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Original Article

Cyclosporine A improves adhesion and invasion of mouse preimplantation embryos via upregulating integrin β3 and matrix metalloproteinase-9

Yuan-Hua Huang[1,2], Yan-Lin Ma[2], Lin Ma[2], Ji-Long Mao[2,3], Yu Zhang[2], Mei-Rong Du[1], Da-Jin Li[1,2]

[1]*Laboratory for Reproductive Immunology, Hospital and Institute of Obstetrics and Gynecology, Fudan University Shanghai Medical College, Shanghai 200011, China;* [2]*Affiliated Hospital of Hainan Medical College, Haikou, China;* [3]*Guiyang College of Traditional Chinese Medicine, Guiyang, China*

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Abstract: Our previous study has demonstrated cyclosporin A (CsA) promotes the migration and invasiveness of human first-trimester trophoblast cells in vitro. Here, we further investigated the effect of CsA on the early implantation in vitro of mouse embryo. Female C57 mice were superovulated and mated, and then two-cell embryos were harvested from the oviducts and sequentially cultured in vitro in G1 and G2 media with 0, 0.1, 1.0 or 10 μM of CsA. Blastocyte formation, blastocyte cell number and apoptosis, embryo hatching were assessed in 4-6 dpc. The adhesion and stretching growth of hatched embryos in laminin coated dishes were evaluated from 5 dpc to 8 dpc, and the expressions of implantation serine proteinase 1 (ISP1), integrin (itg) β3 and matrix metalloproteinase (MMP)-9 were determined by real time PCR and immunofluorescence, respectively. We showed there was no significant difference in blastocyst formation rates, hatching rates, number of whole embryonic cells, apoptotic cells, and distribution of inner cell masses (ICMs) and trophoblasts (TB) between the CsA- and control-treated groups. Expression of ISP1 mRNA was unaffected on 5 dpc. After hatching, adhesion rate of 7 dpc significantly increased in 0.1 and 1.0 μM of CsA treatment, and embryo area of 8 dpc stretch growing on laminin were increased in 1.0 μM of CsA. The mRNA and protein expression of itgβ3 and MMP-9 on 7 dpc blastocyst were up-regulated. In conclusion, CsA in low dosage up-regulates itgβ3 and MMP-9 expression, and enhances embryonic adhesion and invasion, which is beneficial to the embryo implantation.

Keywords: Cyclosporine A, integrin, MMP, pre-implantation embryos

Introduction

In vitro fertilization and embryo transfer (IVF-ET) has been an important therapeutic to deal with infertility since the first IVF-ET baby was born in 1978. After controlled ovarian hyperstimulation was introduced into clinical protocol, multiple embryos transfer, and innovated embryonic culture were used, clinical pregnant rate increased from around 10% to more than 40%. However, the current overall embryo implantation rate remains at around 15-20%, which is lower than natural embryo implantation rate of 30%. At the same time, multiple pregnancy was 20 times higher than that of physiological natural pregnancy. Enhancing implantation potential and restricting the numbers of embryos transferred are tactics in IVF-ET programs.

The successful implantation of embryo depends on embryo hatching, trophoblast development, proper maternal-fetal cross-talking and immune regulation. The invasion potential of trophoblast is essential to embryo implantation. The blastocysts adhere to and invade into endometrium via cross-talk between trophoblasts and endometrium. The trophoblasts show invasive phenotype 10-15 hr after hatching to obtain ability of cell-to-cell adhesion and cell-to-cellular matrix degradation [1]. Integrin (Itg) αvβ3 and laminin expressed by both trophoblast cells and uterine cavity epithelium are the key molecular events in implantation [2, 3]. The interaction of

Table 1. Primers of RT-PCR for different genes used in this study

mice gene		primer sequence	length of amplified fragment (bp)
GAPDH	forward	GGTGCTGAGTATGTCGTGGA	248
	reverse	CCTTCCACAATGCCAAAGTT	
OCT-4	forward	CACGAGTGGAAAGCAACTCA	246
	reverse	AGATGGTGGTCTGGCTGAAC	
CDX-2	forward	AAACCTGTGCGAGTGGATG	170
	reverse	CCAGCTCACTTTTCCTCCTG	
ISP-1	forward	GTACCGTGTTCATGGCCTCT	205
	reverse	TGACTTTGGATGCAGTGAG	
Itgβ-3	forward	TGACATCGAGCAGGTGAAAG	224
	reverse	GAGTAGCAAGGCCAATGAGC	
MMP-9	forward	CGTCGTGATCCCCACTTACT	225
	reverse	AACACACAGGGTTTGCCTTC	

Table 2. The effect of CsA on blastocyst formation of embryos

CsA (μM)	Embryo number (2 dpc)	Blastocyst number (5 dpc)	Blastocyst formation rate (%±SE)
0	689	469	68.07±2.15
0.1	682	460	67.45±2.18
1.0	693	460	66.38±2.20
10	690	472	68.41±2.14

The blastocysts on 2 dpc were cultured in the media containing different concentrations of CsA. A: The blastocysts on 5 dpc were counted. The data are from 2754 blastocysts and χ^2 test was used, $p>0.05$.

trophoblast-derived itg αvβ3 to the cavity epithelium-derived laminin and αvβ3 presents two-way adhesions at the beginning of implantation. Matrix metalloproteinase (MMP)-9 is one of main MMPs that express stably in preimplantation embryo [4, 5]. After adhesion to cavity epithelium of endometrium, trophoblasts break down extra-cellular matrix (ECM) by MMP-9, migrate and "embedding" itself into the endometrium.

Cyclosporine (CsA) is an immunosuppressive agent widely used in patients who have organ transplantation or suffer from autoimmune diseases. Our previous studies have showed that CsA had dual roles in pregnancy, i.e. inducing maternal-fetal immunotolerance and improving biological behavior of trophoblast, resulting in improved pregnant outcome of abortion-prone mouse model [6]. CsA up-regulated expression and activity of MMP-9 by human primary trophoblasts [7]. These data indicated that CsA might improve embryonic implantation in IVF-ET.

In the present study, we first observed the effect of CsA on blastocyte formation, blastocyte cell number and apoptosis, embryo hatching. Then we evaluated the adhesion and stretching growth of hatched embryos in laminin coated dishes. Finally, we investigated the expressions of implantation serine proteinase 1 (ISP1), itg β3 and MMP-9. Our study indicated that CsA might exert regulatory actions on peri-implantation embryo, and shed light on the possible application of CsA in IVF-ET by promoting embryo implantation.

Materials and methods

Animal

Inbred mice (C57, National Resource Center in mutant mice, based in Nanjing University, China) were housed with light from 7:00 to 19:00 and dark from 19:00 to 7:00. The female mice of two-three weeks old were superovulated by an i.p. injection of 10 IU pregnant mare serum gonadotrophin (PMSG) per mouse at 14:00 and then an i.p. injection of 10 IU human chorionic gonadotrophin (hCG) per mouse in 48 hr. After hCG injection, the mice were mated in pare with males of 6-8 weeks old. The vaginal plug was checked 8:00 next morning. This was deemed to be Day 1 post mating (1 dpc) when vaginal plugs appeared and the female mice were separated afterward. The following days called 2 dpc, 3 dpc, and so on. The use of animals in these procedures has been approved

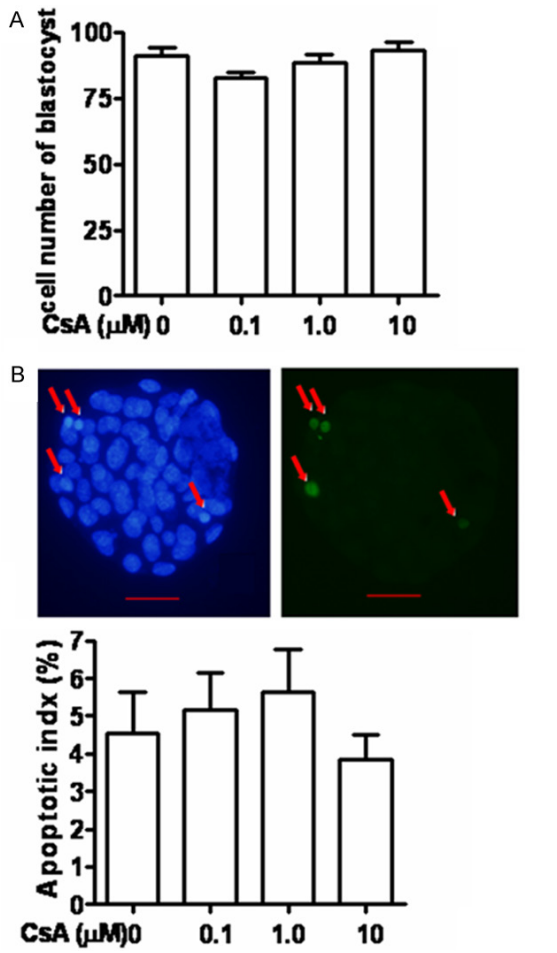

Figure 1. Evaluation of total embryo cells, apoptosis cells and ICM cells by fluorescence microscopy. The blastocysts on 2 dpc were cultured in the media containing different concentrations of CsA. A: The blastocysts on 5 dpc were washed and cell number was determined by Hoechest 33258 staining. The data are from 176 blastocysts. B: The blastocysts on 5 dpc were washed and apoptosis was determined by using TUNEL and DAPI staining based on nuclear morphology and TUNEL assay. Data in B are from 98 embryos. The representative picture of apoptotic cells were showed in A with red arrows. (Bars: 50 μm).

by The Ethics Committee of Hainan Medical College.

Pre-implantation embryo retrieval and culture

The female mice were killed at 8:00 am on 2 dpc. The oviducts were cut and flushed. Embryos were released from oviducts by tearing the oviducts mechanically with G5.5 needles. The embryos were collected and graded [8], and grade I and II embryos were used for the further research.

Before hatching, the embryos were cultured in 37°C, 5% CO_2 in air, 95% humidity with G1 on 2 dpc and G2 media (Vitrolife) on 3 dpc and afterward sequentially. The media contained 10 mg/ml human albumin and CsA in the different concentrations.

Morphological evaluation of embryos

Morulae were assessed morphologically according its fragmentation, speed of development, and blastomeres (number, uniformity of size, cytoplasm granularity). Blastocyst was scored morphologically according to Gardner DK's description [9] including parameters of stage, trophoblast, and inner cell masses (ICM), and hatching. The development of embryos without CsA was used as control where more than 80% of embryos were 8 blastomeres and more than 60% of embryos developed to blastocysts on 3 dpc. The total cell numbers of 5 dpc blastocysts that developed to stage III, IV and V were counted after bisbenzimide nucleus staining according to the assay introduced by Martin KL [10]. The zona pellucida was removed by putting blastocysts in Tyrodes' solution (Sigma) for about 1 min under stereomicroscope observation. After fixed and permeabilized, the embryos were stained in 50 μg/ml bisbenzimide (Beyotime) at 37°C for 20 min. Embryos were washed thoroughly in PBS before being mounted in glycerol and examined using fluorescence microscope.

Allocation of ICMs and trophoblasts

The blastocysts on 4 dpc developing to stage III and after were used. The allocation of ICMs and trophoblasts was assessed by immunofluorescence. ICM cells were determined by detection of OCT-4 positive cells, and the total cells were counted by DAPI staining. The trophoblast cell numbers were obtained by subtracting ICM cells from total cells.

Cell apoptosis

The apoptotic cells of 5 dpc blastocysts were stained and counted by using terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL). The 5 dpc blastocysts that developed to stage III, IV and V were evaluated by using TUNEL kits (Roche) under their instruction of manual, and the method introduced by Brison DR [11]. Blastocysts treated with DNase I were used as positive control, and

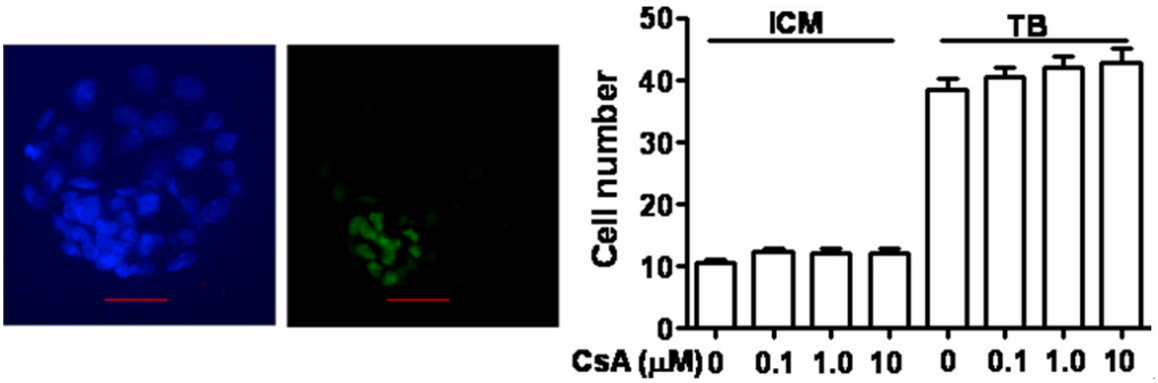

Figure 2. The effect of CsA on the allocation and number of trophoblasts and ICM cells. The blastocysts on 5 dpc were washed and stained for OCT-4 and DAPI. The ICM cells were OCT-4-positive, the trophoblast cell numbers were the total cell number subtract ICM cell number. The data are from 160 blastocysts. TB: trophoblast, ICM: Inner cell masses. (Bars: 50 μm).

Table 3. The effect of CsA on the hatching of blastocysts

CsA (μM)	Number of Blastpcysts (D4, 4 dpc)	Hatched Blastocyst (N, 6 dpc)	% of hatched blastocysts
0	203	183	90.15
0.1	211	189	89.57
1	186	179	96.246
10	199	191	95.98
total	799	742	92.87

The blastocysts on 4 dpc were cultured in the media containing different concentrations of CsA. The blastocysts on 6 dpc were harvested and the number of hatched blastocyst was counted. Data were from 799 blastocysts. and χ^2 test was used, p>0.05.

that without fluoresein-dUTP were as negative control. After TUNEL, the blastocysts were stained with 4',6-diamidino-2-phenylindole (DAPI, Solarbio). Embryos were washed thoroughly in PBS, mounted in glycerol and then examined under fluorescence microscope.

The peri-implantation embryo adhesion and stretching observation

The peri-implantation embryo cultured in the dishes was checked on 7 dpc to assess the embryonic adhesion and the adhesion rate was calculated. The photographs of adhered embryos on 8 dpc were taken and their stretched areas on the dish floor were measured.

Immunofluorescence

The expressions of OCT-4, MMP-9, and itg αvβ3 in embryos were identified by immunofluorescence as described by van Eijk MJ [12] to analyze cell differentiation, allocation.

OCT-4 (4 dpc) was used to identify ICMs. Briefly, after removing of zona pellucida, the embryos were fixed, permeabilized and then incubated in 1:100 rabbit-anti-mouse OCT-4 antibody (Abcam ab19857) for 1 hr in room temperature. After washing, they were then incubated in 1:500 Alexa Fluor 488-conjugated goat-anti-rabbit antibody (Invitrogen A11034) for 1 hr. After washing uncombined second antibody, they were stained in DAPI (10 μg/ml) and mounted in glycerol and examined by using fluorescence microscope. MMP-9 and itgαvβ3 were identified in laminin-coated dish adhered embryos (7 dpc) in situ following the procedure above without zona pellucida digestion. The first antibody was purchased from Abcam and the second from Invitrogen commercially. The stained embryos were checked by confocal laser microscope (Olympus, FV1000).

Reverse transcription polymerase chain reaction and real time polymerase chain reaction (RT-PCR)

The mRNAs of 5 or 7 dpc embryos (10-15 in group) were extracted using RNeasy Plus Micro Kit (Qiagen) following the instruction. Their cDNAs were reversely transcribed by using ImProm-II Reverse Transcription System (Promga). The primers used in RT-PCT were showed in **Table 1**. Brilliant II SYBR Green Q-PCR Master Mix kit (Stratagene) and Mx3000P™ real-time PCR system were used in the experiment. The mRNA abundance of ISP1, itgαvβ3 and MMP-9 were expressed as a ratio to GAPDH values.

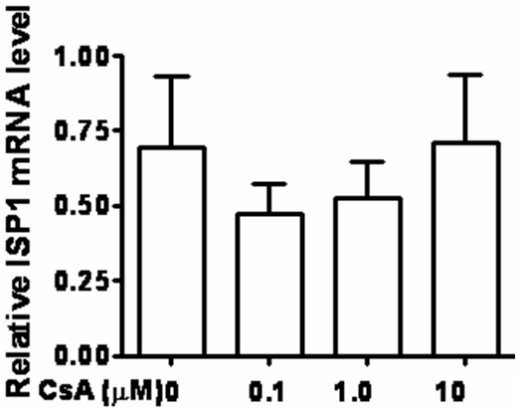

Figure 3. The effect of CsA on blastocyst hatching. The blastocysts on 4 dpc were cultured in the media containing different concentrations of CsA. The blastocysts on 6 dpc were harvested and the number of hatched blastocyst was counted as in **Table 3**. The total RNA was extracted to determine the mRNA level of ISP1 by using RT-PCR. The data were from 799 blastocysts.

Statistical analysis

All results are presented as mean±SEM. Data were analyzed by one-way ANOVA and Bonferroni posttest was used to determine statistical difference between experimental groups (PRISM software version 5.0, GraphPad, San Diego, CA). Proportion was expressed as %±SE, and Chi square tests were used. The difference was proposed to be significant when P<0.05.

Results

The effect of CsA on blastocyst formation, blastocyte cell number and cell apoptosis

We first observed the effect of CsA treatment on blastocyst formation from 2 dpc to 5 dpc. As shown in **Table 2** and **Figure 1**, CsA treatment could not change blastocyst formation rates (**Table 2**) and the cell number of blastocyst (**Figure 1A**) in concentration of 0.1 to 10 μM. Administration of CsA could not affect the apoptosis of blastocyst cells (**Figure 1B**).

The effect of CsA on the allocation and number of trophoblasts and ICM cells, and embryo hatching

Then we determined the allocation of trophoblasts and ICM cells, and embryo hatching. The results in **Figure 2** showed that there is no statistical difference in allocation of trophoblasts and ICM cells. The hatching rates (**Table 3**) and ISP1 (key enzyme for blastocyst hatching) mRNA level (**Figure 3**) were also unaffected by CsA treatment.

Table 4. The effect of CsA on blastocyst adhesion rate

CsA (μM)	Hatched blastocyte number	Adhesioned blastocyst number (7 dpc)	Blastocyst adhesion rate (%±SE)
0	79	60	76.0±4.8
0.1	97	89	91.8±2.8※
1.0	80	75	93.8±2.7※
10	104	87	83.7±3.6

The hatched embryos on 5 dpc were cultured on the laminin-precoated dishes in media containing different concentrations of CsA. On 7 dpc, the adhesioned blastocysts were counted. ※P<0.01, compared with control by using χ^2 test. Data were from 360 embryos.

The effect of CsA on blastocysts adhesion and stretching

The effects of CsA on blastocyst adhesion and stretching growth were investigated to evaluate implantation potential. Laminin (Sigma-Aldrich) was coated on the dish floors to imitate the ECM of endometrium. The 5 dpc hatched blastocysts were treated with G2 media containing different concentration of CsA. On 7 dpc, the adhesion rates of blastocysts were evaluated. The results in **Table 4** demonstrated that blastocyst adhesion rates in 0.1 and 1.0 μM concentration were higher than that of control (P<0.01).

The stretching ability reflects invasion potential of blastocyst [13]. The embryos adhered above were cultured further to 8 dpc, and their areas on the dish floor were measured. It was showed that treatment with 1.0 μM CsA significantly enhanced blastocyst stretching (P<0.05, **Figure 4**), suggesting CsA might promote invasiveness of blastocyst trophoblasts.

CsA upregulated mRNA and protein expression of itg αvβ3 and MMP-9

Itgαvβ3 is the ligand of laminin which play an important role in embryo adhesion in implantation. To further understand the effective mechanism of CsA on implantation, the mRNA of itgβ3 (subunit of itg αvβ3) and its protein expression on 7 dpc were analyzed. It was showed

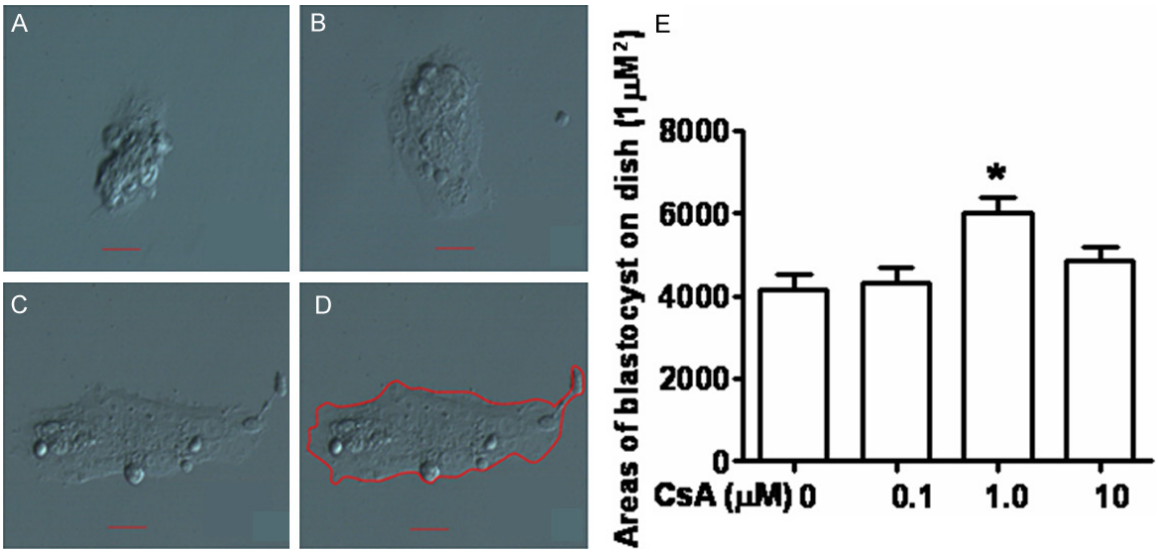

Figure 4. The effect of CsA on the stretching growth of peri-implantation embryo on the laminin-coated dishes. The hatched embryos on 5 dpc were cultured on the laminin-precoated dishes in media containing different concentrations of CsA. On 8 dpc, the stretching area was measured at the back of the dish bottom. The representative pictures are showed in A: poor stretched embryo, B: mild stretched embryo and C: well stretched embryo. D: Embryonic C was measured by a red line for the border area. (Scale: 50 μm). E was from 124 embryos. *P<0.05, compared with control (0 μM CsA).

that both itgβ3 mRNA transcription and protein expression were up-regulated by CsA (**Figure 5**).

The trophoblast cells secrete MMP-9 to break down ECM to make implantation possible. The mRNA and protein of MMP-9 were analyzed when blastocyst of 5 dpc grew in media containing CsA. The results revealed that CsA elevated MMP-9 mRNA levels and protein expression in blastocysts on 7 dpc (**Figure 6**).

Discussion

In 1999, Hajo H et al found that CsA treatment resulted in striking morphological alterations, including membrane ruffling and numerous pseudopodial protrusions, increased cell motility, anchorage-independent (invasive) growth, and cell proliferation of adenocarcinoma cells [14]. These changes were proved to be mediated by transforming growth factor-β (TGF-β) because they could be prevented with monoclonal antibodies directed at TGF-β. The similar changes were also found in other non-malignant cells such as mesangial cell of glomerular [15], gingival fibroblasts [16], and vascular smooth muscle cells [17]. It was also found that CsA treatment (at concentration of 0.1 to 1.0 μM) enhanced proliferation and prohibited apoptosis of trophoblast cells in first-trimester pregnancy in human in vitro [18, 19]. These imply that cyclosporine might change the developing of pre-implantation embryos. Here we demonstrated that the blastocyst formation rate, blastocyst cell number and apoptosis in pre-implantation embryos were not affected by CsA. CsA treatment could not change ICM and trophoblast allocation which was the first cell differentiation in life before embryo contacted to the epithelium of endometrium.

The endometrium is the physical tissue for embryo to implant. But embryo cannot implant when the endometrium has not the ability of reception. The short period that endometrium receives embryo is called "implantation window". And also, the embryo can only invade into endometrium in a special time in its development programme after appearance of syncytiotrophoblast, expression of adhesion molecule on cell surface, secretion of enzymes that essential for syncytiotrophoblast to degrade the ECM of endometrium [20]. Therefore, embryo must obey the settled developmental speed that is not easy to be affected by environment to make them to be synchronous at the time of implantation.

After ovulation, the endometrium changes to the secretory endometrium which is essential for it to gain reception ability with secreting of progesterone at luteal phase. The development of endometrium has an acute schedule. After

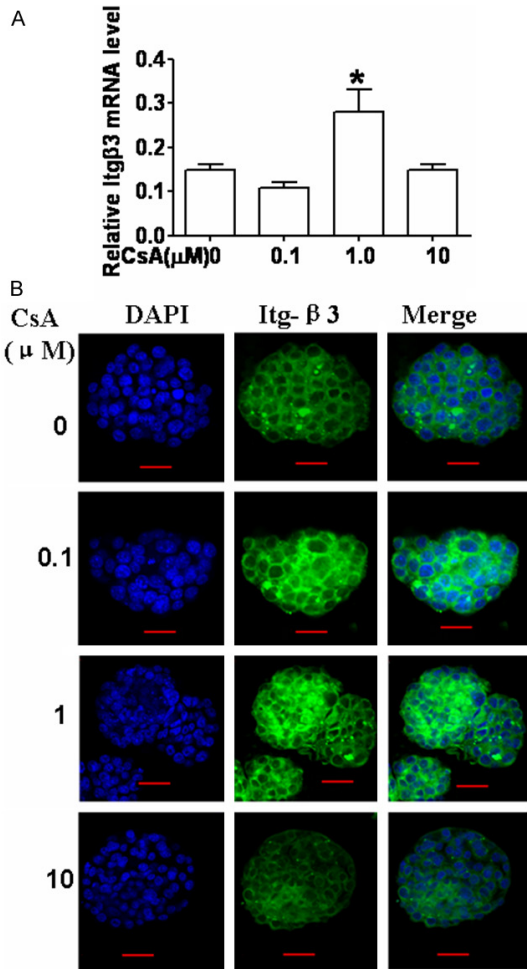

Figure 5. The regulation of CsA on itgβ3 expression of blastocysts. The hatched blastocysts on 5 dpc were cultured in media containing CsA on the laminin-coated dishes. On 7 dpc, itgβ3 mRNA level was determined by RT-PCR (A). The protein level of itgβ3 was detected by fluorescence microscopy (B). The rabbit-anti-mouse itgβ3 antibody and Alexa Fluor 488-congulated second antibody were used. Embryos were observed by confocal laser scanning microscope.

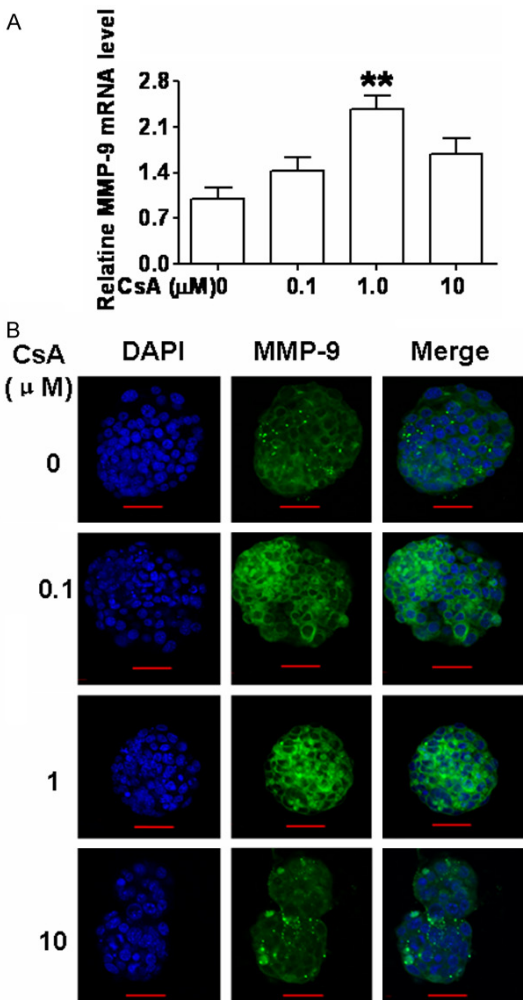

Figure 6. The regulation of CsA on MMP-9 expression of in blastocysts. The hatched blastocysts on 5 dpc were cultured in media containing CsA on the laminin-coated dishes. On 7 dpc, the adhesioned blastocysts were harvest, and MMP-9 mRNA was determined by RT-PCR (A), and MMP-9 protein level was determined by immunofluorescence (B). The rabbit-anti-mouse MMP-9 antibody and Alexa Fluor 488-congulated second antibody were used. Embryos were observed by confocal laser scanning microscope. **P<0.01 compared to the control.

"triggered" by progesterone, it would change in steady speed. The concentration of progesterone and estrogen can affect the development of endometrium [21]. Just the same as endometrium, the embryo has a settled developmental speed to make sure that when arrived in endometrium and meet "implantation window", it has hatched and derived syncytiotrophoblasts, express special adhesion molecules such as integrins [22] and secrete protease such as MMPs [4, 5]. It is understood that the developmental speed of pre-implantation embryo has already settled by mother though the plasma of oocyte in the time of oocyte maturation.

Although our previous studies have demonstrated that CsA could enhance growth and invasion while inhibit apoptosis of human trophoblast cells [18, 19], the present study showed that exposure to CsA did not affect the proliferation of blastocyst cells and developmental speed of pre-implantation embryo. In

fact, early embryo development rate has been set by the maternal oocyte cytoplasm. Thus external material such as CsA can not accelerate embryonic developmental speed, which is favorable to keep embryo-endometrium synch ronization.

Adhesion is one of important steps of embryo implantation during which embryonic trophoblast cells attach to the epithelium via interaction of adhesion molecules, itgαvβ3 and laminin expressed by both trophoblast and end ometrium [2, 3, 23, 24]. At the beginning of implantation, the embryo attached to uterine endometrium through the interaction of integrin and laminin via GRD sequence in laminin, leading to the adhering of embryo to endometrium.

Here, we observed that the adhesion rates of hatched embryos in 0.1 μM and 1.0 μM CsA were significantly increased compared to that of the control. Their stretching areas on laminin were also more extended in 1.0 μM CsA treatment. It was reported that the stretching growing state reflect the embryos invasiveness [25, 26], indicating that exposure to CsA improved the invasiveness of early embryos. In addition, we also found that administration of CsA upregulated the mRNA and protein levels of itgβ3 and MMP-9, the key factors in embryo implantation. All these data suggested that CsA at 0.1 and 1.0 μM could promote hatched embryo implantation.

It was reported that combination of itg αvβ3 and MMP-9 activated MMP-9 and plasminogen activator (PA), which plays targeting roles in embryo implantation [27]. We have reported that the enhancing effects of CsA on proliferation, migration, enzyme expression of human trophoblast cells were appeared in 0.1 μM, most effective in 1.0 μM, then decreased in 10 μM [7, 18, 19]. The same relationship of CsA effect-dosage was also showed in pre-implantation and peri-implantation embryo. We also observed that embryo stretching area and itgβ3 expression in 1.0 μM CsA (**Figures 4** and **5**) were enhanced; MMP-9 expression was upregulated in 1.0 and 10 μM CsA. All the data showed a trend that the effects of CsA on preimplantation and peri-implantation embryo began at 0.1 μM, reached the most effective at 1.0 μM, and decreased at 10 μM although significant difference were not observed. Combine the present result data with our previous reports, our studies showed that CsA could not only promote the implantation of pre-implantation and peri-implantation embryo but also the invasiveness growth of placental trophoblasts. These might be important in potential usage of CsA in reproductive medicine, especially in patient who failed to get pregnant after several good-quality embryo transformations in IVF-ET. This is supported by a report that administration of cyclosporin A to recipients improves the potential of mouse somatic cell nuclear-transferred oocytes to develop to fetuses [28]. In the future, we will focus on the safety and long-term consequences of CsA on the pregnancy.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Meirong Du or Dr. Dajin Li, Laboratory for Reproductive Immunology, Hospital and Institute of Obstetrics & Gynecology, Fudan University Shanghai Medical College, Shanghai 200011, China. Tel: +86 21 63457331; Fax: +86 21 63457331; E-mail: dmrlq1973@sina.cn (MR Du); djli@shmu.edu.cn (DJ Li)

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