

Islet transplantation reverses the effects of maternal diabetes on mouse oocytes

Pan-Pan Cheng¹, Jun-Jie Xia¹, Hai-Long Wang², Ji-Bing Chen², Fei-Yu Wang¹, Ye Zhang², Xin Huang¹, Quan-Jun Zhang³ and Zhong-Quan Qi¹

¹Organ Transplantation Institute of Xiamen University, ²Basic Medical Department of Medical College and ³The Yanwu Branch of Zhongshan Hospital, Xiamen University, Xiamen City, Fujian Province 361005, People's Republic of China

Correspondence should be addressed to Z-Q Qi; Email: oti@xmu.edu.cn

Abstract

Maternal diabetes adversely affects preimplantation embryo development and oocyte maturation. Thus, it is important to identify ways to eliminate the effects of maternal diabetes on preimplantation embryos and oocytes. The objectives of this study were to investigate whether islet transplantation could reverse the effects of diabetes on oocytes. Our results revealed that maternal diabetes induced decreased ovulation; increased the frequency of meiotic spindle defects, chromosome misalignment, and aneuploidy; increased the relative expression levels of *Mad2* and *Bub1*; and enhanced the sensitivity of oocytes to parthenogenetic activation. Islet transplantation prevented these detrimental effects. Therefore, we concluded that islet transplantation could reverse the effects of diabetes on oocytes, and that this technique may be useful to treat the fundamental reproductive problems of women with diabetes mellitus.

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Introduction

Autoimmune destruction of insulin-producing pancreatic β -cells is the main cause of type 1 diabetes mellitus. For many young women with the disease at puberty, treatment and education are often focused on long-term glycaemic control to prevent microvascular damage. The sequelae of microvascular damage, including retinopathy, renal insufficiency, and peripheral neuropathy, are significant. Of equal importance is the effect of hyperglycemia on the reproductive health of women, because the oocyte and the embryo are particularly vulnerable to both acute and chronic exposure to hyperglycemia.

Women with poorly controlled type 1 diabetes often suffer from a series of reproductive problems such as miscarriage, neonatal morbidity and mortality, and congenital malformations (Sadler *et al.* 1988, Becerra *et al.* 1990, Greene 1999). Maternal hyperglycemia adversely affects the progression from the one-cell stage to blastocyst in rodent models (Diamond *et al.* 1989, Lea *et al.* 1996, Moley *et al.* 1998). Emerging evidence has implicated that these effects are associated with compromised oocyte maturation (Colton *et al.* 2002, Doblado & Moley 2007). A decrease in the rate of germinal vesicle breakdown (GVBD; Diamond *et al.* 1989, Colton *et al.* 2002, Chang *et al.* 2005, Kim *et al.* 2007, Ratchford *et al.* 2007) and the number of ova at

metaphase II (MII; Colton *et al.* 2002), both of which are markers of oocyte nuclear maturation, were observed in superovulated oocytes obtained from diabetic mice; furthermore, mitochondrial dysfunction, spindle disorganization, chromosome misalignment, and aneuploidy were observed (Goud *et al.* 2006, Wang *et al.* 2009), indicating the abnormal cytoplasmic maturation of superovulated oocytes obtained from diabetic mice. Since maternal diabetes compromises oocyte maturation and affects the subsequent embryo development, it is necessary to explore the effective ways to reduce damage to oocytes caused by diabetes.

Insulin treatment is the conventional treatment modality for type 1 diabetes. Currently, the mainstays of patient care of women with type 1 diabetes who are seeking pregnancy involve close monitoring and glucose control through dietary modifications and insulin treatment (Gabbe & Graves 2003). Even though improvements in glycaemic control throughout pregnancy have drastically reduced the incidence of spontaneous abortions and congenital malformations in infants delivered by women with diabetes, the incidence of the aforementioned pregnancy complications has increased by three- to fivefold (El-Sayed & Lyell 2001, Baccetti *et al.* 2002). The ability of insulin to curtail delays in oocyte meiotic maturation caused by type 1 diabetic insults has been confirmed in studies

from several different laboratories (Colton *et al.* 2002, Kim *et al.* 2007). Rates of GVBD and ovulation were significantly lower in streptozotocin (STZ)-treated mice than those in control mice. The delay in oocyte meiotic maturation was improved with insulin treatment during superovulation (Colton *et al.* 2002). Kim *et al.* (2007) also observed that insulin reverses the diabetes effect on *in vitro* maturation effects of the chronic diabetes-prone BioBreeding rats. However, we found that the ovulation rate of mice with severe hypoglycemia was lower than that of controls after insulin treatment. In addition, insulin treatment did not prevent or reverse vasculopathy and complications of type I diabetes mellitus (Waxman *et al.* 1993). The quality of life of patients with type I diabetes was affected as well.

Islet transplantation is a promising procedure for the treatment of patients with type I diabetes (Shapiro *et al.* 2000, Sabek *et al.* 2009). This treatment improved overall glucose homeostasis and retard and also reversed the progression of complications in patients with type I diabetes (Fiorina *et al.* 2003a, 2003b, 2005, Venturini *et al.* 2006, Thompson *et al.* 2008). However, little is known about the effects of this treatment on compromised oocyte competence due to diabetes. We therefore hypothesized that islet transplantation can prevent the damages to oocytes induced by diabetes. The objective of this study was to test this hypothesis. Therefore, we assessed the number of ovulated oocytes, spindle morphology, karyotypic status, relative expression of *Mad2* and *Bub1*, and susceptibility of oocytes to activating stimuli, after islet transplantation. In order to avoid immunological rejection, we performed syngraft islet transplantation (islet transplantation for short).

Results

Effects of islet transplantation on weight and blood glucose

As shown in Fig. 1, we kept track of weight and blood glucose of insulin-treated and islet-transplanted mice closely. We observed that insulin-treated mice slightly increased in weight after insulin treatment, while islet-transplanted mice first went through weight loss because of the operation and then gained weight progressively (Fig. 1A). We detected blood glucose every day. As shown in Fig. 1B, insulin-treated mice sometimes went through hyperglycemic or hypoglycemic episodes 2 h after insulin treatment every day. Islet transplantation normalized and stabilized the blood glucose, maintaining the overall glucose homeostasis.

Effects of islet transplantation on ovulation

To determine whether islet transplantation affects the number of ovulated oocytes, we set up four groups: control, diabetic, insulin-treated, and islet-transplanted

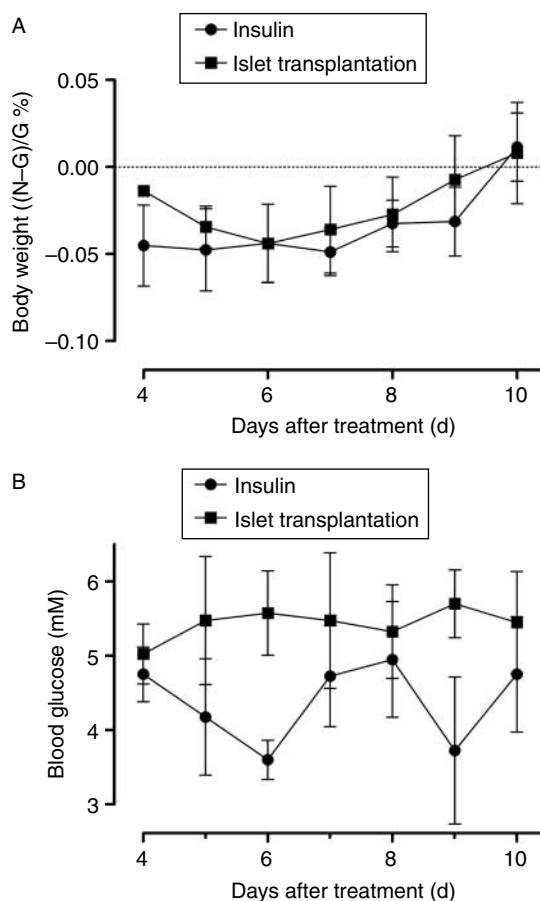


Figure 1 The weight and blood glucose of insulin-treated and islet-transplanted mice since 4 days after STZ injection. (A) The weight of insulin-treated and islet-transplanted mice, shown as body weight $((N-G)/G, \%)$. The letter N represented the weight that we detected every day, and the letter G represented the weight at day 0. (B) The blood glucose of insulin-treated and islet-transplanted mice.

groups. In all groups, the oocytes were retrieved from oviductal ampullae after superovulation with pregnant mare serum gonadotropin (PMSG) and human chorionic gonadotropin (hCG; Fig. 2). We obtained about one-half as many oocytes from diabetic mice as from control mice (Fig. 3). There was no dramatic difference between insulin-treated and diabetic mice. The number of oocytes obtained from islet-transplanted mice was similar to that obtained from control mice. Overall, these results suggest that islet transplantation reversed the effects of maternal diabetes on the number of ovulated oocytes, while insulin treatment could not.

Effects of islet transplantation on spindle morphology

To test for the possible effects of islet transplantation on the organization of the spindle and chromatin in MII oocytes, ovulated MII oocytes were immunolabeled with α -tubulin antibody to visualize the spindle and were co-stained with propidium iodide (PI) to visualize

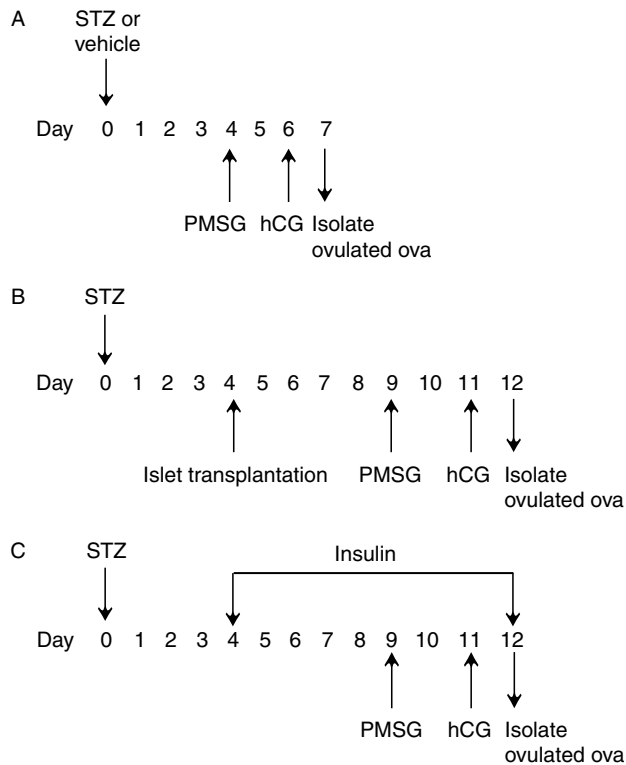


Figure 2 Schematic illustration of diabetes induction, islet transplantation, insulin treatment, and oocyte collection. (A) Diabetes induction. Female mice received an injection of 190 mg/kg streptozotocin (STZ) on day 0. Superovulation began at day 4. Mice were killed and cumulus–oocyte complexes (COCs) were collected 3 days later. (B) Islet transplantation. Four hundred islets were transplanted under the kidney capsule of diabetic B6 mice. Superovulation began at day 9. Mice were killed and COCs were collected 3 days later. (C) Insulin treatment. 1 IU insulin was injected s.c. into diabetic B6 mice for 7 successive days beginning 4 days after STZ administration. Superovulation began at day 7. Mice were killed and COCs were collected 3 days later.

chromosomes. By performing confocal scanning, we found that normal MII oocytes present a typical barrel-shaped spindle, and that chromosomes are well aligned on the metaphase plate (Fig. 4A). In abnormal MII oocytes, spindle defects and chromosome misalignment were readily observed (Fig. 4B). Moreover, a high frequency of spindle defects ($17.1 \pm 1.56\%$) and chromosome misalignment ($9.8 \pm 2.41\%$) was observed in ovulated MII oocytes from diabetic mice compared with the control MII oocytes (Table 1). Conversely, only 5.2 ± 1.17 and $4.5 \pm 2.09\%$ ova displayed disorganized spindle and chromosomes respectively in ovulated MII oocytes from islet-transplanted mice and did not differ from control mice. There was no dramatic difference between insulin-treated and islet-transplanted mice. These results indicate that both insulin treatment and islet transplantation could reverse the effects of maternal diabetes on the organization of spindles and chromatin in MII oocytes.

Effects of islet transplantation on aneuploidy

To further analyze the chromosome morphology of MII oocytes from islet-transplanted mice, we carried out chromosome spread experiments. Normal oocytes have a haploid complement of 20 chromosomes (Fig. 5A), but if the separation of homologous chromosomes or sister chromatids is incorrect during meiosis, then aneuploid oocytes are generated (Fig. 5B). Our analysis was restricted to hyperploidy, because it is less influenced by spreading conditions (Eichenlaub-Ritter & Boll 1989, Cukurcam *et al.* 2003). Our results revealed a significant increase in the incidence of hyperploidy in ovulated oocytes from diabetic mice compared with the ovulated controls (Table 1). The rate of hyperploidy is dramatically reduced in insulin-treated mice compared with diabetic mice, but still significantly higher than in islet-transplanted and control mice. Consistent with the earlier results, in this study, we found that islet transplantation reversed the effect of maternal diabetes on aneuploidy in ovulated oocytes, while insulin treatment had partly reduced the negative influence of diabetes.

Effects of islet transplantation on the relative expression of *Mad2* and *Bub1*

Real-time PCR analysis was used to determine the relative expression levels of *Mad2* and *Bub1* in ovulated oocytes from four groups. As shown in Fig. 6, the relative expression levels of *Mad2* and *Bub1* were significantly higher in diabetic mice than those in control mice, but there were no significant differences among the control, insulin-treated, and islet-transplanted groups. Overall, these results suggested that both insulin treatment and islet transplantation reversed the effects of maternal diabetes on the relative expression levels of *Mad2* and *Bub1*.

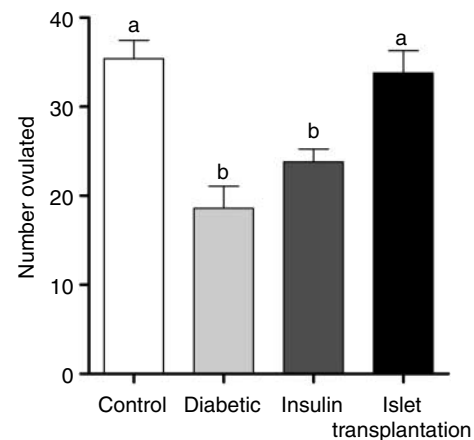


Figure 3 The number of ovulated oocytes in control, diabetic, insulin-treated, and islet-transplanted mice. Superovulated mice from four groups were killed 16 h after human chorionic gonadotropin (hCG) injection. Oocytes were then collected and counted. $N=10/\text{group}$; ^{a,b}values with a common superscript letter did not differ ($P>0.05$).

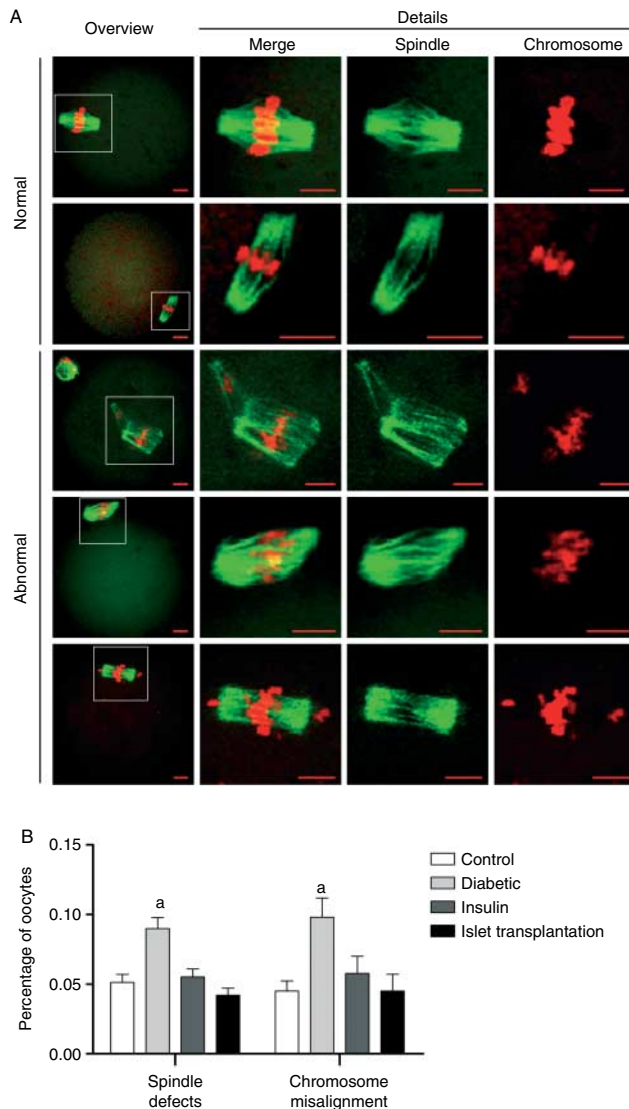


Figure 4 Spindle morphology of ovulated oocytes from control, diabetic, insulin-treated, and islet-transplanted mice. (A) Fluorescent photomicrographs depict normal and abnormal metaphase II (MII) oocytes. Green (Alexa Fluor 488) depicts the microtubules, and red (PI) depicts chromatin. Representative confocal sections are shown. (B) Quantification of ovulated MII oocytes with spindle defects and chromosome misalignment from control and diabetic mice. $N=5/\text{group}$; ^avalues with a common superscript letter represented a difference versus control ($P<0.05$). Scale bars: 10 μm .

Effects of islet transplantation on the susceptibility of oocytes to activating stimuli

To determine whether islet transplantation affected embryo development after parthenogenetic activation of ovulated oocytes, the oocytes collected from four groups were activated by SrCl_2 and subsequently cultured for embryo development. Our results showed that oocytes from the mice of these four groups developed into two-cell and four-cell embryos at a similar rate (Table 2). The blastocyst formation rates in

diabetic mice were significantly higher than those in control mice, while the rates in insulin-treated and islet-transplanted mice were similar to those for the corresponding controls.

Discussion

Mounting evidence has suggested that oocyte quality profoundly affects fertilization, early embryonic survival, establishment and maintenance of pregnancy, fetal development, and even adult disease (Krisher 2004, Sirard *et al.* 2006). Thus, investigation of ways to eliminate the effect of maternal diabetes on oocyte quality may lead to a fundamental solution for reproductive failure in diabetic females. As a treatment for diabetes, islet transplantation has been a hot topic for diabetes research fields (Shapiro *et al.* 2000, 2006). In this study, we investigate whether islet transplantation could reverse the effects of diabetes on oocytes.

Earlier work has shown that ovulation is compromised in diabetic animals (Kirchick *et al.* 1978, Garris *et al.* 1984, Powers *et al.* 1996, Colton *et al.* 2002), and we have confirmed this in the present study. Only approximately one-half as many ova were ovulated by diabetic mice compared with the control mice following a superovulation hormone regimen. However, the insulin-treated mice ovulated fewer compared with the control, while the islet-transplanted mice ovulated similarly to the control.

In order to further study on the effects of islet transplantation on diabetic oocytes, we examined other predictors of oocyte quality. Wang *et al.* (2009) observed an increased frequency of meiotic spindle defects, chromosome misalignment, and aneuploidy in oocytes from diabetic mice, which was confirmed by our laboratory results. In addition, our results revealed that insulin treatment and islet transplantation could normalize meiotic spindle and chromosome alignment, and hence affect the recovery of chromosome segregation.

In order to avoid aneuploidy, meiotic cells have developed a molecular mechanism called spindle checkpoint that underlies faithful chromosome separation in meiosis (Allshire 1997, Hwang *et al.* 1998, Musacchio & Hardwick 2002). As shown in previous research and our results, maternal diabetes increased the aneuploidy rate; therefore, it is possible that maternal diabetes affects the oocyte spindle checkpoint. In support of this hypothesis, the relative mRNA expression levels of *Mad2* and *Bub1* were remarkably higher in ovulated oocytes from diabetic mice when compared with the control mice. In addition, our results revealed that there was no remarkable difference in the relative mRNA expression levels of *Mad2* and *Bub1* among the insulin-treated, islet-transplanted, and control mice. While spindle assembly was affected and *Mad2* and *Bub1* were activated in some of the oocytes from diabetic mice, spindle assembly was normal and *Mad2*

Table 1 Islet transplantation reverses the effects of diabetes on the incidence of aneuploidy in ovulated oocytes.

Group	Number of mice	Number of cells	Aneuploidy
Control	10	47	2 (4.3%) ^a
Diabetic	12	40	7 (17.5%) ^b
Islet-transplanted	10	42	2 (4.8%) ^a
Insulin-treated	10	45	4 (8.9%) ^c

^{a,b,c}Values with a common superscript letter did not differ ($P > 0.05$).

and *Bub1* were not activated in oocytes from islet-transplanted mice.

It is worthwhile to note that *Goud et al. (2006)* pointed out that oocytes obtained from diabetic mice exhibited accelerated aging compared with those from non-diabetic mice, which involved altered spindle structure and premature loss of cortical granules as well as hardening of the zona pellucida. As one of the most prominent manifestations of aged oocytes is an increased susceptibility to activating stimuli (*Kubiak 1989, Lan et al. 2004*), we conducted an experiment to figure out whether oocytes from diabetic mice showed enhanced susceptibility to SrCl_2 . As expected, we identified that the rates of blastocyst formation in diabetic mice were significantly higher than those in the other groups, suggesting that diabetes enhanced the oocyte sensitivity to parthenogenetic activation. Conversely, the rates for blastocyst formation in islet-transplanted mice were similar to those in the control mice, indicating that islet transplantation normalizes oocyte sensitivity to parthenogenetic activation.

Our results showed that insulin treatment could partly cure but not completely reverse the effects of diabetes on oocytes, which were contradictory to earlier studies (*Colton et al. 2002, Kim et al. 2007*). We speculate that there were two reasons for this contradiction. First, the length of exposure of the insulin-treated mice to the diabetic environment in this study was longer than that in earlier studies. In previous experiments, insulin was injected subcutaneously into mice for three successive days beginning 2 days after STZ administration before mice became diabetic (*Colton et al. 2002*), while in this study, the insulin injections began 6 days after STZ injection and the mice maintained diabetic condition for about 3 days. Perhaps the longer the exposure to a diabetic environment, the more difficult it is for the insulin treatment to restore ovulation. Secondly, although under close monitoring and glucose control, insulin-treated mice in this study easily caused hypoglycemia. In other words, our insulin treatment was akin to a clinical setting where diabetic females suffer from abnormal glucose concentration fluctuations despite dietary modifications and insulin treatment. Since oocytes are susceptible to glucose concentration-dependent perturbations in nuclear and cytoplasmic maturation, which lead to poor embryonic development

post-fertilization (*Sutton-McDowall et al. 2010*), we assumed that abnormal glucose concentration fluctuations caused by insulin treatment could be partly responsible for decreased ovulation, as shown in *Fig. 1*.

In conclusion, our results strongly suggest that islet transplantation can reverse the effects of diabetes on oocytes by maintaining the overall glucose homeostasis. And one distinct benefit of islet cell transplantation was that it reduced the risk of oocyte defects being exacerbated by uncontrolled hypoglycemia. Thus, islet cell transplantation may have a specific role in patients who suffer from severe hypoglycemia with lack of warning on insulin therapy in humans. Remarkably, we performed syngraft islet transplantation to avoid immunological rejection. Unfortunately, this is not possible in diabetic humans who receive islet cell transplants and need anti-rejection therapies, as many

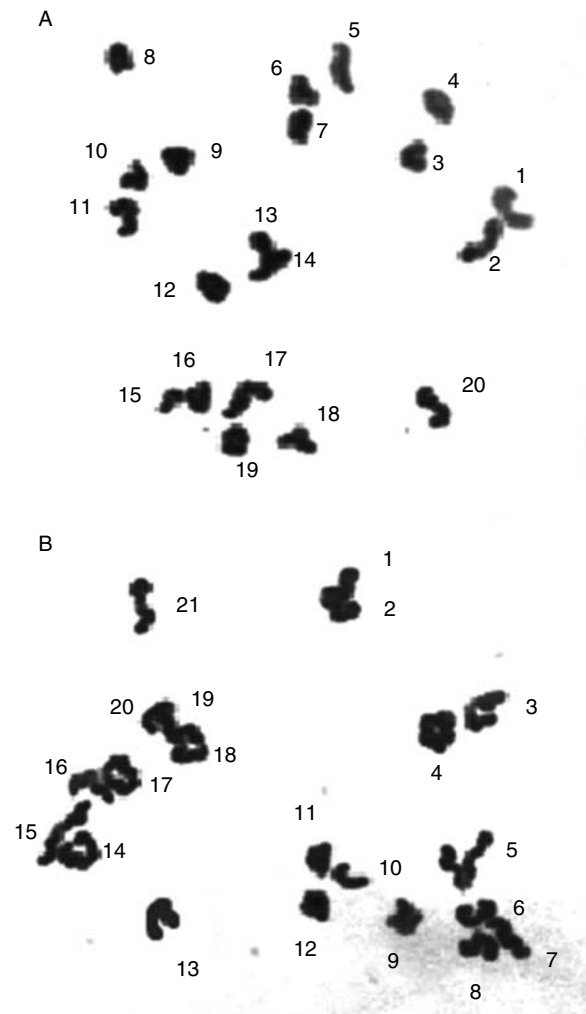


Figure 5 Chromosome spread of ovulated oocytes of control, diabetic insulin-treated, and islet-transplanted mice. Metaphase II (MII) oocytes were stained with PI and examined under a fluorescence microscope. Representative negative fluorescence micrographs: (A) oocytes with a normal haploid complement of 20 chromosomes and (B) oocytes with 21 chromosomes.

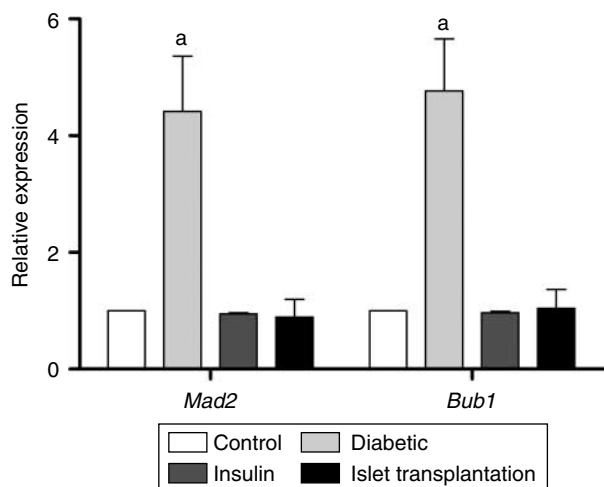


Figure 6 The relative mRNA expression levels of *Mad2* and *Bub1* in ovulated oocytes of control, diabetic, insulin-treated, and islet-transplanted mice, ^avalues with a common superscript letter represented a difference versus control ($P < 0.05$).

of these anti-rejection drugs are known to impair fertility and may cause oocyte defects. This is a major limitation to the extrapolation of these findings to the human situation. Although there are many difficulties with the practice of islet transplantation (Gaglia *et al.* 2005), with continued research into developing renewable islet sources and improved immunosuppression, this may become the most rational and practically feasible therapy for patients with type I diabetes.

Materials and Methods

Chemicals were purchased from Sigma Chemical Co., unless otherwise specified.

Animals

Female mice (C57BL/6), 6–8 weeks old, were maintained in a room with a 12 h light:12 h darkness cycle, with the darkness starting from 1900 h. The mice were obtained from Slac Laboratory Animal Co. Ltd. (Shanghai, China) and handled in accordance with the rules stipulated by the NIH publication 'Principles of Laboratory Animal Care'.

Superovulation and oocyte retrieval

To induce superovulation, C57BL/6 mice were given an i.p. treatment of 10 IU PMSG (Ningbo Sansheng Pharmaceutical Company, Ningbo, China) followed 48 h later by i.p. treatment of 10 IU hCG (Ningbo Sansheng Pharmaceutical Company). Superovulated mice were killed 16 h after hCG injection, and the oviductal ampullae were broken to release the cumulus–oocyte complexes (COCs). The COCs were denuded of cumulus cells by pipetting in M2 medium containing 0.1% (v/v) hyaluronidase, and the oocytes were counted.

Generation of diabetic mice and treatments

A 180–220 mg/kg dose of STZ in 0.1 M citrate buffer (pH 4.4) was injected i.p. to induce diabetes, as described previously (Wang *et al.* 2009). Control mice received an equal volume of the sodium citrate vehicle buffer (0.01 mM, pH 4.4). Blood glucose was measured using a One Touch glucose analyzer (FreeStyle, Abbott). Diabetes onset was defined as two consecutive blood glucose measurements > 16.7 mM. The scheme of superovulation is shown in Fig. 2A.

For the islet transplantation group, transplantation was performed 4 days after STZ administration. Islet transplant was considered functional following two consecutive blood glucose measurements < 8 mM. The time of islet rejection was defined as the first day of two consecutive blood glucose measurements > 11.1 mM. After a week, mice were superovulated and killed (Fig. 2B).

For the insulin treatment group, 1 IU insulin (Sanofi-Aventis Deutschland GmbH, Frankfurt, Germany) was injected s.c. every 24 h into diabetic B6 mice for 7 successive days beginning 6 days after STZ administration. Glucose levels were monitored 2 h after the insulin injection to ensure that blood glucose returned to control levels. The scheme of superovulation is shown in Fig. 2C.

Islet isolation, purification, and transplantation

The donors and recipients were C57BL/6 mice. Donor islets were isolated and transplanted by kidney subcapsular injection as described previously (Xia *et al.* 2010). Briefly, the pancreas was perfused via bile duct cannulation with 3 ml of 1.5 mg/ml collagenase P (Roche) and excised. The pancreas was incubated in a water bath at 37 °C, digested for 20 min, and was then shaken vigorously in cold Hank's balanced salt solution (HBSS). The suspension was allowed to sediment and was washed three times in HBSS. Islets were sorted manually under a microscope, collected in vials of 600 islets, centrifuged at 92 *g* for 2 min, and transplanted using a 1.2 mm diameter venflon (Becton Dickinson, Stockholm, Sweden) under the kidney capsule of diabetic mice.

Immunofluorescence

For spindle and chromosome analyses, oocytes were fixed with 4% (w/v) paraformaldehyde for 30 min and then permeabilized with 0.5% (v/v) Triton X-100 for 20 min. Followed by blocking in 1% (v/v) BSA-supplemented PBS for 1 h, samples were incubated

Table 2 Effect of islet transplantation on embryo development after parthenogenetic activation of ovulated oocytes.

Group	Percentage of two-cell (%)	Percentage of four-cell (%)	Percentage of blastocysts (%)
Normal	77.0 ± 3.8 ^a	66.3 ± 3.8 ^a	49.0 ± 12.2 ^a
Diabetic	81.3 ± 4.6 ^a	76.3 ± 4.4 ^a	58.3 ± 1.2 ^b
Islet-transplanted	77.0 ± 2.1 ^a	72.0 ± 2.0 ^a	47.0 ± 1.5 ^a
Insulin-treated	70.0 ± 2.0 ^a	61.7 ± 3.5 ^a	39.7 ± 2.5 ^a

$N = 6$ /group. ^{a,b}Values with a common letter in their superscripts did not differ ($P > 0.05$).

Table 3 The primer sequences used for qRT-PCR.

Primer name	Sequences
β -actin	Forward: 5'-CATCCGTAAAGACCTCTATGCCAAC-3' Reverse: 5'-ATGGAGCCACCGATCCACA-3'
<i>Mad2</i>	Forward: 5'-AGTTCAGAACTGGTGGTGGTCATC-3' Reverse: 5'-TTCCAACAGTGGCAGAAATGTCA-3'
<i>Bub1</i>	Forward: 5'-ATTGACCATGCGACAGTGAAG-3' Reverse: 5'-GTCATCCCATGGGTCTCAACAG-3'

with Alexa Fluor 488 anti-alpha tubulin (1:50; Ebioscience, San Diego, CA, USA) to visualize the spindle. Nuclear status was evaluated by staining with 10 mg/ml PI in PBS for 10 min. After extensive washing, samples were mounted on slides with anti-fluorescence fade medium (1,4-diazabicyclo-[2,2,2]-octane, DABCO). Finally, the samples were observed under a Zeiss confocal laser scanning microscope (FluoView FV1000, Olympus, Japan).

Chromosome spread

The oocytes were treated with 1% (w/v) sodium citrate for 20 min, individually transferred to a glass slide, and then fixed *in situ* with several drops of 3 parts methanol to 1 part acetic acid. After air-drying, the chromosomes were observed by fluorescence microscopy following staining with 10 mg/ml PI.

Oocyte activation and embryo culture

Oocytes were incubated in activating medium at 37 °C in a humidified atmosphere with 5% CO₂. The activating medium was Ca²⁺-free Chatot–Ziomek–Bavister (CZB) supplemented with 10 mM SrCl₂ and 5 µg/ml CB. The oocytes were incubated in activating medium for 6 h and then in CZB without SrCl₂ at 37 °C in a humidified atmosphere with 5% CO₂ in the air. Assessment of embryo development was performed at 24 h (two-cell stage), 48 h (four-cell stage), and 96 h (blastocysts) after the onset of activation.

Quantitative real-time PCR

Total RNA was isolated from oocytes using the RNeasy kit (Qiagen) according to the manufacturer's protocol. RT and quantitative real-time PCR (qRT-PCR) was performed using commercially available reagents (Toyobo, Osaka, Japan). The StepOne Real-Time PCR System (ABI, Carlsbad, CA, USA) was used to detect amplification, and β -actin was used as a normalizing control. The primer sequences used for the qRT-PCR are listed in Table 3.

Statistical analysis

At least four replicates were conducted for each treatment. Frequencies were arcsine transformed and analyzed with ANOVA, and a Duncan's multiple comparison test was used to locate differences. All analyses were performed using the GraphPad prism (GraphPad, Inc., La Jolla, CA, USA) software. Data are expressed as means \pm s.d., and $P < 0.05$ was considered significant.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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