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Original Contributions

Zinc supplementation prevents cardiomyocyte apoptosis and congenital heart defects in embryos of diabetic mice

Srinivasan Dinesh Kumar [a,b,*], Murugaiyan Vijaya [a], Ramar Perumal Samy [a], S. Thameem Dheen [a], Minqin Ren [c], Frank Watt [c], Y. James Kang [d,e], Boon-Huat Bay [a], Samuel Sam Wah Tay [a,*]

[a] Department of Anatomy, National University Health System, National University of Singapore, Singapore 117597
[b] Lee Kong Chian School of Medicine, Nanyang Technological University, 50 Nanyang Drive, Singapore 637553
[c] Centre for Ion Beam Applications, Department of Physics, Faculty of Science, National University of Singapore, Singapore 117542
[d] Department of Medicine, University of Louisville School of Medicine, Louisville, KY 40292, USA
[e] Regenerative Medicine Research Center, Sichuan University West China Hospital, Sichuan, China

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ABSTRACT

Oxidative stress induced by maternal diabetes plays an important role in the development of cardiac malformations. Zinc (Zn) supplementation of animals and humans has been shown to ameliorate oxidative stress induced by diabetic cardiomyopathy. However, the role of Zn in the prevention of oxidative stress induced by diabetic cardiac embryopathy remains unknown. We analyzed the preventive role of Zn in diabetic cardiac embryopathy by both in vivo and in vitro studies. In vivo study revealed a significant decrease in lipid peroxidation, superoxide ions, and oxidized glutathione and an increase in reduced glutathione, nitric oxide, and superoxide dismutase in the developing heart at embryonic days (E) 13.5 and 15.5 in the Zn-supplemented diabetic group when compared to the diabetic group. In addition, significantly down-regulated protein and mRNA expression of metallothionein (MT) in the developing heart of embryos from diabetic group was rescued by Zn supplement. Further, the nuclear microscopy results showed that trace elements such as phosphorus, calcium, and Zn levels were significantly increased ($P < 0.001$), whereas the iron level was significantly decreased ($P < 0.05$) in the developing heart of embryos from the Zn-supplemented diabetic group. In vitro study showed a significant increase in cellular apoptosis and the generation of reactive oxygen species (ROS) in H9c2 (rat embryonic cardiomyoblast) cells exposed to high glucose concentrations. Supplementation with Zn significantly decreased apoptosis and reduced the levels of ROS. In summary, oxidative stress induced by maternal diabetes could play a role in the development and progression of cardiac embryopathy, and Zn supplementation could be a potential therapy for diabetic cardiac embryopathy.

Introduction

Maternal diabetes has been shown to affect early organogenesis, leading to congenital malformations in various organ systems including the heart [1,2]. Our recent studies revealed that maternal diabetes causes massive cell damage, increase in apoptosis, decrease in proliferation index, and defective heart development in mouse embryos [3,4]. Hyperglycemia causes the cellular oxidative damage by increasing production of ketone bodies, which lead to generation of reactive oxygen species (ROS). The increased level of ROS causes the free radical generation and decreased antioxidants or both [5], which eventually influence the organogenesis during development. Therefore, an efficient antioxidant system including superoxide dismutase (SOD), catalase, glutathione peroxidase (Gpx), reduced glutathione (GSH), and α-tocopherol is critical to the well-being the heart [6]. Zinc (Zn) is known to participate in various cellular functions. It has an antioxidant action in protecting the adult heart from various oxidative stresses. Its abnormal metabolism is related to certain disorders including diabetic complications [7].

In addition to Zn and other antioxidant systems, metallothionein (MT) plays a key role in the scavenging of free radicals. The role of MT as an antioxidant has been extensively studied [8–10]. Zn and its involvement in the synthesis of the antioxidant MT have attracted much attention in diabetes research [11,12].

Abbreviations: EC, endocardial cushion; OFT, outflow tract; NO, nitric oxide; ROS, reactive oxygen species; SOD, superoxide dismutase; TBARS, thiobarbituric acid-reactive substance; MDA, malondialdehyde; GPx, glutathione peroxidase; GSH, reduced glutathione; GSSG, oxidized glutathione; P, phosphorus; Ca, calcium; Fe, iron; Zn, zinc; Cu, copper; Mn, manganese; Mg, magnesium; Se, selenium; STZ, streptozotocin; MT, metallothionein; H9c2 cells, rat embryonic cardiomyoblasts

* Corresponding authors. Fax: +65 67787643.
E-mail addresses: dineshkumar@ntu.edu.sg (S.D. Kumar), anttaysw@nus.edu.sg (S.S.W. Tay).

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Maternal diabetes causes a significant systemic oxidative stress and is often accompanied by Zn deficiency that increases the susceptibility of the embryonic heart to oxidative damage. Therefore, there is a strong rationale to consider Zn supplementation as the therapeutic strategy to prevent or reduce oxidative damage in diabetic cardiac embryopathy.

Nuclear microscopy techniques have the ability to image density variations in relatively thick tissue, map trace elements at the cellular level, and extract quantitative information on these elements [13]. Several groups have shown that some trace elements [such as Zn, copper (Cu), manganese (Mn), and selenium (Se) as cofactors of antioxidant enzymes] play a major role in protecting the insulin-secreting pancreatic beta cells, which are sensitive to free radical damage [14,15]. In addition, the trace element Zn might have an antidiabetic effect by decreasing iron (Fe) levels in the lesion, possibly leading to the inhibition of iron-catalyzed free radical reactions.

The main aim of this study was to understand the oxidative change in the developing heart of embryos from Zn-supplemented diabetic mice, as well as the cellular damage induced by glucose-dependent oxidative stress and the protective role of Zn on H9c2 (embryonic rat cardiomyoblast) cells. Taken together, these results provide further insight into the role of Zn in the prevention of diabetes-induced congenital heart defects, which may give rise to the development of effective treatments against diabetic cardiac embryopathy.

Materials and methods

Animals

The Swiss Albino mice used in the present study were obtained from the Laboratory Animals Centre, Singapore. All procedures involving animals handling was in accordance with Principles of Laboratory Animal Care (NIH publication No. 85-23, revised 1985) and guidelines of the Institutional Animal Care and Use Committee (IACUC), National University of Singapore.

Induction of diabetes mellitus and Zn supplementation

Diabetes mellitus was induced in 8-week-old female mice by an intraperitoneal injection of streptozotocin for three successive days (STZ, 75 mg/kg body weight, Sigma, USA) dissolved in citrate buffer (0.01 M, pH 4.5). Blood glucose level was examined 1 week after STZ injection using Glucometer Elite (Bayer, USA). Mice with nonfasting blood glucose levels exceeding 16 mmol/L were used as diabetic mice. In contrast, control mice maintained the normal blood glucose levels (4–6 mmol/L) before and during pregnancy. Timed mating was carried out by placing four female mice with one normal male mouse in a cage overnight. The day when a copulation plug was observed was counted as embryonic day 0.5 (E0.5). For Zn supplementation diabetic group, Zn sulfate (5 mg/kg body wt) was given intraperitoneally from E0.5 to E13.5 or E15.5 days. Time-mated pregnant mice were divided into three groups: (a) control, (b) diabetes, and (c) diabetes with Zn supplementation. At E13.5 and E15.5, pregnant mice were anesthetized with pentobarbital (150 mg/kg body wt, intraperitoneally) and embryos were collected via Caesarean section. For each experiment, hearts were isolated from embryos from the three groups at different time points. The developmental stage E13.5 corresponds to ~6 weeks of human gestation [16,17]; hence it was chosen as one of the time points. At this stage all 4 chambers of the heart were clearly distinguishable, and the outflow tract (OFT) was evident [18]. In addition, the heart phenotype was apparent in embryos of diabetic mice [3]. Malformed embryos from diabetic mice, normal embryos from diabetic and nondiabetic mice, and normal embryos from Zn-supplemented diabetic mice were used as the diabetic, control, and Zn-supplemented diabetic groups, respectively.

Nitric oxide assay

The total amount of nitric oxide in the heart samples was assessed by the Griess reaction with a colorimetric assay kit (U.S. Biological, Swampscott, MA) that detects nitrite (NO_2^-), a stable reaction product of nitric oxide. Hearts from the embryos of control, diabetic, and Zn-supplemented diabetic mice were collected and homogenized in homogenizing buffer (T-PER; Pierce Biotechnology, Inc., Rockford, IL). Homogenate was centrifuged at 14,000 *g* for 15 min, and the supernatant was collected for the assay. Briefly, 80 μl of samples was added with 10 μl of enzyme cofactor followed by 10 μl of nitrate reductase, according to the manufacturer's instructions, and incubated for 1 h at room temperature (26 °C). Griess reagents (50 μl) A and B were added to the above solution, and the color developed at room temperature after 10 min was measured. The optical density of each of the samples was recorded at 540 nm using a microplate reader (GENios, Tecan Austria GmbH, Grodig/Salzburg, Austria). The nitrite concentration (in μM) was determined from a nitrite standard curve.

Measurement of lipid peroxidation

Heart tissue was homogenized in RIPA homogenizing buffer. The level of lipid peroxidation was estimated by the thiobarbituric acid (TBA) test according to the method described by Ohkawa et al. [19]. Further, the artifactual lipid oxidation is prevented by using butylated hydrotoluene (BHT) during the sample collection. Homogenate was centrifuged at 1600 *g* for 10 min at 4 °C and the supernatant was used for the assay. The amount of 100 μl of supernatant from each sample was taken in a vial and 100 μl of SDS was added and vials were boiled in vigorously boiling water. After an hour, vials were removed and kept in ice bath for 10 min to stop the reaction. The vials were centrifuged at 1600 *g* at 4 °C. The optical density of 150 μl of sample was read at 530 nm using a microplate reader (GENios, Tecan Austria GmbH, Grodig/Salzburg, Austria). The malondialdehyde (MDA) concentration (in μM) was determined from a MDA standard curve.

Detection of superoxide anion

Accumulation of superoxide anion in the developing heart was quantified using a cytochrome reduction assay [20]. Tissue was homogenized and centrifuged at 800 *g*. The supernatant was incubated in the presence of 30 μM succinylated cytochrome *c* and 1 mM NADPH. The change in absorbance at 550 nm was measured. The difference in the amount of reduced succinylated cytochrome *c* in the presence or absence of 0.2 mg/ml superoxide dismutase was used to estimate the amount of superoxide anion by employing an absorbance coefficient 21.1 $M^{-1}\,cm^{-1}$.

Measurements for reduced and oxidized glutathione

A GSH/GSSG kit from U.S. Biological was used to measure reduced glutathione (GSH) and oxidized glutathione (GSSG). The heart tissue was homogenized in 6 vol of 5% metaphosphoric acid with or without 30 mM/L 1-methyl-2-vinyl pyridinium trifluoromethane sulfonate (M2VP), a scavenger of GSH. The homogenate was centrifuged at 1000 *g* for 5 min. For GSH estimation, 5 μl of supernatant was mixed with 355 μl assay buffer containing 100 mM/L sodium phosphate and 5 mM/L EDTA, pH 7.5. For GSSG estimation, 10 μl homogenate with M2VP was mixed with GSSG buffer containing 100 mM/L sodium phosphate and 5 mmol/L EDTA, pH 10.05.