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Addition of Macromolecules to PZM-3 Culture Medium on the Development and Hatching of *In vitro* Porcine Embryos

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ABSTRACT : In this study, we conducted various experiments in order to develop enhanced cultural conditions for *in vitro*-produced porcine embryos. All embryos were produced by *in vitro* maturation (IVM) and fertilization (IVF) of immature oocytes from abattoir-derived ovaries. In experiment 1, we cultured IVF embryos in 4 different groups, namely, 0% bovine serum albumin (BSA), 3% BSA, 0.05% Polyvinyl alcohol (PVA), and 0.5% Polyvinylpyrrolidone (PVP) added to the basal fluid cultural medium, Porcine zygote medium 3 (PZM-3). The rates of embryo development were higher in the group where the PZM-3 media had been supplemented with 3% BSA than the other groups. While not statistically significant, the percent of blastocysts and hatched blastocytes were 6.9% and 25.0% in the 3% BSA group vs. 1.2-6.4% and 0-16.7% in the other groups, respectively. In experiment 2, we added 10% fetal bovine serum (FBS) to PZM-3 on day 0 of culture and observed the development rate of blastocysts per day of culture from days 0 to 5. The development rate of blastocysts was higher at 15.6% on day 4 than on any other day, and was significantly higher than on day 0 or day 1 ($p<0.05$). The development rate of hatched blastocysts was 26.7% on day 4, and was higher than on any other day. In experiment 3, we cultured IVF embryos with different fluid culture media, grouped as 1) PZM-3+0.3% BSA (day0-day7); 2) PZM-3+0.3% BSA→(day-4) PZM-3+10% FBS; 3) PZM-3+0.3% BSA→PZM-3+0.3% BSA+(day-4) FBS 10%; and 4) PZM-3+0.3% BSA+10% FBS (day0-day7). The development rates of blastocysts and hatched blastocysts were 21.5% and 53.1% in group 3, respectively, which was significantly higher than group 4 with respect to blastocyst development (5.2%, $p<0.05$) but not hatched blastocysts (14.3%). The total cell number (TCN) of blastocysts in group 3 was higher at 37.8±16.1 than the other groups at 16.8±4.4 - 30.1±10.9; however, this was not significantly different. The results of this study showed that PZM-3 containing 0.3% BSA and supplemented with FBS during the later stage of culture on day 4 resulted in better TCNs and an increased rate of hatched blastocysts. (**Key Words :** Porcine Embryos, Macromolecule, Blastocysts, Cell Number)

INTRODUCTION

In vitro production (IVP) of porcine embryos is one of the major aspects of the study of mammalian embryogenesis and is widely used in veterinary medicine, animal husbandry, and biotechnology as a means for production of individuals with superior genetic characters by embryo transplantation, development of micro-manipulation techniques (Lee et al., 2007), and stem cell studies.

Many scientists have studied IVP of porcine embryos for a number of years. Iritani et al. (1978) successfully performed *in vitro* fertilization (IVF) of porcine embryos in 1978 and Mattioli et al. (1989) succeeded in birthing piglets by IVF embryo transplantation. Although IVF and embryo production using *in vitro* matured (IVM) porcine oocytes have been successful, their developmental potential is still lower than that of embryos produced *in vivo*. There are a number of major factors on the *in vitro* culture of mammalian embryos such as *in vitro* maturity level of immature oocytes (Gupta et al., 2005; Park and Kim, 2007), IVF process, culturing method, among others.

For the culture of porcine embryos, a number of different culture media for pig embryos such as Whitten's medium (Beckmann et al., 1993), North Carolina State University 23 (NCSU-23) (Petters et al., 1993), and Beltsvile embryo culture medium 3 (BECM-3) have been developed (Dobrinsky et al., 1996); NCSU-23 medium is

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now widely in use. Recently, Porcine Zygote Medium 3 (PZM-3) has been developed as a new medium for the *in vitro* culture (IVC) of pig embryos (Yoshioka et al., 2002). Moreover, it has been reported that porcine embryos from a number of different origins have a higher developmental potential in PZM-3 compared with NCSU-23 or BECM-3 medium (Im et al., 2004).

Culture medium for porcine embryos usually consists of simple minerals, energy sources, amino acids, pH stabilizer, trace elements, and antibiotics. In addition, studies regarding the effects of growth factors (Makarevich et al., 2006), antioxidants (Lee et al., 2004; Jang et al., 2005), or chelators added to basal culture media on embryo development are being steadily conducted.

Supplementation of basic culture medium with macromolecular components remains the easiest and most practical system for routine IVP of porcine embryos. Defined media with polyvinylpyrolidone (PVP, Cholewa and Whitten, 1970) and polyvinylalcohol (PVA, Bigger et al., 1997; Lee and Funkui, 1996; Luo et al., 2006), semidefined media with bovine serum albumin (BSA, Bigger et al., 1997), and relatively undefined media with fetal bovine serum (FBS, Dobrinsky et al., 1996) have all been added to mammalian embryo media as energy sources.

The serum contains not only a variety of energy sources (Choi et al., 2002; Rizos et al., 2003) and growth factors (Saito et al., 1984; Ogawa et al., 1987) needed for *in vitro* embryonic development, but also plays a role as a barrier for oval adhesion and hardening under *in vitro* conditions (Kane, 1987). However, the influential ingredients of serum are not clearly known, although it has been reported that serum added to early stage porcine or bovine embryos can be dangerous to *in vitro* embryo development and can seriously reduce the development of hatched blastocysts (Wang and Day, 2002).

In contrast, one report indicated that addition of serum to later stage embryos produced *in vitro* could accelerate the development of blastocysts, resulting in an 80% hatch rate (Thompson et al., 1998; Choi et al., 2002). Thus, it appears that the addition of serum has a biphasic effect, in that the addition of serum to IVP embryos inhibits the development of blastocysts at early stages of growth, but promotes development at later stages.

In addition, it has been reported that serum contains both inhibitors of embryonic development (Ogawa and Marrs, 1987) and malformation inducing factors (Chatot et al., 1984); however, since the major components of serum can differ based on different sampling methods, PVA and PVP as BSA alternative are recognized for their role as simpler and consistent stimulus-inducing materials.

Therefore, until now, comparative studies that have investigated the effects of addition of various macromolecules on the development and hatching of porcine embryos have been performed using IVC media, but not PZM-3 media. Accordingly, in the present study, we researched the effects on the development and hatching of porcine *in vitro* embryos by addition of various macromolecules to PZM-3 medium and serum addition at later stages of culture in order to establish enhanced culture conditions in order to better understand the process of basic molecular metabolism with respect to the IVP of porcine embryos.

MATERIALS AND METHODS

Chemicals and media

Unless otherwise stated, all chemicals used in this study were purchased from Sigma Chemical CO. (St. Louis, MO, USA). Solutions were expressed as percent dilutions (v:v) and all media used for IVM, IVF and IVC were pre-warmed at 39°C in a 5% CO_2 atmosphere at maximum humidity 4 h prior to use.

IVM procedure

Ovaries were collected from prepubertal gilts at a local slaughterhouse and transported to the laboratory within 2 to 3 hours in saline supplemented with 25 μg/ml gentamicin at 25-30°C. Cumulus oocyte complexes (COCs) were obtained by aspiration from follicles 2 to 6-mm in diameter using an 18-gauge needle connected to a 10 ml disposable syringe. Only COCs with compact cumulus cell layers and evenly granulated ooplasm were selected. According to the method of Bavister et al. (1983) the COCs were washed 3 times in HEPES-Tyrode-albumin-lactate-pyruvate medium (TALP medium) supplemented with 25 mM HEPES and 3 mg/ml BSA. Groups of 50 COCs were then placed into 500 μl of BSA-free NCSU-23 solution containing 0.57 mM cysteine, 10% porcine follicular fluid (pFF), 2.5 mM β-mercaptoethanol, 10 ng/ml epidermal growth factor (EGF), 10 IU/ml human chorionic gonadotropin (hCG) and 10 IU/ml pregnant mare serum gonadotropin (PMSG) in each well of a 4-well multidish (Nunc, Roskilde, Denmark). After allowing 22 h for maturation, the oocytes were washed twice in the same maturation medium without PMSG and hCG and cultured in this medium for 22 h at 39°C in a 5% CO_2 atmosphere at maximum humidity.

IVF procedure

Diluted porcine semen was produced by the Dabby A.I center and was kept at 17°C for 5 days. The semen was layered on top of a discontinuous Percoll density gradient (2 ml 45% percoll over 2 ml 90% percoll) in a 15 ml tube and centrifuged at 500×g for 20 min at room temperature. The spermatozoa were collected in the bottom fraction and were

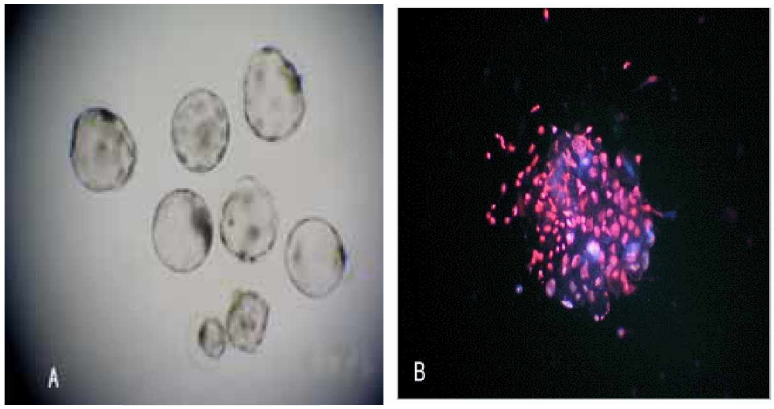

Figure 1. Photographs of porcine blastocysts derived by *in vitro* maturation, fertilization and culture (A). Differentially stained porcine blastocysts with blue nuclei representing the inner cell mass (ICM) and pink to red nuclei representing outer cells (TE)(B).

washed three times: twice in D- PBS containing 1 mg/ml BSA, 100 μg/ml penicillin and 75 μg/ml streptomycin at 500×g for 5 minutes and once in mTBM. The spermatozoa were then diluted with modified Tris-buffered medium (mTBM) to give a final concentration of 3×10^6 spermatozoa/ml.

After the IVM period, oocytes were briefly treated with 0.1% hyaluronidase in Dulbecco's phosphate-buffered saline (D-PBS, Gibco, USA) supplemented with 1 mg/ml BSA to remove cumulus cells and then washed 2 to 3 times with mTBM containing 1 mg/ml BSA and 2.5 mM caffeine sodium benzoate (Abeydeera et al., 1997). After washing, groups of 25 to 30 oocystes were placed in 48 μl droplets of mTBM in 60 mm petri dishes that had been covered with warm mineral oil. Two μl of the spermatozoa suspension were added to each fertilization drop, resulting in a final concentration of 2.5×10^5 spermatozoa/ml. Oocytes and spermatozoa were then incubated together for 6 h at 39°C in a 5% CO_2 atmosphere at maximum humidity.

IVC procedure

After completion of the sperm-oocyte coincubation, presumptive zygotes (at 0 culture days) were washed 2-3 times in PZM-3 supplemented with 3 mg/ml BSA. Thirty oocytes were then transferred to 50 μl of PZM-3 in a 60 mm petri dish, covered with warm mineral oil, and cultured for 168 h at 39°C in a 5% CO_2 atmosphere at maximum humidity.

Blastocyst differential staining

The zona pellucida of the balstocysts were removed with a 0.5% protease solution and washed 4 to 5 times in TL-HEPES solution supplemented with 0.1% PVA (TL-PVA). Zona-free blastocysts were incubated in a 1:5 dilution of rabbit anti-bovine whole serum in TL-PVA medium for 1 h. After washing an additional 5 times in TL-PVA medium, blastocysts were reincubated in a 1:10 dilution of a guinea pig complement in TL-PVA medium supplemented with 4 μg/ml propidium iodide (PI) and 4 μg/ml bisbenzimide for 1 h. Pesumptive blastocysts that had been stained were mounted on slides and the cells were counted under a fluorescence microscope (Olympus, Tokyo, Japan). The bisbenzimide stained inner cell mass (ICM) nuclei fluoresced blue, while trophectoderm (TE) nuclei, which stained with both bisbenzimide and PI, fluoresced red or pink (Figure 1).

Experimental designs

Experiment 1 : Addition of different macromolecular components

Oocytes at 6 h post-insemination were randomly allocated and cultured in PZM-3 supplemented with i) PZM-3 with no addition of BSA, ii) PZM-3+0.3% BSA, iii) PZM-3+10% FBS, iv) PZM-3+0.05% PVA, v) PZM-3+ 0.5% PVP.

Experiment 2 : Addition period of FBS

Oocytes at 6 h post-insemination were randomly allocated and cultured in PZM-3 (0 days of culture) supplemented with 10% FBS from day 0 to 5.

Experiment 3 : Stepwise addition of FBS

Oocytes at 6 h post-insemination were randomly allocated and cultured in PZM-3 (0 days of culture). The treatment groups were: 1) PZM3+0.3% BSA from days 0 to 7, 2) PZM3+0.3% BSA from days 0 to 4 and then 10% FBS from day 4 to 7, 3) PZM-3+0.3% BSA from days 0 to 7 with addition of 10% FBS at day 4, and 4) PZM-3+0.3% BSA+10% FBS from days 0 to 7.

Statistical analysis

We chose to use the x^2-test for statistical analysis of the experimental results and one-way analysis of variance (ANOVA) to evaluate cell numbers using SPSS (version 12.0). P values of less than 0.05 were considered significant.

Table 1. Development of *in vitro* produced porcine embryos grown in PZM-3 supplemented with different macromolecules

Supplementation	No. of examined oocytes	No. (%) of embryo developed to		
		≥2-cell	Blastocysts	Hatched blastocysts
Control	81	49 (60.5)	1 (1.2)	0 (0.0)
0.3% BSA	116	82 (70.7)	8 (6.9)	2 (25.0)
10% FBS	110	77 (70.0)	6 (5.5)	1 (16.7)
0.05% PVA	110	76 (69.1)	7 (6.4)	0 (0.0)
0.5% PVP	110	68 (61.8)	2 (1.8)	0 (0.0)

Control: no supplementation of BSA, FBS, PVA or PVP.

Table 2. Effects of addition period of 10 % fetal bovine serum on development of *in vitro* produced porcine embryos

Group[1]	No. of examined oocytes	No. (%) of embryo developed to		
		≥2-cell	Blastocysts	Hatched blastocysts
Control	96	62 (64.6)	13 (13.5)^{b}	2 (15.4)
Day 0	89	55 (61.8)	4 (4.5)^{a}	0 (0.0)
Day 1	91	58 (63.7)	5 (5.5)^{ab}	1 (20.0)
Day 2	96	70 (72.9)	10 (10.4)^{abc}	0 (0.0)
Day 3	96	64 (66.7)	9 (9.4)^{abc}	2 (22.2)
Day 4	96	65 (67.7)	15 (15.6)^{c}	4 (26.7)
Day 5	96	65 (67.7)	16 (16.7)^{c}	4 (25.0)

$^{a, b, c}$ Within the same columns, values with different superscripts differ significantly ($p<0.05$).

Control: PZM-3−0.3% BSA.

[1] At 6 h post insemination, oocytes were randomly allocated and cultured in 0.3% BSA containing PZM-3 supplemented with 10% FBS from day 0 to 5.

Table 3. Effect of successive or stepwise serum supplements on development of *in vitro* produced porcine embryos

Group[1]	No. of examined oocytes	No. (%) of embryo developed to		
		≥2-cell	Blastocysts	Hatched blastocysts
1	149	115 (77.2)	32 (21.5)^{b}	2 (6.3)^{a}
2	151	122 (80.8)	35 (23.2)^{b}	13 (37.1)^{b}
3	149	120 (80.5)	32 (21.5)^{b}	17 (53.1)^{b}
4	134	112 (83.6)	7 (5.2)^{a}	1 (14.3)^{ab}

$^{a, b}$ Within the same columns, values with different superscripts differ significantly ($p<0.05$).

[1] Group 1: PZM-3−0.3% BSA, Group 2: PZM-3+0.3% BSA→(day-4) PZM-3+10% FBS, Group 3: PZM-3−0.3% BSA+(day-4) 10% FBS, Group 4: PZM-3−0.3% BSA−10% FBS.

RESULTS

Experiment 1 : Different macromolecular supplements

Effects of addition of macromolecules on the development and hatching rates of IVP porcine embryos are shown at the Table 1. The cleavage rate (≥2-cell) was similar among treatments (60.5-70.7%). The development rates of blastocysts and hatched blastocysts were 6.9% vs. 1.2-6.4% and 25.0% vs. 0-16.7% in the group supplemented with 0.3% BSA group than in other groups, respectively; however, the difference was not significant.

Experiment 2 : Addition period of FBS

The effect of the addition period of FBS on the development and hatching of IVP porcine embryos is shown in the Table 2. After *in vitro* culture of IVF porcine embryos, the cleavage rate was similar to that obtained in experiment 1. The rate of blastocyst development was significantly higher between the day 4 and 5 groups than the day 0 and 1 groups ($p<0.05$); however the rate of hatched blastocysts was not significantly different among treatments.

Experiment 3 : Stepwise addition of FBS

The development and hatching rates of IVP embryos in PZM-3 medium with stepwise addition of FBS by different culture design are shown at Table 3. The cleavage rate of the oocytes were very similar in all of the groups treated with stepwise addition of FBS (77.2-83.6%). The rate of blastocyst development was significantly higher in Group 1 (21.5%), Group 2 (23.2%), or Group 3 (21.5%) than in group 4 (5.2%) ($p<0.05$). The rates of hatched blastocysts, however, were significantly higher in Group 2 (37.1%) and Group 3 (53.1%) than in Group 1 (6.3%) ($p<0.05$).

The cell numbers within the blastocysts of the porcine embryos cultured with the media treated with stepwise addition of FBS in different culture conditions are shown in Figure 2. The cell number of the ICM at 2.0±1.0 was smaller in Group 4 than in other groups, but not significantly different. The TE cell number was relatively larger at 35.4±15.1 in Group 3 than 27.4±9.7 in Group 1 or 27.0±10.2 in Group 2. Indeed, the TE cell number was significantly higher in Group 3 than in Group 4 (14.2±6.4) ($p<0.05$). The total cell number (TCN) was similar among

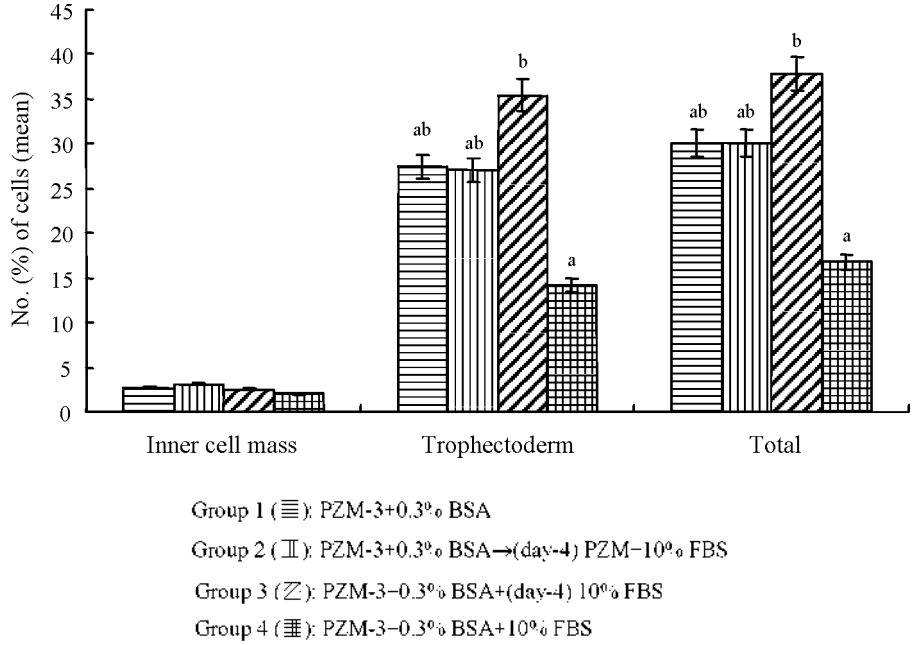

Figure 2. Effect of successive or stepwise serum supplements on cell number of porcine blastocyst developed from *in vitro* produced embryos. [a, b] Within the same columns, values with different superscripts differ significantly ($p<0.05$).

Groups 1, 2 and 3 (30.1±10.1, 30.1±10.9, and 37.8±16), but was significantly different between Group 3 and Group 4 (16.8±4.4).

DISCUSSION

BSA has always been added to the basal culture media of embryos, and has been reported to have effects on early stage embryos of *in vitro* derived hamster (Mckiernan et al., 1992), the development of porcine embryos (Rho et al., 2002), the cell number of *in vitro* derived bovine embryos (Kricher et al., 1999) and also to have defensive effects against toxic materials within the culture media (Kane, 1987). However, the chemical compositions in culture media vary greatly due to different preparations or batches. This variety of chemical compositions is considered to be the major reason why medium supplemented with BSA can be harmful on the development of *in vitro* derived embryos (Mckiernan et al., 1992; Gardner, 1994). Therefore, recently, macromolecules like PVP, PVA, transferin, collagen, or insulin have been used as alternatives to BSA, whereby the medium supplemented with these macromolecules is considered to be defined medium for IVC of *in vitro* derived embryos, as it can be helpful to establish the chemical composition of culture medium and increase the reproducibility of results.

Some studies have reported that the addition of BSA is not effective for the development of IVP embryos (Takagi et al., 1991; Shamsuddin et al., 1994; Abeydeera et al., 2000; Kim et al., 2004) and that macromolecules like PVA can serve as materials for nitrogen fixation and protein synthesis in early staged mouse IVP embryos and can also be used to control the composition of defined medium or for identification of mechanisms by specific material. PVA can also be used as an alternative to BSA, serving as materials or nutrients for fixed nitrogen required for early staged embryos (Choi et al., 1999).

In this study, we investigated the rates of the development and hatching of IVF porcine embryos grown in PZM-3 media supplemented with either BSA (0.3%), FBS (10%), PVA (0.05%) and PVP (0.5%). There were no significant differences with respect to embryo development in the four treatment groups. These results suggest that PVP, PVA or FBS can all be used as an alternative to BSA for supplementation of the basal medium (Table 1).

On the other hand, serum consists of many different components and to has a biphasic effect that clearly improves or inhibits embryo development depending on the stage of embryo to which it is added. Indeed, it has been reported by many scientists that addition of FBS to early staged embryos can be dangerous for development of *in vitro* blastocysts (Bavister, 1995; Lee et al., 1998), although addition of FBS to later staged embryos is reportedly helpful for blastocyst development (Lonergan et al., 1998) and improves rates of hatched blastocysts (Wang et al., 1997).

In the present study, we found that FBS was indeed helpful for embryo development when added at a later development stage (after day 4) of IVC, rather than at earlier stages (Table 2). In addition to serum, BSA has

always been added to the basal medium for IVP embryos and has been reported to be an effective means of enhancing embryo development. Consistent with these findings, we found that mixed addition of FBS with BSA on day 4 of culture was more effective for development of hatched blastocysts than by addition of BSA alone (Table 3).

Kaaekuahiwi et al. (1990) reported that the reason why addition of serum increases the rates of hatched blastocysts is that serum contains a plasminogen that degenerates the zona pellucida to facilitate hatching.

In general the IVP blastocysts observed by optical microscopic show no superficial destruction or deformity of blastomores even though some have outstanding reduction in development capacity or metabolism (Massip et al., 1993). In cases where such embryos are transplanted, the implantation may be successful, but the rate of abortion is high (Overstr, 1996). Accordingly, in this study, we investigated the cell numbers of blastocysts by staining to evaluate the quality of the blastocysts prior to embryo transplantation (Figure 2). The cell number of *in vivo* derived blastocysts is reported to be around 57 at day 6 (Yoshioka et al., 2002), 93 at day 7 (De la Fuente et al., 1997) and around 175 at the blastocyst stage just before hatching (Davis et al., 1985). The average cell number of the blastocysts in this study was smaller, compared with the cell number of *in vivo* produced blastocysts, but the TCN was relatively higher in group 3 than in the other groups. This result is consistent with the report suggesting that addition of FBS to *in vitro* derived embryos at later culture stage is helpful for increasing the TCN (Wang and Day, 2002).

In conclusion, we suggest replacing BSA with PVP, PVA or FBS for the purposes of supplementing PZM-3 basal medium and adding a 0.3% BSA and FBS mixture during a later culture stage day to improve embryo development and TCN.

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