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Abstract: Regardless of the presence of sperm-borne oocyte-activating factors, activation of bovine oocytes with exogenous activation stimuli is required for further development after intracytoplasmic sperm injection (ICSI). The present study was designed to develop a new activation regimen for improving the blastocyst yield after ICSI of bovine oocytes harvested from ovaries stored at 10-12 oC for 24 h. Following ICSI, oocytes were treated with 5 μM ionomycin for 5 min, 7% ethanol for 5 or 10 min, ionomycin followed by ethanol (5 or 10 min), ionomycin followed by 10 μg/mL Cycloheximide for 5 h, or ionomycin followed by 1.9 mM 6-dimethylaminopurine for 3 h. Across the activation regimens, the cleavage rates of ICSI oocytes (45-77%) were higher than those of parthenogenetically activated oocytes (11-21%; $P<0.05$). Activating the ICSI oocytes with ionomycin plus ethanol improved the blastocyst yield (29-30%) comparing to the non-treated oocytes (12%; $P<0.05$) but the other regimens did not (9-18%; $P>0.05$). The higher blastocyst yields were due to increasing the proportion of ICSI oocytes that passed through the early post-fertilization events until cleavage. None of the regimens have any adverse effect on the quality of the blastocysts regarding the total cell number or the proportion of the inner cell mass cells. Thus, a

new activation regimen composed from two triggers for single calcium increase has been proven effective to improve the blastocyst yield after bovine ICSI using oocytes harvested from stored ovaries.

*** Revision Notes**

Dear Dr. John P. Kastelic,

Thank you very much for prompt reply and accepting our manuscript for publication in Theriogenology. We have replied for each comment from Co-editor and the reviewer-2 in point-by-point basis.

Sincerely yours,
Shinichi Hochi (Corresponding author)
Hany Abdalla (First author)

Co-editor

1- P value has been added to the Abstract (Lines 27,29).
2- The number of the references in Introduction section has been reduced.

Reviewer #2

1- Regarding omitting the two groups (5 min ethanol group and ionomycin followed by 5 min ethanol group); We do not think that the presence of these two groups causes any difficulty to recognize the superiority of ionomycin and ethanol combination. Since activation of 7% ethanol for 5 min 4 hr after ICSI is a commonly applied method for bovine ICSI, presence of such group must be important to declare the superiority of the new combination (ionomycin and ethanol) over the methods of ethanol alone under the same experimental circumstance regarding oocyte quality, injection skill, culture condition, and so on. For the ionomycin followed by 5 min ethanol, the efficiency of this method to improve the cleavage and the blastocyst yield was similar to that of ionomycin followed by 10 min ethanol, making it possible to choose the method in which oocytes are exposed to minimum exogenous stimuli.
2- The time of 2nd polar body detection has been added in footnote of Tables 1 and 2.
3- Figure legend (Line 435) has been corrected.

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Revised

A combined treatment of ionomycin with ethanol improves blastocyst development of bovine oocytes harvested from stored ovaries and microinjected with spermatozoa

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Running head: Bovine oocyte activation after ICSI

Abstract

Regardless of the presence of sperm-borne oocyte-activating factors, activation of bovine oocytes with exogenous activation stimuli is required for further development after intracytoplasmic sperm injection (ICSI). The present study was designed to develop a new activation regimen for improving the blastocyst yield after ICSI of bovine oocytes harvested from ovaries stored at 10–12 ^{o}C for 24 h. Following ICSI, oocytes were treated with 5 μM ionomycin for 5 min, 7% ethanol for 5 or 10 min, ionomycin followed by ethanol (5 or 10 min), ionomycin followed by 10 μg/mL Cycloheximide for 5 h, or ionomycin followed by 1.9 mM 6-dimethylaminopurine for 3 h. Across the activation regimens, the cleavage rates of ICSI oocytes (45–77%) were higher than those of parthenogenetically activated oocytes (11–21%; $P<0.05$). Activating the ICSI oocytes with ionomycin plus ethanol improved the blastocyst yield (29–30%) comparing to the non-treated oocytes (12%; $P<0.05$) but the other regimens did not (9–18%; $P>0.05$). The higher blastocyst yields were due to increasing the proportion of ICSI oocytes that passed through the early post-fertilization events until cleavage. None of the regimens have any adverse effect on the quality of the blastocysts regarding the total cell number or the proportion of the inner cell mass cells. Thus, a new activation regimen composed from two triggers for single calcium increase has been proven effective to improve the blastocyst yield after bovine ICSI using oocytes harvested from stored ovaries.

Keywords: Bovine ICSI; Blastocyst yield; Ionomycin; Ethanol; Stored ovary.

1. Introduction

Intracytoplasmic sperm injection (ICSI) has been used to investigate fundamental aspects of the fertilization process [1,2], to overcome some forms of male infertility in human [3] and to produce transgenic animals [4]. The ICSI procedure has resulted in birth of live offspring in many species [5]. Since the first attempt of bovine ICSI [6], the developmental potential of the ICSI oocytes into blastocysts in vitro or live calves in vivo still low, unstable and far from satisfactory regardless of the numerous efforts [7–16]. Beside the technical difficulties caused by darkness of the ooplasm, large size of the sperm heads, and elasticity and toughness of the oolemma, the inability of the mechanical stimulation and/or the injected spermatozoon itself to induce proper oocyte activation after ICSI [17] may be responsible for this low blastocyst yields.

It has been reported that the pattern of calcium oscillations in bovine ICSI oocytes was abnormal [18] and the inactivation of the maturation promoting factors (MPF) occurred temporary [15]. This led to a hypothesis that the release and/or the activation of the sperm-borne oocyte activating factor (SOAF) are compromised after ICSI. This hypothesis is supported by the low blastocyst yields after ICSI without any exogenous activation stimuli [8,10,11,13–16]. To improve the blastocyst yields after bovine ICSI, additional exogenous activation stimuli that induce intracellular calcium spike such as direct current [19], calcium ionophore [20], ionomycin [8,13,14,16,17] or ethanol [9–11,15,16] have been applied. However, the monotonic calcium increase triggered by these stimuli [21,22] was insufficient to completely inactivate the MPF due to re-accumulation of the cyclin B [23] and brought the oocytes to arrest again at the M-III stage [17,23,24]. To avoid this phenomenon, these triggers have been accompanied with other chemicals that can directly or indirectly inactivate the MPF without changing the intracellular calcium profiles.

Cycloheximide (CHX) as a protein synthesis inhibitor [13] or 6-dimethylaminopurine (6-DMAP) as a protein kinase inhibitor [8,14,16] are often used for this purpose.

Great disadvantage for using the protein kinase inhibitors or the protein synthesis inhibitors is that these inhibitors do not specifically inhibit the activity of a particular kinase or the synthesis of a specific protein that control the cell-cycle progression. But they inhibit the activity of several kinases or the synthesis of several proteins that may be involved in other cell functions, whose inhibition may have a deleterious effect on the subsequent cellular events after oocyte activation [25]. Moreover, the calcium oscillations triggered by the sperm cells function not only in inducing resumption of meiosis but also in many other events [26]. For example, recruitment of specific maternal RNAs [27,28] which is essential for activation of zygotic genome [29] and may be extended to other unknown functions. In addition, using bovine oocytes harvested from ovaries stored for 26–30 h resulted in the great reduction of the blastocyst yield after ICSI [30], though the developmental potential of these oocytes after in vitro fertilizaton (IVF) or somatic cell nuclear transplantation was not impaired [30,31]. Immediate transportation of bovine ovaries to the laboratories is restricted in Japan as a result of special examination applied to avoid spreading of bovine spongiform encephalopathy. If the oocytes harvested from the stored ovaries have similar developmental potential to those harvested from freshly collected ovaries, application of ovarian storage can be extended to overcome a long distance between the slaughterhouse and the laboratory or to control the time schedule of the experiment.

Therefore, the present study was designed to develop a new activation regimen without using either protein synthesis or protein phosphorylation inhibitors, that would be efficient to improve the blastocyst yield after sperm microinjection of bovine oocytes harvested form stored ovaries.

2. Materials and Methods

2.1. Chemicals and media

Unless otherwise stated, all chemicals used in this study were purchased from Sigma-Aldrich Chemicals (St. Louis, MO, USA). Modified Brackett and Oliphant (mBO) medium (IVF100; Institute for Functional Peptides, Yamagata, Japan) was used for sperm preparation. Hepes-buffered TCM-199 (Earle's salt; Gibco BRL, Grand Island, NY, USA) supplemented with 0.2 mM sodium pyruvate and 50 μg/mL gentamycin sulfate, referred hereafter as TCM-199, was supplemented with 10% fetal bovine serum (FBS; SAFC Biosciences, Lenexa, KS, USA), 0.002 AU/mL FSH (Kawasaki-Mitaka Pharmaceutical, Kanagawa, Japan) and 1 μg/mL 17ß-estradiol for in vitro maturation (IVM) medium, or with 3 mg/mL bovine serum albumin (BSA) with or without 1000 IU/mL hyaluronidase for denuding or handling oocytes in atmosphere condition, respectively. For short-term culture of oocytes before, during and after ICSI, TCM-199 supplemented with 5% FBS was used. The modified synthetic oviduct fluid (mSOF) [32] supplemented with 30 μL/mL essential amino acids solution (x 50, Gibco-0270), 10 μL/mL non-essential amino acids solution (x 100, Gibco-11140) and 5% FBS was used as culture medium for parthenogenetic and ICSI oocytes.

2.2. Oocyte collection and maturation

Abattoir-derived bovine ovaries were transported to the laboratory in 10–12 ºC saline within 24 h after slaughter. The contents of 2–8 mm follicles were aspirated and oocytes surrounded with at least two layers of compact cumulus cells were matured for 22 h at 38.5 ºC under 5% CO_2 in air. Oocytes were freed from the cumulus cells by a brief Vortex-mixing in the TCM-

199/BSA/hyaluronidase medium. Oocytes with an extruded first polar body were defined as matured, and cultured in the TCM-199/FBS at 38.5 ºC under 5% CO_2 in air until they were used for ICSI (<4 h).

2.3. Intracytoplasmic sperm injection (ICSI)

Commercially available frozen semen from a Japanese Black bull (2–3 x 10^7 sperm cells per 0.5-mL straw) was thawed in water bath at 37 ºC for 30 sec, and the content was layered on percoll density gradient consisting of 2 mL of 45% percoll above 2 mL of 90% percoll in a 15-mL conical tube. The tube was centrifuged for 20 min at 700 g, and the pellet was resuspended in mBO medium supplemented with 5 mM theophylline and washed twice for 5 min at 300 g each. The sperm pellet was treated with 5 mM Dithiothreitol in mBO medium for 20 min at 37 ºC. The sperm cells were washed twice by mBO medium for 5 min at 300 g each. Immediately before ICSI, 2 μL from the sperm suspension were mixed with 8 μL of M2 medium containing 10% polyvinylpyrrolidone (PVP) [33].

The ICSI was performed with a piezo-driven micromanipulator (PMAS-CT150; PrimeTech, Ibaraki, Japan), according to the method described previously [11]. Briefly, single spermatozoon was immobilized by applying several piezo pulses to the midpiece before being aspirated tail first into a blunt-ended injection pipette with an outer diameter of 7–9 μm. An oocyte was held with the holding pipette as the polar body was located either at 6 or 12 o'clock position. The zona pellucida was drilled by several piezo pulses (speed 2, intensity 2). The spermatozoon was repositioned to the tip of the injection pipette, and the injection pipette was advanced mechanically deep into the center of the oocyte, stretching the oolemma extensively. Upon application of a single piezo pulse (speed 2,

intensity 2), the oolemma was punctured at the pipette tip. The sperm was gently injected into the ooplasm and the injection pipette was withdrawn.

2.4. Experimental design for oocyte activation

Sperm-injected oocytes were allocated to one of the following treatments: (1) Non-treated; Injected oocytes were incubated in 100-μL microdrops of the TCM-199/FBS medium at 38.5 ºC under 5% CO_2 in air for 4 h. (2) Ethanol alone [10]; Injected oocytes were incubated for 4 h as described above, and then treated with 7% ethanol in TCM-199 supplemented with 1 mg/mL PVP for 5 or 10 min. (3) Ionomycin alone; Injected oocytes were treated with 5 mM ionomycin in PBS for 5 min immediately after ICSI. (4) Ionomycin + ethanol; Injected oocytes were first treated with ionomycin immediately after ICSI as described above, and incubated in the TCM-199/FBS medium for 4 h. Next, the oocytes were treated with 7% ethanol for 5 or 10 min, as described above. (5) Ionomycin + CHX [13]; Injected oocytes were first treated with ionomycin immediately after ICSI as described above, and followed by incubation in culture medium containing 10 μg/mL CHX for 5 h. (6) Ionomycin + 6-DMAP [24]; Injected oocytes were first treated with ionomycin immediately after ICSI as described above, and incubated in the TCM-199/FBS medium for 3 h. Next, the oocytes were incubated in culture medium containing 1.9 mM 6-DMAP for an additional 3 h. The presence of the second polar body was recorded immediately before transferring the oocytes into culture medium (4 h after ICSI), except for the oocytes treated with ionomycin + CHX (5 h after ICSI when the oocytes were released from the CHX treatment) and ionomycin + 6-DMAP (3 h after ICSI when the oocytes were exposed to the 6-DMAP-containing medium).

For parthenogenetic development, matured oocytes were incubated in TCM-199/FBS medium until they reach the same age of the sperm-injected oocytes at the time of activation, and then

treated with ionomycin alone, ethanol alone for 5 or 10 min, ionomycin + ethanol for 5 or 10 min, ionomycin + CHX, or ionomycin + 6-DMAP, as described above. In addition, matured oocytes were injected with an appropriate volume of sperm holding medium and handled as the non-treated group.

2.5. In vitro culture and assessment of ICSI-derived blastocysts

After activation treatments, the sperm-injected, sham-injected or parthenogenetically-activated oocytes were transferred to the mSOF medium and cultured at 39 ^{o}C under 5% CO_2, 5% O_2 and 90% N_2. The Day-0 was defined as to the day of ICSI or parthenogenetic activation. The cleavage rates were recorded on Day-3 and the developmental rates into blastocysts were recorded up to Day-8.

Differential cell staining for the inner cell mass cells (ICM) and the trophoectoderm cells (TE) was applied for qualitative analysis of ICSI-derived Day-7 expanding blastocysts, as reported previously [34]. Briefly, the TE cells of the harvested blastocysts were permeabilized and stained via incubating the embryos in PBS containing 0.5% Triton X-100 and 100 μg/mL propidium iodide (PI) for 30 sec at 38 ^{o}C. The ICM cells of the blastocysts were then counterstained via incubating the embryos in 25 μg/mL Hoechst 33342 dissolved in ethanol for 2–3 h at 4 ^{o}C. The blastocysts were washed once in glycerol containing 100 mg/mL 1,4-diazabicyclo[2.2.2]octane as an antifade before being mounted on a glass slide with the same medium. Under the epifluorescence microscope, ICM cell nuclei labeled with bisbenzimide appeared blue and the TE cell nuclei labeled with PI and bisbenzimide appeared pink.

2.6. Statistical analysis

Five to nine replicates were performed in each ICSI group and four to six replicates in each parthenogenetic group. Proportional data for extrusion of the second polar body, cleavage, development into blastocysts and ICM ratio were arcsin-transformed and analyzed by one-way ANOVA. Differences among the means in different groups were compared by Bonferroni's post-hoc multiple comparison test. A value of $P<0.05$ was considered statistically significant.

3. Results

In vitro developmental potential of bovine ICSI oocytes with or without different activation treatments is shown in Table 1. Treating the oocytes with ionomycin immediately after ICSI improved the proportions of the oocytes extruding the second polar body (71–84%) compared to those in the other groups (45–58%). The cleavage rates of ICSI oocytes treated with ionomycin plus ethanol or CHX (69–78%) were higher than that of non-treated oocytes (39%). However, treating the oocytes with ethanol alone, ionomycin alone or ionomycin plus 6-DMAP did not improve the cleavage rate (45–61%). Although the proportions of the cleaved zygotes developed into blastocysts until Day-8 were statistically similar among all groups (19–43%), these proportions were obviously lower in ionomycin alone, ionomycin + CHX or ionomycin + 6-DMAP groups (19, 24 or 21%, respectively) than in ionomycin + ethanol groups (38–43%). Moreover, ionomycin plus ethanol treatment significantly improved the proportions of ICSI oocytes developing into blastocysts until Day-8 (29–30%) compared to non-treated oocytes (12%). Quality analysis of Day-7 expanding blastocysts by differential cell counting (Figure 1) indicates that there were no significant differences in the total cell number or the ratio of the ICM cells between the blastocysts derived from non-treated oocytes (128 and 37%, respectively) and those derived after different activation

205 treatment (108–135 and 30–39%, respectively), though a significant difference was found in the ratio of the ICM cells between ionomycin + 10 min ethanol and ionomycin + 5 min ethanol groups.

Parthenogenetic development of bovine oocytes after physical or chemical activation treatments is shown in Table 2. Based on the observation immediately before treatments with ethanol alone, no spontaneous extrusion of the second polar body occurred in matured oocytes at 210 least up to 26 h after IVM. Sham injection with sperm-holding medium resulted in extrusion of the second polar body in 20% of oocytes 4 h after the injection which was lower than those extruding the polar body after ionomycin treatment (54–77%). Very few sham-injected oocytes (3%) cleaved while higher proportions of oocytes (11–21%) cleaved after different activation regimens. In all groups, parthenogenetic development into blastocysts was rarely observed. Under the same 215 activation regimen, higher cleavage rate and blastocyst yield were noted in ICSI oocytes (Table 1) compared to parthenogenetic oocytes (Table 2).

4. Discussion

220 In the present bovine ICSI study, the blastocyst yield achieved after a combined activation treatment with ionomycin and ethanol (30%) is comparatively higher compared to that reported by Matsukawa *et al.* [30] (8%) where the oocytes were harvested from stored ovaries. This blastocyst yield is comparable to or even higher than those in many reports where bovine oocytes harvested from freshly collected ovaries were used for ICSI [8,10,11,13–16]. Simultaneously with the ICSI 225 experiments in the present study, high blastocyst yield (40 ± 3%, 90/223) was obtained after routine IVF protocol, suggesting that oocytes harvested from stored ovaries carry sufficient in vitro developmental potential after IVF and ICSI if they were subjected to appropriate activation regimen. Although oocytes collected from fresh ovaries were not applicable to this study, it is reasonable to

postulate that the new activation regimen reported here could be efficient to improve the ICSI-derived blastocyst yield from those oocytes.

Comparison between sperm-injected and sham-injected oocytes regarding the polar body extrusion, cleavage rate and blastocyst development confirms that the physical stimulation is not sufficient to induce oocyte activation in bovine species. The blastocyst yield in non-treated group (12%) is higher than those (0–8%) reported previously by several laboratories [8,10,11,14–16], but comparable to or even lower than those (15–23%) in other reports [12,13]. However, blastocysts/ cleavage rate in our study (30%) is comparable to that reported by Wei and Fukui [12] (32%) which indicates that a failure in the early post-fertilization events -from fertilization until cleavage- is the main determiner to the successful development after bovine ICSI. Application of additional activation stimuli to the bovine ICSI oocytes have been considered important for decondensation of sperm heads, pronuclear formation, cleavage and embryonic development [8,35]. Since induction of abnormally high frequent and/or prolonged calcium elevation may trigger premature termination of embryonic development [36], the additional activation stimuli better to mimic the mechanism induced during normal fertilization without affecting other oocytes metabolic pathway. Ooplasmic injection of a promising SOAF candidate, phospholipase C zeta or its cRNA [37] was applied as a more physiological approach, but complicated steps required for isolation of this substance or preparation of its cRNA as well as difficulty to control the injected volumes make this method far from practical.

A simplest regimen proved effective to improve blastocyst production after bovine ICSI, using only a trigger for inducing a single calcium increase in oocytes, was to treat the oocytes 4 h after ICSI with 7% ethanol solution for 5 min [10,11,15,16]. Our blastocyst yield by this protocol (13%) was comparable to those reported by some previous literatures (14–21%) [10,11,15] but lower than that reported by Oikawa *et al.* (29%) [16]. This protocol was originally applied to the ICSI oocytes

that extruded the second polar body; in another word, the oocytes that received activation stimuli - from the sperm- enough for the resumption of meiosis. Fujinami *et al*. [15] reported that bovine oocytes showed re-elevation of MPF at 6 h after sperm injection, and that treating those oocytes with ethanol prevents this re-elevation. Our unsatisfactory blastocyst yield by this protocol was probably due to the lower proportion of ICSI oocytes extruding the second polar body at the time of ethanol activation (45–46%) compared to that (60–70%) reported by Horiuchi *et al.* [11]. In order to increase the ICSI oocytes that extruded the second polar body at the time of ethanol treatment, oocytes were treated with 5 mM ionomycin immediately after ICSI. This attempt significantly improved the Day-8 blastocyst yields (29–30% of overall oocytes) without significant improvement in the blastocyst yield per cleaved zygote (38–43% versus 30% in non-treated control). This suggests that, in bovine ICSI, the early post-fertilization events from fertilization up to the first cleavage have a great impact on the successful development of the embryos.

Another activation regimen proved effective to improve blastocyst yield after bovine ICSI is to combine ionomycin with 6-DMAP [8,14,16]. In the present study, neither the cleavage rate nor the blastocyst yield was improved regardless of the improved proportion of the second polar body extrusion after this treatment (Table 1). This result was contradictory to some previous reports [8,14,16,] but in agreement with the report by Keskintepe *et al.* [38]. Using CHX instead of 6-DMAP improved the cleavage rate but not the blastocyst yield, which is in agreement with the report by Galli *et al*. [13]. Moreover, both activation regimens (ionomycin plus CHX or 6-DMAP) resulted in obviously low blastocyst yield per cleaved zygote (21–23%). Thus, the significantly lower blastocyst yields from the ICSI oocytes treated with ionomycin plus CHX or 6-DMAP (Table 1) may be reflected by the adverse effects of the inhibitors employed here (CHX and 6-DMAP) on the activity of other kinases or the synthesis of the other proteins involved in other cell functions

rather than regulation of cell cycle [39]. Using more specific kinase inhibitors for cell cycle regulation [40] may avoid such side effects of CHX or 6-DMAP.

One disadvantage of the application of exogenous activation is to increase the possibility of the parthenogenetic development [41]. In the present study, only a few blastocysts (1–2%) were obtained during 8-days culture after chemical or physical parthenogenetic treatments (Table 2). In addition, we have a similar result after parthenogenetic activation of sham-injected oocytes with ionomycin and ethanol (data not shown). Chemicals such as ethanol, ionomycin or calcium ionophore combined with or without CHX were not sufficient to induce parthenogenetic development in bovine oocytes [42,43]. Induction of diploidization by combining the calcium oscillation-inducing activators with cytochalasin or 6-DMAP was necessary to improve the parthenogenetic development of bovine oocytes [24,44,45]. This may explain the complete failure or very low parthenogenetic developmental rate after different activation protocols in the present study. However, relatively high proportions of parthenogenetic development have been reported after activation of sham-injected bovine oocytes with calcium oscillations-inducible chemicals without diploidization [8,14,16]. Although parthenogenetic development in our system was very low, analysis of karyotype in the ICSI-derived bovine blastocysts may be helpful.

Undoubtedly, the most precise evidence for the quality of the in-vitro produced bovine embryos is their ability to develop into full-term calves after transfer to recipient animals. However, the total cell number of the blastocysts, the ratio of ICM cells, as well as the developmental kinetics until blastocoele formation are often used as alternative parameters to assess the quality of the blastocysts [46]. In the present study, there was no significant difference in such in vitro parameters between the ICSI-derived blastocysts produced without any activation treatments and those produced with different activation regimens (Figure 1), suggesting that all activation regimens employed here have no adverse effect on the embryonic development and differentiation in vitro.

In conclusion, activation of bovine oocytes, harvested from ovaries stored at 10–12 ^{o}C for 24 h, after ICSI by a combined treatment of ionomycin with ethanol (rather than CHX or 6-DMAP) improved the blastocysts yield without any adverse effect in their quality.

Acknowledgements

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Figure legend

Figure 1: Differential cell counting of Day-7 expanding blastocysts derived from bovine ICSI. Gray columns refer to the total cell number while black columns refer to the ratio of the inner cell mass (ICM) cells (Mean ± SE). EtOH: Ethanol, CHX: Cycloheximide, 6-DMAP: 6-dimethylaminopurine. Values with different letters (a-b) indicate significant difference ($P < 0.05$).

Table 1

In vitro development of sperm-injected bovine oocytes after different activation treatments.

Activation protocols *	Cultured No.	Extruded PB $ No. (%)	Cleaved No. (%)	Developed to blastocysts No.	(%; Per cleaved)	(%; Per cultured)
Non-treated	189	88 (45 ± 6) [c]	77 (39 ± 5) [d]	24	(30 ± 4)	(12 ± 2) [b]
EtOH (5 min)	201	92 (46 ± 6) [c]	95 (45 ± 5) [cd]	31	(30 ± 6)	(13 ± 3) [b]
EtOH (10 min)	197	116 (58 ± 3) [bc]	122 (61 ± 8) [abd]	38	(29 ± 6)	(18 ± 4) [ab]
Ionomycin	196	154 (79 ± 2) [ab]	90 (47 ± 4) [bd]	17	(19 ± 1)	(9 ± 1) [b]
Ionomycin + EtOH (5 min)	197	149 (74 ± 3) [ab]	135 (67 ± 7) [abc]	57	(43 ± 5)	(29 ± 5) [a]
Ionomycin + EtOH (10 min)	196	165 (84 ± 3) [a]	153 (77 ± 6) [a]	58	(38 ± 6)	(30 ± 5) [a]
Ionomycin + CHX	207	149 (71 ± 6) [ab]	148 (73 ± 4) [ab]	34	(24 ± 5)	(18 ± 3) [ab]
Ionomycin + 6-DMAP	185	134 (73 ± 3) [ab]	109 (57 ± 4) [abd]	19	(21 ± 5)	(11 ± 2) [b]

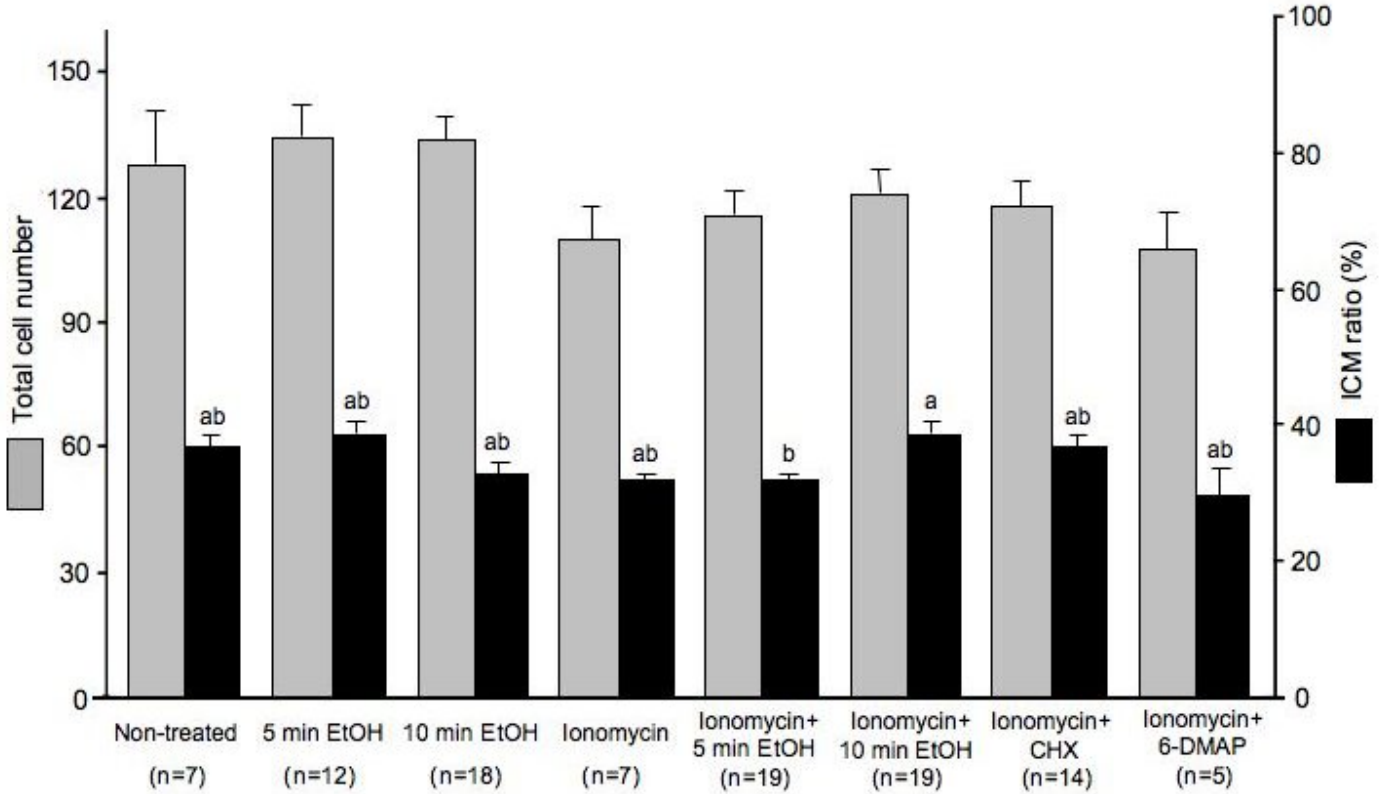
(%): Percentages are expressed as Mean ± SE of at least five replicates per treatment. [a-d] Values with different superscripts within columns are significantly different ($P < 0.05$). Abbreviations: $ PB: Polar body, observed at 3–5 h after ICSI. * EtOH: Ethanol; with exposure time in parenthesis. CHX: Cycloheximide. 6-DMAP: 6-dimethylaminopurine.

Table 2

Parthenogenetic development of bovine oocytes after different activation treatments.

Activation protocols*	Examined No.	Extruded PB[$] No. (%)	Cleaved No. (%)	Developed to blastocysts No. (%)
Sham injection	96	19 (20 ± 5) [b]	3 (3 ± 1)	0 (0 ± 0)
EtOH (5 min)	102	---	22 (21 ± 8)	1 (1 ± 1)
EtOH (10 min)	96	---	13 (15 ± 6)	1 (1 ± 1)
Ionomycin	92	62 (67 ± 4) [a]	14 (15 ± 1)	0 (0 ± 0)
Ionomycin + EtOH (5 min)	93	70 (77 ± 6) [a]	17 (19 ± 4)	0 (0 ± 0)
Ionomycin + EtOH (10 min)	96	66 (69 ± 3) [a]	12 (11 ± 4)	1 (1 ± 1)
Ionomycin + CHX	109	59 (54 ± 7) [a]	19 (18 ± 5)	1 (1 ± 1)
Ionomycin + 6-DMAP	87	57 (65 ± 2) [a]	18 (20 ± 5)	1 (2 ± 2)

(%): Percentages are expressed as Mean ± SE of at least four replicates per treatment. [a,b] Values with different superscripts within columns are significantly different ($P < 0.05$). Abbreviations: [$] PB; Polar body, observed at 3–5 h after activation. * EtOH: Ethanol; with exposure time in parenthesis. CHX: Cycloheximide. 6-DMAP: 6-dimethylaminopurine.


Total cell number
ICM ratio (%)
150
120
90
60
30
0
100
80
60
40
20
0
ab
ab
ab
ab
b
a
ab
ab
Non-treated (n=7)
5 min EtOH (n=12)
10 min EtOH (n=18)
Ionomycin (n=7)
Ionomycin+ 5 min EtOH (n=19)
Ionomycin+ 10 min EtOH (n=19)
Ionomycin+ CHX (n=14)
Ionomycin+ 6-DMAP (n=5)

(Figure 1)