RESEARCH ARTICLE

Resveratrol prevents embryonic oxidative stress and apoptosis associated with diabetic embryopathy and improves glucose and lipid profile of diabetic dam

Chandra K. Singh[1], Ambrish Kumar[1], David B. Hitchcock[2], Daping Fan[3], Richard Goodwin[3], Holly A. LaVoie[3], Prakash Nagarkatti[1], Donald J. DiPette[3] and Ugra S. Singh[1]

[1] Department of Pathology, Microbiology and Immunology, School of Medicine, University of South Carolina, Columbia, SC, USA
[2] Department of Statistics, University of South Carolina, Columbia, SC, USA
[3] Department of Cell Biology and Anatomy, School of Medicine, University of South Carolina, Columbia, SC, USA

Received: September 17, 2010
Revised: November 26, 2010
Accepted: November 30, 2010

Scope: Diabetic embryopathy, a consequence of diabetic pregnancy, is associated with increase in embryonic oxidative stress and apoptosis, which lead to severe embryonic damage at early stage of organogenesis.

Methods and results: This study investigated if resveratrol, found in red grapes and blueberries, may prevent diabetes-induced oxidative stress and apoptosis in embryos and have beneficial effects in diabetic dams. A rodent model of diabetic embryopathy was used. Diabetes was associated with lowered reduced glutathione levels (26.98%), increased total thiol (100.47%) and lipid peroxidation (124.73%) in embryos, and increased blood sugar (384.03%), cholesterol (98.39%) and triglyceride (1025.35%) in diabetic dams. Increased apoptosis (272.20%) was also observed in the embryos of diabetic dams. Administration of resveratrol (100 mg/kg body weight (b.w.)) during pregnancy prevented both oxidative stress and apoptosis in embryos. Resveratrol reduced embryonic maldevelopment by improving embryo weight (41.23%), crown rump length (16.50%) and somite number (11.22%). It further improved the glucose (33.32%) and lipid (cholesterol 41.74%, triglyceride 60.64%) profile of the diabetic dams, which also represents the protective role of resveratrol in diabetes.

Conclusion: Resveratrol was found to prevent embryonic oxidative stress and apoptosis. It also improved glucose and lipid profile of diabetic dams, indicating the beneficial effects in diabetic pregnancy.

Keywords:
Apoptosis / Diabetic embryopathy / Oxidative Stress / Resveratrol

1 Introduction

Foetal development during gestation is a complex process mainly influenced by maternal environment. Epidemiological studies in humans and experiments in rodent embryos have shown that there is an increased risk of foetal malformations, spontaneous abortions and developmental delay in diabetic pregnancies [1, 2]. They also show a direct correlation between the degree of maternal hyperglycaemia and the incidence and severity of foetal abnormalities during the first trimester [2, 3]. The incidences of developmental defects in foetuses due to diabetes are shown to vary from 4.2 to 13.4% compared with ~1% in the general population [4–6].

Maternal diabetes-induced malformations have been detected in all major organ systems, but the central nervous and the cardiovascular systems are the most susceptible [7]. The diabetic condition induces teratogenic effects in the developing embryo and causes diabetic embryopathy [8]. Increased level of oxidative stress followed by apoptosis has shown to be associated with diabetic embryopathy [8, 9].

Correspondence: Dr. Ugra S. Singh, Department of Pathology, Microbiology and Immunology, 6311 Garners Ferry Road, Columbia, SC 29209, USA
E-mail: ugra.singh@uscmed.sc.edu
Fax: +1-803-733-1515

Abbreviations: **b.w.,** body weight; **C,** control (non-diabetic); **CR,** control treated with resveratrol; **D,** diabetic; **DR,** diabetic treated with resveratrol; **FPLC,** fast protein liquid chromatography; **GSH,** reduced glutathione; **HNE,** 4-hydroxy-2-nonenal; **LPO,** lipid peroxidation; **RSV,** resveratrol (3,5,4′-trihydroxy-trans-stilbene); **SOD,** superoxide dismutase; **STZ,** streptozotocin

The most commonly used animal models for studies of diabetic embryopathy are streptozotocin (STZ)-induced diabetes in rats and mice [10, 11]. Studies have shown that embryonic phenotypes obtained from such animal models resemble the malformations seen in human infants [12].

Even though the incidence of congenital malformations in diabetic pregnancies has been reduced by intensive insulin treatment and glucose monitoring, it is still two- to six-fold higher than those in normal pregnancies [3, 13]. Many studies have shown that ~50% of congenital malformations can be prevented by folic acid supplementation before and during pregnancy [14]. However, complete protection by any drug or supplement remains elusive. These findings indicate that additional agents are required to prevent embryonic malformations in the diabetic embryopathy.

Resveratrol (RSV) is a phytoalexin found in grapes and berries, with high levels in red grapes. Recent studies suggest that RSV, by activating SIRT1, safely mimics the effects of dietary restriction in laboratory animals [15]. RSV also improves insulin sensitivity, lowers plasma glucose and increases mitochondrial capacity in diet-induced obese mice [16]. In obese Zucker rats, a genetic model of type 2 diabetes, RSV increases the expression of glucose transporter GLUT-4 [17]. RSV crosses the blood–brain barrier and protects the brain from traumatic brain injury [18]. Whether RSV prevents oxidative stress and apoptosis associated with embryonic maldevelopment in diabetic pregnancy is not known. Using a rodent model of diabetic embryopathy, we examined the effects of RSV on diabetes-induced embryonic oxidative stress and apoptosis. We analysed the role of RSV in improving the embryonic oxidative stress and foetal outcome as well as glucose and lipid profiles of the diabetic dam.

2 Materials and methods

2.1 Experimental animal model

The present study was performed using a protocol approved by University of South Carolina Institutional Animal Care and Use Committee. Adult, age-matched (60 days old) female Sprague–Dawley rats (Charles River, Wilmington, MA, USA) of average weight 200–230 g were placed with proven breeder male Sprague–Dawley rats for breeding just before the end of the daily light cycle. The following morning, each female was examined for the sperms in vaginal smears. The day sperms were first observed was defined as the 0 day of gestation, or embryonic day 0 (E0).

2.2 Induction of diabetes

On day E1, STZ (Sigma, St. Louis, MO, USA; 65 mg/kg b.w. dissolved in 100 mM citrate buffer, pH 4.5) was administered intraperitoneally (i.p.) to induce diabetes [10]. After 48 h (E3) of STZ administration, blood was drawn from the rats by tail pricking, and glucose levels were measured using TRUE2go glucose-monitoring system (HOMEdiagnostics, FL, USA). Rats with blood glucose concentrations >250 mg/dL were considered to be diabetic.

2.3 Experimental plan

Four groups of pregnant rats (12 rats for each group) were used to complete this study; non-diabetic rats (C, control), control treated with RSV (CR), diabetic (D), and diabetic treated with RSV (DR). RSV (Sigma; 100 mg/kg b.w. dissolved in water) was administered by gavage feeding for 10 days (from day E3 to E12). Every second day, at the same time, b.w. and glucose concentration were measured. On gestation day 12, the pregnant rats were anaesthetised using isoflurone (1 mL in bell jar apparatus) for 2 min and then sacrificed. Embryos were collected and studied to assess embryonic apoptosis and oxidative stress. Three embryos from each pregnancy were used for immunohistochemistry and TUNEL assays and the remaining embryos (~9) for oxidative stress, Western blot analysis and to score developmental delay analysis.

2.4 Apoptosis detection

Embryo sections from cranial neural tube region were processed for the detection of nuclear DNA fragmentation associated with apoptosis using the DeadEnd^{TM} Fluorometric TUNEL System assay kit (Promega, WI, USA). Labelled DNA ends with FITC-dUTP were counterstained with DAPI to stain nuclear DNA and examined under Nikon E-600 fluorescence microscope (Nikon Instruments, CA, USA).

2.5 Protein analysis by Western blot

Total embryonic tissues were homogenised in 1× RIPA buffer containing 1 mM PMSF and Western blot was performed as noted previously [19]. Primary antibodies used were caspase-9, caspase-7, cleaved caspase-3 and β-actin (Cell Signalling, MA, USA), caspase-8 and superoxide dismutase (SOD)-2 (Santa Cruz Biotechnology, CA, USA). The secondary antibodies used were goat anti-rabbit IgG-horseradish peroxidase and goat anti-mouse IgG-horseradish peroxidase from Santa Cruz Biotechnology. The blot was stripped and re-probed with β-actin to check for loading differences.

2.6 Immunohistochemical staining

Immunohistochemistry was performed as described previously [19] with embryonic cranial neural tube sections

representing midbrain region. Primary antibodies (cleaved caspase-3, Cell Signalling; 4-hydroxy-2-nonenal (HNE), Abcam, MA, USA) were detected with secondary antibodies conjugated to FITC or Rhodamine (Santa Cruz Biotechnology; Invitrogen, CA, USA). Dual immunostaining was performed for simultaneous localisation of cleaved caspase-3 with TUNEL apoptotic nuclei and HNE adduct formation. Briefly, paraffin-embedded sections were dewaxed and hydrated in DEPC-treated solutions before microwaving in 100 mM Tris-HCl buffer (pH 10.0) containing 5% urea for 25 min. Slides were incubated in ice-cold permeabilisation solution (0.1% Triton X-100 in 0.1% sodium citrate solution), and non-specific proteins blocked with 5% rat serum (Sigma) in PBS for 2 h at room temperature. Sections were incubated with anti-active caspase-3 (Cell Signaling) for overnight at 4°C. TUNEL solution (Promega) of enzyme and label (rTdT incubation buffer) was applied to sections for 1 h at 37°C. Finally, sections were incubated with Rhodamine-labelled rat anti-rabbit IgG (Invitrogen) for 2 h, followed by DAPI staining. For dual staining of HNE adduct formation with cleaved caspase-3, sections were simultaneously incubated with both primary antibodies followed by detection with secondary antibodies (Rhodamine and FITC labelled). Labelled sections were mounted in Vectashield mounting media (Vector Laboratories, USA) and visualised with Nikon E-600 fluorescence microscope.

2.7 Oxidative stress and antioxidative analysis

Total embryonic tissues were homogenised in buffer containing 100 mM Tris-HCl (pH 7.4), 1 mM EDTA, 0.05% Triton X-100, and 0.5 mM PMSF, sonicated (Branson Sonifier 450; duty cycle 50 and time 10 s), and centrifuged. The supernatant was immediately used to measure the total thiol, lipid peroxidation (LPO), reduced glutathione (GSH), and SOD activity. Total thiol in the embryonic tissue homogenates was estimated using 5,5′-dithiobis-(2-nitrobenzoic acid) reagent [20]. Formation of thiobarbituric acid reactive substance as a product of the LPO was estimated [21]. For GSH quantification, the GSH-Glo^{TM} Glutathione Assay kit (Promega) was used. SOD activity was determined using a kit from Sigma. All the spectrophotometric readings were taken in Spectramax spectrophotometer (Molecular Devices, CA, USA).

2.8 Serum analysis

After the animals were sacrificed, blood samples were collected and serum was isolated without disturbing the blood clot. This serum sample was used to measure insulin, cholesterol, and triglycerides. Serum insulin was measured using an ultra-sensitive rat insulin ELISA kit (Crystal Chem, IL, USA). Serum cholesterol and triglyceride concentrations were measured by colorimetric methods using a kit from Raichem (Cliniqa, CA, USA). Triglycerides and total cholesterol profiling were assayed by fast protein liquid chromatography (FPLC, AKTA purifier, GE Healthcare, USA) with a flow rate of 0.5 mL/min.

2.9 Analysis of embryonic developmental delay

On gestation day 12, embryos were collected and studied under a stereomicroscope to score embryonic developmental delay with regard to altered size (crown rump length) and somite number as described previously [22, 23]. Embryo weight was also measured to assess the outcome of diabetic pregnancy.

2.10 Data analysis

Comparisons of means across the treatments (C, CR, D, and DR) were made for all the data sets using the ANOVA *F*-test (except for the data set of GSH, which required a Kruskal–Wallis test). For several of the data sets (cholesterol, triglycerides, serum insulin, LPO, apoptotic nuclei, and glucose), a natural logarithm transformation was employed. For the glucose data, measurements were made at each of the five time points, so a two-factor ANOVA model was used with treatment as one factor and time as the other. Since five comparisons were made simultaneously, a Bonferroni correction was used, ensuring an experiment-wise error rate of at most 0.05.

3 Results

3.1 RSV reduces diabetes-induced embryonic apoptosis

Apoptosis commonly occurs during a variety of developmental processes in mammals [24]. It has been reported that diabetes-induced apoptosis in embryos during development may be one of the mechanisms leading to embryo malformation [25]. The TUNEL assay was performed to detect apoptosis in the cranial neural tube region. DNA fragmentation, as evidenced by fluorescein-12-dUTP incorporation (green fluorescence), indicative of the occurrence of apoptosis, was seen in all the embryo samples but in the case of the diabetic group it was increased almost 3.5-fold (Fig. 1A). Representative sections were from midbrain region (Fig. 1B). Apoptotic nuclei were counted in the cranial neural tube region and mean data of five embryos of different pregnancy were plotted (Fig. 1C). RSV treatment prevented excess apoptosis in the embryo of diabetic dams. The pattern of apoptosis in embryos of controls treated with RSV was unchanged.

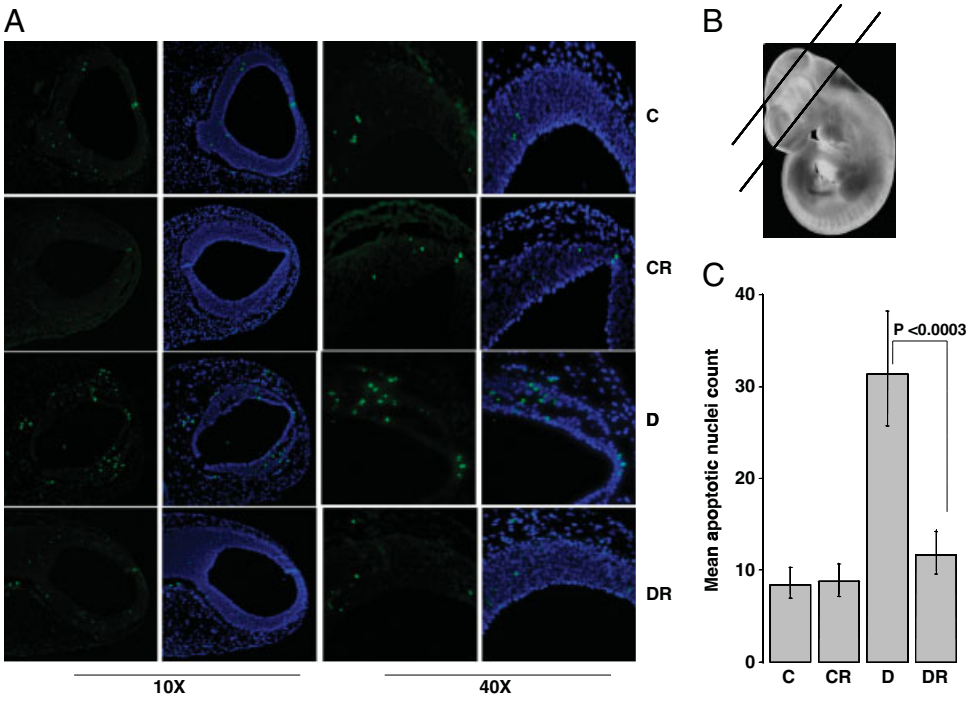

Figure 1. Resveratrol reduces apoptosis in E12 embryos of diabetic dams. TUNEL staining was performed to determine the effect of RSV on diabetes-induced apoptosis in the neural tubes. Immunofluorescent photomicrographs were taken at a magnification of 10 × (which covers the complete cranial neural tube region) and 40 × (which focuses on small region of cranial neural tube) (A). At both magnifications, each field was imaged with FITC alone and FITC+DAPI to localise the apoptotic nuclei. The representative sections are from midbrain region and their approximate location is indicated in (B). For apoptotic nuclei, three sections *per* embryo were counted from five rats of different pregnancy and mean data were plotted (C). Approximately 3.5 times higher numbers of apoptotic nuclei were found in embryos of diabetic dams; however, a significantly reduced number of apoptotic nuclei were found in embryos of diabetic dams treated with RSV, almost equivalent to the control. The number of apoptotic nuclei of control and control treated with RSV remained unchanged. Error bars represent ±2 SEs for each estimated mean.

3.2 RSV inhibits diabetes-induced activation of caspases

After confirmation of apoptosis by TUNEL assay in embryonic neural tube region, we analysed the expression of caspases responsible for activation and execution of apoptotic processes. Activated forms of caspase-8 and caspase-9 were increased in the embryos of diabetic dams, which shows that both apoptotic pathways (intrinsic and extrinsic) are involved [19, 26]. There was an increased expression of cleaved caspase-3, which might be involved in the execution of apoptotic processes [25]. RSV treatment significantly suppressed the activation of all three caspases in embryos of diabetic dams (Fig. 2A). Caspase-7 expression was unchanged in all the groups.

Cleaved caspase-3 expression was further analysed in cranial neural tube region by immunohistochemistry. Diabetes significantly increased the level of cleaved caspase-3 in the embryonic neural tube region. However, RSV treatment prevented the increase and restored it to the levels similar to control (Fig. 2B). RSV alone had no effect as evident from control treated with RSV. Further, dual immunostaining was performed to co-localise the cleaved caspase-3 with TUNEL apoptotic nuclei. Cleaved caspase-3 was localised in the cytoplasm, whereas the nucleus contained little or no staining for this enzyme. TUNEL assay demonstrated DNA strand breaks in many apoptotic cells of the neural tube and cleaved caspase-3 appeared in most of the cells. These findings suggest that activation of caspase-3 is an early event of apoptosis before DNA fragmentation in the embryos of diabetic dams (Fig. 2C). There were few cells which stained positive for either cleaved caspase-3 or TUNEL. Dual immunostaining with normalised levels of cleaved caspase-3 and TUNEL-positive cells further confirmed the protective effect of RSV in the embryos of diabetic dams.

3.3 RSV decreases embryonic oxidative stress and improves antioxidant status

Oxidative stress is suggested to be the main cause of diabetes-induced apoptosis leading to malformation in the embryos and its severity depends on foetal antioxidative capacity. We, therefore, assessed the oxidative stress and antioxidant level in whole embryos by evaluating LPO, total thiol levels, reduced GSH levels, SOD levels and activity, and HNE presence.

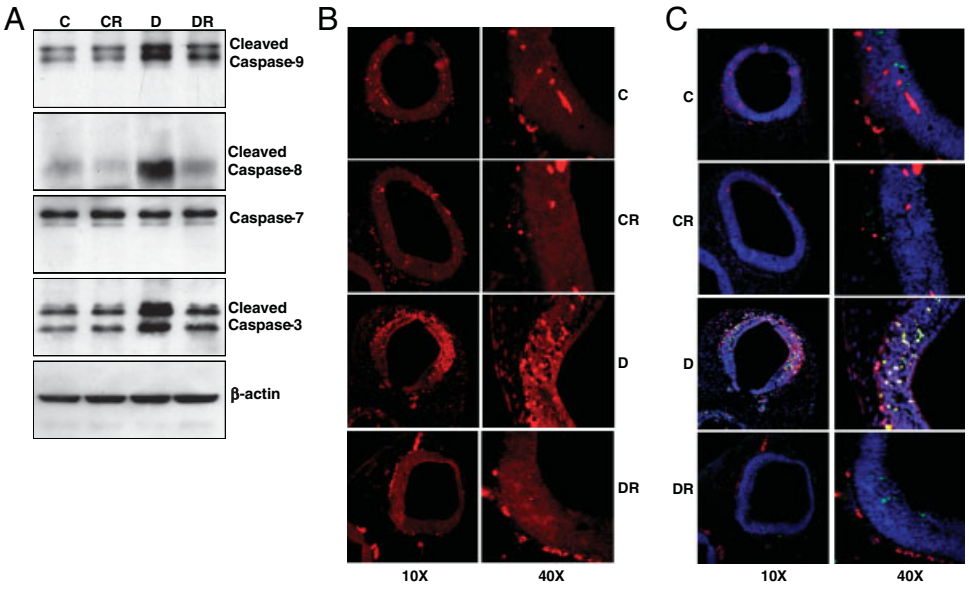

Figure 2. Western blot analysis to assess the activation of caspases. Total embryonic protein was prepared using 1 × RIPA lysis buffer and immunoblotted with caspase-9, caspase-8, caspase-7, and cleaved caspase-3 antibodies and finally reprobed and blotted with β-actin antibody to determine the loading differences (A). Diabetes increases the active forms of caspase-9 and caspase-8 as well as increases the level of cleaved caspase-3 for execution of apoptotic processes. RSV treatment significantly suppresses the activation of caspases in embryos of diabetic dams. Caspase-7 expression remained unchanged. Cleaved caspase-3 expression in cranial neural tube region was further confirmed by immunohistochemistry (B). It was detected with Rhodamine-labelled secondary antibody (immunofluorescent photomicrographs at 10 × and 40 ×, respectively). Further dual immunostaining was performed to compare the activated caspase-3 with TUNEL-positive cells and counterstained with DAPI to visualise the nuclear DNA (C). Both were co-localised in most of the cells of neural tube and neural crest. RSV significantly reduced the level of cleaved caspase-3. RSV alone had no effect as evident from control treated with RSV. Red-stained cells represent activated caspase-3, green colour represents TUNEL-positive cells, and yellow colour represents cells positive for both cleaved caspase-3 and TUNEL.

3.3.1 Lipid peroxidation

Malondialdehyde formed as a result of LPO in embryos of diabetic dams was found two-fold higher in comparison to the control and RSV-treated control groups (Fig. 3A). RSV treatment normalised the LPO level in embryos of diabetic dams.

3.3.2 Total Thiol

Embryos of diabetic dams were also found with elevated levels of total thiol, but RSV treatment significantly inhibited this effect (Fig. 3B).

3.3.3 Reduced GSH

GSH, a non-protein thiol, is an antioxidant found in eukaryotic cells and plays a role in the detoxification of H_2O_2 and LPO. Therefore, a change in GSH levels in the embryos of diabetic group may also indicate the antioxidant status. There was a reduced level of GSH in the embryos of diabetic group. However, after RSV treatment, an improved level of GSH was found, almost equivalent to the control (Fig. 3C). A slight numerical increase in GSH level was also found in the embryos of control dams treated with RSV but it was not statistically significant.

3.3.4 SOD expression and activity

SOD catalyses the oxido-reduction of reactive oxygen species. SOD-2 expression was determined by Western blot and no changes were observed in any of the treatment or control groups (Fig. 3D). SOD activity was also measured and found unchanged in all the groups (data not shown), indicating that SOD expression and its activity remain unaffected under the diabetic conditions and even after RSV treatment.

3.3.5 Immunohistochemistry of HNE

It is well documented that oxidative stress induces the formation of electrophilic aldehydes (4-hydroxynoneal and 4-oxo-2-nonenal) as by-products of peroxidation of lipid membranes [27]. HNE exerts various biological effects in various cell types, such as alterations in cell proliferation, cell-cycle procession, and apoptosis [27, 28]. To assess and localise the pattern of oxidative stress in the

embryos, immunolocalisation of HNE was performed. In embryos of diabetic dams, the whole cranial neural tube region stained positive implying increased oxidative stress status (Fig. 3E). Less intense staining of HNE was found in the embryos of diabetic dams treated with RSV, control treated with RSV, and in the controls signifying normal oxidative stress status during pregnancy. Further dual immunostaining was performed to analyse the pattern of HNE adduct formation with activation of caspase-3. Most of the neural tubes and neural crest cells were positively immunostained, indicating high susceptibility toward oxidative stress. Cleaved caspase-3 (or activated caspase-3) was also co-localised with HNE in most of these cells (Fig. 3F). RSV treatment normalised the level of HNE adduct formation as well as caspase-3 activation.

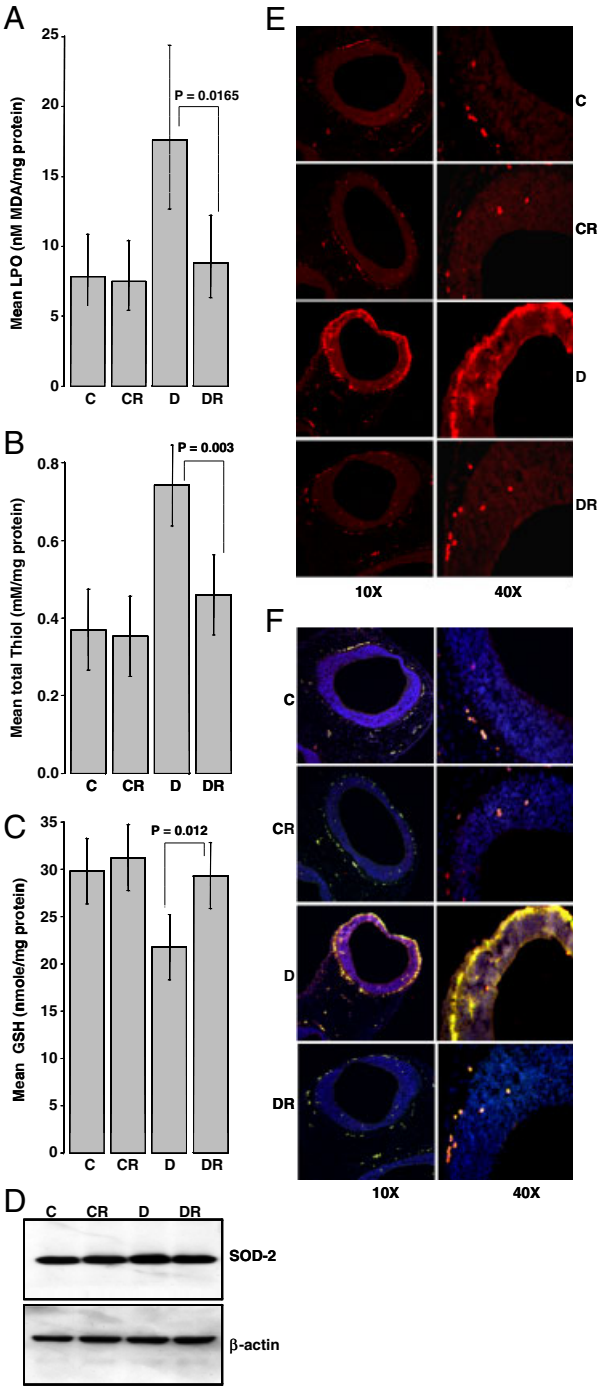

Figure 3. Diabetes-induced oxidative stress analysis and anti-oxidative effect of resveratrol. Total embryonic proteins were extracted and assayed for lipid peroxidation (LPO), total thiol, and reduced glutathione (GSH). To assess LPO, thiobarbituric acid reactive substance formation in tissue homogenates was evaluated by the amount of malondialdehyde (MDA) formed (A). Total thiol and reduced GSH levels in tissue homogenates were assessed with 5,5′-dithiobis-(2-nitrobenzoic acid) reagent (B and C). Superoxide dismutase expression was analysed by Western blot but no change in the expression was observed (D). RSV significantly suppressed LPO and total thiol level in the embryo of diabetic dams. GSH level was decreased in the embryos of diabetic dam but normalised after RSV treatment. All the parameters in the control treated with RSV group were unchanged. Error bars represent ±2 SEs for each mean. Further, consequences of oxidative stress were analysed by immuno-histochemistry for 4-hydroxy-2-nonenal (HNE) adduct formation (E), and HNE co-localisation pattern was also assessed in reference to cleaved caspase-3 by dual immunostaining and counterstained with DAPI (F). RSV significantly suppressed HNE adduct formation in embryos of diabetic dams. Red colour represents cells with HNE adduct formation, green colour represents activated caspase-3, and yellow colour represents cells for both HNE adduct and cleaved caspase-3.

3.4 RSV improves blood glucose status in diabetic dams during the gestation period

Simultaneously, the health status of diabetic dams was also evaluated in terms of blood glucose, insulin status, and lipid accumulation. Initial non-fasting blood glucose of all rats was ~85 mg/dL. After STZ administration, in the case of diabetic dams a continuous increase in blood glucose was observed and at gestation day E12, it was 410.31 ± 20.71 mg/dL; whereas in the case of diabetic dams treated with RSV, a continuous decrease in the blood glucose was monitored from the day E7 onward and at E12 it was 280.92 ± 22.52 mg/dL (Fig. 4A). The blood glucose level of controls treated with RSV remained normal (82.86 ± 3.07 mg/dL) similar to the untreated controls (83.27 ± 1.24 mg/dL). Diabetes severely affected the body weight of pregnant rats as observed after 2 days of STZ injection (~10% decrease) and it remained low in comparison to control and control treated with RSV group. RSV treatment did not affect the body weights of either the control or diabetic dams (data not shown). Insulin plays a major role in glucose homeostasis. We performed ELISA for insulin in the serum of different groups and found lower levels of insulin in the diabetic group compared with control groups. However, a statistically significant increase in the

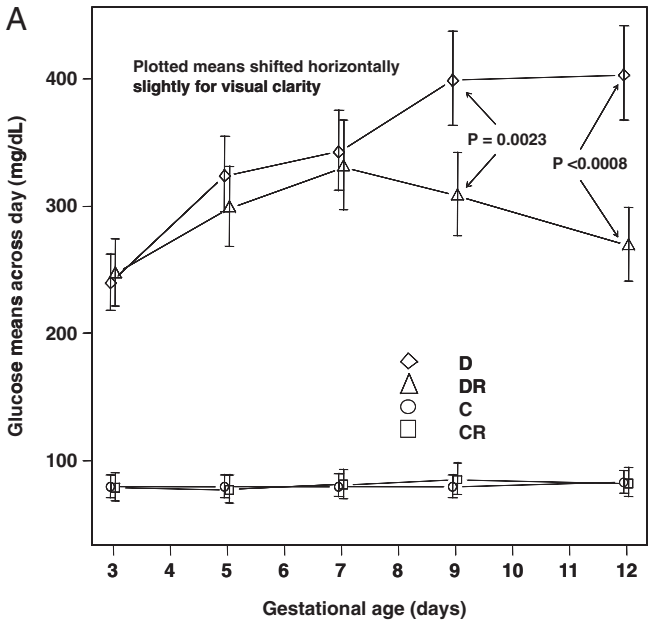

Figure 4. Resveratrol lowers blood glucose and increases insulin level in the diabetic dam. Blood glucose of diabetic rat during gestation was significantly lowered by RSV treatment (A). The effect was more pronounced from day 7 onward. RSV significantly increased insulin level in diabetic dams (B), although it was much lower compared with control groups. Error bars represent ± 2 SEs for each estimated mean.

insulin level was found in RSV-treated diabetic group [29], although it was much lower in comparison to control groups (Fig. 4B).

3.5 RSV improves serum lipid profile in the diabetic dam

Diabetes affects lipid metabolism [30]. Storage of blood samples at 4°C revealed accumulation of lipid droplets in the serum of diabetic rats. However, a significant decrease in the accumulation of lipid droplets was found in the RSV-treated diabetic group (data not shown). To address this observation, we measured cholesterol and triglycerides levels in the serum [31]. Diabetes increased serum cholesterol of diabetic dams and RSV treatment significantly normalised it (Fig. 5A). Similarly, serum triglyceride levels were very high in diabetic dams and RSV significantly reduced them (Fig. 5B). The cholesterol and triglyceride levels of RSV-treated controls remained unaffected. The lipoprotein profile of serum for cholesterol and triglyceride was also analysed by FPLC. It was found that RSV significantly reduced the content of very low-density lipoprotein (VLDL) in diabetic dams. Interestingly, RSV increased high-density lipoprotein (HDL) in controls with slight increase in VLDL (Fig. 5C and D).

3.6 RSV improves outcome of diabetic pregnancy by preventing embryonic development delay

From the above results, it was confirmed that RSV prevents embryonic oxidative stress and apoptosis and concurrently improves glucose homeostasis as well as lipid profile. To determine the effects of RSV on embryonic development, 12-day-old embryos were analysed to score the improvement in developmental delay. To define developmental delay, three different parameters including weight of embryo, crown rump length, and somite number were analysed [2, 23]. All embryos were morphologically analysed under a stereomicroscope to measure the size of embryo (crown rump length) and count the somite number. Embryos of diabetic dams were found with reduced weight, crown rump length, and somite number; however, RSV treatment showed a significant improvement in all three parameters (Fig. 6A–C). RSV alone did not affect the development of embryos as evident from embryo weight, crown rump length, and somite number data of control-treated RSV.

The present study was completed with 12-day-old embryos of four groups of pregnant rats; each represents data of 12 rats. Four diabetic dams in which complete embryo resorption was found (blood sugar >500 mg/dL) were excluded from the experiments and equal number of diabetic dams with viable embryos were included. Individual embryo resorption, generally 1–2 embryos/litter, was noticed equally in all the groups. The number of embryos *per* pregnancy in all the groups was in the range of 9–15. Diabetic condition, or RSV treatment, did not affect the number of embryos *per* pregnancy. The result was quantitatively analysed in reference to diabetic (D) *versus* diabetic treated with RSV (DR) and in each case the DR group mean was significantly greater (or less depending on the direction of interest) than the diabetic mean. We compared the mean $\log_e$ glucose for diabetic and diabetic treated with RSV, separately at all the five time points. The diabetic-treated RSV mean was significantly lower than the diabetic mean at gestation days 9 and 12.

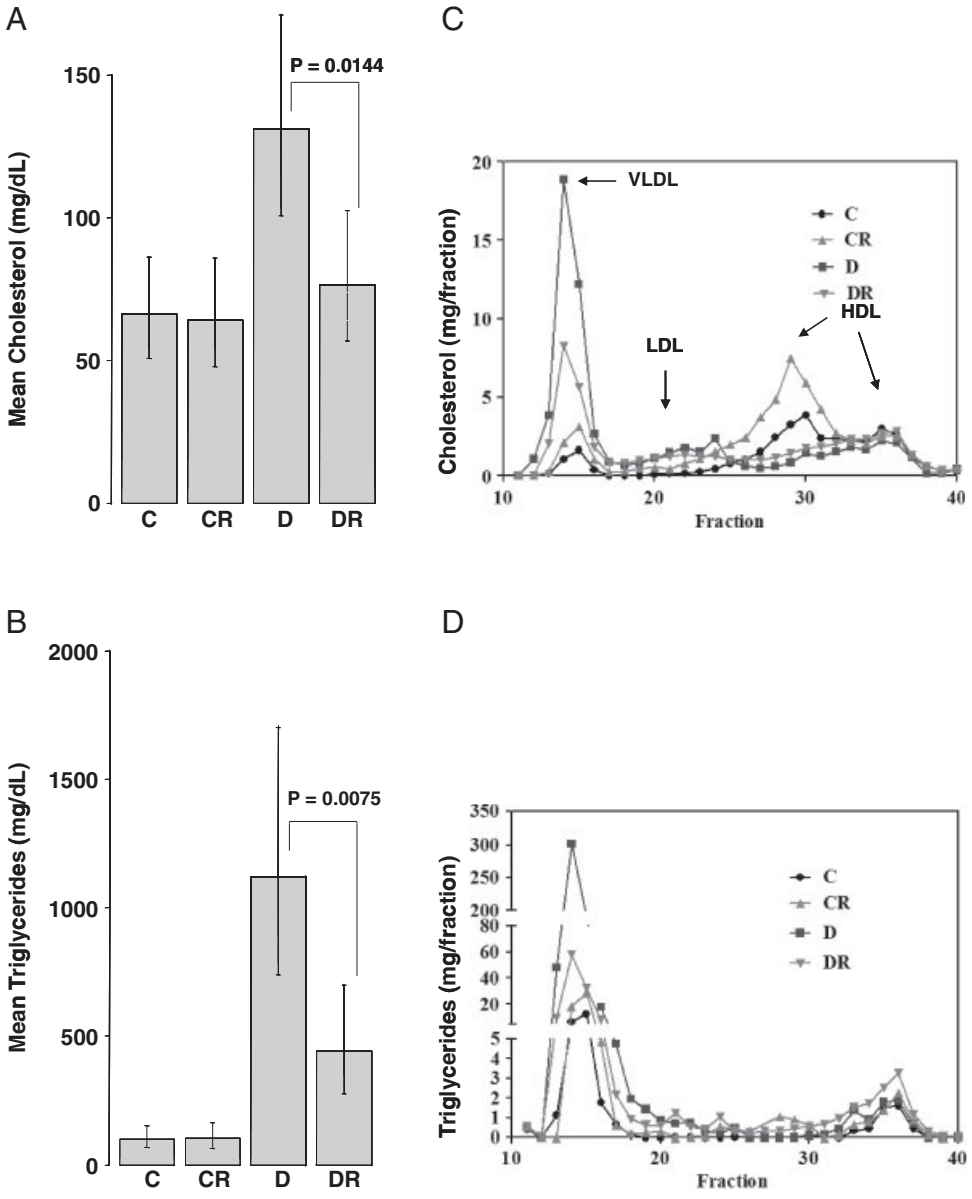

Figure 5. Resveratrol reduces serum lipid accumulation in diabetic dams. Blood from rats was collected after sacrifice and serum was isolated. Cholesterol and triglyceride were measured in the serum by colorimetric methods (A and B). The lipoprotein profile was assessed using same serum sample by FPLC (C and D). RSV significantly decreased lipid accumulation including cholesterol and triglyceride in diabetic dams and increased HDL. Error bars represent ± 2 SEs for each estimated mean.

4 Discussion

RSV has shown promising results for the treatment of many diseases and recently it has shown protective effects against diabetes in animal models as well as in human trials [32–35]. Our current findings build upon this previous work to test the protective role for RSV in a previously unexplored model of diabetic embryopathy. In the present study, we tested the hypothesis that RSV would prevent embryonic oxidative stress and apoptosis associated with diabetic embryopathy and also improve the sugar levels and lipid profile of the diabetic dams. The RSV dose was selected on the basis of previous studies and RSV bioavailability [32, 36]. We showed that gavage feeding of RSV (100 mg/kg b.w.) not only prevented oxidative stress and apoptosis in whole embryos associated with diabetic embryopathy but also improved glucose homeostasis and lipid metabolism of diabetic dams.

Embryos of diabetic dams showed a decrease in their weight and protein content. These changes are likely related to important alterations in protein turnover due to hyperglycaemia-induced oxidative stress followed by excessive apoptosis [8, 9, 25]. Apoptosis, or programmed cell death, is the principal mechanism utilised by multicellular organisms to orchestrate tissue morphogenesis during development and to maintain homeostasis in adulthood. During embryogenesis, apoptosis is utilised ubiquitously to eliminate unwanted or excess cells in the organism. Therefore, any disturbance in the normal pattern of apoptotic cell death causes marked abnormalities in tissue

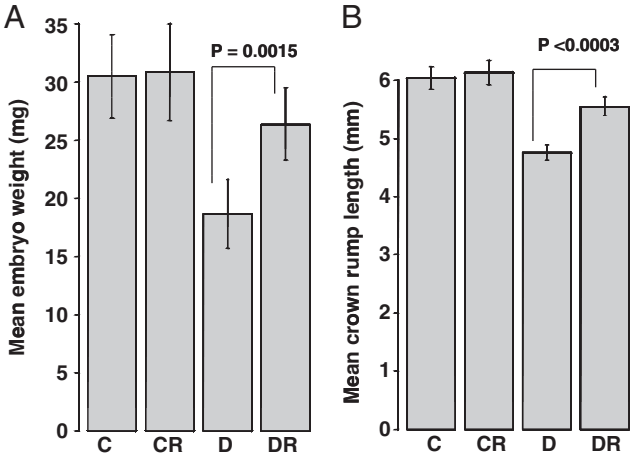

Figure 6. Resveratrol improves outcome of diabetic pregnancy by preventing embryonic development delay. Embryos isolated from all the groups (C, CR, D, DR) were analysed under a stereomicroscope. Resveratrol significantly improved embryo weight (A), crown rump length (B), and somite number (C). Resveratrol alone did not affect embryo development as evident from control treated with RSV data. Error bars represent ± 2 SEs for each estimated mean.

morphogenesis [24]. We found increased apoptosis in the embryos of diabetic dams as evident from the number of apoptotic nuclei, cleaved caspase-3 data, and their co-localised pattern (Figs. 1 and 2). Previous reports also support the embryonic apoptosis activation *via* cleaved caspase-3 [19, 26, 37]. We also studied either the extrinsic or the intrinsic apoptotic pathways responsible for the activation of caspases. Stimulation of the extrinsic and intrinsic pathway leads to the activation of caspase-8 or caspase-9, respectively, at the apoptosome. Following the activation, the initiator caspases then cleave and activate the executioner caspases-3 and caspase-7. We found the involvement of both apoptotic pathways (extrinsic and intrinsic) as evident from increased expression of cleaved portion of caspase-8 and caspase-9, followed by the activation of cleaved caspase-3 (Fig. 2). Activated caspase-3 induces multiple cellular events that trigger a range of downstream apoptotic events such as cell shrinkage, membrane blebbing, and DNA fragmentation. Caspase-7 expression remains unaltered, showing no involvement in diabetes-induced embryonic apoptosis. However, after RSV treatment normalised pattern of apoptosis as well as normal level of caspases was found.

There is considerable evidence that hyperglycaemia-induced oxidative stress is one of the main causes of diabetic complications [8, 25]. In mammals, reactive oxygen species are generated during metabolism and increased during diabetes inducing oxidative stress and apoptosis. Further antioxidant mechanisms (which limit the damaging effects of free radicals) determine the susceptibility and severity of oxidative stress. Increased levels of LPO and total thiol and decreased level of GSH indicate oxidative stress under diabetic conditions (Fig. 3A–C). GSH is a scavenger of hydroxyl radicals and singlet oxygen and functions as a substrate for GSH peroxidase, a hydrogen-peroxide-quenching enzyme. High LPO level in embryo clearly reflects enhanced peroxide formation due to free-radical-mediated destruction of lipids. Of the aldehydes that originate from the peroxidation of cellular membrane lipids, HNE is the major aldehyde. HNE is highly reactive toward free sulfydryl groups of proteins producing thioether adducts that further undergo cyclisation to form hemiacetals [27]. Immunohistochemistry performed with cranial neural tube section for HNE and dual immunostaining of HNE with cleaved caspase-3 confirms the increased HNE adduct formation and their co-localisation with activated caspase-3 in the embryos of diabetic dams (Fig. 3E and F). RSV might work as strong antioxidant, preventing changes in all the parameters responsible for oxidative stress. However, SOD level was unaltered. We found normalised level of LPO, total thiol, GSH as well HNE adduct formation in embryos of diabetic dam treated with RSV.

The blood sugar lowering capacity of RSV is now established as evident from human clinical trial study as well as published studies [29, 31, 34, 35]. In our studies, RSV also decreased the level of sugar from gestation day 7 onward, and at gestation day 12 it decreased the sugar level by 33.32% (Fig. 4A). The glucose lowering effect of RSV may not be only due to the increased serum insulin (Fig. 4B) as RSV is also known to regulate the expression of GLUT-4 [17, 38]. PI3-Akt signalling is reported to be involved in anti-hyperglycaemic effect of RSV in STZ-induced diabetic rats [29]. STZ targets β-cells in the pancreas to reduce the production of insulin. The observed effects of diabetes on the embryos may not be due to STZ as the reported serum half-life of STZ after an intravenous injection (200 mg/kg b.w. in mouse) is ~5 min with no drug measurable by 2 h [39].

Cholesterol and triglyceride concentrations are generally raised in pregnancy and are considered as a key foetal fuel. But in case of diabetic pregnancies, excess increase may contribute to the embryo malformation [30, 40]. In our studies diabetes increased the level of cholesterol by 2-fold and triglycerides by 12-fold (Fig. 5A and B). RSV significantly decreased the cholesterol and triglycerides level in the serum of diabetic dams and increased HDL level as is evident from the lipoprotein profile of control dams (Fig. 5). Recently, RSV was shown to attenuate the expression of HMG-CoA reductase (a rate-limiting enzyme for cholesterol synthesis and a target for statin molecules) mRNA in high fat diet fed hamsters [41]. Statins are used to lower the

cholesterol and triglycerides in human beings, but they are not recommended for pregnant and nursing mother [42]. RSV may be a safe substitute to regulate cholesterol and triglycerides under these disease conditions.

Our results indicate that RSV prevents diabetes-induced apoptosis and oxidative stress in embryos and also concurrently improves the health of diabetic dams as indicated by lower blood sugar, cholesterol, and triglycerides levels. We also observed improved outcome of diabetic pregnancy as evident from improved embryo weight, crown rump length, and somite number in the embryo of diabetic dam (Fig. 6A–C). The improvement in developmental delay by RSV might be because of improved maternal environment as well as direct effects on the embryos as evident from normalised embryonic antioxidant status and apoptosis level. It may be a suitable substitute for or potentially used in combination with insulin and folic acid during diabetic pregnancies as well as for nursing mother.

Diabetes increases oxidative stress, which may impair gene expression during development, and variability in gene expression determines the level of embryonic malformation [43]. There might be variability in the level of oxidative stress and apoptosis (because of individual foetal antioxidative capacity), which may lead variability/severity in embryos maldevelopment of diabetic dams. Here, RSV might be working *via* its antioxidative potential preventing oxidative stress-induced impairment in gene expression, which leads to normal embryonic development [44]. Although RSV dose is high, it was well tolerable as evident from the control treated with RSV data. High dose of RSV is now acceptable for many studies including human trial as evident from published literature [32, 34, 36, 45]. Taken together, present studies demonstrated that RSV possesses significant blood sugar, cholesterol, and triglyceride lowering activity, antioxidative capacity without any observable side effects preventing diabetes-induced embryonic oxidative stress and apoptosis.

This research was supported in part by NIH R21AA016121 (US) 5P01AT003961-03 (PN), and Health Sciences Distinguished Professorship (DJD).

The authors have declared no conflict of interest.

5 References

[1] Rosenn, B., Miodovnik, M., Combs, C. A., Khoury, J., Siddiqi, T. A., Glycemic thresholds for spontaneous abortion and congenital malformations in insulin-dependent diabetes mellitus. *Obstet. Gynecol.* 1994, *84*, 515–520.

[2] Wilson, G. N., Howe, M., Stover, J. M., Delayed developmental sequences in rodent diabetic embryopathy. *Pediatr. Res.* 1985, *19*, 1337–1340.

[3] Miller, E., Hare, J. W., Cloherty, J. P., Dunn, P. J. *et al.*, Elevated maternal hemoglobin A1c in early pregnancy and major congenital anomalies in infants of diabetic mothers. *N. Engl. J. Med.* 1981, *304*, 1331–1334.

[4] Suhonen, L., Hiilesmaa, V., Teramo, K., Glycaemic control during early pregnancy and fetal malformations in women with type I diabetes mellitus. *Diabetologia* 2000, *43*, 79–82.

[5] Casson, I. F., Clarke, C. A., Howard, C. V., McKendrick, O. *et al.*, Outcomes of pregnancy in insulin dependent diabetic women: results of a five-year population cohort study. *Br. Med. J.* 1997, *315*, 275–278.

[6] Galindo, A., Burguillo, A. G., Azriel, S., Fuente Pde, L., Outcome of fetuses in women with pregestational diabetes mellitus. *J. Perinat. Med.* 2006, *34*, 323–331.

[7] Becerra, J. E., Khoury, M. J., Cordero, J. F., Erickson, J. D., Diabetes mellitus during pregnancy and the risks for specific birth defects: a population-based case–control study. *Pediatrics* 1990, *85*, 1–9.

[8] Ornoy, A., Embryonic oxidative stress as a mechanism of teratogenesis with special emphasis on diabetic embryopathy. *Reprod. Toxicol.* 2007, *24*, 31–41.

[9] Chappell, J. H., Jr., Wang, X. D., Loeken, M. R., Diabetes and apoptosis: neural crest cells and neural tube. *Apoptosis* 2009, *14*, 1472–1483.

[10] Reece, E. A., Wu, Y. K., Zhao, Z., Dhanasekaran, D., Dietary vitamin and lipid therapy rescues aberrant signaling and apoptosis and prevents hyperglycemia-induced diabetic embryopathy in rats. *Am. J. Obstet. Gynecol.* 2006, *194*, 580–585.

[11] Sugimura, Y., Murase, T., Oyama, K., Uchida, A. *et al.*, Prevention of neural tube defects by loss of function of inducible nitric oxide synthase in fetuses of a mouse model of streptozotocin-induced diabetes. *Diabetologia* 2009, *52*, 962–971.

[12] Freinkel, N., Diabetic embryopathy and fuel-mediated organ teratogenesis: lessons from animal models. *Horm. Metab. Res.* 1988, *20*, 463–475.

[13] Evers, I. M., de Valk, H. W., Visser, G. H., Risk of complications of pregnancy in women with type 1 diabetes: nationwide prospective study in the Netherlands. *Br. Med. J.* 2004, *328*, 915.

[14] Mills, J. L., Signore, C., Neural tube defect rates before and after food fortification with folic acid. *Birth Defects Res. A Clin. Mol. Teratol.* 2004, *70*, 844–845.

[15] Pearson, K. J., Baur, J. A., Lewis, K. N., Peshkin, L. *et al.*, Resveratrol delays age-related deterioration and mimics transcriptional aspects of dietary restriction without extending life span. *Cell Metab.* 2008, *8*, 157–168.

[16] Lagouge, M., Argmann, C., Gerhart-Hines, Z., Meziane, H. *et al.*, Resveratrol improves mitochondrial function and protects against metabolic disease by activating SIRT1 and PGC-1alpha. *Cell* 2006, *127*, 1109–1122.

[17] Lekli, I., Szabo, G., Juhasz, B., Das, S. *et al.*, Protective mechanisms of resveratrol against ischemia-reperfusion-induced damage in hearts obtained from Zucker obese rats: the role of GLUT-4 and endothelin. *Am. J. Physiol. Heart Circ. Physiol.* 2008, *294*, H859–H866.

[18] Virgili, M., Contestabile, A., Partial neuroprotection of *in vivo* excitotoxic brain damage by chronic administration of the red wine antioxidant agent, trans-resveratrol in rats. *Neurosci. Lett.* 2000, *281*, 123–126.

[19] Guleria, R. S., Pan, J., Dipette, D., Singh, U. S., Hyperglycemia inhibits retinoic acid-induced activation of Rac1, prevents differentiation of cortical neurons, and causes oxidative stress in a rat model of diabetic pregnancy. *Diabetes* 2006, *55*, 3326–3334.

[20] Hu, M. L., Measurement of protein thiol groups and glutathione in plasma. *Methods Enzymol.* 1994, *233*, 380–385.

[21] Ohkawa, H., Ohishi, N., Yagi, K., Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Anal. Biochem.* 1979, *95*, 351–358.

[22] Zabihi, S., Wentzel, P., Eriksson, U. J., Altered uterine perfusion is involved in fetal outcome of diabetic rats. *Placenta* 2008, *29*, 413–421.

[23] Wentzel, P., Gareskog, M., Eriksson, U. J., Folic acid supplementation diminishes diabetes- and glucose-induced dysmorphogenesis in rat embryos *in vivo* and *in vitro*. *Diabetes* 2005, *54*, 546–553.

[24] Brill, A., Torchinsky, A., Carp, H., Toder, V., The role of apoptosis in normal and abnormal embryonic development. *J. Assist. Reprod. Genet.* 1999, *16*, 512–519.

[25] Sun, F., Kawasaki, E., Akazawa, S., Hishikawa, Y. *et al.*, Apoptosis and its pathway in early post-implantation embryos of diabetic rats. *Diabetes Res. Clin. Pract.* 2005, *67*, 110–118.

[26] Zhao, Z., Yang, P., Eckert, R. L., Reece, E. A., Caspase-8: a key role in the pathogenesis of diabetic embryopathy. *Birth Defects Res. B Dev. Reprod. Toxicol.* 2009, *86*, 72–77.

[27] Kruman, I., Bruce-Keller, A. J., Bredesen, D., Waeg, G., Mattson, M. P., Evidence that 4-hydroxynonenal mediates oxidative stress-induced neuronal apoptosis. *J. Neurosci.* 1997, *17*, 5089–5100.

[28] Ong, W. Y., Lu, X. R., Hu, C. Y., Halliwell, B., Distribution of hydroxynonenal-modified proteins in the kainate-lesioned rat hippocampus: evidence that hydroxynonenal formation precedes neuronal cell death. *Free Radic. Biol. Med.* 2000, *28*, 1214–1221.

[29] Chi, T. C., Chen, W. P., Chi, T. L., Kuo, T. F. *et al.*, Phosphatidylinositol-3-kinase is involved in the anti-hyperglycemic effect induced by resveratrol in streptozotocin-induced diabetic rats. *Life Sci.* 2007, *80*, 1713–1720.

[30] Lindegaard, M. L., Damm, P., Mathiesen, E. R., Nielsen, L. B., Placental triglyceride accumulation in maternal type 1 diabetes is associated with increased lipase gene expression. *J. Lipid Res.* 2006, *47*, 2581–2588.

[31] Rivera, L., Moron, R., Zarzuelo, A., Galisteo, M., Long-term resveratrol administration reduces metabolic disturbances and lowers blood pressure in obese Zucker rats. *Biochem. Pharmacol.* 2009, *77*, 1053–1063.

[32] Kennedy, D. O., Wightman, E. L., Reay, J. L., Lietz, G. *et al.*, Effects of resveratrol on cerebral blood flow variables and cognitive performance in humans: a double-blind, placebo-controlled, crossover investigation. *Am. J. Clin. Nutr.* 2010, *91*, 1590–1597.

[33] Pervaiz, S., Holme, A. L., Resveratrol: its biologic targets and functional activity. *Antioxid. Redox Signal.* 2009, *11*, 2851–2897.

[34] Elliott, P. J., Walpole, S., Morelli, L., Lambert, P. D. *et al.*, Resveratrol/SRT-501. *Drugs Fut.* 2009, *34*, 291.

[35] Palsamy, P., Subramanian, S., Resveratrol, a natural phytoalexin, normalizes hyperglycemia in streptozotocin-nicotinamide induced experimental diabetic rats. *Biomed. Pharmacother.* 2008, *62*, 598–605.

[36] Wenzel, E., Soldo, T., Erbersdobler, H., Somoza, V., Bioactivity and metabolism of trans-resveratrol orally administered to Wistar rats. *Mol. Nutr. Food Res.* 2005, *49*, 482–494.

[37] Gareskog, M., Cederberg, J., Eriksson, U. J., Wentzel, P., Maternal diabetes *in vivo* and high glucose concentration *in vitro* increases apoptosis in rat embryos. *Reprod. Toxicol.* 2007, *23*, 63–74.

[38] Penumathsa, S. V., Thirunavukkarasu, M., Zhan, L., Maulik, G. *et al.*, Resveratrol enhances GLUT-4 translocation to the caveolar lipid raft fractions through AMPK/Akt/eNOS signalling pathway in diabetic myocardium. *J. Cell Mol. Med.* 2008, *12*, 2350–2361.

[39] Schein, P. S., Loftus, S., Streptozotocin: depression of mouse liver pyridine nucleotides. *Cancer Res.* 1968, *28*, 1501–1506.

[40] Gupta, S., Arora, S., Trivedi, S. S., Singh, R., Dyslipidemia in pregnancy may contribute to increased risk of neural tube defects – a pilot study in north Indian population. *Ind. J. Clin. Biochem.* 2009, *24*, 150–154.

[41] Cho, I. J., Ahn, J. Y., Kim, S., Choi, M. S., Ha, T. Y., Resveratrol attenuates the expression of HMG-CoA reductase mRNA in hamsters. *Biochem. Biophys. Res. Commun.* 2008, *367*, 190–194.

[42] Kazmin, A., Garcia-Bournissen, F., Koren, G., Risks of statin use during pregnancy: a systematic review. *J. Obstet. Gynaecol. Can.* 2007, *29*, 906–908.

[43] Salbaum, J. M., Kappen, C., Neural tube defect genes and maternal diabetes during pregnancy. *Birth Defects Res. A Clin. Mol. Teratol.* 2010, *88*, 601–611.

[44] Ates, O., Cayli, S. R., Yucel, N., Altinoz, E. *et al.*, Central nervous system protection by resveratrol in streptozotocin-induced diabetic rats. *J. Clin. Neurosci.* 2007, *14*, 256–260.

[45] Cottart, C. H., Nivet-Antoine, V., Laguillier-Morizot, C., Beaudeux, J. L., Resveratrol bioavailability and toxicity in humans. *Mol. Nutr. Food Res.* 2010, *54*, 7–16.

