/L to 1 g/L. We shall henceforth refer to such formulations as M16_{-0-0.4} and M16_{-0-0.1} respectively throughout the text.

The mean number of Ca²⁺ oscillations obtained with M16_{-0-0.4} (see record Fig. 5E) was not statistically different from those obtained with M16-MgSO₄ (57.8 ± 22.2 vs 54.0 ± 35; Table 2). However, with M16_{-0-0.1} (see record Fig. 5F), the number of Ca²⁺ oscillations increased significantly from 57.8 ± 22.2 to 83.2 ± 28.2 ($P = 0.042$, Table 2); the duration and the frequency were not significantly different but the TlbO decreased significantly from 8.2 s ± 3.8 s to 4 s ± 1.9 s ($P = 0.009$; Table 2). These results suggest that [BSA]₀ has a slight but significant effect on the Ca²⁺ response when the Ca²⁺ response is exacerbated.

The TlbOs were always highly correlated with the number of Ca²⁺ oscillations whatever the interindividual variabilities of the Ca²⁺ responses

To better envision whether the TlbO coefficient correlates with the number of Ca²⁺ oscillations and can describe egg functioning whatever the culture media, we plotted a fitting line through all data ($n = 134$) with

TlbO on the X-axis and the numbers of Ca²⁺ oscillations on the Y-axis (Fig. 7). The plot shows that the numbers of Ca²⁺ oscillations was a decreasing function of the TlbO with a high correlation coefficient ($R = 95.3\%$). It showed a constant relationship between TlbO and the number of Ca²⁺ oscillations for every egg whatever their interindividual Ca²⁺ response variabilities (Fig. 7).

Developmental responses following ICSI in the presence of M16 and KSOM

Rate of egg activation and cleavage following ICSI

Among 777 oocytes subjected to monospermic ICSI and cultured *in vitro* in M16 or KSOM media, 653 survived (84%). No eggs swelled or fragmented when replaced immediately after ICSI in M16 or KSOM at 37°C. All surviving eggs formed PNs by 4 h following ICSI, but 53 of them (8.1%) displayed a single PN. The cleavage rate from the 'M16' and 'KSOM' groups to the 2-cell stage was respectively equal to 98.2% (160/163) and 97.0% (96/99) (Table 3). The cleavage rate of *in vivo* fertilized eggs cultured in KSOM was 100% (24/24; Table 3).

Rate of survival to term

Among ICSI eggs cultured in M16 ('M16' eggs), 216 eggs (2-PN or 2-cell) were transferred into 27 recipients. Thirteen of them (48.2%) gave birth to 42 live pups (40.4%) but 2 died within the first week.

Figure 7 The TlbO coefficient is always correlated with the number of Ca²⁺ oscillations whatever the interindividual variability among the Ca²⁺ responses. All data were plotted on a Cartesian graph with TlbO coefficient on the X-axis and the total number of Ca²⁺ oscillations on the Y-axis. Every color-coded dot point is the cross point between the TlbO and the number of oscillations for every single egg ($n = 134$ eggs). For every experimental group ($n = 11$), the range of TlbO coefficients is represented in the bottom part of the graph by a color-coded line with the mean value as a dot point and the standard error of the mean by upward triangles. On the right side of these lines at the same level, are the name of the group and the average TlbO values. We can see that the projection of the TlbO coefficient along the X-axis for any egg inside any group, on the regression line ($y = (a + b \times x)/(1 + c \times x + d \times x^2)$ with $a = 191.455$, $b = 5.834$, $c = 0.494$, $d = 0.001$) predicts with accuracy the number of Ca²⁺ oscillations that are displayed on the first column on the left to the Y-axis. The second column represents the experimental values ordered by increasing value from the bottom. We can see that the predictive values are very close to the experimental data. This graph makes it possible to see that the TlbO coefficient has predictive power whatever the interindividual variabilities of the egg functioning. In every culture medium formulation, the Ca²⁺ response appears determined by a common functional logic.

Table 3 Survival rate to term for 'M16', 'KSOM' and 'control' groups.

	Eggs transferred	Cleavage (%)	No. recipients	No. pregnant (%)	Live pups from transferred (%)	Live pups from pregnant (%)	Gestational length $\pm$ s.d.	Litter size $\pm$ s.d.	♂/♀ ratio	No dead (%)
'M16' D ₀ (2PN)	56	–	7	4 (57.1)	14 (25.0)	14 (43.7)	19.5 $\pm$ 0.5	3.5 $\pm$ 1.6	0.5	1
'M16' D ₁ (2C)	160	–	20	9 (45.0)	28 (17.5)	28 (38.9)	20.2 $\pm$ 0.4	3.1 $\pm$ 0.7	1.17	1
'M16'total'	216	(98.2)	27	13 (48.2)	42 (19.4)^a	42 (40.4)^c	20 $\pm$ 0.5	3.2 $\pm$ 1.1	0.90	2 (4.7)
'KSOM' D ₀ (2PN)	104	–	13	7 (53.8)	24 (23.1)	24 (42.9)	19.9 $\pm$ 0.4	3.4 $\pm$ 2.3	1	1
'KSOM' D ₁ (2C)	96	–	12	8 (66.7)	17 (17.7)	17 (26.6)	20.5 $\pm$ 0.5	2.1 $\pm$ 1.3	1.5	1
'KSOM'total'	200	(97.0)	25	15 (60.0)	41 (20.5)^b	41 (34.2)^d	20 $\pm$ 0.5	2.7 $\pm$ 2.0	1.17	2 (4.8)
Control D ₀ (2PN)	24	–	3	3 (100)	18 (75)	18 (75)	20 $\pm$ 0	6.0 $\pm$ 1.6	0.63	0
Control D ₁ (2C)	24	–	3	2 (66.6)	8 (33.3)	8 (50)	20 $\pm$ 0	4 $\pm$ 1	1.67	0
CONTROL	48	(100)	6	5 (83.3)	26 (54.2)^{a,b}	26 (65.0)^{c,d}	20 $\pm$ 0	5.2 $\pm$ 1.9	0.86	0
Total	464		58	33 (56.9)	109 (23.5)	109 (43.9)	20 $\pm$ 0.5	3.3 $\pm$ 1.8	1.02	4 (3.7)

The same letter is used to denote statistical difference. ^a(*** P < 0.001); ^b(*** P < 0.001); ^c(P = 0.014); ^d(*** P < 0.001) by χ^2 test. The proportion of litter size were not significantly different between groups, is presented in bold, with Z-test and Yates correction applied for calculations.

Among ICSI eggs cultured in KSOM ('KSOM' eggs), 200 eggs (2-PN or 2-cell) were transferred into 25 recipients. Fifteen of them (60%) gave birth to 41 live pups (34.2%) but 2 died within the first week (Table 3).

Regarding *in vivo*-fertilized egg ('control' eggs), 48 eggs (2-PN or 2-cell) were transferred into 6 recipients. Five of them (83.3%) gave birth to 26 live pups (65.0%) (Table 3).

The rates of live pups from the number of eggs transferred were not significantly different between 'M16' and 'KSOM' but both of them were significantly lower than the 'control' (19.4% and 20.5% vs 54.2%; P < 0.001; χ^2 test, Table 3), as well as the rate against the number of eggs transferred into the pregnant recipients (40.4% and 34.2% vs 65%; P < 0.001; χ^2 test, Table 3). The mean litter sizes at birth between experimental 'M16', 'KSOM' and 'control' were not statistically different (3.2 $\pm$ 1.1; 2.7 $\pm$ 1.9 and 5.2 $\pm$ 1.7 respectively by Z-test; Table 3). Regarding the sex ratio, there is no significant difference between the proportions of males and females in every group (Z test). No statistical impact of the day of egg transfer i.e. D₀ at 2PN stage or D₁ at 2C stage on the rate of live pups or the pregnancy rates was detected (Table 3).

Postnatal growth until the 8th week of age

The growth of the 'M16' females from the 1st to the 8th week of age was always significantly higher than that of the 'KSOM' females whatever the day of transfer (D₀ or D₁; Table 4). However, the females issued from 'control eggs' that were fertilized *in vivo* but cultured in KSOM before their transfer had a higher growth rate than the females issued from the 'KSOM eggs' (see diagram Figs 1, 8A and Table 4). Regarding the males, the 'control eggs' tended to have a higher growth than 'M16 eggs' after week 3, but no statistical difference were detected between 'KSOM' and 'M16' groups (Fig. 8B and Table 4).

Organs allometry of mice at the 8th week of age

Post-mortem examination of selected organs (hearts, livers, lungs, kidneys, brains) and white fat around the kidneys and gonads at the 8th week showed that 'M16' females had more than twice the fat deposit than that measured in 'KSOM' females at the 8th week, i.e. 285.2 $\pm$ 140.9 vs 135.3 $\pm$ 49.5 mg respectively (Table 5). In contrast, we found that the 'M16' males had an average of 26% more fat deposit than the 'KSOM' males (but not statistically significant), and a significant increase in the lung:bodyweight ratio (Table 5). Interestingly, 'M16' females displayed significantly smaller brains and smaller brains relative to their body mass than 'KSOM' females while 'M16' males also displayed significantly smaller brains and smaller brains relative to their body mass than the 'control' (Fig. 9A, B and Table 5).

Figure 8 The postnatal growths. (A) The weights of female offspring are plotted with vertical bars representing the standard deviation of the data. The medium is represented by a color code. Solid lines represent the average growth of animals issued from transfer at both D₀ and D₁. Dashed lines represent the growth of animals issued from D₀ or D₁. Dashed lines and solid lines of the same color are confounded, showing that the prolonged culture period from D₀ to D₁ has no impact on the growth whatever the culture media. (B) Male growth with the same color code as in (A). Statistical tests were done for females and males separately with 2-way ANOVA (3 media × 2 stages) for every week with all pairwise multiple comparison procedures (Bonferroni *t*-test). Stars represent the level of statistical significance ($\alpha = 5\%$). The plots are from Table 4 data.

Discussion

The near linear increment of the time delay between oscillations appears to be a common characteristics of the Ca²⁺ responses at fertilization in mice

Experimental requirements for analyzing the sensitivity of the Ca²⁺ response to external media

Exploiting the parameters of Ca²⁺ signaling as a quantitative measurement of egg functioning confronts, the high plasticity of the Ca²⁺ signaling machinery (Yu 2008, Wakai *et al.* 2011, Swann 2013) and its extreme sensitivity to perturbations due to methodologies and technical settings that can lead to misinterpretations (Swann 2013). We used a microfluidic chamber (Ozil & Swann 1995, Ozil *et al.* 2006) to keep eggs spiking in a constantly refreshed environment throughout the period of egg activation. Moreover, fertilization by ICSI is known to perturb Ca²⁺ homeostasis by causing extra Ca²⁺ influx due to the puncture of the plasma membrane (Tesarik & Sousa 1995, Sato *et al.* 1999, Yanagida *et al.* 2001). As a quality check of ICSI procedure, we systematically caught the initial rise of the Ca²⁺ oscillation. Then, if the initial Ca²⁺ oscillation appeared normal (Deguchi *et al.* 2000, Saunders *et al.* 2002, Ozil *et al.* 2005), i.e. always occurring within a similar time span observed after fertilization, i.e. 3–4 min following ICSI (Figs 3 and 5) (Lawrence *et al.* 1997), Ca²⁺ recording was validated and continued. With our ICSI protocol, this first large Ca²⁺ oscillation was always followed by a series of shorter transient Ca²⁺ oscillations (Figs 3 and 5) that also displayed a couple of super-oscillations on the top (data not shown). This experimental setting makes it possible to record the whole series of Ca²⁺ oscillations and discard artifactual responses due to ICSI or malfunctioning of the technical set-up.

Analysis of the Ca²⁺ responses in the presence of different media makes it possible to identify a new metrics: the TlbO parameter

The near linear increment of the time delay between oscillations appears to be a common characteristic of the Ca²⁺ responses at fertilization in mice, whatever the culture media diversity. This finding (see examples Fig. 2) suggests that the Ca²⁺ responses, once fertilization has occurred, have a predictable nature, which is dependent on the culture media. Furthermore, the fact that all eggs were activated clearly shows that medium formulation is capable of supporting higher numbers of Ca²⁺ oscillations than the minimum needed for the activation of the developmental processes (i.e. 4 oscillations in M16, data not shown). Moreover, the fact that an empirical regression line most closely describes the association between TlbO and the total number of Ca²⁺ oscillations (Fig. 7) strongly suggests that Ca²⁺ influx, which is known to be the trigger of Ca²⁺ oscillations (Miyazaki 2007, Takahashi *et al.* 2013) is faithfully coupled with an unknown mechanism that adds a delay in ascending order before triggering the next oscillation and causes cessation of oscillations when the time interval reaches a maximum. In addition, we can see that the characteristics of the TlbO coefficient with its *y*-intercept makes it possible to predict not only the total number of oscillations but also the total duration of the

Figure 9 Normalized and centered polar plots of the relative organ:bodyweight ratio. (A) Female organs: 'M16' females display a significant increase in fat weight associated with a decrease in brain weight in comparison with the 'KSOM'. (B) Male organs: 'M16' males display a significant increase in lung and a significant decrease in brain in comparison to the 'Control'. The medium is represented by a color code. Data from females and males were centered-reduced together.

Ca^{2+} response and therefore the average frequency for every single record without the need of recording the whole Ca^{2+} response (see legend Fig. 2 for an example of calculation).

We exploited this new metrics to firstly gauge the functional changes induced by different medium and secondly to better understand what sort of Ca^{2+} mechanism determines the linkage between the TiO_2 , the number of Ca^{2+} oscillations, the total duration, the frequency and some compounds of the medium.

External $[\text{Mg}^{2+}]$ has a major effect on the time increment between Ca^{2+} oscillations (TiO_2)

Surprisingly, doubling the free $[\text{Ca}^{2+}]$ in M16 from 1.16 to 2.44 mM (Table 2) did not significantly increase the mean frequency to the level displayed in KSOM because the means remained significantly different (3.6 ± 2 vs 5.6 ± 1.6 ($P = 0.031$); Fig. 4B). Moreover, dispermic fertilization, which is thought to double the amount of the $\text{PIC}\zeta$ and increase the frequency as previously shown by Faure and coworkers did not significantly increase the mean number of oscillations and the mean duration in both the M16 and the KSOM (Fig. 4C and D); it increased the mean frequency in M16 (2.7 ± 0.4 – 3.6 ± 2 ; $P < 0.001$; Fig. 4C) but not in KSOM (Fig. 4D) (Faure et al. 1999). This shows that the effect of polyspermy on the Ca^{2+} response is media formulation dependent (see further below). However, regarding the pace, some reports have shown that whatever the degree of polyspermy, the frequency of Ca^{2+} oscillations tends to increase during Ca^{2+} recording (Faure et al. 1999). At this stage, it is difficult to understand why the Ca^{2+} response would accelerate before stopping after some time. In our hand, acceleration of Ca^{2+} oscillations only occurred when eggs were exposed to UV light that was not sufficiently attenuated by ND filters. In this case, Ca^{2+} oscillations started to accelerate slowly, they never stopped and very often the resting $[\text{Ca}^{2+}]_i$ level increased and eggs ended up by dying suggesting that acceleration could originate from a detrimental effect of the UV

light when the frequency of data acquisition is high; here, 0.5 Hz for several hours. Therefore, comparison of frequency with previous reports can be done when records did not display acceleration of Ca^{2+} oscillations. For example, our results fit well with a recent report showing that when ICSI eggs were subsequently injected with a single equivalent dose of $\text{PIC}\zeta$, about doubling the dose of $\text{PIC}\zeta$, the increase in number of Ca^{2+} oscillations in KSOM was only marginal (Sanusi et al. 2015).

Therefore, we can see that despite the extreme sensitivity of $\text{PIC}\zeta$ to tiny $[\text{Ca}^{2+}]_i$ elevation (Rice et al. 2000, Kouchi et al. 2005, Nomikos et al. 2005, Yu 2008, Swann 2013), neither the doubling of the free $[\text{Ca}^{2+}]_o$, nor dispermic fertilization are capable of significantly getting over some unknown functional constraints imposed by the formulation of M16 or KSOM medium that make the Ca^{2+} responses different.

In contrast, when the Mg^{2+} ion was removed from the M16, the Ca^{2+} responses were dramatically changed (Table 2). These results draw attention to the role of external $[\text{Ca}^{2+}]_o$ and $[\text{Mg}^{2+}]_o$ and their ratio in controlling the Ca^{2+} response. Mg^{2+} is known to be a natural weak Ca^{2+} flux blocker (Iseri & French 1984, Lansman et al. 1986, Hartzell & White 1989) and well-known inhibitor of some members of the TRP cationic non-selective plasma membrane channels, which are expressed in mouse oocytes (Carvacho et al. 2013, 2016).

It has been proposed that Ca^{2+} influx mostly supports Ca^{2+} oscillations by ensuring the replenishment of the Ca^{2+} stores in advance of the next oscillation suggesting that refilling the Ca^{2+} stores during the time course of the Ca^{2+} response is the pacemaker of the oscillations (Takahashi et al. 2013, Wakai et al. 2013). The M-shape pattern of the time course of $[\text{Ca}^{2+}]_i$ oscillations seen in Fig. 5A, E and F clearly shows that high frequency runs down the Ca^{2+} oscillation peak amplitude while subsequently, when the frequency is slowed down, the peak amplitude is restored. It thus appears that the mechanism that sets the pace might not require the full replenishment of the Ca^{2+} stores. In addition, the M-shape pattern suggests that when the intensity of

Table 4 Animal weight from the 1st to the 8th week of age according to 3 media, 2 days of transfer and 2 genders.

	Stage/week	1st	2nd	3rd	4th	5th	6th	7th	8th
M16 females	D ₀ (2PN); n = 9	6.30 ± 0.42	10.17 ± 1.97	13.19 ± 1.87	17.15 ± 1.84	19.67 ± 1.86	20.94 ± 1.45	22.33 ± 1.14	22.56 ± 1.49
	D ₁ (2C); n = 12	6.16 ± 0.97	10.55 ± 1.38	13.00 ± 1.75	17.68 ± 1.69	20.43 ± 1.56	21.35 ± 1.33	22.25 ± 1.30	23.49 ± 1.81
	Total; n = 21	6.22 ± 0.8	10.38 ± 1.6	13.09 ± 1.7	17.45 ± 1.7	20.10 ± 1.7	21.18 ± 1.4	22.28 ± 1.2	23.09 ± 1.7
Statistical ≠ between stage D ₀ and D ₁		ns	ns	ns	ns	ns	ns	ns	ns
KSOM females	D ₀ (2N); n = 12	5.29 ± 0.49	8.79 ± 1.00	11.36 ± 1.44	15.28 ± 1.86	18.29 ± 1.38	19.49 ± 1.46	20.19 ± 1.78	21.08 ± 1.76
	D ₁ (2C); n = 6	5.32 ± 0.47	8.61 ± 0.72	10.96 ± 1.21	15.28 ± 1.21	18.2 ± 0.80	18.77 ± 0.68	19.67 ± 0.87	20.36 ± 0.96
	Total; n = 18	5.30 ± 0.5	8.73 ± 0.9 ^a	11.23 ± 1.3	15.28 ± 1.6	18.27 ± 1.2	19.25 ± 1.3 ^b	20.03 ± 1.5	20.84 ± 1.5
Statistical ≠ between stage D ₀ and D ₁		ns	ns	ns	ns	ns	ns	ns	ns
Control females	D ₀ (2PN); n = 11	5.82 ± 0.56	9.48 ± 0.68	12.35 ± 0.91	16.56 ± 1.18	19.55 ± 2.00	20.93 ± 2.06	21.69 ± 2.48	22.57 ± 2.52
	D ₁ (2C); n = 3	5.45 ± 0.46	9.69 ± 0.39	12.31 ± 0.99	16.88 ± 0.87	19.74 ± 0.35	20.80 ± 0.59	21.74 ± 0.92	22.08 ± 1.94
	Total; n = 14	5.74 ± 0.5	9.52 ± 0.6 ^a	12.34 ± 0.9	16.63 ± 1.1	19.59 ± 1.8	20.90 ± 1.8 ^b	21.71 ± 2.2	22.46 ± 2.3
Statistical ≠ between stage D ₀ and D ₁		ns	ns	ns	ns	ns	ns	ns	ns
Statistical ≠ between media into female groups		M16 > KSOM (***P < 0.001)	M16 > KSOM (***P < 0.001)	M16 > KSOM (***P < 0.001)	M16 > KSOM (***P < 0.001)	M16 > KSOM (***P < 0.004)	M16 > KSOM (***P < 0.001)	M16 > KSOM (***P = 0.001)	M16 > KSOM (**P = 0.002)
M16 males	D ₀ (2PN); n = 5	6.30 ± 0.33	10.22 ± 1.00	12.84 ± 1.18	17.90 ± 2.16	20.83 ± 2.24	23.25 ± 2.58	24.65 ± 2.93	25.46 ± 3.16
	D ₁ (2C); n = 14	5.69 ± 1.17	9.95 ± 1.47	13.21 ± 1.89	19.06 ± 2.06	22.90 ± 2.50	25.20 ± 2.67	26.92 ± 2.96	27.79 ± 3.19
	Total; n = 19	5.86 ± 1.0	10.02 ± 1.3	13.11 ± 1.7	18.76 ± 2.1	22.36 ± 2.5	24.69 ± 2.7	26.32 ± 3.0	27.18 ± 3.3
Statistical ≠ between stage D ₀ and D ₁		ns	ns	ns	ns	ns	ns	ns	ns
KSOM males	D ₀ (2P); n = 12	5.69 ± 1.05	9.47 ± 1.96	12.76 ± 2.83	18.17 ± 3.24	23.26 ± 2.06	25.02 ± 2.45	26.65 ± 2.34	28.49 ± 2.18
	D ₁ (2C) n = 9	4.99 ± 0.81	8.85 ± 1.24	11.52 ± 1.90	16.85 ± 1.89	21.88 ± 1.23	23.69 ± 1.53	25.45 ± 1.82	26.38 ± 1.87
	Total; n = 21	5.39 ± 1	9.22 ± 1.6	12.23 ± 2.5	17.60 ± 2.8	22.67 ± 1.8	24.45 ± 2.2	26.13 ± 2.1	27.59 ± 2.3
Statistical ≠ between stage D ₀ and D ₁		ns	ns	ns	ns	ns	ns	ns	ns
Control males	D ₀ (2PN); n = 7	5.94 ± 0.62	9.84 ± 0.47	13.43 ± 0.83	19.79 ± 1.88	24.30 ± 2.27	26.60 ± 1.75	27.49 ± 1.78	29.11 ± 1.90
	D ₁ (2C); n = 5	5.87 ± 0.51	10.22 ± 0.34	13.62 ± 0.52	19.88 ± 1.01	23.64 ± 1.72	26.35 ± 1.65	28.14 ± 1.05	28.92 ± 1.15
	Total; n = 12	5.92 ± 0.6	10.0 ± 0.5	13.50 ± 0.7	19.83 ± 1.5	24.02 ± 2.0	26.49 ± 1.6	27.76 ± 1.5	29.03 ± 1.6
Statistical ≠ between stage D ₀ and D ₁		ns	ns	ns	ns	ns	ns	ns	ns
Statistical ≠ between media into male groups		ns	ns	ns	Ctrl > M16 (*P = 0.025)	Ctrl > M16 (*P = 0.042)	Ctrl > M16 (*P = 0.043)	ns	ns

Data were analyzed with two way ANOVA with all pairwise comparison with Bonferroni *t*-test correction for every week and female and male animals separately.

Table 5 Mean organ weight (g) and organ/body weight ratios of female ($n = 12$ for control; $n = 7$ for M16 and 13 for KSOM) and male offspring ($n = 12$ for control $n = 7$ for M16 and 10 for KSOM) at 8th week of age.

	Heart	Liver	Lung	Kidney	Brain	Fat
A. Female						
Organ weight (mg) noticed						
'Control'	109.0 ± 10.8	1263.5 ± 306^a	140.9 ± 15.3	285.0 ± 43.0	483.4 ± 17.7	193.3 ± 85.3
'M16'	115.2 ± 13.5	1188.9 ± 139	148.9 ± 10.2	305.2 ± 44.8	449.5 ± 59.0	285.2 ± 140.9^b
'KSOM'	105.0 ± 6.1	1024.5 ± 107^a	136.1 ± 13.7	281.0 ± 20.3	473.7 ± 18.1	135.3 ± 49.5^b
Organ:body weight ratio (×100)						
'Control'	0.490 ± 0.052	5.600 ± 0.959	0.633 ± 0.056	1.272 ± 0.103	2.184 ± 0.234	0.844 ± 0.300
'M16'	0.503 ± 0.049	5.183 ± 0.436	0.650 ± 0.028	1.333 ± 0.182	1.972 ± 0.300^c	1.233 ± 0.551^d
'KSOM'	0.507 ± 0.034	4.94 ± 0.41	0.658 ± 0.074	1.357 ± 0.105	2.292 ± 0.169^c	0.643 ± 0.200^d
B. Male						
Organ weight (g)						
'Control'	149.5 ± 10.7	1612.7 ± 234.5	152.5 ± 13.8 g	470.1 ± 68.9	499.5 ± 25.8^{f, g}	549.7 ± 199.7
'M16'	136.0 ± 18.7	1510.4 ± 261.2	166.4 ± 13.9^e	435.6 ± 82.7	435.5 ± 40.8^f	607.6 ± 363.4
'KSOM'	137.3 ± 7.2	1564.7 ± 140.3	150.1 ± 8.7^e	430.9 ± 43.05	462.83 ± 17.76^g	481.7 ± 176.5
Organ:body weight ratio (×100)						
'Control'	0.515 ± 0.024	5.552 ± 0.722	0.525 ± 0.036^h	1.618 ± 0.206	1.724 ± 0.104ⁱ	1.903 ± 0.731
'M16'	0.480 ± 0.048	5.300 ± 0.265	0.590 ± 0.050^h	1.529 ± 0.151	1.550 ± 0.195ⁱ	2.049 ± 0.992
'KSOM'	0.504 ± 0.039	5.726 ± 0.393	0.546 ± 0.019	1.581 ± 0.185	1.698 ± 0.106	1.756 ± 0.628

Statistical analysis for organ weight was performed by one-way ANOVA with pairwise multiple comparison procedures and Bonferroni *t*-test correction for female and male animals separately. Letters in bold indicate significant difference between groups; ^a(* $P = 0.025$); ^b(* $P = 0.03$); ^c(* $P = 0.016$); ^d(** $P = 0.002$); ^e(* $P = 0.038$); ^f(*** $P < 0.001$); ^g(* $P = 0.015$); ^h(** $P = 0.002$); ⁱ(* $P = 0.029$). See the Radar Plot Fig. 9.

the Ca^{2+} influx is not inhibited by external Mg^{2+} , it can trigger oscillations with partially empty stores that release little Ca^{2+} . It might also override the downregulation of the InsP_3R channels by persistent Ca^{2+} oscillations that have been shown to be responsible of the cessation of Ca^{2+} oscillations (Brind *et al.* 2000, Malcuit *et al.* 2005, Lee *et al.* 2006) because here the total duration increased from 3 h 19 ± 36 min in M16 to 4 h 14 min ± 31 min in M16 without Mg^{2+} (Fig. 6A).

From these observations, we can conclude that the external $[\text{Mg}^{2+}]_o/[\text{Ca}^{2+}]_o$ ratio has a critical function in controlling the pace of oscillations probably through the intensity of transmembrane Ca^{2+} influx.

In contrast, removing external H_2PO_4^- , which is a powerful Ca^{2+} complexing agent (Zoetewij *et al.* 1993, Dutka *et al.* 2005, Allen *et al.* 2011), has no apparent impact on either the free Ca^{2+} in M16 or the Ca^{2+} response (Fig. 6B and Table 2), even when the Ca^{2+} response is exacerbated by the lack of Mg^{2+} in media (Fig. 5A, E and F and Table 2).

However, switching both $[\text{Mg}^{2+}]_o$ and $[\text{KH}_2\text{PO}_4]_o$ between M16 and KSOM shows that $\text{M16}_{\text{X-KSOM}}$ cannot mimic KSOM response (Fig. 6D) while $\text{KSOM}_{\text{X-M16}}$ does mimic M16 response (Fig. 6C). Therefore, it is possible that high external (BSA), which is 4 times higher in M16 than that in KSOM, can contribute to slow down the pace of the Ca^{2+} response by a still unknown mechanism (Fig. 5E, F and Table 2).

Overall, these observations strongly suggest that the free external $[\text{Mg}^{2+}]_o/[\text{Ca}^{2+}]_o$ ratio is a strong determinant of the evolution in the time increment between oscillations and their number. It remains to be understood the mechanism by which external $[\text{Mg}^{2+}]_o/[\text{Ca}^{2+}]_o$ ratio determines the parameters of the Ca^{2+} response.

External $[\text{Mg}^{2+}]_o/[\text{Ca}^{2+}]_o$ ratio appears responsible for causing a cumulative delay in triggering Ca^{2+} oscillations

Regarding the role of Mg^{2+} , some reports have demonstrated that Mg^{2+} ions that penetrate into the pore when the plasma membrane Ca^{2+} channel is activated can experience stabilization from ligand groups inside the channel (Lansman *et al.* 1986). Such intra-channel stabilization of Mg^{2+} ions might be responsible for the progressive blockage of Ca^{2+} permeation each time Ca^{2+} entry is stimulated following every Ca^{2+} oscillation (Mohri *et al.* 2001, Takahashi *et al.* 2013). Therefore, we hypothesize that accumulation of Mg^{2+} inside the channel lumen after every Ca^{2+} influx might cause a cumulative blockage of Ca^{2+} permeation from outside. More time would then be required to elevate the $[\text{Ca}^{2+}]_i$ toward the threshold level above which Ca^{2+} oscillation is triggered (see diagram Fig. 10). The cessation of the Ca^{2+} oscillations might also correspond to the time when the intra channel pores have accumulated a sufficient amount of Mg^{2+} to fully block any Ca^{2+} permeation into the cytosol (diagram Fig. 10). In the absence of external Mg^{2+} , the Ca^{2+} influx is not inhibited (Fig. 5A, E and F) and thus might override several mechanisms that are known to stop Ca^{2+} oscillations such as: (i) the depletion of Ca^{2+} stores (Takahashi *et al.* 2013, Wakai *et al.* 2013); (ii) the progressive downregulation of the desensitization of IP_3R (Jellertette *et al.* 2000) and (iii) sequestration of $\text{PLC}\zeta$ in PN (Marangos *et al.* 2003). Evidence of this precedence is the fact that oscillations still occurred when PNs were visible (data not shown).

In mice, studies have indicated that divalent-selective ion channels such as TRPM7 and TRPM6 are involved

Figure 10 A schematic diagram illustrating how external Mg²⁺ could progressively run down Ca²⁺ influx during the time course of the Ca²⁺ oscillations. In gray the diagram proposed by Swann (2013), Yu (2008) shows a positive feedback loop taking over the mechanism of oscillation when PLC ζ is introduced into the egg at fertilization. In black two additional feedbacks upstream of PLC ζ activation by the rise in [Ca²⁺]_i; (1) a Ca²⁺ influx following the Ca²⁺ release from intracellular stores increases the [Ca²⁺]_i. (2) Accumulation of external Mg²⁺ inside the lumen of the open Ca²⁺ channels might cause a progressive blockage of Ca²⁺ permeation, which postpones the occurrence of the next oscillation. We hypothesize that the downregulated intensity of the Ca²⁺ influx after every Ca²⁺ oscillation slows down the rise of the [Ca²⁺]_i before the next oscillation. More time is required for the [Ca²⁺]_i to reach the threshold beyond which Ca²⁺ oscillation is triggered. As a consequence, the pacemaker phase (in black) increases over time as shown by decomposing every phase of the Ca²⁺ oscillations from the Ca²⁺ record taken from Fig. 2 (see text). When Ca²⁺ permeation blockage through the channels reaches a maximum, Ca²⁺ oscillations stop. The hypothetical mechanism of ABCI might constitute a counting mechanism that is [Mg²⁺]_o/[Ca²⁺]_o ratio and Ca²⁺ channel property dependent.

in the control of Mg²⁺ homeostasis (Komiya *et al.* 2014) and are required for early development in the mouse (Liu *et al.* 2011, Carvacho *et al.* 2016). The overall results suggest that an accumulative blockage of Ca²⁺ influx (here named ABCI) induced by external Mg²⁺ might explain the time step that builds on the TlbO coefficient (see diagram Fig. 10). Moreover, it is possible that this singular phenomenon helps interpreting the fact that the slight increase in frequency caused by dispermic fertilization (Fig. 4C and D) correlates with a slight decrease in total duration. It is possible that any increase in frequency due to higher dose of PLC ζ , other things being equal, will accelerate the termination of the Ca²⁺ response because the full blockage of the Ca²⁺ influx by Mg²⁺ will occur earlier due to the hypothetic ABCI mechanism as can be seen on Fig. 4C and D. It would be interesting to see whether the termination of the Ca²⁺ oscillations is associated with the saturation of divalent-selective ion channels by Mg²⁺. Another possible demonstration of the ABCI model will require measuring the evolution of the duration of the pacemaker phase that precedes every Ca²⁺ oscillation during the time course of the Ca²⁺ response (Fig. 10). However, regarding the large interindividual variabilities of the Ca²⁺ responses in all groups (Table 2), further studies should determine what the respective roles played by the sperm in delivering various dose of PLC ζ are (Kashir *et al.* 2014) and that of the mature oocytes with their varying number of functional divalent-selective ion channels (Carvacho *et al.* 2016) that might contribute to building up the diversity of the Ca²⁺ responses at fertilization.

Do the development stages and adult phenotype correlate with the Ca²⁺ response at fertilization?

Developmental stages

In contrast to the change of the Ca²⁺ responses between M16 and KSOM, no difference was found in the rate of egg activation and cleavage to 2-cell or in the rate of live pups by pregnant recipients between the media (Table 3). Despite the fact that these rates of live pups were comparable with those obtained when ICSI eggs were transferred at the 2-cell stage (Kuretake *et al.* 1996, Lacham-Kaplan *et al.* 2003), the survival rate by eggs transferred remains significantly low compared with eggs fertilized *in vivo* but cultured in KSOM (19.4% and 20.5% vs 54.2% respectively, $P < 0.001$, Table 3) as well as the survival rate among the pregnant recipient (40.4% and 34.2% vs 65% respectively, Table 3). However, given the range of the number of Ca²⁺ oscillations in 'M16' (4–14) and 'KSOM' (9–31), it is impossible to determine if 'M16' or 'KSOM' offspring are issued from similar or different Ca²⁺ responses (the TlbOs overlap, Fig. 7). The lower survival rates are not in either case due to aberrant Ca²⁺ signaling induced by ICSI (Table 2). We assume that the use of cytochalasin for a couple of minutes at room temperature before ICSI that made the plasma membrane malleable or the short time spent in ICSI media (see 'Materials and methods' section) were not detrimental because the effect of cytochalasin is highly reversible (Balakier & Tarkowski 1976) and ICSI medium was instantly removed when eggs were placed in the microfluidic chamber at 37°C. It remains possible

that other phenomena related to ICSI, not mirrored in the Ca^{2+} response, such as the delay between the full disintegration of the plasma and nuclear membranes of the sperm once injected into the cytosol (Kuretake *et al.* 1996, Knott *et al.* 2003, Yanagimachi 2005, Morozumi *et al.* 2006) have an impact. Further studies focusing on synchronization of Ca^{2+} oscillations at different frequencies with the remodeling of the sperm nucleus by using sequential media with different $[\text{Mg}^{2+}]_o/[\text{Ca}^{2+}]_o$ ratios during the fertilization period might help understand the role of Ca^{2+} oscillations in sperm cell remodeling and perhaps improve the survival rate to term after ICSI.

Adult phenotype

The growth rates (Fig. 8 and Table 4) clearly show sex-specific effects of culture media, which have already been widely described and studied in the mouse (Fernandez-Gonzalez *et al.* 2004, Watkins *et al.* 2007, Banrezes *et al.* 2011, Donjacour *et al.* 2014, Feuer *et al.* 2014a) and in some reports in human following IVF (Ceelen *et al.* 2008a,b, Kleijkers *et al.* 2014). Though the present results contribute to narrowing the time window down to the first 4 h during which culture media affects the postnatal response in a sex-specific manner.

The experimental design (Fig. 1) demonstrates that the differences in the female growth rates were mostly induced by the nature of the external media during the short period between fertilization and PNs formation for the following reasons:

1. The differences in growth between 'M16' and 'KSOM' were due neither to the litter size, which were similar (Table 3) nor to the duration of the culture period in M16 after the PN stage because the growth rate issued from eggs transferred at D_0 or D_1 are similar in all cases (Table 4).
2. The differences between 'M16' and the 'control' were small (Fig. 8), showing that after PNs formation, the use of the M16 for experimental eggs or KSOM for 'control' for a limited time in culture had no apparent impact on the growth.
3. The difference between 'M16' and 'KSOM' groups is highly significant. Therefore, given points 1 and 2, it appears that the most sensitive time window to the formulation of the culture medium is the short time period between fertilization and the PNs stage.

Hence, these results confirm an earlier study that the period of egg activation is a very sensitive time window where any functional perturbation might have developmental consequences (Ozil *et al.* 2006, Ducibella & Fissore 2008) including the survival rate to term.

Regarding organ allometry, the present results show that the weight of several organs and the

organ:bodyweight ratios issued from animals of the 'M16' group, in particular the brain, were significantly lower than the 'control' and the 'KSOM' group (Fig. 9 and Table 5). Regarding the female-specific fat deposit in the presence of M16 (Fig. 9 and Table 5), our observations are consistent with other reports on IVF of mice issued from eggs cultured in Whitten media (Feuer *et al.* 2014b). This group has shown that IVF and prolonged *in vitro* culture to blastocyst stage in Whitten media increases postnatal fat accumulation and glucose metabolism in female mice. They revealed broad changes in metabolic homeostasis characterized by systemic oxidative stress and mitochondrial dysfunction (Feuer *et al.* 2014b), but they did not record Ca^{2+} signaling at fertilization. At this stage, we can only suggest that these long-term impacts are mostly linked with the initial 4-h period following fertilization. A preliminary study on differential miRNA expression at PNs stage following fertilization by ICSI suggests that early transcription of maternal mRNA is indeed associated with the intensity of Ca^{2+} activity (Barrey *et al.* 2014) and reinforces previous suspicions regarding the role of Ca^{2+} oscillations at fertilization in gene expression (Ozil *et al.* 2006).

For this reason, any empirical intervention at fertilization in human IVF or prolonged *in vitro* culture should be avoided as previously mentioned (van Blerkom *et al.* 2015) until extensive scrutiny of long-term phenotypic and epigenetic consequences are carried out in mice or other animal models.

Conclusion and perspectives

The major findings of this study are: (i) the time intervals between oscillations increase almost linearly during the time course of egg activation; (ii) the TiB_0 coefficient that measures the time increments between two successive oscillations varies according to the external $[\text{Mg}^{2+}]_o/[\text{Ca}^{2+}]_o$ ratio; (iii) the external Mg^{2+} might cause an accumulative blockage of Ca^{2+} influx (ABCI) during the time course of Ca^{2+} oscillations; (iv) the ABCI might constitute a reliable counting mechanism that stops oscillations when the incremental phenomena has totally blocked Ca^{2+} ions permeation by stabilization of Mg^{2+} inside the lumen of divalent-selective ion channels; (v) the adult phenotypes after fertilization by ICSI appear highly sensitive to culture media formulation during the short time window between the time of ICSI and the time of PNs formation.

In addition to providing new insight into the mechanism of Ca^{2+} oscillations, the apparent faithful mechanism of ABCI that sets the total numbers of Ca^{2+} oscillations, offers a physiological mean to tune at will the number of Ca^{2+} oscillations by varying external

[Mg²⁺]_o/[Ca²⁺]_o ratio in media during egg activation. For this aim, we looked at the Ca²⁺ response induced by human IVF commercial media such as COOK and VITROLIFE. These modern media have differences in pyruvate, lactate and amino acids but higher [Mg²⁺] and lower [Ca²⁺] than M16 or KSOM (i.e. 1.5 mM Mg²⁺ and 1.1 mM Ca²⁺ for Cook and 1.7 mM Mg²⁺ and 1.1 mM Ca²⁺ for VITROLIFE (Morbeck *et al.* 2014). In our hands, COOK and VITROLIFE media generated 5 ± 0.8 oscillations (*n* = 12) and 5 ± 1.4 oscillations (*n* = 15) respectively (unpublished data). Therefore, it clearly appears that high concentration of Mg²⁺ locks up the number of Ca²⁺ oscillations to few Ca²⁺ oscillations with low variability, whereas removing Mg²⁺ will unleash the regime of Ca²⁺ oscillations with greater interindividual variability. Since the redox parameters have been shown to have postnatal impacts (Banrezes *et al.* 2011), the present results highlight the critical role of medium formulation during the period of fertilization. Yet, we do not know what the range of Ca²⁺ responses is when eggs are fertilized *in vivo* and why they have higher survival rate (Table 3). A very recent report shows that some *in vivo*-fertilized eggs with sperm derived from *Plcz1*^{-/-} males that fail to trigger Ca²⁺ oscillations can develop to term albeit at greatly reduced efficiency (Hachem *et al.* 2017). However, since it has been shown that low ionic strength media has the capability to trigger monotonic large Ca²⁺ release from internal stores in non-fertilized eggs (Ozil & Swann 1995), it might be possible that the oviductal fluid has some unknown but specific properties that make the Ca²⁺ response different from *in vitro* fertilization. As we have no access to the Ca²⁺ response *in vivo*, testing the survival rate and health of mice fertilized *in vitro* in media with various combinations of [Mg²⁺]_o/[Ca²⁺]_o and pyruvate/lactate ratio could make it possible to determine the best combination of compounds that optimizes the production of ATP (Campbell & Swann 2006) and sets the best NADH and FADH₂ redox potential (Dumollard *et al.* 2009, Banrezes *et al.* 2011). Media formulations gauged by TlB_o could become an active means to better understand the role of early Ca²⁺ oscillations and the mitochondrial response in the developmental processes, and probably improve the survival rate and health of ART children across the life course (Feuer & Rinaudo 2017).

It remains to be learned whether the ABCI mechanism is present in other animal and human species at fertilization.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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References

- Allen DG, Clugston E, Petersen Y, Roder IV, Chapman B & Rudolf R 2011 Interactions between intracellular calcium and phosphate in intact mouse muscle during fatigue. *Journal of Applied Physiology* **111** 358–366. (doi:10.1152/japplphysiol.01404.2010)
- Balakier H & Tarkowski AK 1976 Diploid parthenogenetic mouse embryos produced by heat-shock and Cytochalasin B. *Journal of Embryology and Experimental Morphology* **35** 25–39.
- Banrezes B, Sainte-Beuve T, Canon E, Schultz RM, Cancela J & Ozil JP 2011 Adult body weight is programmed by a redox-regulated and energy-dependent process during the pronuclear stage in mouse. *PLoS ONE* **6** e29388. (doi:10.1371/journal.pone.0029388)
- Barrey E, Banrezes B, Sainte-Beuve T, Vaiman A & Ozil J-P 2014 Early differential miRNA expression following *in vitro* fertilization of mouse eggs incubated in two standard culture media. Abstract book in *FEBS Journal* **281** (Supplement 1) 264; EMBO Meeting 30th August–4th September 2014, Paris.
- Biggers JD 1998 Reflections on the culture of the preimplantation embryo. *International Journal of Developmental Biology* **42** 879–884.
- Brind S, Swann K & Carroll J 2000 Inositol 1,4,5-trisphosphate receptors are downregulated in mouse oocytes in response to sperm or adenophostin A but not to increases in intracellular Ca(2+) or egg activation. *Developmental Biology* **223** 251–265. (doi:10.1006/dbio.2000.9728)
- Campbell K & Swann K 2006 Ca²⁺ oscillations stimulate an ATP increase during fertilization of mouse eggs. *Developmental Biology* **298** 225–233. (doi:10.1016/j.ydbio.2006.06.032)
- Carvacho I, Lee HC, Fissore RA & Clapham DE 2013 TRPV3 channels mediate strontium-induced mouse-egg activation. *Cell Reports* **5** 1375–1386. (doi:10.1016/j.celrep.2013.11.007)
- Carvacho I, Ardestani G, Lee HC, McGarvey K, Fissore RA & Lykke-Hartmann K 2016 TRPM7-like channels are functionally expressed in oocytes and modulate post-fertilization embryo development in mouse. *Scientific Reports* **6** 34236. (doi:10.1038/srep34236)
- Ceelen M, van Weissenbruch MM, Vermeiden JP, van Leeuwen FE & Delemarre-van de Waal HA 2008a Cardiometabolic differences in children born after *in vitro* fertilization: follow-up study. *Journal of Clinical Endocrinology and Metabolism* **93** 1682–1688. (doi:10.1210/jc.2007-2432)
- Ceelen M, van Weissenbruch MM, Vermeiden JP, van Leeuwen FE & Delemarre-van de Waal HA 2008b Growth and development of children born after *in vitro* fertilization. *Fertility and Sterility* **90** 1662–1673.
- Deguchi R, Shirakawa H, Oda S, Mohri T & Miyazaki S 2000 Spatiotemporal analysis of Ca(2+) waves in relation to the sperm entry site and animal-vegetal axis during Ca(2+) oscillations in fertilized mouse eggs. *Developmental Biology* **218** 299–313. (doi:10.1006/dbio.1999.9573)

- Donjacour A, Liu X, Lin W, Simbulan R & Rinaudo PF 2014 In vitro fertilization affects growth and glucose metabolism in a sex-specific manner in an outbred mouse model. *Biology of Reproduction* **90** 80.
- Ducibella T, Huneau D, Angelichio E, Xu Z, Schultz RM, Kopf GS, Fissore R, Madoux S & Ozil JP 2002 Egg-to-embryo transition is driven by differential responses to Ca^{2+} oscillation number. *Developmental Biology* **250** 280–291. (doi:10.1006/dbio.2002.0788)
- Ducibella T & Fissore R 2008 The roles of Ca^{2+} , downstream protein kinases, and oscillatory signaling in regulating fertilization and the activation of development. *Developmental Biology* **315** 257–279. (doi:10.1016/j.ydbio.2007.12.012)
- Dumollard R, Carroll J, Duchon MR, Campbell K & Swann K 2009 Mitochondrial function and redox state in mammalian embryos. *Seminars in Cell and Developmental Biology* **20** 346–353. (doi:10.1016/j.semcdb.2008.12.013)
- Dupont G & Combettes L 2016 Fine tuning of cytosolic Ca^{2+} oscillations. *F1000Research* **5** F1000.
- Dutka TL, Cole L & Lamb GD 2005 Calcium phosphate precipitation in the sarcoplasmic reticulum reduces action potential-mediated Ca^{2+} release in mammalian skeletal muscle. *American Journal of Physiology: Cell Physiology* **289** C1502–C1512. (doi:10.1152/ajpcell.00273.2005)
- Faure JE, Myles DG & Primakoff P 1999 The frequency of calcium oscillations in mouse eggs at fertilization is modulated by the number of fused sperm. *Developmental Biology* **213** 370–377. (doi:10.1006/dbio.1999.9388)
- Fernandez-Gonzalez R, Moreira P, Bilbao A, Jimenez A, Perez-Crespo M, Ramirez MA, Rodriguez De Fonseca F, Pintado B & Gutierrez-Adan A 2004 Long-term effect of in vitro culture of mouse embryos with serum on mRNA expression of imprinting genes, development, and behavior. *PNAS* **101** 5880–5885.
- Feuer SK, Donjacour A, Simbulan RK, Lin W, Liu X, Maltepe E & Rinaudo PF 2014a Sexually dimorphic effect of in vitro fertilization (IVF) on adult mouse fat and liver metabolomes. *Endocrinology* **155** 4554–4567. (doi:10.1210/en.2014-1465)
- Feuer SK, Liu X, Donjacour A, Lin W, Simbulan RK, Giritharan G, Piane LD, Kolahi K, Ameri K, Maltepe E *et al.* 2014b Use of a mouse in vitro fertilization model to understand the developmental origins of health and disease hypothesis. *Endocrinology* **155** 1956–1969.
- Feuer SK & Rinaudo PF 2017 Physiological, metabolic and transcriptional postnatal phenotypes of in vitro fertilization (IVF) in the mouse. *Journal of Developmental Origins of Health and Disease* **8** 403–410.
- Hachem A, Godwin J, Ruas M, Lee HC, Buitrago ME, Ardestani G, Bassett A, Fox S, Navarrete F, de Sutter P *et al.* 2017 PLC ζ is the physiological trigger of the Ca^{2+} oscillations that induce embryogenesis in mammals but offspring can be conceived in its absence. *Development* **144** 2914–2924.
- Hartzell HC & White RE 1989 Effects of magnesium on inactivation of the voltage-gated calcium current in cardiac myocytes. *Journal of General Physiology* **94** 745–767. (doi:10.1085/jgp.94.4.745)
- Iseri LT & French JH 1984 Magnesium: nature's physiologic calcium blocker. *American Heart Journal* **108** 188–193. (doi:10.1016/0002-8703(84)90572-6)
- Jellerette T, He CL, Wu H, Parys JB & Fissore RA 2000 Down-regulation of the inositol 1,4,5-trisphosphate receptor in mouse eggs following fertilization or parthenogenetic activation. *Developmental Biology* **223** 238–250. (doi:10.1006/dbio.2000.9675)
- Kashir J, Nomikos M, Lai FA & Swann K 2014 Sperm-induced Ca^{2+} release during egg activation in mammals. *Biochemical and Biophysical Research Communications* **450** 1204–1211. (doi:10.1016/j.bbrc.2014.04.078)
- Kimura Y & Yanagimachi R 1995 Intracytoplasmic sperm injection in the mouse. *Biology of Reproduction* **52** 709–720. (doi:10.1095/biolreprod52.4.709)
- Kleijkers SH, van Montfort AP, Smits LJ, Viechtbauer W, Roseboom TJ, Nelissen EC, Coonen E, Derhaag JC, Bastings L, Schreurs IE *et al.* 2014 IVF culture medium affects post-natal weight in humans during the first 2 years of life. *Human Reproduction* **29** 661–669. (doi:10.1093/humrep/deu025)
- Knott JC, Kurokawa M & Fissore RA 2003 Release of the Ca^{2+} oscillation-inducing sperm factor during mouse fertilization. *Developmental Biology* **260** 536–547. (doi:10.1016/S0012-1606(03)00251-3)
- Komiya Y, Su LT, Chen HC, Habas R & Runnels LW 2014 Magnesium and embryonic development. *Magnesium Research* **27** 1–8.
- Kono T, Jones KT, Bos-Mikich A, Whittingham DG & Carroll J 1996 A cell cycle-associated change in Ca^{2+} releasing activity leads to the generation of Ca^{2+} transients in mouse embryos during the first mitotic division. *Journal of Cell Biology* **132** 915–923. (doi:10.1083/jcb.132.5.915)
- Kouchi Z, Shikano T, Nakamura Y, Shirakawa H, Fukami K & Miyazaki S 2005 The role of EF-hand domains and C2 domain in regulation of enzymatic activity of phospholipase C ζ . *Journal of Biological Chemistry* **280** 21015–21021. (doi:10.1074/jbc.M412132000)
- Kuretake S, Kimura Y, Hoshi K & Yanagimachi R 1996 Fertilization and development of mouse oocytes injected with isolated sperm heads. *Biology of Reproduction* **55** 789–795. (doi:10.1095/biolreprod55.4.789)
- Kurokawa M & Fissore RA 2003 ICSI-generated mouse zygotes exhibit altered calcium oscillations, inositol 1,4,5-trisphosphate receptor-1 down-regulation, and embryo development. *Molecular Human Reproduction* **9** 523–533. (doi:10.1093/molehr/gag072)
- Lacham-Kaplan O, Shaw J, Sanchez-Partida LG & Trounson A 2003 Oocyte activation after intracytoplasmic injection with sperm frozen without cryoprotectants results in live offspring from inbred and hybrid mouse strains. *Biology of Reproduction* **69** 1683–1689. (doi:10.1095/biolreprod.103.018184)
- Lansman JB, Hess P & Tsien RW 1986 Blockade of current through single calcium channels by Cd^{2+} , Mg^{2+} , and Ca^{2+} . Voltage and concentration dependence of calcium entry into the pore. *Journal of General Physiology* **88** 321–347. (doi:10.1085/jgp.88.3.321)
- Lawrence Y, Whitaker M & Swann K 1997 Sperm-egg fusion is the prelude to the initial Ca^{2+} increase at fertilization in the mouse. *Development* **124** 233–241.
- Lee B, Vermassen E, Yoon SY, Vanderheyden V, Ito J, Alfandari D, De Smedt H, Parys JB & Fissore RA 2006 Phosphorylation of IP3R1 and the regulation of $[\text{Ca}^{2+}]_i$ responses at fertilization: a role for the MAP kinase pathway. *Development* **133** 4355–4365. (doi:10.1242/dev.02624)
- Liu W, Su LT, Khadka DK, Mezzacappa C, Komiya Y, Sato A, Habas R & Runnels LW 2011 TRPM7 regulates gastrulation during vertebrate embryogenesis. *Developmental Biology* **350** 348–357. (doi:10.1016/j.ydbio.2010.11.034)
- Malcuit C, Knott JC, He C, Wainwright T, Parys JB, Robl JM & Fissore RA 2005 Fertilization and inositol 1,4,5-trisphosphate (IP3)-induced calcium release in type-1 inositol 1,4,5-trisphosphate receptor down-regulated bovine eggs. *Biology of Reproduction* **73** 2–13. (doi:10.1095/biolreprod.104.037333)
- Marangos P, FitzHarris G & Carroll J 2003 Ca^{2+} oscillations at fertilization in mammals are regulated by the formation of pronuclei. *Development* **130** 1461–1472. (doi:10.1242/dev.00340)
- Miao YL, Stein P, Jefferson WN, Padilla-Banks E & Williams CJ 2012 Calcium influx-mediated signaling is required for complete mouse egg activation. *PNAS* **109** 4169–4174. (doi:10.1073/pnas.1112333109)
- Miyazaki S 1995 Calcium signalling during mammalian fertilization. *Ciba Foundation Symposium* **188** 235–247.
- Miyazaki S 2007 Intracellular calcium oscillations in mammalian eggs at fertilization. *Journal of Physiology* **584** 713–714. (doi:10.1113/jphysiol.2007.144238)
- Mohri T, Shirakawa H, Oda S, Sato MS, Mikoshiba K & Miyazaki S 2001 Analysis of $\text{Mn}^{2+}/\text{Ca}^{2+}$ influx and release during Ca^{2+} oscillations in mouse eggs injected with sperm extract. *Cell Calcium* **29** 311–325. (doi:10.1054/ceca.2000.0196)
- Morbeck DE, Krisher RL, Herrick JR, Baumann NA, Matern D & Moyer T 2014 Composition of commercial media used for human embryo culture. *Fertility and Sterility* **102** 759.e759–766.e759. (doi:10.1016/j.fertnstert.2014.05.043)
- Morozumi K, Shikano T, Miyazaki S & Yanagimachi R 2006 Simultaneous removal of sperm plasma membrane and acrosome before intracytoplasmic sperm injection improves oocyte activation/embryonic development. *PNAS* **103** 17661–17666. (doi:10.1073/pnas.0608183103)
- Nomikos M, Blayney LM, Larman MG, Campbell K, Rossbach A, Saunders CM, Swann K & Lai FA 2005 Role of phospholipase C- ζ domains in Ca^{2+} -dependent phosphatidylinositol 4,5-bisphosphate hydrolysis and cytoplasmic Ca^{2+} oscillations. *Journal of Biological Chemistry* **280** 31011–31018. (doi:10.1074/jbc.M500629200)
- Ozil JP & Swann K 1995 Stimulation of repetitive calcium transients in mouse eggs. *Journal of Physiology* **483** 331–346. (doi:10.1113/jphysiol.1995.sp020589)

- Ozil JP & Huneau D 2001 Activation of rabbit oocytes: the impact of the Ca²⁺ signal regime on development. *Development* **128** 917–928.
- Ozil JP, Markoulaki S, Toth S, Matson S, Banrezes B, Knott JG, Schultz RM, Huneau D & Ducibella T 2005 Egg activation events are regulated by the duration of a sustained [Ca²⁺]_{cyt} signal in the mouse. *Developmental Biology* **282** 39–54. (doi:10.1016/j.ydbio.2005.02.035)
- Ozil JP, Banrezes B, Toth S, Pan H & Schultz RM 2006 Ca²⁺ oscillatory pattern in fertilized mouse eggs affects gene expression and development to term. *Developmental Biology* **300** 534–544. (doi:10.1016/j.ydbio.2006.08.041)
- Politi A, Gaspers LD, Thomas AP & Hofer T 2006 Models of IP₃ and Ca²⁺ oscillations: frequency encoding and identification of underlying feedbacks. *Biophysical Journal* **90** 3120–3133. (doi:10.1529/biophysj.105.072249)
- Rice A, Parrington J, Jones KT & Swann K 2000 Mammalian sperm contain a Ca(2+)-sensitive phospholipase C activity that can generate InsP(3) from PIP(2) associated with intracellular organelles. *Developmental Biology* **228** 125–135. (doi:10.1006/dbio.2000.9929)
- Sanusi R, Yu Y, Nomikos M, Lai FA & Swann K 2015 Rescue of failed oocyte activation after ICSI in a mouse model of male factor infertility by recombinant phospholipase C ζ . *Molecular Human Reproduction* **21** 783–791. (doi:10.1093/molehr/gav042)
- Sato MS, Yoshitomo M, Mohri T & Miyazaki S 1999 Spatiotemporal analysis of [Ca²⁺]_i rises in mouse eggs after intracytoplasmic sperm injection (ICSI). *Cell Calcium* **26** 49–58. (doi:10.1054/ceca.1999.0053)
- Saunders CM, Larman MG, Parrington J, Cox LJ, Royse J, Blayney LM, Swann K & Lai FA 2002 PLC ζ : a sperm-specific trigger of Ca(2+) oscillations in eggs and embryo development. *Development* **129** 3533–3544.
- Saunders CM, Swann K & Lai FA 2007 PLC ζ , a sperm-specific PLC and its potential role in fertilization. *Biochemical Society Symposia* **74** 23–36.
- Swann K & Yu Y 2008 The dynamics of calcium oscillations that activate mammalian eggs. *International Journal of Developmental Biology* **52** 585–594. (doi:10.1387/ijdb.072530ks)
- Swann K 2013 Measuring Ca²⁺ oscillations in mammalian eggs. *Methods in Molecular Biology* **957** 231–248.
- Swann K & Lai FA 2013 PLC ζ and the initiation of Ca(2+) oscillations in fertilizing mammalian eggs. *Cell Calcium* **53** 55–62. (doi:10.1016/j.ceca.2012.11.001)
- Takahashi T, Kikuchi T, Kidokoro Y & Shirakawa H 2013 Ca(2+)_i influx-dependent refilling of intracellular Ca(2+)_i stores determines the frequency of Ca(2+)_i oscillations in fertilized mouse eggs. *Biochemical and Biophysical Research Communications* **430** 60–65. (doi:10.1016/j.bbrc.2012.11.024)
- Tesarik J & Sousa M 1995 Key elements of a highly efficient intracytoplasmic sperm injection technique: Ca²⁺ fluxes and oocyte cytoplasmic dislocation. *Fertility and Sterility* **64** 770–776. (doi:10.1016/S0015-0282(16)57853-4)
- van Blerkom J, Cohen J & Johnson M 2015 A plea for caution and more research in the ‘experimental’ use of ionophores in ICSI. *Reproductive BioMedicine Online* **30** 323–324. (doi:10.1016/j.rbmo.2015.02.002)
- Vanden Meerschaut F, Leybaert L, Nikiforaki D, Qian C, Heindryckx B & De Sutter P 2013 Diagnostic and prognostic value of calcium oscillatory pattern analysis for patients with ICSI fertilization failure. *Human Reproduction* **28** 87–98. (doi:10.1093/humrep/des368)
- Wakai T, Vanderheyden V & Fissore RA 2011 Ca²⁺ signaling during mammalian fertilization: requirements, players, and adaptations. *Cold Spring Harbor Perspectives in Biology* **3** a006767.
- Wakai T, Zhang N, Vangheluwe P & Fissore RA 2013 Regulation of endoplasmic reticulum Ca(2+) oscillations in mammalian eggs. *Journal of Cell Science* **126** 5714–5724. (doi:10.1242/jcs.136549)
- Watkins AJ, Platt D, Papenbrock T, Wilkins A, Eckert JJ, Kwong WY, Osmond C, Hanson M & Fleming TP 2007 Mouse embryo culture induces changes in postnatal phenotype including raised systolic blood pressure. *PNAS* **104** 5449–5454.
- Whittingham DG 1971 Culture of mouse ova. *Journal of Reproduction and Fertility* **14** 7–21.
- Yanagida K, Katayose H, Hirata S, Yazawa H, Hayashi S & Sato A 2001 Influence of sperm immobilization on onset of Ca(2+) oscillations after ICSI. *Human Reproduction* **16** 148–152. (doi:10.1093/humrep/16.1.148)
- Yanagimachi R 2005 Intracytoplasmic injection of spermatozoa and spermatogenic cells: its biology and applications in humans and animals. *Reproductive BioMedicine Online* **10** 247–288. (doi:10.1016/S1472-6483(10)60947-9)
- Yu Y, Saunders CM, Lai FA & Swann K 2008 Preimplantation development of mouse oocytes activated by different levels of human phospholipase C ζ . *Human Reproduction* **23** 365–373. (doi:10.1093/humrep/dem350)
- Zoetewij JP, van de Water B, de Bont HJ, Mulder GJ & Nagelkerke JF 1993 Calcium-induced cytotoxicity in hepatocytes after exposure to extracellular ATP is dependent on inorganic phosphate. Effects on mitochondrial calcium. *Journal of Biological Chemistry* **268** 3384–3388.

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