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ORIGINAL ARTICLE

Xeno-oestrogenic activity in serum as marker of occupational pesticide exposure

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Background: An increasing number of currently used pesticides are reported to possess oestrogen-like properties or to disturb the endocrine system in other ways.

Objectives: To investigate if xeno-oestrogenic activity in serum can be used as a biomarker of the combined exposure to pesticides with oestrogen-like properties in an occupational setting.

Methods: Serum samples were obtained from two separate cohorts representing non-pregnant and pregnant female greenhouse workers in Denmark. Serum samples from 270 non-pregnant women and 173 pregnant women were analysed for xeno-oestrogenic activity. A fraction containing major xeno-oestrogens but without pharmaceutical and endogenously produced oestrogens was isolated from each serum sample by solid-phase extraction and tested for oestrogenic response in a MCF-7 cell proliferation assay. The pesticide exposure for each woman was categorised as low, medium or high based on information collected by detailed interviews of the women and the employers.

Results: In both cohorts, an exposure-associated increase in the xeno-oestrogenic activity in serum was demonstrated. Among the pregnant women, the association between pesticide exposure and xeno-oestrogenic activity in serum was statistically significant for women who had been at work within the last week, while no association was observed for women who had not been at work during the most recent week.

Conclusions: The study illustrates the usefulness of this biomarker for qualitative assessment of the combined exposure to mixtures of oestrogen-like pesticides. Although the individual pesticides responsible for the xeno-oestrogenic response were not identified, the study demonstrates that, even within highly-controlled greenhouse operations, occupational exposure to oestrogen-like pesticides can result in detectable impacts on hormonal activity in the blood.

Exposure situations are often complex and involve several potentially interacting chemicals. Compounds with the same mode of action are likely to be additive.^{1–3} Development and use of exposure biomarkers is a valuable tool in environmental epidemiology, and a promising approach to complex exposures is to use biomarkers that integrate the response from several substances that affect the same target.

Based on strong evidence from studies in wildlife and laboratory animals, there is increasing concern that exposure to endocrine disrupting chemicals may impair reproductive capability, disturb fetal development, and promote carcinogenesis in humans.^{4–6} A biomarker assessing the total exposure to xeno-oestrogens in serum samples was initially developed by Soto and Sonnenschein^{7–8} and this methodology has since been refined and used for measurement of the xeno-oestrogenic response in adipose tissue⁹ or in serum samples^{10–12} from populations with increased exposure to persistent, lipophilic pollutants. The approach used in this biomarker assay includes extraction of oestrogens from serum samples followed by separation of xeno-oestrogens from endogenous oestrogens, and the oestrogenic activity of the xeno-oestrogen fraction (XE fraction) is then determined *in vitro*. The focus so far has been on lipophilic, non-polar pollutants that are easily separated from the endogenous and pharmaceutical oestrogens. However, several polar substances, including some of the currently used pesticides, are also thought to possess oestrogenic properties *in vitro*^{13–15} and *in vivo*.¹⁶ The aim of this study was to investigate whether the biomarker approach for serum xeno-oestrogenic activity could be used to assess the mixed exposure to potentially oestrogenic pesticides in an occupational setting. We chose to examine female greenhouse workers in Denmark,

where exposures generally are well controlled, although current safety procedures are not specifically aimed at preventing exposures to endocrine disruptors.

METHODS

Study design and participants

The study is based on two independent cohorts of female greenhouse workers.

Cohort 1: non-pregnant female greenhouse workers

A cohort of female greenhouse workers was established from February 1996 to December 1997 in a cross-sectional study design. A letter of invitation was sent to all greenhouse owners on the island of Funen, Denmark to obtain permission to visit the greenhouses and to invite the female employees for participation in the study. Among the 220 greenhouse horticultures on Funen, 100 (45%) of the owners agreed to participate after telephone contact, whereas 35% did not meet the inclusion criteria (no female employees), and 20% chose not to participate. Thus, 69% of the eligible employers agreed to participate in the study. All female employees at the 100 greenhouse horticultures were informed orally and in a letter of invitation about the project. Among the women working in the greenhouses 602 agreed to participate and gave written consent; based on union records and information from employers, the participants correspond to about 75% of the eligible women. Interview-assisted questionnaires and blood

Abbreviations: HPLC, high performance liquid chromatography; E2, 17 β -oestradiol; PPE, personal protective equipment; RPE, relative proliferative effect; XE fraction, xeno-oestrogen fraction

samples were collected at the workplace. Blood samples were successfully obtained from 571 (95%) of the participants.

Cohort 2: pregnant greenhouse workers

Pregnant women employed in greenhouses on Funen were recruited consecutively from July 1996 to October 2000 at the Department of Occupational and Environmental Medicine at Odense University Hospital. A previous survey (unpublished data) indicated that approximately 50% of all pregnant women working in greenhouses on Funen were referred by their general practitioner to the hospital department to receive advice regarding working conditions during pregnancy. In an attempt to increase the percentage of referred pregnant greenhouse workers, information on the study was sent to all general practitioners and owners of greenhouse horticulture industries on Funen. During the inclusion period, a total of 336 pregnant greenhouse workers were referred to the department. After oral and written information about the study, acceptance and written consent was obtained from 289 women (representing 314 pregnancies); that is, 93% of the total number referred. One woman was included with three separate pregnancies, 23 women participated with two separate pregnancies, and the remaining 265 women participated only once. All interviews were performed at the department of Occupational and Environmental Medicine. In most cases the women were in first trimester at the time of referral with a mean of gestational week 7 (range 1.5–26). After the interview the women were asked to deliver a blood sample, which were collected at the hospital immediately after the interviews. Blood samples representing 239 of the 314 pregnancies (76%) were successfully obtained.

The study was conducted according to the Helsinki II Declaration and was approved by the regional ethical review committee and the Danish Data Protection Agency. In both cohorts the women were included after providing informed written consent.

Exposure information

The participants of both cohorts underwent a structured interview with detailed questions about working conditions during the last three months. The information included average weekly working hours, a description of job functions and average weekly time used for each function, personal handling of pesticides (for example, application and mixing), re-entry intervals before entering greenhouses where pesticides had been applied, procedure for handling of plant cultures recently treated with pesticides, and the use of personal protective equipment. The women were also asked about the type of pesticide products and frequency of use both in the relevant working areas and in the greenhouse as a whole. Further, the women were asked about educational background, smoking habits, non-occupational exposure to pesticides (used at home for pets or during gardening), and use of pharmaceutical hormones including oral contraceptives or other products containing oestrogen.

The information about use of pesticides obtained from the interview was supplemented with information from the employers. We asked the employer for detailed information on the use of pesticides during the last three months. For the cohort of non-pregnant women this information was, in most cases, provided as copies of the logbooks, in which all pesticide use (date of application, area treated, trade name of the pesticide product and the concentration applied) must be registered according to Danish law. For the cohort of pregnant women, the employers were contacted by telephone to obtain more detailed information about actual and recent pesticide use in the area where the woman had been working.

Based on the information from the women and the employers, the pesticide exposure level for each woman was categorised as low, medium or high. All exposure ratings were performed in parallel and independently by two toxicologists with special expertise in horticulture working conditions and these ratings were completed before the assessment of xeno-oestrogenic activity in the serum samples. Initially, all work functions were scored according to potential pesticide contact. Application and mixing of pesticides and dipping of cuttings directly into pesticide solutions were assigned a high score. Re-entry activities with intensive plant contact (for example, nipping cuttings or propping up the plants) had a medium score, and re-entry activities with minimal plant contact (for example, moving of pot plants and packing) had a low score. Scores for the different work functions were developed in collaboration with representatives of greenhouse owners and the Danish labour union for greenhouse workers. Exposure ratings for each woman were obtained by combining the work function scores with information about the average weekly time spent on the different work functions, the use of personal protective equipment (PPE) and the information about when and how often pesticides had been applied in the working area or on the plants handled by the woman. Hence, for women who had direct contact with pesticides by applying, mixing or dipping cuttings in pesticides, the exposure level was rated as high or medium depending on the exact procedure, the duration of the process, and the use of PPE. For the re-entry activities, the exposure was rated as high if pesticides were used frequently (more than once a week) in the working area and the woman often handled treated plants without using gloves and had other work functions with intensive plant contact. Exposure was rated as low if pesticides had not been applied in the working area and the woman had not handled plants that had been treated with pesticides within the last three months. The low exposure groups can therefore be considered as internal non-exposed control groups. Exposures were rated as medium if pesticides were used in the working area only infrequently (less than once a week), if intensive plant contact occurred or if pesticides were used frequently, but without contact with plants within 24 h after treatment (table 1).

Blood samples

Venous blood samples were collected into plain Venoject tubes (Terumo Europe NV, Leuven, Belgium) during the day time (between 09:00 and 16:00). One hour after sampling, the blood samples were centrifuged to separate the serum. Within 4 h after sampling, the serum was frozen in 1 ml aliquots in Cryo tubes (Greiner, Frickenhausen, Germany) at -80°C until use.

Due to the labour intensity of the biomarker method, subgroups of samples were selected for analysis. From the cohort of non-pregnant women, 270 serum samples (out of 571) were selected. The samples include all blood samples obtained from the low and high exposure groups. From the medium exposure group, 119 serum samples (out of 420) were selected to represent smokers, non-smokers, and users and non-users of oral contraceptives within this exposure group. For the 270 women included in the analysis of xeno-oestrogenic activity, the median age was significantly lower in the medium and high exposure groups than in the control group (table 2). There were significantly fewer smokers in the medium exposure group and more users of oral contraceptives in the high exposure group compared to the control group. Only a few women from each exposure group used hormone-containing pharmaceuticals (mainly hormone replacement therapy) other than oral contraceptives.

From the cohort of pregnant women, blood samples from 173 pregnancies were analysed for xeno-oestrogenic activities. The

Table 1 Criteria for rating exposure levels

| Work function | Low (controls) | Medium | High |
|---------------------------|--|---|--|
| Application of pesticides | No pesticides applied | Infrequently irrigating plants, dipping of cuttings in pesticides using PPE | Mixing and spraying pesticides OR frequently irrigating plants AND/OR dipping cuttings in pesticides without using PPE |
| Re-entry | No pesticides applied in the working area AND no handling of treated plants within the last 3 months | 1. Pesticides applied in working area >1/week AND intensive contact to treated plants using PPE, OR 2. Pesticides applied in working area >1/week AND minor contact with treated plants with or without using PPE, OR 3. Pesticides applied in working area <1/week AND intensive contact to treated plants with or without using PPE | Pesticides applied in working area >1/week AND intensive contact to treated plants without using PPE |

PPE, personal protective equipment.

samples represent all blood samples obtained from women whose pregnancies resulted in one or more live born children. Among these women, there was no significant difference in median age and number of smokers between the exposure groups. Gestational week at sampling was significantly lower in the highly exposed group (table 2), possibly due to earlier referral. Only three women, all in the medium exposure group, used hormone-containing pharmaceuticals (progesterone supplement).

Analytical methods

Xeno-oestrogenic activity in serum samples

Xeno-oestrogenicity in serum was measured as previously described¹⁰ with minor modifications in order to allow inclusion of pesticides relevant to these cohorts. Briefly, xeno-oestrogens were extracted from 4 ml of serum by solid-phase extraction (OASIS HLB 6cc 500 mg extraction cartridge from Waters, Milford, MA, USA). The extracted compounds were separated by high performance liquid chromatography (HPLC) to collect a fraction including major xeno-oestrogens (XE fraction), while avoiding pharmaceutical and endogenous oestrogens. This fraction was then tested for oestrogenic response in the MCF-7 cell proliferation assay. In our previously described method,¹⁰ we sampled the HPLC fraction eluting within the first 5.5 min. In the present study, we expanded the sampling period to 8.0 min in order to include more hydrophilic pesticides. This fraction included, in addition to the compounds previously

described,¹⁰ a wide range of pesticides, for example, dieldrin (1.75 min), endosulfan (1.83+3.87 min), chlorpyrifos (1.94 min), deltamethrine (2.07+3.22 min), methoxychlor (2.18+2.72 min), methiocarb (5.36 min), vinclozolin (5.8 min), pirimicarb (6.19 min), and fenarimol (7.76 min). As before,¹⁰ subfractions of serum from the pregnant women were excluded to avoid pregnancy-related hormones. In the present study, an additional subfraction from 6.2–7.1 min was eliminated to avoid pregnenolone. To calibrate and adjust time windows for collection of the HPLC fractions, a standard mixture, including vinclozolin, fenarimol, 5 α -dihydroprogesterone, pregnenolone, testosterone and oestrone was run before each sample series.

Testing of the XE fraction in the MCF-7 cell proliferation assay was performed as previously described.¹⁰ To allow determination of both an agonistic and an antagonistic response in the proliferation assay, the serum extracts were analysed with and without concomitant addition of 10 pM 17 β -oestradiol (E2). This E2 concentration induces approximately half of the maximum response in the proliferation assay. Due to insufficient serum volume, 15 serum samples (1 control and 14 exposed) were not tested together with E2.

The MCF-7 cell proliferation assay results are expressed as the number of cells after six days of incubation with the test substance. This response was adjusted for the result of negative control samples (cells treated with oestrogen-free medium supplemented with solvent only) in each analytical run to

Table 2 Distribution of age, smoking, oral contraceptive usage, hormone pharmaceutical usage*, and gestational week at sampling among three exposure groups of female greenhouse workers

| | Low (controls) | Medium | High |
|--------------------------------|------------------|-------------------|-------------------|
| Non-pregnant women | | | |
| Sample size, n | 58 | 119 | 93 |
| Age (years) | | | |
| Median (25th–75th percentiles) | 42.6 (33.0–48.6) | 34.2 (27.7–43.9)† | 29.3 (23.6–41.2)† |
| Smokers, n (%) | 20 (34.5) | 19 (16.0)† | 31 (33.3) |
| Oral contraceptive use, n (%) | 12 (20.7) | 23 (19.3) | 38 (40.9)† |
| Hormone treatment, n (%) | 6 (10.3) | 5 (4.2) | 5 (5.3) |
| Pregnant women | | | |
| Sample size, n | 29 | 88 | 56 |
| Age (years) | | | |
| Median (25th–75th percentiles) | 26.7 (25.2–28.5) | 27.3 (24.8–30.3) | 27.5 (25.7–29.7) |
| Smokers, n (%) | 13 (44.8) | 27 (30.7) | 16 (28.6) |
| Hormone treatment†, n (%) | 0 | 3 (3.4) | 0 |
| Gestational week at sampling | | | |
| Median (25th–75th percentiles) | 5.9 (5.1–8.3) | 6.6 (5.1–8.7) | 5.2 (4.3–7.0)† |

*Hormone containing pharmaceuticals other than oral contraceptives.
†p<0.05 compared to low exposed controls.

Table 3 Xeno-oestrogenic activity* in serum from female greenhouse workers exposed to different levels of pesticides

| | Median RPE (25th–75th percentiles) | | |
|---|------------------------------------|----------------|----------------|
| | Low (controls) | Medium | High |
| Non-pregnant women | 3.2 (1.8–4.9) | 4.0 (2.2–6.3) | 3.8 (2.7–4.9) |
| n | 58 | 119 | 93 |
| Pregnant women (all) | 6.0 (2.5–8.1) | 7.2 (4.7–11.2) | 7.6 (5.4–11.8) |
| n | 29 | 88 | 56 |
| Pregnant women who had been working during the previous week | 5.2 (0.6–6.8) | 8.8 (5.5–15.3) | 7.7 (5.3–11.0) |
| n | 12 | 29 | 29 |
| Pregnant women who had <i>not</i> been working during the previous week | 6.3 (2.9–8.3) | 6.3 (3.7–9.5) | 7.0 (5.4–12.2) |
| n | 16 | 55 | 24 |

RPE (relative proliferative effect) = $[(PE_{\text{test sample}} - 1)/(PE_{\text{maxE2}} - 1)] \times 100$, where PE is the response normalised to controls; n, number of serum samples analysed.

*Data represent RPE obtained for the XE fractions isolated from 4 ml of serum and tested in the MCF-7 cell proliferation assay.

account for differences in the initial seeding density. All serum extracts were analysed in triplicate and the coefficient of variation (CV) within each run was below 5%. The inter-assay variation for all the series was 14%. All results are given as the relative proliferative effect (RPE) compared to the maximum E2 response:

$$\text{RPE} = [(PE_{\text{test sample}} - 1)/(PE_{\text{maxE2}} - 1)] \times 100$$

where PE is the response normalised to controls. Thus, the RPE describes the efficacy of the test sample to induce cell proliferation relative to that of E2. Within each cohort the samples were analysed without information on exposure status or other predictors and in random order.

Statistics

Pesticide exposure was categorised as low (control), medium, or high as described above. Potential confounders identified from a priori considerations of factors that might influence xeno-oestrogenic response included age, smoking, actual use of oral contraceptives (non-pregnant women) or other hormone containing pharmaceuticals, and gestational week at sampling (pregnant women). Differences in the distribution of these covariates between the exposure groups were compared by using the non-parametric Mann-Whitney test. Data on xeno-oestrogenic and xeno-antioestrogenic activities as well as age and gestational week at sampling were not normally distributed, and the median and interquartile range (25th–75th percentiles) was therefore calculated. Further, negative values of the xeno-oestrogenic and anti-oestrogenic activities were substituted by 0.01 RPE. The data, along with age and gestational week, were then transformed using the natural logarithm to approach a normal distribution. A multiple linear regression analysis was performed taking into account the potential confounders mentioned above. Pesticide exposure, smoking, and oral contraceptive and other hormone pharmaceutical usage were entered into the model as dummy variables.

RESULTS

The data from the two cohorts of female greenhouse workers were analysed separately as the HPLC fraction containing pregnenolone had been removed from the serum samples from the pregnant women, but not from the non-pregnant women.

For all women, re-entry activities (such as moving or packing pot plants or nipping cuttings) constituted the main work functions. Besides, 17% of the women in the cross-sectional cohort and 23% in the cohort of pregnant women reported to have been directly involved in applying pesticides mainly by irrigating fungicides or growth retardants.

In both cohorts of female greenhouse workers, an exposure-associated increase in xeno-oestrogenic activity was observed (tables 3 and 4). Of the possible confounders included in the multiple regression analyses, only gestational week at sampling significantly affected (increased) the xeno-oestrogenic activity in serum. After confounder adjustment, the xeno-oestrogenic activity was still positively associated with the level of pesticide exposure in both cohorts (table 4), although the xeno-oestrogenic activity in the medium exposed group of pregnant women was not significantly higher than the control group. Gestational week at sampling was lower for the group of pregnant women with high pesticide exposure compared to the medium and low exposure groups (table 2). This could lead to a lower xeno-oestrogenic response in the high exposure group and hence underestimate the difference between the groups and might be one explanation for the more marked effect of confounder adjustment among the pregnant women (table 4). Additionally, most of the pregnant women (77%) had been absent from work for some time before blood sampling, in accordance with the general recommendation to refrain from possibly hazardous procedures until the working conditions had been assessed by the occupational health specialists. The exposure-free period varied from a few days to several weeks. As the pesticides used in the working areas are non-persistent and assumed to be metabolised and excreted within few days, the regression analyses were repeated separately for those women who had been at work and those who had *not* been at work within the last week before blood sampling. For eight women information about last working day was missing, and they were therefore excluded from this analysis. A strong association between xeno-oestrogenic activity and pesticide exposure was seen for the women who had been at work within the last week, while no such association was observed for the women not at work during the most recent week.

Irrespective of pesticide exposure level, the response obtained from serum samples from non-pregnant women was lower than the response obtained from serum samples from pregnant women (table 3). This tendency was also seen when the serum samples were tested together with 10 pM E2 to assess antagonistic or potentiating effects. For the pregnant women, the response obtained in the presence of E2 was higher than the response induced by E2 alone (table 5). However, for the non-pregnant women, the E2-induced response was reduced, thus indicating an anti-oestrogenic effect of the serum extract. For none of the two cohorts did the response in the presence of E2 differ between the pesticide exposure groups (table 5). Multiple linear regression analyses, taking into account the confounders

Table 4 Unadjusted and adjusted results* from linear regression analyses of xeno-oestrogenic activity in serum among female greenhouse workers exposed to pesticides compared to unexposed/low exposed greenhouse workers

| | Change in xeno-oestrogenic activity (%) β (95% CI) | | |
|---|--|------------------|------------------|
| | Low (controls) | Medium | High |
| Non-pregnant women | | | |
| Unadjusted results | Reference | 41 (12 to 70) | 45 (15 to 75) |
| Adjusted† results | | 58 (26 to 90) | 57 (22 to 92) |
| Pregnant women (all) | | | |
| Unadjusted results | Reference | 76 (9 to 143) | 176 (105 to 247) |
| Adjusted‡ results | | 60 (-6 to 126) | 204 (133 to 275) |
| Pregnant women who had been working during the previous week | | | |
| Unadjusted results | Reference | 409 (308 to 510) | 429 (328 to 530) |
| Adjusted‡ results | | 403 (301 to 505) | 567 (463 to 671) |
| Pregnant women who had <i>not</i> been working during the previous week | | | |
| Unadjusted results | Reference | -11 (-103 to 81) | 94 (-10 to 198) |
| Adjusted‡ results | | -24 (-116 to 68) | 87 (-17 to 191) |

*The data shown in the table are % change in xeno-oestrogenic activity in the high and medium exposed groups compared to the low exposed group (reference value).

†Adjusted for age, use of oral contraceptives, use of hormone containing pharmaceuticals (other than oral contraceptives), and smoking.

‡Adjusted for age, gestational week at sampling, use of hormone containing pharmaceuticals (other than oral contraceptives) and smoking.

mentioned before, also did not indicate any exposure related effect on the serum response after co-exposure with E2 (data not shown).

DISCUSSION

The present study demonstrates an exposure-related increase in xeno-oestrogenic activity of serum obtained from two cohorts of women occupationally exposed to pesticides in greenhouses. This finding is difficult to relate to individual substances, because the major part of currently used pesticides has not yet been investigated for endocrine disrupting activities. However, screening studies using cellular assays indicate that a considerable part of these pesticides possess endocrine disrupting properties, whether by activating the oestrogen receptor, blocking the androgen receptor and/or by interfering with enzymes involved in the metabolism of steroid hormones^{13 15 17}. Although the potencies of the pesticides as hormone agonists or antagonists are low when compared to the natural ligands, effects of pesticides with the same mode of action are likely to be additive, and the integrated response from mixed pesticide exposure is therefore of interest. Additive effects of oestrogenic

acting pesticides have been demonstrated in vitro in several studies.^{3 18} If the concentration-response curves are sigmoid, a combined effect of low concentrations of several oestrogenic pesticides can induce a marked response, even though the response induced by the individual compounds may be below the detection limit.

This study demonstrates the usefulness of this biomarker for qualitative exposure assessment of oestrogenic pesticides at the group level. The biomarker has the additional advantage of being fairly independent of confounders. However, the wide variation in xeno-oestrogenic activity within the groups, including the control group, suggests that the biomarker cannot be used for quantitative exposure assessment at the individual level. Although some of the inter-individual variation within the exposed groups could be explained by individual differences in exposure level and pesticides used, some of the variation is likely the result of exposure to oestrogenic compounds from other sources—for example, food and cosmetics.

In general, the xeno-oestrogenic response did not differ much between the medium and high exposure groups,

Table 5 Xeno-oestrogenic response* when serum samples from female greenhouse workers were tested together with E2

| | % of the response induced by 10 pM E2 alone, median (25th–75th percentiles) | | |
|---|---|---------------|---------------|
| | Low (controls) | Medium | High |
| Non-pregnant women | 75 (63–83) | 77 (59–104) | 76 (65–90) |
| n | 57 | 106 | 92 |
| Pregnant women (all) | 131 (124–141) | 133 (122–145) | 134 (123–147) |
| n | 29 | 88 | 56 |
| Pregnant women who had been working during the previous week | 132 (124–142) | 135 (122–147) | 133 (123–148) |
| n | 12 | 29 | 29 |
| Pregnant women who had <i>not</i> been working during the previous week | 129 (122–140) | 132 (122–143) | 133 (123–147) |
| n | 16 | 55 | 24 |

n, number of serum samples analysed.

*Results represents data obtained for the XE fractions isolated from 4 ml of serum and tested together with 10 pM E2 in the MCF-7 cell proliferation assay. They are expressed as percentages of the response obtained by 10 pM E2 alone.

presumably because exposure rating is fraught with difficulties when individual behaviour characteristics play a major role. For example, individuals considered highly exposed with direct contact with pesticides or newly treated plants may not necessarily be highly exposed if they are more aware of the hazard and adjust their work habits to minimise the exposure.

For all exposure levels, the xeno-oestrogenic responses in pregnant women were considerably higher than those from the non-pregnant women. Likewise, a lower response from non-pregnant women was obtained when the serum was tested together with 10 pM E2, thus supporting a lower responsiveness of the cells. As the exposure situation for the two cohorts was very similar, the reason for this difference could be attributed to slight differences in assay sensitivity over time. The serum samples from the cohort of non-pregnant women were analysed approximately one year before the samples from the cohort of pregnant women. Although the procedure is standardised and the response is normalised to control (unexposed cells) and to the maximal response induced by E2 (positive control), some change in the responsiveness of the assay over time is unavoidable. An additional consideration is that, although all known endogenous compounds with oestrogenic activity were removed from the serum, some unidentified endocrine metabolites produced during pregnancy could remain in the serum fraction analysed and have contributed to a higher response. This possibility would then also explain the tendency of an increased xeno-oestrogenic activity at increasing gestational week. A tendency towards higher xeno-oestrogenic responses among pregnant women compared to non-pregnant women was also observed within the control group from our previous study,¹⁰ where xeno-oestrogenic activity in serum samples from pregnant Faroese women exposed to polychlorinated biphenyls and other persistent pollutants was compared with the xeno-oestrogenic activity among Danish pregnant and non-pregnant controls. Hence, fractionating of the serum samples from pregnant women may have to be further refined to avoid interference from pregnancy-related hormonal substances. In contrast, among non-pregnant women, the use of oral contraceptives or other hormone-containing pharmaceuticals did not affect the xeno-oestrogenic response, thus indicating that all pharmaceutical oestrogens had been effectively removed from the serum.

The difference in response level between the two cohorts did not prevent the ability to demonstrate an exposure-related increase in xeno-oestrogenic activity in the two different cohorts. Among the pregnant women the presence of an exposure-related increase in xeno-oestrogenic activity only in serum from the women who had been at work during the previous week strongly supports the notion that the response is directly related to the occupational pesticide exposure. The absence of exposure-related differences when co-tested with E2 is in agreement with the fact that only one pesticide so far has been identified as an oestrogen antagonist.¹⁰

The exact pesticides responsible for the xeno-oestrogenic activity cannot be identified from this study. The women were exposed to a wide range of pesticides, of which only a minor part has been tested for endocrine disrupting properties. In a sub-analysis, women with medium or high exposure to known pesticides with demonstrated oestrogenic properties (chlorpyrifos, deltamethrin, endosulfane, fenarimol, methiocarb, tochloro-fos-methyl)¹³ had higher xeno-oestrogenic activity than unexposed controls (data not shown). However, the response of these women did not differ from the one seen in the group of women exposed to other pesticides—that is, including substances with unknown oestrogenic potentials.

Worldwide, many women of child-bearing age are working in occupations using pesticides and they are therefore potentially

exposed to endocrine disrupting compounds. Since the developing fetus is particularly vulnerable to endocrine disrupting substances, it is crucial to protect pregnant women against such exposures. This study demonstrates that occupational exposure to oestrogenic pesticides does occur, even under circumstances where strict controls and hygiene procedures must be respected. Therefore, pregnancy outcomes and the health status of children of women potentially exposed to pesticides in their workplace should be carefully monitored to ensure that appropriate measures are taken to prevent adverse health effects. Better guidance in the use of protective equipment, especially in relation to re-entry activities, as well as prolonged re-entry intervals, is expected to reduce the exposure levels.

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Main messages

- Most currently used pesticides have yet to be examined for endocrine disrupting activities, although cell-based assays indicate that a considerable number may possess endocrine disrupting properties.
- A biomarker method for assessing xeno-oestrogenic activity of human serum can be applied to estimate the combined impact of mixed exposures to pesticides.
- Examination of two groups of female greenhouse workers has shown an exposure-related increase in xeno-oestrogenic activity in serum.
- A clear association between pesticide exposure and xeno-oestrogenic activity in serum was observed among women who had been at work in greenhouses during the previous week, while no such association was observed for those absent during the past week or longer. This finding supports the validity of the association between occupational pesticide exposure and xeno-oestrogenic response in serum, and suggests that it is primarily dependent on recent exposures.
- The results obtained show that occupational exposure to pesticides may cause endocrine changes, despite strict controls and hygiene procedures used in greenhouses.

Policy implications

- The developing fetus is considered particularly vulnerable to endocrine disrupting substances, and female workers must therefore be protected from exposure to such compounds, including pesticides with oestrogen-like activities.
- Many pesticides have been shown to possess endocrine disrupting properties in cell-based studies, and their possible impact on human health deserves attention.
- Improved safety at work, including use of protective equipment in relation to re-entry activities, as well as prolonged re-entry intervals, may reduce the exposure level to approved pesticides.

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