Effects of Chemical Migrants from Two Widely Used Plastics on Reproduction in Mice

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Two plastic items were investigated for toxicity, due to chemical migrants, on reproduction and subsequent pregnancy outcomes. Extraction of high-density polyethylene (HDPE) food oil jerrycans, and polyvinyl chloride (PVC) blood bags was carried out. HDPE and PVC were extracted with sesame oil and normal saline, respectively. The extracts were prepared daily and administered (50 ml/kg b.w.) into pregnant Swiss albino mice from gestation day 0 until delivery. Control groups received the pure vehicles that were subjected to the same conditions of extraction and extracts. Pregnancy weight gain, gestation period, litter size, stillbirths and offspring sex ratio were recorded. Blood sex hormones (progesterone, estradiol and prolactin) were assayed for each pregnancy trimester. Birth weight, growth rate and sex hormone levels [females: follicle stimulating hormone (FSH), leutenizing hormone (LH) and estradiol (E_2); males: testosterone] were monitored in offspring. ELISA was applied to assay hormones. HDPE caused significant ($p \leq 0.01$) stillbirth. Blood hormone levels in dams and offspring for both treatments indicated no significance. PVC treatment exhibited negative effects on all parameters. In conclusion, HDPE is leachable and could affect reproduction, as indicated by the stillbirth incidence. PVC sample might not be toxic at the conditions of the experiment. Oil-plastic extract could exhibit a pronounced effect on pregnancy outcomes in contrast with the aqueous one.

Key words —— high-density polyethylene, polyvinyl chloride, migrant, extract, pregnancy, mice

INTRODUCTION

The usage of plastics over the past century has enabled humanity to make huge technological advances. Probably, no industry has undergone such rapid growth and development as the plastics industry. A plastic material, in fact, is a group of chemicals blended with the parent polymer through certain industrial processes. These chemicals, collectively known as additives, include plasticizers, coloring agents, flame retardants, stabilizers, antistatic agents, blowing agents, and fillers. The resulting material is then fabricated into a suitable product for a specific application.[1,2)]

A number of studies are still going on to investigate various toxic effects posed by plastics migrating chemicals, with emphasis on plastics used in biomedical items and packaging of food and drugs. It would be inevitable that any leaching chemical from the plastic container would find its way into the foodstuff or drug stored into that container. Most migrants have a greater leachability rate into oily and fatty media due to their organic nature.[3)] The migrating chemicals can have both an impact on food safety and an impact on food quality, a matter that also applies to drugs and biologic solutions.[4,5)]

Reproductive and developmental toxicologic aspects of plastics migrants represent an expanded area for *in vitro* and *in vivo* studies.[6)]

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MATERIALS AND METHODS

Animals —— Virgin female and adult male Swiss Webster albino mice of 25–35 g body-weight range were used. The animals were dealt with according to the Animal Care and Use Committee (ACUC)[7)] guidelines for animal care. They were housed in macrolone cages (27 × 21 × 14 cm), with sawdust bedding and had free access to commercial laboratory chow and tap water. The colony room was main-

Table 1. Categories of Pregnant Mice according to Treatment Protocol

Treatment group	Control group	Route
40 mice treated with sesame oil (SO) extract of the HDPE chips.	40 mice given SO (vehicle) only	per os
40 mice treated with normal saline (NS) extract of the PVC chips.	40 mice given NS (vehicle) only	i.p.

tained at 23 ± 1°C and relative humidity of 45–55%, and a 12-hr light/dark cycle (under fluorescent light). Animals were quarantined for a period of 6 days to check their suitability for the study, on the basis of good physical condition and freedom from clinical signs of disease or injury during this period. The animals were subsequently mated for breeding. During the mating period, mice were housed in the order of one male plus two females (triad breeding) per cage, between 7:00 p.m. and 8:00 a.m. Males were then removed and females were checked for vaginal copulatory plug; the day the plug was observed was designated gestational day (GD) 0. Each mated female was then placed in an individual cage under the same conditions until delivery, unless otherwise stated. On day 4 postpartum, litters were culled randomly to eight pups and left with dams up to weaning (at 21 day age), after which they were separated according to sex.

Study Materials —— Two types of plastics were used for this study:

High-density polyethylene (HDPE) jerrycans (manufactured by a local vegetable oil company to package their product), Jeddah, Saudi Arabia.

Polyvinyl chloride (PVC) Blood collection bags [obtained from KAAU Hospital (KAAUH)], Jeddah, Saudi Arabia.

Preparation of the Plastic Extracts —— The extraction of the plastic samples, under this study, followed the method described in the United States Pharmacopoeia (USP).[8] According to the method samples were cut into small chips (0.5 cm in the larger dimension). Extraction of each sample was carried out on the basis of 4 g of the plastic material per 20 ml extraction medium, using 0.9% NaCl (normal saline solution) for the PVC sample and sesame oil for HDPE. Extraction was carried out into glass-stoppered flasks in the oven, with incubation at 50°C for 72 hr. Both extraction media were subjected to the same conditions of extraction and extracts. The extracts were prepared daily and cooled to room temperature before being administered into pregnant mice according to the protocol of animals' treatment below. The normal saline solution and its corresponding extract were subjected to sterilization, prior to intraperitoneal (i.p.) injection of animals, *via* microfilteration with sterile Millex®-GS 0.22 μm filter unit (Carrigtwohill Co., Cork, Ireland), inside a biological safety cabinet (NUAIRE Inc., Minnesota, U.S.A., Model NU-201).

Treatment of Animals —— Two groups of pregnant dams, 40 animals each, received the extracts in a daily dose equivalent to 50-ml/kg-body weight,[8,9] starting from GD 0. Animals receiving the PVC extract were injected i.p. Those receiving HDPE extract were treated orally (per os). During the treatment course of each group, 10 animals were sacrificed to collect blood samples for the assay of pregnancy hormones at the end of each trimester (*i.e.*, 6, 12, 18 days from the beginning of gestation), with a total of 30 animals sacrificed for the three trimesters. The remaining 10 continued the daily treatment until delivery. Forty animals were used as a control group for each treatment. Control groups received the extraction media (vehicles) under the same conditions of treatment and were dealt with similarly.

In summary, the 4 groups of pregnant mice were categorized according to Table 1.

Blood samples were collected in vials containing the anticoagulant ethylenediaminetetraacetic acid (EDTA) and then subjected to centrifugation (at room temperature), using micro-centrifuge (model 235B, Fisher Scientific Company, U.S.A.) at 3900 rpm for 15 min. Plasma specimens harvested were stored in stoppered and labeled Erlenmeyer vials at –20°C until analysis for sex hormones.

Litters produced by the surviving treated and control subgroups, corresponding to the above 4 groups, were subjected to a battery of tests (see parameters below) to monitor prenatal and perinatal effects of the plastics extracts on offspring.

Parameters —— According to Dixon[10] and Hood,[11] the following parameters were monitored as pregnancy outcomes for all dam groups:

Weight gain in the pregnant dams (weighing the animals twice a week).

Assay of blood sex hormones during pregnancy: estradiol (E_2), progesterone and prolactin like hu-

man chorionic gonadotropin (hCG) in humans, prolactin performs the luteotropin function of supporting the corpus luteum until the placenta produces amounts of progesterone sufficient to support pregnancy.[12,13]

Gestation period.

Litter size (number of pups per litter).

Stillbirths.

Sex ratio (number of males/number of females).

The offspring of all groups were monitored for:

Growth rate (weighing the offspring twice a week, from delivery up to weaning).

Assay of blood sex hormones in the adult offspring:

Females: Follicle stimulating hormone (FSH), Leutenizing hormone (LH), E_2.

Males: Testosterone.

Sex Hormones Assay —— Blood levels of sex hormones were determined with enzyme-linked immunosorbent assay (ELISA) method.[14] Assay kits for these hormones were obtained from Diagnostic Systems Laboratories, Inc., Webster, Texas, U.S.A. Bioelisa Reader, Model EL_x800 and automatic Bioelisa Washer, Model EL_x50 (Bio-Tek Instruments Inc., U.S.A.) were used. The Reader was calibrated to plot the mean absorbance readings for the standards supplied for each hormone, yielding a calibration curve from which it reads the concentration values of unknowns. Assays were conducted using protocols supplied by the manufacturer for each individual hormone.

Student's *t*-test was used to analyze data obtained for all parameters to determine statistical significance.[15]

Gas Chromatography/Mass Spectrometry (GC/MS) Analysis of the Plastic Extracts —— Plastic extracts under this study were subjected to qualitative analysis to identify the plastic leachables. According to the method applied by Simoneau and Hannaert,[16] the extract (prepared at the experimental conditions of this study) of HDPE was extracted with acetonitrile (HPLC grade, Fisher Scientific Company), whereas the extract of PVC, on the other hand, was extracted with *n*-hexane (HPLC grade, Fluka Company, Switzerland).

A GC/MS-QP2010 Series instrument (Shimadzu Corporation, Analytical Instruments Division, Kyoto, Japan) was used for GC/MS analysis.

Chromatographic Separations were Performed under the Following Conditions: Capillary column: Rtx-5MS (Restek Corp., U.S.A.), 30 m × 250 μm i.d., film thickness: 0.25 μm.

Injection: 1.0 μl, using the pulsed split mode (with split ratio of 20).

Injector temperature: 225°C.

Interface temperature: 250°C.

Mass spectrometer ion source temperature: 280°C.

Ion source: electron ionization (EI), positive ion mode.

Carrier gas: helium (under pressure of 66 kPa, and purge flow of 3.0 ml/min).

Temperature programme: from 40 to 280°C at 48°C/min, with an initial isotherm of 2 min and a final isotherm of 6 min.

Total run time: 13 min.

Electron energy: 70 eV.

Full-scan mass range: 50–350 amu.

Mass spectra were interpreted by comparison with the mass spectral library software of the National Institute of Standards and Technology (NIST).

RESULTS

Pregnancy Outcomes

HDPE Extract: The weight gain in the HDPE-extract-treated dams during gestation period was comparable to that of the control (Table 2).

The pregnancy outcomes shown in Table 3 indicate a highly significant ($p \leq 0.01$) number of stillbirths, without significant changes in the other parameters.

The growth rate recorded for the offspring of the treated group (Table 4) indicated insignificant difference from the control.

PVC Extract: The weight gain in dams during pregnancy, pregnancy outcomes and offspring growth rate, presented in Tables 5–7, did not show any significant changes, in contrast to corresponding controls, with regard to each parameter.

Blood Sex Hormone Levels

Statistical analysis of hormonal data for both treatments did not elicit significant changes in the concentrations of the blood sex hormones assayed in either dams or their adult offspring (Tables 8 and 9). FSH and LH in the adult female offspring of all dams were undetectable by the assay method applied. Due to the fact that pituitary gonadotropin secretion controls ovarian steroidogenesis, FSH and LH can be assessed by indirect assays that reflect their influence on the target ovarian tissues.[17] Thus, the insignificant result indicated by estradiol in the adult

Table 2. Effect of SO Extract of HDPE, Administered Daily (50 ml/kg, per os) During Gestation Period, on Maternal Weight Gain (in g)

	Days of Gestation			
Treatment	0[a)]	3	6	9
SO Vehicle (Control)	29.09 ± 0.85	30.01 ± 0.74	31.83 ± 0.76	35.38 ± 0.77
HDPE-SO Extract	30.56 ± 0.42	31.29 ± 0.57	33.40 ± 0.65	36.79 ± 0.97
LSD	1.77	1.90	1.99	2.55
	Days of Gestation			
Treatment	12	15	18	
SO Vehicle (Control)	39.20 ± 1.07	43.38 ± 1.16	47.20 ± 0.89	
HDPE-SO Extract	40.67 ± 1.16	44.37 ± 1.05	46.86 ± 0.99	
LSD	3.40	2.28	3.27	

Values represent mean ± SEM, n = 7/group. *a*) Day 0 of gestation period. LSD: Least significant difference.

Table 3. Effects of SO Extract of HDPE, Administered Daily (50 ml/kg, per os) During Gestation Period, on the Pregnancy Outcomes

Treatment	Gestation Period (Days)	Litter Size	Number of Stillbirths	Sex Ratio (M/F)
SO Vehicle (Control)	21.63 ± 0.18	7.00 ± 1.05	0.00	1.04 ± 0.13
HDPE-SO Extract	20.86 ± 0.34	5.86 ± 0.83	1.86 ± 0.63*	1.26 ± 0.35
LSD	0.86	2.72	1.03	1.01

Values represent mean ± SEM, n = 7/group. Highly significant difference from the control (*$p \leq 0.01$). LSD: Least significant difference.

Table 4. Prenatal Effect of SO Extract of HDPE, Administered Daily (50 ml/kg, per os) to Mice During Gestation Period, on the Offspring Growth Rate (in g)

Treatment	Age (days)				
	0[a)]	3	6	9	12
SO Vehicle (Control)	1.59 ± 0.08	3.09 ± 0.14	5.74 ± 0.17	7.08 ± 0.32	8.50 ± 0.36
HDPE-SO Extract	1.49 ± 0.10	2.70 ± 0.31	4.48 ± 0.49	6.65 ± 0.39	9.12 ± 0.39
LSD	0.33	0.73	1.60	1.39	0.88
Treatment	Age (days)				
	15	18	21	24	27
SO Vehicle (Control)	9.97 ± 0.36	12.26 ± 0.48	14.86 ± 0.70	18.18 ± 0.89	20.97 ± 0.96
HDPE-SO Extract	10.74 ± 0.46	12.31 ± 0.47	14.61 ± 0.39	17.25 ± 0.38	19.91 ± 0.47
LSD	1.71	1.77	2.03	3.36	2.63

Values represent mean ± SEM, n = 8/group. *a*) Body weight taken at birth. LSD: Least significant difference.

female offspring might be considered an indirect measurement for these two gonadotropins, denoting that they did not undergo significant changes.

It should be noted that although 10 animals were used for each subgroup at the start of experiment, some had died during the treatment. All data relating to the lost dams were excluded from results. With regard to offspring data, the number of animals in Tables depends on litter size and the culling afterwards.

Migrants Detected in the Plastic Extracts

The HDPE-sesame oil (SO) extract yielded three migrants: isovaleric acid, butanal and di-n-butylphthalate (DBP), whereas the detected migrants in the PVC-normal saline (NS) extract were only two: 2-ethyl-1-hexanol and Ethyl citrate (Figs. 1 and 2).

Table 5. Effect of NS Extract of PVC, Administered Daily (50 ml/kg, i.p.) During Gestation Period, on Maternal Body Weight Gain (in g)

Treatment	Days of Gestation			
	0[a)]	3	6	9
NS Vehicle (Control)	28.35 ± 0.59	27.90 ± 0.53	29.68 ± 0.56	31.10 ± 0.47
PVC-NS Extract	28.06 ± 0.74	27.46 ± 0.69	29.29 ± 0.71	30.58 ± 0.68
LSD	2.00	1.84	1.91	1.73
Treatment	Days of Gestation			
	12	15	18	
NS Vehicle (Control)	36.28 ± 0.61	39.88 ± 0.72	46.08 ± 0.95	
PVC-NS Extract	35.67 ± 0.71	39.38 ± 0.76	45.30 ± 0.88	
LSD	1.99	2.23	2.76	

Values represent mean ± SEM, n = 8/group. *a*) Day 0 of gestation period. LSD: Least significant difference.

Table 6. Effects of NS Extract of PVC, Administered Daily (50 ml/kg, i.p.) to Mice During Gestation Period, on the Pregnancy Outcomes

Treatment	Gestation Period (days)	Litter Size	Number of Stillbirths	Sex Ratio (M/F)
NS Vehicle (Control)	19.00 ± 0.00	11.63 ± 0.77	0.25 ± 0.16	1.28 ± 0.18
PVC-NS Extract	18.90 ± 0.10	10.10 ± 0.43	0.50 ± 0.50	1.35 ± 0.28
LSD	0.15	1.79	0.99	0.69

Values represent mean ± SEM, n = 8/group. LSD: Least significant difference.

Table 7. Effect of NS Extract of PVC, Administered Daily (50 ml/kg, i.p.) during Gestation Period, on the Offspring Growth Rate (in g)

Treatment	Age (days)				
	0[a)]	3	6	9	12
NS Vehicle (Control)	1.37 ± 0.02	3.28 ± 0.12	4.60 ± 0.08	6.64 ± 0.17	7.61 ± 0.17
PVC-NS Extract	1.41 ± 0.03	3.38 ± 0.12	4.88 ± 0.09	6.95 ± 0.11	7.96 ± 0.10
LSD	0.08	0.36	0.26	0.42	0.41
Treatment	Age (days)				
	15	18	21	24	27
NS Vehicle (Control)	9.73 ± 0.26	12.06 ± 0.25	16.13 ± 0.29	19.37 ± 0.31	22.45 ± 0.35
PVC-NS Extract	9.83 ± 0.14	12.40 ± 0.13	16.55 ± 0.20	19.75 ± 0.29	22.61 ± 0.36
LSD	0.60	0.57	0.74	0.90	1.07

Values represent mean ± SEM, n = 8/group. *a*) Body weight taken at birth. LSD: Least significant difference.

DISCUSSION

Based on the insignificant change in the peripheral blood's sex hormones (the seemingly absence of endocrine modulation), accompanied by significant stillbirth (in the treated group only), we hypothesized that an inflammatory process, caused by migrants in the HDPE-oil extract, could be behind this incidence of stillbirth *via* immunogenic response. The mention of this kind of response towards plastic migrants and environmental toxicants in the placenta is extensive in literature.[18–20)] Published data indicate that plastics migrants may cause alterations in reproductive behavior and contribute to subfecundity, infertility, pregnancy loss, growth retardation, intrauterine fetal demise, birth defects, and ovarian failure *via* complex mechanisms that may involve hormonal and/or immune disruption, DNA adduct formation, altered cellular proliferation, or inappropriate cellular death.[21)]

Like Many foreign compounds, migrants contained in the HDPE extract might have crossed the placenta. These agents might be lipophilic to traverse more easily and attain the maternal-fetal equilibrium

Table 8. Effects of SO Extract of HDPE, Administered Daily (50 ml/kg, per os) to Pregnant Mice, on the Level of Blood Sex Hormones in the Dams (During 1st, 2nd and 3rd Trimesters[a)]) and Their Adult Offspring

Treatment	Dams					
	Progesterone (ng/ml)			Estradiol (pg/ml)		
	1st	2nd	3rd	1st	2nd	3rd
SO Vehicle (Control)	41.56 ± 4.32	88.61 ± 5.53	128.62 ± 4.89	35.84 ± 3.15	46.94 ± 6.59	52.88 ± 5.83
HDPE-SO Extract	45.05 ± 6.07	81.29 ± 12.91	112.39 ± 4.79	39.11 ± 3.70	51.55 ± 4.88	44.85 ± 3.06
LSD	17.92	46.66	27.24	9.12	15.05	14.31
Treatment	Dams			Adult Offspring		
	Prolactin (ng/ml)			Females	Males	
	1st	2nd	3rd	Estradiol(pg/ml)	Testosterone (ng/ml)	
SO Vehicle (Control)	1.64 ± 0.07	1.72 ± 0.04	1.90 ± 0.05	28.25 ± 2.27	7.59 ± 0.99	
HDPE-SO Extract	1.78 ± 0.05	1.94 ± 0.30	1.94 ± 0.09	33.22 ± 4.53	8.42 ± 0.59	
LSD	0.21	0.37	0.19	10.48	2.21	

Values represent mean ± SEM (dams: n = 7/group; offs.: n = 8/group). *a*) Six, twelve, eighteen days of pregnancy, respectively. LSD: Least significant difference.

Table 9. Effects of NS Extract of PVC, Administered Daily (50 ml/kg, i.p.) to Pregnant Mice, on the Level of Blood Sex Hormones in the Dams (During 1st, 2nd and 3rd Trimesters[a)]) and Their Adult Offspring

Treatment	Dams					
	Progesterone (ng/ml)			Estradiol (pg/ml)		
	1st	2nd	3rd	1st	2nd	3rd
NS Vehicle (Control)	56.96 ± 5.23	73.32 ± 4.32	123.00 ± 7.43	33.02 ± 4.19	35.65 ± 5.34	43.31 ± 6.34
PVC-NS Extract	49.65 ± 6.75	61.76 ± 5.44	113.54 ± 8.55	32.54 ± 3.22	37.39 ± 3.36	47.77 ± 3.71
LSD	18.05	14.71	24.08	11.17	13.11	15.14
Treatment	Dams			Adult Offspring		
	Prolactin (ng/ml)			Females	Males	
	1st	2nd	3rd	Estradiol (pg/ml)	Testosterone (ng/ml)	
NS Vehicle (Control)	1.96 ± 0.14	1.99 ± 0.04	1.79 ± 0.09	34.74 ± 2.74	11.07 ± 1.72	
PVC-NS Extract	1.87 ± 0.09	1.83 ± 0.07	1.61 ± 0.08	42.71 ± 6.54	10.13 ± 1.66	
LSD	0.34	0.16	0.25	14.62	5.32	

Values represent mean ± SEM (dams: n = 7/group; offs.: n = 8/group). *a*) Six, twelve, eighteen days of pregnancy, respectively. LSD: Least significant difference.

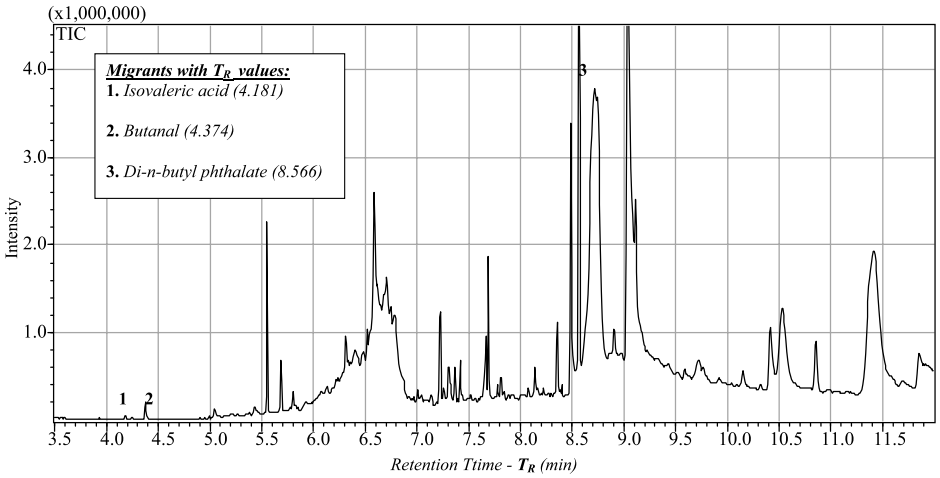

Fig. 1. GC/MS of HDPE Migrants in Sesame oil Extracted with Acetonitrile

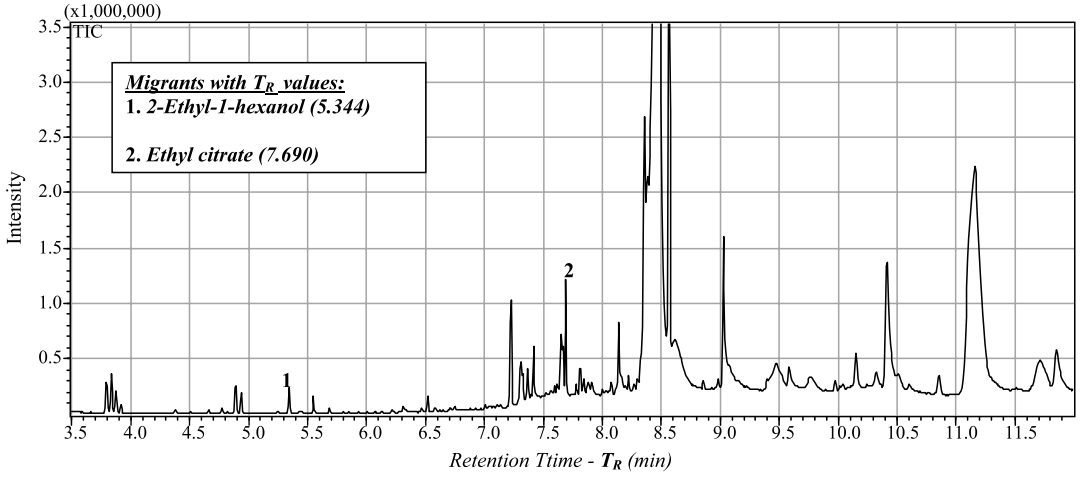

Fig. 2. GC/MS of PVC Migrants in Normal Saline Extracted with *n*-Hexane

more rapidly.[22)]

A chemical insult in the placenta might induce adverse changes in it s vasculature due to ischemic necrosis, leukocyte infiltration, fibrin deposits, cellular debris and vascular thrombosis that would mostly be ensued by a reduced blood flow to the fetus, leading to complications like abortion, stillbirth (fetal death), or fetal growth retardation.[23)]

Phelps *et al*.[24)] investigated the immunogenic reactions towards plastic migrants. These researchers found that the silicone-based catheters they implanted in the jugular vein in rats resulted in an immune response that lead to the obstruction of the catheter tip. The obstruction was due to the formation of masses that were composed of numerous platelets and granulocytes (neutrophils), beside the formation of a sheath that covered the intravascular portion of the implanted catheters, the components of which were identified to be endothelial cells, smooth muscle cells, fibroblasts and numerous collagen fibers. They concluded that tissue responses to the plastic migrating chemicals were behind this local intravascular inflammatory reaction, which is consistent with our hypothesis that the chemical agent(s) contained in the HDPE extract and/or relating metabolite(s), have induced such inflammatory reactions in the placenta, presumably by blockage of placental capillary network, thereby inducing intrauterine hypoxia and malnutrition to the fetuses in the affected labyrinthine zones. This could be justified by delivery of normal live births, by these dams, with a neonatal growth rate comparable to the control offspring observed in this study.

The extract of PVC sample did not elicit significant alterations in the pregnancy outcomes in this subacute study. A probable reason for this finding might be attributed to the reduced tendency of the sample toward aqueous extraction.[25)] Faouzi *et al*.[26)] found that blood stored in PVC bags gave rise to a higher extraction rate of leachable agents due to the lipid portion it contains. Trissel[5)] showed that leachability of PVC could occur even into sterile water. Based on these facts, the current finding does not exclude toxicity of PVC material of these blood bags, because patients, such as those on massive transfusions, normally receive migrants from these bags at regular and continuous doses in a chronic treatment, regardless of their concentrations.

Migrants detected by the GC/MS analysis of extracts of the two plastic samples covered by this study are in agreement with published work.[27–29)]

In conclusion, the results of this study emphasizes the need to search into toxic effects posed by plastics, especially when these man-made materials come in contact with foodstuffs of fatty nature, beside the avoidance of food oil packaging in plastics, unless migration-resistant materials are fabricated.

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