DOES SMOKING OF DIABETIC RAT MOTHERS’ IN PRE_PREGNANCY PERIOD EXERT EFFECTS ON FETAL OUTCOME?

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ABSTRACT

BACKGROUND: The potential effects of cigarette smoke exposure on reproductive outcomes are a major scientific and public health concern. Maternal hyperglycemia during early pregnancy is associated with increased risk of abnormalities in the offspring which are 2-5-fold higher than that of the normal population. THE AIM OF THE STUDY: Is to evaluate the effect of smoking in pre-pregnancy period of diabetic mothers on fetal development. MATERIALS AND METHODS: Sixty diabetic female rats were randomly distributed in three experimental groups, according to presence or absence of cigarette smoke exposure: group I; twenty diabetic rats exposed to filtered air that serve as negative control group(D), group II; twenty diabetic rats exposed to cigarette smoke before and during pregnancy (DS) and serves as positive control and group III; twenty diabetic rats exposed only to cigarette smoke in pre-pregnancy period (DSPP). Diabetes was induced by streptozotocin and exposure to cigarette smoke for six weeks. At day 21 of pregnancy, rats were killed for maternal biochemical determination and reproductive outcomes, and fetal anomaly analyses. Data were analyzed by tests according to symmetrical or asymmetrical distribution (data considered significant if p<0.05). RESULTS: The exposure to smoking reduced triglycerides and VLDL concentrations, no change in oxidants level, reduced anterior phalanges, metatarsus, and caudal vertebrae numbers in fetuses from these rats. CONCLUSION: It is necessary to cease smoking long before planning pregnancy since stopping smoking only when pregnancy is detected may not contribute effectively to fully adequate embryo-fetal development.

KEY WORDS: Rat, diabetes, cigarette, smoke, pregnancy, anomaly, oxidative stress

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INTRODUCTION

Diabetes mellitus (DM) is a serious medical problem whose prevalence has increased dramatically. DM is a metabolic disorder characterized by hyperglycemia, insufficient insulin secretion and receptor insensitivity to endogenous insulin. In pregnancies complicated by diabetes, hyperglycemia and lipid metabolism alterations are associated with both maternal and fetal complications causing reproductive abnormalities that enhance spontaneous abortion, congenital anomalies, and neonatal morbidity and mortality. Previous studies have suggested that the teratological impact of a diabetic environment partly depends on excess of reactive oxygen species (ROS) in the embryo as a consequence of either: increased free oxygen radical formation or decreased capacity of ROS-scavenging enzymes or both. DM is a risk factor for atherosclerotic diseases, which can be aggravated by the presence of arterial hypertension, dyslipidemia and cigarette consumption. It is a well known fact that cigarette smoking constitutes a significant health hazard. It has long been established that maternal smoking during pregnancy has adverse consequences for the mother and the child. The toxic effect of maternal exposure to tobacco smoke on the fetal metabolic status is indicated by the significant disturbance of the antioxidant status in newborns exposed in utero to tobacco smoke. In addition, induction of oxidative stress and detrimental histopathological changes has been observed in the lungs and liver of breast-fed rat pups whose mothers were exposed to nicotine. A significant negative correlation has been observed between the number of cigarettes smoked per day by pregnant women and newborns’ weight, body mass index, length and head circumference. Experimental diabetes induced by cytotoxic drug administration, such as streptozotocin, during the adult life of laboratory animals causes severe diabetic clinical status that reproduces uncontrolled human DM1. Severe diabetes (glycemia superior to 300 mg/dL) causes maternal hypertriglyceridemia and hypercholesterolemia, which compromises maternal-fetal metabolism during pregnancy in rats. It causes increased maternal oxidative stress, increased rate of pre and post-implantation embryonic losses and intrauterine growth retardation (IUGR). Diabetic rats exposed to cigarette smoke during pregnancy presented placentomegaly, increased placental index, small fetuses for pregnancy age and decreased liver glycogen concentrations. Some scientists verified the association of severe diabetes and exposure to cigarette smoke before and during rat pregnancy was related to the incidence of hypertriglyceridemia, and this result was due to the diabetes and not to exposure to smoke. There was no alteration to protein metabolism at term pregnancy. Diabetes and cigarette smoke exposure led to the activation of the antioxidant system in an attempt to detoxify the organism in face of high lipid peroxidation, which can be characterized by the determination of reactive substances to thiobarbituric acid. However, there are no reports concerning the association of diabetes and cigarette smoke exposure in rats unexposed to tobacco smoke during pregnancy (pre-pregnancy exposure), thus reproducing the status of gravid women who stopped smoking when their pregnancy was positively diagnosed. Hence, The Aim of This Study was to evaluate the effects of such association on the maternal organism and on the development of offspring.

MATERIALS AND METHODS

Animal Grouping

Sixty Adult albino rats (200-250 g) were obtained from the animal house, Umm Al-Qura University, were used throughout the experiment and received balanced diet with...
free access to water. All animal procedures were performed according to approved protocols and in accordance with the recommendations for the proper care and use of laboratory animals. Animals were divided into three groups; group I: consisted of twenty diabetic rats exposed to filtered air (D) that serve as negative control group, group II: consisted of twenty diabetic rats exposed to cigarette smoke before and during pregnancy (DS) and serves as positive control and (group III): consisted of twenty diabetic rats exposed only to cigarette smoke before pregnancy (pre-pregnancy) (DSPP).

2. Methods
2.1 Animals and experimental groups
Eight-weeks-old female and ten-weeks-old male albino rats, weighing approximately 190g and 220g respectively, were obtained from UQU, animal House. During the two-week acclimatization and the experimental exposure periods, rodents were maintained in an experimental room under controlled conditions of temperature (22-25 ºC), no humidity, and a 12-hour light/dark cycle with normal diet and bottles water. A total of 60 rats were randomly distributed into three groups (each group consisted of 20 rats) as follows:

GROUP 1: consisted of twenty diabetic rat exposed to filtered air (D) that serve as negative control group.

GROUP 2: consisted of twenty diabetic rats exposed to cigarette smoke before and during pregnancy (DS) and serves as positive control.

GROUP 3: consisted of twenty diabetic rats exposed only to cigarette smoke before pregnancy (pre-pregnancy) (DSPP).

2.2 Experimental design
The experimental design for severe diabetes and cigarette smoke exposure in rats was performed according to Lima et al. 27

2.2.1 Cigarette smoke or filtered-air exposure procedure
Eight-weeks-old females white albino rats were placed into whole-body exposure chambers and exposed to filtered air (D) or to cigarette smoke (DS and DSPP) for 30 minutes, twice a day [20 cigarettes/day], for two months (DS and DSPP). During exposure, the temperature was maintained at 22-25 ºC with no humidity.

2.2.2 Induction of diabetes
Approximately six weeks after filtered air or cigarette smoke exposure, diabetes was induced by streptozotocin (STZ - SIGMA Chemical Company, St. Louis, Millstone). STZ was dissolved in citrate buffer (0.1M, pH 6.5) and administered by intravenous injection at a dose of 40 mg/kg body weight to all groups.

Inclusion criteria
Any rat developed diabetic state which confirmed by glycemia > 300 mg/dL seven days after STZ injection by a One-Touch Ultra Johnson & Johnson glucometer. Values were expressed in milligrams per deciliter (mg/dL) 22.

2.2.3 Mating Procedure
All female rats were mated overnight to non-diabetic male rats unexposed to cigarette smoke. The morning when sperm was found in the vaginal smear was designated gestational day 0. The mating procedure consisted of 2 consecutive weeks, a period comprising approximately three estral cycles, until a replicate number of groups were obtained.

Exclusion criteria
All the non-mated female rats after this period were considered to be infertile and discarded from the study. The rats were then distributed into experimental groups (D, DS and DSPP).

2.2.4 Pregnancy Period
Glycemia was measured at days 0 and 21 of pregnancy in all experimental groups. At day 21 of pregnancy, the female rodents were
weighed for estimation of body weight gain (maternal weight at day 21 minus maternal weight at day 0 of pregnancy) and anesthetized with sodium pentobarbital (Hypnol® 3%) for laparotomy and collection of blood samples for biochemical determinations. The uterus was removed and weighed, and the ovaries and uterine contents examined to determine the number of corpora lutea and implantation sites, resorptions (embryonic death), and number and position of viable fetuses. The rate of embryonic loss before implantation was calculated as: (number of corpora lutea – number of implantations) × 100/number of corpora lutea, and used as a measure of failing conception effect or pre-implantation loss. The percentage of embryonic loss after implantation was calculated as: (number of implantations – number of live fetuses) × 100/number of implantations, which was used as a measure of abortifacient effect or for identification of post-implantation loss. In the lack of visible implantation sites, the uterine corns were stained with a preparation of 10% ammonium sulphate. Immediately after exploratory laparotomy, all viable fetuses and placentas were weighed for determination of the placental index (placental weight/fetal weight).

2.3 Biochemical analysis
Blood samples were divided in two ways: one portion of blood was put into anticoagulant-free test tubes and in others containing anticoagulant.

2.3.1 Assay for the lipid and protein profile
The blood samples without anticoagulant were kept at low temperature for 30 minutes (min) and then centrifuged for 10 min at 4°C. The supernatant was collected as serum and stored at -80°C for determination of total cholesterol (and fractions), triglycerides and proteins by using a Wiener assay kit.

2.3.2 Assay for oxidative stress status
Another portion of blood was placed into tubes containing anticoagulant and then centrifuged for 10 min at 4°C for assaying the oxidative stress status. The oxidative stress biomarkers estimated were superoxide dismutase (SOD), and malonaldehyde (MDA) as an index of lipid peroxidation.

MDA ESTIMATION
MDA and antioxidants were estimated in washed erythrocytes. MDA determination was measured by thiobarbituric acid (TBA). Briefly, 1.0 mL of washed erythrocytes were added to the test tube containing 1.0 mL of 3.0% sulphone salicylic acid, agitated for 10 seconds (sec), centrifuged for 3 min and kept in rest for 15 min. The sample was diluted to 500:1 of 0.67% TBA solution. The mixture was heated to 80°C for 30 min the results were expressed as nM of MDA per gram of hemoglobin (nM/g Hb).

SOD ESTIMATION
SOD activity was determined from its ability to inhibit pyrogallol auto-oxidation. The reaction mixture (1.0 mL) consisted of 5.0 mM Tris (hydroxymethyl) aminomethane (pH 8.0), 1.0 mM EDTA, bidistilled water and 20L of the sample. The reaction was initiated by the addition of pyrogallol (final concentration of 0.2 mM), and absorbance was measured by a spectrophotometer with a wavelength of 420 nm (25°C) for 5 min. Enzymatic activity units were defined as SOD units able to produce 50% of pyrogallol oxidation inhibition. All data were expressed in units of SOD per miligram of hemoglobin. Activity was measured on a spectrophotometer. One unit of activity was equal to the micromolar of substrate reduced per gram of hemoglobin.

HEMOGLOBIN DETERMINATION: Hb was performed to calculate oxidative stress biomarkers.
2.4 Fetal anomaly analysis
The fetuses were weighed and analyzed for incidence of external anomaly. After external analysis, half of the fetuses were fixed in Bodian’s fluid and serial sections were prepared for visceral examination as described by Wilson\textsuperscript{32}. The remaining fetuses were prepared for skeleton examination by the staining procedure of Staples and Schnell\textsuperscript{33}.

2.5 Ethical considerations
Ethical clearance was obtained from The Ethics Committee for Experimental Animal Research of UQU, Faculty of Pharmacy that approved the protocols used in this study.

2.6 Statistical analysis
1. The results of oxidative stress (MDA and SOD) were reported as mean ± standard deviation (SD) and analyzed by ANOVA multiple comparisons.
2. Other convenient statistical analyses were performed as:
   a. The glycemic level results were presented as mean ± standard error of mean (SEM).
   b. The reproductive outcome data were analyzed by Poisson regression (for implantation sites, live fetuses, reabsorption).
   c. Tukey’s multiple comparison (for litter, maternal, fetal and placental weights) and by negative binomial distribution (for external, skeletal and visceral anomaly frequencies). Statistical significance was considered as p < 0.05. All data were evaluated according to help of the statistical professional.

RESULTS
Sixty rats involved in the study, all rats developed diabetes and forty (40) only were exposed to cigarette smoke G2 (DS) and G3 (DSPP) presented no alteration (p>0.05) in glycemia at the beginning or end of pregnancy as compared to groups D and DS (Table 1). There was no statistical significant difference (P > 0.05) in glucose level among each group either at beginning or end of the study. There were statistical significant difference (P < 0.5) of G1 (D) in comparison with G 2 (DS) and G 3(DSPP) as shown in (Table 1).

<table>
<thead>
<tr>
<th>Groups</th>
<th>G1 (D) N=20</th>
<th>G2 (DS) N=20</th>
<th>G3 (DSPP) N=20</th>
</tr>
</thead>
<tbody>
<tr>
<td>Variables</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 0</td>
<td>420.62 ± 58.63</td>
<td>512.75 ± 41.84</td>
<td>493.64 ± 54.67</td>
</tr>
<tr>
<td>Day 21</td>
<td>469.83 ± 52.99</td>
<td>530.14 ± 91.32</td>
<td>555.07 ± 64.51</td>
</tr>
<tr>
<td>P value at day0 and day 21</td>
<td>P &gt; 0.5</td>
<td>P &gt; 0.5</td>
<td>P &gt; 0.5</td>
</tr>
<tr>
<td>P value</td>
<td>G1 Vs G2</td>
<td>G1 Vs G3</td>
<td>G1 Vs G3</td>
</tr>
<tr>
<td></td>
<td>P &lt; 0.5 *</td>
<td>P &lt; 0.5 *</td>
<td>P &lt; 0.5 *</td>
</tr>
</tbody>
</table>

G1 (D): Diabetic rats exposed to filtered air
G2 (DS) Diabetic rats exposed to cigarette smoke before and during pregnancy
G3 (DSPP) Diabetic rats exposed only to cigarette smoke just before pregnancy (pre-pregnancy)
Values are expressed as mean ± SD, n=number per each group.
* P < 0.5: Significant statistically difference; while N.S. P > 0.5: non significant value

In G2 (DS) and G3 (DSPP) females, the implantation sites and the live fetus number were reduced; litter weight and maternal weight gain decreased; and the pre-implantation loss rate also increased (p<0.05) in relation to the diabetic group. No statistically significant differences (p>0.05) were found.
for corpora lutea or resorption rates, fetal and placental weight or post-implantation loss rate in groups G2 (DS) and G3 (DSPP) as compared with G1 (D) as shown in Table 2.

**Table (2)**

<table>
<thead>
<tr>
<th>MRD</th>
<th>Groups N=60</th>
<th>G1 (D) N=20</th>
<th>G2 (DS) N=20</th>
<th>G3 (DSPP) N=20</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of corpora lutea</td>
<td>12.89 ± 3.11</td>
<td>11.74 ± 2.94</td>
<td>12.29 ± 2.96</td>
<td></td>
</tr>
<tr>
<td>No. of implantation sitesa</td>
<td>12.64 ± 3.08</td>
<td>7.58 ± 3.69*</td>
<td>8.15 ± 3.98*</td>
<td></td>
</tr>
<tr>
<td>No. of live fetusesa</td>
<td>10.86 ± 1.19</td>
<td>6.42 ± 4.28*</td>
<td>7.16 ± 4.03*</td>
<td></td>
</tr>
<tr>
<td>No. of resorptionsa</td>
<td>1.54 ± 3.07</td>
<td>1.11 ± 1.83</td>
<td>0.90 ± 0.79</td>
<td></td>
</tr>
<tr>
<td>Litter weight</td>
<td>70.33 ± 8.95</td>
<td>47.11 ± 20.88*</td>
<td>45.89 ± 23.91*</td>
<td></td>
</tr>
<tr>
<td>Maternal Weight Gain (MWG) b</td>
<td>104.78 ± 17.00</td>
<td>46.62 ± 48.66*</td>
<td>71.98 ± 26.87*</td>
<td></td>
</tr>
<tr>
<td>MWG – Litter weight b</td>
<td>280.82 ± 30.6</td>
<td>245.94 ± 40.50*</td>
<td>260.98 ± 21.90</td>
<td></td>
</tr>
<tr>
<td>Fetal weight b</td>
<td>4.47 ± 0.62</td>
<td>3.38 ± 1.75</td>
<td>4.26 ± 1.15</td>
<td></td>
</tr>
<tr>
<td>Placental weight b</td>
<td>0.63 ± 0.12</td>
<td>0.65 ± 0.22</td>
<td>0.64 ± 0.25</td>
<td></td>
</tr>
<tr>
<td>Pre-implantation loss (%)c</td>
<td>6.08</td>
<td>34.69*</td>
<td>35.30*</td>
<td></td>
</tr>
<tr>
<td>Post-implantation loss (%)c</td>
<td>10.87</td>
<td>27.35</td>
<td>18.61</td>
<td></td>
</tr>
</tbody>
</table>

G1 (D): Diabetic rats exposed to filtered air
G2 (DS) Diabetic rats exposed to cigarette smoke before and during pregnancy
G3 (DSPP) Diabetic rats exposed only to cigarette smoke before pregnancy (pre-pregnancy)

Values are expressed as mean ± SD, n=number per each group.

*Significant statistically difference compared to D. ap<0.05 – Zero – Inflated Poisson; b p < 0.05 – Normal distribution (Tukey test), and c p<0.05 – Gamma distribution. while N.S. P> 0.5: non significant value

The biochemical parameters evaluated at day 21 of pregnancy are presented in Table (3). Group 3 (DSPP) presented a significant decrease in VLDL and triglycerides levels as compared with group1 (D) where p<0.05. There were no alterations (p>0.05) in HDL, cholesterol, protein and MDA levels or SOD enzymatic activity among groups.

**Figure (1):** A photograph of an adult normal female rat abdomen and pelvis after dissection of the anterior abdominal wall showing: 1. Left uterine horn 2. Kidney 3. Colon with a fecal pellet inside 4. Liver 5. Spleen 6. Right uterine horn.
Figure (2): photograph of an adult Litter from an affected dam, showing abnormal hemorrhagic in all visceral contents in fetuses/neonates of pregnant diabetic rats after pre conceptional exposure to smoking.

Figure (3): A photograph of Litter from an affected dam, showing several gross, visceral and skeletal anomalies in fetuses/neonates of pregnant diabetic rats after pre conceptional exposure to smoking.

Figure (4): A photograph of Litter from an affected dam, rat; fetuses with different sizes (delayed development) in fetuses/neonates of pregnant diabetic rats after pre conceptional exposure to smoking.
Figure (5 A, B, & C): Figure (5A): A photograph of normal fetus and placenta of rat fetus. Figure (5B): A photograph of pale rat fetus of pregnant diabetic rats with delayed development from an affected diabetic dam after pre-conceptional exposure to smoking; hemorrhagic placenta (arrow). Figure (5C): A photograph of macerated rat fetus from an affected dam found dead at gestation day.

The biochemical parameters evaluated at day 21 of pregnancy are presented in Table (3). Group 3 (DSPP) presented a significant decrease in VLDL and triglycerides levels as compared with group 1 (D) where p<0.05. There were no alterations (p>0.05) in HDL, cholesterol, protein and MDA levels or SOD enzymatic activity among groups.

### Table (3)

**Biochemical measurements among different groups of the study**

<table>
<thead>
<tr>
<th>Variables</th>
<th>Groups N=60</th>
<th>G1 (D) N=20</th>
<th>G2 (DS) N=20</th>
<th>G3 (DSPP) N=20</th>
</tr>
</thead>
<tbody>
<tr>
<td>HDL (mg/dL)a</td>
<td>72.45 ± 75.67</td>
<td>94.62 ± 37.9</td>
<td>76.98 ± 64.89</td>
<td></td>
</tr>
<tr>
<td>VLDL (mg/dL)a</td>
<td>312.33 ± 132.65</td>
<td>224.82 ± 136.40</td>
<td>181.52 ± 125.42*</td>
<td></td>
</tr>
<tr>
<td>Triglycerides (mg/dL)a</td>
<td>1572.65 ± 667.80</td>
<td>1122.12 ± 687.0</td>
<td>911.21 ± 606.94*</td>
<td></td>
</tr>
<tr>
<td>Total Cholesterol (mg/dL)a</td>
<td>321.62 ± 196.60</td>
<td>235.97 ± 90.81</td>
<td>285.12 ± 170.24</td>
<td></td>
</tr>
<tr>
<td>Protein (g/dL)a</td>
<td>12.9 ± 3.7</td>
<td>10.44 ± 4.61</td>
<td>12.82 ± 6.9</td>
<td></td>
</tr>
<tr>
<td>MDA (nM/g Hb)a</td>
<td>748.54 ± 842.6</td>
<td>606.91 ± 681.99</td>
<td>738.76 ± 521.51</td>
<td></td>
</tr>
<tr>
<td>SOD (mU/g Hb)a</td>
<td>12.23 ± 11.86</td>
<td>17.55 ± 9.97</td>
<td>22.41 ± 20.51</td>
<td></td>
</tr>
</tbody>
</table>

G1 (D): Diabetic rats exposed to filtered air
G2 (DS): Diabetic rats exposed to cigarette smoke before and during pregnancy
G3 (DSPP): Diabetic rats exposed only to cigarette smoke before pregnancy (pre-pregnancy)
Values are expressed as mean ± SD, n=number per each group.
*Significant statistically difference compared to D. ap<0.05 – Zero – Inflated Poisson; b p < 0.05 – Normal distribution (Tukey test), and c p<0.05 – Gamma distribution. while N.S. P> 0.5: non significant value.

No statistically significant differences (p>0.05) were found concerning the frequency of external, skeletal or visceral anomalies in fetuses from all studied groups of rats (Table 4).

### Table (4)

**Frequency of external, skeletal and visceral anomalies among different groups of the study**

<table>
<thead>
<tr>
<th>Anomalies</th>
<th>Groups N=60</th>
<th>G1 (D) N=20</th>
<th>G2 (DS) N=20</th>
<th>G3 (DSPP) N=20</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. External anomalies</td>
<td>144 (13)</td>
<td>122 (15)</td>
<td>111 (13)</td>
<td></td>
</tr>
<tr>
<td>Number fetuses examined (litter)</td>
<td>144 (13)</td>
<td>122 (15)</td>
<td>111 (13)</td>
<td></td>
</tr>
<tr>
<td>Total number of fetuses (%) with alteration</td>
<td>0 (0.0%)</td>
<td>0 (0.0%)</td>
<td>0 (0.0%)</td>
<td></td>
</tr>
<tr>
<td>2. Skeletal anomalies</td>
<td>65 (13)</td>
<td>48 (15)</td>
<td>47 (13)</td>
<td></td>
</tr>
<tr>
<td>Number of fetuses examined (litter)</td>
<td>65 (13)</td>
<td>48 (15)</td>
<td>47 (13)</td>
<td></td>
</tr>
<tr>
<td>Total number of fetuses (%) with alteration</td>
<td>57 (87.7%)</td>
<td>45 (93.7%)</td>
<td>46 (97.8%)</td>
<td></td>
</tr>
<tr>
<td>Abnormally shaped vertebral centrum</td>
<td>13 (20%)</td>
<td>12 (25.0%)</td>
<td>19 (40.4%)</td>
<td></td>
</tr>
<tr>
<td>------------------------------------</td>
<td>----------</td>
<td>------------</td>
<td>------------</td>
<td></td>
</tr>
<tr>
<td>Supernumerary rib</td>
<td>33 (50.77%)</td>
<td>20 (41.67%)</td>
<td>20 (42.55%)</td>
<td></td>
</tr>
<tr>
<td>Abnormally shaped sternebrae</td>
<td>130 (200%)</td>
<td>112 (254.1%)</td>
<td>170 (367.7%)</td>
<td></td>
</tr>
<tr>
<td>Sternebrae agenesis</td>
<td>5 (7.7%)</td>
<td>2 (4.1%)</td>
<td>2 (4.2%)</td>
<td></td>
</tr>
<tr>
<td>Angulated sternebrae</td>
<td>1 (1.5%)</td>
<td>5 (10.4%)</td>
<td>0 (0.0%)</td>
<td></td>
</tr>
<tr>
<td>Palatine agenesis</td>
<td>10 (15.4%)</td>
<td>0 (0.0%)</td>
<td>0 (0.0%)</td>
<td></td>
</tr>
</tbody>
</table>

3. Visceral anomalies

<table>
<thead>
<tr>
<th>Number of fetuses (%) with alteration</th>
<th>65 (13)</th>
<th>53 (15)</th>
<th>46 (13)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total number of fetuses (%)</td>
<td>35 (53.8%)</td>
<td>38 (71.7%)</td>
<td>31 (67.4%)</td>
</tr>
<tr>
<td>Enlarged bladder</td>
<td>1 (1.5%)</td>
<td>2 (3.7%)</td>
<td>2 (4.3%)</td>
</tr>
<tr>
<td>Cardiomegaly</td>
<td>1 (1.5%)</td>
<td>1 (1.8%)</td>
<td>0 (0.0%)</td>
</tr>
<tr>
<td>Hydronephrosis</td>
<td>13 (20.0%)</td>
<td>14 (26.4%)</td>
<td>7 (15.2%)</td>
</tr>
<tr>
<td>Hydroureter</td>
<td>25 (38.4%)</td>
<td>37 (69.8%)</td>
<td>30 (65.2%)</td>
</tr>
</tbody>
</table>

G1 (D): Diabetic rats exposed to filtered air
G2 (DS): Diabetic rats exposed to cigarette smoke before and during pregnancy
G3 (DSPP): Diabetic rats exposed only to cigarette smoke before pregnancy (pre-pregnancy)

Values are expressed as mean ± SD, n=number per each group.
* P < 0.5: Significant statistically difference.
In the G2 (DS) and G3 (DSPP) rat groups, a statistically significant reduction in the number of anterior phalanges and metatarsus was found as compared the group one (D). There was a reduction (p<0.05) in the number of caudal vertebrae in group 3 (DSPP) in relation to group D (Table 5).

Table (5)

**Ossification sites in fetuses among different groups of the study**

<table>
<thead>
<tr>
<th>O.S.</th>
<th>Groups</th>
<th>G1 (D) N=14</th>
<th>G2 (DS) N=20</th>
<th>G3 (DSPP) N=20</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anterior phalange</td>
<td>2.89 ± 0.10</td>
<td>2.46 ± 0.08*</td>
<td>2.07 ± 0.02*</td>
<td></td>
</tr>
<tr>
<td>Metacarpus</td>
<td>3.88 ± 0.08</td>
<td>3.90 ± 0.15</td>
<td>3.8 ± 0.15</td>
<td></td>
</tr>
<tr>
<td>Posterior phalange</td>
<td>0.51 ± 0.04</td>
<td>0.39 ± 0.09*</td>
<td>0.06 ± 0.02</td>
<td></td>
</tr>
<tr>
<td>Metatarsus</td>
<td>4.42 ± 0.08</td>
<td>4.12 ± 0.15*</td>
<td>3.04 ± 0.15*</td>
<td></td>
</tr>
<tr>
<td>Number of caudal vertebra</td>
<td>4.14 ± 0.08</td>
<td>4.09 ± 0.19</td>
<td>3.15 ± 0.09*</td>
<td></td>
</tr>
<tr>
<td>Sternebra</td>
<td>5.99 ± 0.11</td>
<td>5.97 ± 0.22</td>
<td>5.86 ± 0.21</td>
<td></td>
</tr>
</tbody>
</table>

G1 (D): Diabetic rats exposed to filtered air
G2 (DS): Diabetic rats exposed to cigarette smoke before and during pregnancy
G3 (DSPP): Diabetic rats exposed only to cigarette smoke before pregnancy (pre-pregnancy)

Values are expressed as mean ± SD, n=number per each group.
* P < 0.5: Significant statistically difference; while N.S. P> 0.5: non significant value
*p<0.05 – Significant statistically difference compared to D (Negative binominal distribution)
DISCUSSION

In the present study, severe diabetes was induced by streptozotocin (40 mg/Kg) in the adult phase of all rats. The female rodents of the experimental groups showed higher blood glucose level than 300mg/dL, which is compatible with severe diabetes. Cigarette smoke exposure significantly exacerbated hyperglycemia in group 2 (DS) and group 3 (DSPP). A study conducted on smoker diabetic and non-diabetic patients investigated the effect of cigarette on blood glucose level at different moments. Both groups showed increased blood glucose level after smoking. However, such increase was more accentuated in the diabetic group. After cigarette smoke exposure, there was a mobilization of catecholamines, somatrophic-hormone stimulation and cortisol production, leading to increased glycemia, as also observed in our study. The analysis of maternal reproductive performance was aggravated in the Diabetes/cigarette smoke association, regardless of the exposure moments. The number of implanted embryos and of live fetuses that developed was smaller in the groups of rats submitted to the association of two variables, thus contributing to the weight reduction of offspring. At full term pregnancy, these rats then showed reduced maternal weights as compared to those in early pregnancy, thus showing that the litter weight interfered with maternal weight. However, the rats that stopped being exposed to cigarette smoke during pregnancy showed similar body weights to those in the diabetic group (control). The main mechanisms used to explain such gain after the interruption of cigarette smoke exposure include increased energy expenditure, reduced resting metabolic rate and reduced physical activity, as observed by Pistelli et al., who reported that smoking is associated with body-weight reduction whereas interruption is associated with weight gain. The embryonic loss rate prior to implantation depends on the number of corpora lutea and implantation sites. In this study, it was observed that the number of implantation sites was smaller in group 2 (DS) and group 3 (DSPP), leading to increased percentages of pre-implantation losses in the respective groups. For a newly formed embryonic fixation (embryonic implantation) to occur in the gravid endometrium there must be a synchronism between the maternal organism and embryonic development. Hyperglycemia leads to alterations in the intrauterine environment, and when associated with smoking, it is suggested that the process of blastocyst fixation to the gravid endometrium is aggravated, since smoking is related to disorders in gametogenesis, ovulation, ovocyte transportation by the uterine tubes and the process of implantation of the fertilized cell, which results in fertility reduction, thus confirming our findings. Smoker women’s fertility rates are reduced and the risk for abortion and preterm childbirth is greater. Studies have shown weakened ovary functioning and disorders in sexual hormone metabolism, resulting in complications during pregnancy or early menopause and osteoporosis. Cigarette smoke contains more than 4,000 chemical products, and its metabolites exert toxicity in both the circulatory system and target organs. The levels of toxic substances from cigarettes in reproductive tissues or fluids are significantly higher than in serum, suggesting that the toxic substances from tobacco accumulate in reproductive organs, thus contributing to impair the embryonic implantation process. The rats that were not submitted to tobacco smoke exposure in pregnancy continued to show fetal viability reduction, similarly to those which continued being exposed during the whole experiment. Although increased post-implantation loss rates were observed, such rates were not significant after the diabetes-tobacco-smoke association due to the heterogeneity between the rat percent values. The reduced number of live fetuses found in diabetic rats exposed to cigarette smoke was...
not due to the presence of anomalies, since our data showed no significant increases in external or internal (skeletal or visceral) anomalies in group 2 (DS) and group 3 (DSPP), thus showing that cigarette smoke exposure did not exacerbate what hyperglycemia had already affected in the organogenesis processes. Contrarily, Nishimura and Nakai\textsuperscript{42} observed that when nicotine, one of the major components of tobacco, was administered, purely and in high doses (25 mg/Kg), to pregnant rats, it led to the onset of anomalies in the lower limbs and fetal mortality. Deformities and fetal death were not reported by another study using the administration of nicotine in lower doses (12 mg/Kg)\textsuperscript{43}. The onset of anomalies associated with cigarette smoke may, then, be related to the level of nicotine administered. Nevertheless, the literature also reports contradictory data concerning a possible association between smoking and the onset of congenital anomalies. Currently, there is a general consensus that smoking does not seem to be an important factor in anomaly induction\textsuperscript{44}. In the present study, cigarette smoke exposure did not cause alteration to the mean of fetal weights as observed in other studies. However, the classification of weights performed by our research group characterized intrauterine growth restriction as confirmed by the increased percentage of fetuses classified as small for pregnancy age\textsuperscript{23}. It is known that decreased weight at birth presents a direct relation between the number of cigarettes smoked, carbon-monoxide concentration and cigarette tar content. However, despite the numerous investigations, the mechanism by which smoking interferes with fetal development remains unclear. The offspring of women who smoked during pregnancy are born with tobacco combustion products in their organisms which are harmful to their development. Such newborns usually present lower birth weight, are less developed and more vulnerable to diseases, including asthma and even cancer\textsuperscript{45}.

Data have shown an association between abnormal fetal growth and both “active” or “passive” cigarette smoke exposure during gestation. Esposito et al.\textsuperscript{46} identified a temporal window of vulnerability during the pre/peri-implantation period of embryonic development for in utero smoke-exposure-induced low birth weight. Further, these studies documented the establishment of a viable animal model with which to test hypotheses regarding the cellular and molecular mechanisms underlying gestational low birth weight induced by tobacco-smoke exposure. The intrauterine growth restriction can also be confirmed by a decreased number of ossification centers in fetuses from diabetic mothers exposed to cigarette smoke. Aliverti et al.\textsuperscript{47} identified ossification points in rats, which were compatible with pregnancy age, with the purpose of simulating the somatic development of newborns. However, the mechanisms responsible for alterations in bone development have not been clarified. During this early period of gestation, embryonic cells are highly proliferative and segregate. They polarize to form the two primary cell populations of the embryo and the placenta, and the cells forming the embryo begin to undergo a myriad of processes associated with implantation\textsuperscript{48}. One example of the alteration caused by cigarette is the fact that nicotine, the principal component of cigarette smoke, has been found to delay embryonic implantation\textsuperscript{49}. A balanced intrauterine environment is essential for the conceptus normal development. The presence of diabetes in pregnancy changes the intrauterine hormonal and metabolic environment, which results in congenital anomalies and neonatal hypoglycemia\textsuperscript{50}. At present, it is known that the diabetic syndrome exacerbates the triglycerides levels and oxidative stress\textsuperscript{22}. In this study, the rats that stopped smoking group (DSPP) showed decreased triglyceride and VLDL levels and no change in oxidants levels’ concentration. However, these beneficial effects were not sufficient to stop the lipid peroxidation
observed in these animals by MDA concentration, similarly to the diabetic group, thus contributing to the presence of an adverse intrauterine environment for embryofetal development. Such uterine alterations could reflect on the reduced number of implantations and live fetuses. Different results from studies in which pregnant rodents were exposed to cigarette smoke have been equivocal due to differences in exposure conditions, dosing times and concentrations of total suspended particulates\textsuperscript{51-53}. Interpretation of such studies is further complicated by the fact that cigarette smoke contains over 4,000 identifiable compounds, and approximately 600 additives\textsuperscript{54}.

**CONCLUSION**

Despite the benefits stemming from smoking interruption during the pregnancy of diabetic rats, such improvement was insufficient to avoid metabolic alterations and provide an adequate intrauterine environment for embryofetal development. This has been confirmed by \textit{intrauterine growth restriction and reduction in ossification sites}. Therefore, these results suggest that it is necessary to \textit{cease smoking long before planning pregnancy} since stopping smoking only when pregnancy is detected may not contribute effectively to fully adequate embryofetal development.

**ACKNOWLEDGMENT**

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