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Systems for Production of Calves from Hanwoo (Korean Cattle) IVM/IVF/IVC Blastocyst
Ⅲ. Vitrification and One-Step Dilution of Hanwoo Blastocyst

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ABSTRACT

This study was to examine whether Hanwoo IVM /IVF /IVC blastocyst can be successfully survived *in vitro* / *in vivo* after vitrification and one-step dilution. For vitrification, blastocysts were serially exposed in glycerol (G) and ethylene glycol (EG) mixtures [10% (v /v) G for 5 min., 10% G plus 20% EG (v /v) for 5 min., and 25% G plus 25% EG (v /v) for 30 sec.] which is diluted in 10% FBS added D-PBS. And then they were loaded in the straw, placed in cold nitrogen vapor for 3 min. and plunged into LN_2 (−196℃). One-step dilution within the straw was done in 25 ℃ and 36 ℃ water for about 5 min. and 3 min., respectively. Recovered embryos after one-step dilution were cultured in cumulus cell mono-layered drop for 48 h or were transferred into recipient cows. When the embryo survival *in vitro* was assessed to re-expanded and hatched rates at 24 h and 48 h after one-step dilution, the results of vitrified group (85.4, 43.8%) was high, although these results were significantly lower than normal development (100.0, 63.3%) of control group, respectively ($P<0.001$, $P<0.05$). When *in vitro* survival of vitrified groups according to developmental stage was compared, the results of fast developed embryos (expanded blastocyst and early hatching blastocyst stage) were significantly higher than those of delayed developed one (early blastocyst stage) after one-step dilution (early hatching: 88.0, 48.0%; expanded: 81.1, 45.3%; early: 66.7, 14.3%) ($P<0.05$). Also, in case of *in vitro* survival of vitrified groups according to embryo age (day 7, 8 and 9), when embryo age was younger, *in vitro* survival was significantly higher (day 7: 67.3, 34.5%; day 8: 76.9, 40.7%; day 9: 60.9, 23.9%) ($P<0.05$). Finally, when *in vivo* development potential of vitrified and one-step diluted Hanwoo blastocysts was examined, 4 of 8 recipient (50%) cows became confirmed pregnant. These results demonstrated that our vitrification and one-step dilution technique can be applied easily and effectively on field trial without the equipment and embryological skills required for conservative dilution and transfer.

(Key words: Hanwoo IVM /IVF /IVC blastocyst, Vitrification, One-step dilution *In vitro*/*In vivo* survival)

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I. INTRODUCTION

Embryo transfer has become a routine procedure in cattle breeding programs, and cryopreservation of embryos plays an integral role in such programs. Among the freezing methods, direct transfer would be practical and acceptable procedures for use under on-farm conditions (Dochi et al., 1998).

The direct transfer method make possible to eliminate technical errors associated with the procedure from thawing and dilution. Study on direct transfer was firstly demonstrated by Leibo group in 1984, "one-step"™ straw method, in which bovine embryos were slowly frozen using glycerol and diluted only in the straw before being transferred into recipient cows. Besides, many researchers reported the practicability of direct transfer methods (Massip et al., 1984; Suzuki et al., 1990; Voelkel and Hu, 1992; Dochi et al, 1995). However, most of them used the conservative method in freezing step although they selected the simple one-step dilution in thawing step by necessity. Recently, vitrification has been widely used and is now regarded as a potential alternative to conservative slow freezing. Vitrification has potential advantages over conventional freezing in that it takes only a few seconds for cooling embryos, and there is no extra-cellular crystallization, which is one of the major causes of cell injury (Rall and Fahy, 1985). Thus, if Hanwoo IVM /IVF /IVC blastocysts were vitrified and one-step diluted at freezing and thawing, their practical application in the field would be much easier. In this study, as a preliminary test for practical embryo transfer program, we tested whether the Hanwoo IVM /IVF /IVC blastocyst can be successfully survived *in vitro* /*in vivo* after vitrification and one-step dilution.

II. MATERIALS AND METHODS

1. Production of Hanwoo IVM/IVF/IVC Blastocysts

Chemicals were obtained from Sigma Chemical Company (St. Louis, MO) and media from GIBCO (Grand Island, NY), unless otherwise stated. The culture procedures employed in the production of preimplantation embryos from Hanwoo follicular oocytes were as outlined by Park et al. (1998). Briefly, Hanwoo ovaries were obtained from a slaughterhouse, and cumulus-oocyte complexes (COCs) were aspirated from visible follicles (2~6 mm in diameter).

The COCs were then washed with HEPES -buffered Tyrode's medium and cultured in maturation medium composed of TCM199+10% fetal bovine serum (FBS) supplemented with 0. 2 mM sodium-pyruvate, 1 μg /ml follicle-stimulating hormone, 1 μg /ml estradiol-17β, and 25 μg /ml gentamycin sulfate at 39℃, 5% CO_2 incubator. After incubation for 22~24 h in IVM medium, the COCs were inseminated using highly motile sperm recovered from frozen-thawed Hanwoo bull semen separated on a discontinuous percoll column. Fertilization was assessed as cleavage rate (≥2-cell) after 44±2 h co-incubation with the sperm. For *in vitro* culture, cleaved embryos were cultured in CR1 (Rosenkrans et al., 1993) medium supplemented with 3 mg /ml fatty acid-free BSA and then transferred into 10% FBS added CR1 medium at day 4 after IVF. For the study, day 8 blastocysts produced *in vitro* after IVF were mainly used and they were classified into early, expanded and early hatching stage according to their developmental morphology (Kim et al., 1996) (Fig. 1). Also, expanded blastocysts produced *in vitro* from day 7 to day 9 after IVF were applied to comparison of developmental capacity after freezing and one

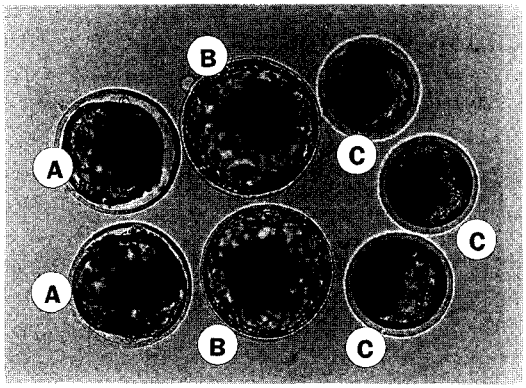

Fig. 1. Classification of Hanwoo IVM/IVF/IVC blastocysts. Embryos were divided into early (C), expanded (B) and early hatching (A) stage according to blastocoele expansion and zona thickness. × 150.

-step dilution.

2. Vitrification Procedures

Freezing was slightly modified from the method described by Agca et al. (1998). Solutions used for vitrification were prepared in modified Dulbecco's phosphate buffered saline (D-PBS) supplemented with 10% FBS. Vitrification solutions were mixtures of glycerol (G) and ethylene glycol (EG). Selected blastocysts for experiments were first exposed to 10% (v/v) G for 5 min, then were transferred to 10% G plus 20% EG (v/v) for 5 min, and finally were transferred to 25% G plus 25% EG (v/v) (G25EG25). And then they were transferred using pasteur pipette into vitrification solution section of 0.25 ml French mini straw (IMV, L'Aigle, France) and heat-sealed within 30 to 45 sec. Plastic 0.25 ml straws were prepared as follows. Briefly, a 7.5 cm length was filled with 0.5 mol sucrose solution (prepared in m-DPBS containing 10% FBS) followed by a 0.5 cm air bubble, 1.0 cm G25EG25, 0.5 cm air bubble. The remaining part of the straw was filled with 0.5 mol sucrose. Before being plunged into LN_2, straw was placed horizontally on styroform box which included LN_2 and exposed by the cold nitrogen vapor for 3 min. Average embryo numbers loaded in each straw were about three to five.

3. Thawing and One-step Dilution Procedures

Thawing and one-step dilution were done as described by van Wagtendonk-de Leeuw et al. (1997). Vitrified straws were thawed in air of 25 to 27℃ for 10 sec and then water of 25℃ until all ice had disappeared.

One-step dilution of thawed straws was done as follows. Straws were held at the plugged end and yield single columns of air and liquid. Straws were placed vertically (plug-end down) 36℃ water for 3 min and then in 25℃ water for about 1 min (plug-end up). Straws were then held at the end opposite to the plug in 25℃ water for 3 min.

4. *In vitro* Culture after One-step Dilution

After dilution procedures were finished, the heat seals were removed by cutting with scissors, contents of straws were poured into petri-dish and then recovered embryos were placed in an co-culture drop (10μl) to examine the *in vitro* survival for 48 h. Cumulus cell drop was prepared with cumulus cells recovered from *in vitro* matured Hanwoo oocytes before IVF treatment.

5. Embryo Transfer

Vitrified and one-step diluted embryos were transferred directly into recipients by placing the straw into an embryo transfer gun. This embryo transfer into uterus of recipient cows was carried out within 10 min after dilution. Recipient cows were prepared as follows. They were multiparous and were received embryos either 7

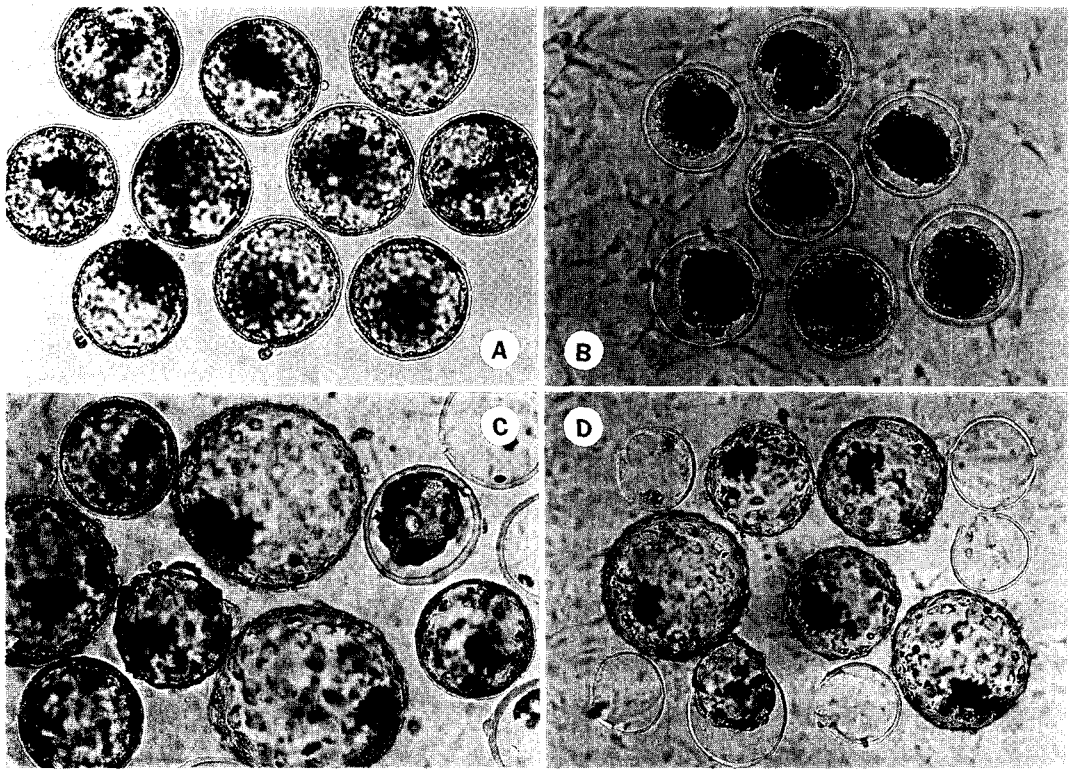

Fig. 2. Survival morphology of vitrified Hanwoo blastocysts after one-step dilution. When vitrified IVM/IVF/IVC Hanwoo blastocysts (A) were one-step diluted in the straw, embryos were indicated shrink morphology before being introduced into culture drop (B). After culture for 24 h (C) and 48 h (D), survived blastocysts were showed normal development (A-C: ×150, D: ×80).

or 8 day standing estrus during natural estrus cycles. Estrus was monitored by artificial insemination technicians. Recipients were palpated for the presence of a well-formed corpus luteum and no abnormal structures on the ovary or uterus immediately before one-step dilution and transfer of the embryo.

6. Assessment of Embryo Survival after Thawing

In case of *in vitro* survival, the post-thawing survival of embryos was observed every 24 h under microscope and judged as morphological survivors if they were expanded into blastocysts within the first 24 h of culture (Fig. 2C), and hatched out totally within the next 48 h (Fig. 2D). In case of *in vivo* survival, pregnancies were confirmed at first when recipient cows did not return to the subsequent estrus cycle, and later by manual palpation per rectum on day 45 and 90.

7. Experimental Design

First, to examine the usability of vitrification and one-step dilution, *in vitro* development of freezing-thawing group was compared with that of control group. Second, to compare *in vitro* survival of vitrified groups according to develop-

mental stage, classified day 8 Hanwoo blastocysts were thawed and one-step diluted. Third, to compare *in vitro* survival of vitrified groups according to embryo age, day 7, 8 or 9 expanded Hanwoo blastocysts were thawed and one-step diluted. Last, to examine the potential of *in vivo* development after vitrification and one-step dilution, Hanwoo blastocysts were transferred into uterus of recipient cows.

8. Statistical Analysis

The significance of difference among treatment group in each experiment was compared with Chi-square test using SAS Institute software package (SAS Institute Inc., Cary, NC, USA, 1985).

Ⅲ. RESULTS

1. Hanwoo Embryo Production *in vitro*

In a total of 10 replicates, 2,992 Hanwoo oocytes were subjected to *in vitro* maturation and fertilization procedures. On day 2 a total of 2,541 (84.9%) embryos were cleaved. The total number of embryos that developed to blastocysts day 7 after IVF was 1,278 (50.3%). Also, when they were classified to early, expanded and beyond hatching blastocyst stage, 470 (18.5%), 572 (22.5%) and 236 (9.3%) were produced, respectively (Table 1).

2. *In vitro* Survival of Vitrified Day 8 Hanwoo IVM/IVF/IVC Blastocysts

To examine the usability of vitrification and one-step dilution, *in vitro* development of freezing-thawing group was compared with that of control group. When the embryo survival *in vitro* was assessed to re-expanded and hatched rates at 24 h and 48 h after one-step dilution, as shown in Table 2, the results of vitrified group (85.4, 43.8%) was high, although these results were significantly lower than normal development (100.0, 63.3%) of control group, respectively ($P<0.001$, $P<0.05$).

Table 1. Development of Hanwoo follicular oocytes at day 8 after IVF (r=10)

No. of oocytes	No.(%) of ≥2-cell	No.(%) of development to			
		≥Bla.*	ErB	EdB	≥HgB
2,992	2,541 (84.9)	1,278 (50.3)	470 (18.5)	572 (22.5)	236 (9.3)

*Bla.; Blastocyst, ErB; Early blastocyst,
EdB; Expanded blastocyst, HgB; Hatching blastocyst.

Table 2. *In vitro* survival of vitrified Day 8 Hanwoo IVM/IVF/IVC blastocysts after one-step dilution (r=20)

Treatment	No. of blastocyst examined	No (%). of blastocysts survived		
		24 h later	48 h later	
		≥ EdB or Re-edB*	≥ HgB	HdB
Control	120	120 (100.0)[a]	106 (88.3)[c]	76 (63.3)[c]
Vitrified	89	76 (85.4)[b]	68 (76.4)[d]	39 (43.8)[d]

* EdB; Expanded blastocyst, Re-edB; Re-expanded blastocyst,
HgB; Hatching blastocyst, HdB; Hatched blastocyst
Means in the column without common superscripts are significantly different ($p<0.001$)[a-b], ($p<0.05$)[c-d].

3. *In vitro* Survival of Vitrified Hanwoo IVM/IVF/IVC Blastocysts according to Developmental Stage

To compare *in vitro* survival of vitrified group according to developmental stage, classified day 8 Hanwoo blastocysts were thawed and one-step diluted. When the treatment groups were divided into three, as indicated in Table 3, fast developed embryo groups (expanded blastocyst and early hatching blastocyst stage) showed the better resistance to cryoppreservation than delayed developed embryo group (early blastocyst stage) at 24 h and 48 h after one-step dilution (early hatching: 88.0, 48.0%; expanded: 81.1, 45.3%; early 66.7, 14.3%) ($P<0.05$). However, the highest survival rates among the developmental stage were obtained in early hatching blastocysts.

4. *In vitro* Survival of Vitrified Hanwoo IVM/IVF/IVC Blastocysts according to Embryo Age

To compare *in vitro* survival of vitrified groups according to embryo age, day 7, 8 or 9 expanded Hanwoo blastocysts were thawed and one-step diluted. In Table 4, when embryo age was younger, *in vitro* survival was significantly higher (day 7: 67.3, 34.5%; day 8: 76.9, 40.7%; day 9: 60.9, 23.9%) ($P<0.05$). However, day 8 expanded embryos, which age showed vigorous blastocyst production, were the highest sur-

Table 3. *In vitro* survival of vitrified Day 8 Hanwoo IVM/IVF/IVC blastocysts according to developmental stage after one-step dilution

Blastocyst stage	No. of blastocyst examined	No (%). of blastocysts survived		
		24 h later	48 h later	
		≥EdB or Re-edB*	≥HgB	HdB
Early hatching	50	44 (88.0)^{a}	41 (82.0)^{c}	24 (48.0)^{a}
Expanded	53	43 (81.1)^{a,b}	38 (71.7)^{c}	24 (45.3)^{a}
Early	21	14 (66.7)^{b}	7 (33.3)^{d}	3 (14.3)^{b}

*EdB; Expanded blastocyst, Re-edB; Re-expanded blastocyst,
HgB; Hatching blastocyst, HdB; Hatched blastocyst
Means in the column without common superscripts are significantly different ($p<0.05$)$^{a-b}$, ($p<0.01$)$^{c-d}$.

Table 4. *In vitro* survival of vitrified Hanwoo IVM/IVF/IVC blastocysts according to embryo age after one-step dilution

Embryo age (day)	No. of blastocyst examined	No (%). of blastocysts survived		
		24 h later	48 h later	
		≥EdB or Re-edB*	≥HgB	HdB
7	55	37 (67.3)	33 (60.0)^{a}	19 (34.5)^{a,b}
8	108	83 (76.9)	75 (69.4)^{a}	44 (40.7)^{a}
9	92	56 (60.9)	39 (42.4)^{b}	22 (23.9)^{b}

*EdB; Expanded blastocyst, Re-edB; Re-expanded blastocyst,
HgB; Hatching blastocyst, HdB; Hatched blastocyst
Means in the column without common superscripts are significantly different ($p<0.05$)$^{a-b.}$

vival *in vitro* among the treatment groups.

5. *In vivo* Survival of Vitrified Hanwoo IVM/IVF/IVC Blastocysts after One-step Dilution

To examine the possibility of *in vivo* development after vitrification and one-step dilution, 2 Hanwoo blastocysts per each were transferred into uterus of 8 recipient cows. After transfer of thawed /one-step diluted blastocysts into recipient cows, 4 of 8 recipients (50%) did not return to the subsequent estrus cycle, and all of them were found pregnant by manual palpation at 45 days after transfer.

Ⅳ. DISCUSSION

These results demonstrate that vitrification and one-step dilution can be applied as an practical technique when cryopreserved Hanwoo IVM /IVF /IVC blastocysts were used in the field. The application of vitrification and one-step dilution to on-farm transfer reduces the equipment and embryological skills required for dilution and transfer, yields a considerable savings of time per transfer (Massip et al., 1984; Suzuki et al., 1990; Voelkel and Hu, 1992; Dochi et al, 1995, 1998). Like this, recent most attempts to improve the cryopreservation of bovine embryos have been directed the simplification of freezing and thawing procedures. In this study, we endeavored to find the simple and effective freezing and thawing method of Hanwoo IVM / IVF /IVC blastocyst for on-farm transfer. At present, vitrification has been widely used and is now regarded as a potential alternative to conservative slow freezing. Vitrificaton has potential advantages over conventional freezing in that it takes only a few seconds for cooling embryos, and there is no extracelluar crystallization, which is one of the major causes of cell injury (Rall and Fahy, 1985). In this study, vitrification using glycerol and ethylene glycol mixture as cryoprotectants showed stable *in vitro* survival after thawing and one-step dilution. In the results, *in vitro* survival of vitrification and one-step dilution group was high (≥re-expansion at 24 h: 85.4%, ≥hatching at 48 h: 76.4% and hatched at 48 h: 43.8%), although these results were significantly lower than normal development (100.0, 88.3 and 63.3%) of control group, respectively (24 h: $P<0.001$, 48 h: $P<0.05$). Also, fast developed embryos (expanded blastocyst and early hatching blastocyst stage) produced at day 8 showed the better resistance to cryopreservation than delayed developed one (early blastocyst stage) at 24 h and 48 h after one-step dilution (early hatching: 88.0, 48.0%; expanded: 81.1, 45.3%; early: 66.7, 14.3%) ($P<0.05$). Recently, the pregnancy rate obtained in field trial of vitrified and one-step diluted bovine embryos produced *in vitro* was reported 36% by routine nonsurgical transfers (Vajta et al., 1997). In this study, although there was a few in count, after transfer of one-step diluted blastocysts, 4 of 8 recipient (50%) cows did not return to the subsequent estrus cycle, and all of them were found pregnant by manual palpation at 45 days after transfer. But, for accurate examination on *in vivo* survival of vitrified and one-step diluted Hanwoo blastocysts, a number of embryo transfer in field should be continued in the future. However, by this preliminary test for practical ET program, we confirmed that the Hanwoo IVM /IVF /IVC blastocysts can be successfully survived *in vitro /in vivo* after vitrification and one-step dilution.

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요 약

체외생산된 한우 배반포기배로부터 송아지 생산을 위한 체계

Ⅲ. 한우 배반포기배의 초자화 동결과 1단계 융해

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본 실험은 체외 생산된 한우 배반포기배가 초자화 동결과 1-단계 융해 후 체외/체내에서 성공적으로 생존할 수 있는지의 여부를 확인하고자 실시하였다. 초자화 동결은 glycerol (G) 또는/과 ethylene glycol (EG), 그리고 10% FBS가 들어있는 D-PBS 동결액을 이용하여 배반포기배를 10% G에서 5분, 10% G와 20% EG 혼합액에서 5분 그리고 25% G와 25% EG 혼합액에서 30초간 노출시켰으며, straw에 넣은 후 질소 증기에 3분간 쐬고 액화질소에 침지함으로써 완성하였다. 1-단계 융해는 straw 자체를 25 ℃와 36 ℃에 각각 5분과 3분간 처리함으로써 이루어졌으며, 융해 후 회수된 난자는 단층배양이 유도된 난구세포 소적에서 48시간 배양하거나 대리모인 소에 이식하였다. 체외 생존능 평가를 융해 후 24시간째의 재팽창율과 48시간째의 부화율로 조사하였을 때, 대조군의 정상 발달 (100.0, 63.3%) 보다는 유의하게 낮았지만 초자화 동결군의 결과 (85.4, 43.8%) 는 높게 나타났다 ($P<0.001$, $P<0.05$). 발달단계에 따른 체외생존능을 조사하였을 때, 빠르게 발달된 배반포기배가 느리게 발달하는 난자군보다 유의하게 높은 생존율을 나타내었다 (부화초기: 88.0, 48.0%; 팽윤: 81.1, 45.3%; 초기: 66.7, 14.3%) ($P<0.05$). 또한, 배반포기배가 생산된 나이(체외수정 후 7, 8, 9일)에 따른 초자화동결군의 체외생존능을 조사하였을 때, 배의 나이가 어릴수록 체외생존능은 유의하게 높은 경향을 나타내었다 (7일: 67.3, 34.5%; 8일: 76.9, 40.7%; 9일: 60.9, 23.9%) ($P<0.05$). 초자화동결된 한우배반포기배의 1-단계 융해 후 체내생존 가능성을 확인하고자 8마리의 소에 이식하였을 때, 4마리가 임신된 것을 확인하였다. 따라서, 이상의 높은 생존율을 고려하여 볼 때, 본 연구에서 사용된 초자화동결과 1-단계 융해는 기존의 동결란을 융해 이식할 때 필요로 했던 실험장비나 고도의 숙련된 기술 없이도 실험현장에서 쉽게 이용할 수 있으면서 효율적인 결과를 얻을 수 있는 방법이라는 것을 알 수 있었다.

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