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EFFECT OF OOCYTE AGE AND ACTIVATION AGENTS ON IN VITRO DEVELOPMENT OF MOUSE PARTHENOGENETIC EMBRYOS

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ABSTRACT

The present study was conducted to explore the effect of oocyte age and activation agents on initiation and development of mouse parthenogenetic embryos. A total of 943 mouse oocytes were divided into 9 treatments based on a completely randomized design with 3 x 3 factorial patterns; with the first factor was oocyte ages: 14-, 17- and 20-hr-old (were calculated based on the time of oocytes collection after the administration of hCG) and the second factor was activation agents (ethanol, calcium ionophore A23187, strontium chloride). The oocytes were exposed to 5 µg/ml cytochalasin B and then cultured in potassium simplex optimization medium at 37°C under 5% CO_2 up to 5 days. The results showed that the highest number of good quality oocytes was obtained by 14-hr after the administration of hCG. The level of activation and 2 pronuclei (2 PN) increased significantly in 17- and 20-hr-old oocytes ($P \leq 0.05$). The highest rate of embryonic development and blastocyst cell counts produced by 17-hr-old oocytes activated by strontium chloride ($P \leq 0.05$). It is concluded that initiation and in vitro development of mouse parthenogenetic embryos are affected by the age of oocytes and activation agents, with strontium chloride is the best activation agent especially in 17-hr-old oocytes.

Keywords: activation agent, oocytes age, parthenogenetic embryos, mouse
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INTRODUCTION

Normally, after ovulation from the ovarian follicles, mammalian oocytes are unable to develop to the next stage but arrest at metaphase of meiosis II. In an in vivo environment, oocytes will enter the fallopian tube through fimbriae and continue to move towards the ampulla, a place where the oocytes will meet with sperm. If the oocytes are successfully fertilized by sperm, the oocytes will undergo activation characterized by exocytosis of cortical granules, extrusion of second polar body (PB), and formation of pronuclei (Gordo *et al*., 2002) to produce a single-cell embryo or zygote. In the in vitro environment, oocytes may also be activated by parthenogenesis, which does not involve sperm for their subsequent development. Intracellular calcium content of parthenogenetic-activated oocytes will increase to play a role in inducing cortical granules exocytosis and activating calmodulin-dependent protein kinase II. Exocytosis of cortical granules works in preventing polyspermy during fertilization, while calmodulin-dependent protein kinase II functions to stimulate cyclin B destruction, p34cdc2 kinase inactivation and M-phase promoting factor (MPF) destruction. MPF destruction causes activation of oocytes, allowing the cell cycle is continued to the next stage (Ito *et al*., 2003).

Ethanol, calcium ionophore and strontium chloride are three of the several parthenogenetic activation agents commonly used for activation of mammalian oocytes (Tatone *et al*., 2002; Rogers *et al*., 2006; Nalley and Hine, 2015; Nikiforaki *et al*., 2016; Hebisha and Mahmoud, 2017). Calcium ionophore has a high permeability to pass through cell membranes. Oocytes exposure to calcium ionophore causing an increasing in intracytoplasmic calcium concentration from both calcium influx and endoplasmic reticulum (Vasilev *et al*., 2012). Both calcium ionophore and ethanol can cause single calcium transient (Rybouchkin *et al*., 1996, Rogers *et al*., 2006), but have not been reported to cause spontaneous calcium oscillations (Ferrer-Buitrago *et al*., 2017). Also, their ability for activation and development of embryos was greatly variable (Vanden Meerschaut *et al*., 2014). On the other hand, strontium chloride causes calcium oscillation (Rogers *et al*., 2006), which is essential for the inactivation of MPF and prevents its rebinding (Ducibella *et al*., 2002, Tóth *et al*., 2006). The different characteristics among the three activation agents may result in differences in activation and development rates of parthenogenetic embryos.

Activation and development of parthenogenetic embryos rates are also influenced by oocytes age. Different MPF contents between each age group of oocytes cause differences in the embryo activation and development levels (Seidel *et al*., 1976; Tian *et al*., 2002).

Data on the effects of the three activation agents in various age groups of oocytes on the level of activation and development of parthenogenetic embryos in mice are

rarely reported. Thus, this study aimed at exploring the effect of oocytes age and activation agents on activation rates, preimplantation embryonic development, and blastocyst cell counts.

MATERIALS AND METHODS

Animal: A total of 50 Swiss Webster female mice aged 8 to 12 weeks were used in this study. Mice were maintained at the Laboratory of Reproductive Biology and Animal Health, Faculty of Animal Husbandry, Nusa Cendana University. The room was maintained on 12:12-hr photo period (lights on 06.00 AM), and free access to the food and water.

Superovulation and Collection of Oocytes: Female mice were injected intraperitoneally with 5 IU pregnant mare's serum gonadotropin (Folligon, Intervet, Holland) followed by an injection of 5 IU human chorionic gonadotrophin (hCG; Chorulon, Intervet, Holland) 48 hr later. Fourteen-, 17- or 20-hr post hCG injection, mice were humanely killed (mice were decapitated 45 min following an intraperitoneal injection of 100 mg / kg Ketamine hydrochloride (Sigma-Aldrich, NMID686C-50MG) with 10 mg / kg xylazine hydrochloride supplement (Sigma-Aldrich, X1251-1G; Ko *et al*., 2019). The oviduct was cut off, cumulus-oocyte complexes (COC) were removed by cutting the wall of the ampulla oviduct and placed in Dulbecco's phosphate buffer saline solution (PBS; gibco, 21600-051-10x1L) supplemented 1.0 mg / ml of bovine serum albumin (BSA; Sigma, A2153-50G). Cumulus cells were dissociated from the oocytes by placing COC in PBS solution (gibco, 21600-051-10x1L) containing 0.03% hyaluronidase (sigma, H4272-30MG). Good quality oocytes (Oocytes are surrounded by a compact cumulus, the cytoplasm is homogeneous, intact zona pellucida, and contained first polar body; Lasienë *et al*., 2009) were used for parthenogenesis activation.

Oocytes Activation: Denuded oocytes from all three age groups (14-, 17-, 20-hr) were examined using an inverted microscope with 400x magnification (Axio Observer A1, Carl Zeiss-germany). The good quality oocyte (oocytes with intact zona pellucida, solid cytoplasm, and contained first polar body) were selected for activation with 7% ethanol (Merck, 1.00983.1000) for 7 minutes, 6 µM calcium ionophores A23187 (Sigma, C7522-10MG) for 4 minutes, or 10 mM strontium chloride (sigma, 255521-100G) for 2.5 hr, and then were exposed to 5 µg / ml cytochalasin B (Sigma, C6762-5MG) for 4 hr at 37ºC, 5% CO_2 (New Brunswick, Galaxy 170R CO_2 incubator, Eppendorf) (Nalley and Hine, 2015).

Oocytes were grouped into following categories to evaluate the effect of oocyte age and activation agents: 1) activated oocytes (oocytes with one or two pronuclei and one PB), 2) abnormal oocytes including oocytes with two pronuclei and fragmented oocytes, 3) dead oocytes, and 4) Metaphase II oocytes (Kishigami and Wakayama (2007). The ratio was calculated by dividing the number of oocytes in each category by the total oocytes treated.

Culture of Embryo: The activated oocytes were washed three times in PBS (gibco, 21600-051-10x1L) and then cultured in potassium simplex optimization medium supplemented by 10% fetal bovine serum (*Sigma,* A2153-50G) at 37ºC under 5% CO_2 (New Brunswick, Galaxy 170R CO_2 incubator, Eppendorf) up to 5 days. The development rate of the embryo was calculated at each stage (2-cell, 4-cell, morula, blastocyst, and hatched blastocyst), by dividing the number of embryos at each stage by total activated oocytes in each treatment group. Embryo quality was calculated based on the amount of blastocyst, inner cell mass (ICM) and trophectoderm cells. Total ICM and trophectoderm cells were determined by exposed blastocyst to rabbit anti-mouse serum antibody (Sigma, M5774-2ML) for 1-hr and complement sera from guinea pig (Sigma, S1639-5ML) for 30 minutes, and followed by staining using Hoechst 34580 (Sigma, 63493-5MG) - Propidium iodide (Sigma, 81845-25MG). ICM cells located on the inside of the embryo were blue, whereas trophectoderm cells on the outside were red (Hine *et al*., 2008).

Experimental Design and Statistical Analysis: The study used a completely randomized design with 3 x 3 factorial patterns with 6 replicates. The first factor was oocytes age consisting of 14-, 17-, and 20-hr, and the second factor was activation agents consisting of ethanol, calcium ionophore A23187, and strontium chloride. The results of the study were expressed as mean ± standard deviation. Data were analyzed with analysis of variance and continued with Duncan's multiple range test. Analysis using SPSS 20.0 for windows software.

RESULTS

Oocytes Quality at Different Age: Data on oocytes quality is shown in Table 1. Mostly oocytes (79.4 - 93.0%) were qualified as of good quality, while the rest were extra cytoplasmic abnormalities (dark zona pellucida and large perivitelline space), intracytoplasmic abnormalities (dark or granular cytoplasm and cytoplasmic fragments) and no first PB. The highest number of good quality oocytes was obtained by 14-hr-old. However, statistically, there was no significant difference ($P>0.05$) with 17-hr-old, but both had significantly higher ($P\leq0.05$) good quality of oocytes compared to 20-hr-old oocytes. The lack of good quality oocytes in 20-hr-old oocytes was mainly due to the high percentage of intracytoplasmic abnormalities, which exceeds threshold of 10%.

ECA: extracytoplasmic abnormalities; ICA: intracytoplasmic abnormalities; PB: polar body. Different

superscripts in the same column showed significant differences (P≤0.05). The results of the study were displayed as mean ± standard deviation.

Activation Rate of Mouse Oocytes: There were 943 good-quality oocytes used for the activation of parthenogenesis, consisting of 357 of 14-hr-old, 294 of 17-hr-old, and 292 of 20-hr-old oocytes. Oocytes in each age group were activated with ethanol, calcium ionophore, or strontium chloride. The level of oocytes activation was shown in Table 2. In general, the highest activation rate was found in 20-hr-old, -followed by 17-hr-old, and 14-hr-old oocytes were the lowest. However, there was no significant difference (P>0.05) in the activation rate between 20-hr-old with 17-hr-old, but both were significantly different (P≤0.05) from 14-hr-old; those results were the same for the three activation agents.

Based on the percentage of oocytes with 2 PN, 17- and 20-hr-old oocytes produced the highest results when they were activated with calcium ionophore and strontium chloride. Statistically, 17- and 20-hr-old oocytes activated with strontium chloride and calcium ionophore resulted in a higher percentage of 2 PN oocytes (P ≤0.05) than 14-hr-old oocytes, and also higher (P ≤0.05) compared with activated by ethanol at all oocyte ages. The lowest percentage of oocytes with 2 PN was produced by the ethanol treatment group in all age groups, ranging from 65.0 to 73.0%, and this was mainly due to the high percentage of metaphase II oocytes (on 14-hr-old oocytes), and dead oocytes (on 20-hr-old oocytes).

Table 1. Quality of mouse oocytes (%) at different ages

Oocytes age (hr)	No. of oocytes	ECA oocytes	ICA oocytes	Oocytes with no first PB	good quality Oocytes
14	384	3 (0.8±0.86) [c]	4(1.0±0.81) [c]	20(5.2±1.83) [a]	357(93.0±1.69) [a]
17	326	13(4.0±1.43) [b]	11(3.4±1.36) [b]	8(2.5±0.87) [b]	294(90.0±2.51) [a]
20	368	25(6.8±1.89) [a]	44(12.0±2.80) [a]	7(1.9±1.39) [b]	292(79.4±4.83) [b]

Table 2. Oocytes activation rate (%) and mean (± standard deviation) at different ages and activation agents.

Activation agents	Oocytes age (hr)	No. oocytes	Activation rate	2 PN oocytes	1 PN oocytes (%)	MII oocytes (%)	Dead oocytes (%)
Ethanol	14	120	84 (70.0 ± 5.58)[c]	78 (65.0 ± 3.51)[d]	6 (5.0 ± 2.72)[c]	34 (28.3 ± 4.10)[a]	2 (1.7 ± 2.84)[b]
	17	99	83 (83.8 ± 11.18)[b]	72 (72.7 ± 7.16)[c]	11 (11.1 ± 5.17)[ab]	10 (10.1 ± 5.43)[c]	6 (6.7 ± 6.78)[ab]
	20	100	87 (87.0 ± 8.03)[ab]	73 (73.0 ± 4.30)[c]	14 (14.0 ± 4.16)[a]	2 (2.0 ± 3.13)[d]	11 (11.0 ± 6.96)[a]
Calcium ionophore	14	119	91 (76.5 ± 4.22)[c]	87 (73.1 ± 4.72)[c]	4 (3.4 ± 3.09)[c]	24 (20.2 ± 6.34)[b]	4 (3.4 ± 3.88)[b]
	17	99	92 (92.9 ± 2.06)[a]	85 (85.9 ± 4.23)[ab]	7 (7.1 ± 4.35)[bc]	2 (2.0 ± 3.06)[d]	5 (5.1 ± 2.55)[ab]
	20	97	90 (92.8 ± 1.42)[a]	84 (86.6 ± 5.92)[a]	6 (6.2 ± 5.54)[bc]	0 (0.0 ± 0.00)[d]	7 (7.2 ± 1.42)[ab]
Strontium chloride	14	118	103 (87.3 ± 2.63)[b]	95 (80.5 ± 5.47)[b]	8 (6.8 ± 5.55)[bc]	10 (8.5 ± 1.28)[c]	5 (4.2 ± 2.59)[ab]
	17	96	90 (93.8 ± 5.01)[a]	86 (89.6 ± 3.14)[a]	4 (4.2 ± 5.77)[c]	3 (3.1 ± 3.19)[d]	3 (3.2 ± 3.19)[b]
	20	95	90 (94.7 ± 5.76)[a]	87 (91.6 ± 3.61)[a]	3 (3.2 ± 4.14)[c]	1 (1.1 ± 3.14)[d]	4 (4.2 ± 3.79)[ab]

PN: pronucleus; MII: metaphase II; Different superscripts in the same column show significant differences (P≤0.05).

Embryos Development Rate: The development rate of parthenogenetic embryos decreased significantly, especially in oocytes activated with ethanol in all age groups. This was reflected by the low blastocyst rate ranging from 0.0 to 8.3%. This result was much lower than those oocytes activated with strontium chloride with range 14.9 to 33.7% (Table 3). 17-hr-old oocytes produced higher rates of embryo development than 14- or 20-hr-old (P≤0.05); this applied to all the three activation agents. Overall, 17-hr-old oocytes activated with strontium chloride resulted in a higher rate of embryonic development (P ≤0.05) compared to other treatment groups.

The number of blastocyst cells: There were 64 blastocysts used in this experiment, consisting of 8 blastocysts from the ethanol group, 26 blastocysts from the calcium ionophore group and 30 blastocysts from the strontium chloride group. The small amount of blastocyst of the ethanol group was due to the low blastocyst rate. The number of blastocyst cells in the ethanol group ranged from 34.0 to 40.8 cells, less than calcium ionophore (41.0 to 51.4 cells) and strontium chloride (46.8 to 56.9). Activation of strontium chloride or calcium ionophore in 17-hr-oocyte resulted in a higher number of blastocyst cells ($P \leq 0.05$) compared to other treatment groups. On the other hand, there was no significant difference ($P>0.05$) for the number of trophectoderm cells (Tabel 4).

Table 3. Comparison of development rate (%) and mean+/- SD of parthenogenetic embryos among various oocyte ages and activation agents.

Activation agents	Oocytes age (hr)	2 PN oocytes	2-cells	4-cells	Morula	Blastocyst	Hatched blastocyst
Ethanol	14	78	52 (66.7 ± 4.00)^{cd}	31 (39.7 ± 8.68)^{e}	9 (11.5 ± 7.02)^{de}	2 (2.6 ± 4.63)^{ef}	0 (0.0 ± 0.00)^{d}
	17	72	53 (73.6 ± 3.62)^{c}	37 (51.4 ± 9.39)^{d}	17 (23.6 ± 5.94)^{c}	6 (8.3 ± 0.44)^{de}	2 (2.8 ± 4.30)^{d}
	20	73	38 (52.1 ± 2.31)^{e}	19 (26.0 ± 2.87)^{f}	4 (5.5 ± 4.53)^{e}	0 (0.0 ±0.00)^{f}	0 (0.0 ± 0.00)^{d}
Calcium ionophore	14	87	63 (72.4 ± 6.96)^{c}	44 (50.6 ± 3.04)^{d}	23 (26.4 ± 2.51)^{c}	11 (12.6 ± 2.10)^{cd}	3 (3.5 ± 3.94)^{cd}
	17	85	70 (82.4 ± 6.63)^{b}	57 (67.1 ± 6.64)^{b}	30 (35.3 ± 6.20)^{b}	19 (22.4 ± 4.50)^{b}	9 (10.6 ± 4.78)^{b}
	20	84	53 (63.1 ± 5.34)^{d}	41 (48.8 ± 3.07)^{d}	14 (16.7 ± 6.61)^{d}	6 (7.1 ± 3.97)^{de}	1 (1.2 ± 2.40)^{d}
Strontium chloride	14	95	78 (82.1 ± 6.62)^{b}	59 (62.1 ± 5.53)^{bc}	35 (36.8 ± 5.27)^{b}	22 (23.2 ± 6.69)^{b}	10 (10.5 ± 2.04)^{b}
	17	86	79 (91.9 ± 4.67)^{a}	68 (79.1 ± 4.91)^{a}	41 (47.7 ± 5.10)^{a}	29 (33.7 ± 7.61)^{a}	18 (20.9 ± 8.18)^{a}
	20	87	64 (73.6 ± 6.89)^{c}	51 (58.6 ± 5.06)^{c}	23 (26.4 ± 5.56)^{c}	13 (14.9± 5.84)^{c}	7 (8.1 ± 5.86)^{bc}

PN: pronucleus. Different superscripts in the same column show significant differences ($P \leq 0.05$).

Table 4. Comparison of the number of mouse blastocyst cells (mean+/-SD) produced from various age oocytes and activation agents.

Activation agents	Oocytes age (hr)	No. blastocyst	No. Blastocyst cells		
			ICM cells	Trophectoderm cells	Total
Ethanol	14	2	7.5 ± 0.71^{d}	26.5 ± 2.12^{e}	34.0 ± 2.83^{e}
	17	6	10.3 ± 1.37^{c}	30.5 ± 4.59^{de}	40.8 ± 4.45^{d}
	20	-	-	-	-
Calcium ionophore	14	10	11.5 ± 1.84^{bc}	34.2 ± 7.90^{bcd}	45.7 ± 8.18^{cd}
	17	10	13.4 ± 1.71^{ab}	38.0 ± 5.16^{abc}	51.4 ± 3.86^{abc}
	20	6	9.3 ± 1.75^{cd}	31.7 ± 2.94^{cde}	41.0 ± 2.90^{d}
Strontium chloride	14	10	13.1 ± 1.85^{b}	39.1 ± 3.73^{ab}	52.2 ± 2.68^{ab}
	17	10	15.5 ± 2.80^{a}	41.4 ± 5.19^{a}	56.9 ± 4.38^{a}
	20	10	10.7 ± 1.16^{c}	36.1 ± 5.97^{abcd}	46.8 ± 5.83^{bcd}

ICM: inner cell mass. Different superscripts in the same column show significant differences ($P \leq 0.05$).

DISCUSSIONS

The purpose of this study was to explore the effect of oocytes age and activation agents on activation and embryo development rate of mouse parthenogenetic embryos. Oocytes aged 14, 17 and 20 hr and three types of activation agents namely ethanol, calcium ionophore A23187 and strontium chloride were used. In general, 14- and 17-hr-old oocytes displayed a higher quality compared to 20-hr-old. On the other hand, the highest abnormality was yielded by 20-hr-old-oocyte. This

indicated that the older oocytes have a lower quality compared to the younger ones. Poor quality oocytes were characterized by abnormalities in granules, darker cytoplasmic color, non-rounded oocytes shape, large perivitelline space, presence of fragments in a first PB (Ebner *et al*., 2001). According to Igarashi *et al*. (2015), aged oocytes also have chromosomal abnormalities, and cellular organelle dysfunction, especially mitochondria and endoplasmic reticulum, and both of them were major contributing factors to the low quality of aged oocytes (Eichenlaub-Ritter *et al*., 2004; Bentov *et al*., 2011; Eichenlaub-Ritter *et al*., 2011; Duncan *et al*., 2012) and embryos (Igarashi *et al*., 2015). Chromosomal abnormalities observed in aged oocytes can cause miscarriages and defects in offspring. Aside, aged oocytes are more sensitive to oxidative stress that damages intracellular components such as deoxyribonucleic acid, proteins, lipids, and mitochondria (Igarashi *et al*., 2015). Therefore, it is reasonable that aged oocytes exhibit lower quality than the younger ones. In the natural fertilization process, the impacts of oocytes aging are observed in terms of decreased rate of fertilization and embryo development, and increased offspring abnormality (Miao *et al*., 2009; Van Blerkom, 2011; Lord *et al*., 2013).

The activation potential was determined by activating oocytes from each age group with ethanol, calcium ionophore, and strontium chloride. A high activation level was performed by 17- and 20-hr-old oocytes. This indicated that the level of activation increased with the increasing age of oocytes (Otaegui *et al*., 1999, Meo *et al*., 2004). The results of this study in line with studies in Wistar rats conducted by Krivokharchenko *et al*. (2003) and Mizutani *et al*. (2004). This phenomenon was caused by the decrease in maturation promoting factors (MPF) levels of old oocytes (Kikuchi *et al*., 2000; Tian *et al*., 2002), which plays role in inducing M-phase in eucaryotic cells including oocytes.

Although they displayed a high level of activation, old oocytes (20-hr-old) performed lower embryonic development and a smaller number of blastocyst cells in all activation agents. Observations on 72 hr of culture showed that embryos from old oocytes produced fewer blastomeres (5 to 6 cells) compared to the younger ones i.e. 7 to 8 cells (data not shown). These data indicated the slow rate of cell division in embryos from old oocytes. The results of this study provided two basic indications: young oocytes displayed low activation rates but relatively high embryonic development rates, whereas old oocytes have high activation but low embryonic development rate. The results of this study were in line with several previous studies. Kharche and Birade (2013) reported that the response of oocytes activation and development of bovine embryos were influenced by the age of oocytes and the activation agents used. Old oocytes decreased the fertilization rate (Maure and Foote, 1971) and subsequent embryonic development.

The level of activation and development of mouse embryo were also influenced by the activation agent. Strontium chloride was better in oocytes activation, embryo development, and blastocyst cell amount, which are higher than ethanol and calcium ionophore. This study confirmed data from the previous study by Loren and Lacham-Kaplan (2006) that using 10 mM strontium chloride in mouse oocytes injected with spermatids resulted in oocytes activation levels and blastocyst rates (96 and 37%) higher than ethanol i.e. 94% and 4%. A high rate of embryonic development was also performed when strontium chloride was used for mouse oocytes activation, with a blastocyst rate reaching 60.42% compared to calcium ionophore + 6-DMAP and 8% ethanol, 23.28 and 13.86%, respectively (Idris *et al*., 2013).

This study proved that strontium chloride is more suitable for mouse oocytes activation in all age groups. This may be related to the better ability of the agent in triggering calcium oscillations compared to other activation agents (Loren and Lacham-Kaplan, 2006). According to Kline (1996), strontium can induce the release of repetitive intracellular calcium like the normal fertilization performed by sperm. Calcium oscillations induced by strontium results in more superior embryonic development (Lacham-Kaplan *et al*., 2003) and higher blastocyst rates (Bos-Mikich *et al*., 1997).

Several studies have shown that the effectiveness of strontium also depended on species, concentration, and duration of incubation. Tateno *et al*. (1997) found a low activation rate when strontium chloride was used for hamster oocytes activation. In rat, oocytes were arrested in the metaphase III stage (Hayes *et al*., 2001), but in other studies, using 2 mM strontium for 15 minutes were effective for inducing rat oocytes activation (Krivokharchenko *et al*., 2003).

The results of this study confirm that activation and development rate of mouse parthenogenetic embryos are affected by the age of oocytes and activation agents, with strontium chloride is the best activation agent especially in 17-hr-old oocytes.

Conclusions: The activation and development rate of mouse parthenogenetic embryos were influenced by oocytes age and the activation agent used. Activation of 17-hr-old oocytes with strontium chloride produced the highest activation and development rate of parthenogenetic embryos.

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