Maternal antioxidants prevent beta cell apoptosis and promote formation of dual hormone-expressing endocrine cells in male offspring following fetal and neonatal nicotine exposure

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Abstract

Aim—Fetal and neonatal nicotine exposure causes beta cell oxidative stress and apoptosis in neonates, leading to adult-onset dysglycemia. The goal of this study was to determine whether an antioxidant intervention could prevent nicotine-induced beta cell loss.

Methods—Nulliparous female Wistar rats received daily subcutaneous injections of either saline or nicotine bitartrate (1.0 mg/kg/d) for 2 weeks prior to mating until weaning. Nicotine-exposed dams received either normal chow or diet containing antioxidants (1000 IU/kg vitamin E, 0.25% w/w coenzyme Q10 and 0.1% w/w alpha-lipoic acid) during mating, pregnancy and lactation; saline-exposed dams received normal chow. Pancreas tissue was collected from male offspring at 3 weeks of age to measure beta cell fraction, apoptosis, proliferation and the presence of cells co-expressing insulin and glucagon.

Results—The birth weight of the offspring born to nicotine-exposed dams receiving dietary antioxidants was significantly reduced. Most interestingly, the antioxidant intervention to nicotine-exposed dams prevented the beta cell loss and apoptosis observed in nicotine exposed male offspring whose mothers did not receive antioxidants. Male pups born to nicotine-treated mothers receiving antioxidants also had a trend towards increased beta cell proliferation and a significant increase in islets containing insulin/glucagon bi-hormonal cells relative to the other two treatment groups.

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Author Disclosure Statement

No competing financial interests exist.

Conclusion—This study demonstrates that exposure to maternal antioxidants protects beta cells from the damaging effects of nicotine thus preserving beta cell mass.

Keywords

antioxidant therapy; beta cell mass; insulin/glucagon bi-hormonal cells; nicotine; pancreas development

Introduction

Type 2 diabetes occurs when the beta cell is unable to produce sufficient insulin to maintain normoglycemia and mounting evidence suggests that this may be due to a decreased beta cell mass.^{1,2} For example, pancreatic beta cell mass is reduced by 40–60% in patients with type 2 diabetes,^{3–5} and this deficit is evident before diagnosis of the disease.⁴ In animal models, restoration of beta cell mass following experimental destruction results in normoglycemia that can persist until adulthood.⁶ Therefore, therapeutic interventions that restore beta cell mass and/or prevent further beta cell loss are of particular interest for the management and prevention of type 2 diabetes.

Several lines of evidence support the hypothesis that oxidative stress, an imbalance between the production of reactive oxygen species (ROS) and the cellular antioxidant defense system, plays an essential role in the development of type 2 diabetes.^{7–10} Pancreatic beta cells are known to be particularly sensitive to oxidative damage due to their relatively low expression of antioxidant enzymes compared to other cell types.^{11,12} Indeed, oxidative stress in the pancreas is associated with beta cell dysfunction and increased beta cell apoptosis.^{7–10} Our laboratory has previously demonstrated that exposure to nicotine, the major addictive component of cigarette smoke, during pregnancy and lactation results in increased beta cell apoptosis and reduced beta cell mass in the male offspring at birth and weaning.¹³ Moreover, in our animal model nicotine-induced beta cell deficits in the neonate are associated with increased oxidative stress in the pancreas including elevated levels of protein carbonyls (indicative of oxidative damage to pancreatic proteins) and increased ROS levels in isolated islets.¹⁴ These findings are consistent with the considerable evidence both *in vivo* and *in vitro* showing that exposure to cigarette smoke or nicotine alone results in increased oxidative stress in fetal, neonatal and adult tissues.^{15–22} In animal models of adult onset type 2 diabetes, treatment of the affected animal with antioxidants protects beta cell mass and prevents beta cell apoptosis.^{23–25} Moreover, antioxidant vitamins have been shown to prevent nicotine-induced oxidative stress *in vitro*.²¹ However, the ability of antioxidants to protect beta cells during critical windows of pancreatic development has not been critically examined. Therefore the goal of this study was to test the hypothesis that maternal administration of an antioxidant cocktail during fetal and neonatal pancreatic development protects against nicotine-induced beta cell damage in the male offspring.

Methods

Maintenance and treatment of animals

All animal experiments were approved by the Animal Research Ethics Board at McMaster University, in accordance with the guidelines of the Canadian Council for Animal Care. Nulliparous 200–250g female Wistar rats (Harlan, Indianapolis, IN, USA) were maintained under controlled lighting (12:12 L:D) and temperature (22°C) with *ad libitum* access to food and water. Two weeks prior to mating the dams were randomly assigned to receive either saline (n=10; SC) or nicotine (n=20). Dams were injected with 1.0 mg/kg/day nicotine bitartrate (Sigma Aldrich, St. Louis, MO, USA) or saline subcutaneously for 14 days prior to mating, and during pregnancy until weaning (postnatal day 21; PND21). The dose of nicotine used in this animal model resulted in maternal serum cotinine concentrations of 136ng/ml,²⁶ which is within the range of cotinine levels reported in women who are considered “moderate smokers” (80 to 163 ng/mL)²⁷ and serum cotinine concentrations of 26 ng/ml in the nicotine-exposed offspring at birth²⁶ which is also within the range (5 to 30 ng/ml) observed in infants nursed by smoking mothers.²⁸ In addition, this dose of nicotine has been shown to increase markers of oxidative stress in the offspring.¹⁴ Nicotine-exposed dams were further randomized to receive either normal diet (nicotine chow–NC; n=10) or diet supplemented with an antioxidant cocktail (nicotine antioxidant–NA; n=10) starting 2 weeks prior to mating until the end of lactation (i.e., postnatal day 21; PND21). For this study we opted to only treat the nicotine-exposed dams with the antioxidant cocktail. The maintenance of a healthy oxidative balance is particularly important during pregnancy,²⁹ therefore we predicted that an antioxidant intervention in healthy, saline-treated dams without the presence of a pro-oxidant would cause undesirable side effects. Indeed, antioxidants have been shown to protect beta cells, but only in the presence of a pro-oxidant; antioxidant treatment of healthy, unstressed beta cells led to *decreased* beta cell function and viability.^{30,31}

For the antioxidant supplementation group, coenzyme Q10 (0.25% w/w), alpha-lipoic acid (0.1% w/w) and vitamin E acetate (1000 IU/kg) were added to standard rodent diet (Teklad Global 16% Protein Rodent diet; Harlan Teklad, Madison WI) by the manufacturer. Strobel et al.³² have reported that in male Wistar rats, consumption of a diet supplemented with 1000 IU vitamin E/kg diet and 0.16% w/w alpha lipoic acid resulted in plasma levels of vitamin E which are consistent with those reported in pregnant women.³³ Similarly, rats consuming a diet supplemented with 0.2% CoQ10 had serum CoQ10 levels which are representative of human serum levels in pregnant women.^{34,35} Information regarding serum levels of alpha lipoic acid in humans is difficult to obtain due to the short half life of this compound.³⁶ We chose to provide a combination of antioxidants: 1) because antioxidants function optimally as reduction-oxidation (redox) couples³⁷ and 2) to target different pathways of oxidative stress. Vitamin E (alpha-tocopherol) is a lipophilic free radical scavenger that prevents ROS-induced cellular damage including lipid peroxidation.³⁸ Furthermore, vitamin E has been shown to prevent nicotine-induced oxidative stress in the placenta.²¹ Coenzyme Q10 (CoQ10) is a lipid soluble antioxidant found at high levels in the mitochondria³⁹ and in combination with vitamin E has been shown to reduce lipid peroxidation in pancreatic mitochondria of diabetic rats.⁴⁰ Finally, alpha-lipoic acid is an

amphiphilic antioxidant scavenger that has been shown to be cytoprotective in pancreatic beta cells under conditions of oxidative stress.^{31,41}

Maternal body weight and food consumption were monitored biweekly for the duration of the study. Two weeks after the initiation of treatment, dams were mated (1:1) with age-matched Wistar rats and were monitored daily for confirmation of breeding (i.e. the presence of sperm in a vaginal flush). The day that a positive sign of copulation was observed was designated gestational day 0 (GD0). Dams were allowed to deliver normally. For each dam, gestation length, litter size, birthweight, sex, and the number of stillbirths were recorded. From these data the mating success rate ($[\# \text{ of females mated} / \# \text{ of females cohabited}] * 100$), pregnancy success rate ($[\# \text{ of dams delivering a litter} / \# \text{ dams with a confirmed mating}] * 100$), the live birth index ($[\# \text{ of live offspring} / \# \text{ of offspring delivered}] * 100$) and the sex ratio ($\# \text{ of male offspring} / \# \text{ of female offspring}$) were calculated. Litter size was culled to eight at birth (postnatal day 1; PND1) to assure uniformity of litter size between treated and control litters. Pups were weighed weekly during lactation and at 3 weeks of age (PND21), male offspring were euthanized by CO₂ asphyxiation. Pancreas tissue and fat pads (mesenteric, epididymal, and perirenal) were collected, weighed and processed for subsequent analysis.

Systemic antioxidant capacity and oxidative stress

To determine the effects of maternal dietary antioxidant supplementation during pregnancy and lactation on systemic antioxidant capacity, we measured oxygen radical antioxidant capacity (ORAC) in maternal and neonatal serum at weaning (PND21) using a commercially available kit according to the manufacturer's directions (Oxford Biomedical Research, Rochester Mills, MI).

To assess oxidative damage to pancreatic proteins, the presence of protein carbonyl groups in the pancreas (mitochondrial fraction) of the male offspring was quantified using the OxyBlot™ Protein Oxidation Detection Kit (Chemicon International, Temecula CA) as previously described.¹⁴ Briefly, to obtain mitochondrial proteins, the Compartmental Protein Extraction Kit (K3013010; Biochain Institute Inc., Hayward, CA) was used according to manufacturer's instructions. Protein samples were then prepared with the Oxyblot™ Kit, according to manufacturer's instructions. Densitometric analysis of immunoblots was performed using ImageJ 1.37v software (National Institutes of Health, Bethesda, MD).

Beta cell fraction, apoptosis and regeneration

The beta cell fraction (i.e., the proportion of the pancreas tissue containing insulin positive cells), beta cell proliferation and apoptosis in the male offspring (n=6 per group) were quantified as follows. For determination of beta cell fraction, immunohistochemical detection of insulin was performed on four paraffin-embedded sections (5µm) per animal, separated by a minimum of 40µm, as previously described,¹³ using a polyclonal, guinea pig anti-swine insulin primary antibody (1:150 dilution) (DakoCytomation, Carpinteria, CA), the Vectastain anti-rabbit kit (Vector Laboratories, Burlingame, CA), and diaminobenzadine (Sigma Aldrich, St. Louis, MO) as the chromogen. For all sections, the whole pancreas was analyzed by combining measurements from up to 25 fields per section.

Immunopositive cells were identified using Image Pro Plus Version 5.1 software (Media Cybernetics, Inc., Silver Spring MD) for automated cell counting. The beta cell fraction was calculated as the ratio of beta cell area (immunopositive staining) to total pancreas area (immunopositive staining plus pancreas counterstaining) $\times 100$.

Detection of beta cell apoptosis was performed using a triple immunofluorescent staining protocol as previously described.¹³ Briefly, insulin was detected using a polyclonal, guinea pig anti-swine insulin antibody (1:150; DakoCytomation, Carpinteria, CA), followed by an anti-rabbit Alexa Fluor 594 secondary antibody (1:400; Invitrogen, Carlsbad CA). Next, tissues were subjected to the terminal deoxynucleotidyltransferase mediated dUTP nick end labelling (TUNEL) assay according to manufacturer's instructions (Roche Applied Science, Laval, QC). Finally, nuclei were counterstained with DAPI (Sigma Aldrich, St. Louis, MO) and tissue sections were imaged with a Leica DMRA2 microscope using Openlab software version 4.0.2 (Improvision, Waltham MA). Images were analyzed with Image Pro Plus Version 5.1 software (Media Cybernetics, Inc., Silver Spring MD); five islets per section were quantified and reported as the percentage of TUNEL⁺ beta cells.

To measure beta cell proliferation, sections were subjected to 15 minutes of heat-induced epitope retrieval at 95°C using a 10 mmol/l citrate buffer, pH 6.0. Pancreas sections were then incubated with guinea pig anti-insulin (1:1000; Linco, Billerica, MA) and mouse anti-proliferating cell nuclear antigen (PCNA; 1:100; BD Biosciences, Mississauga, ON), followed by a mixture of goat anti-guinea pig Alexa Fluor 488 (1:1000; Invitrogen, Carlsbad CA) and goat anti-mouse Alexa Fluor 594 (1:1000; Invitrogen, Carlsbad CA). Images were captured using an Axiovert 200 microscope (Carl Zeiss, Toronto, ON, Canada) connected to a digital camera (Retiga 2000R, QImaging, Surrey, BC, Canada) controlled with Openlab 5.2 software (Perkin Elmer, Waltham, MA, USA). Images were analyzed with Image Pro Plus Version 5.1 software (Media Cybernetics, Inc., Silver Spring MD); five islets per section were quantified and reported as the percentage of PCNA⁺ beta cells.

Recent studies have suggested that beta cell regeneration may also occur through the formation of neogenic beta cells arising from pre-existing alpha cells. During this regenerative process, an intermediate cell type arises that co-expresses both insulin and glucagon, and contributes to the recovery of beta cell mass.^{42,43} Therefore, we investigated the possibility that our antioxidant cocktail may preserve beta cell mass in nicotine-exposed animals by promoting the formation of new beta cells from pre-existing alpha cells by determining the expression of double-positive insulin/glucagon intermediate cell types in pancreatic islets of the offspring. The presence of cells co-expressing insulin and glucagon was assessed using the same staining procedure as with beta cell proliferation, but combining guinea pig anti-insulin (1:1000; Linco, Billerica, MA) and mouse anti-glucagon (1:1000; Sigma Aldrich, St. Louis, MO), followed by goat anti-guinea pig Alexa Fluor 488 and goat anti-mouse Alexa Fluor 594 secondary antibodies (1:1000; Invitrogen, Carlsbad CA). Images were analyzed with Image Pro Plus Version 5.1 software (Media Cybernetics, Inc., Silver Spring MD); five islets per section were quantified and reported as the percentage of islets containing insulin/glucagon co-positive cells. To confirm the presence of bi-hormonal cells in pancreatic islets, pancreas tissue from the offspring (SC: n=4, NC and NA: n=5 per group) was collected and processed for electron microscopy as previously

described.⁴⁴ Grids were examined with a JEOL 1200EX transmission electron microscope (JEOL Ltd., Tokyo, Japan) and representative photographs were taken at 12000x magnification. Bihormonal cells were identified by the presence of both typical insulin and glucagon granules within the same cell. Glucagon granules are clearly distinguishable from insulin granules by their smaller size, increased density and absence of a clear halo surrounding the dense core.

Statistical Analysis

All statistical analyses were performed using SigmaStat (v.3.1, SPSS, Chicago, IL, USA). The results are expressed as mean $\pm$ SEM. Data were tested for normality as well as equal variance, and when normality or variance tests failed, data were analyzed using Kruskal-Wallis one-way ANOVA on ranks. Otherwise one-way ANOVA followed by appropriate *post-hoc* tests were performed when significance was indicated ($p < 0.05$). Categorical variables (mating success rate and pregnancy success rate) were compared using Fisher's exact test. For all outcomes at birth (i.e., litter size, live birth index, sex ratio and birth weight) the statistical unit was the litter.

Results

Pregnancy and birth outcomes

There was no significant effect of treatment on maternal food consumption prior to pregnancy or during pregnancy (data not shown). Furthermore, pregnancy weight gain did not differ between the three groups (SC 140 ± 7 g; NC 136 ± 6 g; NA 124 ± 6 g; $p = 0.287$). Neither fertility nor pregnancy outcomes were affected by either nicotine treatment or the antioxidant diet; no differences were observed in time to copulation, mating success, pregnancy success rate or gestation length between treatment groups (Table 1).

Antioxidant supplementation to nicotine-treated dams resulted in a significant decrease in birth weight of the pups relative to those exposed to nicotine alone; however the birth weight of NA animals was not significantly different from SC controls (Table 1). There were no significant differences in litter size, live birth index or the sex ratio between the treatment groups.

Postnatal growth and adiposity

Offspring of nicotine-exposed dams receiving dietary antioxidants had significantly reduced weight gain from birth to weaning (SC 39.3 ± 0.8 g; NC 41.2 ± 1.1 g; NA 33.7 ± 1.2 g) relative to both SC and NC offspring ($p < 0.001$) despite no differences in maternal food consumption during the same period (food consumption [kcal/100g body weight/d]: SC 53.5 ± 0.8 ; NC 53.3 ± 1.7 ; NA 51.4 ± 1.4 ; $p = 0.469$). Consequently NA offspring were significantly lighter at weaning (Figure 1A); an effect which may be attributable, in part, to a reduction in absolute and relative fat pad weights (Figures 1B and 1C).

Oxidative stress

There was no effect of either nicotine exposure or antioxidant supplementation on systemic antioxidant capacity (i.e., ORAC) in either the dams or the offspring (data not shown).

Although protein carbonyl levels in the pancreas of male nicotine-exposed offspring were increased by 37% above levels in control offspring, this did not reach statistical significance (optical density SC = 7075 ± 1322 ; optical density NC = 9708 ± 691 ; optical density NA = 7591 ± 819 ; $p=0.231$)

Beta cell mass, apoptosis and proliferation

Compared to animals whose mothers were exposed to saline (SC), the male offspring of nicotine-exposed dams (NC) had a significant reduction in pancreatic beta cell fraction at weaning, an effect that was attributed to increased beta cell apoptosis and not decreased beta cell proliferation (Figure 2). Antioxidant supplementation to nicotine-exposed dams prevented this nicotine-induced beta cell loss, such that the beta cell fraction was not significantly different between the NA and SC (control) pups (Figure 2). The protection of beta cell mass by antioxidant supplementation in nicotine-exposed animals appears to be attributed to several mechanisms, including a significant reduction in beta cell apoptosis compared to NC offspring, as well as a non-significant trend ($p=0.07$) towards increased beta cell proliferation (Figure 2). Moreover, antioxidant supplementation to nicotine-treated dams resulted in a significant increase in the percentage of pancreatic islets containing bi-hormonal cells (i.e., expressing both insulin and glucagon in the same cell) (Figure 3).

Discussion

In this study we have demonstrated that the addition of an antioxidant cocktail to the diet of nicotine-exposed dams during pregnancy and lactation prevented beta cell loss in the male nicotine-exposed offspring. Although other studies using adult rodent models of diabetes have reported beneficial effects of antioxidant therapies on the preservation of beta cell mass and islet insulin content,^{23,25} this is the first study to show a protective effect of antioxidant treatment on beta cell mass when the treatment is initiated during fetal development and continued until weaning (i.e., during pancreatic development).

We have previously demonstrated that the reduction in beta cell mass in nicotine-exposed male offspring is a result of increased mitochondrial-mediated apoptosis.⁴⁵ Therefore the antioxidants selected for this study (i.e., vitamin E, CoQ₁₀ and alpha-lipoic acid) were chosen because they have been shown to improve mitochondrial function, prevent mitochondrial-mediated apoptosis and work cooperatively as redox couples.^{37,46–51} Furthermore, vitamin E, CoQ₁₀ and alpha-lipoic acid have all been shown to preserve beta cell mass and/or reduce pancreas specific oxidative damage in rodent models of type 2 diabetes.^{23–25,40} Although the antioxidant cocktail did not affect systemic antioxidant capacity (i.e., ORAC levels), there is some evidence to suggest that it may have reduced pancreas specific oxidative damage in the male offspring. We have previously demonstrated that fetal and neonatal exposure to nicotine results in increased oxidative damage to pancreatic proteins (i.e., protein carbonyl formation).¹⁴ Similarly in this study, although not statistically significant, protein carbonyl levels were 37% higher in NV offspring than controls, whereas the NA offspring had no evidence of increased pancreatic protein carbonyl levels. We designed the antioxidant cocktail to contain constituents known to prevent mitochondrial-mediated apoptosis, the main pathway of beta cell apoptosis following

nicotine exposure. As predicted, pups born to nicotine-exposed mothers who received the antioxidant cocktail had reduced beta cell apoptosis relative to offspring whose mothers received nicotine alone. Therefore, the prevention of apoptosis may be one pathway by which the antioxidants in this study preserved beta cell mass.

However, there also appear to be other mechanisms involved in the protective effects of this antioxidant diet on beta cells. For instance, antioxidant supplementation to nicotine-exposed dams resulted in a 1.7-fold increase in the proportion of beta cells expressing PCNA ($p=0.07$) relative to male offspring whose mothers received normal chow, an effect which may have contributed to the preservation of beta cell mass in these animals. In addition, we also observed a significant increase in the proportion of islets containing cells that co-express both insulin and glucagon. Using lineage-tracing experiments, Thorel and colleagues recently demonstrated that following beta cell ablation, a transient population of bi-hormonal cells, originating from pre-existing alpha cells, appears in pancreatic islets.⁴³ The authors speculated that these cells likely represent emergent beta cells that contribute to beta cell regeneration. These results were confirmed by Chung et al., who demonstrated the presence of intermediate cells co-expressing insulin and glucagon within one week following beta cell ablation.⁴² Although we cannot comment on the origin of the double-positive endocrine cells that arise in our animal model following antioxidant treatment, we do speculate that this may be an alternative mechanism through which antioxidants promote beta cell regeneration following nicotine-induced beta cell loss.

The antioxidant cocktail used in this study successfully protected the developing pancreas from nicotine-induced beta cell loss. However, the observed reduction in birth weight and increased incidence of very low birth weight offspring (i.e., SGA, < 2 standard deviations below the average birth weight of SC pups) in the NA group limits the usefulness of this specific combination of antioxidants in human populations. We speculate that the low birth weight in the NA group may be attributable to the presence of vitamin E in the diet as CoQ₁₀ and alpha-lipoic acid have both safely been used during pregnancy with no reported adverse effects on birth weight^{52,53} whereas studies in humans have reported an increased incidence of low birth weight and perinatal death in women receiving high dose vitamins C and vitamin E during pregnancy.^{54,55} However, other clinical trials have failed to demonstrate any adverse effects of high dose vitamin C and E supplementation on birth weight.^{56,57} Moreover, Sen and Simmons⁵⁸ did not find any evidence of reduced birth weight when pregnant rats were given an antioxidant supplement including vitamin E, although the level of vitamin E supplementation in their study was significantly lower than in our study (360 IU/kg vs 1000 IU/kg). Interestingly, this lower level of vitamin E supplementation was able to prevent oxidative stress and the development of abnormal glycemic control in the offspring of obese dams (i.e., a condition leading to increased oxidative stress in the offspring).⁵⁸ Therefore, future studies should examine whether or not CoQ₁₀ and alpha-lipoic acid alone or in combination with a lower dose of vitamin E will protect the developing beta cells without affecting birth weight and neonatal growth.

In male animals exposed to nicotine during fetal and neonatal life, the loss of beta cell mass leads to the development of dysglycemia in adulthood.^{13,59} Similarly, children born to women who smoke during pregnancy are at an increased risk of developing type 2 diabetes

during adult life.⁶⁰ In this study we have demonstrated that the addition of an antioxidant cocktail to the diet of nicotine-exposed dams during pregnancy and lactation prevented the loss of beta cell mass in male nicotine-exposed offspring. Since a reduced beta cell mass contributes to the onset of type 2 diabetes,⁶¹ we propose that an antioxidant intervention during pregnancy may attenuate the increased risk of developing type 2 diabetes in those children born to smoking mothers. However, the long-term effects of this intervention on postnatal beta cell function remain to be determined. Regardless, an antioxidant intervention may also be a beneficial therapeutic strategy for protecting developing pancreatic beta cells in other conditions of oxidative stress during pregnancy, such as maternal diabetes, maternal obesity, infection and inflammation. However, the safety of these antioxidant interventions would have to be carefully evaluated before recommendation for use by pregnant women, especially considering the reports of reduced birth weight in clinical trials following antioxidant use during pregnancy.

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References

1. Leahy JL. Pathogenesis of type 2 diabetes mellitus. *Arch Med Res.* 2005; 36:197–209. [PubMed: 15925010]
2. Rhodes CJ. Type 2 diabetes-a matter of beta-cell life and death? *Science.* 2005; 307:380–384. [PubMed: 15662003]
3. Sakuraba H, Mizukami H, Yagihashi N, Wada R, Hanyu C, Yagihashi S. Reduced beta-cell mass and expression of oxidative stress-related DNA damage in the islet of Japanese Type II diabetic patients. *Diabetologia.* 2002; 45:85–96. [PubMed: 11845227]
4. Butler AE, Janson J, Soeller WC, Butler PC. Increased beta-cell apoptosis prevents adaptive increase in beta-cell mass in mouse model of type 2 diabetes: evidence for role of islet amyloid formation rather than direct action of amyloid. *Diabetes.* 2003; 52:2304–2314. [PubMed: 12941770]
5. Yoon KH, Ko SH, Cho JH, et al. Selective beta-cell loss and alpha-cell expansion in patients with type 2 diabetes mellitus in Korea. *J Clin Endocrinol Metab.* 2003; 88:2300–2308. [PubMed: 12727989]
6. Bouwens L, Rومان I. Regulation of pancreatic beta-cell mass. *Physiol Rev.* 2005; 85:1255–1270. [PubMed: 16183912]
7. Kajimoto Y, Kaneto H. Role of oxidative stress in pancreatic beta-cell dysfunction. *Ann NY Acad Sci.* 2004; 1011:168–176. [PubMed: 15126294]
8. Kaneto H, Kawamori D, Matsuoka TA, Kajimoto Y, Yamasaki Y. Oxidative stress and pancreatic beta-cell dysfunction. *Am J Ther.* 2005; 12:529–533. [PubMed: 16280646]
9. Donath MY, Ehses JA, Maedler K, et al. Mechanisms of beta-cell death in type 2 diabetes. *Diabetes.* 2005; 54(Suppl 2):S108–113. [PubMed: 16306327]
10. Drews G, Krippeit-Drews P, Dufer M. Oxidative stress and beta-cell dysfunction. *Pflugers Arch.* 2010; 460:703–718. [PubMed: 20652307]

11. Lenzen S, Drinkgern J, Tiedge M. Low antioxidant enzyme gene expression in pancreatic islets compared with various other mouse tissues. *Free Radic Biol Med*. 1996; 20:463–466. [PubMed: 8720919]
12. Tiedge M, Lortz S, Drinkgern J, Lenzen S. Relation between antioxidant enzyme gene expression and antioxidative defense status of insulin-producing cells. *Diabetes*. 1997; 46:1733–1742. [PubMed: 9356019]
13. Bruin JE, Kellenberger LD, Gerstein HC, Morrison KM, Holloway AC. Fetal and neonatal exposure to nicotine and postnatal glucose homeostasis: Identifying critical windows of exposures. *J Endocrinol*. 2007; 194:171–178. [PubMed: 17592031]
14. Bruin JE, Petre MA, Lehman MA, et al. Maternal nicotine exposure increases oxidative stress in the offspring. *Free Radic Biol Med*. 2008; 44:1919–1925. [PubMed: 18343235]
15. Zhao Z, Reece EA. Nicotine-induced embryonic malformations mediated by apoptosis from increasing intracellular calcium and oxidative stress. *Birth Defects Res B Dev Reprod Toxicol*. 2005; 74:383–391. [PubMed: 16193507]
16. Crowley-Weber CL, Dvorakova K, Crowley C, et al. Nicotine increases oxidative stress, activates NF-kappaB and GRP78, induces apoptosis and sensitizes cells to genotoxic/xenobiotic stresses by a multiple stress inducer, deoxycholate: relevance to colon carcinogenesis. *Chem Biol Interact*. 2003; 145:53–66. [PubMed: 12606154]
17. Wetscher GJ, Bagchi M, Bagchi D, et al. Free radical production in nicotine treated pancreatic tissue. *Free Radic Biol Med*. 1995; 18:877–882. [PubMed: 7797095]
18. Ozokutan BH, Ozkan KU, Sari I, Inanc F, Guldur ME, Kilinc M. Effects of maternal nicotine exposure during lactation on breast-fed rat pups. *Biol Neonate*. 2005; 88:113–115. [PubMed: 15925865]
19. Husain K, Scott BR, Reddy SK, Somani SM. Chronic ethanol and nicotine interaction on rat tissue antioxidant defense system. *Alcohol*. 2001; 25:89–97. [PubMed: 11747978]
20. Yildiz D. Nicotine, its metabolism and an overview of its biological effects. *Toxicol*. 2004; 43:619–632. [PubMed: 15109883]
21. Gallo C, Renzi P, Loizzo S, et al. Potential therapeutic effects of vitamin E and C on placental oxidative stress induced by nicotine: an in vitro evidence. *Open Biochem J*. 2010; 4:77–82. [PubMed: 20676222]
22. Halima BA, Serra K, Kais R, Salwa E, Najoua G. Indicators of oxidative stress in weanling and pubertal rats following exposure to nicotine via milk. *Hum Exp Toxicol*. 2010; 29:489–496. [PubMed: 19900975]
23. Kaneto H, Kajimoto Y, Miyagawa J, et al. Beneficial effects of antioxidants in diabetes: possible protection of pancreatic beta-cells against glucose toxicity. *Diabetes*. 1999; 48:2398–2406. [PubMed: 10580429]
24. Song KH, Lee WJ, Koh JM, et al. Alpha-Lipoic acid prevents diabetes mellitus in diabetes-prone obese rats. *Biochem Biophys Res Commun*. 2005; 326:197–202. [PubMed: 15567171]
25. Karatug A, Bolkent S. The potential role of combined antioxidant treatment on pancreas of STZ-diabetic mice. *Exp Toxicol Pathol*. 2011; doi: 10.1016/j.etp.2011.08.012
26. Holloway AC, Kellenberger LD, Petrik JJ. Fetal and neonatal exposure to nicotine disrupts ovarian function and fertility in adult female rats. *Endocrine*. 2006; 30:213–216. [PubMed: 17322582]
27. Eskenazi B, Bergmann JJ. Passive and active maternal smoking during pregnancy, as measured by serum cotinine, and postnatal smoke exposure. I. Effects on physical growth at age 5 years. *Am J Epidemiol*. 1995; 142:S10–S18. [PubMed: 7572982]
28. Luck W, Nau H. Nicotine and cotinine concentrations in serum and urine of infants exposed via passive smoking or milk from smoking mothers. *J Pediatr*. 1985; 107:816–820. [PubMed: 4056988]
29. Dennery PA. Effects of oxidative stress on embryonic development. *Birth Defects Res C Embryo Today*. 2007; 81:155–162. [PubMed: 17963268]
30. Schroeder MM, Belloto RJ Jr, Hudson RA, McNerney MF. Effects of antioxidants coenzyme Q10 and lipoic acid on interleukin-1 beta-mediated inhibition of glucose-stimulated insulin release from cultured mouse pancreatic islets. *Immunopharmacol Immunotoxicol*. 2005; 27:109–122. [PubMed: 15803864]

31. Lee BW, Kwon SJ, Chae HY, et al. Dose-related cytoprotective effect of alpha-lipoic acid on hydrogen peroxide-induced oxidative stress to pancreatic beta cells. *Free Radic Res.* 2009; 43:68–77. [PubMed: 19358002]
32. Strobel NA, Peake JM, Matsumoto A, Marsh SA, Coombes JS, Wadley GD. Antioxidant supplementation reduces skeletal muscle mitochondrial biogenesis. *Med Sci Sports Exerc.* 2011; 43:1017–24. [PubMed: 21085043]
33. Chelchowska M, Ambroszkiewicz J, Gajewska J, Laskowska-Klita T, Leibschang J. The effect of tobacco smoking during pregnancy on plasma oxidant and antioxidant status in mother and newborn. *Eur J Obstet Reprod Biol.* 2011; 155:132–136.
34. Kunitomo M, Yamaguchi Y, Kagota S, Otsubo K. Beneficial effect of coenzyme Q10 on increased oxidative stress and nitrate stress and inflammation and individual metabolic components developing in a rat model of metabolic syndrome. *J Pharmacol Sci.* 2008; 107:128–137.
35. Haruna M, Matsuzaki M, Ota E, et al. Positive correlation between maternal serum coenzyme Q10 levels and infant birth weight. *Biofactors.* 2010; 36:312–318. [PubMed: 20641057]
36. Teichert J, Hermann R, Ruus P, Preiss R. Plasma kinetics, metabolism, and urinary excretion of alpha-lipoic acid following oral administration in healthy volunteers. *J Clin Pharmacol.* 2003; 43:1257–1267. [PubMed: 14551180]
37. Tarnopolsky MA. The mitochondrial cocktail: rationale for combined nutraceutical therapy in mitochondrial cytopathies. *Adv Drug Deliv Rev.* 2008; 60:1561–1567. [PubMed: 18647623]
38. Stahl W, Sies H. Antioxidant defense: vitamins E and C and carotenoids. *Diabetes.* 1997; 46(Suppl 2):S14–S18. [PubMed: 9285493]
39. Viallalba JM, Parrado C, Santos-Gonzalez M, Alcain FJ. Therapeutic use of coenzyme Q10 and coenzyme Q10-related compounds and formulations. *Expert Opin Investig Drugs.* 2010; 19:535–554.
40. Sena CM, Nunes E, Gomes A, et al. Supplementation of coenzyme Q10 and alpha-tocopherol lowers glycated hemoglobin level and lipid peroxidation in pancreas of diabetic rats. *Nutr Res.* 2008; 28:113–121. [PubMed: 19083397]
41. Singh U, Jialal I. Alpha-lipoic acid supplementation and diabetes. *Nutr Res.* 2008; 66:646–657.
42. Chung CH, Hao E, Piran R, Keinan E, Levine F. Pancreatic β -cell neogenesis by direct conversion from mature alpha-cells. *Stem Cells.* 2010; 28:1630–1638. [PubMed: 20653050]
43. Thorel F, Nepote V, Avril I, et al. Conversion of adult pancreatic alpha-cells to beta-cells after extreme beta-cell loss. *Nature.* 2010; 464:1149–1154. [PubMed: 20364121]
44. Bruin JE, Petre MA, Raha S, et al. Fetal and neonatal nicotine exposure in Wistar rats causes progressive pancreatic mitochondrial damage and beta cell dysfunction. *PLoS One.* 2008; 3:e3371.
45. Bruin JE, Gerstein HC, Morrison KM, Holloway AC. Increased pancreatic beta cell apoptosis following fetal and neonatal exposure to nicotine is mediated via the mitochondria. *Toxicol Sci.* 2008; 103:362–370. [PubMed: 18203686]
46. Tharakan B, Holder-Haynes JG, Hunter FA, Childs EW. Alpha lipoic acid attenuates microvascular endothelial cell hyperpermeability by inhibiting the intrinsic apoptotic signaling. *Am J Surg.* 2008; 195:174–178. [PubMed: 18096126]
47. Naderi J, Somayajulu-Nitu M, Mukerji A, et al. Water-soluble formulation of Coenzyme Q10 inhibits Bax-induced destabilization of mitochondria in mammalian cells. *Apoptosis.* 2006; 11:1359–1369. [PubMed: 16830230]
48. Moon Y, Lee KH, Park JH, Geum D, Kim K. Mitochondrial membrane depolarization and the selective death of dopaminergic neurons by rotenone: protective effect of coenzyme Q10. *J Neurochem.* 2005; 93:1199–1208. [PubMed: 15934940]
49. Papucci L, Schiavone N, Witort E, et al. Coenzyme q10 prevents apoptosis by inhibiting mitochondrial depolarization independently of its free radical scavenging property. *J Biol Chem.* 2003; 278:28220–28228. [PubMed: 12736273]
50. Moreira PI, Santos MS, Sena C, et al. CoQ10 therapy attenuates amyloid beta-peptide toxicity in brain mitochondria isolated from aged diabetic rats. *Exp Neurol.* 2005; 196:112–119. [PubMed: 16126199]

51. de Arriba G, de Hornedo JP, Rubio SR, et al. Vitamin E protects against the mitochondrial damage caused by cyclosporin A in LLC-PK1 cells. *Toxicol Appl Pharmacol.* 2009; 239:241–250. [PubMed: 19523970]
52. Al Ghafli MH, Padmanabhan R, Kataya HH, Berg B. Effects of alpha-lipoic acid supplementation on maternal diabetes-induced growth retardation and congenital anomalies in rat fetuses. *Mol Cell Biochem.* 2004; 261:123–135. [PubMed: 15362495]
53. Teran E, Hernandez I, Nieto B, et al. Coenzyme Q10 supplementation during pregnancy reduces the risk of pre-eclampsia. *Int J Gynaecol Obstet.* 2009; 105:43–45. [PubMed: 19154996]
54. Xu H, Perez-Cuevas R, Xiong X, et al. An international trial of antioxidants in the prevention of preeclampsia (INTAPP). *Am J Obstet Gynecol.* 2010; 202:239.e1–239.e10. [PubMed: 20207239]
55. Poston L, Briley AL, Seed PT, Kelly FJ, Shennan AH. Vitamin C and vitamin E in pregnant women at risk for pre-eclampsia (VIP trial): randomised placebo-controlled trial. *Lancet.* 2006; 367:1145–1154. [PubMed: 16616557]
56. Villar J, Purwar M, Meriardi M, et al. World Health Organization multicentre randomised trial of supplementation with vitamins C and E among pregnant women at high risk for pre-eclampsia in populations of low nutritional status from developing countries. *BJOG.* 2009; 116:780–8. [PubMed: 19432566]
57. Chappell LC, Seed PT, Briley AL, et al. Effect of antioxidants on the occurrence of pre-eclampsia in women at increased risk: a randomized trial. *Lancet.* 1999; 354:810–816. [PubMed: 10485722]
58. Sen S, Simmons RA. Maternal antioxidant supplementation prevents adiposity in the offspring of western diet-fed dams. *Diabetes.* 2010; 59:3058–3065. [PubMed: 20823102]
59. Somm E, Schwitzgebel VM, Vauthay DM, et al. Prenatal nicotine exposure alters early pancreatic islet and adipose tissue development with consequences on the control of body weight and glucose metabolism later in life. *Endocrinology.* 2008; 149:6289–6299. [PubMed: 18687784]
60. Montgomery SM, Ekblom A. Smoking during pregnancy and diabetes mellitus in a British longitudinal birth cohort. *BMJ.* 2002; 324:26–27. [PubMed: 11777801]
61. Matveyenko AV, Butler PC. Relationship between β -cell mass and diabetes onset. *Diabetes Obes Metab.* 2008; 10 (Suppl 4):23–31. [PubMed: 18834430]

Significant findings of this study

An antioxidant intervention during pregnancy protects fetal beta cells from the damaging effects the pro-oxidant chemical nicotine. This beta cell protection occurs via a reduction in beta cell apoptosis, and increased expression of endocrine cells co-expressing glucagon and insulin.

What this study adds

Since children born to women who smoke during pregnancy are at an increased risk of developing type 2 diabetes, these results provide evidence that an antioxidant intervention during pregnancy for these women may protect the developing fetal pancreas.

Figure 1. Body weight (1A) and fat pad weight at weaning. Total (1B) and relative (as a proportion of body weight) fat pad weight (1C) are presented. All data are presented as the mean $\pm$ SEM. Data with different superscripts are significantly ($p < 0.05$) different from each other.

Figure 2.

Beta cell fraction (beta cell area/total pancreas area $\times$ 100), apoptosis (percentage of TUNEL⁺ beta cells; TUNEL = terminal dUTP-mediated nick end labeling) and proliferation (percentage of PCNA⁺beta cells; PCNA = proliferating cell nuclear antigen) at postnatal day 21. Representative photographs of immunohistochemical staining of beta cells are provided for SV, NV and NA offspring at postnatal day 21. Brown staining indicates insulin-positive beta cells. All data are presented as the mean $\pm$ SEM.

Figure 3.

A) Quantification of cells co-expressing insulin and glucagon at postnatal day 21 (expressed as the percentage of islets with co-positive cells). Representative photographs of two islets with bi-hormonal cells are provided in panels C and D. Glucagon-positive cells are shown in green, insulin-positive cells in red and bi-hormonal cells in yellow, indicated by either white arrows (single cells) or white boxes (bi-hormonal cell clusters). Co-localization of insulin and glucagon granules in the same cell was confirmed by electron microscopy in panel B (red and green arrows indicate glucagon and insulin granules, respectively, located adjacent within the same islet cell). All data are presented as the mean $\pm$ SEM.

Table 1**Pregnancy and birth outcomes**

Outcome measure	Saline (SC)	Nicotine (NC)	Nicotine + Antioxidant (NA)
Time to copulation (days)	2.9 ± 0.5	2.8 ± 0.5	3.1 ± 0.3
Mating success rate (%)	100	100	90
Pregnancy success rate (%)	100	93	100
Gestation length (days)	21.1 ± 0.2	21.0 ± 0.1	21.3 ± 0.2
Live birth index (%)	100	99.6 ± 0.4	95.6 ± 3.7
Litter size (N)	13.4 ± 1.1	14.1 ± 1.0	15.4 ± 1.0
Sex ratio (M/F)	1.1 ± 0.2	1.1 ± 0.2	1.5 ± 0.2
Birthweight (g)	6.0 ± 0.3	6.1 ± 0.1	5.2 ± 0.2 [†]

Data are presented as mean ± SEM. Values with a † are significantly (p<0.05) different from nicotine-exposed animals randomized to receive normal diet.