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Optimizing Electrical Activation of Porcine Oocytes by Adjusting Pre- and Post-Activation Mannitol Exposure Times

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Contents

Modifying electrical activation conditions have been used to improve *in vitro* embryo production and development in pigs. However, there is insufficient information about correlations of porcine embryo development with oocyte pre- and post-activation conditions. The purpose of this study was to compare the developmental rates of porcine oocytes subjected to different mannitol exposure times, either pre- or post-electrical activation, and to elucidate the reason for the optimal mannitol exposure time. Mannitol exposure times around activation were adjusted as 0, 1, 2 or 3 min. Blastocyst development were checked on day 7. Exposure of oocytes to mannitol for 1 or 2 min before electrical activation produced significantly higher blastocyst rates than exposure for 0 or 3 min. There was no significant difference in blastocyst rates when activated oocytes were exposed to mannitol for 0, 1, 2 or 3 min after electrical activation. While exposure of oocytes to mannitol for 1 min pre- and 3 min post-activation showed significantly higher blastocyst development than 0 min pre- and 0 min post-activation. It also showed higher maintenance of normal oocyte morphology than exposure for 0 min pre- and 0 min post-activation. In conclusion, exposure of oocytes to mannitol for 1 min pre- and 3 min post-activation seems to be optimal for producing higher *in vitro* blastocyst development of porcine parthenogenetic embryos. The higher blastocyst development is correlated with higher maintenance of normal morphology in oocytes exposed to mannitol for 1 min pre- and 3 min post-activation.

Introduction

Oocytes undergo activation through oscillation of intracellular calcium which is a pivotal process in embryo development (Solter 2000). For porcine embryo development, several kinds of activation methods (electrical, chemical and a combination of the two) have been reported but the electrical activation method is most widely used for its simplicity, ease and efficiency (Prochazka et al. 1992; Machaty et al. 1997; Wang et al. 1998; Zhu et al. 2002; Yi and Park 2005; Che et al. 2007; Im et al. 2007; Koo et al. 2008; Kang et al. 2009; Park et al. 2010, 2011, 2012; Kim et al. 2013). Specifically, electrical activation of porcine oocytes is carried out in a buffered solution containing a sugar like mannitol, inositol or sorbitol (Liu and Moor 1997; Sato et al. 2005; Koo et al. 2008). Among these, mannitol is the most popular sugar for electrical activation of porcine oocytes (Im et al. 2007; Koo et al. 2008).

It has been reported that exposure of oocytes to electrical stimuli during manipulation has a detrimental effect on further embryo development, and thus, oocytes need a threshold for stimulation and the subsequent calcium oscillations; exceeding or paucity of this threshold will alter the development of the resulted embryos (Collas et al. 1993). It was reported that development of parthenogenetic porcine oocytes could be hampered by reactive oxygen species generated by electrical activation (Koo et al. 2008). In addition, increased apoptosis in electrically activated porcine embryos is the main cause of decreased developmental competence (Hao et al. 2004). Even though the electrical activation method is still widely used for porcine embryo parthenogenenesis (Im et al. 2007; Koo et al. 2008; Park et al. 2011), the developmental competence of the embryos could be corrupted by this approach. Furthermore, rhesus monkey spindle transfer experiments were successful when Sendai virus was used for fusion of ooplasm and metaphase spindle instead of electrical stimuli (Tachibana et al. 2009). It is thought that exposure of oocytes to electrical stimuli could be a major cause of poor embryo development, and therefore, optimization of the electrical activation conditions is needed.

Washing embryos through serial dilution of mannitol solution before and after electrical activation already has been widely used protocol (Eakin and Hadjantonakis 2006). The embryos would float initially and gradually sink to the bottom of the plate. It takes about 30 s–1 min. However, it is also known that long time exposure to mannitol can be harmful to embryonic development. This study was carried out to examine the developmental rates of porcine oocytes subjected to different mannitol exposure times either pre- or post-electrical activation and to elucidate the possible reason for the optimal mannitol exposure time.

Materials and Methods

Chemicals

All chemicals and reagents used in this study were purchased from Sigma-Aldrich Corp. (St Louis, MO, USA) unless otherwise stated.

In vitro maturation of porcine oocytes

Preparation of porcine oocytes was carried out according to our previous method (Kang et al. 2009) with minor modifications. Briefly, pig ovaries were collected from a local slaughterhouse and transported to the laboratory in 0.9% (w/v) NaCl solution at 30°C.

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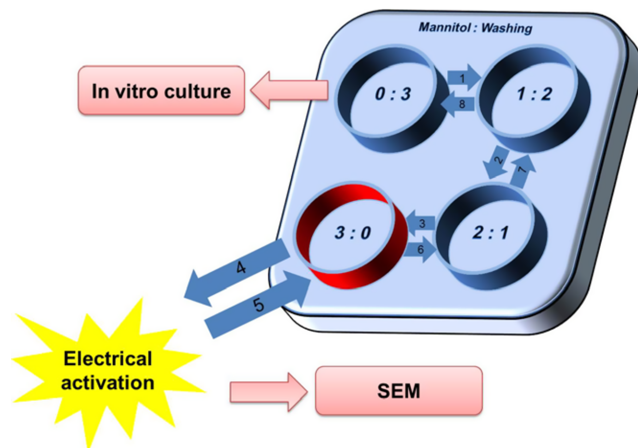

Scheme 1. Oocytes were sequentially pre-equilibrated with each solution (mannitol : washing ratio = 0 : 3, 1 : 2 and 2 : 1, respectively) for 1 min and with 3 : 0 ratio solution for 0–3 min. Electrically activated embryos were sequentially post-equilibrated in solution (mannitol : washing ratio = 3 : 0) for 0–3 min and with 2 : 1, 1 : 2 and 0 : 3 ratio solution for 1 min. The exposure time in the red coloured well (mannitol : washing ratio = 3 : 0) before and after electrical activation was a critical parameter for this study. The comparison of pre-activation (Fig. 1a), post-activation (Fig. 1b) and combined activation mannitol exposure time (Figs 1c and 2a,b) was carried out using this red coloured well. Activated embryos were cultured or fixed for analysis of developmental competence and ultrastructure

Table 1. Composition of mannitol solution

Substrates	Concentrations
D-Mannitol	0.26 M
$MgCl_2$	0.1 mM
$CaCl_2$	0.1 mM
HEPES	0.5 mM
BSA	0.05%

HEPES, *N*-(2-hydroxyethyl)-piperazine-*N*'-(2-ethanesulphonic acid); BSA, Bovine serum albumin.

Cumulus–oocyte complexes (COCs) were aspirated from follicles with an 18-gauge needle attached to a 10-ml syringe. Compact COCs with evenly granulated cytoplasm were selected for maturation. *In vitro* maturation (IVM) medium consisted of tissue culture medium–199 (Invitrogen, Carlsbad, CA, USA) supplemented with 10 ng/ml epidermal growth factor, 4 IU/ml of pregnant mare serum gonadotropin (Intervet International B.V, Boxmeer, Netherlands) and 4 IU/ml of human chorionic gonadotropin (Intervet International B.V) and 10% porcine follicular fluid. Incubation of COCs was at 39°C in a humidified atmosphere of 5% CO_2 and 20% O_2. After 22 h of culture, COCs were washed and then cultured in fresh IVM medium without hormones for a further 22 h. At 44 h of culture, oocytes were denuded using 0.1% hyaluronidase with repeated pipetting.

Electrical activation and *in vitro* culture

Denuded oocytes were sequentially pre-equilibrated with each solution (mannitol : washing ratio = 0 : 3, 1 : 2 and 2 : 1, respectively) for 1 min and with 3 : 0 ratio solution for 0–3 min (Scheme 1). Electrical activation was carried out in a chamber between two electrodes using mannitol solution (Table 1) and a BTX Electro-Cell Manipulator 2001 (BTX Inc., San Diego, CA, USA). The parameters for electrical activation were 2 kV/cm, 1-DC pulse and 30-μs. Electrically activated embryos were post-equilibrated in solution (mannitol : washing ratio = 2 : 1, 1 : 2 and 0 : 3, respectively) for 1 min with each solution and with 3 : 0 ratio solution for 0–3 min (Scheme 1). 0 min exposure with 3 : 0 ratio solution means skip of well (3 : 0 ratio solution). Post-mannitol-exposure time was fixed at 3 min in case of comparison of pre-mannitol-exposure time (Fig. 1a). Pre-mannitol-exposure time was fixed at 1 min in case of comparison of post-mannitol-exposure time (Fig. 1b). '0/0' means pre-mannitol exposure for 0 min (3 : 0 ratio solution) and post-mannitol exposure for 0 min (3 : 0 ratio solution) and '1/3' means pre-mannitol exposure for 1 min (3 : 0 ratio solution) and post-mannitol exposure for 3 min (3 : 0 ratio solution) (Figs 1c and 2). Activated embryos were cultured in Porcine Zygote Medium-3 (Funakoshi Co., Tokyo, Japan) in 5% CO_2, 5% O_2 at 39°C. Blastocyst development was examined at day 7 of *in vitro* culture.

Scanning electron microscopy

The scanning electron microscopy (SEM) was carried out at the National Instrumentation Center for Environmental Management (NICEM, Seoul, Korea). Three groups of samples were used for SEM (control – *in-vitro*-matured oocytes, 0/0 and 1/3). In brief, samples were first fixed with 2% paraformaldehyde and 2% glutaraldehyde in 0.05 M sodium cacodylate buffer (pH 7.2) at 4°C for 2 h. Fixed samples were washed three times using 0.05 M sodium cacodylate buffer (pH 7.2) at 4°C for 10 min. Secondary fixation was carried out using 1% osmium tetroxide in 0.05 M sodium cacodylate buffer (pH 7.2). Secondary fixed samples were washed twice using distilled water at room temperature (RT). Dehydration was carried out using a sequence of 30%, 50%, 70%, 80%, 90% and 100% ethyl alcohol at RT for 10 min each. Dehydrated specimens were mounted on metal stubs and further dried for 2 days at RT. Gold coating was carried out at 20 mA for 200 s. SEM images (×1 000 or ×2 000) were collected using field emission SEM (JEOL, Tokyo, Japan).

Statistical analysis

ANOVA and *t*-test (PRISM ver 4.0; Graphpad Software Inc., La Jolla, CA, USA) were used for statistical analysis. Significant difference was considered when $p < 0.05$.

Results

Effect of pre- and post-activation mannitol exposure time on porcine blastocyst development

Exposure of oocytes to mannitol for 1 or 2 min before electrical activation showed higher blastocyst formation rates (33.62 ± 3.51%, n = 71; and 32.75 ± 5.93%, n = 71, respectively) than exposure for 0 (10.93 ±

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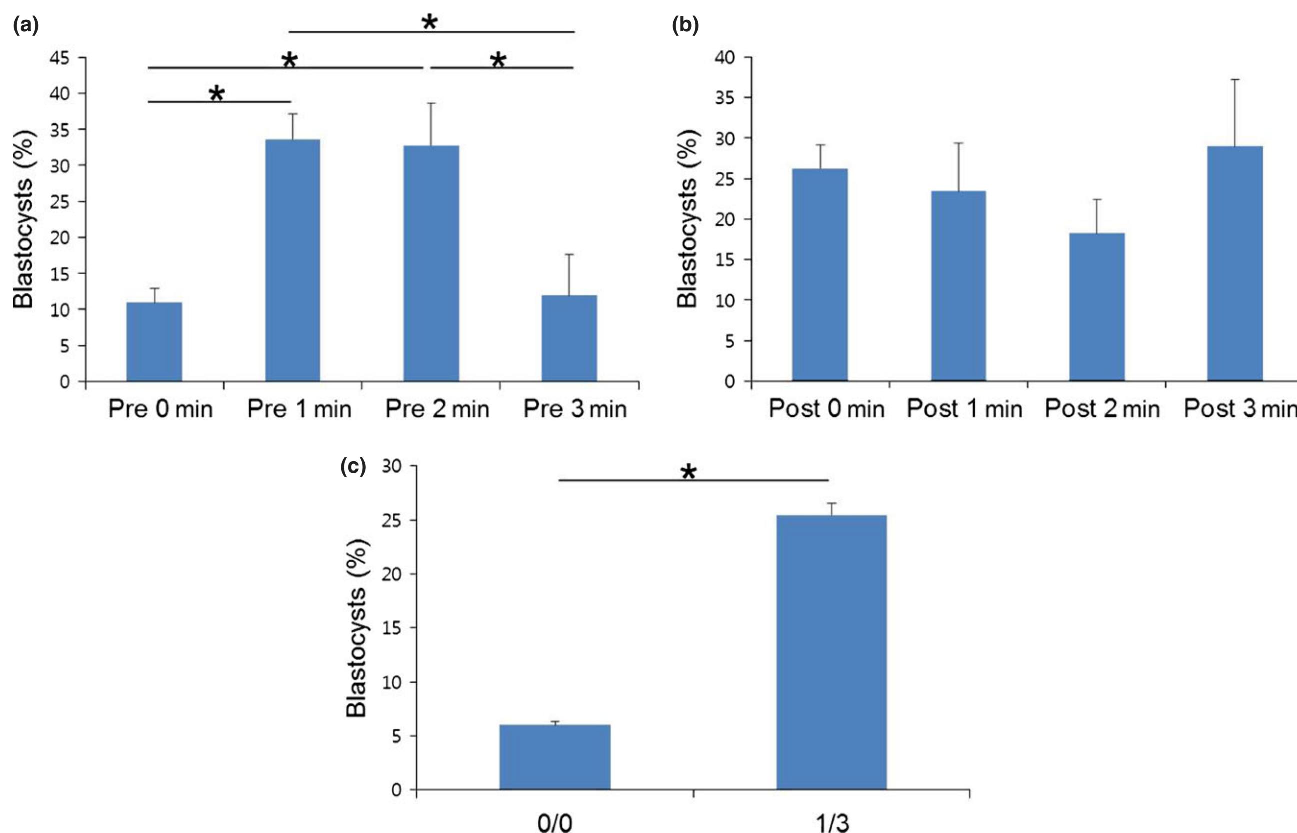

Fig. 1. Effect of pre- and post-activation mannitol exposure time on porcine blastocyst development. (a) Exposure of oocytes to mannitol for 1 or 2 min before electrical activation showed higher blastocyst rates than exposure for 0 or 3 min. Error bars indicate the standard error derived from quantification of at least three independent experiments (*$p < 0.05$). (b) No significant difference in blastocyst rate was observed when activated oocytes were exposed to mannitol for 0, 1, 2 or 3 min. Error bars indicate the standard error derived from quantification of at least three independent experiments. (c) Exposure of oocytes to mannitol for 1 min pre- and 3 min post-activation showed higher blastocyst development than 0 min pre- and 0 min post-activation. Error bars indicate the standard error derived from quantification of at least three independent experiments (*$p < 0.05$)

1.93%; n = 72) or 3 min (11.92 ± 5.70%; n = 70) ($p < 0.05$) (Fig. 1a). There was no significant difference in blastocyst development rates when activated oocytes were exposed to mannitol for 0 (26.22 ± 2.96%; n = 115), 1 (23.42 ± 5.90%; n = 116), 2 (18.25 ± 4.21%; n = 115) or 3 min (28.94 ± 8.27%; n = 115) ($p > 0.05$) (Fig. 1b). While exposure of oocytes to mannitol 1 min pre- and 3 min post-activation showed higher blastocyst developmental rates than 0 min pre- and 0 min post-activation (25.44 ± 1.08% vs 6.04 ± 0.29%; n = 51 vs 50) ($p < 0.05$) (Fig. 1c).

Effect of pre- and post-activation mannitol exposure time on oocyte ultrastructure

SEM of oocytes of the 0/0 group showed higher distortion or breakage of activated oocytes than the control or 1/3 groups. The 1/3 group showed similar distortion and breakage rates of activated oocytes with the control group (Fig. 2a). The quantification data also indicated that the control and 1/3 groups are similar, but 0/0 was morphologically different; the ratio of breakage, distortion and normal was 0%, 37.5% and 62.5%, respectively (control oocytes; n = 8), 41.7%, 50.0% and 8.3%, respectively (0/0 oocytes; n = 12) and 5.6%, 27.8% and 66.7%, respectively (1/3 oocytes; n = 18) (Fig. 2b).

Discussion

In this study, we compared blastocyst developmental rates of porcine oocytes subjected to different mannitol exposure times either pre- or post-electrical activation and elucidated the reason for optimal exposure times. Exposure of *in vitro* matured oocytes to mannitol for 1 or 2 min before electrical activation showed significantly higher blastocyst development rates than exposure for 0 or 3 min. There was no significant difference in blastocyst rate when activated oocytes were exposed to mannitol for 0, 1, 2 or 3 min. However, exposure of oocytes to mannitol for 1 min pre- and 3 min post-activation showed significantly higher blastocyst developmental rates than 0 min pre- and 0 min post-activation. Exposure of oocytes to mannitol for 1 min pre- and 3 min post-activation showed higher maintenance of cytoskeleton integrity than 0 min pre- and 0 min post-activation.

In general, cumulus-free oocytes were equilibrated with mannitol before and after electrical activation to lower the damaging effects of this process on oocytes or activated embryos (Koo et al. 2008; Kang et al. 2009). However, this is just a conventional procedure, and there is no previous report comparing the relationship between mannitol exposure times, either pre- or post-electrical activation, and blastocyst development. Here,

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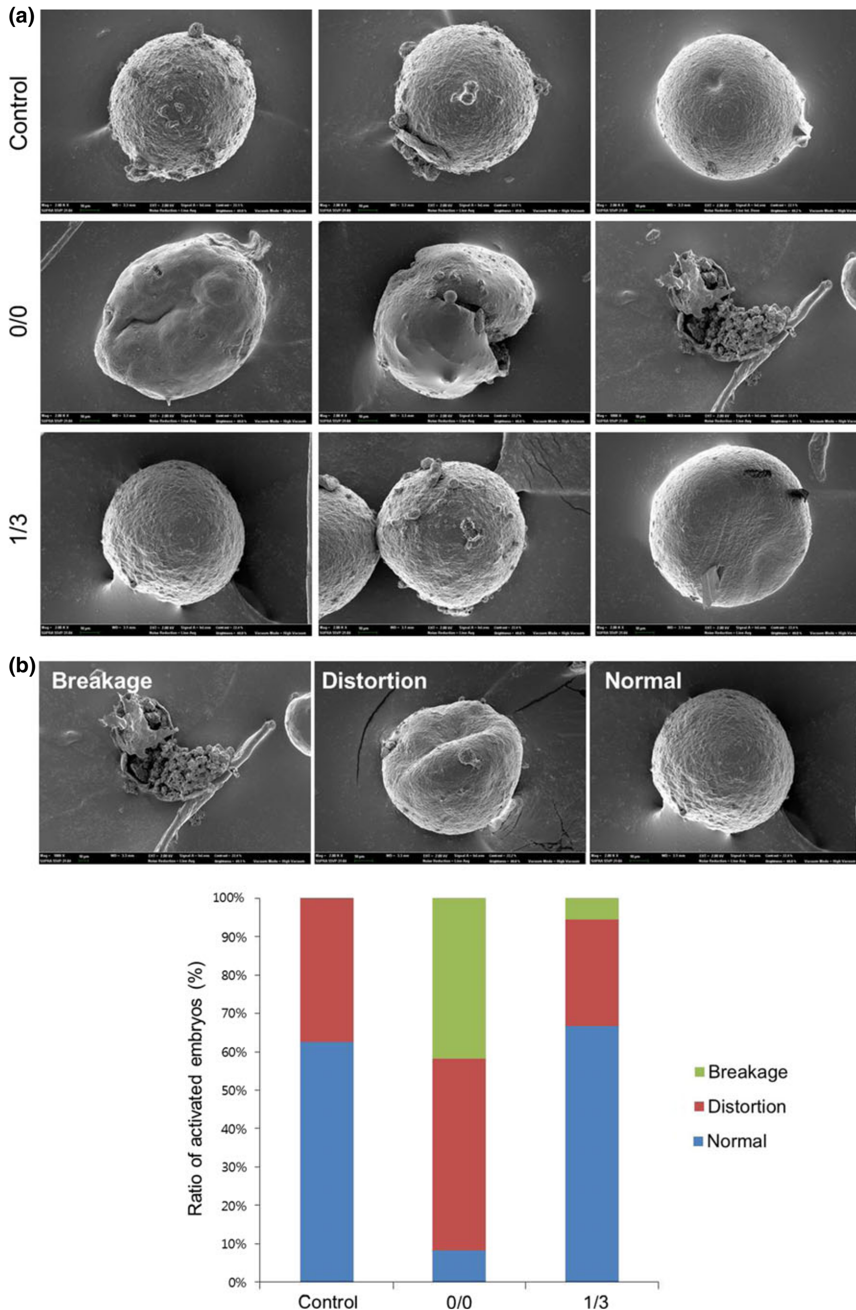

Fig. 2. Effect of pre- and post-activation mannitol exposure time on ultrastructure. (a) SEM image of the 0/0 group represents examples of higher distortion or breakage of activated oocytes than the control or 1/3 group. However, the 1/3 group showed similar distortion or breakage frequencies of activated oocytes as the control group. (b) Representative images of breakage, distortion and normal (Upper). The quantification data also indicated that the control and 1/3 groups show similar patterns, but the 0/0 group shows a different pattern (Lower)

we show that exposure of mature oocytes to mannitol before electrical activation is a more critical factor for blastocyst development than mannitol exposure after electrical activation (Fig. 1a,b). The best of the pre-activation mannitol exposure times was 1 or 2 min, but a significant decrease in blastocyst development was found at 0 or 3 min (Fig. 1a). It is thought that this optimal pre-activation mannitol exposure time may be attributed to batch variations in porcine blastocyst development because it is difficult to perform electrical activation of hundreds of porcine oocytes within 1 or 2 min. The optimal combination of pre- and post-activation mannitol exposure times was 1 and 3 min (1/3). It is thought that the results obtained from different post-mannitol exposure times (1/3 vs 0/0; Fig. 1c) are valid because post-mannitol exposure time did not significantly affect blastocyst development (Fig. 1b). To our knowledge, there is no previous information on the optimal pre- and post-activation mannitol exposure times. Thus, it is suggested that this result is informative for technical improvements of research fields related to porcine parthenogenesis.

When oocytes were stimulated electrically in a non-equilibrated state (0/0), the ratio of distortion or breakage of activated embryos was greatly increased (Fig. 2a,b). However, this detrimental effect of high voltage electrical stimuli could be overcome by exposure of porcine oocytes to mannitol for 1 min pre- and 3 min post-activation (1/3) (Fig. 2a,b). The cytoskeleton is divided into microfilaments, microtubules and intermediate filaments, and representative proteins are actin, tubulin and keratin (Liang and MacRae 1997).

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Previous studies have shown that normal distribution of F-actin in porcine oocytes was significantly decreased by irreversible damage of microfilaments during cryopreservation (Wu et al. 2006). The developmental competence of porcine oocytes was hampered by this cytoskeletal damage (Wu et al. 2006). It is reasonable to speculate that destruction of the ultrastructure of porcine oocytes in the 0/0 group by improper suboptimal mannitol exposure time is correlated with destruction of cytoskeleton-related oocyte proteins. It is important to further identify the destroyed cytoskeletal proteins and the mechanisms of destruction caused by improper mannitol exposure times.

In conclusion, exposure of oocytes to mannitol for 1 min pre- and 3 min post-activation seems to be optimal for higher *in vitro* blastocyst development in porcine parthenogenesis. The higher blastocyst development is due to increased maintenance of normal morphology in oocytes exposed to mannitol both pre- and post-activation. In future, this informative methodology could be applied to improve cloning in pigs or in other species in which an electrical stimulus is used for the crucial step of oocyte activation.

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Conflict of interest

The authors declare no conflicts of interest.

Author contributions

D. Kwon designed study, collected data, analysed data, drafted paper and revised paper. I.M. Saadeldin helped in the technical support and draft manuscript and revised paper. S.J. Kim, S.J. Park, J.T. Kang, H.J. Park, J.H. Moon and O.J. Koo helped in the technical support. G. Jang analysed data. B.C. Lee designed study, analysed data, drafted paper and revised paper and in final approvement of submission.

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