

CLINICAL CORNER: COMMUNICATION

Comparison of pregnancy outcomes in natural cycle IVF/M treatment with or without mature oocytes retrieved at time of egg collection

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The objective of this study is to compare the pregnancy and live birth rates of a natural cycle *in vitro* fertilization (IVF) combined with *in vitro* maturation (IVM) treatment (natural cycle IVF/M) by the presence or absence of mature oocytes retrieved. Infertile women were divided into two groups: (A) patients with mature oocytes found at retrieval and (B) patients with only immature oocytes at retrieval. Patients of group A were further divided into three subgroups: (A1) mature oocytes retrieved from both the leading and the small follicles, (A2) mature oocytes retrieved from the leading follicles only, and (A3) mature oocytes retrieved from the small follicles only. Pregnancy and implantation rates were compared. The results indicate that the clinical pregnancy rates were 40.1% (126/314) and 34.5% (19/55) for groups A and B, respectively. There were no differences in pregnancy rates among the subgroups: A1 = 44.0% (66/150), A2 = 34.9% (30/86), and A3 = 38.5% (30/78). In addition there were no differences in implantation rates among the groups (16.2% = 139/859, 15.0% = 22/147, 16.8% = 69/410, 14.7% = 34/232, and 16.6% = 36/217, respectively). However, the live birth and miscarriage rates were significantly different between the group A and group B (29.6% = 93/314 vs. 16.4% = 9/55 and 26.2% = 32/126 vs. 52.6% = 10/19, respectively). In conclusion, for natural cycle IVF/M treatment, although the clinical pregnancy rates are not different regarding the retrieval of mature oocytes or the time of the egg retrieval, the live birth rate is higher ($P < 0.05$) when the mature oocytes are obtained at the time of the egg retrieval.

Keywords IVF, IVM, natural cycle, oocytes, pregnancy

Abbreviations IVF: *in vitro* fertilization; IVM: *in vitro* maturation; PCOS: polycystic ovary syndrome; OHSS: ovarian hyperstimulation syndrome; COCs: cumulus-oocyte complexes; GV: germinal vesicle; ET: embryo transfer.

Introduction

In recent years protocols of stimulation for *in vitro* fertilization (IVF) treatment have undergone considerable changes. As indicated previously [Edwards 2007a; 2007b], two modified forms of treatment are attracting attention. One is minimal stimulation IVF using a low dosage of gonadotropin, and another is *in vitro* maturation (IVM). Immature oocyte retrieval followed by IVM of these oocytes is gaining worldwide attention as treatment for women with infertility. Immature oocyte retrieval followed by IVM was shown initially to be a successful treatment for infertile women with polycystic ovary syndrome (PCOS), because there are numerous antral follicles within the ovaries in this group of patients making them prone to develop ovarian hyperstimulation syndrome (OHSS) [Chian et al. 2000].

Apart from these two attractive protocols, there has been an increasing interest in natural cycle IVF primarily because it is more comfortable for patients, has fewer side effects, and eliminates the potential for possible long-term effects of repeated ovarian stimulation with gonadotropin-releasing hormone and gonadotropin [Brinton et al. 2005]. However, the classical protocol of natural cycle IVF treatment is encumbered by a number of other problems, including an increased risk of a failure of oocyte retrieval during egg collection and an absence of an embryo for transfer. Nevertheless, there has been a resurgence of interest in natural cycle IVF treatment in recent years because the efficiency of IVF technology has improved markedly [Nargund et al. 2001; Marieke-Lukassen et al. 2003].

The strategy that has been successfully explored using natural IVF is the one combined with IVM (natural cycle IVF/M) for women without PCOS [Chian et al. 2004; Lim et al. 2007a and b; Lim et al. 2009], indicating that a selective group of ovulatory women can benefit from natural cycle IVF/M with acceptable pregnancy rates [Lim et al. 2007a]. More recently, we reported that natural cycle IVF/M treatment might be offered to more than 50% of the total infertile

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women who were seeking infertility treatment, and with more than 40% pregnancy rate [Lim et al. 2009]. However, it is still unclear whether during natural cycle IVF/M treatment there are differences in pregnancy and implantation rates between the patients with and without the presence of mature oocytes obtained at the time of the retrieval. Therefore, the goal of this study was to assess whether the efficiency of natural cycle IVF/M treatment is influenced by the stage of maturity of the oocytes obtained at the time of the retrieval.

Results

Table 1 summarizes patient characteristics while Table 2 shows the cycle outcome of group A (patients with mature oocytes) and group B (patients without mature oocytes). In group A, a total of 739 mature oocytes were obtained at the time of retrieval from 314 cycles of natural IVF/M with an average of 2.4 ± 1.6 per patient. Of these 739 mature oocytes, 644 (87.1%) were fertilized and 610 (94.7%) cleaved. There were also 2,780 immature oocytes

of which 1,826 (65.7%) matured *in vitro* and 1,457 (79.7%) were fertilized. In group B, a total of 515 immature oocytes were retrieved from 55 cycles with an average of 9.4 ± 4.9 per patient. Of these 515 immature oocytes, 363 (70.5%) were matured *in vitro* and 294 (81.0%) were fertilized. There were no differences between the two groups in terms of the total fertilization ($81.9\% = 2,101/2,565$ vs. $81.0\% = 294/363$) and embryo cleavage ($92.1\% = 1,934/2,101$ vs. $89.1\% = 262/294$) rates. Furthermore, the quality of embryos was not different between groups. Following embryo transfer, the clinical pregnancy rates and implantation rates between the groups were not different (40.1% (126/314) versus 34.5% (19/55) and 16.2% (139/859) versus 15.0% (22/147) in group A and group B, respectively). However, the live births per embryo transfer ($29.6\% = 93/314$ vs. $16.4\% = 9/55$) and miscarriage per clinical pregnancy ($26.2\% = 33/126$ vs. $52.6\% = 10/19$) rates were significantly different between group A and group B.

Table 3 shows the pregnancy outcomes based on the mature oocytes retrieved from the different sized follicles at the time of egg retrieval. Whether the mature oocytes were retrieved from leading follicles or small follicles, there were no differences in fertilization ($81.8\% = 1,054/1,288$, $81.8\% = 508/621$, and $82.2\% = 539/656$, respectively) and embryo cleavage ($91.9\% = 969/1,054$, $93.9\% = 477/508$ and $90.5\% = 488/539$, respectively) rates among those subgroups. Also the quality of embryos were not different among groups assessed by morphology. In addition, there were no statistically significant differences in terms of the clinical pregnancy ($44.0\% = 66/150$, $34.9\% = 30/86$, and $38.5\% = 30/78$), implantation ($16.8\% = 69/410$, $14.7\% = 34/232$, and $16.6\% = 36/217$), live birth per embryo transfers ($30.7\% = 46/150$, $26.7\% = 23/86$, and $30.8\% = 24/78$), and miscarriage per

Table 1. Patient characteristics.

	Group A	Group B
No. of patients (cycles)	283 (314)	53 (55)
Age (mean \pm SD)	31.2 ± 3.6	30.4 ± 3.3
Years of infertility (mean \pm SD)	2.9 ± 1.3	2.9 ± 1.0
Cause of infertility		
Tubal factor (%)	46.3	34.0
Male factor (%)	29.3	39.6
Unexplain (%)	5.7	1.9
Other (%)	18.7	24.5

Table 2. Comparison of pregnancy and live birth rates of natural cycle IVF/M treatment in women with or without mature oocytes collected at the time of egg retrieval.

Groups	A	B	P value
No. of patients (cycles)	283 (314)	53 (55)	–
Age (mean \pm SD)	31.2 ± 3.6	30.4 ± 3.3	NS
No. of mature oocytes retrieved (mean \pm SD)	739 (2.4 ± 1.6)	–	–
No. of immature oocytes retrieved (mean \pm SD)	2,780 (8.9 ± 5.0)	515 (9.4 ± 4.9)	NS
No. of oocytes matured <i>in vitro</i> (%)	1,826 (65.7)	363 (70.5)	NS
Total numbers of oocytes matured (mean \pm SD)	2,565 (8.2 ± 3.5)	363 (6.6 ± 3.5)	NS
No. of oocytes fertilized (%)	2,101 (81.9)	294 (81.0)	NS
No. of <i>in vivo</i> matured oocytes fertilized (mean \pm SD)	644 (2.1 ± 1.4)	–	–
No. of <i>in vivo</i> matured oocytes cleaved (mean \pm SD)	610 (1.9 ± 1.4)	–	–
No. of <i>in vitro</i> matured oocytes fertilized (mean \pm SD)	1,457 (5.1 ± 1.4)	294 (4.9 ± 2.2)	NS
No. of <i>in vitro</i> matured oocytes cleaved (mean \pm SD)	1,324 (4.2 ± 2.6)	262 (4.8 ± 2.7)	NS
No. of zygotes cleaved (%)	1,934 (92.1)	262 (89.1)	NS
No. of embryos transferred (mean \pm SD)	859 (2.7 ± 0.4)	147 (2.7 ± 0.5)	NS
No. of clinical pregnancies (%)	126 (40.1)	19 (34.5)	NS
No. of embryos implanted (%)	139 (16.2)	22 (15.0)	NS
Live births per cycle (%)*	93 (29.6)	9 (16.4)	0.043
Singleton	74	9	–
Twins	18	–	–
Triplets	1	–	–
Miscarriage rate per clinical pregnancies (%)*	33 (26.2)	10 (52.6)	0.019

Group A: The patients with mature oocytes retrieved at egg retrieval.

Group B: The patients without mature oocytes retrieved at egg retrieval.

* Significantly different between groups.

Table 3. Comparison of pregnancy and live birth rates based on the presence of mature oocytes retrieved from the leading or non-leading follicles at the time of egg retrieval.

Subgroups of A	A1	A2	A3	P value
No. of patients (cycles)	137 (150)	82 (86)	76 (78)	–
Age (mean ± SD)	31.6 ± 3.8	31.2 ± 3.2	31.0 ± 3.5	NS
No. of mature oocytes retrieved (mean ± SD)	477 (3.2 ± 1.5)	89 (1.0 ± 0.2)	173 (2.2 ± 1.6)	–
No. of immature oocytes retrieved (mean ± SD)	1,221 (8.1 ± 4.6)	808 (9.4 ± 5.4)	751 (9.6 ± 5.2)	NS
No. of oocytes matured <i>in vitro</i> (%)	811 (66.4)	532 (65.8)	483 (64.3)	NS
Total numbers of oocytes matured (mean ± SD)	1,288 (8.6 ± 3.5)	621 (7.2 ± 3.4)	656 (8.4 ± 3.5)	NS
No. of oocytes fertilized (%)	1,054 (81.8)	508 (81.8)	539 (82.2)	NS
No. of <i>in vivo</i> matured oocytes fertilized (mean ± SD)	417 (2.8 ± 1.5)	81 (1.0 ± 2.3)	146 (2.0 ± 1.3)	–
No. of <i>in vivo</i> matured oocytes cleaved (mean ± SD)*	395 (2.6 ± 1.4)	80 (0.9 ± 0.4)	135 (1.7 ± 1.3)	–
No. of <i>in vitro</i> matured oocytes cleaved (mean ± SD)	574 (3.8 ± 2.6)	397 (4.6 ± 2.7)	353 (4.5 ± 2.5)	NS
No. of zygotes cleaved (%)	969 (91.9)	477 (93.9)	488 (90.5)	NS
No. of embryos transferred (mean ± SD)	410 (2.7 ± 0.4)	232 (2.7 ± 0.5)	217 (2.8 ± 0.4)	NS
No. of clinical pregnancies (%)	66 (44.0)	30 (34.9)	30 (38.5)	NS
No. of embryos implanted (%)	69 (16.8)	34 (14.7)	36 (16.6)	NS
Live births per cycle (%)*	46 (30.7)	23 (26.7)	24 (30.8)	NS
Singleton	41	15	18	–
Twins	5	8	5	–
Triplets	0	0	1	–
Miscarriage rate per clinical pregnancies (%)*	20 (30.3)	7 (23.3)	6 (20.0)	NS

Group A1: Mature oocytes were retrieved from both leading and small follicles.

Group A2: Mature oocytes were retrieved from the leading follicles only.

Group A3: Mature oocytes were retrieved from the small follicles only.

* All embryos that produced from *in vivo* matured oocytes were transferred.

clinical pregnancy (30.3% = 20/66, 23.3% = 7/30, and 20.0% = 6/30) rates among those subgroups.

Discussion

Although the clinical pregnancy rates showed no differences whether the mature oocytes were retrieved or not, the results of this study demonstrated that for natural cycle IVF/M treatment, the live birth rate was higher ($P < 0.05$) when the mature oocytes were obtained at the time of the retrieval. The results of the present study also indicated that there might be a higher miscarriage rate when the transferred embryos were produced from the immature oocytes only.

In previous publications, it has been demonstrated that HCG-priming obtained better results in terms of oocyte maturation *in vitro* and clinical pregnancy rates than the non-priming group of infertile women with PCOS [Chian et al. 2000]. Further studies confirmed that even though the leading or dominant follicle was recruited, the administration of HCG did not affect the quality of oocytes retrieved from the follicles of smaller size [Chian et al. 2002; Chian et al. 2004]. In a previous study, we also demonstrated the existence of LH/HCG receptor in the cumulus cells from the small follicles when the HCG-priming IVM treatment cycles were performed for patients with PCOS [Yang et al. 2005].

An important observation of this study is that during natural cycle IVF/M it is impossible to predict the stage of oocyte maturity according to the follicular size. In fact, as seen in patients of group A, many *in vivo* mature oocytes were harvested from the small size follicles (<12 mm in diameter) than in the leading follicles (≥12 mm in diameter). This observation should spur renewed interest in studying

the process of folliculogenesis and the resumption of meiosis during natural cycles. One should also use caution in relying on a particular size of the follicle to predict oocyte maturity.

Lim et al. [2009] have shown that the optimal sized follicles could be between 12 to 14 mm in diameter for natural cycle IVF/M treatment, which can prevent premature ovulation [Lim et al. 2007a]. In this study, surprisingly, we have discovered that some mature oocytes can be retrieved from relatively small follicles (between 8 to 10 mm in diameter). We have demonstrated that the embryo development from *in vivo* matured oocytes had better quality than *in vitro* matured oocytes, resulting in a higher clinical pregnancy rate in the natural cycle IVF/M treatment with *in vivo* matured oocytes compared to the cycles without *in vivo* matured oocytes [Lim et al. 2007b]. It has also been reported that *in vivo* matured oocytes from polycystic ovary syndrome (PCOS) infertile women had significantly higher clinical pregnancy rates compared to patients without *in vivo* matured oocytes retrieved [Son et al. 2008]. In the present study, interestingly, the data shows that there were no significant differences in terms of the clinical pregnancy and implantation rates of the natural cycle IVF/M treatment with or without *in vivo* matured oocytes retrieved at the time of egg retrieval (Table 2).

A limiting factor of this study is that patients in group A had embryos transferred that were derived from both *in vivo* and *in vitro*-matured oocytes, rendering the assessment of the impact on pregnancy and implantation rates of *in vivo* matured oocytes versus the *in vitro* matured impossible. Although we do not know exactly the embryos produced from the *in vivo* matured oocytes from the leading follicles to the small follicles (Table 3), it seems that it was most

likely the embryos of Subgroup A1 produced for transfer were from the *in vivo* matured oocytes, because the total number of *in vivo* matured oocytes were 417 (2.8 ± 1.5) versus 410 (2.7 ± 0.4) embryos transferred. Nevertheless, we can confirm that the pregnancy and implantation rates in group B were from the embryos produced from the immature oocytes matured *in vitro* because there were only immature oocytes obtained at the time of egg retrieval in group B.

It is important to note that the quality of embryos was not always better from *in vivo* matured oocytes subject to natural cycle IVF/M treatment, especially the *in vivo* matured oocytes retrieved from relatively small follicles. This result contrasts the previous observation that the quality of embryos derived from *in vivo* matured oocytes is superior to the embryos produced from *in vitro* matured oocytes [Son et al. 2008]. This raises the issue of the state of the *in vivo* matured oocytes derived from the small follicles after HCG-priming triggered completion of nuclear maturation.

It is generally accepted that only the dominant or leading follicles respond to LH surge triggering resumption of meiosis and ovulation [Gougeon 1986; Gougeon and Testart 1990]. Even though there are LH/HCG receptors in the cumulus and granulosa cells from the relatively small follicles [Yang et al. 2005; Jin et al. 2005], it is not clear how the small follicles react to the physiological LH surge. Clearly, it is important to determine the time of HCG injection in the natural cycle IVF/M treatment in order to retrieve more mature oocytes from the leading follicles. The results presented in this study further demonstrate that the optimal sizes of follicles are between 12 to 14 mm in diameter in order to achieve a better clinical outcome with natural cycle IVF/M treatment.

Opinions diverge about HCG-priming IVM treatment. Indeed, some investigators believe that HCG-priming may not be beneficial to oocyte maturation from small follicles. This suggests that HCG priming alone may not have a significant effect on clinical outcome [Fadini et al. 2009]. Therefore, the different sizes of follicles and the way they respond to HCG-priming must be considered. First, the *in-vivo* matured oocytes were from both the dominant follicle and small follicles. Second, the GVBD oocytes from small follicles were derived from relatively small follicles but respond to HCG-priming. Third, the GV stage oocytes derived from small follicles did not respond to HCG-priming. It has been believed that the oocyte quality correlates with the size of follicles. Fadini et al. [2009] reported that FSH plus HCG priming obtained a better clinical outcome with modified IVM treatment.

Interestingly, there are no significant differences in terms of clinical pregnancy rates between group A ($40.1 = 126/314$) and group B ($34.5\% = 19/55$) independent of retrieving mature oocytes (Table 2). In fact, an average of 1.9 ± 1.4 embryos derived from *in vivo* matured oocytes were transferred in group A compared to group B in which no embryo was produced from *in vivo* matured oocytes transfer. In addition, the implantation rates were not different between these two groups. Surprisingly there was a higher miscarriage rate in group B as compared to group A

(Table 2), indicating that the embryos produced from *in vitro* matured oocytes may be associated with a higher miscarriage rate. We cannot reconcile the higher miscarriage rate for *in vitro* matured oocytes. However, further analysis of group A with subgroups by the mature oocytes obtained from the different sizes of follicles indicated that there were no differences among groups in terms of clinical pregnancy, implantation, live birth, and miscarriage rates (Table 3).

In conclusion, although the clinical pregnancy rates are not different in terms of mature oocytes being retrieved or the time of egg retrieval with natural cycle IVF/M treatment, the live birth rate is higher ($P < 0.05$) when the transferred embryos were produced from the *in vivo* matured oocytes. Natural cycle IVF/M may be the most suitable treatment for younger women who have regular menstrual cycles.

Materials and Methods

Patients

A total of 336 patients with a minimum of a 2 year history of infertility underwent 369 cycles from April 2005 to December 2009. All patients had regular menstrual cycles and a normal uterus. The reasons of infertility were tubal factor, male factor, and unexplained (Table 1). The treatment protocol was approved by the Institutional Review Board (IRB) of Maria Fertility Hospital, Seoul, South Korea, and informed written consent was obtained from all patients.

Natural cycle IVF/M treatment

The protocol for natural cycle IVF/M (Lim-Chian protocol) was described previously [Lim et al. 2009]. In brief, the treatment cycle was initiated based on an ultrasound scan on d 3-5, and repeated on d 7-9 and then at 1-3 d interval until the leading follicle reached 12-14 mm in diameter. At this point 10,000 IU of human chorionic gonadotropin (HCG; IVF-C[®]; LG Inc., Seoul, Korea) was administered intramuscularly and the oocyte retrieval was performed 36 h later. The minimum endometrial thickness had to be more than 6 mm when HCG was administered.

Transvaginal ultrasound-guided aspiration was performed using a 17-gauge double lumen needle (COOK, Eight Mile Plains, Queensland, Australia) for the aspiration of the leading follicles and then with the use of a 19-gauge single lumen needle (COOK) for the aspiration of small follicles. A portable aspiration pump was connected to the aspiration needle with a pressure < 100 mmHg. The aspirates were collected in tubes (10 mL) containing pre-warmed heparinized Ham's F-10 medium buffered with HEPES.

Cumulus-oocyte complexes (COCs) from the leading follicle (≥ 12 mm in diameter) and small follicles (< 12 mm in diameter) were evaluated for maturity under a stereo-microscope with 'sliding' technique (Chian et al. 2000). In brief, by letting COCs slowly slide down from one side to another at the bottom of the tissue culture dish (60 x 15 mm, Falcon) it is possible to observe the oocyte cytoplasm, and assess whether it contains a germinal vesicle (GV). To avoid the possibility of missing COCs from small follicles, the

remaining follicular aspirates were filtered using a Cell Strainer ($\text{\O}70\text{-}\mu\text{m}$, Falcon, Becton Dickinson & Company, NJ, USA), and washed three times with HEPES buffered Ham's F-10 medium supplemented with recombinant human serum albumin. If the COCs did not contain a GV, they were denuded of the cumulus cells using 0.03% hyaluronidase (Sigma, St Louis, MO, USA) in HEPES buffered Ham's F-10 medium and mechanical pipetting. Oocyte maturation was assessed by the presence of the first polar body (1PB) in the perivitelline space (PVS).

In vitro fertilization (IVF) and in vitro maturation (IVM)

The mature oocytes collected at the time of retrieval were subjected to insemination 2 or 3 h later by intracytoplasmic sperm injection (ICSI). The immature oocytes (Metaphase-I and germinal vesicle stage) were transferred into an organ culture dish (60 x 15 mm, Falcon; Franklin Lakes, NJ, USA) containing 1 mL of maturation medium (Maria Fertility Hospital, Korea) supplemented with 30% of the patients' own serum (inactivated at 56° for 30 min) with a final concentration of 0.6 IU/ml recombinant human FSH (Gonal-F; Merck Serono, Seoul, Korea), 0.1 IU/mL human chorionic gonadotropin (IVF-C; IVF-C°; LG Inc., Seoul, Korea), and 10 ng/mL recombinant human epidermal growth factor (Invitrogen, Seoul, Korea) at 37°C in 6% CO₂, 5% O₂, and 89% N₂ with high humidity for maturation in culture. Three h after culture, the metaphase-I oocytes were re-checked again for their maturity. If the oocytes became mature, the oocytes were denuded from the cumulus cells using 0.03% hyaluronidase and mechanical pipetting, and then inseminated by ICSI 2 h later. The remaining immature oocytes were cultured overnight, and then all COCs were denuded of the cumulus cells. At this point, *in vitro* matured oocytes were inseminated by ICSI 2 h after denuding. Fertilization was assessed 17-19 h after ICSI to detect the appearance of two distinct pronuclei and two polar bodies. The zygotes were then cultured in 20 μL of embryo culture medium (Maria Fertility Hospital, Korea) for further development.

Embryo transfer and endometrial preparation

Embryo transfer (ET) was performed on d 3 or 4 after oocyte retrieval. Before transfer, all embryos for each patient were pooled together and selected for transfer. The developed embryos were graded according to the following criteria: grade 1, even-sized and symmetrical blastomeres with no obvious fragmentation, grade 2, uneven sized blastomeres, or a total cytoplasmic mass containing <10% fragmentation, grade 3, 10%-50% cytoplasm fragmentation, and grade 4, >50% cytoplasmic fragmentation. Grade 1 and grade 2 embryos were considered good quality embryos.

For endometrial preparation, 6 mg Estradiol Valerate (Progynova®, Schering, Korea) was administered daily and started on day of oocyte retrieval, and luteal support with 100 mg progesterone in oil (Progest®, Samil Pharm. Co., Ansan, Korea) administered daily started on day of initial ICSI. Serum pregnancy test was obtained on d 15 or 16 after oocyte retrieval, and clinical pregnancy was determined

by visualization of a gestational sac and fetal heart beat on ultrasound 6 w after ET.

Group design

Based on whether mature oocytes were obtained or not at the time of retrieval, the patients were divided into two groups: patients with mature oocytes (group A) and patients with immature oocytes only (group B). In addition, patients of group A were further subdivided into subgroup A1: if mature oocytes were retrieved from both the leading and the small follicles, subgroup A2: if the mature oocytes were retrieved from the leading follicles only, and subgroup A3: if the mature oocytes were retrieved from the small follicles only.

Statistical analysis

The data were analyzed through and agreement with Kappa statistics using StatsDirect (version 1.9.14 for Windows; StatsDirect Ltd, Cheshire, UK). Evaluation of the differences among groups, clinical pregnancy, implantation, live birth, and miscarriage rates were analyzed by χ^2 -test. Results are expressed as mean values with 95% confidence interval; the differences were considered statistically significant if $P < 0.05$.

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