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Levels of DNA Adducts in the Blood and Follicular Fluid of Women Undergoing In Vitro Fertilization Treatment and Its Correlation with the Pregnancy Outcome

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Abstract This study is designed to investigate the impact of DNA damage on pregnancy and fertilization rate outcome in a sub-sample of women undergoing IVF treatment. Blood and follicular fluid samples (n = 60) were analyzed for DNA adducts. While no BPDE-DNA adducts were detected, other unknown lipophilic adducts were seen in blood and follicular fluid. Women who failed to achieve pregnancy had higher DNA adducts in follicular fluid than those who succeeded ($p < 0.05$). Follicular fluid cotinine levels were associated with DNA adduct levels in blood and follicular fluid ($p < 0.05$). Evaluation of DNA damage resulting from oxidative stress could have a role in predicting IVF success rate.

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Chemicals can alter the reproductive function in all species leading to hormonal and/or immune disruption; DNA adducts formation, altered cellular proliferation and inappropriate cellular death (Sharara et al. 1998). Bulky DNA lesions; originating from reactive oxygen species (ROS), are the most frequent damage which could block DNA replication and transcription. This might lead to the development of various health disorders (Wang 2008). Environmental pollutants such as heavy metals, polycyclic aromatic hydrocarbons (PAH) and pesticides were shown to cause oxidative DNA damage (Valavanidis et al. 2006). Benzo[a]pyrene (B[a]P), a potent carcinogen of the polycyclic aromatic hydrocarbon group is present in cigarette smoke. It has a tendency to bind DNA covalently giving rise to 7β,8α-dihydroxy-9α,10α-epoxy-7,8,10-tetrahydrobenzo[a]pyrene-BPDE-DNA adducts (Boysen and Hecht 2003). Zenzes (2000) found DNA adducts in the ovarian cells of women exposed to cigarette smoke. A recent study by Neal et al. (2008) showed that B[a]P reaches the follicular fluid and that its level was higher in smokers. They suggested that this might have a toxic effect on follicular development and subsequent negative impact on fertility. Cotinine, the main metabolite of nicotine and a reliable biomarker of tobacco smoke was also seen at higher levels in follicular fluids of women smokers compared to non-smokers (Zenzes 2000).

Our previous study provided evidence that Saudi women might be exposed to a variety of pollutants from various sources (Al-Saleh et al. 2008). In general, the implantation rates after in vitro fertilization (IVF) was estimated to be as

low as 20% (Coskun et al. 2000). Apparently, exposure to environmental pollutants might have a role in the etiology of such low rates.

The purpose of this study is to measure bulky lipophilic DNA adducts including aromatic adducts such as BPDE-DNA by ^{32}P-postlabeling technique and relate it to pregnancy outcome and fertilization rate in a sub-sample of women undergoing IVF treatment.

Materials and Methods

The data evaluated in this study originated from samples and questionnaires collected for a previous project "Exposure to environmental pollutants and its effect on the outcome of in vitro fertilization outcome" (Al-Saleh et al. 2008). Blood and follicular fluid samples were collected from 619 women undergoing IVF treatment at the IVF-embryo transfer unit, King Faisal Specialist Hospital and Research Centre (KFSH&RC) between 12/01/2002 and 16/10/2003. The mean age of these women was 31.76 ± 5.12 years old. Only a sub-sample of 67 women was selected for this study because DNA adducts procedure is lengthy and time-consuming. The selection was based on every 10th sample. Women with positive beta-human chorionic gonadotropin (β-hCG) were considered pregnant. Pregnancy was subdivided into biochemical (positive β-hCG, negative ultrasound), abortion or ongoing. Written informed consent was obtained from each participant and approved by the Research Ethic Committee of KFSH&RC.

Analyses of lead, cadmium, mercury, *p,p*'-dichloro-2, 2-bis (*p*-chlorophenyl) ethylene (DDE) and cotinine in blood and follicular fluid samples were measured as described previously (Al-Saleh et al. 2008, 2009). Serum and follicular fluid B[a]P levels were determined by a modified method of Sirimanne et al. (1996) using the Alliance Waters 2690 High Performance Liquid Chromatography (HPLC) system and a Hewlett Packard, Model 1046A fluorescence detector. The analytical recovery for spiked serum and follicular fluid with B[a]P at various concentration levels (0.8–5.0 µg/L) were 101.3%–104.9% and 99.7%–101.2%, respectively.

DNA was isolated from blood lymphocytes and follicular fluid using the PureGene kit (Gentra Systems, Minneapolis, MN). For ^{32}P-postlabeling, DNA (10–25 µg) was digested with a mixture of micrococcal nuclease (Sigma Chemical Co., St. Louis, MO) and spleen phosphodiesterase (Boehringer Mannheim Corp., Indianapolis, IN). The enriched adducts were labeled by T4 polynucleotide kinase (10U/µl) in the presence of molar excess of commercial [γ-^{32}P]ATP (20 µCi; 7000 Ci/mmol specific activity) (ICN Pharmaceutical, Inc., Costa Mesa, CA). Then they were separated by multi-directional polyethyleneimine (PEI)-cellulose-thin layer chromatography (TLC). The solvent system used to resolve the adducts was: Dimentional-1 (D1) = 1 M sodium phosphate, pH 6.0; D3 = 4 M lithium formate/8.5 M urea, pH 3.5; D4 = 0.8 M lithium formate/8.5 M urea/0.5 M Tris–HCl, pH 8.0; and D5 = 1 M sodium phosphate, pH 6.0. To evaluate the levels of adducts, an aliquot of diluted DNA digest (2 ng) was also labeled in parallel with adducts and normal nucleotides converted to 5′-monophosphate were resolved using 0.5 M acetic acid (Gupta and Arif 2001). The positive standard of BPDE-modified DNA adducts was run in parallel in all the experiments for identification purposes. Adduct diagonal radioactive zones (DRZs) were visualized, quantified by Typhoon 8600 variable mode imager and then followed by ImageQuant software (Molecular Dynamics, Sunnyvale, CA, USA). The relative adduct labeling (RAL) was calculated as follows: RAL = count per minute (*cpm)* in adducts/*cpm* in total nucleotides × 1/dilution factor. Adducts were expressed as adducts per 10^{10} nucleotides (Arif et al. 2004). Figure 1 shows radiogram of negative DNA control with no background adduct level.

Data are reported as means ± SE. Women with successful pregnancy or fertilization outcome constitute controls and the rest as cases. The fertilization rate was defined as the percentage of the fertilized eggs to the number of eggs. Although large number of confounding variables were collected originally in the IVF project, we were not able to perform logistic regression analysis because insufficient number of cases. We had to add a value of 1 to the adduct data in order to account for values of zero (0). Differences between groups were tested for significance using the Mann–Whitney *U* test. Spearman rank correlation coefficients (*r*) were calculated to evaluate the closeness of relationship between two continuous variables. Values were considered significant at $p < 0.05$.

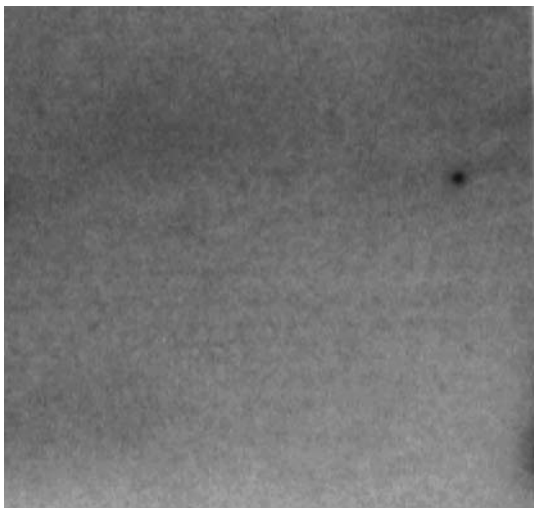

Fig. 1 Radiogram of negative DNA control

Results and Discussion

In this study, heavy metals (lead, cadmium and mercury), *p,p*-DDE and cotinine were detected in both blood and follicular fluid of women (Table 1). The levels of *p,p*-DDE, lead, cadmium and mercury in the blood were of higher magnitude (5.45, 5.96, 1.56 and 1.47, respectively) than their counterparts in the follicular fluid. All our participants had blood lead and follicular fluid levels less than the US occupational blood lead exposure limits (40 μg/dL). The threshold limit of cadmium's clinical importance (1 μg/L) was found in the blood and follicular fluid of 10.8% and 7.5% women, respectively. While 13.4% and 7.5% of the women had, respectively blood and follicular fluid mercury levels that exceeded the EPA safety limit of ≥5.8 μg/L. *p,p'*-DDE (the major metabolite of DDT) was detected in 81.3% of the serum and 49.3% of the follicular fluid samples of our participants, but the majority were below the detection limits (DLs). Only 30.5% of the women had *p,p'*-DDE levels in the serum above the DL of 1.63 μg/L, while 6% had follicular *p,p'*-DDE above the DL of 1.35 μg/L. Though, the levels of these environmental contaminants were low, their presence particularly in the follicular fluid might raise some question about their possible negative effects on the ovarian function. As a biomarker of active or passive smoking exposure, cotinine was measured in the blood and follicular fluid of our participants. Based on self-reporting, 2 women and the husbands of 14 women were active smokers. Serum cotinine was high in one woman only who is an active smoker (16.17 μg/L) and the rest were in the range of 0–0.03 μg/L. On the other hand, 17 women (30.4%) had detectable cotinine in follicular fluid, ranging between 1.62 and 35.21 μg/L (1.66 ± 2.17 μg/L). Zenzes et al. (1996) found cotinine levels in 50 nonsmoker women were 4.2 ± 2 μg/L. This seems to be comparable with our results. Our study shows that the level of cotinine in serum was 4.6-fold less than in follicular fluid. According to Zenzes (2000), cotinine interacts directly and incorporates into the cells of follicle as well as the developing oocyte leading to detrimental consequences after conception. No B[a]P was found in the serum or follicular fluid of our participants. It is clear

Table 1 Descriptive statistics of the variables

Measured variables	n	Mean ± SE	Median	Range
Women's age (years)	67	31.90 ± 0.695	32.0	20–44
Age when started menstruating in years	67	12.84 ± 0.151	13.0	10–16
Days of menstrual cycle	67	5.64 ± 0.129	6.0	3–7
Women's BMI (Kg/m^2)	67	28.826 ± 0.614	28.35	17.75–40
Number of good embryo	67	1.81 ± 0.330	1.0	0–17
Number of fair embryo	67	0.52 ± 0.107	0	0–3
Number of poor embryo	67	1.22 ± 0.236	1.0	0–9
Total number of embryo	67	3.54 ± 0.445	3.0	0–20
Number of eggs	67	8.91 ± 0.857	7.0	0–30
Number of fertilized eggs	67	4.76 ± 0.561	4.0	0–23
Number of transferred embryo	67	1.54 ± 0.126	2.0	0–4
Number of implanted embryo	67	0.13 ± 0.047	0	0–2
Number of biochemical pregnancy	67	0.03 ± 0.021	0	0–1
Number of aborted	67	0.03 ± 0.021	0	0–1
Blood cadmium levels (μg/L)	65	0.547 ± 0.042	0.519	0–1.508
Blood lead levels (μg/dL)	67	3.159 ± 0.274	2.782	0.674–12.219
Blood mercury levels (μg/L)	67	3.604 ± 0.497	3.438	0–30.345
Serum *p,p*-DDE levels (μg/L)	59	1.653 ± 0.288	0.830	0–8.82
Follicular cadmium levels (μg/L)	67	0.350 ± 0.062	0.237	0–2.902
Follicular lead levels (μg/dl)	67	0.530 ± 0.068	0.379	0–3.731
Follicular mercury levels (μg/L)	67	2.454 ± 0.593	1.398	0–38.302
Follicular *p,p*-DDE levels (μg/L)	67	0.302 ± 0.060	0.303	0–2.424
Serum cotinine (μg/L)	45	0.360 ± 0.359	0	0–16.17
Follicular cotinine levels (μg/L)	56	1.657 ± 0.728	0	0–35.21
Total DNA adducts in blood (per 10^{10} nucleotides)	60	148.136 ± 58.56	21.50	0–2435.1
Total DNA adducts in follicular fluid (per 10^{10} nucleotides)	60	553.825 ± 217.81	0	0–8896.9

that cotinine can be detected in follicular fluid of women irrespective of their smoking status.

The absence of BPDE-DNA adduct in our women might result from either low levels of B[a]P exposure or genetic polymorphisms (Boysen and Hecht 2003). On the other hand, other unknown DNA adducts were seen in both blood and follicular fluid samples (Fig. 2). The levels of the average adducts in follicular fluid (553.83 ± 217.81 per 10^{10} nucleotides) were significantly higher than in blood samples (148.14 ± 58.56 per 10^{10} nucleotides) with p-value of 0.019. Different lipid characteristics between serum and follicular fluid could also account for these differences (Neal et al. 2008). A significant positive correlation was observed between the blood and follicular fluid total adducts ($r = 0.445$, $p = 0.001$). There was large inter-individual variations in DNA adduct levels which might be attributed to variations in their metabolic activation/detoxification profiles and/or DNA repair pathways. Ketelslegers et al. (2006) showed that analysis of multiple genetic polymorphisms can give better explanation of observed inter-individual variation in the levels of DNA adducts.

There are many theories regarding the adverse effects of pollutants on the reproductive system. For example, Agarwal et al. (2003) emphasized the role of ROS in the pathophysiology of human reproduction for both male and female. They showed that follicular fluid ROS, at low concentrations, may be a potential marker in predicting the outcome of IVF treatment. When we looked at the possible effect of heavy metals, DDE and smoking on the formation of DNA adducts, positive correlations were seen only between the follicular cotinine and blood DNA adducts

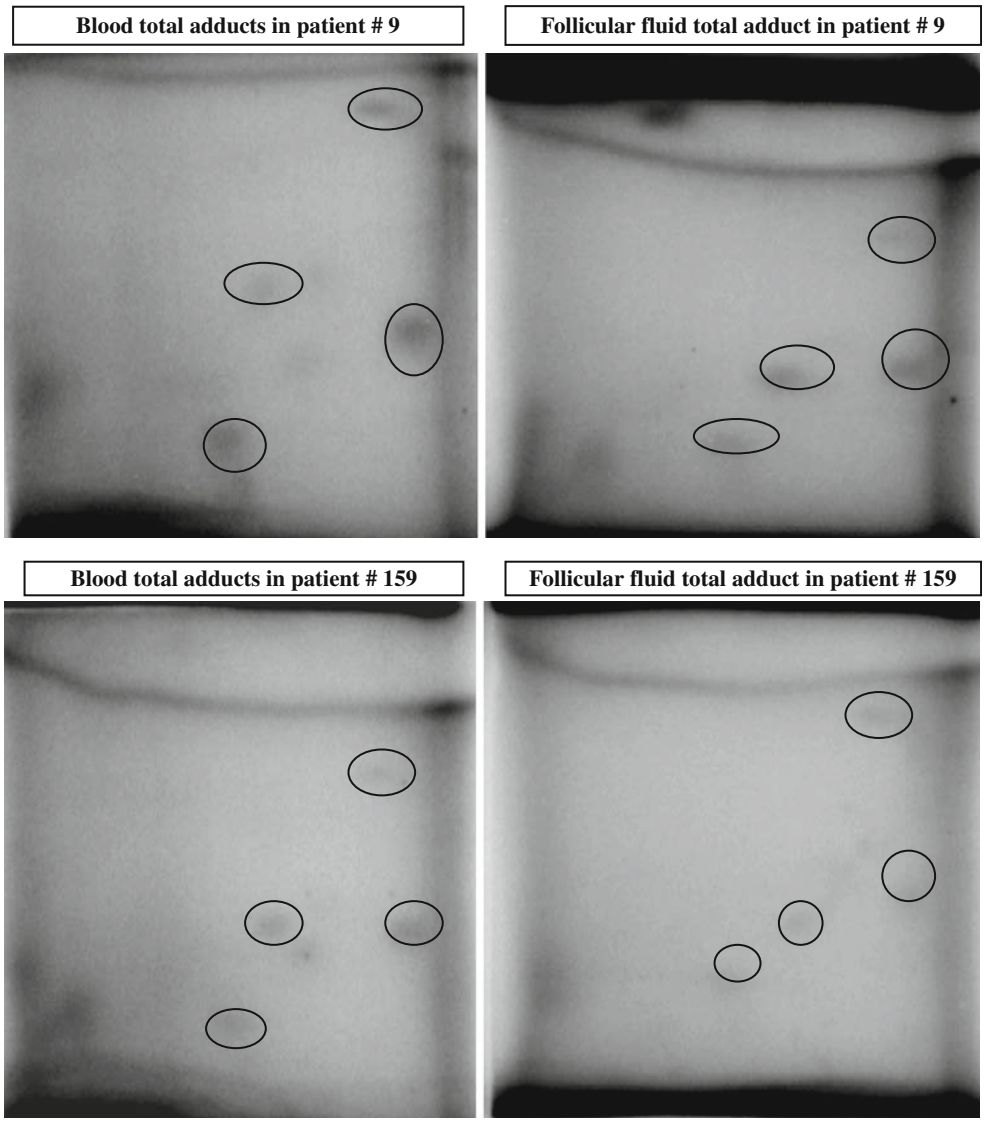

Fig. 2 ^{32}P-postlabelling adduct maps of DNA taken from the blood and follicular fluid of two participants with high total DNA adducts

($r = 0.361$, $p = 0.011$) and follicular fluid DNA adducts ($r = 0.334$, $p = 0.018$). Though most women were non-smokers, it seems that they were exposed to passive smoking. Siddiqui et al. (2000) estimated that 34.9% Saudi males are smokers. This may have played a role in induction of DNA adducts in both matrixes. Husgafvel-Pursiainen (2004) reviewed human biomarker studies that were conducted among non-smokers with involuntary exposure to tobacco smoke. The presence of DNA adducts, urinary metabolites of carcinogens, urinary mutagenicity, SCEs and gene mutations suggest that the risk of genetic alterations in passive smokers might be as equal as smokers. Exposure to metals and tobacco smoke has been reported to induce intra-follicular oxidative stress that may lead to DNA damage (Zenzes 2000). Though no association was seen, one should not exclude the fact that lead, cadmium, mercury and DDT are known to exert some adverse effect on female reproductive health (Mendola et al. 2009).

In this study, we tested the impact of DNA adducts in the blood and follicular fluid of IVF women on the pregnancy and fertilization outcome. Using Mann-Whitney U test, we found that cases and controls of the pregnancy outcome were significantly different with regard to follicular DNA adduct levels but not blood. Women who failed to achieve pregnancy had higher DNA adducts in their follicular fluid (468.13 ± 245.07 per 10^{10} nucleotides, $n = 27$) than those who succeeded (295.58 ± 286.28 per 10^{10} nucleotides, $n = 20$) with a p-value of 0.016 (Fig. 3a). The levels of DNA adducts in blood samples of women who failed pregnancy were higher (137.04 ± 80.71 per 10^{10} nucleotides, $n = 29$) than those who succeeded (78.31 ± 48.92 per 10^{10} nucleotides, $n = 18$), though it was not significant ($p = 0.245$). Surprisingly, the levels of DNA adducts in the

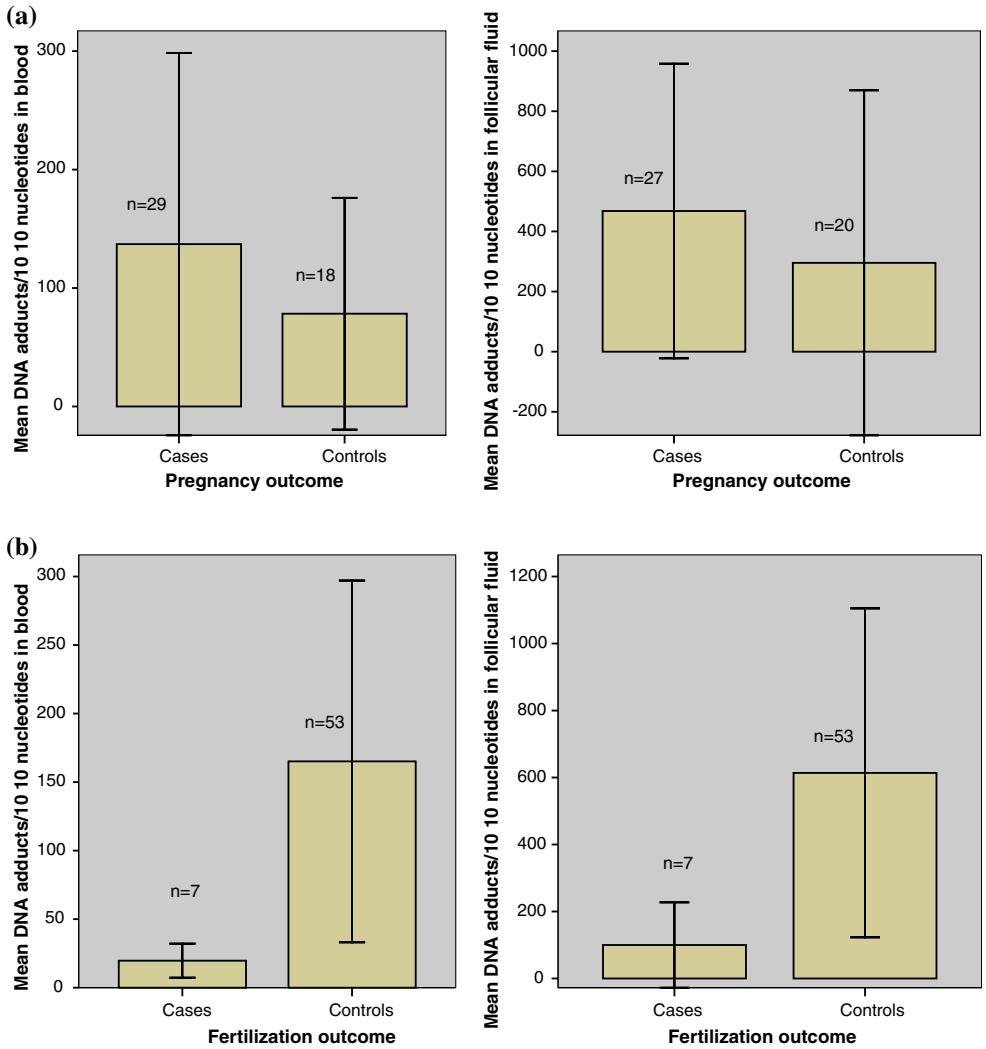

Fig. 3 The levels of adducts in the blood and follicular fluid of women ($n = 60$) undergoing IVF treatment as classified by their **a** pregnancy outcome; and **b** fertilization rate. *Error bars* show mean ± SE

blood and follicular fluid of women who failed fertilization were lower (19.71 ± 6.21 per 10^{10} nucleotides, n = 7 and 99.85 ± 63.66 per 10^{10} nucleotides, n = 7, respectively) than those who succeeded (165.10 ± 66.01 per 10^{10} nucleotides, n = 53 and 613.78 ± 245.52 per 10^{10} nucleotides, n = 53, respectively), but were not significant with *p*-values of 0.804 and 0.512, respectively (Fig. 3b). The formation of adducts in follicular fluid may be a potential source of DNA damage which might prevent egg fertilization or the development of embryo. Wiener-Megnazi et al. (2004) regarded follicular fluid as a biological "window," reflecting metabolic and hormonal processes occurring in the microenvironment of the maturing oocyte before ovulation, and also as a predictor of outcome parameters such as fertilization, embryo cleavage, and pregnancy rates in IVF.

The exact origin and nature of these unknown adducts remain problematic despite the sensitivity of the method. It is; therefore, important to characterize these adducts in order to identify environmental factors that play a role in the etiology of IVF failure or infertility problems.

Though our sample is small, our findings support the hypothesis that the presence of lipophilic DNA adducts in follicular fluid could affect IVF outcome parameters leading to lower pregnancy success rate. It seems to us that evaluation of follicular fluid oxidative stress status in women undergoing IVF treatment could have a role in predicting its success rate. It may be recommended to examine the potential of oxidative stress biomarkers in revealing unexplained infertility in women before deciding to embark on IVF treatment.

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