JPSA
Japan Poultry Science Association

http://www.jstage.jst.go.jp/browse/jpsa
doi:10.2141/jpsa.2023001
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Egg Development After *In Vitro* Insemination in Japanese Quail (*Coturnix japonica*)

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In vitro fertilization has been widely used to produce offspring in several mammalian species. We previously successfully produced Japanese quail chicks using intracytoplasmic sperm injection (ICSI), whereas *in vitro* insemination was not successful. This may be due to the difficulties associated with mimicking the sperm-egg fusion process and subsequent events in physiological polyspermic fertilization *in vitro*. In the present study, we observed egg development after *in vitro* insemination and investigated the inactivation of metaphase-promoting factor (MPF) and cytostatic factor (CSF), which are downstream of the Ca^{2+} signaling pathway in the egg, due to fertilizing sperm. We found a sperm number-dependent increase in hole formation caused by sperm penetration of the perivitelline membrane, the extracellular coat surrounding the egg. Egg development was observed following *in vitro* insemination; however, the developmental rate and stages after 24-h culture were inferior to those of ICSI eggs, even when insemination was performed with a high number of sperm (2×10^4). We also noted the downregulation of inositol 1,4,5-trisphosphate receptor-1, ryanodine receptor-3, cyclin B1, and c-MOS, which are important regulatory components of MPF and CSF in the egg, which was dependent on the number of sperm used for insemination. However, the decreases observed in these components did not reach the levels observed in the ICSI eggs. Collectively, the present results suggest that a sperm number higher than 2×10^4 is required for the progression of the Ca^{2+} signaling pathway, which initiates subsequent egg development in Japanese quail.

Key words: Blastoderm, Cytostatic factor, *In vitro* insemination, Japanese quail, Metaphase-promoting factor

J. Poult. Sci., 60: jpsa.2023001, 2023

Introduction

In vitro fertilization (IVF) is one of the most conventional methods of assisted reproductive technology worldwide. The development of *in vitro* insemination techniques has resulted in significant advances in the study of fertilization, with a focus on sperm-egg fusion in mammalian species (Miyado *et al*., 2000; Inoue *et al*., 2005; Jin *et al*., 2011; Ohto *et al*., 2016). Intracytoplasmic sperm injection (ICSI) has also been widely used in human infertility clinics (Palermo *et al*., 1992) and in basic biology research, such as in the production of transgenic mice (Perry *et al*., 1999).

Although IVF has been attempted in birds, limited information is currently available because of the difficulties associated with obtaining a sufficient number of eggs. Howarth (1970) and Nakanishi *et al*. (1990) reported the presence of multiple sperm and the subsequent formation of male pronuclei in the germinal disc of the ovum 2–3 h after *in vitro* insemination in chicken. Tanaka *et al*. (1994) produced viable chicks by implanting ova

Received: April 12, 2022, Accepted: June 29, 2022
Available online: January 25, 2023
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that were inseminated *in vitro* into the oviducts of recipient hens. We previously demonstrated the binding and penetration of multiple sperm into an isolated perivitelline membrane (pvm), the extracellular coat surrounding the egg, in Japanese quail (Sasanami *et al*., 2012; Ichikawa *et al*., 2017). We also successfully produced quail chicks using ICSI and subsequent *ex ovo* culture without implantation into the oviduct (Mizushima *et al*., 2014). Furthermore, we identified phospholipase Czeta1 (PLCZ1), citrate synthase (CS), and aconitase2 (ACO2) as sperm factors released from numerous sperm into the ooplasm to induce fertilization signaling in quail eggs, and demonstrated that the introduction of these factors into ICSI eggs with a single sperm via complementary RNA (cRNA) microinjection mimicked the complete polyspermic fertilization process. However, it is important to note that ICSI omits several important steps in fertilization, such as the sperm-egg fusion process (Mizushima *et al*., 2014).

When ovulated, avian eggs are arrested at metaphase of the second meiotic division (MetII). In mammalian eggs, MetII arrest is maintained by cytostatic factor (CSF), which prevents the ubiquitin-dependent degradation of cyclin B (CCNB), the regulatory subunit of MetII-promoting factor (MPF), by inhibiting anaphase-promoting complex/cyclosome (APC/C) activities. At the time of fertilization, sperm break this arrest by inducing a series of Ca^{2+} spikes (Ca^{2+} oscillations), which persist in the pronuclear formation stage of the fertilization process (Jones, 2005). *PLCZ1* was originally isolated from mice as a sperm-specific factor responsible for inducing Ca^{2+} oscillations (Saunders *et al*., 2002). During fertilization, PLCZ1-generated Ca^{2+} response pathways are mediated by calmodulin-dependent protein kinase II (CaMKII), which directly phosphorylates Erp1, a component of the CSF, thereby inducing the degradation of CCNB (Tatone *et al*., 2002; Tung *et al*., 2005; Schmidt *et al*., 2005; Madgwick *et al*., 2006; Shoji *et al*., 2006; Inoue *et al*., 2007). In addition, CaMKII activates the degradation of c-MOS, the product of the proto-oncogene *c-mos*, which plays a pivotal role in the regulation of CSF activity (Sagata *et al*., 1988). Several studies have implicated the involvement of mitogen-activated protein kinase (MAPK) via MAPK/extracellular signal-regulated protein kinase kinase (MEK) as a downstream factor of c-MOS (the Mos-MEK-MAPK pathway) (Tunquist and Maller, 2003; Yamamoto *et al*., 2008).

We previously investigated the unique Ca^{2+} pattern in fertilizing eggs related to polyspermic fertilization in Japanese quail, and revealed that PLCZ1 was responsible for the induction of the initial transient Ca^{2+} increase, whereas CS and ACO2 were required for long-lasting spiral-like Ca^{2+} oscillations (Mizushima *et al*., 2014). Furthermore, we demonstrated that these initial transient Ca^{2+} increases and spiral-like Ca^{2+} oscillations are mediated by inositol 1,4,5-trisphosphate receptor (ITPR)1/ITPR3 and ryanodine receptor-3 (RYR3), respectively (Mizushima *et al*., 2022). Although two unique Ca^{2+} signaling pathways are considered to inactivate MPF and CSF after fertilization, the role of each pathway in the initiation of meiosis has not yet been elucidated. Therefore, the aim of the present study was to investigate the developmental progression of quail eggs during *ex vivo* culture following *in vitro* insemination. We also examined the expression of MPF and CSF in quail eggs at MetII and the downregulation of Ca^{2+} signaling pathways after ICSI or *in vitro* insemination.

Materials and Methods

Animals

All experimental procedures for the care and use of animals were approved by the Animal Care and Use Committees of Shizuoka University (approval number: 2018A-5), Hokkaido University (approval number: 14-0135), and Shimane University (approval number: MA-30-13).

Male and female Japanese quail, *Coturnix japonica*, 8–20 weeks of age (Quail Cosmos, Tahara, Japan and Muroran Uzuraen, Muroran, Japan), were individually maintained under a photoperiod of 14 h light:10 h dark (lights on at 05:00) with *ad libitum* access to water and a commercial diet (Muroran Uzuraen). A total of 100 female quail were used in this study, of which 20 were used for observation of hole formation and 80 for IVF studies.

In Vitro Insemination and ICSI

Ejaculated semen was collected from male quails immediately before copulation with a teaser female prior to ejaculation (Kuroki and Mori, 1997), and suspended in 1 mL of Hanks' balanced saline solution (HBSS, GE Healthcare Japan Corporation, Hino, Japan). Unfertilized eggs were recovered from the anterior magnum between 0.5 and 2 h after egg oviposition (Mizushima *et al*., 2014) and washed in phosphate-buffered saline (PBS) with gentle rotation at 41.5°C for 1 h after the removal of adhesive albumen. Washed eggs were transferred to 20-mL plastic cups containing 10 mL HBSS heated at 41.5°C, and 100 μL (2×10^2, 2×10^3, or 2×10^4 sperm) of the sperm suspension was placed on the germinal disc. After 30 min incubation at 41.5°C, the eggs were cultured in Dulbecco's modified Eagle's medium as previously described by Ono *et al*. (1994) and Mizushima *et al*. (2014).

ICSI-treated eggs served as the control group to observe normal development for comparison. All procedures used for the preparation of sperm-derived egg activation factors (*PLCZ1*, *CS*, and *ACO2* cRNAs) and microinjections were performed as previously described (Mizushima *et al*. 2014, 2022).

Staging of Embryo Development and Whole-Mount DAPI Staining

After 24 h of culture, the stages of blastoderm development were classified under a stereomicroscope according to the classification system of Eyal-Giladi and Kochav (1976). Blastoderms were then dissected from the yolk, fixed in an ethanol/glacial acetic acid (3:1) solution, and stained with DAPI, as previously described (Mizushima *et al*., 2009), to discriminate between normal and pseudo-development (Olszańska *et al*., 2002; Mizushima *et al*., 2009).

Observation of Holes on the pvm After In Vitro Insemination

Thirty minutes after *in vitro* insemination, a piece of the pvm, approximately 5 mm in diameter, located on the germinal disc was cut and washed with PBS. The pvm was then transferred

onto a glass slide and stained with Schiff's reagent after fixation with 3.7% (v/v) formaldehyde in PBS. Holes on the pvm were photographed at 200× magnification under a light microscope (E800; Nikon, Tokyo, Japan), and the number of holes per 3-mm^2 area was calculated from Image J software.

Western Blot Analysis of CCNB1, c-MOS, ITPR1, and RYR3

To investigate the progression of molecular signaling in the meiotic resumption of fertilized eggs, germinal discs were collected after 3 h culture and dissolved in intracellular-like medium (120 mM KCl, 0.1 mM EGTA, 10 mM Na-β-glycerophosphate, 0.2 mM PMSF, 1 mM DTT, and 20 mM HEPES–NaOH, pH 7.5) by homogenization according to the method described by Mizushima *et al*. (2022). The supernatant was collected by centrifugation at 10,000 ×*g* for 10 min, which was used as the egg extract. The protein concentrations in the samples were measured using a bicinchoninic acid protein assay kit (Wako Pure Chemical Industries Ltd., Osaka, Japan).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed under reducing conditions as previously described (Laemmli, 1970) using an 8% or 12% (w/v) gel for resolving and a 5% (w/v) polyacrylamide gel for stacking. Ten micrograms of proteins were loaded per lane, resolved by SDS-PAGE, and transferred onto a polyvinylidene fluoride membrane (Millipore, Bedford, MA, USA). After incubation with blocking buffer using a detector block (SeraCare Life Sciences, Milford, MA, USA), the membrane was incubated at 4°C overnight with a rabbit anti-human cyclin B1 antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) or rabbit anti-c-MOS antibody (Bioss, Inc., Tokyo, Japan). Rabbit anti-rat ITPR1 antibody (Alomone Labs Ltd., Jerusalem, Israel) and mouse anti-chicken RYR antibody (GeneTex, Inc., Irvine, CA, USA) were used to detect ITPR1 and RYR3, respectively. An anti-chicken γ-tubulin (TUBG) monoclonal antibody (Sigma-Aldrich, St. Louis, MO, USA) was used as the internal control. Immunoreactive signals were visualized using a chemiluminescent technique (Millipore) with a goat anti-rabbit or donkey anti-mouse secondary antibody coupled with horseradish peroxidase. Chemiluminescence was detected using a LAS 3000 system (GE Healthcare, Madison, WI, USA). The visualized blots were digitized using ImageJ 1.48v software (National Institutes of Health, Bethesda, MD, USA).

Statistical Analysis

Protein expression levels were normalized to TUBG protein levels as an internal control. Data are expressed as mean ± standard deviation and were analyzed for significant differences using analysis of variance. The means were compared between groups using Tukey's *post-hoc* test. Differences were considered statistically significant at $P < 0.05$.

Results

Development of Quail Eggs and Hole Formation on the pvm After In Vitro Insemination

The number of holes in eggs inseminated with different sperm concentrations increased in a sperm number-dependent manner (Fig. 1). Consistent with this result, blastoderm development also improved with an increase in the number of sperm used for insemination. As shown in Table 1, none of the eggs inseminated with 2×10^2 sperm developed after culture for 24 h. In contrast, 2 out of 10 eggs developed into stages III-V with insemination of 2×10^3 sperm, and 6 out of 14 eggs (43%) showed blastoderm development between stages V and VIII after 24 h of culture following insemination with 2×10^4 sperm.

Downregulation of MPF and CSF Expression in Quail Eggs After ICSI

To investigate the role of MPF and CSF in the maintenance of MetII arrest in quail eggs, the protein expression of CCNB1 and c-MOS, which are indicators of the regulatory subunits of MPF and MAPK signaling, respectively, was examined. Western blot analysis showed that anti-CCNB1 and anti-c-MOS reacted with proteins of approximately 50 kDa and 43 kDa, respectively (Fig. 2A), corresponding with the sizes of the frog and mouse counterparts (Castro *et al*., 2001; Cao *et al*., 2020). These signals were not detected when the blots were incubated with normal rabbit IgG. We detected immunoreactive ITPR1 and RYR3, which may be involved in the induction of transient Ca^{2+} increases and spiral-like Ca^{2+} oscillations, respectively. Bands of approximately 270 kDa and 500 kDa were detected using anti-ITPR1 and anti-RYR3 antibodies, respectively. These bands also did not react with normal rabbit or mouse IgG antibody.

To investigate the inactivation of CCNB1 and c-MOS during egg activation, we examined the expression levels of these proteins 3 h after ICSI, together with the expression of sperm-

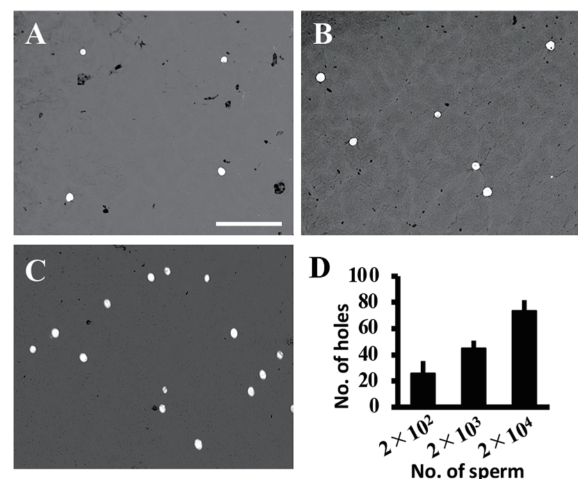

Fig. 1. **Hole formation on the pvm located on the germinal disc following *in vitro* insemination with different sperm numbers.** (A–C) The pvm was isolated from an egg incubated with 2×10^2 (A), 2×10^3 (B), or 2×10^4 (C) sperm, subjected to Schiff's staining, and observed under a light microscope. (D) The number of holes per 3 mm^2 area was calculated and expressed as mean ± SD. Values were obtained from three samples. Scale bar = 200 μm.

Table 1. **Blastoderm development after *in vitro* insemination at 24 h of culture**

No. of sperm inseminated	No. of eggs		No. of embryos								
			Developed to the stage* of								
	Treated	Developed (%)	II	III	IV	V	VI	VII	VIII	IX	X
ICSI	6	5 (83)							2	1	2
2×10^2	7	0 (0)									
2×10^3	10	2 (20)		1		1					
2×10^4	14	6 (43)				2	1	2	1		

*Developmental stages were determined according to the classification system of Eyal-Giladi and Kochav (1976)

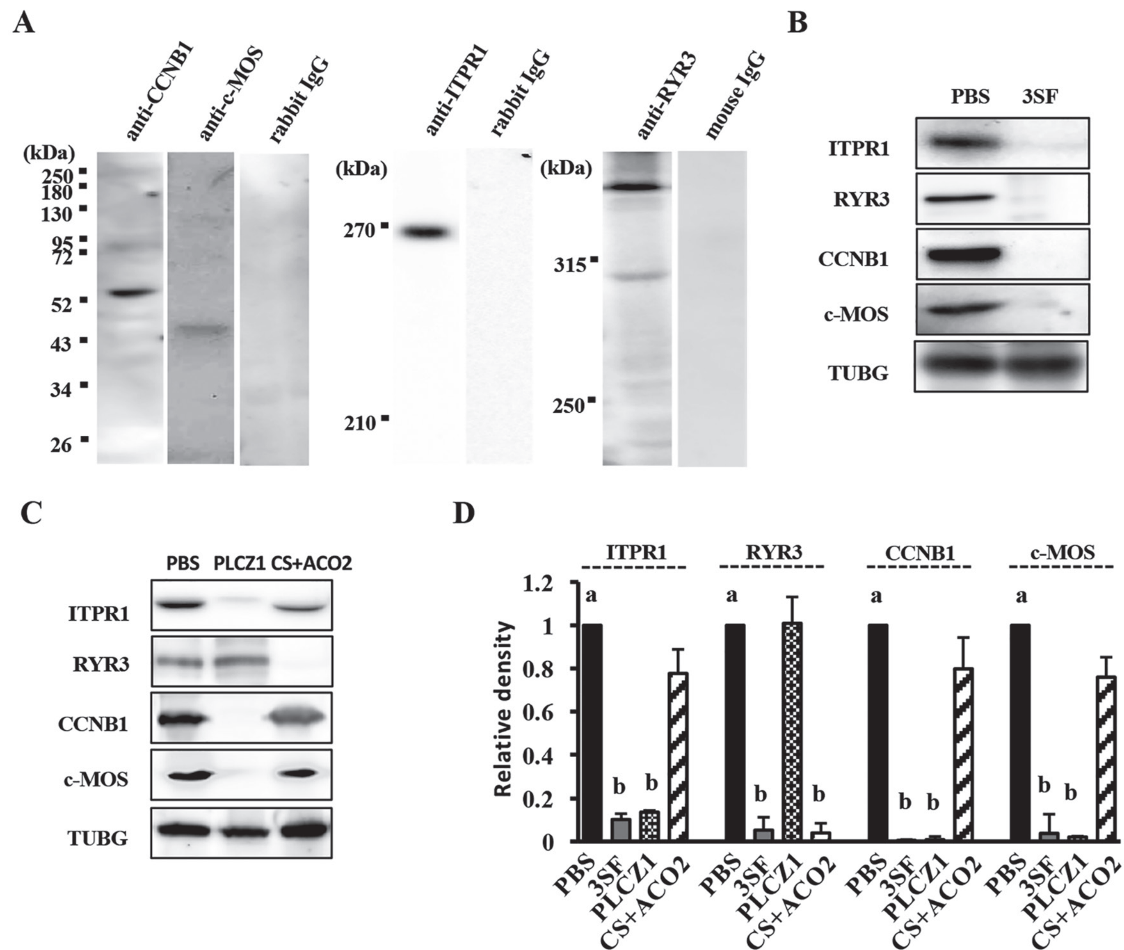

Fig. 2. **Presence of CCNB1, c-MOS, ITPR1, and RYR3 proteins in quail eggs and their down-regulated expression after ICSI.** (A) Detection of CCNB1, c-MOS, ITPR1, and RYR3 proteins in ovulated quail eggs. (B) Western blot analysis 3 h after ICSI with injection of PLCZ1, CS, and ACO2 cRNA or the solvent alone. (C) Western blot analysis 3 h after ICSI with PLCZ1 cRNA alone, CS and ACO2 cRNAs, or the solvent alone. (D) Quantification of immunoreactivity after ICSI. Band intensities were quantified and expressed as the means ± standard deviations of three samples. Values with different letters are significantly different ($P < 0.01$).

borne egg-activating factors. As shown in Fig. 2B and 2D, the intensities of the bands for CCNB1 and c-MOS proteins were both significantly weaker than those from eggs injected with solvent (control) 3 h after ICSI. This degradation was also observed for ITPR1 and RYR3, consistent with the findings reported by Mizushima *et al*. (2022). Similar degradation of CCNB1 and c-MOS proteins was observed 3 h after ICSI with *PLCZ1* cRNA, whereas it did not occur in ICSI eggs with injection of *CS* and *ACO2* cRNAs (Fig. 2C and 2D). These results indicate that the initial PLCZ1-induced Ca^{2+} wave was responsible for the inactivation of MPF and CSF during initial egg development in quail. Downregulation of RYR3 expression was only induced

by injection of *CS* and *ACO2* cRNAs, as previously reported by Mizushima *et al*. (2022).

MPF and CSF Activities in Quail Eggs After In Vitro Insemination

Figure 3A–C show changes in ITPR1, RYR3, CCNB1, and c-MOS protein expression levels in eggs 3 h after *in vitro* insemination with different sperm concentrations. Figure 3D shows the quantification of the changes observed in the band intensities for these proteins presented as the relative density to the internal control TUBG. Decreases in ITPR1 and RYR3 protein levels were dependent on the number of sperm used for insemination (Fig. 3A–D). However, these degradation levels were lower than those of eggs treated with ICSI. In addition, down-regulation of CCNB1 and c-MOS protein expression was observed when insemination was performed using 2×10^4 sperm, which was also less than the degree of downregulation observed in ICSI-treated eggs (Fig. 3C and 3D).

Discussion

The present study revealed the blastoderm development in ovulated Japanese quail eggs inseminated *in vitro*. The overall rate of embryo development after *in vitro* insemination was 20–43%, which is similar to or higher than the rates reported by Olszańska *et al*. (2002) and Batellier *et al*. (2003), with 15% of inseminated eggs developing in quail or chicken. Furthermore, egg development to blastoderm stage VIII was improved over that achieved in previous studies (Stage VI; Olszańska *et al*., 2002; Batellier *et al*., 2003). These results indicate that eggs that ovulate spontaneously and are subsequently recovered from the oviduct are an appropriate source for avian IVF. However, despite the advantage of conventional *in vitro* insemination in retaining the physiological conditions to a greater extent, the rate and progression of embryo development with *in vitro* insemination were markedly lower than those of eggs treated with ICSI, as reported by Mizushima *et al*. (2014). This may be due to a failure in breaking meiotic arrest, given that the incidence of CCNB1

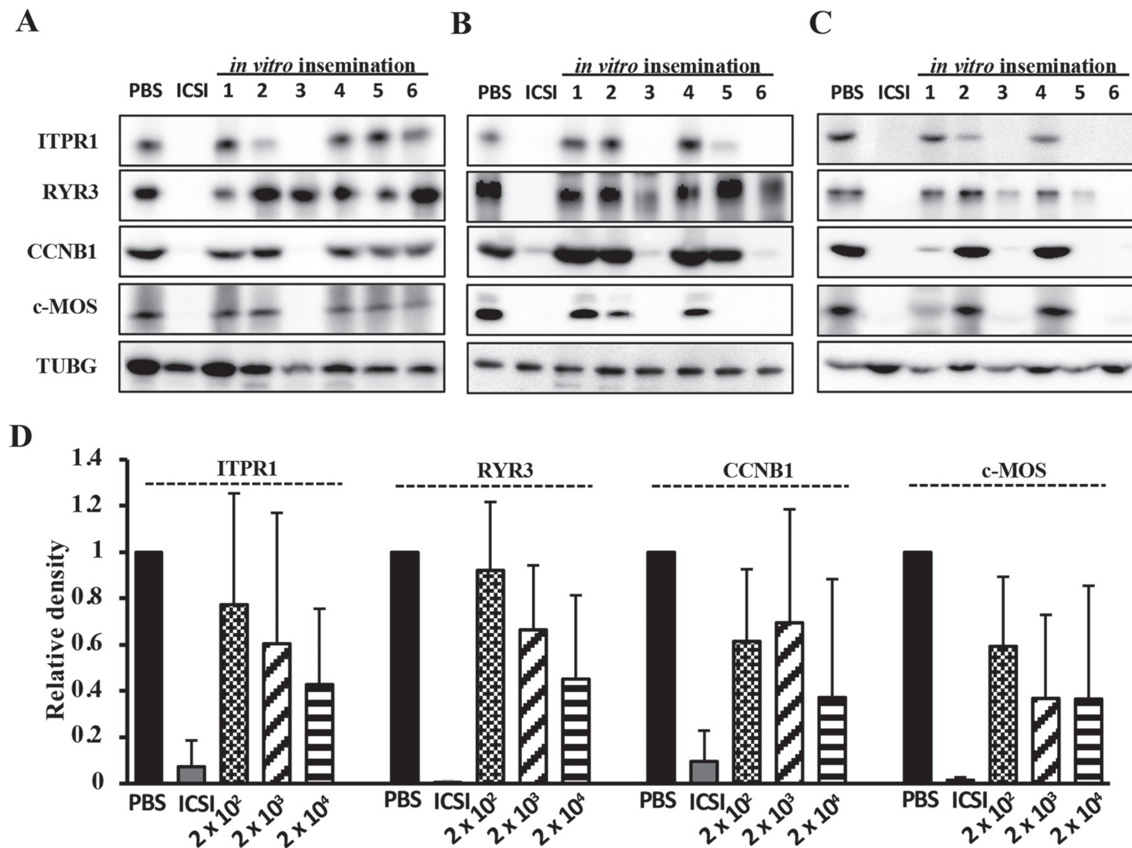

Fig. 3. **Changes in ITPR1, RYR3, CCNB1, and c-MOS protein expression levels in quail eggs following *in vitro* insemination with different sperm numbers.** (A–C) Western blot analysis 3 h after in vitro insemination with 2×10^2 (A), 2×10^3 (B), or 2×10^4 (C) sperm, respectively. Numbers 1–6 represent each of the six eggs treated with sperm. Egg extracts after ICSI or PBS treatment were used as the control. (D) Quantification of immunoreactivity after in vitro insemination. Band intensities were quantified and are expressed as means ± standard deviations. The sample number of PBS- and ICSI-treated eggs is three each. Band intensities in these three samples were measured in each membrane.

breakdown of all *in vitro* insemination groups was lower than that of the ICSI eggs. The fertilization rate of only 12–20% using ICSI with a single sperm reported by Hrabia *et al*. (2003) was improved to more than 70% by the endowment of three sperm factors contained in 200 sperm, which generated an initial transient Ca^{2+} increase and subsequent long-lasting spiral-like Ca^{2+} oscillations in the ooplasm (Mizushima *et al*., 2014). This finding indicates that the average of 70 holes made by sperm on the pvm after insemination with 2×10^4 sperm is insufficient to induce an increase in $[Ca^{2+}]i$ in quail eggs. RYR3, which is essential for inducing spiral-like Ca^{2+} oscillations, was not completely degraded in five out of six eggs after insemination, even with 2×10^4 sperm, suggesting that the processing of events related to cell cycle progression in fertilizing eggs was incomplete in the *in vitro*-inseminated eggs. Although it is technically difficult to clarify the position and number of sperm in the germinal disc after *in vitro* insemination, further detailed observations of spatiotemporal changes in Ca^{2+} increases in avian eggs following natural fertilization or *in vitro* insemination are required.

This study also provides the first demonstration that PLCZ1-generated transient Ca^{2+} increases were sufficient to disrupt MPF activity in Japanese quail eggs, based on evidence of the degradation of CCNB1 and ITPR1. The induction of CCNB1 degradation was confirmed by the microinjection of *PLCZ1* cRNA alone (Fig. 2C and 2D). We previously reported a higher rate of female pronuclear formation following microinjection of *PLCZ1* cNRA alone, and no female pronuclear formation by *CS* and *ACO2* cRNAs injection, which supports this hypothesis (Mizushima *et al*., 2009, 2014, 2022). In mouse eggs microinjected with a green fluorescent protein (GFP)-fused *CCNB1* cRNA chimeric construct, degradation of GFP-fused CCNB1 was reported approximately 10 min after the first sperm-induced Ca^{2+} spike (Nixon *et al*., 2002). In Japanese quail eggs, the degradation of ITPR1 was noted within 30 min after *PLCZ1* cRNA microinjection, when transient Ca^{2+} increases were terminated, suggesting that the initiation of immediate CCNB1 degradation ensures meiotic exit (Mizushima *et al*., 2022). Although limited information is currently available on the degradation of CCNB1 in avian eggs, c-MOS destruction is clearly accelerated by PLCZ1, which is consistent with ITPR1 degradation, indicating the presence of APC/C-mediated ubiquitination.

In summary, the present results demonstrate blastoderm development by *in vitro* insemination in ovulated Japanese quail eggs. Since delayed development of *in vitro*-inseminated eggs was observed, sperm numbers higher than 2×10^4 may be required for the progression of the Ca^{2+} signaling pathway to achieve full-term development to hatching in Japanese quail.

Acknowledgments

This study was supported by a Grant-in-Aid for Scientific Research (C) (General) (19K06363 to SM).

Author Contributions

Yoshinobu Ichikawa, Shusei Mizushima, and Tomohiro Sasanami conducted the experiments and wrote the paper. Noritaka Hirohashi helped in analyzing the data and writing the paper. All the authors approved the final manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

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