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CLINICAL CORNER: CASE REPORT

Successful pregnancy after $SrCl_2$ oocyte activation in couples with repeated low fertilization rates following calcium ionophore treatment

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Abstract

This report describes a successful pregnancy and delivery following oocyte activation with strontium chloride ($SrCl_2$) in couples with repeated complete fertilization failure or low fertilization rates even after calcium ionophore treatment. Eight infertile couples who showed complete fertilization failure or low fertilization rates after conventional intracytoplasmic sperm injection (ICSI) and calcium ionophore treatment. When the results of fertilization were not satisfactory in the cycles, the oocytes were artificially activated by $SrCl_2$ for the next attempts. Oocyte activation with $SrCl_2$ significantly increased the fertilization rates, when compared with conventional ICSI or calcium ionophore treatment (61.7% vs. 20.0% or 25.3%, respectively). There was significant increase in the proportions of good-quality cleaved embryos (50.0% vs. 0% or 12.5%, respectively). The rate of surplus embryos that developed to blastocyst stage increased in $SrCl_2$-treated oocytes, when compared with that in ICSI with or without calcium ionophore treatment (25.7% vs. 0% or 9.1%, respectively). Five successful pregnancies were attained after oocyte activation with $SrCl_2$, of which eight healthy children were born. Physical and mental development of the children were normal from birth to 60 months. These results suggest that $SrCl_2$ in treatment should be considered as an effective method for artificial oocyte activation (AOA) to improve fertilization rates and embryo quality in cases with complete fertilization failure or low fertilization rates after calcium ionophore treatment.

Abbreviations: $SrCl_2$: strontium chloride; ICSI: intracytoplasmic sperm injection; AOA: artificial oocyte activation; IVF: *in vitro* fertilization; AFF: autologous follicular fluid

Keywords

AOA, calcium ionophore, fertilization failure, ICSI, $SrCl_2$

History

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Introduction

Intracytoplasmic sperm injection (ICSI) has enabled fertilization of oocytes from patients whose partners have extremely low numbers of viable sperm and a very low probability of achieving fertilization *in vitro*. However, we occasionally encountered unusual cases in which fertilization failed despite the sperm being properly injected into the oocyte, and it has been a difficult problem to solve for a long time. The reasons for this phenomenon may be a partial or complete inability of the spermatozoa to activate the oocytes, deficiency of sperm protamine, or the inability of the oocytes to decondense spermatozoa [Nasr-Esfahani et al. 2007; Sakkas et al. 1996; Schmiady et al. 1996]. When the oocytes were activated using electroporation [Mansour et al. 2009; Yanagida et al. 1999; Zhang et al. 1999], calcium ionophore [Battaglia et al. 1997; Chi et al. 2004; Eldar-Geva et al. 2003; Kim et al. 2001; Kyono et al. 2009; Moaz et al. 2006; Tejera et al. 2008; Terada et al. 2009; Tesarik and Sousa 1995], calcium ionophore and puromycin [Lu et al. 2006; Murase et al. 2004; Nakagawa et al. 2001], calcium chloride and ionophore [Rybouchkin et al. 1997], or ionomycin [Nasr-Esfahani et al. 2008], followed by ICSI in women whose oocytes could not be fertilized in previous IVF cycles, some of them could form pronuclei and achieved childbirth.

The calcium ionophore was found to be an excellent candidate especially for improving fertilization, embryo quality, and pregnancy in women who showed complete fertilization failure or low fertilization rates. Thus, calcium ionophore is commonly used for artificial oocyte activation (AOA), but the result is not satisfactory in some patients. Yanagida et al. [2006] reported oocyte activation by strontium chloride ($SrCl_2$) in a patient with a low fertilization rate, and the patient achieved higher fertilization rate and successful first pregnancy and delivery using fresh embryos and frozen-thawed embryos transfer. Kyono et al. [2008] reported that five healthy children were born following ICSI using $SrCl_2$ oocyte activation in nine patients. The children showed a normal physical development profile from birth until 12 months. Furthermore, several studies have continually reported that through the same method infertile patients could be successfully treated yielding pregnancies and

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deliveries [Chen et al. 2010; Kim et al. 2012; Yang et al. 2012]. Therefore, AOA with $SrCl_2$ could be an effective alternative or option for patients with complete fertilization failure or low fertilization rates in common with calcium ionophore treatment. In the present study, we evaluated the efficacy and safety of AOA with $SrCl_2$ in couples with complete fertilization failure or low fertilization rates despite ICSI with calcium ionophore treatment in previous cycles.

Results

Case 1

A 25-year-old woman and a 33-year-old man had primary female factor infertility. The woman displayed polycystic ovary syndrome and bilateral tubal obstruction, and semen examination was normal (volume, 3.0 ml; count, 25×10^6/ml; motility, 53.0%; abnormal morphology, 25.0%). All other female and male diagnostic parameters were normal.

At the first cycle, ten metaphase II (MII) oocytes were retrieved and used for conventional ICSI. Motile sperms without obvious abnormal morphology were injected into the oocytes. None of the oocytes were fertilized until the next 2 days. At the second to fifth cycles, the oocytes were exposed to a calcium ionophore. The fertilization rates were 38.5% (5/12), 0% (0/6), 35.7% (5/14), and 10.0% (4/40), respectively, but pregnancy was not achieved.

At the sixth cycle, the ovaries were stimulated by the long protocol using a GnRH agonist, and 26 MII oocytes were obtained, allowing us to perform ICSI. After sperm injection, all of the oocytes were stimulated using $SrCl_2$ for activation. A total of 16 out of 26 activated oocytes were fertilized (61.5%) and developed into well-cleaved embryos. Three well-cleaved embryos (ten-, nine-, and eight-cell stage) were selected and transferred on Day 3. Subsequently, two gestational sacs were identified on ultrasound. The patient delivered dizygotic female and male twins (both 1,900 g; both 46, XX and XY) at 33 weeks and 6 days of gestation by caesarean section. Physical and mental development assessment of the children were normal from birth to 60 months.

Case 2

A 38-year-old woman and a 38-year-old man had primary infertility of unknown etiology. Semen examination was normal (volume, 2.0 ml; count, 50×10^6/ml; motility, 53.0%; abnormal morphology, 20.0%), and all other female and male diagnostic parameters were normal. Artificial inseminations using the husband's spermatozoa were attempted twice, but pregnancy could not be achieved.

At the first cycle, three MII oocytes were retrieved and used for conventional ICSI. One was fertilized (33.3%) and transferred, but pregnancy was not achieved. At the second cycle, five MII oocytes were retrieved and treated with ICSI, followed by activation with a calcium ionophore. One fertilized (20.0%), but the embryo displayed arrested (two-cell stage) development, so embryo transfer was cancelled.

At the third cycle, the ovaries were stimulated with GnRH using the antagonist protocol and five ooytes were collected, including four MII oocytes. After ICSI, all of the oocytes were stimulated by $SrCl_2$ activation. All activated oocytes fertilized (100%) and developed into well-cleaved embryos. Three well-cleaved embryos (12-, and two eight-cell stage) were selected and transferred on Day 3. Subsequently, two gestational sacs were identified on ultrasound. The patient delivered dizygotic male twins (2,800 g and 2,100 g; both 46, XY) at 37 weeks and 2 days of gestation by caesarean section. Physical and mental development assessment of the children were normal from birth to 48 months.

Case 3

A 33-year-old woman and a 33-year-old man had primary infertility caused by male factor. Semen examination showed severe oligoasthenoteratozoospermia with >90% immotile and teratozoospermatozoa (volume, 4.5 ml; count, 0.2×10^6/ml; motility, 10.0%; abnormal morphology, more than 90.0%), and all other female and male diagnostic parameters were normal.

As the first cycle was performed at another clinic, no data were available except that the fertilization had failed. At the second and third cycles, the oocytes were exposed to the calcium ionophore after ICSI. The fertilization rates were 25.0% (3/12) and 33.3% (3/9), respectively. However, pregnancy was not achieved.

At the fourth cycle, the ovaries were stimulated by the long protocol using a GnRH agonist. Nine oocytes were collected, including eight MII oocytes. After ICSI, oocytes were activated by $SrCl_2$ and six of the eight oocytes were fertilized (75.0%). Two well-cleaved embryos (eight-cell stage) and one poorly cleaved embryo (six-cell stage with $\geq$50% fragmentation) were selected and transferred on Day 3, but pregnancy was not achieved. At the fifth cycle, the ovaries were stimulated with GnRH antagonist protocol and three oocytes were collected, including two MII oocytes. After ICSI, all of the oocytes were stimulated using $SrCl_2$ for activation. All activated oocytes were fertilized (100%) and developed into well-cleaved embryos. Two well-cleaved embryos (ten-, and eight-cell stage) were transferred on Day 3. Subsequently, a gestational sac was identified on ultrasound. The patient delivered a female (2600 g; 46, XX) at 38 weeks and 4 days of gestation. Physical and mental development assessment of the child was normal from birth to 36 months.

Two years later, this couple attempted the same procedure again. At the sixth cycle, the ovaries were stimulated with GnRH antagonist protocol and six ooytes were collected (including four MII oocytes), which were then activated by $SrCl_2$. All activated oocytes were fertilized (100%), and one well-cleaved embryo (eight-cell stage) and two poorly cleaved embryos (eight-cell stage with $\geq$50% fragmentation) were transferred on Day 3. Subsequently, a gestational sac was identified on ultrasound. The patient delivered a male (2860 g; 46, XY) at 38 weeks of gestation. Physical and mental development assessment of the child was normal from birth to 12 months.

Case 4

A 36-year-old woman and a 38-year-old man had primary infertility caused by male factor. Semen examination showed

severe oligoasthenoteratozoospermia with 100% teratozoospermatozoa (volume, 1.5 ml; count, 2×10^6/ml; motility, 10%; abnormal morphology, 100%), and all other female and male diagnostic parameters were normal.

At the first cycle, which we performed, 13 MII oocytes were retrieved and used for conventional ICSI. Motile sperms were injected into oocytes. None of the oocytes were fertilized until the next 2 days. At the second to fourth cycles, the oocytes were exposed to a calcium ionophore. The fertilization rates were 33.3% (4/12), 33.3% (3/9), and 25.0% (3/12), respectively, but pregnancy was not achieved.

At the fifth cycle, the ovaries were stimulated by the long protocol using a GnRH agonist, and 12 oocytes were collected (including ten MII oocytes), which were then activated by $SrCl_2$ and fertilization rates increased to 80.0% (8/10) and developed into well-cleaved embryos. Three well-cleaved embryos (eight-cell stage) were selected and transferred on Day 3. Subsequently, two gestational sacs were identified on ultrasound. The patient delivered dizygotic male twins (2,600 g and 2,200 g; both 46, XY) at 37 weeks and 3 days of gestation. Physical and mental development assessment of the children were normal from birth to 24 months.

Overall, $SrCl_2$ treatment significantly increased the fertilization rates, when compared with conventional ICSI or calcium ionophore treatment (61.7% (66/107) vs. 20.0% (14/70) or 25.3% (40/158), respectively). The number of good-quality embryos having <5% cytoplasmic fragmentation significantly increased after $SrCl_2$ treatment, when compared with conventional ICSI or calcium ionophore treatment on Day 3 (50.0% (33/66) vs. 0% (0/14) or 12.5% (5/40), respectively). The rate of surplus embryos that developed to the blastocyst stage was markedly increased following $SrCl_2$ treatment, when compared with that following conventional ICSI or calcium ionophore treatment (25.7% (9/35) vs. 0% (0/1) or 9.1% (1/11), respectively). Pregnancy and implantation rates were 41.7% (5/12) and 25.8% (8/31), compared with conventional ICSI (0% (0/15) and 0% (0/29)) or calcium ionophore treatment (0% (0/9) and 0% (0/13)), respectively. As well as, all infants born thereafter displayed normal karyotype and no congenital abnormalities. The clinical outcomes of eight couples included in this study are shown in Table 1.

Discussion

Modified ICSI is considered to be the most powerful tool in assisted reproductive technology. Although the fertilization rate of ICSI is typically considered to be the highest among the assisted reproduction techniques presently being performed, the rate of complete fertilization failure after ICSI has been reported to be 1.29–3% [Esfandiari et al. 2005; Liu et al. 1995]. Complete fertilization failure or low fertilization rates after ICSI can occur repeatedly in some couples. It is known that oocyte activation does not occur in approximately 70% of the unfertilized oocytes after ICSI, despite accurate injection of the spermatozoon into the cytoplasm of the oocyte [Yanagida 2004].

Several reports have demonstrated the efficacy of chemical substances used after ICSI in couples who experienced complete fertilization failure or low fertilization rates in previous cycles of conventional ICSI. Such AOA in humans has been performed using a variety of electrical stimulation protocols and chemical substances. Among them, calcium ionophore has been used for oocyte activation in humans since the 1990s. Tesarik and Testart [1994] introduced the activation of human oocytes with calcium ionophore after ICSI, and Hoshi et al. [1995] reported the first pregnancy using ICSI with calcium ionophore. Calcium ionophore treatment is the most commonly applied method for oocyte activation in clinical trials, and it has demonstrated efficacy and safety for a long time. This treatment causes a single transient increase in the intracellular concentration of calcium ion (Ca^{2+}) in the oocytes, and consequently, this transduction pathway induces oocyte activation through signal transduction mechanisms. This function is called ''trigger'' [Swann and Ozil 1994]. When human eggs are fertilized physiologically, intracellular Ca^{2+} concentration is increased after sperm-egg fusion, followed by calcium oscillation, which continues for 3–4 hours [Tesarik et al. 2000]. The oscillation function is dependent on the release of sperm associated oocyte activation factors that sustain repetitive Ca^{2+} release from intracellular stores of oocyte. In these cases, fertilization failure may be caused by spermatozoal or oocyte factors or both. Thus, heterologous ICSI is useful to evaluate the causes, whether it is due to sperm- or oocyte-related factors [Heindryckx et al. 2005]. Furthermore, Bos-Mikich et al. [1995] demonstrated that calcium oscillation during mitosis

Table 1. Clinical outcomes of ICSI combined with AOA in a total of eight couples.

Variable	Conventional ICSI	ICSI with calcium ionophore	ICSI with $SrCl_2$
ICSI cycles (n)	9	15	12
Maternal age (median years)	31.5	32.0	33.3
No. of oocytes (mean ± SD)	83 (9.2 ± 4.8)	180 (12.0 ± 10.1)	146 (12.2 ± 10.9)
No. of MII oocytes (mean ± SD)	70 (7.8 ± 2.5)	158 (10.5 ± 9.0)	107 (8.9 ± 6.9)
No. of oocytes fertilized (%)	14 (20.0)[a]	40 (25.3)[a]	66 (61.7)[b]
No. of embryos cleaved (%)	13 (92.5)	31 (77.5)	63 (95.5)
No. of good-quality embryos (%)	0 (0.0)[a]	5 (12.5)[a]	33 (50.0)[b]
No. of embryo transferred (mean ± SD)	13 (1.4 ± 1.3)	29 (1.9 ± 1.3)	31 (2.6 ± 0.7)
No. of blastocysts developed (%)	0/1 (0.0)	1/11 (9.1)	9/35 (25.7)
No. of embryos implanted (%)	–	–	8 (25.8)
No. of clinical pregnancies (%)	–	–	5 (41.7)
No. of miscarriage rates (%)	–	–	0 (0.0)

[ab]Different letter superscripts denote significant differences ($p < 0.01$) among values within a row. ICSI: intracytoplasmic sperm injection; AOA: artificial oocyte activation.

and the exit from meiosis increases the cell number of the inner cell mass in blastocysts. It has been shown that calcium trigger and oscillation play a significant role in oocyte activation and embryo development. However, the problem of a complete fertilization failure or low fertilization rate after calcium ionophore treatment still needs to be solved.

Strontium (Sr^{2+}) can be used in place of a calcium ionophore for oocyte activation, and has been found to promote calcium oscillation in mice oocytes [Kline and Kline 1992; Zhang et al. 2005]. Kline and Kline [1992] reported the egg activation effect of Sr^{2+} is superior to the calcium ionophore, because Sr^{2+} treatment induces more calcium oscillations. Suganuma et al. [2005] suggested that when compared with calcium oscillation patterns observed with calcium ionophore treatment, the patterns with $SrCl_2$ treatment appears to be more similar to those that occur during spontaneous fertilization. Thus, $SrCl_2$ is considered to be the most efficient agent for oocyte activation by inducing calcium oscillations in mice. However, it is not known whether if $SrCl_2$ is as useful for oocyte activation as calcium ionophore treatment in humans. Recently, several studies reported that $SrCl_2$ treatment of infertile patients resulted in successful pregnancies and deliveries [Chen et al. 2010; Kim et al. 2012; Kyono et al. 2008; Yanagida et al. 2006; Yang et al. 2012]. Furthermore, the physical and mental development of these children from birth to 12 months were normal [Kyono et al. 2008]. These results show that $SrCl_2$ treatment is useful for activating human oocytes that frequently fail to get fertilized in ICSI.

In the present study, although no problems were found with the semen of the eight males in semen analysis, the possibility cannot be eliminated that some of males had a defective sperm associated oocyte activation factor, resulting in oocyte activation failures. In addition, we could not identify whether a sperm- and oocyte-related deficiency existed in each couple, because the heterologous ICSI had not been performed due to ethical considerations. Based on our findings, eight infertile couples failed to achieve fertilization and pregnancy with previous treatments, but AOA with $SrCl_2$ resulted in four women successfully delivering eight babies (two singleton pregnancies and three twin pregnancies). For these eight couples, mean fertilization rates after $SrCl_2$ activation significantly increased to 61.7% from 20.0% or 25.3% ($p<0.01$). The frequency of good-quality embryos on Day 3 improved from 0% or 12.5% to 50.0% ($p<0.01$). This suggests that $SrCl_2$ treatment could be a better alternative for oocyte activation for couples who experienced complete fertilization failure or low fertilization rates even after calcium ionophore treatment in previous trials.

According to the infant health care assessment conducted by the NHIS in Korea, the physical and mental development of children from birth to 60 months was normal. We will continuously monitor the development of these children until 72 months, because long-term safety is an important consideration to recommend AOA using $SrCI_2$ as a safe and effective method for fertilization of unfertilized oocytes in infertile couples. Therefore, it is hoped that the use of AOA using $SrCI_2$ for oocyte activation will be understood in the same way as calcium ionophore with regard to safety. Although the safety of $SrCl_2$ treatment has been clarified, further studies are needed, because the long-term effects of ICSI with $SrCl_2$ on the resulting babies and children remain largely unknown. It is important that the established protocol is often revised, and that the role of ICSI in infertility therapy is continually re-evaluated [Varghese et al. 2007]; the same also applies to the use of AOA with ICSI.

In conclusion, these results suggest that $SrCl_2$ in treatment should be considered as an activator in couples who have complete fertilization failure or low fertilization rates after calcium ionophore treatment in previous cycles. It was an effective option especially for repeated fertilization failure or low fertilization rates and low quality embryo. However, the mechanism and genetic safety of oocyte activation induced by $SrCl_2$ treatment is not clear. Therefore, further study and tests are required to confirm the safety of $SrCl_2$ treatment in oocyte activation for clinical application. Although this treatment seems to be safe for use in humans according to the clinical outcomes thus far, patients must be informed regarding the potential risks of the prescribed fertility treatment and the possible long-term health implications on the child.

Materials and Methods

Patients

This case series study was approved by the Maria Fertility Hospital Institutional Review Board. The risks and benefits of the treatments and the possible implications for the future health of their children were explained in detail, and informed consent was obtained from each couple. The couples visited our IVF center between March 2008 and February 2012, and a total of eight couples who showed complete fertilization failure or low fertilization rates (ranging from 0% to 37.5%; mean = 20.0%) after conventional ICSI in the first cycles were included in this study. In the next cycles, the oocytes were exposed to 10 μM of calcium ionophore A 23187 (Sigma, St Louis, MO) for 30 min after ICSI. When the results of fertilization rates were not satisfactory in the cycles (ranging from 0% to 40.0%; mean = 25.3%), the oocytes were artificially activated by $SrCl_2$ for the next attempts. The couples who succeeded in achieving live births are described in detail.

Ovarian stimulation, preparation of gametes, and ICSI

Ovarian stimulation was conducted using a combination of gonadotrophin-releasing hormone (GnRH) agonist (leuprorelin; Lucrin Depot, Abbott Laboratories, Spain), GnRH antagonist (cetrorelix; Cetrotide, Serono, Switzerland), and human menopausal gonadotrophin (HMG) (Merional, Institute Biochemique SA, Switzerland). An injection of 10,000 units of human chorionic gonadotrophin (HCG) (IVF-C, LG Life Science, Korea) was administered when the dominant follicle reached a mean diameter of 17–18 mm. Oocytes retrieval were performed 36 h after HCG administration. Transvaginal ultrasound-guided aspiration was performed with a 19-gauge needle (COOK, Eight Mile Plains, Queensland, Australia). The retrieved oocytes were cultured for several hours in MRC#D01 medium (Fertilization medium, Biosupply Co., Korea). Immediately before ICSI, the oocytes were denuded with 40 IU/ml hyaluronidase (Sigma, St Louis, MO) and mechanically pipetted.

Strontium chloride treatment of oocytes

Injected oocytes were activated by $SrCl_2$ (Sigma-Aldrich, St Louis, MO) treatment for approximately 30 min after ICSI. Strontium chloride treatment was performed as described by Swann and Ozil [1994]. For artificial activation, the oocytes were exposed to 10 mM of $SrCl_2$ in MRC#OW medium (Oocyte washing medium, Biosupply Co., Korea) for 60 min in an atmosphere of 6% CO_2, 5% O_2, and 90% N_2. The oocytes were subsequently rinsed several times in MRC#D01 medium.

Oocyte culture and embryo transfer

Fertilization was assessed 18 h after insemination by the appearance of two distinct pronuclei and two polar bodies. The zygotes were co-cultured with autologous cumulus cells in 10 μl of MRC#D16 medium (Cleavage and blastocyst medium, Biosupply Co., Korea) supplemented with 20% autologous follicular fluid (AFF) [Yoon et al. 2001]. The AFF was collected from follicles that produced healthy mature oocytes with a clear corona radiata. The AFF was used for culture after inactivation at 56°C for 30 min and sterilization with a 0.22 μm filter, followed by centrifuging for 15 min at 3,000 rpm.

Embryonic development was assessed on Day 3 of culture according to the regularity of blastomeres, the percentage and pattern of anucleate fragments, and all dysmorphic characteristics of the embryos. In this study, the embryo was defined as ''good-quality'' if it developed into at least eight blastomeres, had <5% of anucleate fragments, and showed no apparent morphologic abnormalities. The embryos were transferred on Day 3 after oocyte retrieval via transcervical route, in a standard manner. After embryo transfer, the surplus embryos were further cultured until Day 6, and the embryos that developed to the blastocyst stage were cryopreserved through the vitrification method [Lee et al. 2006].

Assessment of infant health care

Prior to the infant health care assessment, informed consent was obtained from the couples. This assessment was according to the standard set by the National Health Insurance Service (NHIS) in Korea. The infant health and oral cavity care questionnaire, physical measurement (height, body weight, head circumference, BMI, etc.), development screening test (exercise, language personal-social, self-care, problem solving, etc.), health education and counseling (injury prevention, nutrition, sleeping, etc.), which assessed infant health care in terms of physical and mental health, was completed by pediatricians as described in Supplements 1–4. The children were checked at birth and at 6, 12, 24, 36, 48, and 60 months.

Statistical analysis

The statistical analysis was carried out with SPSS 12.0 software (SPSS Inc., Chicago, IL, USA). Quantitative data are presented as the mean ± standard deviation (SD) and properties are used for categorical variables. Mean values were compared by student *t*-tests and proportions were compared by the chi-squared test or Fisher's extracted test. A probability value of $p < 0.01$ was considered as statistically significant.

Declaration of interest

The authors, Jun-Woo Kim, Sang-Don Kim, Seong-Ho Yang, San-Hyun Yoon, Jae-Hoon Jung, and Jin-Ho Lim have no declarations of interest.

Author contributions

All the authors participated in designing the study, patients' enrollment, analysis of results, preparation of manuscript and approved of the final manuscript. Conceived and designed the study: J-WK, S-HY; Recruited and treated patients: S-DK, J-HJ; Analyzed the data: J-WK, S-DK, S-HY, J-HJ; Wrote the manuscript: J-WK, S-DK; Corrected, revised, and approved the final version: S-HY, J-HJ, J-HL.

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