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Association between uterine toxicity induced by chlorpyrifos and downregulation of heparin-binding epidermal growth factor and *L-selectin* genes

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Abstract

Various factors are effective in reducing the fertility rate. This experiment aimed to investigate chlorpyrifos (CPF), an organophosphate, that could alter the structure of the uterus and the molecules involved in parental and fetal. CPF was injected intraperitoneally in thirty mice for five days in a week (six weeks). The animals were euthanized on the 5th day of gestation, then their blood and uterus were collected for biochemical and histopathological assays. Exposure to CPF resulted in a significant reduction in maternal weight gain and the number of litters. Alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP) were significantly increased in blood serum of the CPF group compared with the control. The number of uterus glands, endometrium thickness, and the uterine cavity were changed following CPF injection. Additional investigation indicated that the expressions of *L-selectin, L-selectin ligand*, and *heparin-binding epidermal growth factor (HB-EGF)* as initial adhesion of mice blastocysts and maternal endometrium biomarkers were downregulated in the CPF group. Nevertheless, any mortality and abnormal clinical symptoms were not observed in the treated mice. This study revealed a potential molecular mechanism of continuous CPF-induced toxicity in fetal-maternal attachment without clinical symptoms.

Introduction

Implantation of embryo within uterus is the first step after successful fertilization of the oocyte. Placental formation is achieved by the adhesion of the blastocyst to the endometrium.[1] During a limited period and specific location, the proper interaction between the uterus as a receptor and the blastocyst as a receiver takes place.[2]

About 10 to 15 hr after the embryo hatches, first sign of conversion trophoblast mouse blastocyst to specific invasion cells appears in the zona pellucida site. At that time, the trophectoderm cells change and connect to the extracellular matrix.[3]

Following the appropriate endometrium thickness for the acceptance of the blastocyst, the specific blastocyst adherence molecules will be released and attached to the luminal epithelium.[4] During this period, the connection mechanism between mother and fetus take place by some adhesion molecules such as integrins, leukemia inhibitory factor (LIF), *L-selectins*, homeobox A10 (HOXA10), glutaredoxin (Glrx), glycodelinA (GlyA), and heparin-binding epidermal growth factor (HB-EGF).[2,5] Proper expression of these factors in the initial four - six days of gestation in mice leads to complete embryo placement, appropriate fetal attachment, and placenta formation without failure.[6]

Selectin family as a group of important cell adhesion molecules contains three glycoproteins L, P, and E, with a similar structure identified on the leukocytes, platelets, and endothelial cells, respectively.[1,7] Previous investigations have shown that *L-selectin* expression on the blastocyst and its interaction with oligosaccharide ligands on the surface of maternal lumina are the most essentials to the fetus-uterus attachment. The *L-selectin* ligands are located in the luminal and glandular epithelium of the uterus, and the expression is increased during the uterus acceptance.[8,9] There are some ligands for *L-selectin*, such as podocalyxin-like (PODXL), glycosylation-dependent cell adhesion molecule-1 (GlyCAM-1), and mucosal addressin cell adhesion molecule-1 (MadCAM-1) that PODXL is present at the time of uterus acceptance, which helps to stabilize embryonic connectivity.[6]

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The heparin-binding epidermal growth factor is one of the epidermal growth factor (EGF) family cytokines that cooperates with other molecules during embryonic development. It is expressed in the maternal glandular and luminal epithelium 6 to 7 hr before embryo attachment in humans and less than this in mice.[2,10] In the first step, blastocysts send some signals for uterus receptivity; subsequently, HB-EGF is expressed to respond to it, which is not dependent on sex hormones. As previous studies have established, in pseudo-pregnant mice with natural releasing sexual hormones, the HB-EGF expression does not occur.[10]

Previous studies indicate that environmental contaminants were one of the major causes of human infertility, so it is critical to understand the pollutant effects.[11] Based on previously published studies, chlorpyrifos (CPF) exposure can cause infertility or congenital disabilities in male and female reproductive organs. [12,13] Additionally, some adverse effects such as the nervous system and immune system disorders with dysfunction of pivotal organ physiology observe due to CPF exposure.[11]

To create an appropriate animal model for evaluating the effects of CPF toxin, oral and injection methods can be used. Based on experimental studies, prescribed a wide range of CPF (82.00 - 504 mg kg^{-1}) in mice orally for one - four weeks leads to various symptoms such as unconsciousness, muscle paralysis, sweating, haziness, and abdominal pains. [11] Additionally, injection of CPF in pregnant rats with a low dose (1.00 mg kg^{-1}) did not elicit any mortality, while in high-dose induction (5.00 mg kg^{-1}), the mortality rate increased.[14]

The liver is a vital organ in the biotransformation of food, drugs, and toxins, so liver enzymes can be used as biomarkers to detect toxicity effects caused by pesticides.[15,16] The assessment of alanine Aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP), is useful for the early detection of liver dysfunction followed by CPF toxicities.[17]

Additionally, CPF has consequences on the placenta and embryonic development that require more investigation to characterize the toxin effects.[12] Many studies have focused on variations of pregnant females exposed to organophosphates while few researches have examined the impact of these pesticides on the uterus. Based on previous studies, in the human cytotrophoblast isolated cells exposed to CPF, the apoptosis rate of the placenta stromal cell can be increased leading to the disruption of implantation and abortion.[14]

The inhibition of adhesive molecules like *L-selectin*, *L-selectin ligand*, and *HB-EGF* is one of the most critical risk factors for falling mouse uterine acceptance rate. So, the effect of CPF on infertility can be assessed by analyzing the rate of mentioned protein expression. Based on our knowledge, investigations on the implantation window and their related molecules with a long time and low dose prescription of CPF have not been studied. On the other hand, embryo attachment evaluation in humans is ethically and realistically impossible; researchers need to induce suitable animal models to investigate the toxin effects on the female reproductive system.

Materials and Methods

Selection of animals and CPF administration. In this study, 30 females outbred NMRI mice about 10-weeks old (24.00 - 28.00 g) were purchased from the Animal Facilities and Husbandry Department, Pasteur Institute of Iran. After the incubation period and confirmation of healthy condition, all mice were divided into three groups: treatment received 3.00 mg kg^{-1} CPF (Sigma-Aldrich, Darmstadt, Germany), control (without any injection), and sham received 40.00 μL kg^{-1} dimethyl sulfoxide (Sigma-Aldrich). All mice were transferred to the laboratory following the initial examination and assurance of health conditions. The animals were housed in controlled situations as 12:12 hr dark:light cycle at a temperature of 21.00 ˚C in a type III polycarbonate mouse cage with stainless steel wire bar lid. The mice had access to adequate and appropriate water in polycarbonate bottles and were fed with standard rodent pellets. All mice weighed on the first and the last day of the experiment before euthanasia. The stoke solution for injection in 40 mice was prepared as followed: 3.00 mg CPF (Sigma-Aldrich) dissolved in 40.00 μL dimethyl sulfoxide (Sigma-Aldrich) then diluted in normal saline to prepare the low dose of toxin. As a result, 100 μL of prepared stock was injected intraperitoneally in each mouse every day for six weeks (five days per week; I.P).[18,19] The mice in the sham and the treatment groups received equivalent doses of dimethyl sulfoxide, while the mice in the control group did not receive any injections.[20] Followed the final injection, females mated with the healthy untreated males. After confirmation of pregnancy by observation of vaginal plaque, days of embryo formation were calculated. Ethical approval for the study was obtained from the Islamic Azad University Science and Research Branch (IR.IAU.SRB.REC.1398.060).

Blood sample collection and biochemical assays. The blood samples to isolate sera were obtained via heart puncture on the 5th day of gestation, under general anesthesia by 100 mg kg^{-1} ketamine (Alfasan, Woerden, Netherlands) and 18.00 mg kg^{-1} xylazine (Alfasan).[21] Liver enzymes AST, ALT, and ALP levels were measured by an auto-analyzer (Alpha Classic; Sanjesh Company, Tehran, Iran), using diagnostic kits (Pars Azmun, Tehran, Iran).[22]

Uterine tissue collection. On the 5th day of gestation, after anesthesia, the blood samples were obtained by cardiac puncture then mice were euthanized in a CO_2 chamber, and embryos were removed by uterine flushing.[23] Briefly, the uterus was separated using an

insulin syringe containing normal saline to wash it without causing rupture. Using the loop microscope (Nikon, Fujisawa, Japan) the number of placenta of each uterus counted. The obtained uterus samples were fixed in 10.00% formalin solution for tissue processing, and in other micro tubes, the uterus was stored at – 80.00 ˚C for more studies.[24]

Histological studies of the uterus. The fixed uterus samples were dehydrated by alcohol series, clarified in xylene (Sigma-Aldrich), and embedded in paraffin. Serial paraffin sections (a thickness of 4.00 - 6.00 µm) were prepared for Hematoxylin and Eosin (H&E) staining. The further histological evaluation was done by an optical microscope (Nikon) to determine the gland numbers and thickness of the uterus. Finally, Image J Software (National Institutes of Health, Bethesda, USA)evaluated uterine thickness and gland numbers in all sections.[25]

Real-time PCR (RT-PCR) studies: quantitative analysis of gene expression. The RT-PCR evaluated the expression levels of *HB-EGF, L-selectin*, and *PODXL* genes. Uterine RNA was extracted according to Qiagen (Hilden, Germany) protocol from all samples by using QIAzol reagent (Qiagen). The RNA samples were treated with DNase I, then the RNA samples were reverse-transcribed into cDNA using fast cDNA synthesis, enabling sensitive real-time kit (Qiagen) protocol and oligo (dT) primers. Each PCR reaction performed using the PCR master mix and SYBER Green (RealQ Plus 2x Master Mix Green – Amplicon, Odense, Denmark), on ABI Step One (Applied Biosystems, Foster, USA), according to the manufacturer's protocol. Forty cycles were considered for each Real-Time PCR and temperatures of each cycle were set at 94.00 ˚C for 30 sec, 58.00 ˚C for 30 sec, and 72.00 ˚C for 30 sec. Specific forward and reverse primer sequences were designed for the genes studied using NCBI (CinnaGen, Tehran, Iran) as shown in Table 1. Beta-actin is used as a housekeeping gene, which is amplified in the same run for each gene. Data were analyzed based on delta-delta CT from the device, and the normalization of data was done by β-actin as a reference gene.

Western blotting. Briefly, the uterus samples were homogenized by radioimmunoprecipitation assay buffer (Sigma-Aldrich) containing protease inhibitor. Then, proteins are transferred to a polyvinylidene fluoride (PVDF) membrane. The membranes were incubated by blocking solution (5.00% non-fat milk). Then, the specific primary antibodies against *L-selectin* and HB-EGF were used on a swing shaker for 120 min. Proteins were incubated with horseradish peroxidase-linked (HRP) secondary antibody for 90 min at room temperature on a shaker (IKA, Staufen, Germany). Then, protein expression was confirmed by using chemiluminescence on radiographic film. The band intensities acquired from each protein extract normalized against the corresponding band values of the house-keeping protein GAPDH. The protein band density was quantified using ImageJ software.[25] The following antibodies were used: *L-selectin* (B-8): sc-390756, a mouse monoclonal antibody, HB-EGF (H-1): sc-365182, a mouse monoclonal antibody, and Glyceraldehyde- 3-phosphate dehydrogenase (GAPDH) (6C5): sc-32233 for primary antibodies. The secondary antibody was m-IgGκ BP-HRP: sc-516102.

Statistical analysis. Statistical analysis among three groups was conducted by One-Way ANOVA, followed by the Tukey post hoc test multiple. A $p < 0.05$ was considered to represent statistical significance. Results presented as the means ± standard error (SE). The Kolmogorov-Smirnov test was selected for the normal distribution of mice weight gain during the study and assessed by the Paired Sample T-test. All of the figures were evaluated by Image J software. By using the Prism Software (version 5.04; Graph Pad Software Inc., San Diego, USA) and SPSS (version 27; IBM, Armonk, USA), differential genes and protein expression values were evaluated.

Results

Effects of CPF on body weight and the number of litters. As shown in Figure 1A, in comparison with the CPF group, a higher significant increment in the weight gaining of the sham and the control group was observed ($p < 0.001$). Despite all mice being pregnant, the number of litters in the treatment group have shown a significant decrease ($p < 0.001$) compared to the other groups. (Fig. 1B). The mean ± SE for the CPF group was 8.20 ± 0.51 whereas in the control and the sham group were 13.30 ± 0.49 and 11.70 ± 0.65.

Table 1. Specific forward and reverse primer sequences used in RT-PCR.

Gene name	Primer sequence	Product size (bp)	Annealing temperature (˚C)	GenBank® accession
HB-EGF	F 5′- AACAACCCTGACCCTCCCACT-3′	203	63.11	NM-010415.2
	R 5′- CTCTTCTTCCCTAACCCCTTTCC-3′		60.05	
L-selectin	F 5′- CTAAGGAGGACTGTGTGGAGA-3′	173	58.19	NM_011346.2
	R 5′- GATGGAGGTGTGATTGTTGATAG-3′		57.00	
PODXL	F 5′- CTAATCCTCCCTCCCCACTTC-3′	191	58.95	NM_013723.3
	R 5′- TCACACACCATTCTCAACTCCA-3′		59.56	
β-actin	F 5′- TCAGAGCAAGAGAGGCATCC-3′	187	59.17	NM-007393.5
	R 5′- GGTCATCTTCTCACGGTTGG-3′		58.27	

HB-EGF: heparin-binding epidermal growth factor; *L-selectin*; *PODXL:* podocalyxin-like; bp: base pair.

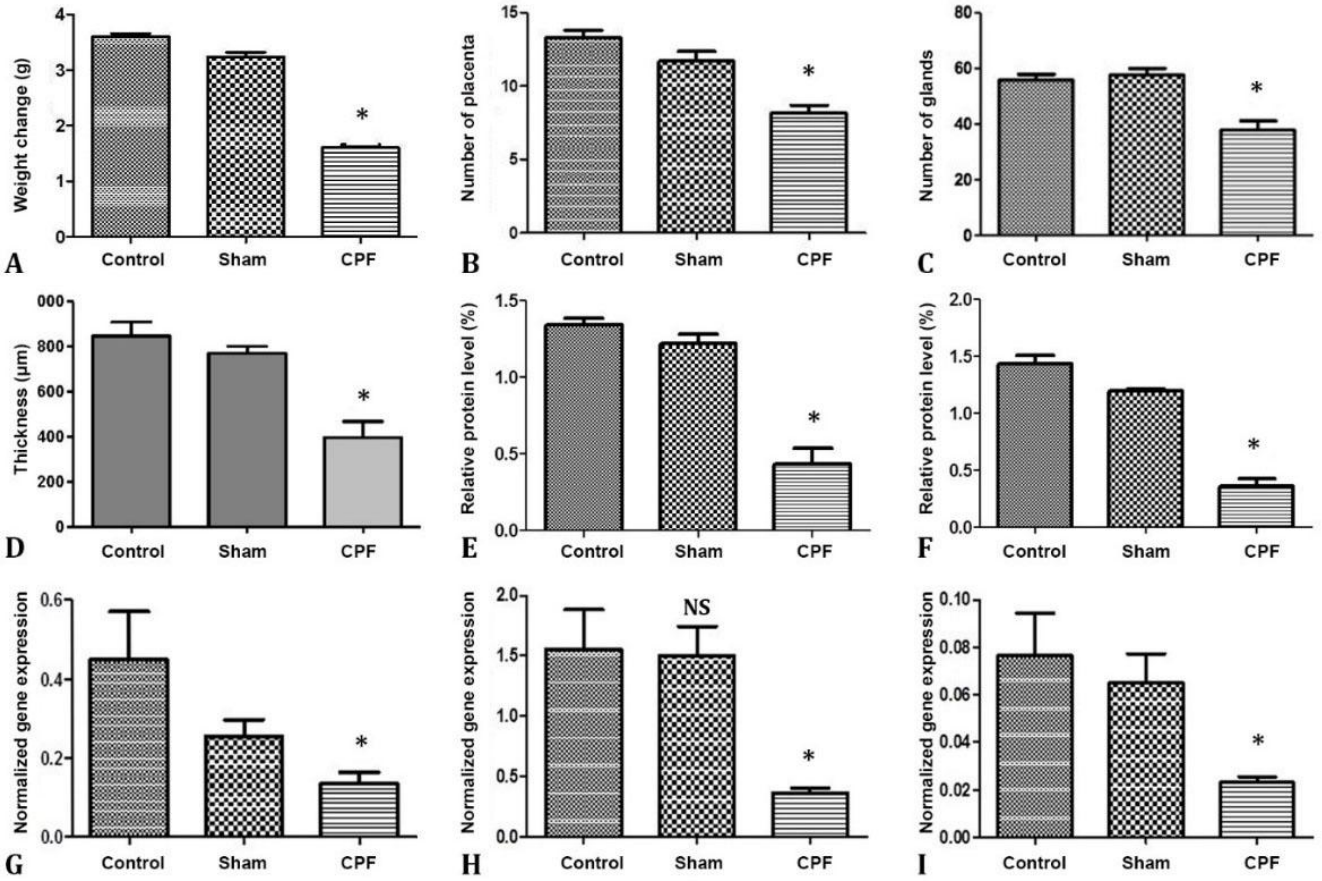

Fig. 1. A) The weight of examined mice was calculated at the beginning of the study and the end of the injection. The weight change showed in this Figure, the significant increase of weight observed in the control and sham group in comparison with the treatment (CPF) (* $p < 0.001$); **B)** The number of placenta in all three groups counted. The effect of CPF on treated mice was highly significant (* $p < 0.001$). **C)** Significant decrease in gland numbers of CPF groups compared with others (* $p < 0.001$); **D)** the thickness of the endometrium decreased in the treatment group (* $p < 0.01$). **E)** Quantification of protein levels of HB-EGF in the CPF group compared with the control (*$p < 0.001$) and, **F)** expression of L- selectin in CPF (* $p < 0.001$) in contrast with the control and the sham by Western blotting; **G**, **H**, and **I)** The gene expression patterns for three representative genes including *HB-EGF, L-selectin* and *PODXL* on the 5th day of gestation in three groups (control, sham, and CPF) were assessed by RT-PCR. Quantitative RT-PCR analysis of all RNAs were significant in CPF groups for *HB-EGF* (* $p < 0.05$); *L-selectin* (* $p < 0.01$), and *PODXL* (* $p < 0.05$).

Liver enzymes. According to the results, the concentration of ALT in the treatment group was significantly higher than the control group ($p < 0.001$) and the sham ($p < 0.01$), even though there were not any differences between the control and the sham group ($p > 0.05$). The serum level of AST in the CPF group also increased compared with the control group ($p < 0.01$) and the sham ($p < 0.05$). Additionally, ALP concentration in the CPF received group was significantly higher than the control group ($p < 0.05$) but in comparison to the sham group this enhancement was not statistically significant ($p > 0.05$). There were no significant changes in the sham group compared with the control group in both AST and ALP levels ($p > 0.05$).

Histopathological examination. The histopathological changes followed by CPF induction were evaluated by endometrial glands, uterine cavity, the edge of the uterine epithelium, and thickness of the uterus (Fig. 1D). Some abnormalities in the epithelial surfaces of the uterus were detected in the uterus of the CPF group such as the inconsistency in the endometrium cells and basal membrane, with a decrease in the thickness of the endometrium, myometrium and perimetrium. However, hyperplasia and hypertrophy of luminal and glandular epithelium and neutrophil infiltration in endometrium were not observed in the CPF group (Fig. 2). The numbers of secretory glands in the CPF group compared with the control and the sham decreased ($p < 0.001$; Fig. 1C) but in the control and sham group, the glands were distended and their epitheliums become prepare for secretion which was misses in CPF sections. In contrast, well-developed epithelium and uterine glands in the lamina propria were observed in the control and the sham groups. The CPF group's uterine cavity was larger than the others, but the uterus thickness was reduced compared to the control groups ($p < 0.01$) and the sham ($p < 0.01$; Fig. 1D).

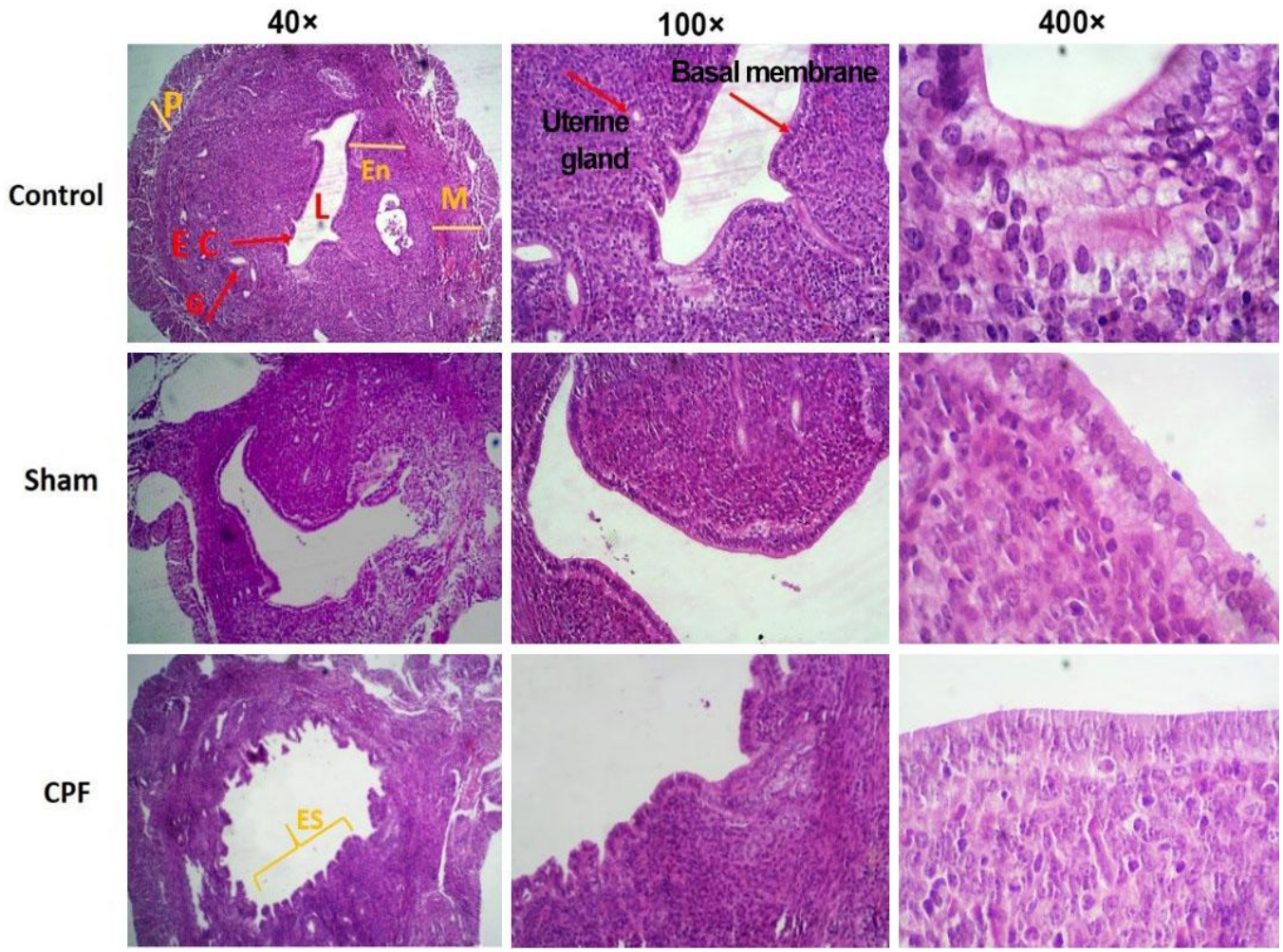

Fig. 2. A) Uterus samples of mice with Hematoxylin and Eosin staining in control, sham, and the treatment group after six weeks with a dose of 3.00 mg kg^{-1} (CPF). ES: epithelial surfaces, EC: epithelial cell, L: lumen, M: myometrium, P: perimetrium, G: gland, and En: endometrium. Some inconsistency in the epithelial surfaces in CPF group had been showed by ES.

Western blotting / real-time PCR. We surveyed whether CPF exposure changed the level of *HB-EGF* and *L-selectin* related to the uterus function (Fig. 3). Based on the results HB-EGF in the treatment group decreased significantly compared with the control and the sham groups ($p < 0.001$; Fig. 1E). A comparison of the L-selectin protein level in the treatment group showed a significant decrease in comparison with the others ($p < 0.001$; Fig. 1F). Next, the mRNA expressions of *L-selectin, PODXL*, and *HB-EGF* were studied by Real-Time PCR analysis with applied β-actin as a reference gene (Fig. 1). In the uterus samples of the CPF-treated mice, the mean ± SE was calculated for the expression level of *HB-EGF* (0.13 ± 0.02) in contrast with the control group (0.44 ± 0.12) and the sham (0.25 ± 0.04). It appeared that the expression level of *HB-EGF* was lower in the treatment group than in the control group ($p < 0.05$), but this difference was not statistically significant in the sham group and the CPF group (Fig. 1G). Expression of *L-selectin* in the treatment group was significantly lower than the control ($p < 0.01$) and sham ($p < 0.05$), as the mean ± SE in the CPF, the sham, and the control groups were 0.36 ± 0.03, 1.50 ± 0.24, and 1.55 ± 0.32. In contrast, the results in sham group were not significantly different from control group (Fig. 1H). The mRNA level of *PODXL* in the treatment group was significantly lower than control group ($p < 0.05$), while the sham group ($p > 0.05$). The results in the sham group were not significantly different from control group (Fig. 1I).

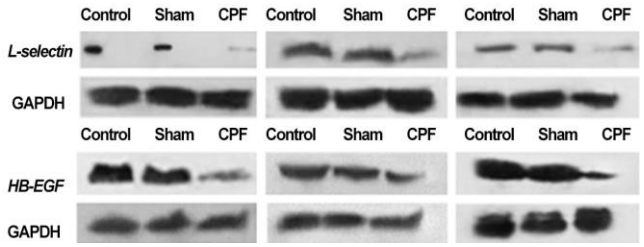

Fig. 3. The blot of HB-EGF and L- selectin protein represent in all three groups with GAPDH.

Discussion

Abortion in mammals can be caused and affected by different factors, including immunological and hormonal problems, infections, and some environmental factors. Despite recent improvements in supported reproduction techniques, the implantation rate for each fetus infrequently exceeds 30.00%. Any disorganization in the uterus structure and inappropriate factors cause disorder in produce relative adhesion molecules and subsequently no attachment between the mother and the embryo.[2]

In recent studies, the CPF effects with high doses of consumption on the reproductive system of mice and rats have been proven.[26]. But the effect of low or medium continuous doses of CPF has not been investigated on the molecules related to the implantation window. Thus CPF, as one of the most extensively used pesticides, was tested on the female reproductive system of mice in this investigation. The selective dose used in this research was the average dose used in previous studies to prevent mortality and recognizable clinical symptoms.[27] The IP injection of 3.00 mg kg^{-1} CPF per day caused toxic effects on the liver but there were no clinical symptoms such as tremors, arching of the back, and respiratory distress mentioned in the other studies.[27]

As described before, the selected dose surprisingly revealed toxic effects on female mice by decreasing in gaining weight and the number of litters. In comparison to the control and sham values, mice maternal weight at the start of the trial and the end of the injection period showed that CPF could affect weight gain, as corroborated by Farag *et al*. and Ambali *et al*.[27,28] Compared to the other groups, the dose used to induce the animal model had adverse effects on mice-weight gaining.

The present study focused on the expression of *HB-EGF, L-selectin*, and *PODXL*, which were essential for stable attachment of the fetus to the uterus. *HB-EGF* binds to its receptors on the embryo surface which is necessary for the regulation of fetal-uterine adherent molecules.[29] Moreover, this protein is essential for follicular development, embryo implantation, ovulation, and early development.[30] Researchers have found that *HB-EGF* mRNA has been expressed in the endometrium early pregnancy villi, decidua, and placenta.[31]

Additionally, the endometrium HB-EGF protein and mRNA expression reduced in the CPF exposed mice compared with the control group. These results revealed the impact of CPF on the implantation window stage, which triggered a miscarriage and decreased the amount of litter confirmed by Wang *et al.*[29] In our experimental group, the insufficient thickness of endometrium for healthy pregnancy was correlated with the lower expression of HB-EGF that proved the synergistic action of mentioned factors on the uterus-fetus interactions. The results showed that CPF injection decreased the expression of *L-selectin* and *PODXL* mRNA in comparison to the other groups. Genbacev *et al*. mentioned that L-selectin's binding to its ligands might be the first step in embryo implantation and was an essential regulator for human pregnancy.[32] Also, in line with the findings of Ambali *et al.*, CPF injection caused a reduction in the placenta number.[27] These results suggested that *HB-EGF*, *L-selectin*, and its ligand expression in the endometrium afterward CPF injection may be closely related to the litter numbers reduction and uterus morphology abnormalities.

In control and sham groups, ideal implantation had occurred due to the appropriate function of uterus glands but in CPF treated-mice group, proper implantation did not happen because the number of functional uterus glands was inadequate. As Hirota described, these glands were responsible for attracting the embryo to attach to the uterus. These reductions lead to a decrease in litter numbers as described before.[4] In histopathological assessments, increasing the uterine cavity and decreasing the uterus' thickness, followed by using CPF, was recorded. All this data is in accordance with other researchers who evaluated lead acetate as one of the most critical environmental toxins, which decreased the thickness of the uterus.[33]

One study to evaluate the antioxidant effect of green tea against the oxidative stress of CPF on the liver of male rats found that 6.75 mg kg^{-1} CPF could cause a significant increase in hepatic ALT and AST after 28 consecutive days.[17] According to the results, a significant elevation in the liver enzymes was observed in CPF received group compared to the others. These findings are consistent with the previous reports on fish and rats, in which toxin exposure caused a significant enhancement in the liver enzymes level.[16,17] Additionally, any statistical differences in measured factors was not observed between the control and the sham group. It confirmed that the DMSO concentration did not affect the pregnancy rate. Therefore, the selected dose of DMSO as a CPF solvent did not have any effect on the evaluated data which proved the observed alteration in selected criteria was due to the consumed CPF.

The expression of *HB-EGF, L-selectin*, and *PODXL* was down-regulated by environmental factors, which led to abnormalities in uterine epithelia and glands. The prenatal CPF exposure in mice caused liver enzymes' elevation, reduction of maternal gaining weight, and the number of litters. Thus, the present results suggest that environmental factors sometimes have no noticeable consequences. However, their side effects on the fetus and fertility due to the long period of consumption can reveal by bimolecular assessments in the implantation window phase.

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Conflict of interest

All authors declared that they have no conflict of interest.

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