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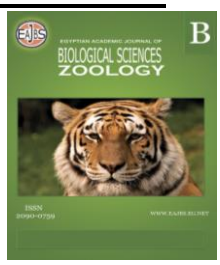

Exposure to Lufenuron During the Third Gestational Period Induces Genotoxicity and Oxidative Stress Effects in Pregnant Albino Rats and Their Fetuses

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ABSTRACT

The last few decades witnessed massive increments in the accumulation of agrochemicals and insecticides residues in the environment, poisoning human and animal food. In our study, we evaluated the genotoxic, and oxidative stress effects of residual-level lufenuron exposure on pregnant rats during the third period of gestation (day 13-19) to both mother and fetuses. The experimental design depended on dividing the females into three groups; control (untreated), low-dose group (orally administered with 0.4 mg/kg lufenuron) and high-dose group (orally administered with 0.8 mg/kg lufenuron). In comet assay, the liver cells of lufenuron-treated pregnant dams and their fetuses showed significant DNA damage compared to control. Cell cycle arrest was also detected in liver cells at G0/G1 phase through flow cytometry. The oxidative stress induced by lufenuron in liver cells of mothers and fetuses was detected by the increased lipid peroxidation as indicated by the elevated malondialdehyde (MDA) levels- and decreased levels of enzymatic antioxidants (glutathione peroxidase [GPx] and superoxide dismutase [SOD]). In conclusion, the obtained results point out the high potential of chronic exposure to lufenuron residual concentrations during the third period of gestation for exerting genotoxic, and oxidative stresses on pregnant rats and their fetuses.

INTRODUCTION

The race in the food industry has forced insecticides into the agricultural system as an indispensable component for the fierce competition in increasing crop yields and food production (Alexandratos and Bruinsma, 2012). Naturally, while these competitions focused on increasing production, they inadvertently or intentionally neglected the harmful impact of these chemicals on the environment and human health (El–Seedy *et al*., 2006).

The last few decades witnessed massive increments in the accumulation of agrochemicals and insecticides residues contaminating the human environment and poisoning his food and animals (EEA, 2013; Carvalho *et al*., 2009; Carvalho *et al*., 2003; Kale *et al*., 1999). Strikingly, some insecticides are evaporated after application and carried to other areas where they are condensed by cooler climates leading to their distribution to new areas far away from the areas they were originally applied (Li and

Jin, 2013; Simonich and Hites, 1995). As an inevitable result of these unethical practices, insecticides residues were detected in many kinds of crops, human and animal food and water (Chourasiya *et al*., 2015; Witczak and Abdel-Gawad, 2014). In Egypt, the presence of insecticides residues exceeding the allowed maximum residual limit along with unregistered pesticides has been reported in several crops (Mustapha *et al*., 2017; Mansour *et al*., 2009a; Mansour *et al*., 2009b; Ahmed *et al*., 2002) .

Some of these insecticides were proven to have the ability to cross the placental barrier, hence affecting fetuses (Ostrea *et al*., 2009; Whyatt *et al*., 2009; Bradman *et al*., 2003). Maternal exposure to chemical pollutants, including insecticides, was ranked as the second most important cause of infant mortality in developing countries (Cremonese *et al*., 2014; Gorini *et al*., 2014; Kurinczuk *et al*., 2010). The accumulated Evidence over the past twenty years suggests that exposure to insecticides induce genetic damage and mutations to human and might be a pivotal contributor to many types of human cancers (Marrs, 2012; Axelson, 2006; Korsloot *et al*., 2004).

Single-cell gel electrophoresis or comet assay is one of the most important methods for the assessment of genotoxicity caused by environmental chemicals. It is also a rapid, sensitive, and inexpensive test for the detection of DNA damage. One of the modifications of this assay, alkaline comet assay, is considered the most common method for measuring DNA damage in eukaryotic cells (Neri *et al*., 2015). It was extensively used for *invivo* and *invitro* assessment of genotoxicity caused by chemical compounds in mice (Sasaki *et al*., 2000), *Drosophila melanogaster* (Eid *et al*., 2017), human germ cells (Pandir, 2016) , and zebrafish (Gülsoy *et al*., 2015).

DNA damage or cell cycle failure initiates cell cycle arrest. Severe DNA damage or irreversible failure of the cell cycle eventually leads the cell to progress toward apoptosis. Exposure to certain DNA-damaging chemicals or conditions activates phase-specific checkpoints leading to cell cycle arrest (Enoch and Norbury, 1995). Cytotoxic compounds, exposure to radiation, or cell toxic drugs can induce G2/M phase accumulation (Wu *et al*., 2006; Bonelli *et al*., 1996). Human cell lines (erythroleukemia and breast cancer) suffered from cell cycle perturbations represented in G2/M phase arrest and progression to apoptosis after treatment with aniline THDA and morphine, respectively (Chen *et al*., 2017; Wu *et al*., 2006). Measuring cells at a single time point is the simplest flow cytometry assay amongst the recently developed methods for cell cycle analysis (Larsen *et al*., 2001; Darzynkiewicz *et al*., 1976).

Exposure to insecticides was also found to induce oxidative stress and lipid peroxidation. However, the trans-generational effects of maternal exposure to insecticides are still under-studied (Ndonwi *et al*., 2019). Although the effects of human exposure to insecticides during the gestational period are insufficiently studied, however, accumulating evidence suggests that exposures during prenatal and postnatal periods may affect the metabolism of the adult metabolism and his biochemical homeostasis (Tukhtaev *et al*., 2012).

A few previous reports suggested that even low doses of lufenuron -an acylurea insect growth regulator (IGR) that was classified safe- may lead to teratogenic, mutagenic, and genotoxic effects in vertebrates and *Drosophila melanogaster* (Eid *et al*., 2017; Pener and Dhadialla, 2012). This study aims to evaluate the ability of lufenuron to cross the placenta and affect rat fetuses. The main goal is to assess the genotoxic, and oxidative stress effects of residual levels of lufenuron on both pregnant albino rats and their fetuses when exposed during the third period of gestation.

MATERIALS AND METHODS

Tested Chemical:

Lufenuron is an acylurea insecticide with the chemical formula(1-[2,5-Dichloro-4-(1,1,2,3,3,3-hexafluoropropoxy)phenyl]-3-(2,6difluorobenzoyl) urea). The desired doses were prepared directly before administration by dissolving 5% emulsifiable concentrate (Syngenta, Egypt) in distilled water to reach 0.4 and 0.8 mg in 0.5 ml of the solution.

Experimental Animals:

Fifteen males and thirty virgin females sprague-Dawley rats weighing between 140 to 180 g were procured from the animal house of the National Research Center, Egypt. Animals were checked by a vet for common problems as obesity and breathing problems and were observed closely for signs of stress, pain, illness, and injury. Before the onset of the experiment, animals were allowed to acclimate in the laboratory environment with free access to food and water for one week. Light-dark photoperiod was maintained at 12:12 h at a controlled environment temperature of 22±1 °C and relative humidity of 55±5% (Nasrin *et al*., 2012). All experiments and procedures were performed in accordance with relevant guidelines/regulations of the ethical committee. The experiments were consented to by The Institutional Animal Care and Use Committee (IACUC), Faculty of Science, Cairo University with the approval number (CU/I//F/29/18).

Crosses and Treatments:

Every two females were caged with one male and left for mating overnight. The females were daily checked for the vaginal plug and a vaginal smear (which signifies the first day of gestation) was prepared (Burdan *et al*., 2011). Pregnant females were isolated and randomly classified into three groups, each containing ten females.

The control and treated groups were orally administrated with the corresponding treatment of distilled water and lufenuron, respectively, once daily at the same time over the seven days of the third gestation period (from 13^{th} to 19^{th} gestational day) as follows:

- **Control group**: Pregnant rats received an equivalent volume of vehicle (distilled water) by gavage.
- **Low dose group (LD)**: Pregnant rats were treated with a low dose of lufenuron (0.4 mg /kg of body weight) by gavage.
- **High dose group (HD)**: Pregnant rats were treated with a high dose of lufenuron (0.8 mg/kg of body weight) by gavage.

The doses under investigation were selected based on the maximum residue levels (MRL) in some crops according to European Food Safety Authority (EFSA) report (**2017**).

Assessment of DNA Fragmentation By Comet Assay (SCGE):

Liver tissues were cut into small pieces and gently homogenized in 1 ml PBS. The extent of DNA strand breaks in the liver tissues of mothers and fetuses from the three groups was evaluated by alkaline comet as previously described by Tice *et al.* (2000). Comets were analyzed using an Axio fluorescence microscope (Carl Zeiss, Germany) with an excitation filter at 524 nm and a barrier filter at 605 nm. Tail length, percentage of migrated DNA, and tail moment were measured by Komet 5.0 analysis system developed by Kinetic Imaging, Ltd. (Liverpool, United Kingdom) connected to a charge-coupled device (CCD) camera.

Cell Cycle Analysis By Flow Cytometry Through PI Staining:

Liver tissues from mothers and fetuses of all groups were weighed, rinsed in phosphate-buffered saline, and minced using a pair of scissors into 1-mm fragments then

digested using collagenase (1 ml/0.25 g tissue) (Meng *et al*., 2016; Seglen, 1976). Cell cycle analysis by flow cytometry through PI staining was performed according to the method previously described by Allen and Davies (2007). Attune flow cytometer (Applied Biosystem, USA) was used to analyze the stained cells.

Estimation of Oxidative Stress Markers:

Hepatic activities for MDA, GPX, and SOD were measured using a UV-2100 spectrophotometer (Qualitest, USA) according to Ohkawa *et al.* (1979), Paglia and Valentine (1967), and Nishikimi et al. (1972), respectively by using the appropriate kits (Biodiagnostic, Egypt).

Statistical Analysis:

Statistical analysis was performed using the SPSS software version 22. One-way analysis of variance was used to study the effects of both treatment types on the studied parameters. Post-hoc Duncan′s multiple range test was conducted to study the similarities in the studied variables among the experimental groups.

RESULTS

Assessment of DNA Fragmentation By Comet Assay (Single-cell gel electrophoresis [SCGE]):

The extent of DNA damage in liver cells of mothers and fetuses in lufenuron-exposed rat groups was measured using single-cell gel electrophoresis. The liver cells of both mothers and fetuses exposed to a low or high dose of lufenuron showed a significant increase in DNA damage ($P < 0.05$) as represented by the measured tail length, tail DNA%, and tail moment, relative to the control group (Fig. 1). The obtained results revealed a significant increase in tail length and tail moment recorded for liver cells of mothers in the LD group and their fetuses, relative to the control group. The highly significant increase in tail length and moment in the liver cells obtained from mothers in the HD group and their fetuses implies a massive DNA damage caused by the exposure to a higher dose of lufenuron (Table 1). The analysis of the results shows that the significance of the DNA damage after exposure to lufenuron increased in a concentration-dependent manner.

Table 1: Comet assay parameters obtained by image analysis in hepatic cells.

Group	Maternal			Fetal		
	Tails length (µm)	Tail DNA%	Tail moment	Tails length (µm)	Tail DNA%	Tail moment
Control	1.67±0.10	1.27±0.06	2.12±0.30	1.73±0.11	1.38±0.03	2.39±0.18
Low dose	4.54±0.18 *	4.00±0.11*	18.16±1.05*	2.17±0.16 *	2.39±0.05*	5.18±0.63*
High dose	8.05±0.53 **	6.43±0.04**	51.76±2.07**	4.00±0.23**	3.48±0.07**	13.29±0.33**

*: represents significant difference of tail length at P<0.05 as compared to the control.

**: represents significant difference of tail length at P<0.05 as compared to the low dose

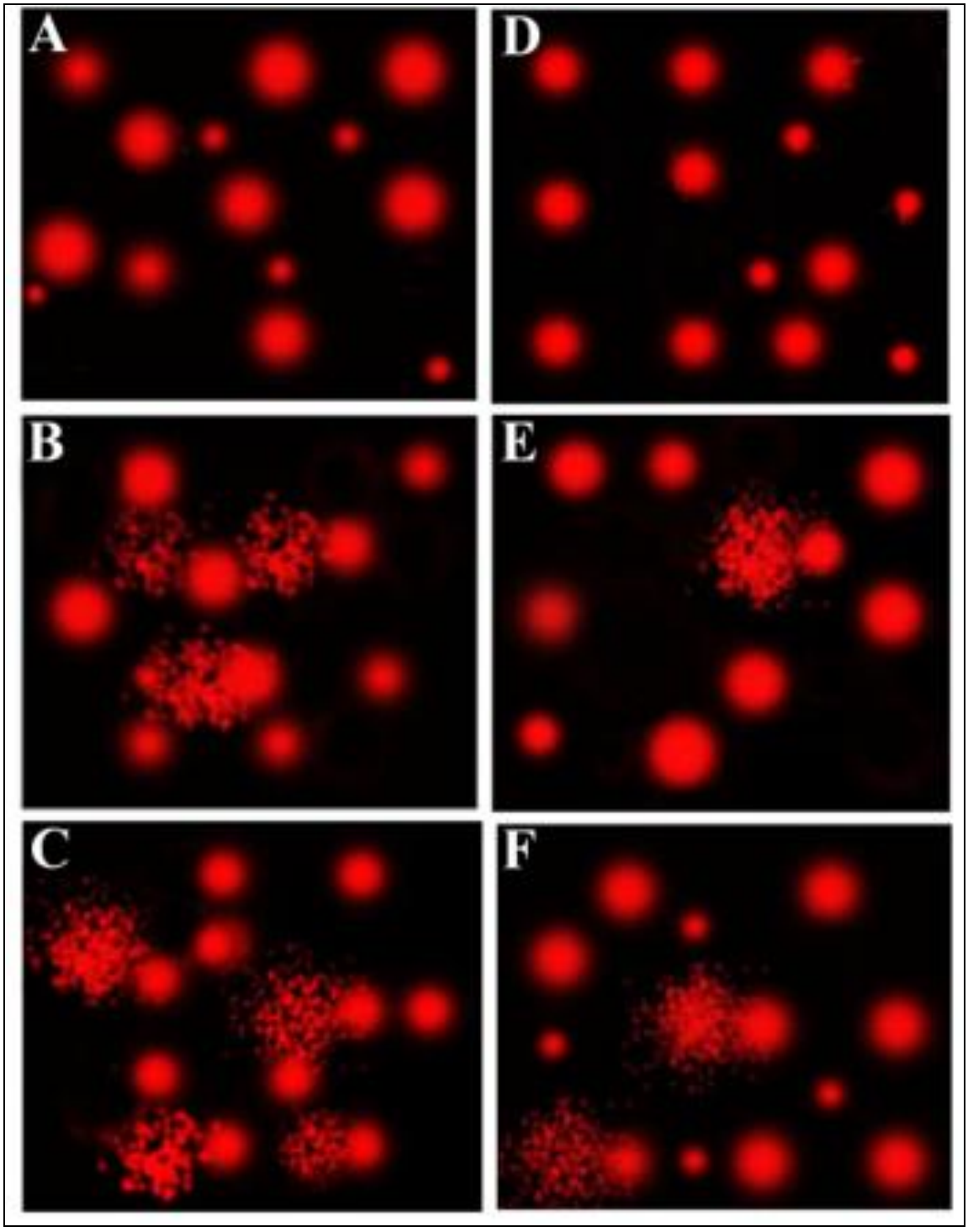

Fig. 1. Photomicrographs that demonstrate DNA damage in liver tissues using comet assay. **A)** Control group maternal cells, **B)** low-dose group maternal cells, **C)** high-dose group maternal cells, **D)** control group fetal cells, **E)** low-dose group fetal cells, and **F)** high-dose group fetal cells.

Cell Cycle Analysis Through Flow Cytometry Using Propidium Iodide (PI) Staining:

The results of cell cycle analysis for liver cells of mothers and fetuses in the treatment groups provided some evidence on the occurrence of cell cycle perturbations (Table 2). Maternal cells obtained from the LD groups showed a significant increase of cell percentage in the G0/G1 phase and a significant decrease in the G2/M phase cell

percentage, relative to the control group. There was no significant effect in the S phase. The cells of HD group on the other hand showed in addition to the significant increase of cell percentage in the G0/G1, a significant decrease in cell percentage in both S and G2/M phases (Fig. 2). Fetal liver cells obtained from the LD and HD groups showed a significant increase in cell percentage in the G0/G1 phase and a significant decrease in the S and G2/M phases, relative to that of the control group (Fig. 3). All the mentioned results suggest a cell cycle arrest at the G0/G1phase in the liver cells of mothers and fetuses obtained from treated groups.

Table 2: Average % of cells in each cell cycle phase in the liver cells.

Group	Maternal			Fetal		
	G0/G1 phase	S phase	G2/M phase	G0/G1 phase	S phase	G2/M phase
Control	49±2.20	17±1.14	33±1.27	54±2.58	18±1.11	27±1.60
Low dose	59±2.34*	17±1.00	24±1.22*	63±3.10*	16±0.96*	20±1.00*
High dose	67±2.10**	14±1.37**	19±1.09**	73±3.24**	14±0.82**	13±0.74**

*: represents significant difference (P<0.05) as compared to the control.
**: represents significant difference (P<0.05) as compared to the low dose.

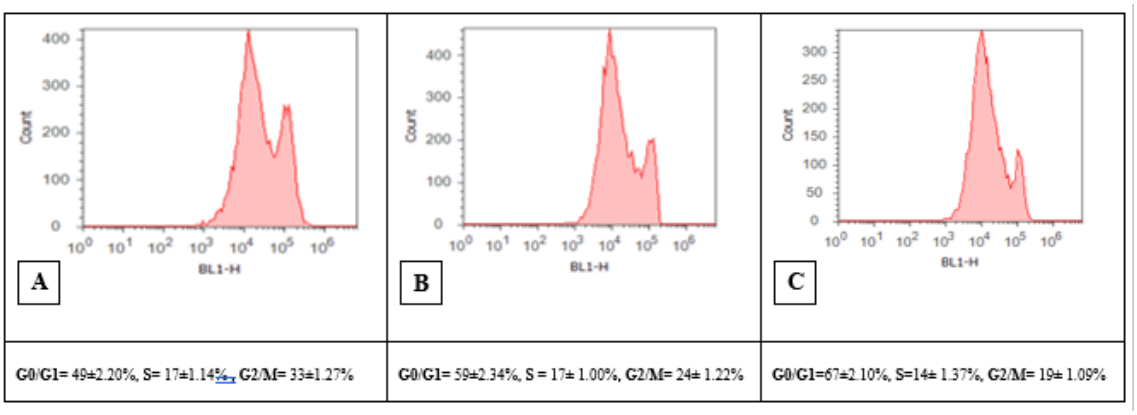

Fig. 2. Cell cycle analysis of maternal liver cells as illustrated by flow cytometry using propidium iodide staining; **A**. Control, **B**. low dose group and **C**. high dose group.

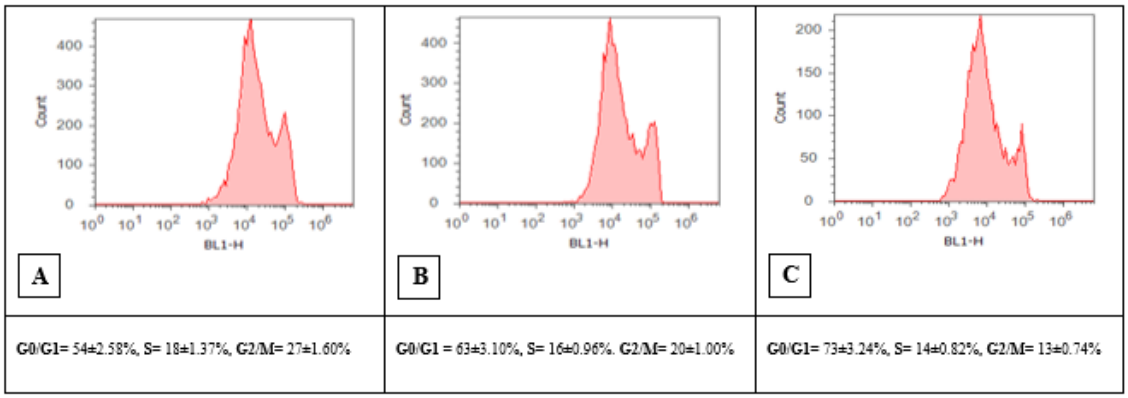

Fig. 3. Cell cycle analysis of fetal liver cells as illustrated by flow cytometry using propidium iodide staining; **A**. Control, **B**. low dose group and **C**. high dose group.

Estimation of Oxidative Stress:

The spectrophotometric analysis for the levels of MDA, GPx and SOD in liver cells obtained from mothers and fetuses of the control, LD, and HD groups revealed a potential of lufenuron exposure for posing oxidative stress on liver cells of the pregnant females and their fetuses in a dose-dependent manner (Table 3). The maternal and fetal

liver MDA level in the LD and HD groups was found to be significantly higher ($P < 0.05$) than that of the control group. The mothers' and fetuses' hepatic GPx and (SOD) activities were significantly decreased in both treatment groups when compared to the control group (Fig. 4).

Table 3: oxidative parameters of liver tissues

Group	Maternal			Fetal		
	G0/G1 phase	S phase	G2/M phase	G0/G1 phase	S phase	G2/M phase
Control	49±2.20	17±1.14	33±1.27	54±2.58	18±1.11	27±1.60
Low dose	59±2.34*	17±1.00	24±1.22*	63±3.10*	16±0.96*	20±1.00*
High dose	67±2.10**	14±1.37**	19±1.09**	73±3.24**	14±0.82**	13±0.74**

*: represents significant difference ($P<0.05$) as compared to the control.
**: represents significant difference ($P<0.05$) as compared to the low dose.

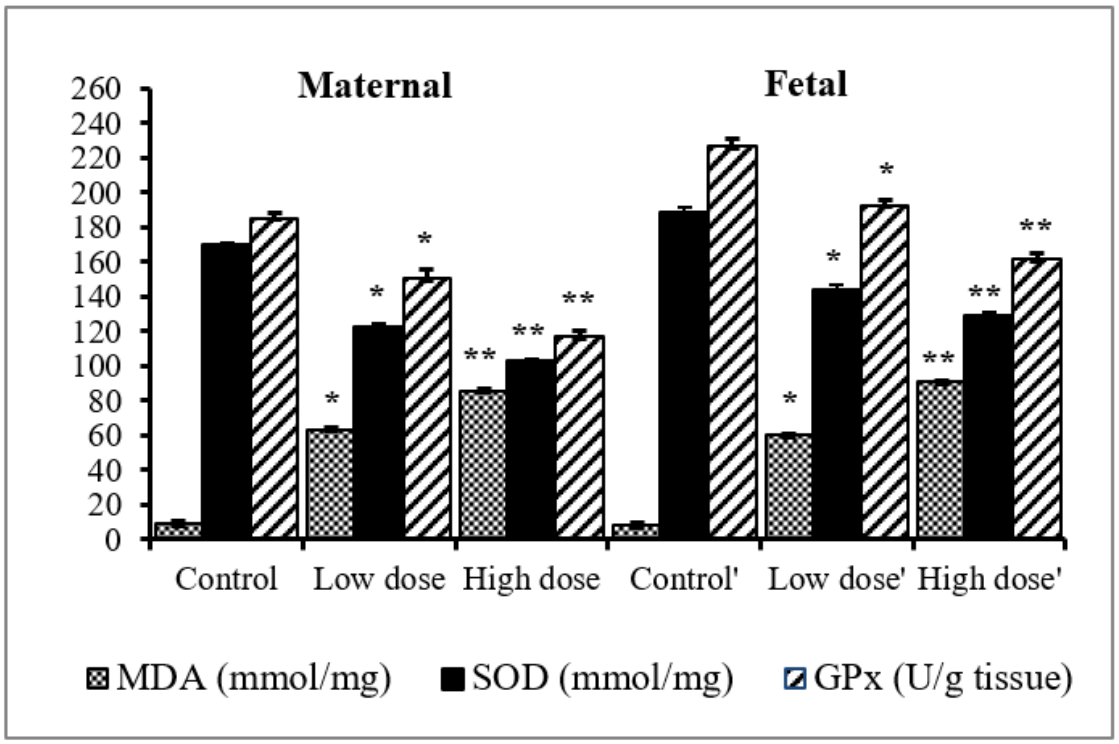

Fig. 4. Histogram representing the estimation of oxidative stress markers. The histogram represents; malondialdehyde (MDA) level in liver cells of mothers and fetuses of the three groups based on the MDA spectrophotometric assay. Superoxide dismutase activity (SOD) in liver cells of mothers and fetuses of the three groups based on the SOD spectrophotometric assay. Glutathione peroxidase activity (GPx) in liver cells of mothers and fetuses of the three groups based on the GPx spectrophotometric assay.

DISCUSSION

Excessive use of insecticides ignoring the safe management protocols, especially in developing countries, led to increased contamination of terrestrial and aquatic ecosystems and exposed humans and other forms of life to the harmful effects of these chemicals (Adjrah *et al*., 2013; Salako *et al*., 2012; Arias *et al*., 2011). The fact that most of the currently approved insecticides may be also toxic to several non-target species, including humans, aggravates the problem (Ilboudo *et al*., 2014). Moreover, the controversy about the excessive use of insecticides eventually led to the development and use of more biologically harmful insecticides, such lufenuron and other IGRs that

were previously considered to be safe (Alves *et al*., 2011; Payá *et al*., 2009).

The major risk factor in using insecticides is probably their genotoxic potential which may lead to long-drawn impacts such as cancer and chronic diseases (Bolognesi *et al*., 2011). The results of the present study suggested the high genotoxic potential of Lufenuron to pregnant albino rats and their fetuses when exposure occurs during the third gestational period. Both tested lufenuron concentrations (0.4 and 0.8 mg) were able to induce significant DNA damage and cell cycle perturbations as indicated by the comet assay and flow cytometry, respectively. In previous studies on *D. melanogaster*, lufenuron was proven to be a potent mutagen to both germline and somatic cells (Abd-Alla *et al*., 2003). It also interfered with the formation of spindle fibers in spermatid cells of mice (Deivanayagam *et al*., 2013) along with the induction of molecular lesions in the mice genome (Abou Gabal, 2006). At the DNA level, comet assay revealed significant damage in hepatocytes of female rats and their fetuses (Basal *et al*., 2020), fruit fly (Eid *et al*., 2017) and freshwater snails (Ibrahim *et al*., 2018) after treatment with lufenuron. The same results were obtained *in vivo* after treatment of mice peripheral blood lymphocytes to carbofuran (Pei et al., 2005).

In this study, the hepatocytes of both mothers and their fetuses showed cell cycle arrest at G_0/G_1 phase after exposure to the two lufenuron doses (0.4 mg/kg and 0.8 mg/kg). Flow cytometry by PI staining used in the current work was conveniently applied in previous studies to measure the quantity of DNA in each phase of the cell cycle (Crowley *et al*., 2016; Lee *et al*., 2015). The effect of insecticides on cell cycle arrest was reported in different cell lines. Cell cycle perturbations were detected in human peripheral blood mononuclear cells and adenocarcinoma human alveolar basal epithelial (A549) cell lines exposed to atrazine, butachlor, chlorpyriphos, and dichlorvos (Kaur *et al*., 2017). Cypermethrin induced G_0/G_1 cell cycle arrest in RAW 264.7 cells in a dose-dependent manner (Huang *et al*., 2016). Thiacloprid-based insecticide treatment resulted in p53-mediated cell cycle arrest at the G_0/G_1 phase, and apoptosis induction in bovine lymphocytes (Schwarzbacherová *et al*., 2019). In a more recent study carried out in our labs, exposure of pregnant albino rats to lufenuron during the organogenesis period led to cell cycle arrest in liver cells of both mothers and fetuses at the G_0/G_1 phase (Basal *et al*., 2020).

The oxidant-mediated responses -including cell death, lipid peroxidation, metabolic perturbations, and deregulation of several signaling pathways- might be considered as a hallmark for the toxic effects of insecticides (Hashimoto *et al*., 2008; Lee *et al*., 2004). Hence, the study of antioxidant enzymes might be used as a valuable tool to evaluate the response of many animal models after exposure to insecticides (Ghelichpour *et al*., 2019 a; Ghelichpour *et al*., 2019b; Yousefi *et al*., 2019; Mirghaed *et al*., 2019; Milić *et al*., 2018; Velasques *et al*., 2016; Mossa *et al*., 2015). In the present study, lufenuron treatment imposed oxidative stress on hepatocytes of the treated mothers and their fetuses. This was clearly manifested by the increased amounts of MDA and decreased levels of GPx and SOD enzymes in the liver cells. A previous study reported that mice treated with lufenuron suffered from increased levels of lipid peroxidation along with the decreased activity of GPx and SOD (Deivanayagam *et al*., 2014). Similar results were obtained in liver cells of mothers exposed to (0.4 and 0.8 mg) lufenuron during organogenesis period as well as their fetuses (Basal *et al*., 2020). Some enzyme biomarkers such as SOD, CAT, and GPx were significantly decreased in fipronil- and glyphosate-treated rats (Milić *et al*., 2018; Mossa *et al*., 2015) as well as in Diazinon treated mice (El-Shenawy *et al*., 2010).

CONCLUSION

The current work pointed out the potential of two residual concentrations of lufenuron (0.4 mg/kg and 0.8 mg/kg) to induce genotoxic stress, cell cycle arrest, and oxidative stress in pregnant albino rats. It also proved that used concentrations also affect the fetuses in the same manner when the exposure occurs during the third gestational period. These results ring a bell that women and their fetuses may be subjected to the same risk.

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ARABIC SUMMARY

التعرض للوفينورون خلال فترة الحمل الثالثة يسبب السمية الجينية و الاجهاد التأكسدي في الجرذان البيضاء الحوامل و أجنتها

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شهدت العقود القليلة الماضية زيادات هائلة في تراكم الكيماويات الزراعية ومخلفات المبيدات الحشرية في البيئة، مما أدى إلى تسمم غذاء الإنسان والحيوان. في دراستنا، قمنا بتقييم التأثيرات السامة للجينات والتأكسدعلي كل من الأم والأجنة نتيجة لتعرض الفئران الحوامل للوفينورون من خلال الفترة الثالثة من الحمل (اليوم 13-19). اعتمد التصميم التجريبي على تقسيم الإناث إلى ثلاث مجموعات. مجموعة التحكم (غير المعالجة)، مجموعة الجرعات المنخفضة (تتلقي عن طريق الفم مع 0.4 مجم / كجم من لوفينورون ومجموعة الجرعات العالية (تدار عن طريق الفم مع 0.8 مجم / كجم من لوفينورون). في اختبار المذنب، أظهرت خلايا الكبد للاناث الحوامل المعالجة بـ لوفينورون وأجنتها تلفًا كبيرًا في الحمض النووي مقارنةً بالمجموعة الغير معالجة. تم اكتشاف توقف دورة الخلية أيضًا في خلايا الكبد في طور G0 / G1 من خلال قياس التدفق الخلوي. تم الكشف عن الإجهاد التأكسدي الناجم عن اللوفينورون في خلايا الكبد للأمهات والأجنة من خلال زيادة بيروكسيد الدهون كما يتضح من ارتفاع مستويات malondialdehyde (MDA) - وانخفاض مستويات مضادات الأكسدة الأنزيمية (glutathione peroxidase [GPx]و([superoxide disutase [SOD] . في الختام، تشير النتائج التي تم الحصول عليها إلى الإمكانات العالية للتعرض المزمن لتركيزات لوفينورون خلال الفترة الثالثة من الحمل لممارسة ضغوط سامة وراثية وأكسدة على الفئران الحوامل وأجنتها.