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Maternal Oxidative Stress, Placental Morphometry, and Fetal Growth in Diabetic Rats Exposed to Cigarette Smoke

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Abstract

The diabetic syndrome affects pregnancy, contributing to placental functional and structural disruptions and impaired fetal development, with many reports indicating tobacco-associated morbidity and perinatal mortality. In our study, an experimental rat model of diabetes and cigarette smoke exposure in pregnant rats was used to determine the impact of the combination of diabetes and exposure to cigarette smoke during pregnancy on maternal oxidative stress biomarkers and placental and fetal development. Diabetes was induced by streptozotocin, and dams were exposed to cigarette smoke by mainstream smoke generated by a mechanical smoking device and delivered into a chamber. Four groups of dams were studied: nondiabetic (C, control) and diabetic (D) exposed to filtered air and nondiabetic (CS) and diabetic (DS) exposed to cigarette smoke prior to and during pregnancy. Maternal oxidative stress biomarkers, placental morphology, and fetal growth were determined close to term. The combination of diabetes and cigarette smoke resulted in elevated maternal blood glucose levels and increased number of small fetuses. Placentas from the DS group showed increased junctional zone and decreased labyrinthine area. The morphological alterations were characterized by extensive vascular congestion, thickness, and hyalinization of the vascular walls, numerous decidual cells with abundant glycogen, and macrophages with cytoplasmic inclusions of hemosiderin. Additionally, they showed increased glycogen accumulation and junctional zone structural derangement with ectopic giant cells. No alterations were observed in maternal oxidative stress status. Thus, our result suggests that diabetes makes pregnant rats more susceptible to the adverse effects of exposure to cigarette smoke on placental morphometry and fetal growth.

Keywords

diabetes, pregnancy, cigarette smoke, placenta, oxidative stress

Introduction

Fetal metabolism and growth are critically dependent on placental nutrient transfer, and fetal demand requires profound changes in maternal metabolism to allow allocation of nutrients to the fetus.[1] Placental transfer of glucose, the primary energy substrate of the fetus, is mediated by facilitative diffusion according to concentration-dependent kinetics under normal conditions.[2] However, exposure to the elevated maternal glucose in diabetes may adversely affect the placental structure and function,[3,4] including increased glycogen deposition and placental weight.[5]

Smoking during pregnancy adversely affects the health of both mother and child and is associated with increased perinatal morbidity and mortality.[6] The components of cigarette smoke have the ability to cross the placental membrane and this compromises fetal development.[7] Maternal smoking has been reported to increase chorionic vascular density already in first trimester of pregnancy,[8] as a compensatory response to the impairment in oxygen carrier capacity associated with smoking. In addition, the increased generation of reactive oxygen species caused by cigarette smoke and the heavy metals, carbon

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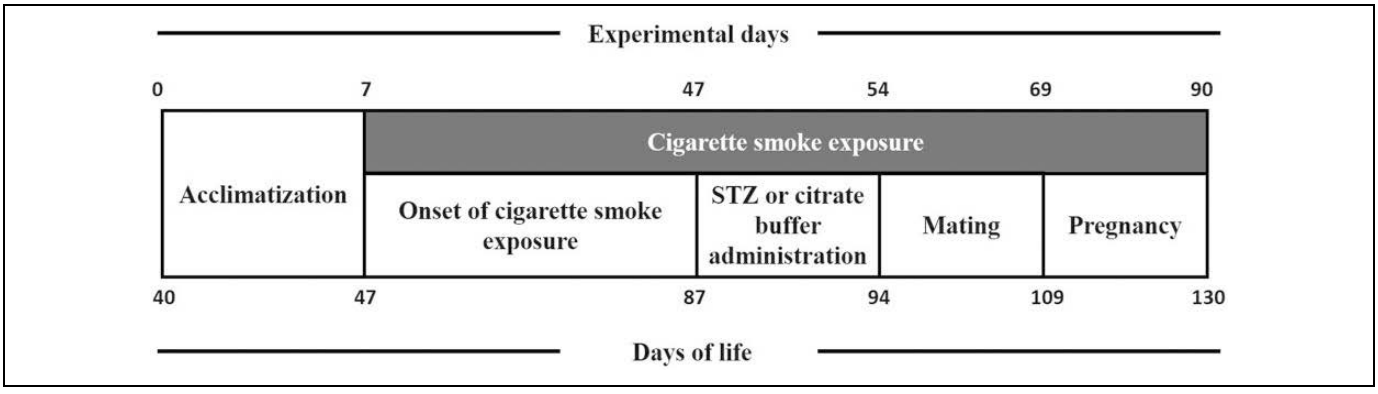

Figure 1. Experimental design used to study the effects of diabetes induction associated with cigarette smoke in rats.

monoxide, and nicotine in cigarette smoke may increase ischemia.[9]

The prevalence of smoking among individuals with and without diabetes is similar. Only about half of patients with diabetes are advised to quit smoking by their health-care providers.[10] In our Diabetes and Pregnancy Service (Botucatu Medical School_UNESP, Brazil), 12.9% of nondiabetic pregnant women and 24.8% of diabetic pregnant women smoked. Although both smoking and diabetes are independent risk factors for pregnancy and neonatal complications, very little is known about how these two adverse effects interact.

In the present study, an experimental rat model of diabetes and cigarette smoke exposure in pregnant rats was used to determine the impact of the combination of diabetes and exposure to cigarette smoke during pregnancy on maternal oxidative stress biomarkers and placental and fetal development.

Materials and Methods

Animals and Experimental Groups

Four-week-old female and male Wistar rats were obtained from São Paulo State University (UNESP), São Paulo, Brazil. During the 2-week acclimatization and the experimental exposure periods, animals (4 rats per cage) were maintained in an experimental room under controlled temperature (22 ± 2°C), humidity (50% ± 10%), in a 12-hour light/dark cycle with ad libitum access to a commercial diet (rat chow) and tap water. Four groups of dams were studied (n= 13 animals/group): nondiabetic (C, control) and diabetic (D) exposed to filtered air and nondiabetic (CS) and diabetic (DS) exposed to cigarette smoke prior to and in the pregnancy period. Procedures and animal handling were performed in accordance with the guidelines provided by the Brazilian College of Animal Experimentation in agreement with the International Guiding Principles for Biomedical Research Involving Animals promulgated by the Society for the Study of Reproduction. All procedures were authorized by the Ethical Committee for Animal Research of the São Paulo State University (UNESP), Brazil (385/2004).

Experimental Design

The experimental approach to induce severe diabetes and to expose rats to cigarette smoke has been described in detail elsewhere.[11] (Figure 1).

Cigarette Smoke Exposure Procedure

Six-week-old female rats placed into whole-body exposure chambers were exposed to filtered air or cigarette smoke for 30 minutes twice a day (equivalent to 20 cigarettes/d, with a carbon monoxide concentration of 193.50 parts/million).[12]

Induction of Diabetes

Approximately 6 weeks after starting cigarette smoke or filtered air exposure, diabetes was induced in the rats with streptozotocin (STZ; Sigma Chemical Company, St Louis, Millstone). Streptozotocin was dissolved in a citrate buffer (0.1 mol/L, pH 4.5) and administered intravenously by injection at a dose of 40 mg/kg of body weight (D and DS). Nondiabetic (C and CS) rats received only citrate buffer. Severe diabetes developed 7 days after STZ injection and was confirmed by a blood glucose concentration superior to 200 mg/dL. The animals were continued to maintain severe hyperglycemia during the whole experimental period.

Mating and Pregnancy

Female rats were mated overnight to nondiabetic male rats unexposed to cigarette smoke. The morning when sperm was observed in the vaginal smear was designated gestational day 0. Nonfasting blood glucose levels (food ad libitum overnight) were monitored in the morning using a conventional glucometer at days 0 and 21 of pregnancy in all experimental groups. At day 21 of pregnancy, the dams were anesthetized with sodium pentobarbital (Hypnol 3%) for laparotomy. Fetuses and their respective placentas were isolated and weighed. Placental efficiency was calculated as the ratio of the fetal to placental weights. One placenta uterine horn per dam was sectioned medial sagittally and fixed in 10% buffered formalin before being processed for paraffin embedding. The

Table 1. Maternal, Fetal, and Placental Analysis From Different Experimental Groups.[a]

Variables	Groups			
	C	CS	D	DS
Dams				
Glycemia (mg/dL) day 0	81.5 (16.7)[b]	86.2 (7.8)[b]	459.3 (65.5)[c]	491.0 (52.1)[d]
Glycemia (mg/dL) day 21	92.7 (10.2)[b]	88.9 (9.3)[b]	480.6 (73.1)[c]	580.1 (33.1)[d]
Fetuses				
Number of live fetuses	12.3 (1.1)[b]	10.2 (2.6)[c]	10.7 (1.1)[c]	6.7 (3.8)[d]
Fetal weight (g)	5.3 (0.3)[b]	5.1 (0.3)[b]	4.3 (0.5)[c]	4.5 (0.4)[c]
SGA (%)	3.6[b]	5.5[b]	36.2[c]	49.2[d]
AGA (%)	94.0[b]	93.2[b]	63.8[c]	50.8[d]
LGA (%)	2.4[b]	1.4[b]	0[b]	0[b]
Placentas				
Placental weight (g)	0.48 (0.02)[b]	0.44 (0.03)[b]	0.63 (0.08)[c]	0.68 (0.18)[c]
Placental efficiency	11.12 (0.74)[b]	11.14 (1.76)[b]	7.27 (1.62)[c]	7.25 (1.56)[c]
Decidual area (mm^2)	0.09 (0.03)[b]	0.07 (0.02)[c]	0.07 (0.03)[c]	0.07 (0.02)[c]
Junctional area (mm^2)	0.29 (0.08)[b]	0.31 (0.13)[c]	0.28 (0.11)[b]	0.37 (0.10)[d]
Labyrinth area (mm^2)	7.42 (1.19)[b]	6.85 (1.41)[c]	6.80 (1.52)[c]	6.39 (1.19)[d]

Abbreviations: AGA, appropriate for gestational age; C, nondiabetic rats exposed to filtered air; CS, nondiabetic rats exposed to cigarette smoke; D, diabetic rats exposed to filtered air; DS, diabetic rats exposed to cigarette smoke; LGA, large for gestational age; SD, standard deviation; SGA, small for gestational age.
[a]Values are expressed as mean (SD). $P < .05$—different letters represent significant difference among groups. Student unpaired *t* test and proportions (%) Fisher exact test.

number of live fetuses was determined and then they were individually classified according to the mean values of fetal weights of the nondiabetic group (C): small for gestational age (SGA) when weight was smaller than C mean (−1.7) standard deviation (SD); appropriate for gestational age (AGA) when weight was included in C mean (±1.7) SD; and large for gestational age (LGA) when weight was greater than C mean (+1.7) SD.[13]

Oxidative Stress Biomarkers

Oxidative stress was analyzed using protocols as we described previously[14] with some modifications. Washed erythrocytes were used to quantify the following biomarkers: thiobarbituric acid reactive species (TBARS; estimated as an index for lipid peroxidation) and sulfhydryl groups (SH) concentrations and the antioxidative activity of the enzymes as superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px).

Placental Morphology and Morphometry

Formalin-fixed placentas were dehydrated in a graded ethanol series and embedded in paraffin according to a standard protocol, sectioned at 5 μm, and mounted on glass slides for hematoxylin and eosin staining. One placenta from each rat was randomly chosen for morphological and morphometrical analyses. The overall morphology and pathology of the placenta (decidual, junctional, and labyrinthine zones) were evaluated under a light microscope in the medial layer. The placental morphometric analyses were performed in a computerized image system coupled to a photomicroscope through a digital camera. Slides were preselected to assure the presence of all placental layers in the sample. From each slide, 6 areas were randomly selected. The areas (mm^2) of placental layers were analyzed. Decidual and junctional zones were evaluated at the magnification of ×50, whereas the labyrinthine region was evaluated at the magnification of ×25.

Statistics

Data are presented as the mean (SD). The statistical differences between the groups were determined. The homogeneity among experimental units is one of the basics of experimental design, and considering that CS, D, and DS are biologically different than C group, and the comparison between DS versus C, D versus C, DS versus C, and DS versus D was evaluated by Student unpaired *t* test. For normal distribution, the proportion data were analyzed by Fisher exact test. SAS software (version 9.3) was applied for all statistical analyses. $P < .05$ was considered as statistically significant.

Results

Blood Glucose Concentrations

The C and CS groups were both normoglycemic. The D and DS groups exhibited hyperglycemia with mean blood glucose levels >450 mg/dL, which were significantly higher than in the control groups. In addition, blood glucose levels in the DS group were significantly higher than in the D groups ($P < .05$; Table 1)

Fetal Parameters

In the diabetic groups, independent of exposure to cigarette smoke, fetal weight was decreased when compared to the nondiabetic groups ($P < .05$; Table 1). There was a decrease in the

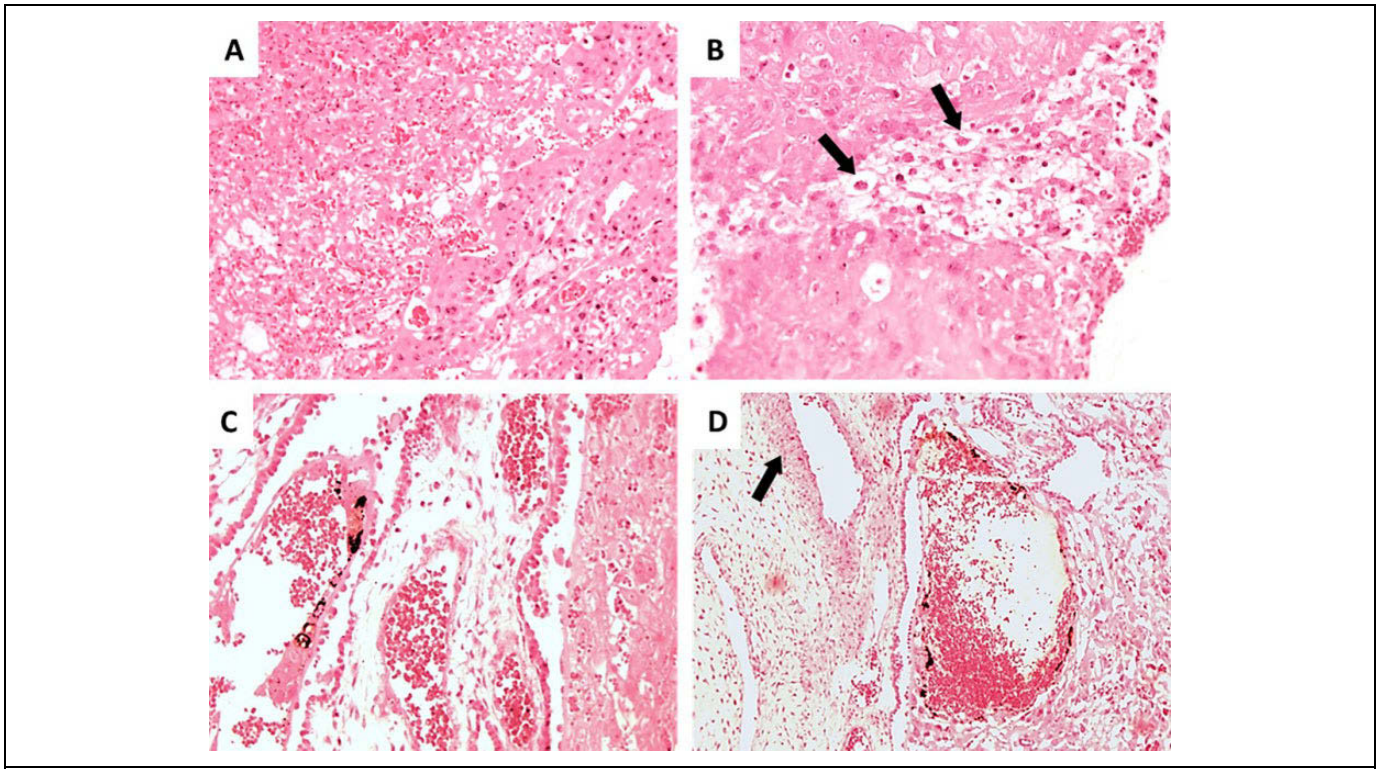

Figure 2. Representative micrographs of the placenta from the different experimental groups. (A) Micrograph from the control group shows the normal histological appearance of the decidual and labyrinthine layers. (B) In contrast, placenta from the D group shows wide areas of decidual cells with clear cytoplasm corresponding to glycogen depots (arrows). (C) Placenta section from the CS group shows vascular congestion and some macrophages in the blood vessels wall with black precipitates in the cytoplasm that correspond to hemosiderin. (D) Vascular congestion, hemosiderin deposits in the vascular wall, and some vessels with mild thickness (arrow) are seen in placenta from the DS group. CS indicates nondiabetic rats exposed to cigarette smoke; D, diabetic rats exposed to filtered air; DS, diabetic rats exposed to cigarette smoke.

number of live fetuses in the CS, D, and DS groups when compared to the control group. The number of live pups in the DS group was significantly lower than in the C, CS, and D groups ($P < .05$; Table 1). The C and CS groups had a higher proportion of fetuses classified as appropriate for gestational age (AGA) than the D and DS groups. There was a higher proportion of SGA fetuses in diabetic groups when compared to nondiabetic groups (C and CS). Moreover, the proportion of SGA fetuses was significantly higher, and the AGA fetuses were lower in the DS group when compared to the D group. The proportion of fetuses LGA was not different among groups ($P < .05$; Table 1).

Placental Parameters

Placental weight was increased in the D and DS groups when compared to the C and CS groups, and the placental efficiency was diminished in the D and DS groups ($P < .05$; Table 1).

In comparison with placentas from the C group (Figure 2A), placentas from the D group showed arteries with wide and fibrous walls with congestion and numerous decidual cells with clear cytoplasm that correspond to glycogen accumulation (Figure 2B). The placentas from the CS group had signs of accentuated vascular congestion and numerous macrophages with brown dark precipitates in the cytoplasm that correspond to hemosiderin (Figure 2C). In the DS group, placentas had extensive vascular congestion, thickness, and hyalinization of the vascular walls and numerous decidual cells with abundant glycogen and macrophages with cytoplasmic inclusions of hemosiderin. Thus, as expected, placenta from the DS group showed a combination of histological changes observed in both the D and CS groups (Figure 2D). Additionally, placentas from the DS group (Figure 3D) showed increased glycogen accumulation and junctional zone structural derangement with ectopic giant cells when compared to the C, CS, and D groups (Figure 3A, B, and C, respectively).

Diabetes and exposure to smoking influenced placental morphometry (Table 1). In comparison with the C group, the decidual area was thinner in the D, CS, and DS groups ($P < .05$), being similar among these groups ($P > .05$), while the junctional area was thicker in the smoking groups CS and DS when compared to the C and D groups ($P < .05$), being the widest in the DS group when compared to others ($P < .05$). In comparison with the C group, the labyrinthine area was thinner in the D, CS and DS groups, with the labyrinthine zone in DS being the thinnest when compared to the C, CS, and D groups ($P < .05$) (Table 1).

Oxidative Stress Biomarkers

The concentrations of TBARS and SH and the SOD and GSH-Px activity were increased in the CS, D, and DS rats when

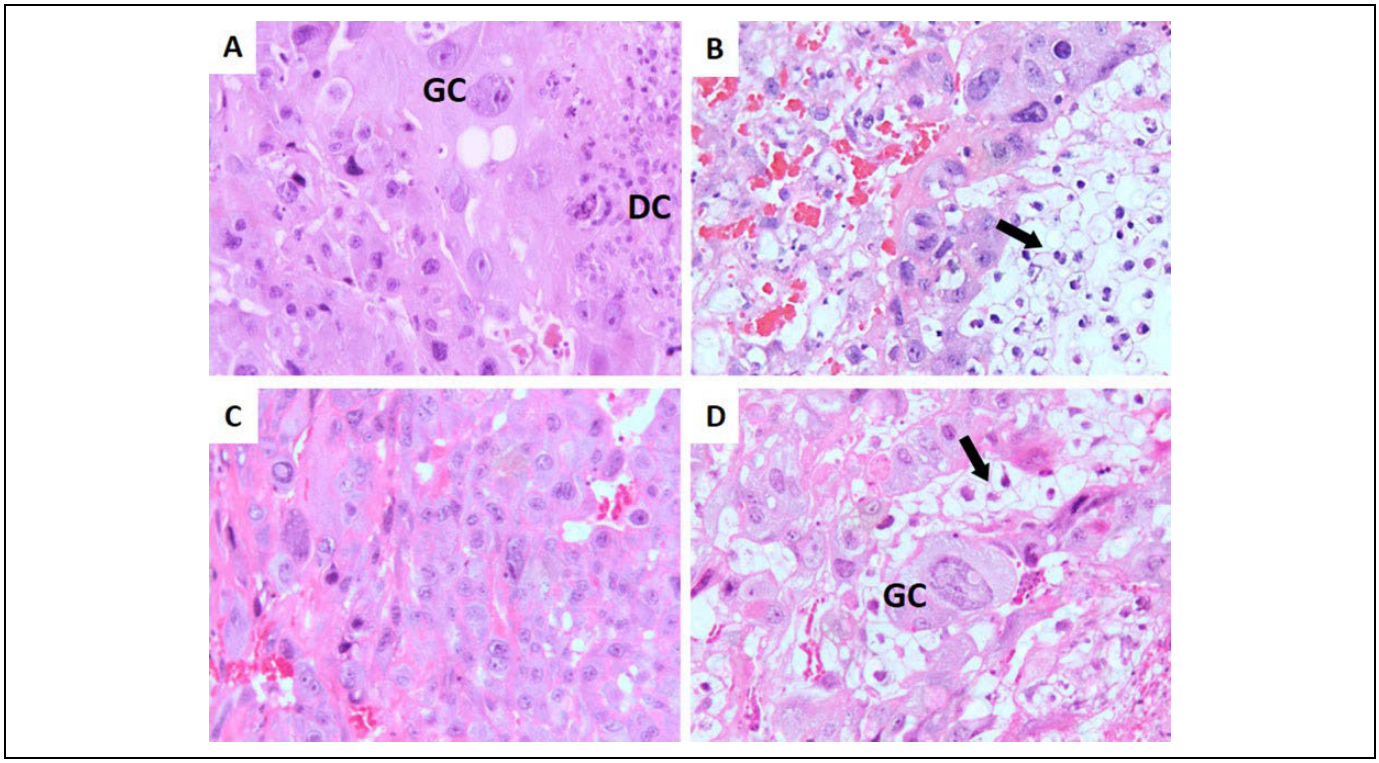

Figure 3. Representative micrographs of the placenta from the different experimental groups. (A) Micrograph from the control group shows the normal histological appearance of the junctional zone. (B) In contrast, placenta from the D group shows wide areas of clear cytoplasm corresponding to glycogen depots (arrow). (C) Placenta section from the CS group shows vascular congestion. D, Structural derangement with glycogen (arrow) depots and ectopic giant cells are present in placental junctional zone from DS group. CS indicates nondiabetic rats exposed to cigarette smoke; (D) diabetic rats exposed to filtered air; DC, decidual cells; DS, diabetic rats exposed to cigarette smoke; GC, giant cells.

Table 2. SOD and GSH-Px Enzymatic Activity and TBARS and SH Concentrations From Different Groups.[a]

	Groups			
Variables	C	CS	D	DS
TBARS (nM/gHb)	50.3 (22.2)[b]	386.8 (368.3)[c]	1165.5 (730.3)[d]	872.3 (678.5)[d]
SOD (UI/mgHb)	2.0 (1.2)[b]	11.2 (9.9)[c]	15.5 (11.5)[d]	20.9 (16.0)[d]
GSH-Px (UI/mgHb)	0.1 (0.1)[b]	0.4 (0.3)[c]	0.3 (0.3)[c]	1.7 (1.6)[d]
SH (μmol/g Hb)	6.4 (7.6)[b]	60.7 (67.2)[c]	37.5 (54.5)[d]	22.1 (7.1)[e]

Abbreviations: C, nondiabetic rats exposed to filtered air; CS, nondiabetic rats exposed to cigarette smoke; D, diabetic rats exposed to filtered air; DS, diabetic rats exposed to cigarette smoke; GSH-Px, glutathione peroxidase; SD, standard deviation; SH, sulfhydryl groups; SOD, superoxide dismutase; TBARS, thiobarbituric acid reactive species.

[a]Values are expressed as mean (SD). $P < .05$—different letters represent significant difference among groups. Student unpaired *t* test.

compared to the C group. The DS group showed no alterations in lipoperoxidation and SOD activity when compared to the D group ($P < .05$; Table 2).

Discussion

The present study showed that pregnant diabetic dams are more susceptible to the adverse effects of exposure to cigarette smoke on placental morphometry and fetal growth. The cigarette smoke exposure elevated blood glucose levels further in diabetic animals and caused placental morphometric and histopathological changes, reduction of live fetuses, and decreased fetal weight suggesting intrauterine growth restriction.

In our study, diabetes and cigarette smoke, in isolation, altered decidual and labyrinthine placental layers, and their combination altered junctional and labyrinthine zones. The importance of these layers for a successful pregnancy is undeniable. The decidualization of uterine tissue is essential to establish successful pregnancy,[15] and embryonic loss was observed by ovariectomy-mediated progesterone withdrawal due to "collapse" of the rat decidua.[16] Decidual cell

differentiation is dependent on the regulatory actions of ovarian steroids, progesterone, and 17 β-estradiol.[17,18] Reduced progesterone levels are present in both hyperglycemia[19] and cigarette smoke exposure[20] and seem to be related to decreased decidual area; however, the association has no influence in morphometry of this layer.

Diabetes reduced the area of the placental labyrinthine zone, and this effect was exacerbated by exposure to cigarette smoke in diabetic dams. It is likely that the combined action of hyperglycemia, heavy metals, and cigarette components (CO and nicotine) adversely affected the labyrinthine zone. The labyrinthine zone of the rodent placenta represents the maternal–fetal exchange area,[21,22] and alterations in the thickness of this area, as seen in our study, may affect oxygen and nutrient transfer. Thus, the decreased labyrinthine thickness may impair placental oxygen and nutrient delivery to the fetus, contributing to the markedly decreased number of live fetuses and decreased fetal weight observed in the diabetic group exposed to cigarette smoke. Additionally, cigarette smoking has been reported to impair placental vasculature and subsequently restrict fetal growth.[23,24] 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD), a type of "dioxin" or "dioxin-like" component in cigarette smoke, suppressed the placental vascular remodeling, including reduced the ratio of the placental labyrinthine zone to the basal zone thickness[25] as verified in our study. Similarly, the TCDD might adversely affect the placental vasculature and possibly contribute to multiple old focal hemorrhages (the presence of macrophages with cytoplasmic hemosiderin).

The combination of diabetes and cigarette smoke exposure also increased the junctional zone area, which may explain the maintained placental weight and placental efficiency despite the decrease in area of the labyrinthine zone. A marked increase exclusively in these rats in the junctional zone and hypertrophy of glycogen cells suggests that this area, in particular, is a direct target of cigarette smoke exposure. The junctional zone secretes an array of signaling molecules and hormones including a number of polypeptide and steroid hormones[26] such as those of the prolactin-like (PRL) family.[27,28] The PRL family members have been divided into classical (5 members), consisting of the placental lactogen subfamily, and nonclassical based on their biological activities (whether they are functioning through the PRL receptor or other signaling pathways, respectively).[28] Classical PRL proteins are involved in the control of protein hormone synthesis,[29] with a possible role in placental morphogenesis.[30] Furthermore, nonclassical PRL family members can also inhibit blood vessel development and may share receptors with proliferin and proliferin-related proteins,[31] which are involved in the placental angiogenic process.[32] Moreover, cigarette smoke exposure can trigger important mechanisms involved in vascular obstructions, tissue ischemia, and impaired angiogenesis.[33] Another potential explanation for the junctional zone hypertrophy diabetic dams exposed to cigarette smoke could be a compensatory mechanism to increase proliferin concentration. However, the exact mechanism by which the junctional zone layer is increased remains to be elucidated.

No direct association among cigarette smoke, diabetes, and increased lipoperoxidation or their SOD activity and thiol group concentration was found. Then it is possible that the uncontrolled hyperglycemia triggers an unbalanced metabolic state, in which cigarette smoke exposure caused no exacerbation of its deleterious effects.

Thus, diabetes and cigarette smoke exposure association causes no additional alterations on maternal oxidative stress. However, this association impairs maternal–fetal exchange in the placental interface, which contributes to reduced fetal growth and number of live fetuses.

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