Oxidative Damage in Embryo and Placenta of Streptozotocin-induced Diabetic Rats
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Abstract
Background: Maternal type 1 diabetes is associated with an increased risk for fetal malformations. The mechanism by which diabetes caused teratogenic disorders is not fully known. Previous studies have demonstrated that many teratogenic diabetic cases were related to free radical oxygen species. This study was conducted to evaluate the effect of maternal diabetes on both embryo and placenta by estimating the oxidative and DNA damage in embryo and placenta of diabetic mellitus-induced rats. The possible role of olive leaves extract of Olea europaea (O. europaea) plant in repairing the damage was also assessed.

Material and Methods: Diabetes mellitus was induced by streptozotocin (STZ) by a single intraperitoneal injection (35 mg/kg b wt). O.europaea leaves water extract was administered orally (550 mg/100g b wt/day) for 5 days before pregnancy and 18 days after. Malondealdehyde (MDA) level, glutathione peroxidase (Gpx) and superoxide dismutase (SOD) activities and glycogen concentration were measured in term embryo and placenta homogenates of diabetic and control rats. Moreover, the evaluation of DNA damage was carried out by the Alkaline Comet Assay using embryos and placentas taken from STZ-induced diabetic and control pregnant rats.

Results: the results showed an elevation in MDA level of the diabetic groups of both embryo and placenta compared to that of the control. This was accompanied by reduction in Gpx and SOD activities indicating oxidative damage. Glycogen level was reduced in diabetic groups of embryo and placenta. Both oxidative and hyperglycemic status were improved in the groups treated with olive leaves water extract. The percentage of tail DNA and tail moment values were also higher in both embryo and placenta of the diabetic-induced rats. DNA damage seems to be partly ameliorated in groups treated with O. europaea leaves water extract.

Conclusion: This study indicated that maternal hyperglycemic condition in diabetic-induced pregnant rats could generate oxidative and DNA damage to embryo and placenta that could be ameliorated by oral doses of olive leaves water extract.

Key words: diabetes mellitus, hyperglycemia, oxidative stress, embryo, placenta, DNA damage, Alkaline Comet Assay, olive leaves.

Introduction
Maternal diabetes constitutes an unfavorable environment for embryonic and fetoplacental development. Despite current treatments, pregnant women with either type 1 or type 2 diabetes are at increased risk of miscarriage, placental abnormalities, and intrauterine malprogramming. The worldwide increase in the incidence of diabetes, in women at reproductive ages brings about growing interest in the use of experimental diabetic models in order to investigate the mechanisms of induction of developmental alterations in maternal diabetes. Experimental models of severe diabetes (glycemia > 300mg/dL), which reproduce the clinical conditions of poorly controlled type-1 diabetes, have been widely used. However, only a few studies have evaluated the repercussions of diabetes on pregnant rats and/or their offspring using models of mild diabetes (glycemia between 120 and 300 mg/dL).

Maternal hyperglycemia during the early stages of development may be sufficient to program changes in physiology and metabolism which are later manifested as adverse effects in diabetic pregnancy. Hence, it is remarkable that glycemic control from the beginning of pregnancy is important for placental and embryofetal development. Oxidative stress plays a pivotal role in cellular injury caused by hyperglycemia. High glucose level can stimulate free radical production. Weak defense system of the body becomes unable to
counterclockwise the enhanced ROS generation and as a result a condition of imbalance between ROS and their protection occurs which leads to domination of the condition of oxidative stress. Oxidative stress represents pathophysiological mechanism lying behind occurrence of different acute and chronic diseases. Pregnancy, mainly due to placenta rich with mitochondria, is also being associated with the state of oxidative stress. Numerous markers have been proposed in order to test oxidative stress in pregnancy state. Phytotherapy is frequently considered to be less toxic and free from side effects than synthetic drugs. In traditional practices, medicinal plants are used to control diabetes mellitus in many countries. This caused an increase in the number of experimental and clinical investigations directed toward the validation of the hypoglycemic and/or anti-diabetic properties of different medicinal plants. Hence, the present study was designed to investigate the possible protective effect of crude water extract of olive leaves of *Olea europaea* plant against oxidative damage in embryo and placenta of STZ-induced diabetes in pregnant rats.

**Material and Methods**

**Animals**

Virgin female albino rats (200 ± 20 gm) were used in this study. Experimental animals were fed on standard rodent diet and were supplied drinking water *ad-libitum*.

**Induction of Diabetes mellitus**

12 hrs-fasted female rats became diabetic (Type1) with a single intraperitoneal injection of STZ (35 mg/kg b wt), from Sigma (USA) diluted in 0.1mol/L of citrate buffer (pH 4.5) according to Holemans *et al.*. Hyperglycemia in female rats before mating was verified by measuring the blood glucose level. A level of more than 250 mg/dl was selected for this study.

**Preparation of olive leaves extract**

Fresh leaves of *O. europaea* were shaded-dried after being thoroughly rinsed with sterile distilled water to remove dust, insecticides, and contaminating material, the olive leaves were ground into fine powder by a mechanical grinder. The powdered plant material was used in preparing the water extract to be administrated orally to female rats for 5 days before mating and for 18 days after pregnancy at a dose of 55 mg / 100 g b wt / day. The dose was calculated with reference to the human therapeutic dose.

**Preparation of tissue homogenates**

Embryos and placentas were quickly extracted from the uterus and weighed separately. Homogenates were prepared by a manual glass homogenizer using a fixed volume of ice cold saline solution. Supernatant was obtained after centrifugation at 4000 rpm for 30 min.

**Experimental design**

Pregnant rats were allocated into 4 groups (5 females each):

- Group I: control untreated pregnant rats.
- Group II: pregnant rats were orally administrated with olive leaves extract.
- Group III: Diabetic pregnant rats.
- Group IV: Diabetic pregnant rats were orally administrated with olive leaves extract.

**Biochemical analysis**

Malondialdehyde (MDA) level (nmol/ mg tissue), Glutathion peroxidase (Gpx) and Superoxide dismutase (SOD) activities (mU/mg tissue and U/mg tissue respectively) in embryo and placenta homogenates were determined using BioVision Lipid Piroxidation Assay Kit. Glycogen level (ug/mg tissue) was estimated in embryo and placenta homogenates according to Cappeln and Jessen. 

**Alkaline Comet Assay**

Evaluation of DNA damage in embryo and placenta was carried out by quantitative and qualitative extent of DNA damage in the cells by measuring the length of DNA migration and the percentage of migrated DNA. Generally, 50 to 100 randomly selected cells were analyzed per sample.

**Statistical Analysis**

Statistical analysis was performed using SPSS statistical package version 17.0. Data was expressed as mean ± SD of the mean. The parameters were analyzed with independent t-test to compare the difference between treated groups and control. The difference between
groups was considered significant at p< 0.05. (18,19)

Results

Embryos and placentas of all groups were used in this study to evaluate the effect of maternal hyperglycemia on the lipid peroxidation end product, malondialdehyde (MDA) level, Glutathione peroxidase (Gpx), Superoxide dismutase (SOD) activities and glycogen content. DNA damage was also assessed by the single cell gel electrophoresis assay (the Comet Assay Technique).

The present results demonstrated a remarkable increase in MDA level (nmol/mg tissue) in embryos of the diabetic group compared to that of the control embryos (Figure 1). However, MDA values in embryos of olive leaves extract treated- diabetic group was improved but not restored to the normal value. Gpx and SOD activities (mU/mg tissue and U/mg tissue respectively) were also assessed (Figures 2,3). Both enzymes showed dramatic elevation in their activities in the embryos of the diabetic group compared to that of control. On the other hand, activities of the two enzymes in embryos in the plant extract group were markedly reduced. Glycogen content (ug/mg tissue) showed non significant change in diabetic group compared to that of control (Figure 4), while elevated in the group orally administrated with olive leaves extract. Changes in placenta MDA of control and diabetic groups are shown in figure (5). MDA level was increased markedly compared to that of the control group while its level was slightly reduced in the placentas of the diabetic – olive leaves treated group. Elevated activity of both Gpx and SOD was recorded in the placenta of the diabetic group which was reduced in diabetic- olive leaves orally administrated groups (Figures 6 and 7). Glycogen content of term placenta of diabetic group was not changed significantly compared to control (figure 8). In olive leaves- treated groups (both control and diabetic) placenta glycogen level was increased above the control level as shown in figure (8).

Analysis of DNA damage

The image analysis software provides a full range of densitometric and geometric parameters describing the complete Comet, as well as the head and Tail DNA portions. Since the Comet Assay reflects the displacement of fluorescence from the head to the tail in damaged cells, the use of % Tail DNA (the percentage of total nuclear DNA that has migrated to the tail) and tail moment (% Tail DNA x length) as the parameter to quantify basal levels of DNA damage, each slide was analyzed in duplicate and 50 cells per slide were scored.

The current study revealed DNA damage in placentas and embryos in diabetic group as shown in table (1) and figure (9). Placentas of pregnant control (C), diabetic (D) and diabetic treated with olive leaves extract (D+O) groups are represented in table (1) and figure (9) as samples 1, 2 and 3 respectively. The percentage value of Tailed DNA of diabetic group treated with olive leaves extract was 13% compared to 4% of the control group while DNA of placenta in diabetic group showed slight repair (10%). Similar observations were recorded for the DNA Tail length (µm), % Tail DNA and Tail moment. Table (1) and figure (9) also demonstrated DNA damage in embryos of the corresponding groups as samples 4, 5 and 6. The percentage value of Tailed DNA was increased to be 16% in group (D) compared to control (6%) while a repair was noticed in embryos of group D+O. Similarly, the increase recorded in DNA Tail length (µm), % Tail DNA and Tail moment in embryos of diabetic group were repaired in the diabetic group treated orally with olive leaves extract.

Discussion

Oxidative stress represents a pathophysiological mechanism lying behind the occurrence of different acute and chronic diseases. Pregnancy, mainly due to placenta rich with mitochondria, is also being associated with the state of oxidative stress. Many markers have been proposed in order to test oxidative stress in pregnancy state (10). Numerous data suggested that lipid peroxidation products and anti oxidative protection components change significantly in pregnancy. (20) Increased intensity of lipid peroxidation in pregnant woman placenta (21,22) has been reported.
However, natural antioxidative response in healthy pregnant women is present at the level that ensures the protection from increased risk and it is a part of pregnancy physiology. Increased level of MDA in diabetics suggests that peroxidative injury may be involved in the development of diabetic complications. The increase in lipid peroxidation is also an indication of decline in defense mechanisms of enzymatic and non enzymatic antioxidants.

Increased MDA level in plasma and many others tissues has been reported in diabetic patients indicating increased lipid peroxidation. The placenta is a provisional organ that executes endocrine, metabolic and nutritional activities essential for the growth and survival of the fetus and for the control of pregnancy. Diabetes during pregnancy has been associated with some of placental disorders. The direct consequences of altered placental function on fetal growth and maturation are not yet fully understood; however, evolving data strengthen the view that ultrastructural placental abnormalities have a role in the developmental and functional disturbances found in the offspring of diabetic pregnancies.

The current results revealed increase in MDA, Gpx and SOD in both placentas and embryos of STZ-induced diabetic rats indicating oxidative damage in their tissues. Spada et al. reported that mild diabetes caused glycemia superior to 120 mg/dL during pregnancy, increased superoxide dismutase, glutathione peroxidase, glutathione reductase activities, and malondialdehyde levels in the blood, and catalase activity in the placenta. Results of the present study are also in agreement with those found by White et al. who found that Cu/Zn SOD was elevated in placentas from diabetic rats.

Normal fetal growth and development depend on a normal placental function. Some types of intrauterine growth restriction in human and animal fetuses have been related to disturbances in placental blood flow and/or defective transport of nutrients from the mother to the fetus. Concerning oxidative damage in embryos, Cederberg et al. found that the mRNA levels of catalase and Mn-SOD were increased in embryos as a response to maternal diabetes. In another study, Forsberg et al. reported that maternal diabetes in vivo induced increased MnSOD expression, concomitant with increased total SOD activity, and a tentative decrease in catalase expression and activity in the embryos.

An association between excess oxygen radical activity and disturbed embryogenesis in diabetic pregnancy has been suggested by Simán and Eriksson. Thiobarbituric acid reactive substances (TBARS) were estimated as a measure of lipid peroxidation in maternal tissue, embryonic tissue, placenta, and fetal brain in the untreated diabetic rats. They reported that an imbalance in the metabolism of free oxygen radicals is involved in the embryonic maldevelopment of diabetic pregnancy.

The present results showed that glycogen level in both embryos and placentas of STZ- induced diabetic group was not changed significantly. In in vitro experiments, Jawerbaum et al. studied glucose, glycogen and triglycerides metabolism in uterine strips and in embryos isolated from controls and diabetic rats at day 10 of pregnancy. They found differences in uterine metabolism of glucose, glycogen and triglycerides in controls and in diabetic rats, but metabolic differences have not been detected between embryos obtained from controls and from diabetic mothers. Also Moley et al. reported that glycogen and 6-phosphogluconate levels were not significantly different in 1-cell mice embryo cultures. In an experiment on diabetic mice, Cifuentes et al. reported that embryonic liver glycogen was not affected by the maternal glycemic status. They explained the role of Glucokinase (GK, hexokinase type IV) as it is required for the accumulation of glycogen in adult liver cells. Paradoxically, mammalian embryonic livers store glycogen successfully in the absence of GK. They described how mammalian embryonic livers, but not adult livers cells, manage to accumulate glycogen in the absence of this enzyme. Hexokinase type I or II (HKI, HKII) substitutes for GK in embryonic livers.

Plant-derived products used in therapeutic purposes usually do not produce any significant
side effects when properly administered. Therefore, plants are good candidates for further investigations aimed at increasing the number of the armamentarium against diabetes mellitus. Olive tree (Olea europaea) leaves have been widely used in traditional remedies in European and Mediterranean countries as extracts, herbal teas, and powder. They contain several potentially bioactive compounds that may have hypoglycemic and antioxidant properties. Poudyal et al. demonstrated that olive leaf, which includes polyphenols such as oleuropein and hydroxytyrosol, was observed to improve cardiac, hepatic and metabolic parameters by decreasing inflammation and oxidative stress in high fat fed rats.

Results of the current study revealed that oral administration of the olive leaves water extract to pregnant rats ameliorated the oxidative damage caused by hyperglycemia in STZ-induced diabetic rats in both embryos and placentas of experimental groups. Multiple animal studies have shown the effectiveness of olive leaf extract is normalizing a variety of cardiovascular, hepatic and metabolic signs, most likely through reversing related chronic inflammation and oxidative stress.

The Single Cell Gel Electrophoresis assay (also known as 'comet assay') is an uncomplicated and sensitive technique for the detection of DNA damage at the level of the individual eukaryotic cell. It was first developed by Östling & Johansson and later modified by Singh et al.. It has since increased in popularity as a standard technique for evaluation of DNA damage/repair, biomonitoring and genotoxicity testing. It involves the encapsulation of cells in a low-melting-point agarose suspension, lysis of the cells in neutral or alkaline (pH>13) conditions, and electrophoresis of the suspended lysed cells. The term "comet" refers to the pattern of DNA migration through the electrophoresis gel, which often resembles a comet.

The present results demonstrated oxidative damage in embryos and placentas of diabetic mothers compared to control. The image analysis results also showed DNA repair in embryos and placentas of diabetic mothers treated orally with olive leaves extract. DNA damage associated with diabetic pregnancy was documented by several authors. Lima et al. demonstrated that DNA damage evidence which was noticed in the maternal repercussions of diabetes is associated with oxidative DNA damage of their newborn. Another study also provided evidence of oxidative damage in diabetic streptozotocin rats as evaluated using the Alkaline Comet Assay. It was also found that increased levels of glycemic status and lipid peroxidation products in diabetes rats, which also supported the hypothesis that enhanced lipid peroxidation could contribute to an increased formation of free radicals in diabetes mellitus.

**Conclusion**

In conclusion, the results of the present study, suggest that maternal STZ-induced hyperglycemia might generate oxidative and DNA damage in term embryos and placentas of diabetic pregnant rats. Daily oral supplementation of olive leaves water extract to female rats prior to and during the gestational period ameliorated the oxidative and DNA damage in both embryos and placentas of the diabetic mothers. The use of this intervention should be further investigated on a large clinical scale in order to justify the above mentioned results concerning the interaction between the plant extracts and the drug used.

**Acknowledgement**

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**References**

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Figure 1: Changes in MDA levels in term embryos of control and diabetic mothers of experimental groups.

Figure 2: Changes in Gpx activity in term embryos of control and diabetic mothers of experimental groups.

Figure 3: Changes in SOD activity in term embryos of control and diabetic mothers of experimental groups.

Figure 4: Changes in Glycogen levels in term embryos of control and diabetic mothers of experimental groups.
Figure 5: Changes in MDA levels in term placentas of control and diabetic mothers of experimental groups.

Figure 6: Changes in Gpx activity in term placentas of control and diabetic mothers of experimental groups.

Figure 7: Changes in SOD activity in term placentas of control and diabetic mothers of experimental groups.

Figure 8: Changes in glycogen levels in term placentas of control and diabetic mothers of experimental groups.
Table 1: DNA damage in cells isolated from term embryos and placentas of control and diabetic mothers of experimental groups.

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>% Tailed</th>
<th>% Untailed</th>
<th>μm Tail length</th>
<th>% Tail</th>
<th>UNIT Tail moment</th>
<th>Groups</th>
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<td>1</td>
<td>4</td>
<td>96</td>
<td>2.31</td>
<td>1.53</td>
<td>3.53</td>
<td>C-Placenta</td>
</tr>
<tr>
<td>2</td>
<td>13</td>
<td>87</td>
<td>4.33</td>
<td>4.05</td>
<td>17.54</td>
<td>D-Placenta</td>
</tr>
<tr>
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<td>10</td>
<td>90</td>
<td>4.27</td>
<td>3.56</td>
<td>15.19</td>
<td>D+O Placenta</td>
</tr>
<tr>
<td>4</td>
<td>6</td>
<td>94</td>
<td>2.31</td>
<td>2.05</td>
<td>4.74</td>
<td>C-Embryo</td>
</tr>
<tr>
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<td>4.22</td>
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<td>D- Embryo</td>
</tr>
<tr>
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<td>92</td>
<td>3.03</td>
<td>4.25</td>
<td>12.87</td>
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</tr>
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</table>

C: control,
D: diabetic,
D+O: diabetic and oil leaves extract

Figure 9: Comet assay obtained by image analysis in cells isolated from term embryos and placentas of control and diabetic mothers of experimental groups.