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High levels estradiol affect blastocyst implantation and post-implantation development directly in mice

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ABSTRACT

Background: Previous studies have demonstrated that high levels of estradiol (E2) impair blastocyst implantation through effects on the endometrium; however, whether high E2 directly affects blastocysts is not well established. The present study sought to clarify the direct impacts of high E2 levels on blastocysts *in vitro*.

Methods: ICR virgin albino mice were used. Using an *in-vitro* 8-day blastocyst culture model, immunofluorescence staining for the estrogen receptor (ER), blastocyst outgrowth assays, differential staining and TUNEL assays of blastocysts, and embryo transfer, we investigated the main outcomes of exposure to different E2 concentrations (10^{-7} to 10^{-4} M) *in vitro* and *in vivo*.

Results: ER α and ER β expression were detected in pre-implantation stage embryos. *In vitro* exposure of blastocysts to 10^{-4} M E2 for 24 h followed by 7 days culture in the absence of E2 caused severe inhibition of implantation and post-implantation development. The late adverse effects of E2 on post-implantation development still occurred at concentrations of 10^{-7} to 10^{-5} M. In addition, blastocyst proliferation was reduced and apoptotic cells were increased following exposure to 10^{-4} M E2. Using an *in vivo* embryo-transfer model, we also

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showed that treatment with high E2 resulted in fewer implantation sites (38% vs. 72% in control) and greater resorption of implanted blastocysts (81% vs. 38% in control).

Conclusion: Exposure to high E2 concentrations *in vitro* is deleterious to blastocyst implantation and early post-implantation development, mainly owing to direct impacts of E2 on implanting blastocysts. In clinical assisted reproductive technique (ART), high serum E2 concentrations not only affects the endometrium, but also affects blastocysts directly at the period of implantation.

At a glance of commentary

Scientific background on the subject

In previous studies, most of data have been demonstrated that high levels of estradiol (E2) impair blastocyst implantation through direct effects on the endometrium; but few information is available regarding direct-impacts high E2 on the blastocysts. The present study sought to clarify the direct impacts of high E2 *in vitro*.

What this study adds to the field

Direct-impacted blastocysts with high E2 concentration *in vitro* are deleterious to blastocyst implantation and early post-implantation development in mice. In clinical ART, high serum E2 concentrations not only affects the endometrium, but also affects blastocysts directly at the period of implantation.

Estradiol (E2), the primary form of estrogen in women of reproductive age, acts as a growth hormone/regulator for a variety of female reproductive organs, including the vaginal lining, cervical glands, lining of the fallopian tubes, the endometrium, and the myometrium [1,2].

E2 has a physiological role in embryonic development and implantation, as demonstrated by a number of *in vitro* studies. Smith et al. demonstrated that mouse embryos, especially blastocysts, may develop an estrogen-sensitive mechanism at different concentrations of E2 (10^{-10} , 10^{-9} , and 10^{-5} M) immediately prior to implantation [3]. Effects of E2 on the metabolism of glucose in the mouse morula and early blastocyst *in vitro* have been investigated by Khurana et al. [4]. It has been hypothesized that during development or implantation of rabbit blastocysts, E2 acts as a local signal from the blastocyst to the uterus [5]. Consistent with this, tamoxifen has been shown to inhibit E2-induced changes in the surface coating of mouse blastocysts in a concentration-dependent manner [6]. E2 produced in the rabbit morula and blastocyst could trigger embryonic differentiation and metabolic functions [7]. The role of E2 in the uterine and endometrial cells for pregnancy has also been well studied [8–11]. However, the physiological concentrations of E2 and its direct role in blastocyst and post-implantation development has not been extensively studied.

It has been proposed that high serum E2 concentrations that may occur during controlled ovarian stimulation (COS)

adversely affect the outcome of *in vitro* fertilization (IVF) [12–14]. However, some investigators have reported the controversial finding that high serum E2 concentrations are not detrimental to the outcome of IVF [15–17]. Most studies have shown that high E2 directly impacts the endometrium or indirectly affects blastocysts during the implantation period [18–21]. Accumulated lines of evidence indicated that E2 does directly impair the embryonic development at cleavage-stage *in vitro*, resulting in a reduction in the number of blastocysts formed and their quality. Furthermore, embryos formed from these *in vitro*-developed blastocysts exhibit decreased adhesion to endometrial cells *in vitro* [22]. Nevertheless, the direct impacts of E2 on blastocysts have not been comprehensively researched.

A number of studies have investigated changes in E2 levels in the peri-ovulation period. In the natural cycle, serum E2 levels decrease between days 1 and 3 post-ovulation. However, E2 levels on post-ovulation days 4–6 (107–260 pg/mL) are still maintained at approximately 80% of the peak levels (133–329 pg/mL) observed before ovulation [23,24]. In ovarian hyper-stimulation syndrome (OHSS), after COS, peak E2 serum levels can reach levels (1590–13,484 pg/mL) 10–400 folds higher than those in the natural cycle [25–28]. After ovulation in COS cycles, serum E2 levels on post-ovulation day5 (~1000–3000 pg/mL) were maintained at ~36%–41% of E2 levels on the day ovulation was triggered [29,30]. This means that serum E2 levels after ovulation in the COS cycle are still higher than those after ovulation in the natural cycle. A few studies have demonstrated a correlation between serum E2 levels and those in the uterine cavity during the menstrual cycle. In Zavy et al. study, the E2 level in uterine cavity increases as the E2 level in serum increases on post-ovulation days in the estrous cycle in mares [31], indicating that, if a high serum E2 level is noted on post-ovulation days, a higher E2 level would occur in the uterine cavity. The interaction between blastocysts and the endometrium during implantation process is affected by high E2 levels in the uterine cavity. The impacts of high E2 concentrations on the endometrium, and possibly on the embryos, were demonstrated in a study by Valbuena et al. [22], which showed that an *in vitro* E2 concentration of 10^{-7} M is equivalent to 27.24 pg/mL of E2 in serum. Compared with the natural cycles, we all know serum estradiol level could be more than 10,000 pg/mL [26–28,32]. Therefore, a very high E2 level could possibly occur in the uterine cavity during the implantation period in OHSS.

Accordingly, we sought to investigate the direct effects of E2 on blastocysts, determine tolerable levels of E2 for blastocysts, and establish the mechanisms underlying the associated adverse effects during the peri-implantation period. In the

present study, the direct impacts of E2 on blastocysts were investigated by using both *in vitro* and *in vivo* models.

Materials and Methods

Animal and blastocyst collection

ICR virgin albino mice were maintained under a 12-h/12-h cycle, with food and water provided *ad libitum*. All animals received humane animal care according to the Guidelines for Care and Use of Experimental Animals. ICR mice (6–8 weeks old) were super-ovulated by injecting 5 IU Pregnant Mare Serum Gonadotropin (Sigma, St. Louis, MO, USA or PMSG; ProSpec, Ness-Ziona, Israel), followed by injection of human chorionic gonadotropin (HCG, 5 IU) 48 h later. Females were then mated overnight with a single fertile male of the same strain. Pregnancy was confirmed by the presence of a vaginal plug next morning. Females mice with vaginal plugs were selected immediately (as the day 1 of pregnancy) for the subsequent experiments. Blastocysts were obtained by flushing the fallopian tubes on the morning of day 4 of pregnancy with pre-warmed Earle's Balanced Salt Solution (EBSS; Sigma) containing 0.3% bovine serum albumin (BSA; Sigma), 1 mM pyruvate sodium, 1 mM glutamine and 2% (v/v) of an antibiotics preparation (100 units penicillin and 100 g streptomycin per ml; Gibco, Grand Island, NY, USA). Blastocysts were collected in uncoated 4-well culture dishes and washed a minimum of three times. At least 100 mice were used to collect more than 1000 blastocysts for subsequent experiments.

Blastocyst culture and definition of developmental stages

Embryos were acquired according to a previously described protocol [33,34]. Briefly, blastocysts acquired from different female mice by flushing the uterine horn on day 4 were expanded, pooled, and randomly selected for experiments. Embryos were cultured in EBSS medium containing 0.3% BSA during the pre-implantation stage, and in CMRL 1066 medium (Sigma) during the post-implantation stage. Both media contained 1 mM glutamine, 1 mM sodium pyruvate, and 50 IU/ml penicillin (Gibco) plus 50 mg/ml streptomycin (Gibco). CMRL 1066 medium containing 20% fetal bovine serum (FBS; Gibco) was used for culturing blastocysts during the first 4 days and then was replaced with medium containing 20% human placental cord serum. Embryos were inspected daily under a dissecting microscope and classified, as described previously [33,34]. Briefly, blastocysts possessing a zona pellucida were defined as Witschi stage 6. Free blastocysts that have shed the zona pellucida and attached lightly to the surface of the culture dish were defined as Witschi stage 7. Implanted blastocysts that had left an inner cell mass on trophoblast outgrowths were defined as Witschi stage 8. Those with an inner cell mass protruding from trophoblast outgrowths forming a small cavity in the center (gastrula) were defined as Witschi stage 9. Embryos in which the gastrula was increased in mass and elongated were defined as Witschi stage 10. Late egg cylinders with two distinguishable parts—the proximal part of the extra-embryonic region and the distal part of the embryonic region (snowman shape)—were defined as Witschi stage 11. Late egg

cylinders with a primitive streak or neurula were defined as Witschi stage 12–13. Somite-stage embryos with a yolk sac enclosing the embryonic shield were defined as Witschi stage 14–15. The well-defined pictures of different developmental stages has been shown in the previous study [34]. In this study, early egg cylinder embryos were defined as embryos that had reached stage 9 or 10 by day 4; late egg cylinder embryos were defined as embryos that had reached stages 11, 12 or 13 by day 6 of culture; and early somite embryos were defined as embryos that had reached stage 14 or 15 by day 8.

Immunofluorescence staining for the ER

Embryos at zygote stage or 2-cell/4-cell/8-cell/morula stage were collected from the oviducts following different timing (20 h, 48 h, 54 h, 65 h and 72 h respectively) after HCG injection. Blastocysts were collected from the uterine cavity following 96 h after HCG injection. Embryos collected at different stages were fixed immediately by incubating in 4% paraformaldehyde for 30 min at room temperature. Samples were permeabilized by incubating with 0.5% Triton X-100 and 1% BSA in PBS, and then were washed three times with PBS containing 0.2% Triton X-100 and 0.3% BSA. Therefore, samples were incubated in blocking buffer containing 4% BSA and 0.2% Triton X-100 in PBS at room temperature for 1 h, followed by incubation overnight at 4 °C with rabbit anti-mouse ER α (PA1-309) and ER β (PA1-311) primary antibodies (Thermo Scientific, Waltham, MA, USA), diluted 1:100. Samples were then incubated at room temperature for 1 h with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG secondary antibody (31635; Thermo Scientific), diluted 1:100, following by counterstaining with bisbenzimidazole (BIS) to label nuclei.

Blastocyst outgrowth assays

Blastocysts were cultured in the absence or presence of E2 (10^{-7} , 10^{-6} , 10^{-5} , or 10^{-4} M) for 24 h and then cultured for 3 days without E2. The number of blastocysts was shown in the legends of [Fig. 3, B]. Thereafter, the culture medium was carefully removed and replaced with 5% hypotonic sodium citrate (30 μ l/well) and blastocysts were incubated at room temperature for 5 min. After evaporating this solution under partial vacuum (200 bar) at 50 °C for 60 min, the expanded cells were fixed using FixDenat fixative at 50 °C for 60 min. The total number of nuclei in outgrowths was assessed after staining with a 4% Giemsa solution (Sigma) at room temperature for 15 min.

Differential staining of blastocysts

Blastocysts with a normal appearance were selected and cultured in the absence or presence of E2 (10^{-7} , 10^{-6} , 10^{-5} , or 10^{-4} M) for 24 h. The morphology of blastocysts was recorded. The proliferation of blastocysts was evaluated by separately counting the number of inner cell mass (ICM) and trophectoderm (TE) cells identified by dual differential staining, which also allows examination of dead cells with fragmented nuclei. This method is based on the impermeability of the TE layer, which protects ICM cells from exposure to the antibody and the complement reaction. The two cell lineages can be distinguished following fluorochrome staining [35].

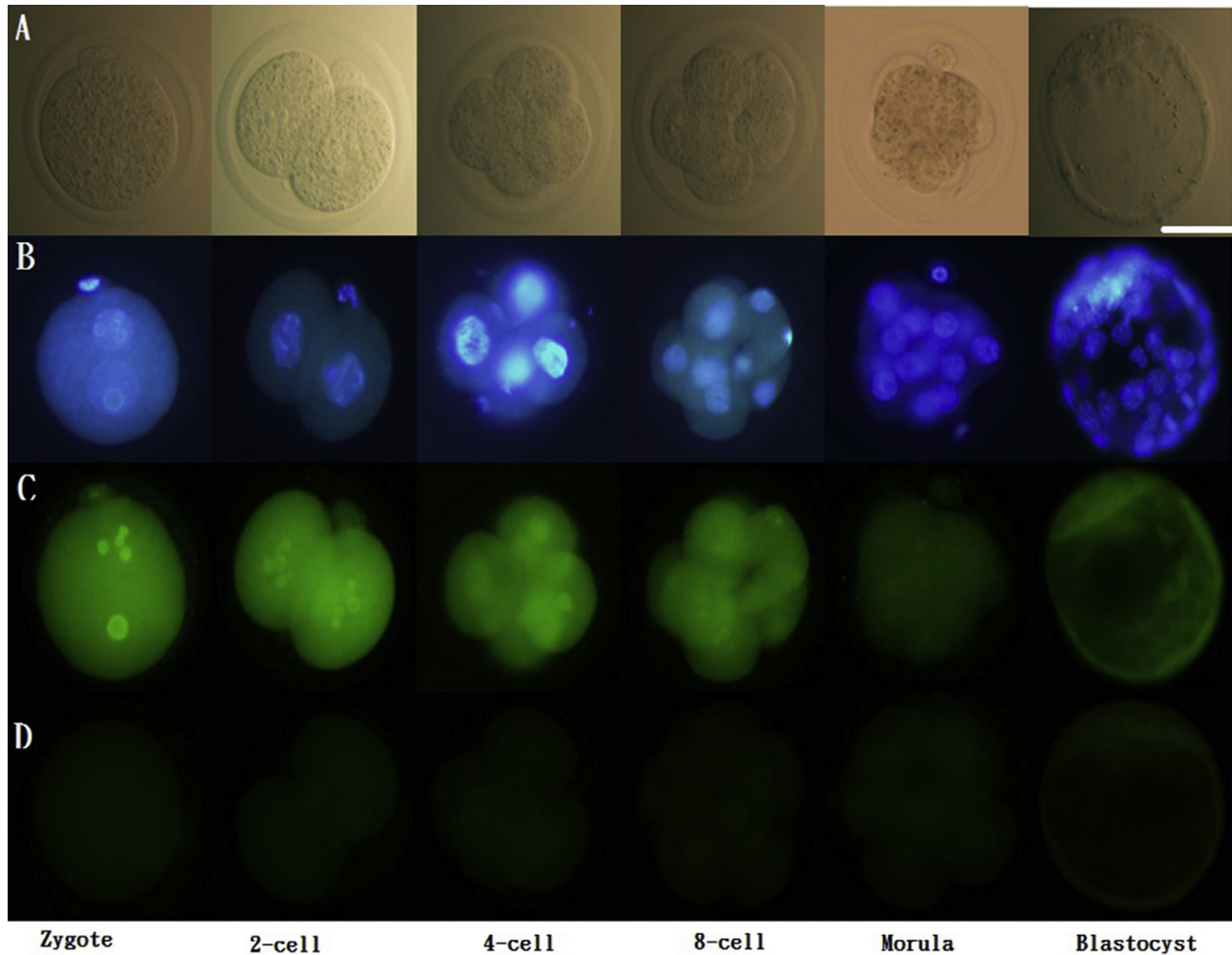


Fig. 1 Immunofluorescence of ER α in pre-implantation embryos. Scattered ER α expression was detected in the cytoplasm as well as the nucleus (co-stained with DAPI) of 1-cell stage embryos to blastocysts. ER α : green; DAPI: blue. Scale bar = 50 μ m (A) Unstained; (B) stained with DAPI; (C) stained with primary and secondary antibodies for ER α ; (D) stained with only secondary antibodies for ER α . Representative images from three experiments using biologically different samples (n \geq 15) are shown.

The zona pellucida was removed by incubating blastocysts in a solution of 0.4% pronase in EBSS (Earle's Balanced Salt Solution, Sigma)-BSA medium containing 0.3% bovine serum albumin (BSA) at 37 °C for 5 min. Denuded blastocysts were exposed to 15% rabbit anti-embryonic cell membrane serum at 37 °C for 30 min and washed with EBSS-BSA medium. Blastocysts were further treated with 10% bovine serum, 2 mg/ml bisbenzimidazole, and 1 mg/ml propidium iodide at 37 °C for 30 min.

The resulting immunolysed blastocysts were gently transferred onto slides, protected from light. Under appropriate UV light excitation, ICM cells that take up bisbenzimidazole but exclude propidium iodide are stained blue, whereas TE cells, which are labeled with both fluorochromes, are stained orange-red. Because it has been shown that multinucleated cells are infrequent in pre-implantation embryos, the number of nuclei was considered an accurate reflection of the number of cells.

TUNEL assay of blastocysts

Blastocysts cultured with different concentrations of E2 (10^{-7} , 10^{-6} , 10^{-5} , and 10^{-4} M) or vehicle for 24 h were collected and

subjected to terminal deoxynucleotidyl transferase UTP nick-end labeling (TUNEL) staining. The embryos were washed in puerarin-free medium, fixed, permeabilized and labeled using an In Situ Cell Death Detection kit (Cat.No.11684817910; Roche, Berlin, Germany), according to the manufacturer's protocol. Bright-field images were obtained using a fluorescence microscope (Olympus BX70, Tokyo, Japan).

Embryo transfer

Blastocysts with a normal appearance were selected and cultured in the absence or presence of E2 (10^{-4} M) for 24 h. Eight untreated control embryos and eight E2-treated embryos were transferred to the left and right uterine horns of each surrogate mice respectively, on day 4 of positive vaginal plug. Ten surrogate mice were analyzed and sacrificed on day 14 post-transfer (18-day fetuses); the frequency of implantation was calculated as the number of implantation sites per number of embryos transferred. The incidence rates of surviving fetuses (number of surviving fetuses/number of implantations) and resorbed fetuses ((number of implantations – number of

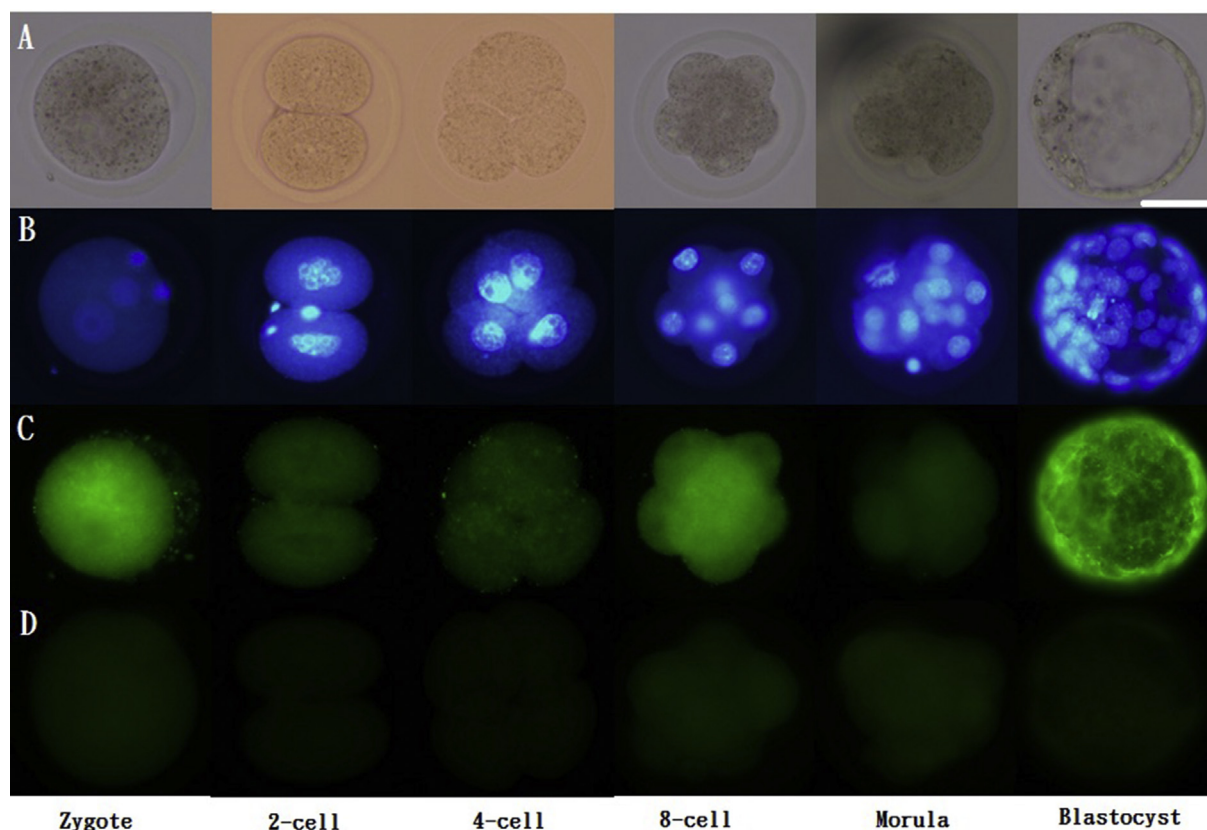


Fig. 2 Immunofluorescence of ER β in pre-implantation embryos. Scattered ER β expression was detected in the cytoplasm as well as the nucleus (co-stained with DAPI) of 1-cell stage embryos to blastocysts. ER β : green; DAPI: blue. Scale bar = 50 μ m (A) Unstained; (B) stained with DAPI; (C) stained with primary and secondary antibodies for ER β ; (D) stained with only secondary antibodies for ER β . Representative images from three experiments using biologically different samples ($n \geq 15$) are shown.

surviving fetuses/number of implantations) were also calculated. The implantation percentage represents the number of implantations per number of transferred embryos $\times 100$. The percentage of resorbed or surviving fetuses represents the number of resorptions or surviving fetuses per number of implantations $\times 100$. The weights of surviving fetuses were measured immediately after dissection.

Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA) and Student's *t*-test using SPSS software (IBM19). The results are presented as means \pm SEMs. *p*-values < 0.05 were considered statistically significant.

Results

ER α and ER β protein expression in pre-implantation embryos

In order to investigate the role of E2 in the embryos, the ER α and ER β would be identified in the embryos firstly. During the pre-implantation stage, both ER α [Fig. 1] and ER β [Fig. 2] were expressed from the 1-cell stage to blastocysts. Strong

expression of ER α was found before the morula stage. ER β was strongly expressed in zygotes, 8-cell embryos, and blastocysts.

Effects of E2 on blastocyst implantation and post-implantation in vitro

Having confirmed the differential expression of ER α and ER β in blastocysts, we further examined the concentration-dependent effects of E2 on blastocyst implantation and post-implantation development *in vitro* for 8 days. In the experiments of 8-day culture, all blastocysts were exposed to different concentrations of E2 (10^{-9} , 10^{-8} , 10^{-7} , 10^{-6} , 10^{-5} , and 10^{-4} M) on first day, and then the blastocysts continued to be cultured without E2 exposure for another 7 days. First, we determined the toxic concentrations of E2 *in vitro*, testing concentrations over a range from 10^{-9} to 10^{-6} M. E2 concentrations of 10^{-9} to 10^{-6} M had no apparent impact on blastocyst implantation or post-implantation embryo development. However, the development of late post-implantation embryos (late egg cylinder stage and early somite stage) was adversely affected at E2 concentrations of 10^{-7} and 10^{-6} M [Table 1]. To further assess concentration-dependent impacts of E2, we performed additional experiments using E2 concentrations of 10^{-7} to 10^{-4} M [Table 2]. The higher E2 concentration (10^{-4} M) exerted a more rapid impact on early-stage embryos, reducing the blastocyst implantation rate nearly in half (57%) compared

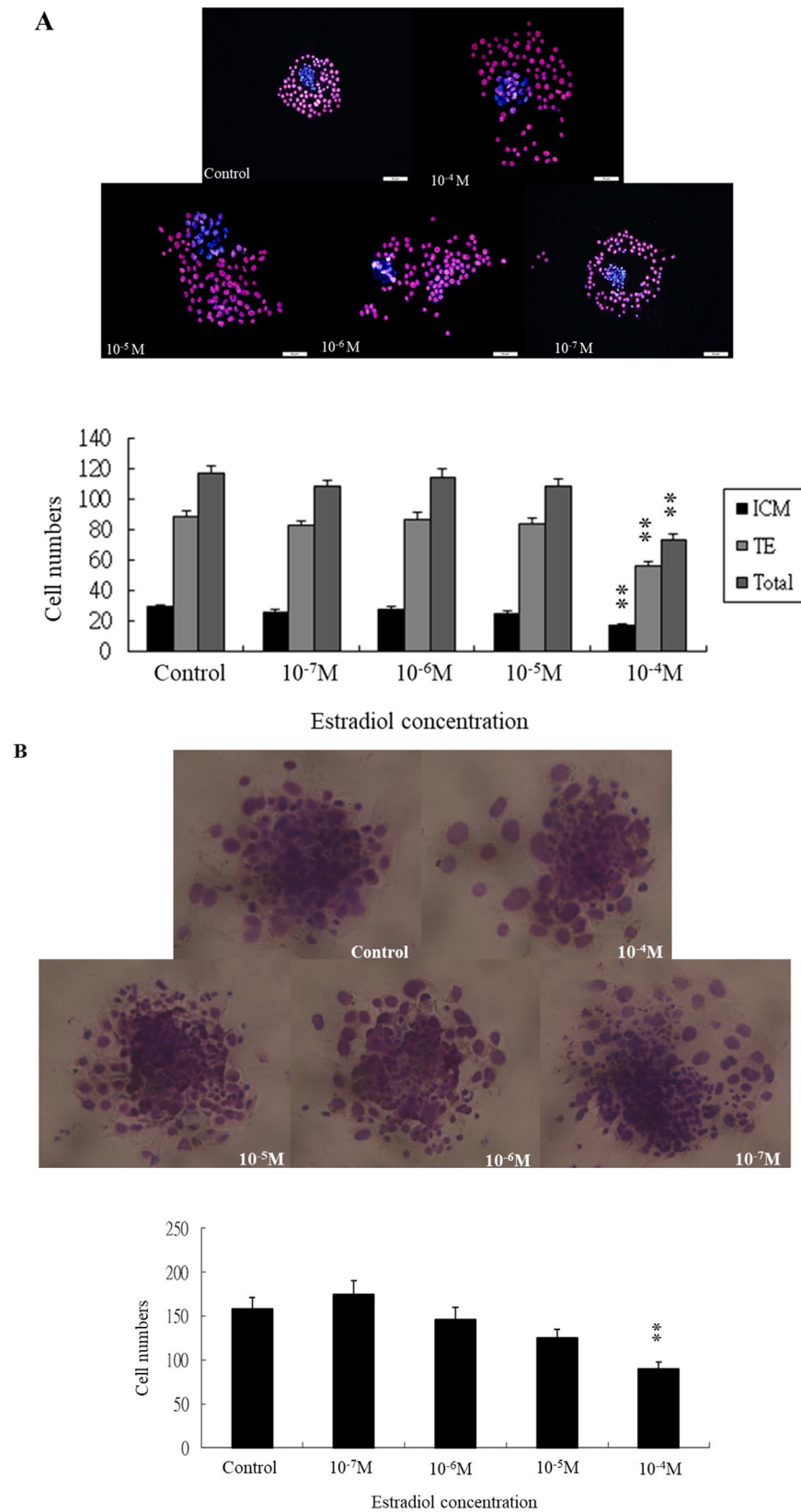


Fig. 3 Effects of E2 on growth of blastocyst cells. (A) Proliferation of blastocyst cells following 24-h exposure to different concentrations of E2, determined by cell counting using a differential staining method. The sample size shown in each group (control:37; 10^{-7} M:38; 10^{-6} M:32; 10^{-5} M:31; 10^{-4} M:38 respectively). (B) Blastocyst cell outgrowth *in vitro* following a 24-h exposure to different concentrations of E2 and culturing for 3 days. Total number of nuclei in outgrowths was assessed after staining with a 4% Giemsa solution. The sample size shown in each group (control:32; 10^{-7} M:34; 10^{-6} M:30; 10^{-5} M:32; 10^{-4} M:31 respectively). Data were recruited from four experiments and analyzed by one-way analysis of variance (ANOVA), ** $p < 0.01$ vs. control.

Table 1 Eight-day development *in vitro* of mouse blastocysts exposed to moderate to high concentration of E2 at the blastocyst stage for 24 h.

Developmental stage	Control group	E2 group			
		10 ⁻⁹ M	10 ⁻⁸ M	10 ⁻⁷ M	10 ⁻⁶ M
Blastocysts	72	56	56	54	58
Hatched/implanted blastocysts (7–8)	69 (96%)	53 (95%)	54 (96%)	53 (98%)	57 (98%)
Early egg cylinder stage (9–10)	43 (60%)	32 (57%)	30 (54%)	31 (57%)	33 (57%)
Late egg cylinder stage (11–13)	36 (50%)	24 (43%)	22 (39%)	14 (26%)*	16 (28%)*
Early somite stage (14–15)	18 (25%)	13 (23%)	13 (23%)	7 (13%)*	4 (7%)*

Data collected from 4 experiments and analyzed by one-way analysis of variance (ANOVA), **p* < 0.05 vs. control.
10⁻⁶ M equivalent to 272.4 pg/mL.

with other groups, which showed implantation rates of 93% (control) to 100% (10⁻⁷, 10⁻⁶, and 10⁻⁵) [Table 2].

Effects of E2 on blastocyst proliferation and outgrowth ability

In order to investigate the impacts of high E2 on cell proliferation of blastocyst *in vitro*, the differential staining and outgrowth assays were used. The numbers of inner cell mass (ICM) and trophoctoderm (TE) cells were both decreased following treatment with 10⁻⁴ M E2 for 24 h [Fig. 3A].

The cell number of blastocyst outgrowth *in vitro* was significantly decreased at the group of 10⁻⁴ M E2, in comparison with other groups [Fig. 3B].

Apoptotic effects of 24-h exposure to E2 at the blastocyst stage

In order to investigate apoptotic effects of high E2 on the blastocyst *in vitro*, the TUNEL assay was used. The total number of apoptotic cells, including inner cell mass (ICM) and trophoctoderm (TE) cells was significantly decreased at the group of 10⁻⁴ M E2, in comparison with other groups [Fig. 4].

E2 disruption of blastocysts in vitro an embryo transfer model

In order to investigate the pregnancy outcome of E2-treated blastocysts in the female uterus, the method of embryo transfer was used. The implantation rate in the E2 group was lower than that in the control group. Specifically, control blastocysts implanted at a rate of ~72% (49 of 68 embryos),

whereas E2-pretreated blastocysts implanted at a rate of ~38% (14 of 37 embryos)—a decrease of ~47% [Table 3]. Moreover, 81% (30 of 37 implants) of implanted embryos failed to develop normally (i.e., were resorbed) in the E2 group compared with 38% (26 of 68 implants) in the control group. The fetal survival rate in the E2 group was also significantly lower than that in the control group. Despite above differences, the body weight of surviving fetuses was similarly distributed between the two groups: ~55% of fetuses in both groups weighed 600–800 mg, and ~45% of fetuses in both groups weighed over 800 mg [Fig. 5].

Discussion

In the study, we have demonstrated that ER α and ER β protein in the pre-implanted embryos, especially the blastocysts. At most, the mRNA expression of ER is noted in the previous studies. We also find the direct impacts of high E2 on the blastocysts firstly, which is not well known in the previous studies.

The detection of ER protein in blastocysts lends further support to possible direct effects of E2 on blastocysts during the pre-implantation period. In one previous study, Hou et al. demonstrated that ER protein is detectable in implanting blastocysts of mouse and early egg cylinder stage embryos developed in culture [36]. In another study, Saito et al. showed that ER α expression was down-regulated in activated blastocysts upon completion of blastocyst implantation in mice [37]. In the current study, we have extended these observations, demonstrating ER α and ER β protein expression in different-stage embryos. Focusing on the blastocyst, we found that

Table 2 Eight-day development *in vitro* of mouse blastocysts exposed to moderate to high concentration of E2 at the blastocyst stage for 24 h.

Developmental stage	Control group	E2 group			
		10 ⁻⁷ M	10 ⁻⁶ M	10 ⁻⁵ M	10 ⁻⁴ M
Blastocysts	44	26	28	26	28
Hatched/implanted blastocysts (7–8)	41 (93%)	26 (100%)	28 (100%)	26 (100%)	16 (57%)*
Early egg cylinder stage (9–10)	24 (55%)	11 (42%)	15 (54%)	11 (42%)	0***
Late egg cylinder stage (11–13)	21 (48%)	7 (27%)*	8 (29%)	7 (27%)*	0***
Early somite stage (14–15)	12 (27%)	2 (8%)*	0**	1 (4%)*	0**

Data collected from 3 experiments, and analyzed by one-way analysis of variance (ANOVA), **p* < 0.05 vs. control, ***p* < 0.01 vs. control, ****p* < 0.001 vs. control.
10⁻⁴ M equivalent to 27,240 pg/mL.

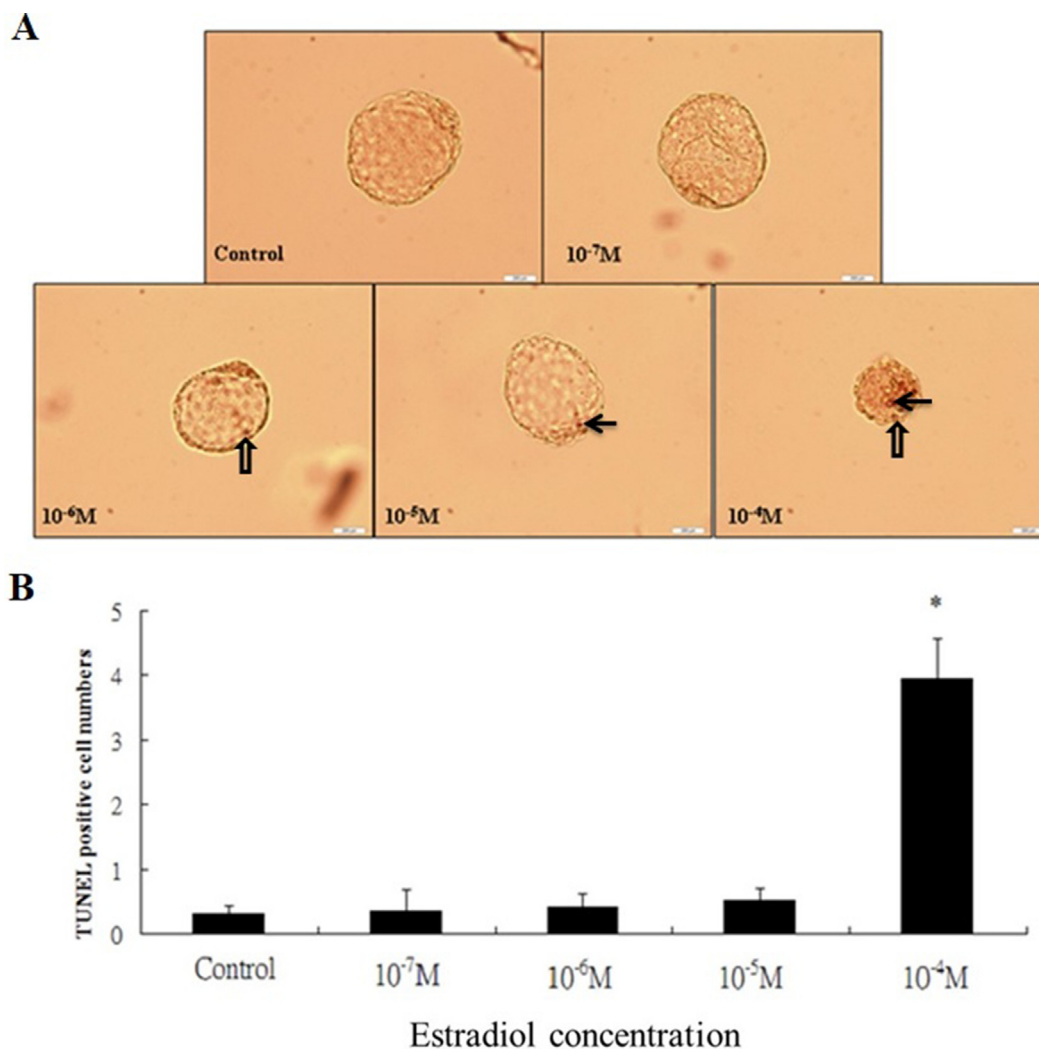


Fig. 4 Apoptotic effects of 24-h exposure to E2 at the blastocyst stage. (A) TUNEL-positive cell shown as arrow in the ICM cells of blastocysts. TUNEL-positive cell shown as blank arrow in the trophoctoderm of blastocysts. (B) Apoptosis of blastocyst cells occurred at E2 levels above a threshold of $10^{-4} M$. * $P < 0.05$ versus the control group. The sample size shown in each group (control:32; $10^{-7} M$:30; $10^{-6} M$:32; $10^{-5} M$:31; $10^{-4} M$:30 respectively). The apoptotic cells were noted using the TUNEL assays. Data were recruited from three experiments and analyzed by one-way analysis of variance (ANOVA).

ER β was more strongly expressed than ER α , suggesting that the actions of E2 on blastocyst implantation and post-implantation development are mediated by ER β . Thus, ER α may be a housekeeping receptor, whereas ER β may be the major receptor responsible for the actions of E2. However, the actual differences of signal intensity of ER α and ER β protein did not be investigated in the study. Blastocyst implantation and development studies designed to confirm this hypothesis

using antagonists selective for ER α or ER β subtypes are ongoing in our laboratory.

Despite our findings and previous reports of ER expression in blastocysts, the actual roles of E2 in blastocysts are still not well investigated. In a study by Valbuena et al. [22], the effects of E2 *in vitro* were investigated at concentrations of $10^{-7} M$ (27.24 pg/mL), $10^{-6} M$ (272.4 pg/mL), $10^{-5} M$ (2724 pg/mL), which could be very close to the serum E2 concentrations

Table 3 Pregnancy outcomes following E2-treated blastocyst transferred into uterus.

Group	Transferred embryo No.	Implantation rate (%)	Implanted embryos	
			Resorption	Surviving fetuses
Control	94	68(72%)	27/68(40%)	41/68(60%)
E2-treated	94	37(39%)*	30/37(81%)*	7/68(19%)*

Data were analyzed by one-way analysis of variance (ANOVA), *** $p < 0.001$ vs. control.

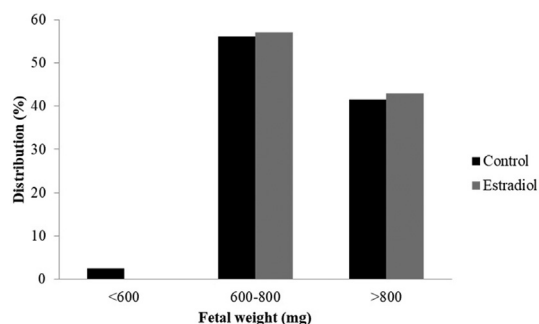


Fig. 5 Neonatal birth weight distribution following E2-treated blastocysts transferred into uterus. E2 exposure and no-exposure at the blastocyst stage for 24 h *in vitro* followed by embryo transfer. 18-day fetuses were collected from the surrogate mice on day 14 post-transfer. Weight distribution of surviving fetuses was analyzed by three subgroup of birth weight. Data were collected from 41 surviving fetuses in the control group and 7 surviving fetuses in the treatment group.

routinely achieved in controlled ovarian stimulation (COS) used for human assisted ART. And the concentration of 10^{-4} M (27240 pg/mL) could occur possibly, but not usually at the condition of OHSS [22,26–28,32]. To determine the toxic concentrations of E2 on the mouse blastocysts *in vitro*, a range of E2 concentration (10^{-9} M to 10^{-4} M) was investigated in the study. Immediate cytotoxic concentration of E2 is 10^{-4} M, which has been shown that cell proliferation and blastocyst outgrowth *in vitro* were significantly inhibited, and revealed an increase in the number of apoptotic cells. The adverse effects of E2 on post-implantation development were most prominent at a concentration of 10^{-4} M, which completely inhibited development from the early egg cylinder stage to the early somite stage. Although E2 concentration of 10^{-7} M to 10^{-5} M did not induce the immediate toxic effects, they still showed the delayed impacts on post-implantation embryonic development (late egg cylinder stage and early somite stage). These observations suggest that E2 levels moderately greater than those observed physiologically may have carry-on effects on the blastocyst. To prevent the direct impacts of high E2 on the blastocyst, control of the serum E2 level (not above 10000 pg/mL) or freezing embryos when E2 reaching 10000 pg/mL would be a sound strategy in clinical ART.

Most previous studies addressing the pharmacological effects of E2 on blastocyst implantation and early post-implantation development have stressed endometrial effects [18–21]. Although one study showed direct effects of a high E2 concentration on pre-implantation embryo development [22], it did not provide clear evidence for an effect of high E2 on blastocyst for implantation. According to another previous study [38], administration of 17β -estradiol at a concentration of 10^{-6} M had negative effects on human trophoblast cell lines, derived from the TE of blastocysts following implantation. Notably, the ER α is also detected in the TE of blastocysts [36]. These findings raise concerns about the potential effects of E2 on the blastocyst, especially on cells of the TE. Human embryonic stem cells are derived from the ICM of the blastocyst, and their differentiation occurs through an

intermediate step involving the formation of embryoid bodies (EBs), which are aggregates of embryonic stem cells. EBs mimic the initial stages of early post-implanted blastocyst development. E2 administration at a concentration of 10^{-7} M exerts effects on endodermal and mesodermal differentiation of human EBs [39]. In our study, we demonstrated a decrease in cell proliferation and an increase in the number of apoptotic cells in whole blastocysts at the E2 concentration of 10^{-4} M, especially in the ICM area, which is the major part of the fetus in post-implantation development. The adverse effects of high E2 on the ICM are compatible with their effects on EBs reported by Kim et al. [39]. These findings demonstrate that E2 has a direct action in the blastocyst for implantation and post-implantation development.

Similar findings were obtained in Valbuena's study [22], which reported that a very high E2 level (10^{-4} M) has a direct impact on pre-implantation embryo development, decreasing the number and quality of blastocysts formed *in vitro*. These findings provide evidence of ER α and ER β in the pre-implantation embryo, a result compatible with our findings of the presence of ER α and ER β in mouse embryos. This previous study also showed that the poor quality of *in vitro* blastocysts resulted in diminished embryo adhesion to endometrial cells *in vitro*. A number of studies have concluded that high levels of E2 reduce the receptivity of the endometrium, but direct effects of E2 on blastocysts were largely unaddressed, possibly owing to limitations of the experimental models used in these studies. Using our *in vitro* model, we have provided evidence that high E2 may directly affect blastocysts, resulting in subsequent adverse effects. In clinical ART, we have found that pregnancy outcomes are higher for thawed embryo transfer cycles than for fresh embryo transfer cycles [40]. The explanation for this improvement does not seem to lie only in avoiding the poor receptivity of endometrium, but also in preventing damage of embryos by a high E2 environment following COS.

In conclusion, we have provided the evidence of direct effects of E2 on the mouse blastocyst. Direct-impacted blastocysts with high E2 concentration ($\geq 10^{-7}$ M) may affect the implantation and post-implantation embryonic development *in vitro* and *in vivo*. In clinical ART, high serum E2 concentrations not only affects the endometrium, but also affects blastocysts directly at the period of implantation.

Conflicts of interest

The authors declare no competing financial or non-financial interests.

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