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Harmful or Not: Trichostatin A treatment of embryos generated by ICSI or ROSI

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Abstract: Trichostatin A (TSA), a histone deacetylase inhibitor, is a known teratogen causing malformations such as vertebral fusions when applied during the postimplantation period; TSA also causes developmental arrest when applied during the preimplantation period. Regardless of these hindrances, we have succeeded in the establishment of an efficient somatic cloning method for the mouse where reconstructed embryos are treated with TSA. To elucidate this apparent discrepancy, we treated fertilized mouse embryos generated either by intracytoplasmic sperm injection (ICSI) or round spermatid injection (ROSI) with 50 nM TSA for 20 h after fertilization as well as parthenogenetic embryos and found that TSA treatment inhibited the preimplantation development of ICSI embryos but not ROSI or parthenogenetic embryos. And, although we often observed hypomorphism following TSA treatment in embryos grown to full term produced by both ICSI (av. of body weight: 1.7 g vs. 1.5 g) and ROSI (1.6 g vs. 1.2 g), TSA treatment reduced the offspring production rate for ICSI from 57% to 34% but not for ROSI from 30% to 36%. Thus, these data indicate that the effects, harmful or not, of TSA treatment on embryonic development depend on their nuclear derivations. Also, the resulting hypomorphism after TSA treatment is a caveat for this procedure in current Assisted Reproductive Technologies.

Keywords: Mouse, teratogenicity, trichostatin A, embryonic development

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Abbreviations

TSA – trichostatin A
ICSI – intracytoplasmic sperm injection
ROSI – round spermatid injection

1 Introduction

As well as other animals, mouse cloning by somatic cell nuclear transfer (SCNT) has been inefficient since the first cloned mouse, "Cumulina," was born in 1997 [1]. Although we have tried several new methods including the methods of oocyte activation [2, 3], inhibition of cytokinesis [3], recloning via nuclear transfer-derived embryonic stem cells (NT-ES cells) [4] and timing of enucleation or injection of nucleus [5], improvement was minimal. We and others have published a new cloning method where embryos are treated with trichostatin A (TSA), a histone deacetylase (HDAC) inhibitor, following nuclear transfer [6–8], which leads to 2-5 times higher success rates for both reproductive and therapeutic cloning [6], suggesting that TSA enhances reprogramming of transferred somatic nuclei in oocytes.

The idea for the improvement of embryonic development by TSA treatment after SCNT originally came from our recent findings that abnormal DNA hypermethylation of spermatid-derived paternal genomes in zygotes following ROSI occurs before the end of the first mitosis and that treatment of ROSI zygotes with TSA results in a significant reduction of DNA methylation level, specifically on spermatid-derived paternal genomes – not maternal ones [9]. This hypermethylation of spermatid-derived genome after ROSI may explain the fact that the offspring production rate of ROSI is significantly lower than that of ICSI [10–14]. The similar phenomenon of abnormal DNA hypermethylation of genomes during preimplantation has been also observed in cloned embryos, which has been believed to be associated with the low developmental potentials of cloned embryos [15–18]. Therefore, if TSA treatment of cloned embryos could similarly remove abnormal DNA methylation, then TSA treatment of cloned embryos was expected to improve further embryonic development. Although the effect of TSA treatment on DNA hypermethylation of cloned genomes has not been demonstrated yet, TSA treatment practically improves mouse cloning technology. This success also opens up great possibilities for application of the TSA method for fertility treatments such as ROSI in a similar way. However, regardless of the success, TSA treatment should be carefully applied because of its teratogenicity.

To date, HDAC inhibitors are widely used both in basic research and medications such as anticonvulsants and anticancer [19]. However, HDAC inhibitors such as valproic acid and TSA are well known potent teratogens using *Xenopus*, zebrafish and mice [20, 21]. TSA, belonging to the group of hydroxamic acids, is the most potent discovered and is a fermentation product of *Streptomyces* [22]. In addition to its teratogenic effects during postimplantation development of mice, TSA treatment at the one- and two-cell stages

causes inhibition of subsequent embryonic cleavage [23]. Understandably, the current belief is that TSA functions as a teratogen, interfering with embryonic development.

The apparent discrepancy between the improvement of clone-embryonic development by TSA and the teratogenicity of TSA prompted us to examine how TSA treatment of fertilized embryos created by ICSI or ROSI affects embryonic development.

2 Materials and methods

2.1 Animals

B6D2F1 mice (C57BL/6 × DBA/2) were used to prepare spermatogenic cells and as oocyte donors. Surrogate females were ICR females mated with vasectomized males of the same strain. All animals were obtained from SLC (Shizuoka, Japan) and were maintained in accordance with the Animal Experiment Handbook at the Riken Centre for Developmental Biology.

2.2 Collection and culture of oocytes

Mature oocytes were collected from the oviducts of 8–12-week-old females that had been induced to superovulate with 5 IU PMSG, followed by 5 IU hCG 48 h later. Oocytes were collected from oviducts approximately 16 h after hCG injection, placed in HEPES-buffered CZB medium, and treated with 0.1% hyaluronidase until the cumulus cells dispersed. The oocytes were then placed in synthetic oviductal medium enriched with potassium (KSOM) containing NEAA and EAA (KSOM AA; Specialty Media, Phillipsburg, NJ, USA) supplemented with 1 mg/ml BSA, covered with paraffin oil (Nacalai Tesque, Kyoto, Japan) and stored at 37 °C (5% CO_2/air).

2.3 Microinsemination with spermatozoa (ICSI) and round spermatids (ROSI)

Collection of spermatogenic cells and injections were performed according to previously described methods [10–13]. For ICSI, the head of each spermatozoon was separated from the tail by applying pulses to the head–tail junction by means of a Piezo-driven pipette (PrimeTech; Ibaraki, Japan). Only the sperm head was injected into each oocyte. For ROSI, oocytes were activated by incubation in Ca^{2+}-free CZB medium containing 5 mM $SrCl_2$ for 20 min. Forty to 80 minutes after activation, the oocytes were injected with the nuclear region of a round spermatid.

2.4 Production of parthenogenetic diploid embryos

Mature oocytes were activated by 5 mM $SrCl_2$ in Ca^{2+}-free CZB medium in the presence of 5 μg/ml cytochalasin B for 6 h and cultured for 4 days in KSOM medium [1, 24].

2.5 TSA treatment and embryo transfer

The oocytes microinjected with sperm or spermatids were cultured in KSOM medium containing 50 nM TSA (Sigma) within 30 min after ICSI or ROSI and then rinsed in KSOM without TSA 20 h after ICSI or ROSI [6, 9]. These embryos were cultured in KSOM until either transfer into surrogate mother (2nd day) or examination of blastocyst formation (4th day). For embryo transfer, approximately 10 two-cell embryos after ICSI or ROSI were transferred to each oviduct of 0.5-day pseudopregnant females (ICR).

2.6 Statistical analysis

The data of the offspring production rates were compared with chi-square test analysis. The data of the offspring weights were analyzed by one-way ANOVA followed by a Tukey multiple comparison test (PRISM Graph Pad version 4; Graph Pad Software, San Diego, CA, USA). Differences at $P < 0.05$ were considered significant.

3 Results

3.1 Effect of TSA treatment on embryos during preimplantation development

To examine the effect of TSA treatment on embryonic development of fertilized embryos, fertilized embryos produced by ICSI or ROSI as well as parthenogenetic embryos were treated with 50 nM TSA for 20 h after injections. These parameters are the maximum allowable while still showing improved cloning rates as we previously reported [6]. As expected from the previous report [23], TSA treatment of ICSI embryos resulted in a significant reduction from 72% to 46% in the developmental rates to expanded blastocyst after 96 h culture (Fig. 1). Surprisingly, neither ROSI nor parthenogenetic embryos showed significant reduction in their developmental rates, but rather an increase from 69% to 85% and 90% to 100%, respectively, even with no statistical significance (Fig. 1). Thus, our data suggest that harmfulness of TSA treatment to preimplantation development is determined by nuclear derivations of embryos.

3.2 TSA treatment of fertilized embryos leads to hypomorphism

Regardless of the reported teratogenicity and toxicity of TSA to embryos, TSA-treated cloned embryos unexpectedly show significantly better development and no apparent abnormalities [6]. In contrast, as expected based on their *in vitro* development, TSA treatment of embryos after ICSI led to significant reduction of the offspring production rates from 57% to 34% (Table 1). Further, when offspring were delivered, hypomorphism was frequently observed among them (Fig. 2A, B). These results indicate that, in contrast to mouse cloning, the TSA treatment of normal fertilized embryos for the first 20 h after

fertilization made a detrimental impact not only for the offspring production rates, but also on the postimplantation development.

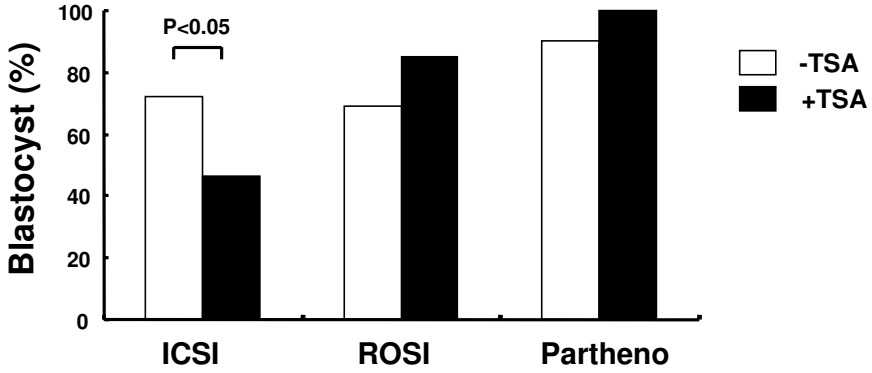


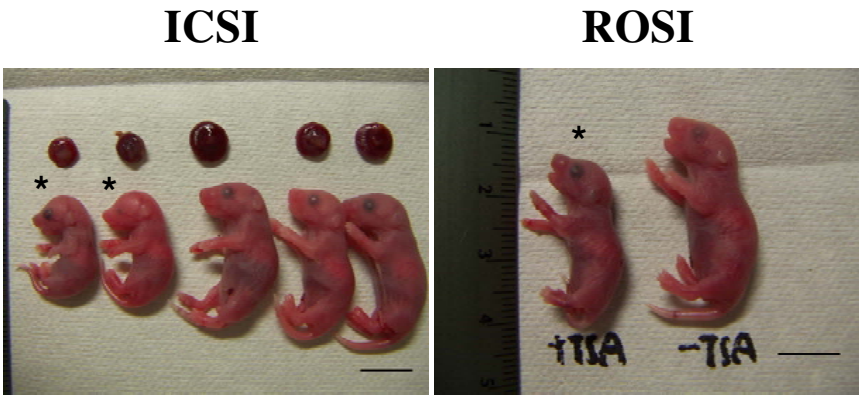
Fig. 1 Dependence of detrimental effect of TSA treatment on the derivatives of treated nuclei. ICSI, ROSI and parthenogenetic embryos were treated with 50 nM TSA (+TSA) or without TSA (-TSA) for 20 h after injection or artificial oocyte activation and then continuously cultured in KSOM medium. After 96 h, developmental rates to expanded blastocysts were calculated. Each experiment used more than 40 embryos and was repeated at least twice.

Male germ cell (Injection type)	Condition of TSA treatment (nM)	No injected oocytes	No (%) PN formation	No (%) 2-cell embryos	No embryos transferred	No (%) live offspring
Sperm (ICSI)	0	81	74(91)	72(97)	49	28(57)^a
	50	79	78(99)	76(97)	56	19(34)^b
Spermatid (ROSI)	0	49	33(67)	32(97)	30	9(30)^b
	50	68	42(62)	42(100)	42	15(36)^b

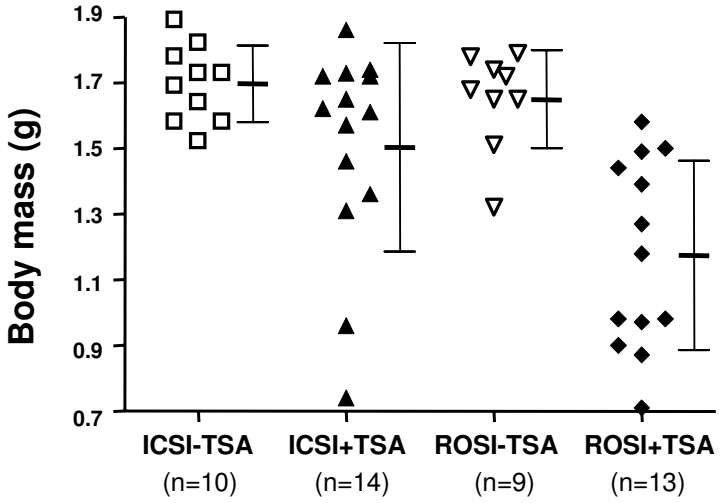
Values with different superscripts are significantly different. a versus b $P<0.05$.

Table 1 20 h TSA treatment after ICSI and ROSI.

Next, we examined the effect of TSA treatment in the development of ROSI embryos. Interestingly, TSA treatment after ROSI also did not reduce but rather increased slightly offspring production to 30% and 36% for controls and TSA treated embryos, respectively (Table 1). On the other hand, TSA treatment after ROSI more frequently resulted in more severe hypomorphism of ROSI offspring (Fig. 2A, B). Actually, on average, 30% body mass (1.65 g vs. 1.17 g) was lost in ROSI embryos by TSA treatment, in contrast with just 10% lost in ICSI. Thus, our data suggest that TSA treatment for 20 h can induce hypomorphism regardless of male germ cell types but the effect of TSA on offspring production rates may differ between male germ cell types.



a)



b)

Fig. 2 Hypomorphism caused by TSA treatment. Either TSA-treated or untreated two cell stage embryos after ICSI and ROSI were transferred into surrogate mothers.
a) Small offspring shown as asterisk were often obtained only after TSA treatment of ICSI or ROSI embryos. Scale bar = 1 cm.
b) Reflecting the hypomorphism of the embryos, the body mass means after TSA treatment were reduced both in ICSI and ROSI. Symbols represent individual samples; bars indicate mean value±SD.

4 Discussion

In this study, we examined how TSA treatment of fertilized embryos affects their subsequent development after using ICSI and ROSI. Then we found that, in terms of preimplantation development, TSA treatment just 20 h after fertilization is harmful to ICSI embryos (which is consistent with the previous report [23]), but not harmful to ROSI and parthenogenetic embryos and also, as our previous report states [6], clearly helpful for somatic cloned embryos. We also observed a similar tendency in their full term develop-

mental rates, that is, the different effect of TSA treatment on the offspring production rates between ICSI and ROSI even if some of the offspring showed hypomorphism. Thus, the detrimental effect of TSA treatment of zygotes on their embryonic development really depends on the nuclear derivations, that is, which of oocyte-, sperm-, spermatid or somatic-derived pronuclei exist in treated zygotes. Therefore, the effect of TSA treatment could be defined by the epigenetic states of the treated nuclei.

What is the mechanism underlying the different effects of TSA treatment among nuclear types on embryonic development? TSA treatment of zygotic embryos after nuclear transfer, ICSI or ROSI leads to hyperacetylated histone H3 and H4 [7, 9, 23, 25], as seen in somatic cells [22, 26]. It currently remains unknown how the hyperacetylation of histones defines the different embryonic developmental rates among nuclear types after TSA treatment. However, one of the possibilities is that acetylation levels of histones or some specific amino-acid residues of them in TSA-treated nuclei may be different and contribute to the varying developmental potentials of TSA-treated embryos carrying unique nuclear types, On the other hand, it has been shown that TSA also triggers selective DNA demethylation depending on the cell type and genomic region in mammalian cells and *Neurospora* [27]. At the same time, it is also reported that the DNA methylation levels of genomes overall are not significantly changed following TSA treatment in somatic cells despite the hyperacetylation of histones [26]. In these points of view, we previously found that ROSI zygotes show DNA hypermethylation of spermatid-derived paternal genomes in zygotes similar to maternal genomes before the end of the first mitosis, which is evidently in contrast with hypomethylation of sperm-derived genomes [9]. The treatment of both ICSI and ROSI zygotes with TSA results in a significant reduction of DNA methylation level of paternal genomes [9] but not maternal genomes [9, 25]. Further, the final DNA methylation levels in paternal genomes after TSA treatment are different even between sperm- and spermatid-derived genomes [9]. Thus, TSA treatment of zygotes can remarkably reduce DNA methylation depending on the origins of transferred nuclei and their genomic regions, most likely according to their epigenetic statuses. Therefore, one of the possibilities is that the different DNA methylation levels in zygotic genomes induced by TSA treatment underlie the different effects of TSA treatment between nuclear derivations on embryonic development.

As already described, the TSA treatment of embryos often caused hypomorphism regardless of types of male germ cells even if the frequency and degree were different between ICSI and ROSI, substantiating the teratogenicity of TSA treatment. These results warn that although TSA treatment significantly improves the mouse cloning technique [6, 7], the safety of this method should be carefully assessed for reproductive technologies including ICSI, ROSI and animal cloning. Regardless, we found that TSA treatment does not harm the blastocyst formation rate nor offspring production rate of ROSI embryos. It is important to point out that the conditions of TSA treatment used in this study were not optimized, that is, TSA 20 h for TSA treatment may be too long or 50 nM TSA may be excessive. Therefore, it may be possible to safely improve the current ROSI technology after careful optimization of conditions of TSA treatment.

In conclusion, our findings elucidate the apparent discrepancy between the improvement of clone-embryonic development by TSA and the teratogenicity of TSA and found a dependency of TSA toxicity on derivatives of treated nuclei. Further, our results provide insight into TSA teratogenicity and will also serve for future improvements of current assisted reproductive technologies (ART).

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