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ORIGINAL ARTICLE

Maternal diabetes increases apoptosis in mice oocytes, not 2-cell embryos

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Abstract Apoptosis may be closely involved in diabetes-induced embryonic malformations. We aimed to investigate the occurrence of apoptosis at an early stage of development, in oocytes and 2-cell embryos of streptozotocin-induced diabetic mice and nondiabetic mice. Diabetic mouse ovarian sections stained with hematoxylin and eosin showed reduced number of growing follicles and delayed oocyte development. Annexin V-positive oocytes were higher in number in diabetic mice than in nondiabetic mice. Quantitative RT-PCR and immunofluorescence analysis revealed the expression of Bax and caspase-3 significantly higher in diabetic than nondiabetic oocytes. In contrast, 2-cell embryos of diabetic and nondiabetic mice showed no annexin V-positive staining. Bax expression was elevated in diabetic 2-cell embryos, but caspase-3 expression did not significantly differ between diabetic and nondiabetic 2-cell embryos. Electron microscopy revealed increased number of swollen mitochondria in diabetic 2-cell embryos. These results suggested that maternal diabetes might increase oocyte apoptosis by a Bax-caspase-3 pathway to play a role in embryonic malformations by delayed oocyte development. Development of 2-cell embryos might be adversely affected by maternal diabetes, but not through Bax-regulated caspase-3 apoptotic pathway.

Keywords Oocyte · 2-cell embryo · Diabetic embryopathy · Apoptosis · Bax · Caspase-3

Introduction

Maternal diabetes during pregnancy is associated with increased risk of growth disturbance and congenital malformation in offspring [1, 2]. In the clinic, the importance of monitoring and glycemic control for pregnant women with diabetes is undisputed, but the optimal strategy for the care of diabetes during pregnancy has yet to be established [3, 4]. Lowering glucose levels during early pregnancy in such women decreases the incidence of malformation and miscarriage but is still significantly higher than among women without diabetes [5, 6], which suggests that the insult of maternal hyperglycemia occurs before organogenesis, possibly during the pre-implantation period and oocyte maturation.

However, most studies of maternal diabetes have focused primarily on the embryo at the stages of blastocyst and the postimplantation embryo. They revealed that diabetes-induced increase in apoptosis occurs in blastocysts and early postimplantation embryos undergoing organogenesis, which might be responsible for diabetic embryopathy [7–9]. Although some studies have reported that maternal diabetes adversely affects oocyte and 2-cell embryo development [10, 11], the precise cell biological mechanisms causing delayed oocyte and 2-cell embryo development have not been clarified.

Apoptosis commonly occurs during various developmental processes in mammals. The Bcl-2 family is widely

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expressed during embryonic development [12]. The pro-apoptotic gene Bax may have a key role in the quality of oocytes and embryos [13]. Caspase-3 is a key pro-apoptotic protein of Bax downstream. The aim of this study was to examine the involvement of the Bax-regulated caspase-3 activation pathway in diabetes-induced inappropriate apoptosis in oocytes and 2-cell embryos.

Results

Effect of maternal diabetes on oocytes and 2-cell embryo development

Morphometric analysis of follicle numbers was performed by counting the number of primordial (Fig. 1a), primary (Fig. 1b), and preantral follicles (Fig. 1c), respectively, in 10 ovarian sections. The number of primordial follicles was not significantly different in diabetic mouse and nondiabetic mouse (Table 1, $P = 0.70$). However, the number of primary follicles was significantly reduced in the diabetic mouse compared with the nondiabetic mouse (Table 1, $P = 0.045$). Similarly, the number of preantral follicles also decreased significantly in the diabetic mouse (Table 1, $P = 0.036$). All developing follicles in diabetic ovarian sections were commonly smaller than those at the same stage in nondiabetic ovarian sections (Fig. 1d, e and Table 1, $P < 0.01$). Furthermore, we measured oocytes diameters excluding the zona pellucida under microscope. Control oocytes ($n = 100$) were 75% larger than 80 μm, while diabetic oocytes ($n = 100$) were 60% larger than 80 μm ($P < 0.05$).

After annexin-V staining, the denuded oocytes and 2-cell embryos were classified into the following three groups: (1) necrotic cells with propidium iodide (PI)-positive red nuclei (Fig. 2a1, a2), (2) viable cells negative for annexin-V staining (Fig. 2b1, b2), and (3) apoptotic cells with homogeneous annexin-V-positive signals in membrane (Fig. 2c1). Diabetic oocytes showed a higher number of annexin-V-positive oocytes than did nondiabetic oocytes (19.6 vs. 7.7%, $P < 0.001$), but 2-cell embryos from diabetic and nondiabetic mice showed no annexin V-positive staining.

Bax and caspase-3 mRNA expression in oocytes and 2-cell embryos

The mRNA expression of both Bax and caspase-3 was significantly higher in diabetic than nondiabetic oocytes (Fig. 3, $P = 0.043$, $P = 0.034$, respectively). Only Bax mRNA expression was significantly higher in 2-cell diabetic than nondiabetic embryos ($P = 0.047$), with no significant difference in the mRNA expression of caspase-3 in 2-cell embryos of the two groups ($P = 0.916$).

Immunofluorescence analysis of Bax and caspase-3 expression in oocytes and 2-cell embryos

In diabetic oocytes, Bax expression and caspase-3 expression were increased 2.6-fold and 1.8-fold than in nondiabetic mice, respectively (Figs. 4a and c, 5, $P < 0.001$ and $P < 0.005$, respectively). As well, Bax expression was higher in 2-cell diabetic embryos, by 26.8%, than in nondiabetic embryos (Fig. 4b, $P < 0.05$). In contrast,

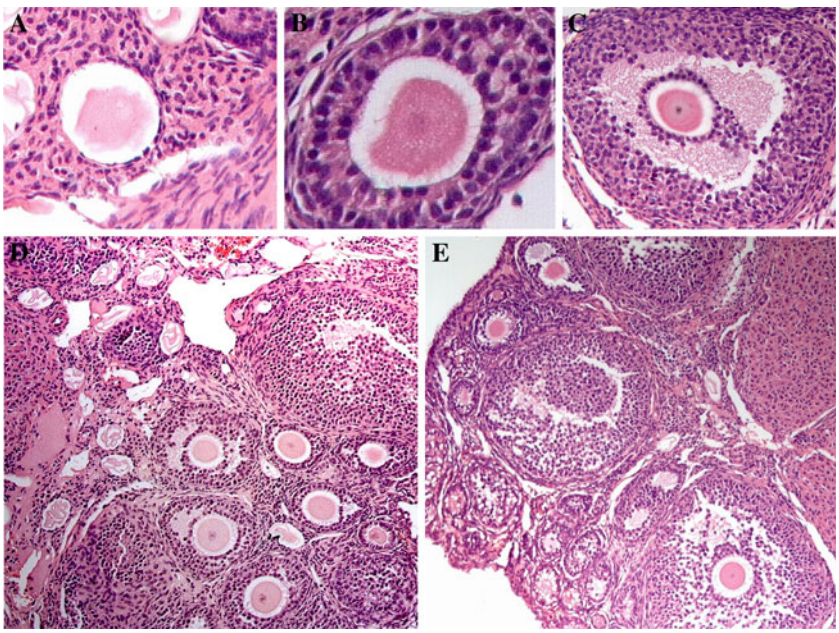

Fig. 1 Ovarian sections stained with hematoxylin and eosin from diabetic and nondiabetic female mice. Primordial follicles (**a**), primary follicles (**b**), preantral follicles (**c**), ovarian section from nondiabetic mouse (**d**), and diabetic (**e**) mouse. (**d**) displays a higher number of larger, more well-developed follicles than (**e**). Original magnificent: (**a**), (**b**), (**c**) ×800; (**d**), (**e**) ×400

Table 1 Mean ± SD number and diameters of primordial, primary, and preantral follicles in every 10th ovarian section

	No of primordial follicles	No of primary follicles*	No of preantral follicles*	Primary follicles diameters (μm)*	Preantral follicles diameters (μm)*
Nondiabetic	234.3 ± 40.1	154.3 ± 21.2	79.0 ± 10.4	85.0 ± 20.8	203.7 ± 93.0
Diabetic	221.3 ± 37.1	107.3 ± 18.5	53.3 ± 9.8	66.7 ± 20.3	152.0 ± 55.2

* $P<0.05$ between diabetic and nondiabetic mice

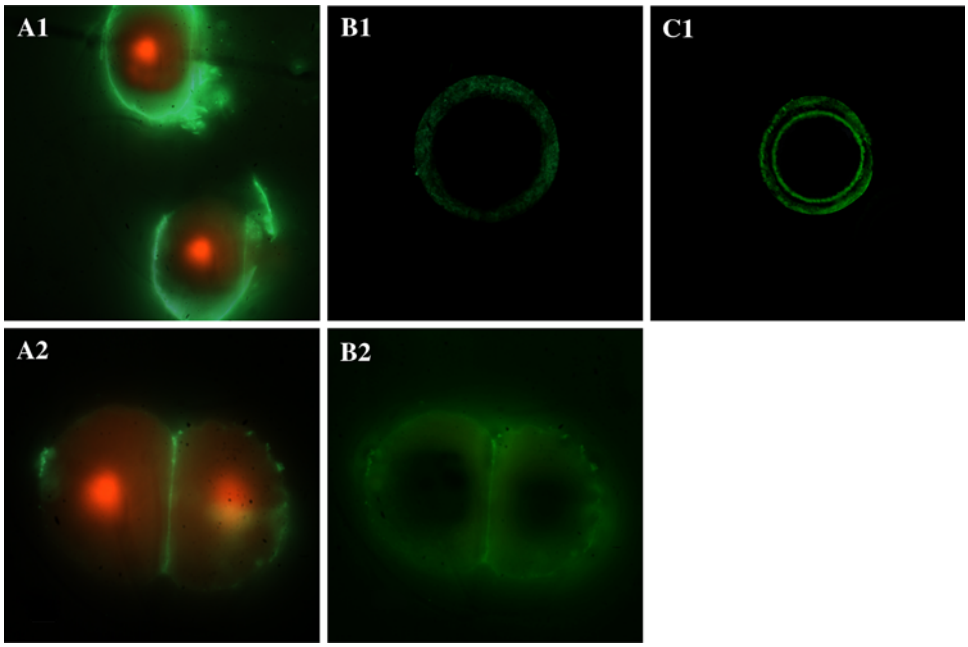

Fig. 2 Propidium iodide and annexin-V staining for apoptosis in oocytes and 2-cell embryos from diabetic and nondiabetic mice. **a1**, **a2** Oocyte and 2-cell embryo, respectively, stained with propidium iodide; **b1**, **b2** oocytes and 2-cell embryo, respectively, negative for annexin V; **c1** occyte stained with annexin V

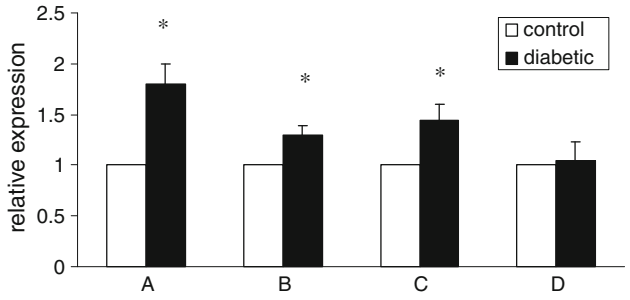

Fig. 3 Relative mRNA expression (mean ± SD) of Bax and caspase-3 in oocytes and 2-cell embryos from diabetic (*black bars*) and nondiabetic mice (*white bars*). Bax expression in **a** oocytes and **b** 2-cell embryos; caspase-3 expression in **c** oocytes and **d** 2-cell embryos. *Error bar* (*) indicates significant differences between two groups ($P < 0.05$)

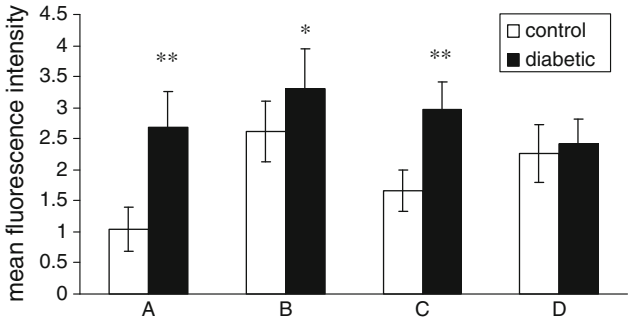

Fig. 4 Quantitative evaluation of mean ± SD fluorescence intensity of Bax and caspase-3 in diabetic (*black bars*) and nondiabetic (*white bars*) oocytes and 2-cell embryos. Bax expression in **a** oocytes and **b** 2-cell embryos; caspase-3 expression in **c** oocytes and **d** 2-cell embryos. * $P < 0.05$, ** $P < 0.005$

caspase-3 expression in 2-cell embryos did not significantly differ between diabetic and nondiabetic mice (Fig. 4d, $P = 0.207$).

The ultrastructural changes of 2-cell embryos

The cytoplasm of embryos was occupied primarily by vesicles and mitochondria (Fig. 6). As a sign of the immature status of cells, all mitochondria showed a hooded morphology and had few cristae. Lysosomes were also evident but were scarce. The proportion of swollen mitochondria was significantly higher in diabetic than nondiabetic 2-cell embryos ($P < 0.001$). However, no embryos showed a typical apoptotic marker, for example an apoptotic body.

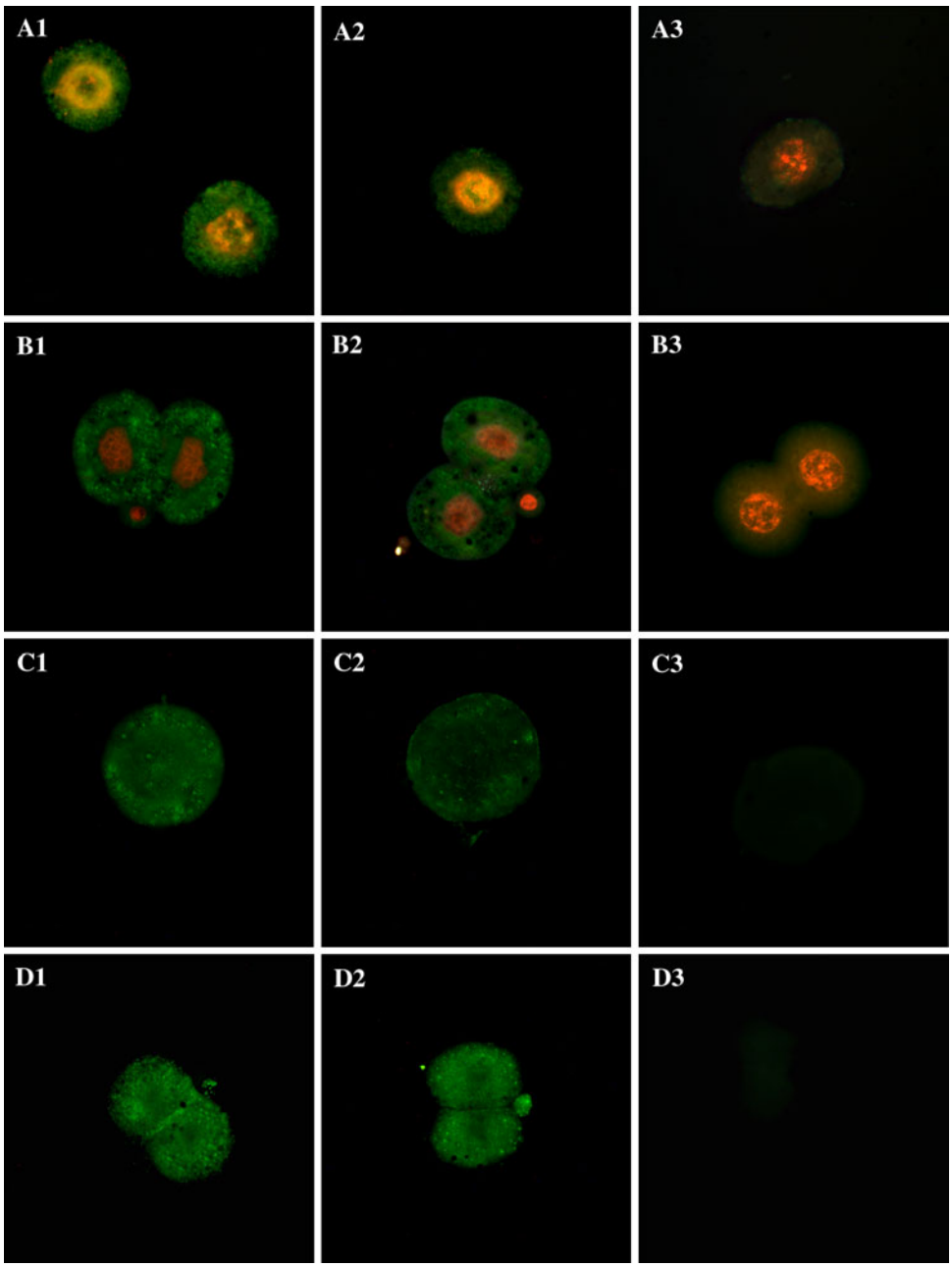

Fig. 5 Detection of BAX (**a–b**) and caspase-3 (**c–d**) in oocytes and 2-cell embryos from diabetic (**a1**, **b1**, **c1**, **d1**) and nondiabetic (**a2, b2**, **c2**, **d2**) mice by immunofluorescence. Picture **a3**, **b3**, **c3**, **d3** are negative controls. Results are representative of two experiments for each protein ($n = 30$ embryos for each experiment). Original magnification ×400

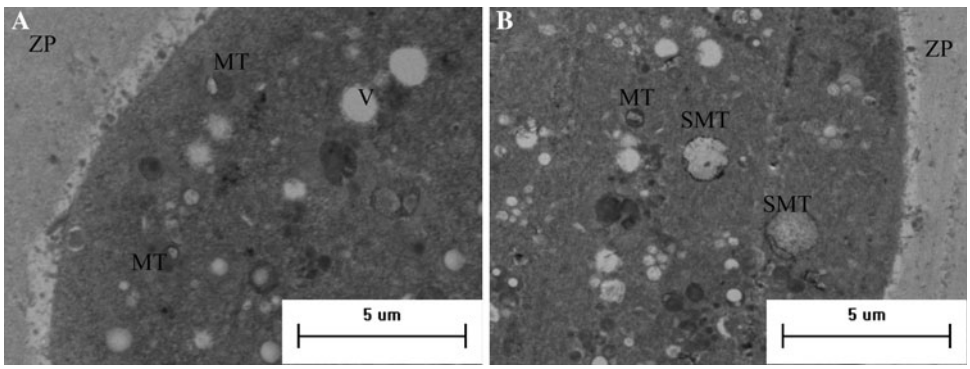

Fig. 6 Ultrastructure of 2-cell embryos from diabetic (**b**) and nondiabetic mouse (**a**) under transmission electron microscopy. *ZP* zona pellucida, *V* vesicle, *MT* mitochondrion, *SMT* swollen mitochondrion

Discussion

Apoptosis may be closely involved in diabetes-induced embryonic malformations. We aimed to investigate the occurrence of apoptosis at an early stage of development, in oocytes and 2-cell embryos of streptozotocin-induced diabetic mice and nondiabetic mice. In this study, diabetic mice showed a higher level of cell apoptosis and an increase of the pro-apoptotic markers Bax and caspase-3 in oocytes than did nondiabetic mice. Exposure of oocytes to a diabetic milieu would result in upregulated Bax to promote a pro-apoptotic state, increased activation of caspase-3 and, ultimately, cell death. Furthermore, hyperglycemia resulted in delayed follicular growth.

Maternal diabetes has a detrimental effect on oocyte maturation and development, as shown by the small sizes of oocytes and developing ovarian follicles and the reduced proportion reaching germinal vesicle breakdown [11]. Similarly, our hematoxylin and eosin (H&E)-stained ovarian sections showed reduced number of growing follicles and delayed oocyte maturation in diabetic mice. Growth retardation of embryos is characteristic of diabetic pregnancies [3, 11]. Lack of control of blood glucose well before the pregnancy diagnosis in women with diabetes mellitus can imply risk to oocytes and offspring.

Generally, apoptosis is considered beneficial during embryonic development and in adult life, but its dysregulation accompanies pathogenesis of many diseases. The effect of maternal diabetic on embryo development is related in part to the increased apoptosis in oocyte [11]. Three cell apoptotic pathways have been identified in oocytes according to their initiator caspase: the death receptor pathway involving TNF, caspase-8 [11, 14, 15], the endoplasmic reticulum stress pathway attributed to activation of caspase-12 [16], and the mitochondrial pathway, in which various signals can trigger the release of harmful proteins by mitochondria into the cytoplasm, leading to activation of caspase-9 and downstream cleavage of caspase-3, -7 or -6 [17, 18].

The mitochondrial pathway is widely involved in oocytes and embryos apoptosis. Bax, an important pro-apoptotic protein, is thought to be important to the quality of oocytes [13]. Microinjection of recombinant Bax into isolated oocytes induces apoptosis, indicating that elevation of cytoplasmic Bax levels triggers apoptosis in female germ cells [19]. However, the precise apoptosis-associated signaling pathway in glucose oocyte toxicity was previously unclear. Ratchford [20] and Colton et al. [21] found adenosine triphosphate (ATP) was decreased in oocytes from STZ-treated mice. It was speculated that the decreased ATP in oocyts may be associated with its increased apoptosis induced by maternal diabetes. In the present study, we assessed the mitochondrial pathway in hyperglycemia-induced apoptosis in oocytes and found the pathway activated during hyperglycemia-induced apoptosis in oocytes with overexpression of Bax and caspase-3. These results suggested that maternal diabetes might increase oocyte apoptosis by a Bax-caspase-3 pathway.

2-cell embryo is an earlier preimplantation stage. We also found that hyperglycemia in vivo modulated the expression of the apoptotic regulatory gene Bax as early as the 2-cell embryo stage in mice. In mammalian blastocysts, diabetic conditions increased the expression of the pro-apoptotic protein Bax [7, 9, 22], which indicates that Bax-mediated apoptosis has an important role in diabetic-induced apoptosis at the blastocyst stage. However, little information is available regarding diabetes-induced apoptosis in 2-cell embryos. The present research demonstrated increased Bax mRNA and protein level in 2-cell diabetic versus nondiabetic embryos. Unexpectedly, 2-cell diabetic and nondiabetic embryos did not differ in caspase-3 expression. Nor did we find annexin V-positive (apoptotic) cells in 2-cell embryos from diabetic mice. These results suggest that Bax-regulated caspase-3 apoptosis did not occur in 2-cell embryos from diabetic mice. However, electron microscopy study revealed an increased number of swollen mitochondria in diabetic 2-cell embryos.

Previous studies have found that maternal diabetes cause mitochondria swelling in rat postimplantation embryo [23]. Swollen mitochondrial matrix has been confirmed to be a result of changes in the mitochondrial permeability transition caused by the opening of the high-conductance permeability transition pore on the inner mitochondrial membrane and resulting in unselective solute fluxes [24]. In this condition, mitochondria can no longer maintain a proton gradient, aerobic ATP synthesis is arrested, a progressive unfolding of the inner mitochondrial cristae occurs, and several apoptosis initiator components are released. However, 2-cell embryo is an earlier stage and it has little metabolic activity until the 12- to 16-cell stage [25] when a shift from oxidative phosphorylation to glycolysis occurs [26]. Therefore, we speculate that up-regulate of Bax and Swellen mitochondria caused by hyperglycemia may not fundamentally impact the energy metabolism in 2-cell embryo.

In summary, we found delayed oocyte maturation and increased Bax-regulated caspase-3 apoptosis in oocytes of diabetic mouse. Oocyte apoptosis may have a role in diabetic embryopathy by delaying development.

Materials and methods

Experimental animals and collection of oocytes and 2-cell embryos

All mouse studies were approved by the Animal Studies Committee of Shantou University Medical College and

conformed to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health. Female immature Kunming mice (age 20–24 days) were used for our experiments. Female mice ($n = 50$) received a single injection of streptozotocin (190 mg/kg, dissolved in sodium citrate buffer, pH 4.4; Sigma Chemical, St. Louis, MO). Four days after injection, blood glucose levels in the tail vein were monitored by use of a glucose analyzer (Johnson, USA). Mice with glucose levels > 240 mg/dl were selected as diabetic animals ($n = 35$). Diabetic and age-matched controls ($n = 38$) received an injection of 10 IU of pregnant mare's serum gonadotropin, then 48 h later, an injection of 10 IU human chorionic gonadotrophin (HCG). Female mice that were not mated ($n = 16$) were killed by cervical dislocation 12 h after HCG administration to obtain oocytes, and the other female mice ($n = 19$) were killed by cervical dislocation 48 h after HCG administration and mated to obtain 2-celled embryos. The number of oocytes and 2-celled embryos collected from diabetic and nondiabetic mice was counted and compared. Oocytes and 2-cell embryos were isolated by puncturing the antral follicles with sterile needles, and then washed through M2 media. Before puncturing, uterine horns with ostia intact were dissected and placed in M2 media that had been equilibrated overnight at 37°C in an atmosphere of 5% CO2.

Annexin-V staining

Staining involved use of an Annexin-V kit according to the manufacturer's instructions (Keygen Biotech, Nanjing, China). Oocytes and 2-cell embryos were stained with annexin-V, a phospholipid-binding protein that detects the translocation of phospholipid phosphatidylserine from the inner to the outer cytoplasmic membrane, which is known to occur during apoptosis. Samples were also stained with PI to distinguish live cells from dead cells. Briefly, cells were washed twice in phosphate-buffered saline (PBS) and stained with 500 μl of binding buffer containing annexin-V-FITC plus PI for 15 min in the dark. Then, cells were washed in 100 μl of binding buffer. Samples were mounted on siliconized slides and observed on fluorescence microscope (Olympus, Japan).

Quantitative RT-PCR

Fluorescence real-time quantitative RT-PCR was used to analyze the mRNA expression of Bax and caspase-3 in diabetic and nondiabetic oocytes and 2-cell embryos. Approximately 100 cells were used for each reaction.

RNA was prepared using Cells-to-cDNA II kit (Ambion, USA) following the instruction manual. After complete removal of medium 100 μl of ice-cold Cell Lysis II Buffer was directly added to the cell. The cells were immediately scraped and pipetted quickly to a cold 1.5 ml microfuge tube in ice. The lysates were processed according to instructions until just before the reverse transcription step, i.e., 75°C for 10 min, DNase I digestion at 37°C for 15 min and 75°C for 5 min. The RNA preparations were stored at −20°C for a short period. Reverse transcription was also performed using the kit according to the instruction with random decamers provided and 5 μl of cell lysate (RNA). Reverse transcription reaction conditions was 30 min at 42°C then 10 min at 95°C. Resultant cDNA was stored at −20°C.

Real-time RT-PCR was performed in a 20 μl reaction buffer containing 10 μl of SYBR® Premix Ex TaqTM (TaKaRa, Inc. Dalian, China) (2×), 0.8 μl of 200 nM of each forward and reverse primer (Table 2), 2 μl cDNA and 7.2 μl H_2O. Thermal cycling conditions were 4 min at 94°C, 35 cycles of 20 s at 94°C, 30 s at 60°C and 30 s at 72°C. Experiments involved use of an ABI 7700 Real Time PCR Instrument (Ambion, USA). β-Actin was used as an endogenous normalizer. Each sample was run in triplicate. Data are expressed as the mean of the relative expression of Bax and caspase-3.

Immunofluorescence labeling

Oocytes and embryos were permeabilized in Tyrode's solution to eliminate zona pellucida and then immediately transferred to PBS. They were fixed in 2% paraformaldehyde for 15 min and then permeabilized in 0.1% Triton X-100 for 15 min. Oocytes and embryos were then blocked for 1 h with normal goat serum in PBS to inhibit nonspecific binding of antibody, then incubated overnight at 4°C in primary antibody (rabbit anti-mouse Bax polyclonal antibody or rabbit anti-mouse caspase-3 polyclonal antibody [Boster, China] or in PBS only [control]). After being washed thrice in PBS, the oocytes and embryos were incubated with secondary antibody (goat anti-rabbit polyclonal IgG-FITC [Boster, China]), for a 1-h incubation at 37°C in the dark. After being washed thrice in PBS, embryos were incubated with PI at 0.01 mg/ml (we omitted

Table 2 Primers designed for amplification of Bax and caspase-3

Gene	Forward (F) and reverse (R) primer sequences	Product size (bp)
Bax	5′-GGATGCGTCCACCAAGAAG (F)	194
	5′-CAAAGTAGAAGAGGGCAACCAC (R)	
Caspase-3	5′-CGATCTGGTACAGACGTG (F)	359
	5′-GCCATGTCATCCTCA (R)	
β-actin	5′-GAGACCTTCAACACCCCAGC (F)	263
	5′-ATGTCACGCACGATTTCCC (R)	

this step in detecting caspase-3 so as to detect Bax). Oocytes and embryos were mounted on slides with the DABCO antifade solution. The embryos were immediately examined using a fluorescence microscope (Olympus, Japan). The mean immunofluorescence intensity was measured by use of Image-Pro Plus 6.0.

H&E-stained ovarian sections

In order to determine the effect of maternal diabetes on oocyte growth, ovary tissues were stained with H&E. We used five diabetic and five nondiabetic mice. After mice were killed by decapitation, isolated ovaries were quickly fixed in 10% formaldehyde for l–1.5 h. Ovaries were dehydrated in a graded ethanol series and cleared with xylene. Ovary tissue was paraffin-embedded and serially sectioned at 4 μm. Sections were deparaffinized in xylene and hydrated by use of an ethanol series (100, 90, 80, 70, and 50%). Then sections were stained with H&E. After being cleared with fresh xylene, sections were mounted with balsam for observation and viewed on light microscopy (Olympus, Japan). Follicle diameters in H&E-stained ovarian sections were measured by the use of IPP (Image-Pro Plus, Media Cybernetics, USA) and compared between diabetic and nondiabetic ovarian sections.

Transmission electron microscopy

2-cell embryos from diabetic or nondiabetic mice were fixed in 2.5% glutaraldehyde, then postfixed in 1% osmic acid for 1 h. Because of the small number of embryos, embryos were embedded in 3% agar for later study. Embryos were dehydrated in a graded ethanol series (50, 70, 80, 95, and 100%) and embedded in epoxy resin. Ultrathin sections were cut with use of a diamond knife and poststained with 1% uranyl acetate in 30% ethanol and then Reynolds lead citrate. Ultrastructure of embryos were examined on transmission electron microscopy (JEOL JEM-1400, Japan).

Statistical analysis

Results were analyzed by Student's *t*-test or chi-square analysis and expressed as means ± S.D. A P-value < 0.05 was considered statistically significant. Statistical analysis involved use of SPSS v13.0 for Windows (SPSS Inc., Chicago, IL).

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