ORIGINAL ARTICLE

Relation between some environmental pollutants and recurrent spontaneous abortion

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Abstract Reproductive health is exquisitely sensitive to characteristics of an individual’s environment including physical, biological, behavioral, cultural and socioeconomic factors. This study was launched to elucidate the effect of the exposure to chemical pollutants as aromatic amines viz. (benzidine, mono-acetyl benzidine, diacetyl benzidine, α,β-naphthylamine) as well as the biological pollutants e.g., human cytomegalovirus (HCMV) as risk factors for recurrent spontaneous abortion (RSA) through determination of MDA as a marker of oxidative stress and determination of some antioxidant markers. The results of the current study revealed that the aborter mothers were being exposed to environmental pollutants as aromatic amines which were manifested by the presence of benzidine, mono-acetyl benzidine, di-acetyl benzidine, α,β-naphthylamine in most of their urine samples, where the level of aromatic amines were more 13.6, 10, 15, and 4-folds than the control group, respectively. Also, the data suggest that in early pregnancy failure there is an increase in markers of oxidative stress and determination of some antioxidant markers. The results of the current study revealed that the aborter mothers were being exposed to environmental pollutants as aromatic amines which were manifested by the presence of benzidine, mono-acetyl benzidine, di-acetyl benzidine, α,β-naphthylamine in most of their urine samples, where the level of aromatic amines were more 13.6, 10, 15, and 4-folds than the control group, respectively. Also, the data suggest that in early pregnancy failure there is an increase in markers of oxidative stress and a probable decrease in maternal antioxidant defenses (22 nmol/ml and 17 mg/l, 550 U/l, respectively). Generation of ROS in large quantities, in the first trimester placenta which has limited antioxidant defenses may cause DNA damage, oxidation of protein and lipid resulting in extensive cell death. Also, it was demonstrated that high elevation of HCMV inhibits cytotrophoblasts proliferation, migration invasion and matrix metalloproteins (MMP) expression. Obviously, placental toxicological responses are partly due to pharmaco/toxico dynamic responses to the chemicals. Conclusively, the aforementioned findings emphasis that, the exposures...
1. Introduction

Human reproduction is not considered as highly efficient biological process. Before the end of the first trimester, 30–50% of pregnancies losses occur at the time of implantation (Gupta, 2007) and 15–20% of clinical pregnancies end in spontaneous abortions (Gupta, 2007). Recurrent pregnancy loss is a frustrating clinical problem both for clinicians and patients (Gupta, 2007). Recurrent pregnancy loss affects 0.5–3% of women in the reproductive age group and between 50% and 60% of recurrent pregnancy losses are idiopathic (Gupta, 2007). One considering factor of recurrent spontaneous abortion (RSA) is environmental pollution (Pandey et al., 2005). The strongest evidence of environmental contaminant exposures interfering with healthy reproductive function in adult female is xenobiotic (Michal et al., 1993). Compounds that can influence hormone function, including pesticides and persistent pollutants, are also associated with risk. The pattern of effects for these endocrine-active compounds is often complex, with no clear dose response, but alterations in function and poor reproductive health outcomes are observed. From a clinical perspective, most modifiable risk appears to be associated with exposures in unique populations (contaminated fish consumers) or occupational group (farm workers) (Michal et al., 1993).

Consumption of contaminated fish has been shown to be an important source of human exposure to a vast and ever-increasing range of compounds, including polycyclic aromatic hydrocarbons (PAH’s) polychlorinated biphenyls (PCBs), dioxins, nitro-aromatics, aromatic amines, organophosphate and organochlorine pesticides, and phthalate ester plasticizers. Such foreign compounds (xenobiotics) are fat soluble and are, therefore, readily taken up from the water, sediment and food sources into the tissues of aquatic organisms (Nunn et al., 1996).

Chemical toxic pollutants are important sources of reactive oxygen species (ROS) in biological systems. Of particular interest to toxicology are xenobiotics that are capable of redox cycling (vis, Transition metals, quinines, dyes, biphenyl herbicides, aromatic amines and aromatic nitro compounds) are known for their redox cycling properties and their potential to cause oxidative stress (Kappus and Sies, 1981). Oxidative stress has also been implicated as an important cause of recurrent pregnancy loss. Loss of antioxidant defenses has been shown to be associated with recurrent pregnancy loss (Simek Naziroglu and Simsk, 1998). Biochemical markers of ROS-induced membrane damage such as lipid peroxidation products, reach high levels immediately before abortion (Sane et al., 1991). It has been proposed that an oxidant/antioxidant imbalance is associated with pregnancy loss (Lagod et al., 2001). Also, the interrelated metabolism of aromatic nitro and amino compounds enables the monitoring of these groups of chemicals via the determination of their metabolites in urine (Weiss et al., 2000). Where, urine is commonly used because it allows continuous noninvasive monitoring and facilitates the investigation of kidney-related toxicity or disease (Jiye et al., 2008).

In this respect, this study was launched to elucidate the effect of the exposure to chemical pollutants as aromatic amines viz. (benzidine, mono-acetyl benzidine, di-acetyl benzidine, α,β-naphthylamine) as well as the biological pollutants e.g., human cytomegalovirus (HCMV) as risk factors for recurrent spontaneous abortion (RSA). Also, determination of MDA as a marker of oxidative stress and determination of some antioxidant markers.

2. Subjects and methods

2.1. Subjects

After the agreement of the ethics committee on this study, the women’s consent is taken before they were included in the study. A total of sixty women were recruited from the department of Obstetric and Gynaecology, El-Shatby University Hospital, Alexandria. Two different groups were evaluated. The first group included 19 healthy fertile women (control group) with an average age of (25–35) and weighed from 60 to 90 kg, had no history of recurrent spontaneous abortion. All had at least one living child and their pregnancy proceeded successfully giving full term healthy neo-borne.

The second group included 36 women with medically unexplained recurrent spontaneous abortion, with an average age of (22–37) and weighed from 62–90 kg with a history of at least two successive spontaneous abortions including abortion up to 20 weeks gestational age. The demographic, medical, and clinical data were collected in each case based on personal interviews and medical reports at the hospital. Detailed medical, surgical, genetic and menstrual histories were taken. Patients were questioned about their use of drugs, tobacco, alcohol and caffeine and whether they have been exposed to occupational hazard. Also, their food habits especially fish consumption were taken in concern. Thyroid dysfunction, diabetes, uterine anomalies and ovarian disease were excluded referring to medical reports by the hospital.

All women included are investigated to assess metabolites of some aromatic amines in urine, lipid peroxide as a marker of oxidative stress, antioxidant enzyme (total glutathione content, and catalase), and serological quantitative assessment of human cytomegalovirus antibodies (IgG).

2.1.1. Collection of fish sample

Saragus saragus and Siganus rivulatus were taken from El-Mex bay at three seasons (summer, winter, spring).

2.2. Sampling

Ten milliliter non-fasting blood sample was withdrawn from every women included in the study on the 21th day after the commencement of menstrual cycle for control group and one month after abortion for the other groups. Sera were separated for all samples by centrifugation and stored at −20 °C until time of assay.
Also, random urine sample was taken from patients within 24 h after normal labor (for control group) and just after consecutive spontaneous abortion for the aborter group.

2.3. Methods

2.3.1. Determination of urinary aromatic amine metabolites
According to Shin (2001), in which 1 ml of urine, 2 g potassium dihydrogen phosphate and 100 μl of diphenylamine was an internal standard. Then, the sample extracted by ethyl ether and 2 μl sample was injected in the GC system.

2.3.2. Determination of blood glutathione content
According to Beulter (1963), where, 0.2 ml of whole blood was added to 1.8 ml of distilled water and 3 ml of precipitating solution was mixed with hemolysate. Then, 2 ml of the filtrate was added to 8 ml of phosphate buffer, and then mixed with 1 ml of DTNB. The optical density was measured at 412 nm.

2.3.3. Determination of plasma catalase activity enzyme level
According to Frederickson et al. (1994), 50 μl of plasma, 0.5 ml phosphate buffer (100 mM, pH 7), and 0.1 ml of diluted 
\( \text{H}_2\text{O}_2 \) were incubated at 25 °C for 1 min, then, the chromogen inhibitor and peroxidase Enzyme were added to incubated mixture for 10 min and measured against blank at 510 nm.

2.3.4. Determination of serum lipid peroxide
According to Satoh (Satoh, 1978), 1 ml serum and 2.5 ml of trichloroacetic acid were centrifuged at 3500 rpm for 10 min. The precipitate was washed with sulfuric acid, then after, 2.5 ml of 0.05 M sulfuric acid and 1 ml of thiobarbaturic acid heating for 30 min then extracts the chromogen with 4 ml of n-butyl alcohol, centrifuged at 3000 rpm and measured at 530 nm.

2.3.5. Gas chromatography mass spectrophotometer identification and quantification of several industrial aromatic amines
According to Diachenko (1979) firstly, the extracted amines from fish flesh with benzene were washed with diluted sulfuric acid (2 × 10^{-4} M) and were cleaned up by silica gel column and were eluted with methylene chloride. The cleaned fish extract and reference compounds were analyzed by HP 6890 mass spectrometer equipped with HP-data system.

2.3.6. Quantitative assessment of HCMV using enzyme-linked immunosorbent assays
Enzyme-Linked Immunosorbent Assay kits (Engvall and Peri- mann, 1971).

2.4. Statistical analysis

The obtained data of the presented study were statistically analyzed using SPSS version 10 software. A difference was considered as significant at \( p \leq 0.05 \).

3. Results

The result represented elevated concentration of two amines in Siganus rivulatus than that present in Saragus saragus (Table 1). The results exhibited that the concentration of benzidine was about 13.6 times more in aborters groups than that of corresponding matched control group. Also, mono, di-acetylated benzidine were elevated in the aborter group by 10 and 15-fold, respectively, more than observed with control. Meanwhile, the urinary metabolite of \( \beta \)-naphthylamine was 3.59-fold than that noticed with the control group, \( \alpha \)-naphthylamine was elevated by 4.1-fold than the corresponding control group. The concentration of benzidine, mono, di-acetylated benzidine and \( \alpha \),\( \beta \)-naphthylamine was highly elevated in aborter group and control group with increased age. Meanwhile, for \( \beta \)-naphthylamine this finding was not in agreement with our result where in both groups (aborter and control group) the concentration not affected with age (Tables 2–6).

Fig. 1 represented the results of lipid peroxide concentration nmol/ml in the sera of healthy control and aborter groups. Statistical analysis of the results using student \( t \)-test exhibit that there was a very high significant increase in the concentration of malondialdehyde (MDA) in the sera of aborter group than that of the control group (\( p < 0.001 \)). The results concerning whole blood glutathione content Fig. 2, revealed that there were highly significant decrease in the concentration of glutathione in the whole blood of aborter group when compared with that of control group (\( p < 0.001 \)). Statistical analysis demonstrated that there was a highly significant decrease in the enzymatic activity of catalase enzyme in the plasma of aborter group than that of the control group (\( p < 0.001 \)) (Fig. 3). To elucidate the

### Table 1

Aromatic amine concentrations (μg/kg d.w) in fish flesh of Mediterranean Sea “El-Mex bay” by Gas chromatography-mass spectrometry in three seasons.

<table>
<thead>
<tr>
<th>Seasons</th>
<th>Type of fish</th>
<th>Aromatic amines concentrations (μg/kg)</th>
<th>Benzidine</th>
<th>( \beta )-Naphthylamines</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Winter</td>
<td>Siganus rivulatus</td>
<td>11594.4</td>
<td>12000.04</td>
<td></td>
</tr>
<tr>
<td>Spring</td>
<td>Siganus rivulatus</td>
<td>8232.09</td>
<td>7830.80</td>
<td></td>
</tr>
<tr>
<td>Summer</td>
<td>Siganus rivulatus</td>
<td>9565.22</td>
<td>13076.00</td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>Siganus rivulatus</td>
<td>9797.2</td>
<td>10969</td>
<td></td>
</tr>
<tr>
<td>Winter</td>
<td>Saragus saragus</td>
<td>5796.84</td>
<td>2768.60</td>
<td></td>
</tr>
<tr>
<td>Spring</td>
<td>Saragus saragus</td>
<td>16811.00</td>
<td>12015.61</td>
<td></td>
</tr>
<tr>
<td>Summer</td>
<td>Saragus saragus</td>
<td>6608.70</td>
<td>4347.83</td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>Saragus saragus</td>
<td>9738.8</td>
<td>6377.3</td>
<td></td>
</tr>
</tbody>
</table>

### Table 2

The relationship between Benzidine concentration and age of control and aborter groups.

<table>
<thead>
<tr>
<th>Control group</th>
<th>Aborer group</th>
</tr>
</thead>
<tbody>
<tr>
<td>16 ≤ age ≤ 28</td>
<td>28 &lt; age ≤ 41</td>
</tr>
<tr>
<td>n</td>
<td>15</td>
</tr>
<tr>
<td>Min–Max</td>
<td>0.005–18.0</td>
</tr>
<tr>
<td>Mean</td>
<td>1.85</td>
</tr>
</tbody>
</table>
results, this study on human cytomegalovirus shows a significant higher level of anti-human cytomegalovirus antibodies (anti-HCMV IgG) concentration (IU/ml) in the sera of aborter group than that in control group ($p < 0.05$), also higher prevalence (IgG + ve%) of anti-HCMV IgG in the aborter group than in control group (Table 7).
4. Discussion

The aquatic environment makes up the major part of our environment and resources; therefore, its safety is directly related to the safety of our health. Fish are excellent subjects for the study of the mutagenic and/or carcinogenic potential of contaminants present in water samples since they can metabolize, concentrate and store waterborne pollutants (Ali and El-Shehawi, 2007). Since fish often respond to toxicants in a similar way to higher vertebrates, they can be used to screen for chemicals that are potentially teratogenic and carcinogenic in humans (Ali and El-Shehawi, 2007). The main application for model systems using fish is to determine the distribution and effects of chemical contaminants in the aquatic environment (Al-Sabti and Metcalfe, 1995). El-Mex bay is contaminated by different pollutants, both of organic and inorganic nature. The most problematic are heavy metals, some organic micropollutants in relation with suspended matter (polychlorinated biphenyls (PCBs), polycyclic aromatic hydrocarbons (PAHs), aromatic amines (AA) and pesticides), nitrogen, phosphorus compounds, and chlorides (Maha, 2008).

The data of the present study revealed that there were high levels of the two studied aromatic amines. Remarkable variations in the concentrations of the two aromatic amines were found in the muscle tissue of Saragus saragus and Siganus rivulatus. This may be due to food habits and species differences. The high levels of toxic elements in fish can confirm the pollution of sea water and the possibilities of the presence of toxic elements (Depledge, 1998). This causes two types of concern: the effects on ecosystems and the risk for human health. Among the effects on normal populations, short-term effects and those that lead to mutations interfering with reproduction and genetic diversity are very significant. The induction of germ cell mutations causes the most severe consequences since they can directly affect the reproductive potential of populations (Depledge, 1998).

The pregnant mother is exposed to a multitude of chemical agents including drugs, food additives, food habits and environmental pollutants. The fetus is connected to the mother by the placenta, through which foreign lipid-soluble chemicals can pass easily (Reynolds and Knott, 1989). It is widely recognized that placenta is not a barrier for foreign compounds, and thus, it is possible that xenobiotic affect fetal development by causing disturbance in the mother’s placenta and/or in the fetus itself (Pacifici and Nottoli, 1995).

In this respect, the herein study was lunched to evaluate the effect of the exposure to chemical pollutants as aromatic amine (benzidine, mono-acetylbenzidine, di-acetylbenzidine, α,β-naphthylamine) as well as the biological pollutants (human cytomegalovirus (HCMV) as risk factors for recurrent spontaneous abortion (RSA) through the determination of urinary metabolites of the studied aromatic amines, quantitative estimation of anti-cytomagalovirus antibodies (IgG) and malondialdehyde (MDA) level as a biomarker of lipid peroxidation in the sera of the aborters group and the corresponding control group.

The results of the current study revealed that the aborters were being exposed to environmental pollutants as aromatic amines that were manifested by the presence of benzidine, mono-acetyl-benzidine, di-acetyl-benzidine, α,β-naphthylamine in most of their urine samples, where the level of aromatic amines were 13.6, 10, 15, and 4-folds more than the control group, respectively. Furthermore, the herein results demonstrated that the levels of urinary excreted aromatic amines were markedly elevated with increasing age. This may be attributed to a progressive accumulation of chemical carcinogens in adipose tissue. It was clearly shown that lipophilic organic xenomolecules such as benz[a]pyrene can accumulate in adipose tissue (Irigary et al., 2006). Therefore, this tissue should be considered as a reservoir for lipophilic xenomolecules including persistent organic pollutants such as dioxin and poly chlorinated biphenyles (PCBs) from which they are released into the plasma, where they can be detected at doses positively correlated with the body mass index (Irigary et al., 2006). Then the present results proved that the biomarker urinary metabolites may represent the early exposure to xenobiot-
ics from 24 to 72 h and also positively correlated with the amount of its accumulation along years of exposure. On the other hand, the level of urinary excreted β-naphthylamine did not show any elevation with increasing age. This is because of the majority of β-naphthylamine is excreted in the urine as glucuronide that is deconjugated prior to analysis (Talaska, 2005).

Also, food habits and poor maternal nutrition during gestation lead to intrauterine fetal growth retardation of developing organism and is associated with a number of unfavorable outcomes for the offspring (Barker et al., 1993). Our results (questionnaire) explain the relation between malnutrition and stress on the pregnant women and may be considered as a risk factor for repeated spontaneous abortion (RSA). Where, stress is a stimulus or succession of stimuli of such magnitude as to tend to disrupt the homeostasis of the organism (Dictionary of Biotechnology, 1997). Meanwhile, chemical toxic pollutants are important sources of reactive oxygen species (ROS) in biological systems. Of particular interest to toxicology are xenobiotics that are capable of redox cycling. Transition metals, quinines, dyes, bi-pyridyl herbicides, aromatic mines and aromatic nitro compounds are known for their redox cycling properties and their potential to cause oxidative stress (Kelly et al., 1998). Oxidative stress and oxidative damages to fundamental biomolecules and to antioxidant defenses of organisms are established fields in environmental toxicology and ecotoxicology (Regoli et al., 2002a,b).

Arylamines and heterocyclic arylamines require metabolic activation to be mutagenic or carcinogenic (Kadlubar and Hammons, 1987). Biochemical activation through N-hydroxylation, followed by sulfation, esterification or acetylation reactions, generating reactive intermediates able to bind to DNA molecules are presently established as the main routes of genotoxicity and carcinogenicity of arylamines (Sugimura, 1997).

In human, the liver plays a major role in xenobiotic metabolism. However, all tissues express at least some drug metabolizing enzymes. In addition to the liver, other major sites for drug metabolism are the gastrointestinal tract, kidneys, lungs, skin and brain (Krisha and Klotz, 1994; Baron and Merk, 2001; Doherty and Charman, 2002). It was shown over 30 years ago that the human placenta also is able to metabolize foreign chemicals during pregnancy (Welch et al., 1968). The placental metabolizing enzymes are already present in early pregnancy (Myllynen et al., 2005). In fact, it seems that the placenta expresses a wider variety of xenobiotic-metabolizing enzymes at the mRNA level during the first trimester than at term (Hakkola et al., 1996a,b). Both during the first trimester and at term, the placenta expresses several CYPs (cytochrome P450) at mRNA levels, although only a few of them have been shown to be functionally active. CYP-mediated metabolism in the placenta is more restricted than hepatic metabolism, but still placental CYP enzymes are capable of metabolizing several drugs and foreign chemicals (Hakkola et al., 1998; Pasanen, 1999). In addition to the CYP enzymes, other phase 1 metabolizing enzymes, such as alcohol dehydrogenase, have also been detected in the placenta (Karl et al., 1988). Among phase 2 enzyme activities, glutathione transferase, epoxide hydrolase, N-acetyltransferase, sulphotransferases, and UDP-glucuronoyl transferase are expressed in human placental tissue (Karl et al., 1988). Even though the metabolic activity of phase 2 enzymes in the placenta is low, at least some of these compounds are capable of xenobiotic metabolism (Hakkola et al., 1998). Human placental enzymes are capable of producing reactive metabolites that can react with DNA, resulting in DNA adducts (Karl et al., 1988). Where, oxidative stress can affect the female fertility potential in number of ways. It may affect the ovulation, fertilization, embryo development and implantation (Agarwal and Shyam, 2004). Oxidative stress in the human placenta is by altering the balance between oxidant (increased MDA levels) and antioxidants (decreased GSH, GSSG and catalase). This can result in cell apoptosis leading to derangements in placental invasion and early abortion (Fiore et al., 2005). The aforementioned finding confirmed and represented good interpretation to the results of the present study which elucidate that there were highly significant increase in the level of MDA in the sera of aborter group than that of the corresponding control group; associated with highly significant decrease in the levels of GSH as well as the enzymatic activity of catalase. Where, antioxidant status in normal pregnancy related diseases is gathering increasing interest in recent years (Sane et al., 1991; Willet et al., 1983).

Some maternal infections, especially during the early gestation, can result in fetal loss or malformations because the ability of the fetus to rest infectious organisms is limited and the fetal immune system is unable to prevent the dissemination of infectious organisms to various tissues. The fetus and/or neonate are infected predominantly by viral but also by bacterial and protozoal pathompetence of the host. In certain high-risk groups, CMV infection may be hogens. Infections with various pathogens cause miscarriage or may lead to congenital anomalies in the fetus while others are associated with neonatal infectious morbidity. Cytomegalovirus (CMV) is more widespread in developing countries and in areas of lower socioeconomic conditions. Therefore, for the majority of people, CMV infection is not a serious problem. The manifestation of acquired CMV infection varies with the age and immuno-competence of the host. In certain high-risk groups, CMV infection may be dangerous.

In general, factors associated with seropositivity include lower socioeconomic status, maternal age more than 30 years, lower level of education and close contact with young children. For most healthy persons who acquire CMV after birth, there are only few or no symptoms and no long-term sequelae. After the primary infection, the virus remains alive but usually dormant within a person’s body for life.

In the herein study, the aborters group showed a significant higher level of anti-HCMV-IgG concentrations in their sera than the control group (p < 0.05). Also, they showed higher prevalence of anti-HCMV-IgG than the control group (100%, 92%, respectively).

Normal placental development depends on the proliferation, differentiation and invasion of CTB stem cells that are derived from the early blastocyst (Cross et al., 1994). Prior to their differentiation into an invasive cell type, these CTB progenitors are a mitotically active cell population that rapidly divides to form cell columns, which function to anchor the placenta to the uterine wall. Hence, cell division or proliferation is a critical requisite step for successful placental formation. In a previous study, HCMV significantly inhibited CTB proliferation (Pereira et al., 2004). The resulting block in proliferation was not due to cytotoxic effects of HCMV that probably provide an early anti-proliferative signal that may consequently
disrupt proper placental establishment (Roth and Fisher, 1999).

5. Conclusion

It was demonstrated that HCMV inhibit cytotrophoblasts proliferation, migration invasion and matrix metalloproteins (MMP) expression. Obviously, placental toxicological responses are partly due to pharmacotoxico dynamic responses to the chemicals. Several pregnancy complications have been associated with oxidative stress. One mechanism of oxidative stress is the uncontrolled production of lipid peroxides. Several foreign chemicals cause oxidative stress in placental tissue. Placental insufficiency, associated with metabolic alterations in the placenta and disorders in the exchange between the maternal body and the fetus, is one of the main causes of miscarriage. An important role in miscarriage is played by an elevated production of reactive oxygen metabolites, which can irreversibly damage various cellular structures. Excess free radicals can cause disorders in protein synthesis; cause enzymes to lose catalytically active groups; distort the activity of glycoproteins, causing changes in the synthesis and activity of hormones and cell membrane receptors, and damage DNA molecules, which frequently lead to irreversible damage to the genome. Therefore, the aforementioned findings emphasise the exposure to environmental chemical and/or biological risk factors is possibly implicated in the pathogenesis of recurrent spontaneous abortion.

References


