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Experimental strategy for translational studies of organophosphorus pesticide neurotoxicity based on real-world occupational exposures to chlorpyrifos

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ABSTRACT

Translational research is needed to understand and predict the neurotoxic consequences associated with repeated occupational exposures to organophosphorus pesticides (OPs). In this report, we describe a research strategy for identifying biomarkers of OP neurotoxicity, and we characterize pesticide application workers in Egypt's Menoufia Governorate who serve as our anchor human population for developing a parallel animal model with similar exposures and behavioral deficits and for examining the influence of human polymorphisms in cytochrome P450 (CYP) and paraoxonase 1 (PON1) enzymes on OP metabolism and toxicity. This population has previously been shown to have high occupational exposures and to exhibit a broad range of neurobehavioral deficits. In addition to observational studies of work practices in the field, questionnaires on demographics, lifestyle and work practices were administered to 146 Egyptian pesticide application workers applying pesticides to the cotton crop. Survey results indicated that the application workforce uses standard operating procedures and standardized equipment provided by Egypt's Ministry of Agriculture, which provides a workforce with a stable work history. We also found that few workers report using personal protective equipment (PPE), which likely contributes to the relatively high exposures reported in these application workers. In summary, this population provides a unique opportunity for identifying biomarkers of OP-induced neurotoxicity associated with occupational exposure.

1. Introduction

Organophosphorus pesticides (OPs) are among the most widely used chemical insecticides in the world (Zaim and Jambulingam, 2007) and continue to be identified in a majority of the samples collected from agricultural workers, even in countries such as the United States where OP use is declining (Arcury et al., 2010). The effects of repeated occupational and environmental exposures to OPs are poorly understood, although human and animal studies consistently identify neurotoxicity as the primary endpoint of concern (Bushnell and Moser, 2006; Costa, 2006). Considered in the context of the documented widespread human exposure to OPs (Barr et al., 2005), these data underscore the need to identify exposures that pose potential risk to human health. However, predicting neurotoxic risk to humans occupationally exposed to OPs has proven difficult for two reasons: (1) a relationship between OP dose and neurobehavioral deficits has yet to be demonstrated in the human research; and (2) the biomarkers typically used to assess OP exposure and/or effect, *viz.*, urinary OP metabolites and blood cholinesterase (ChE) activity, have not been reliably associated with neurobehavioral effects in studies of human occupational or environmental exposures or in chronic low-dose animal studies (Bushnell and Moser, 2006; Colosio et al., 2009;

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Rohlman et al., 2011). The lack of correlation may be because most studies of OP-induced neurobehavioral deficits in human populations have not comprehensively analyzed the pattern and duration or history of exposure, the relationship between external exposure and internal dose, and factors that potentially modify bioeffective dose (Rohlman et al., 2011). Another possibility suggested by experimental evidence of noncholinergic mechanisms of OP-induced neurotoxicity (Bushnell and Moser, 2006; Jett and Lein, 2006; Pope et al., 2005; Pope, 1999) is that biomarkers based on mechanisms *other than ChE inhibition* may be better predictors of OP neurotoxicity than ChE inhibition or may improve predictability if used in combination with ChE inhibition.

The goals of this article are: (1) to describe a strategy for testing traditional and novel biomarkers in parallel human and animal studies to identify the biomarker(s) that best predict OP-induced neurotoxicity following repeated occupational exposures; and (2) to characterize the occupational cohort that serves as the anchor population for these studies, which is pesticide application workers in Egypt's Menoufia Governorate.

1.1. Experimental strategy for identifying biomarkers of OP neurotoxicity

Human research on neurotoxicity from chronic OP exposures has been complicated by the paucity of information on workers' pesticide exposure history and by the fact that most workers are exposed to a complex mixture of pesticides. Consequently, it is difficult to attribute effects exclusively to OP exposures, and the differences between studies may be attributed to different exposure histories between occupational cohorts. Although animal research can address these issues, it has not typically employed exposure and behavioral testing paradigms that are immediately relevant to human exposure and effect patterns, and thus the relationship of data from animal research to human populations is often difficult to interpret. To better determine risks associated with repeated occupational exposures to OPs, there is a need for animal research in which exposures are readily controlled to identify mechanistically based biomarkers, but that research needs to be based on realistic human exposure and effect patterns. In turn, biomarkers identified in animal models then need to be tested in human research to confirm their relevance. These are the fundamental tenets of the research strategy we are pursuing to identify biomarkers that reliably predict OP-induced neurobehavioral deficits in occupationally exposed individuals.

1.1.1. Purpose of research project

The primary goal of this project is to identify biomarker(s) that reliably predict and/or identify individuals at risk for neurotoxic effects following chronic or repeated long-term chlorpyrifos exposure. Biomarkers are parameters or characteristics that can be objectively measured as an indication of exposure or effect. Biomarkers that reliably predict or detect damage to the target organ not only significantly improve identification of at-risk individuals, but facilitate studies to determine the effectiveness of intervention and treatment strategies. We propose to systematically evaluate biomarkers currently used to assess OP exposure and effect, namely, urinary OP metabolites and blood cholinesterase (ChE) inhibition, against novel biomarkers of OP effect that may be mechanistically relevant to OP neurotoxicity, specifically peripheral measures of oxidative stress and inflammation, to determine which best correlate with the occurrence and/or magnitude of neurobehavioral deficits. In addition, we will assess genetic polymorphisms in enzymes that metabolize OPs as biomarkers of susceptibility. To set up the models for these biomarker studies, we are conducting human field research in a cohort of Egyptian pesticide application workers. Exposure data from these human studies will be used to: (1) evaluate current urinary OP metabolites and ChE inhibition as biomarkers of OP neurotoxicity; and (2) develop a parallel animal model using the Long Evans rat, as depicted in Fig. 1, which will be used for initial comparisons of current *versus* novel biomarkers as predictors of OP-induced neurobehavioral deficits.

1.1.2. Selection of a human population

Egyptian pesticide application workers serve to define a specific, real-world workplace exposure pattern and associated adverse health effects. Key reasons for selecting this population as the basis or anchor for this project include: (1) this occupational cohort is exposed to a relatively limited number of pesticides in the workplace, primarily chlorpyrifos (CPF) and limited pyrethroids;

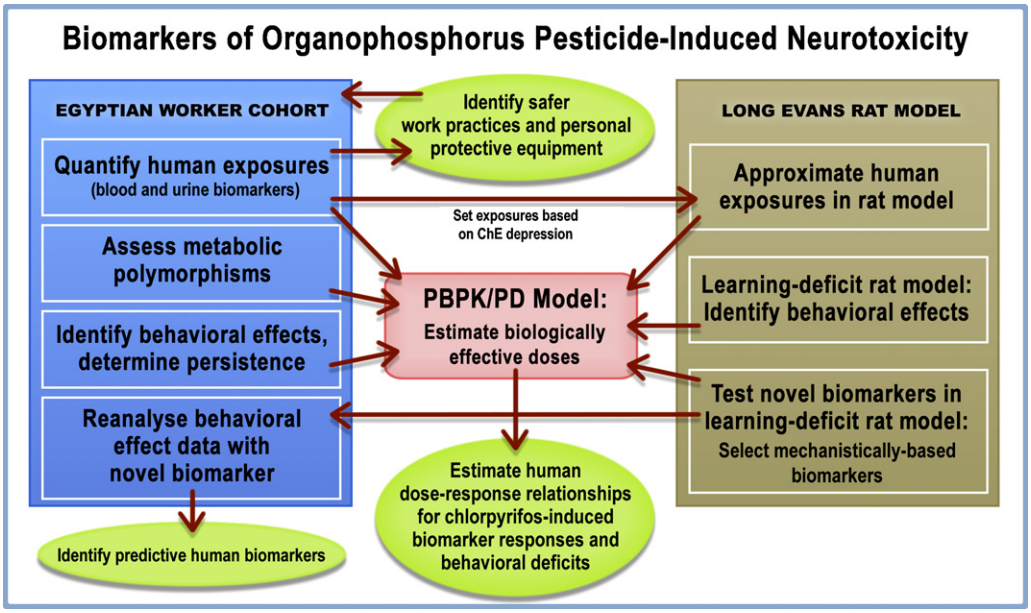

Fig. 1. Schematic depicting a research strategy for identifying predictive biomarkers of organophosphorus pesticide (OP)-induced neurotoxicity. Rectangles represent major study elements or sub-studies; arrows depict inputs or influences.

(2) this population exhibits a broad range of neurobehavioral deficits (Abdel Rasoul et al., 2008; Farahat et al., 2003); and (3) our initial studies confirmed these workers had a wide range of OP exposures that varied according to job category with the highest exposures observed in the job category with the most direct contact with OPs (Farahat et al., 2010, 2011).

1.1.3. Quantifying human exposures to establish comparable dose levels for animal research

To establish the human exposure pattern and identify external exposure and internal dose and dose modifiers of CPF in the Egyptian applicator cohort (see top 2 rectangles on the left in Fig. 1), we have collected blood, urine and saliva samples from pesticide application workers during the CPF application cycle. Urinary levels of the CPF-specific metabolite 3,5,6-trichloro-2-pyridinol (TCPy) are being quantified by negative-ion chemical ionization gas chromatography–mass spectrometry (Farahat et al., 2010, 2011); blood butyrylcholinesterase (BuChE) and acetylcholinesterase (AChE) activities are quantified using Test-Mate kits (EQM Research; Cincinnati, OH) based on the Ellman assay (Ellman et al., 1961; Farahat et al., 2011); and polymorphisms in CYP and PON1 enzymes that activate and detoxify CPF (Foxenberg et al., 2007, 2011) are analyzed by multiplex polymerase chain reaction (PCR) analyses of saliva. Daily urine TCPy levels from workers during periods of CPF application are being used to back-calculate individual CPF exposures utilizing a human physiologically based pharmacokinetic pharmacodynamic (PBPK/PD) model that includes CYP-specific kinetic parameters for CPF metabolism (Foxenberg et al., 2011). In addition to quantifying human exposures, the pharmacodynamics component of the model is being used to predict blood BuChE and AChE activities, which are validated at days when blood was collected from workers and assayed for this effect biomarker. Data from neurobehavioral assessments of pesticide application workers will also be incorporated into the human PBPK/PD model. Finally, CYP and PON-1 polymorphisms and corresponding kinetic parameters will provide a means to evaluate potential genetic modifiers of external exposure and internal dose and facilitate extrapolations to rat studies (central rectangle in Fig. 1).

The exposure data collected in the Egyptian worker cohort are being modeled in a Long Evans rat model to test the relative usefulness of currently used biomarkers of OP exposure and effect (urinary metabolite levels and blood cholinesterase activity) *versus* peripheral biomarkers of oxidative stress and inflammation as predictors of chlorpyrifos-induced neurotoxicity. Oxidative stress will be assessed by quantifying urinary levels of F-isoprostanes and inflammation, by serum levels of inflammatory cytokines and C-reactive protein. Several biological mechanisms have been proposed to explain the lack of association between blood ChE inhibition and OP neurotoxicity following occupational exposures. One is that genetic differences in the expression and/or activity of enzymes that metabolize OPs (Hofmann et al., 2010; Perez-Herrera et al., 2008; Povey et al., 2007) or proteins that scavenge OPs (Lockridge and Masson, 2000) differentially influence peripheral *versus* central outcomes. Another proposal is that ChE inhibition may not be mechanistically related to chronic OP neurotoxicity. While ChE inhibition is considered the primary mechanism responsible for the acute toxicity of OPs (Echbichon and Joy, 1995), experimental evidence suggests that mechanism(s) other than or in addition to ChE inhibition mediate chronic OP neurotoxicity (Bushnell and Moser, 2006; Jett and Lein, 2006; Pope et al., 2005; Pope, 1999). Of the various alternative molecular targets and mechanisms proposed to mediate OP-induced neurobehavioral deficits following repeated low level exposures (Casida and Quistad, 2005; Hernandez et al., 2004; Jett and Lein, 2006; Lockridge and Schopfer, 2010; Pancetti et al., 2007; Soltaninejad and Abdollahi, 2009), oxidative stress and inflammation are of interest because of: (1) the availability of experimentally validated quantitative peripheral biomarkers of oxidative stress and inflammation; (2) evidence that these are mechanistically linked to chronic OP neurotoxicity [reviewed in (Rohlman et al., 2011)]; and (3) these markers correlate well with neurobehavioral deficits observed consequent to neurodegenerative disease (Dziedzic, 2006; Kadiiska et al., 2005a, 2005b; Mrak and Griffin, 2005).

The similarities between human and rat OP neurotoxicity (Bushnell and Moser, 2006; Costa, 2006) justify using a rat model for these studies. To simulate occupational exposures in the Egyptian pesticide application workers, adult male rats were exposed to CPF daily for 4–21 days *via* subcutaneous injection. This route of exposure was selected because we documented that the predominant route of exposure in our occupational cohort is dermal (Fenske and Farahat, unpublished observations). TCPy was quantified in urine samples collected from rats daily, and cholinesterase activity was measured in blood samples collected every 2–3 days. These data have been used to optimize a PBPK/PD model for CPF in rats that is relevant to occupational exposures in humans (Ellison et al., 2011). Our data indicate that exposure of adult male Long Evans rats to CPF at 3 and 10 mg/kg/d s.c., simulate human CPF exposure patterns as confirmed by urinary TCPy levels and blood ChE depression comparable to that observed in the Egyptian pesticide application workers (see arrow between top two rectangles in Fig. 1). For example, blood AChE was inhibited 69 ± 15 and 38 ± 11 percent (mean $\pm$ SD) in rats following daily sc exposure to CPF at 3 and 10 mg/kg/d x 7 days (Ellison et al., 2011). Similar inhibition of blood AChE was observed in the more heavily exposed CPF applicators in the Egyptian cohort (Farahat et al., 2011).

1.1.4. Neurobehavioral studies in the human cohort and animal model

A human neurobehavioral cohort study, for which the data are now collected and are being analyzed, will test the hypotheses that: (1) CPF-induced behavioral deficits are dose-related; and (2) these deficits persist over time and reveal progressive changes over a second application season (third rectangle in left column of Fig. 1). Coincident with the human cohort study, the rat model is being tested using behavioral measures that parallel some of the functional domains shown to be affected in the Egyptian population under study (Farahat et al., 2003) to determine whether the exposure paradigm used in the rat model to simulate the human exposures produces parallel neurobehavioral deficits in rats (middle rectangle in right column of Fig. 1). Based on our preliminary observations (described below), we anticipate that this will be the case, but if similar behavioral deficits are not observed in the human cohort and rodent model, it would raise interesting questions regarding the influence of human genetic polymorphisms and/or co-exposure to pyrethroids on OP-induced neurobehavioral deficits.

Previous studies of Egyptian pesticide application workers have demonstrated impairments in tests such as Trail Making (that reflects complex visual scanning, response speed, and sustained attention) and Digit Symbol-Coding (that reflects visuomotor coordination, motor persistence, response speed and sustained attention) (Abdel Rasoul et al., 2008; Farahat et al., 2003; Joy et al., 2003). Based on these and other (reviewed in Rohlman et al., 2011) studies, we selected the Trail Making and Digit Symbol-Coding tests as well as computer-based measures of attention (Digit Span, Continuous Performance test); discrimination, learning and memory (Match-to-Sample, Reversal Learning); coordination and response speed (Simple Reaction Time, Finger Tapping). Brain imaging studies of humans have implicated frontal-parietal regions in Digit Symbol-Coding tasks (Usui et al., 2009), and there is evidence that frontal damage to humans and to rodents causes

similar impairments in working memory and response inhibition (Robbins, 2007). Although there is not a direct rodent analog of Digit Symbol-Coding tasks with humans, it is possible to examine behaviors that rely on similar neurobiological processes. We investigated Reversal Learning in rats as a behavioral task that parallels our human procedures in its dependence on frontal lobe function. In the rat model, we measured learning and performance from the onset of daily CPF injections, as well as learning and memory function at the end of several weeks of exposure to CPF. Rats were trained to discriminate between a stimulus that signals food and a stimulus that signals no food. Daily CPF administration (0, 3, or 10 mg/kg) began after the discrimination had been acquired. For half of the rats, the discrimination was continued during CPF exposure; for the other half, the discrimination was reversed. This procedure allowed us to assess the effects of chronic exposure to CPF on sustained asymptotic performance, which could be altered by toxicant-induced changes in motivation, attention, or motor behavior, while also examining the effects on acquisition of new reversal learning. Demonstrating a specific deficit in reversal learning indicates that CPF is impairing cognitive processes involved with learning and memory and not affecting performance *via* sensory, motor and motivational effects.

At the end of this task, after 20 days of CPF dosing, rats received a single-trial learning experience (Pavlovian fear conditioning) to evaluate the effects of the cumulative dose of CPF on new memory formation. Single-trial contextual fear conditioning is a widely used assay for hippocampal function. Many experiments have revealed that the same pharmacological and genetic manipulations that cause deficits in contextual fear conditioning also cause deficits in long-term potentiation, a form of synaptic plasticity that is thought to underlie hippocampus-dependent memory (Abel et al., 1997). A demonstration of a deficit in this type of learning would be consistent with biochemical studies that have demonstrated effects of CPF within the hippocampus (Chakraborti et al., 1993). The results of these studies indicate these patterns of CPF exposure result in cognitive deficits in our rat model (Lattal and Lein, unpublished observations).

1.1.5. Identifying novel biomarkers of chlorpyrifos in the rat model

This rat model is being used to test the relative predictability of peripheral biomarkers of inflammation (serum levels of cytokines and C-reactive protein) and/or oxidative stress (urinary levels of F-isoprostanes and biochemical indicators of oxidative stress in the blood) *versus* blood ChE activity and urinary TCPy to predict neurotoxicity in rats (bottom rectangle in right column of Fig. 1). The most predictive biomarker(s) of neurobehavioral deficits identified in the rat studies will then be tested in humans using banked urine or blood samples collected from the same Egyptian cohort at the time of the neurobehavioral studies to determine whether that biomarker, indeed, is a better predictor than urinary metabolites or blood ChE activity (bottom rectangle and oval in left column of Fig. 1). The ovals at the bottom of Fig. 1 reflect the major outputs of the project, which are a better understanding of the factors that influence the expression of biomarkers (central oval) and better prediction of risk associated with repeated occupational exposures to OPs.

1.1.6. Interventions in pesticide application teams

We are also leveraging this research to promote workplace change (top oval in Fig. 1). Egyptian scientists and agricultural engineers have been trained to measure chlorpyrifos exposures and work practices in order to evaluate the effectiveness of personal protective equipment (PPE) and changes in work practices. An in-depth industrial hygiene sampling study was conducted to determine the relative contributions of airborne and dermal exposure to chlorpyrifos during applications, testing the hypothesis that dermal exposures are the primary route of chlorpyrifos exposure. Focus groups have been held with the Egyptian Agricultural Ministry and pesticide application workers to describe the evidence of very high exposures identified in the initial samples (Farahat et al., 2010), compare exposures with those in other countries, and demonstrate the need to minimize pesticide exposures. These data are then used by their staff to evaluate and implement PPE and work practices that reduce exposures. An intervention study has been conducted to evaluate the effectiveness of PPE and work practice changes to reduce dermal contact and thus exposure to pesticides in Egyptian cotton fields during pesticide applications.

2. Longitudinal study

The feasibility of our research strategy and approach is predicated on having a well-characterized human cohort with well-defined exposures and neurobehavioral assessments. Therefore, we conducted a longitudinal study of pesticide workers from Menoufia Governorate. Menoufia is one of the 29 Governorates in Egypt, located in the Nile River delta north of Cairo. Menoufia has 270 field stations in 9 agricultural Districts. Each station is responsible for crop management, including sales of chemicals to farmers, safety training, and pesticide applications (Egyptian Ministry of Agriculture, 2008). Field stations are located close to the agricultural areas and serve as a central meeting place for workers and supervisors, as well as for storage of pesticides and application equipment. Each field station has a team of employees in three job categories: (1) Applicators who apply pesticides from backpack sprayers; (2) Technicians who walk with and direct the path and spray pattern of the applicators; and (3) Engineers who may walk in the fields but more often oversee and direct the application process from the edge of the field, although some are involved in mixing and loading.

Cotton is a national resource in Egypt. The national government oversees the country's production of cotton, which is grown and harvested by independent farmers (Mostafa, 2008). Once farmers sign a contract to plant cotton in their fields, pesticide applications on those fields are under the control of the national Ministry of Agriculture through their District Offices in the individual Governorates. Recently and stretching back for some years, there is a 2-week application period of the OP chlorpyrifos beginning in July, followed by a 2-week period of pyrethroid application, and another 2-week period of chlorpyrifos application, primarily to control the cotton boll weevil. The national Ministry of Agriculture purchases all pesticide application equipment and pesticides and the backpack sprayer equipment is calibrated at the Governorate District Office before distribution to the Governorate field stations.

Herein, we report the methods and results of observational studies of work practices in the field, and of questionnaires administered to document demographics and work history of the occupational cohort.

3. Materials and methods

3.1. Participant enrollment

We obtained approval from the Egyptian Ministry of Agriculture and the Ministry District offices in Menoufia Governorate (equivalent to a county in the US) to contact individual Field Stations where pesticide application teams worked. For logistical purposes we recruited workers from one of the 9 districts in Menoufia Governorate that included workers across the three job categories that comprise a pesticide application team: engineer, technician and applicator. Field stations from the Quesna district were selected based on a recommendation from the Ministry of Agriculture that they were close to the Ministry district offices and

Menoufia University labs and they had a sufficient number of cotton fields to meet the recruiting needs of the study. From these field stations, all prospective participants were invited to participate in the study. Preliminary screening, using a brief questionnaire, was conducted to determine participant eligibility. Inclusion criteria included employment by the Ministry of Agriculture as an engineer, technician, or applicator at the time of recruitment and between 15 and 60 years of age (60 is the Ministry's mandatory retirement age). All participants were males born in Egypt. In June of 2009, we recruited 31 applicators, 59 technicians, and 56 engineers for a total of 146 participants for the study. The engineers and technicians work year-round for the Ministry of Agriculture and are salaried, while the applicators are seasonal workers often hired repeatedly year after year in late June through mid-August for the cotton pesticide application period and paid a daily wage. Twenty-four Ministry of Agriculture workers declined to participate, citing reluctance to donate blood. The response rate was thus 82% for these Ministry of Agriculture workers. All participants provided informed consent and all study activities were approved by the Oregon Health & Science University and Menoufia University Institutional Review Boards.

3.2. Observational data collection

Research team members met with the management at the Ministry of Agriculture in Menoufia Governorate and with Field Station managers to learn about field work practices during pesticide application. To verify the accuracy of the manager statements, observational data were collected on engineers, technicians and applicators over the course of a summer application period; recordings were made of personal protective equipment use, type of clothing, contact with pesticides when mixing and loading pesticides, time in the field during spraying operations, and spraying away or across body when applying pesticides from knapsack sprayers.

3.3. Baseline questionnaires

Structured self-administered questionnaires were used to query demographics, symptoms associated with OP exposure during the past 3 months (drawn from reports in articles on organophosphorus pesticides reviewed by Rohlman et al., 2011), medical history, smoking habits, work history, work practices and personal protective equipment use that may potentially confound the relationship between chlorpyrifos exposure and neurobehavioral deficits. All participants provided written informed consent at the time of enrollment. The baseline questionnaires were administered to participants in June 2009 just prior to initiating pesticide applications during the cotton growing season and prior to baseline neurobehavioral testing.

4. Results

4.1. Work setting and characteristics of Egyptian pesticide workers

In 2007, there were 1068 technicians and engineers assigned to apply pesticides in Menoufia's 270 field stations. The standard procedure is to assign four applicators to each field station, so an estimated 1080 applicators work in the cotton fields during a typical summer in this region. Technicians and engineers have minimal exposure to pesticides during the rest of the year, as their primary tasks are to review lessons learned in the previous season and plan and maintain equipment for pesticide applications in the next season (Hesham et al., 2006). The applicators are seasonal workers, but their off-season activities are varied. There is anecdotal evidence of episodic but not systematic use of pesticides among workers in all three job categories outside their ministry jobs. This was documented by questionnaires given to the workers in 2008 when applications outside work hours were at a peak due to a heavy insect infestation in corn as well as cotton crops. At that time, all applicators ($n = 14$) reported they were involved in private pesticide application outside their Ministry of Agriculture jobs, compared to about 15% of the technicians (2 out of 13) and none of the engineers ($n = 13$). A similar heavy infestation did not occur in 2009, the year the questionnaires reported here were administered.

4.2. Recruitment and demographics

Selected characteristics of the study participants are presented in Table 1. Analysis of variance (ANOVA) was used to compare means between job categories for continuous variables and the Chi-square test for categorical variables. All tests were 2-tailed and an α of 0.05 was used to determine statistical significance. The mean ages for applicators, technicians and engineers were 41.1, 48.3 and 54.0 years, respectively. Mean years of education were higher among engineers (14.5 years) than applicators and technicians (11.7 and 12.3 years, respectively). The differences in age and education observed between the applicators, technicians and engineers were expected, given the nature, degree of responsibility and tasks required for each job. There is also a noteworthy difference in the proportion who report having worked on other farms between the applicators (87.1%) and either the technicians (47.5%) or engineers (50.0%). Again, this difference was anticipated because the applicators are seasonal workers and hold employment elsewhere during other times of the year. This is significant in that farm work may contribute to additional exposure to chlorpyrifos or other pesticides. Applicators may also be employed in different industries outside their Ministry of Agriculture jobs and thus be exposed to other chemicals that could have neurobehavioral effects. To address this issue, we used a questionnaire to ascertain work-related exposure to chemicals,

Table 1
Selected characteristics for Egyptian pesticide application workers, summer 2009.

Characteristic	Applicators ($n = 31$)	Technicians ($n = 59$)	Engineers ($n = 56$)	P-value*
	Mean (SD)			
Age (years)	41.1 (12.0)	48.3 (4.7)	54.0 (4.8)	<0.001
Education (years)	11.7 (2.2)	12.3 (2.0)	14.5 (3.0)	<0.001
Cigarette smoking (pack-years)	10.6 (13.7)	6.8 (13.7)	10.2 (12.9)	0.39
Body Mass Index	26.5 (3.6)	27.0 (3.4)	26.5 (3.5)	0.69
# of years applied pesticides at home past	13.5 (12.1)	9.1 (6.3)	12.0 (10.2)	0.44
	% responding			
Work on farm (yes)	87.1	47.5	50.0	0.004
Reside on farm (yes)	54.8	44.1	57.1	0.62
Reside $\leq$ 25 m from agricultural field	48.4	54.2	73.2	0.11
Mixed or applied pesticides at home in the past 5 years (yes)	67.7	62.7	80.4	0.18
Mixed or applied pesticides outside home (yes)	77.4	49.2	60.7	0.12

* P-values for continuous and categorical variables as determined using ANOVA and Chi-square tests, respectively.

Table 2
Self-reported OP-related symptoms for Egyptian pesticide application workers at baseline summer 2009 and controls summer 2010.

Symptom	Applicators ($n=31$)	Technicians ($n=59$)	Engineers ($n=56$)	Controls ($n=150$)
	% responding			
1. Dizziness (yes)	38.7	40.7	44.6	48.0
2. Felling tense (yes)	38.7	42.4	50.0	52.0
3. Nausea (yes)	25.8	18.6	25.0	22.7
4. Tired (yes)	51.6	42.4	58.9	53.2
5. Sweating (yes)	54.8	42.4	55.4	43.4
6. Difficulty seeing at night (yes)	38.7	27.1	32.1	40.5
7. Forgetful (yes)	45.2	40.7	51.8	48.4
8. Headache (yes)	61.3	54.2	58.9	57.1
9. Loss of appetite (yes)	61.3	47.5	41.1	40.9
10. Fast heart rate (yes)	25.8	28.8	50.0	36.4
11. Difficulty with balance (yes)	22.6	28.8	35.7	26.7
12. Blurred or double vision (yes)	41.9	44.1	48.2	47.2
13. Difficulty concentrating (yes)	48.4	30.5	42.9	40.8
14. Numbness in hands or feet	35.5	30.5	39.3	43.0
15. Loss of consciousness (yes)	19.4	22.0	17.9	23.9
16. Feeling irritable (yes)	41.9	30.5	48.2	41.5
17. Trembling of hands (yes)	12.9	27.1	37.5	31.4
18. Difficulty sleeping (yes)	35.5	42.4	42.9	30.0
19. Difficulty speaking (yes)	9.7	8.5	10.7	14.2
20. Weakness in arms or legs (yes)	19.4	18.6	33.9	21.8
21. Changes in sense of smell or taste (yes)	16.1	13.6	17.9	19.7
22. Feeling depressed	22.6	20.3	23.2	26.2
23. Involuntary movements of arms or legs (yes)	9.7	15.3	21.4	20.2
24. Excessive salivation (yes)	25.8	22.0	28.6	24.2
25. Ringing in your ears (yes)	32.3	37.3	33.9	34.2

such as solvents, to ensure that we could statistically adjust for these potential confounders. The proportion reporting in-home use of pesticides was higher among the engineers (80.4%) compared with either applicators (67.7%) or technicians (62.7%), although the difference did not achieve statistical significance. Based on numerous anecdotal conversations, the home pesticide use did not involve mixing or loading of pesticides for agricultural use, but rather was mainly application of pyrethroid sprays for control of flies and mosquitoes in the home.

4.3. Symptoms and self-reported medical history

Of 25 self-reported symptoms that may be related to OP intoxication (never had symptom in the past 3 months *versus* experienced the symptom one or more times in the past 3 months), the proportion of respondents reporting these symptoms in 2009 is listed in Table 2 by job title. In 2010, the year following the collection of these symptom data, we recruited 150 Ministry of Agriculture workers (mean age was 49.7 years) who were also employed as engineers or technicians but did not apply pesticides for the Ministry and administered the same questionnaire; these individuals are designated as "controls" in the right column of Table 2. In both exposed and control subjects, 52% of the symptoms were reported as occurring in 35% or more of the participants, suggesting that the symptom rates in exposed and control groups are relatively comparable.

As indicated in Table 3, self-reported medical history varied between the three job categories, with a larger percentage of engineers reporting having 7 of the 11 medical conditions queried compared to applicators or technicians. The overall differences between the groups were statistically significant, based on the Chi-square test, for 6 of the conditions (hypertension, diabetes mellitus, heart disease, renal disease, liver disease, epilepsy). While the questions were stated as 'Has a medical doctor ever told you that you have ...', Egyptian physicians report that these results do not necessarily represent physician-diagnosed diseases but may reflect conclusions of the respondents based on blood or urine readings outside the normal range. This is especially prominent in rural areas of Egypt such as where this study was conducted. Alternatively, age alone may explain the higher frequencies of disease in the older engineers. However, one condition that did not differ among the groups was acute pesticide poisoning, which was reported by 16.1% of applicators, 11.9% of technicians, and 10.7% of engineers.

4.4. Work history

Applicators, technicians and engineers form the teams that apply pesticides in the Egyptian cotton fields under the direction of

Table 3
Self-reported medical history for Egyptian pesticide application workers at baseline, summer 2009.

Medical condition	Applicators ($n=31$)	Technicians ($n=59$)	Engineers ($n=56$)	P-value*
	% responding			
Hypertension	9.7	8.5	23.2	0.01
Diabetes mellitus	3.2	3.4	14.3	0.02
Heart disease	0	0	7.1	0.02
Bronchial Asthma	9.7	13.6	12.5	0.58
Skin allergy	19.4	11.9	16.1	0.68
Renal disease	3.2	1.7	16.1	0.002
Liver disease	0	5.1	14.3	0.01
Arthritis	16.1	20.3	28.6	0.14
Epilepsy	0	0	5.4	0.04
Acute pesticide poisoning	16.1	11.9	10.7	0.73
Head injury	22.6	10.2	19.6	0.33

* Chi-square test.

Table 4
Egyptian pesticide application worker self-reported personal hygiene practices baseline, summer 2009.

Behavior	Applicators ($n = 31$)	Technicians ($n = 59$)	Engineers ($n = 56$)	P-value*
	% responding			
When do you usually wash hands:				
Right after applying pesticides	87.1	69.5	80.4	0.57
Before going to bathroom (yes)	12.9	15.3	10.7	0.65
Do not usually wash hands at Work (yes)	6.5	3.4	3.6	0.84
At home after work (yes)	12.9	10.2	7.1	0.73
When do you usually shower/bathe:				
Right after work (yes)	87.1	72.9	69.6	0.22
Before going to sleep (yes)	9.7	17.0	23.2	0.24
The next morning (yes)	3.2	1.7	5.4	0.56
Have you ever mixed pesticides with the following:				
Stick or corn stalk (yes)	54.8	47.5	44.6	0.99
Hand (yes)	16.1	18.6	8.9	0.32
Do not mix pesticides (yes)	25.8	17.0	19.3	0.78
Other (yes)	25.8	17.0	21.4	0.74

* Chi-square test.

Egypt's Agricultural Ministry. The applicators are seasonal workers although they may work for several years in this 'summer' job, and in some cases technicians may be pressed into serving as an applicator. Mean years working in the Ministry of Agriculture were 17.8 (SD = 8.1) for applicators, 20.8 (SD = 7.2) for technicians and 30.0 (SD = 6.2) for engineers.

4.5. Work practices

Teams of 4 applicators led by 3–4 technicians enter the fields in a staggered line so that applicators do not spray each other directly. Applicators often spray in front and then walk past the plants they have just sprayed so that their skin and clothing is wet when they exit the field (Farahat et al., 2010). Ministry technicians and engineers train applicators to use a set pace while walking through the cotton fields during pesticide application to provide a constant application rate to all plants, although technicians may direct applicators to move more slowly in areas of heavy infestation to increase the amount of pesticide applied. Thus, all pesticides, application equipment and procedures used for cotton production are standardized throughout Egypt.

Dermal contact is recognized as the primary route of exposure for OPs in occupational settings (Durham et al., 1972; Hines and Deddens, 2001). Consequently, we queried workers about hand washing and bathing habits as they relate to washing off pesticides and about commonly used methods to mix chlorpyrifos in the field (Table 4). The majority of applicators (87.1%), technicians (69.5%) and engineers (80.4%) reported washing their hands immediately after applying pesticides. The majority of workers reported that they usually shower or bathe after work. None of these hygiene practices were statistically different between the applicators, technicians, or engineers.

There is ample opportunity for a high exposure event in these workplaces during the mixing of pesticide concentrate with water and loading of the solution into backpack sprayers. The concentrated formulation is handled by workers using little or no chemical protective clothing and the loading system is open with the potential for spills and splashes (Farahat et al., 2010). The majority of workers in our study reported that they usually use a stick or a corn stalk to mix pesticides in the field. A few reported using their hands, a work practice that substantially increases their exposure.

We also queried Ministry of Agriculture workers on their usual use of personal protective equipment (PPE) because of its importance in reducing dermal exposure. The most commonly reported PPE used by applicators (39%), technicians (54%), and engineers (54%) was wearing a hat (Table 5). Gloves, goggles, masks and respirators (paper masks in our observations) were used by a minority of workers. Overall, the use of PPE was limited and may explain, in part, the comparatively high exposure previously documented in this population (Farahat et al., 2010, 2011).

5. Discussion

5.1. Workplace setting

The Egyptian pesticide workers provide a population with a relatively consistent exposure history stretching back for many years. The OP chlorpyrifos has been used as the primary application to control the boll weevil in the summer growing season, with application of pyrethroids between chlorpyrifos applications. Moreover there is a constancy of pesticide application procedures across the field sites in this project. The relatively low variability in exposure history and exposure patterns across work teams in Egypt's cotton growing areas supports the assessment of biomarkers in this test population.

5.2. Work practices of Egyptian pesticide application workers

Between a third and half the workers report that they wash and bathe shortly after applying pesticides, but they also report little

Table 5
Egyptian pesticide application worker self-reported personal protective equipment (PPE) use baseline, summer 2009.

PPE	Applicators ($n = 31$)	Technicians ($n = 59$)	Engineers ($n = 56$)	P-value*
	% responding			
Wear hat (always or often)	38.7	54.2	53.6	0.31
Gloves (always or often)	6.5	15.3	28.6	0.02
Goggles (always or often)	16.1	25.4	37.5	0.08
Glasses (always or often)	12.9	18.6	33.9	0.01
Mask over mouth (always or often)	19.4	13.6	19.6	0.43
Mask over mouth and nose (always or often)	29.0	32.2	42.9	0.30

* Chi-square test.

use of PPE that would protect them from OP exposure. The lack of PPE use is consistent with reports from other countries in the region (Issa et al., 2010; Yassin et al., 2002). Safe work practices are not routinely practiced as the workers regularly spray the plants in front of them and then walk through the sprayed plants thereby getting pesticide solution on their skin and clothing (Farahat et al., 2010). That this likely produces high dermal exposures is supported by previous research in this population (Farahat et al., 2010, 2011).

5.3. Project strategy

We have identified three critical questions that need to be answered to predict human risk of OP exposures: (1) What is the relationship between OP dose and neurobehavioral deficits? (2) Are there biomarkers that reliably predict OP-induced neurobehavioral deficits following occupational exposures? (3) What is the potential for genetic variation to modify exposure sensitivity? To answer these questions, we propose a research strategy that employs parallel human and animal studies. The human studies are anchored by Egyptian pesticide workers previously reported to exhibit a broad range of neurobehavioral deficits (Abdel Rasoul et al., 2008; Farahat et al., 2003) and whom we have subsequently confirmed have among the highest reported occupational exposures to an OP (Farahat et al., 2010, 2011). In our animal research, we are using an exposure paradigm that is similar in exposure duration to the 2-week exposure period observed in Egypt's cotton growing areas and employs doses that reduce blood ChE activity to levels similar to those observed in exposed Egyptian pesticide application workers. The animal research is testing both traditional (blood ChE, urinary metabolites) and novel biomarkers to identify optimal biomarkers for predicting OP-induced neurotoxicity. The behavioral tests used in studies of both humans and animals have common threads in testing cognitive and psychomotor performance. One caveat, however, is that this model focuses on chlorpyrifos, despite the documented use of pyrethroids. Thus, discrepancies between the human cohort and the rodent model may indicate a potentially important influence of pyrethroid exposure on chlorpyrifos neurotoxicity, a possibility that can be explored experimentally by adapting the rodent model to more fully capture the human exposure scenario.

6. Conclusions

The project described herein describes a strategy of using parallel studies in humans and animals. A real-world occupational exposure scenario serves as the basis or anchor point for testing traditional and novel biomarkers in a rat model for subsequent evaluation of the most predictive biomarkers in a human population, and for translational studies to identify prevention strategies to reduce human worker exposures. Pesticide applications are controlled by the Ministry of Agriculture and thus have been relatively the same across Egypt for years, providing a more stable exposure history than is seen in most other settings and thus an optimized setting for testing candidate biomarkers. The findings could provide critical data needed to develop predictive biomarkers of chlorpyrifos exposure, biological response and genetic susceptibility. The availability of such biomarkers will facilitate the identification of at-risk individuals as well as the testing of intervention and treatment strategies for chlorpyrifos and potentially other OPs.

Conflict of interest statement

The authors have no conflict of interest to declare.

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References

Abdel Rasoul GM, Abou Salem ME, Mechael AA, Hendy OM, Rohlman DS, Ismail AA. Effects of occupational pesticide exposure on children applying pesticides. Neurotoxicology 2008;29:833–8.
Abel T, Nguyen PV, Barad M, Deuel TA, Kandel ER, Bourtchouladze R. Genetic demonstration of a role for PKA in the late phase of LTP and in hippocampus-based long-term memory. Cell 1997;88:615–26.
Arcury TA, Grzywacz JG, Talton JW, Chen H, Vallejos QM, Galvan L, et al. Repeated pesticide exposure among North Carolina migrant and seasonal farmworkers. Am J Ind Med 2010;53:802–13.
Barr DB, Allen R, Olsson AO, Bravo R, Caltabiano LM, Montesano A, et al. Concentrations of selective metabolites of organophosphorus pesticides in the United States population. Environ Res 2005;99:314–26.
Bushnell PJ, Moser VC. Behavioral toxicity of cholinesterase inhibitors. In: Gupta RC, editor. Toxicology of organophosphate and carbamate compounds. San Diego, CA: Elsevier; 2006, pp. 347–60.
Casida JE, Quistad GB. Serine hydrolase targets of organophosphorus toxicants. Chemico-Biol Interact 2005;157–158:277–83.
Chakraborti TK, Farrar JD, Pope CN. Comparative neurochemical and neurobehavioral effects of repeated chlorpyrifos exposures in young and adult rats. Pharmacol Biochem Behav 1993;46:219–24.
Colosio C, Tiramani M, Brambilla G, Colombi A, Moretto A. Neurobehavioural effects of pesticides with special focus on organophosphorus compounds: which is the real size of the problem. Neurotoxicology 2009;30:1155–61.
Costa LG. Current issues in organophosphate toxicology. Clin Chim Acta 2006;366:1–13.
Durham WF, Wolfe HR, Elliott JW. Absorption and excretion of parathion by spraymen. Arch Environ Health 1972;24:381–7.
Dziedzic T. Systemic inflammatory markers and risk of dementia. Am J Alzheimers Dis Other Demen 2006;21:258–62.
Echbichon DJ, Joy RM. Pesticides and neurological diseases. Boston/London: CRG Press; 1995.
Egyptian Ministry of Agriculture. Website of the Egyptian Ministry of Agriculture. 2008.
Ellison CA, Smith JN, Lein PJ, Olson JR. Pharmacokinetics and pharmacodynamics of chlorpyrifos in adult male Long-Evans rats following repeated subcutaneous exposure to chlorpyrifos. Toxicology 2011;287:137–44.
Ellman GL, Courtney KD, Andres V, Featherstone RM. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem Pharmacol 1961;7:88–95.
Farahat FM, Ellison CA, Bonner MR, McGarrigle BP, Crane AL, Fenske RA, et al. Biomarkers of chlorpyrifos exposure and effect in Egyptian cotton field workers. Environ Health Perspect 2011;119:801–6.
Farahat FM, Fenske RA, Olson JR, Galvin K, Bonner MR, Rohlman DS, et al. Chlorpyrifos exposures in Egyptian cotton field workers. Neurotoxicology 2010;31:297–304.
Farahat TM, Abdelrasoul GM, Amr MM, Shebl MM, Farahat FM, Anger WK. Neurobehavioural effects among workers occupationally exposed to organophosphorous pesticides. Occup Environ Med 2003;60:279–86.
Foxenberg RJ, Ellison CA, Knaak JB, Ma C, Olson JR. Cytochrome P450-specific human PBPK/PD models for the organophosphorus pesticides: chlorpyrifos and parathion. Toxicology 2011;285:57–66.
Foxenberg RJ, McGarrigle BP, Knaak JB, Kostyniak PJ, Olson JR. Human hepatic cytochrome p450-specific metabolism of parathion and chlorpyrifos. Drug Metab Dispos Biol Fate Chem 2007;35:189–93.
Hernandez A, Gomez MA, Pena G, Gil F, Rodrigo L, Villanueva E, et al. Effect of long-term exposure to pesticides on plasma esterases from plastic greenhouse workers. J Toxicol Environ Health A 2004;67:1095–108.
Hesham S, Mazen M, El-Damaty M. Quality of training service for agricultural workers in some Egyptian governorates. Egypt J Agric Res 2006;84:1646–50.
Hines CJ, Deddens JA. Determinants of chlorpyrifos exposures and urinary 3,5,6-trichloro-2-pyridinol levels among termiticide applicators. Ann Occup Hyg 2001;45:309–21.
Hofmann JN, Keifer MC, Checkoway H, De Roos AJ, Farin FM, Fenske RA, et al. Biomarkers of sensitivity and exposure in Washington state pesticide handlers. Adv Exp Med Biol 2010;660:19–27.
Issa Y, Sham'a FA, Nijem K, Bjertness E, Kristensen P. Pesticide use and opportunities of exposure among farmers and their families: cross-sectional studies 1998–2006 from Hebron governorate, occupied Palestinian territory. Environ Health 2010;9:63.
Jett DA, Lein PJ. Non-cholinesterase mechanisms of central and peripheral neurotoxicity: muscarinic receptors and other targets. In: Gupta RC, editor. Toxicology of Organophosphate and Carbamate Compounds. San Diego, CA: Elsevier; 2006, pp. 233–46.

Joy S, Fein D, Kaplan E. Decoding digit symbol: speed, memory, and visual scanning. Assessment 2003;10:56–65.
Kadiiska MB, Gladen BC, Baird DD, Germolec D, Graham LB, Parker CE, et al. Biomarkers of oxidative stress study II: are oxidation products of lipids, proteins, and DNA markers of CCl4 poisoning. Free Radic Biol Med 2005a;38:698–710.
Kadiiska MB, Gladen BC, Baird DD, Graham LB, Parker CE, Ames BN, et al. Biomarkers of oxidative stress study III. Effects of the nonsteroidal anti-inflammatory agents indomethacin and meclofenamic acid on measurements of oxidative products of lipids in CCl4 poisoning. Free Radic Biol Med 2005b;38:711–8.
Lockridge O, Masson P. Pesticides and susceptible populations: people with butyrylcholinesterase genetic variants may be at risk. Neurotoxicology 2000;21:113–26.
Lockridge O, Schopfer LM. Review of tyrosine and lysine as new motifs for organophosphate binding to proteins that have no active site serine. Chemico-Biol Interact 2010;187:344–8.
Mostafa S. Head, Menoufia pest control administration. Shebin El-Kom, Egypt: Menoufia Governorate; 2008.
Mrak RE, Griffin WS. Potential inflammatory biomarkers in Alzheimer's disease. J Alzheimers Dis 2005;8:369–75.
Pancetti F, Olmos C, Dagnino-Subiabre A, Rozas C, Morales B. Noncholinesterase effects induced by organophosphate pesticides and their relationship to cognitive processes: implication for the action of acylpeptide hydrolase. J Toxicol Environ Health B Crit Rev 2007;10:623–30.
Perez-Herrera N, Polanco-Minaya H, Salazar-Arredondo E, Solis-Heredia MJ, Hernandez-Ochoa I, Rojas-Garcia E, et al. PON1Q192R genetic polymorphism modifies organophosphorous pesticide effects on semen quality and DNA integrity in agricultural workers from southern Mexico. Toxicol Appl Pharmacol 2008; 230:261–8.
Pope C, Karanth S, Liu J. Pharmacology and toxicology of cholinesterase inhibitors: uses and misuses of a common mechanism of action. Environ Toxicol Pharmacol 2005;12:433–46.
Pope CN. Organophosphorus pesticides: do they all have the same mechanism of toxicity. J Toxicol Environ Health B Crit Rev 1999;2:161–81.
Povey AC, Jury F, Dippnall WM, Smith AE, Thomson S, Mackness B, et al. GST CYP and PON1 polymorphisms in farmers attributing ill health to organophosphate-containing sheep dip. Biomarkers 2007;12:188–202.
Robbins TW. Shifting and stopping: fronto-striatal substrates, neurochemical modulation and clinical implications. Philos Trans R Soc Lond B Biol Sci 2007;362:917–32.
Rohlman DS, Anger WK, Lein PJ. Correlating neurobehavioral performance with biomarkers of organophosphorous pesticide exposure. Neurotoxicology 2011; 32:268–76.
Soltaninejad K, Abdollahi M. Current opinion on the science of organophosphate pesticides and toxic stress: a systematic review. Med Sci Monit 2009;15:RA75–90.
Usui N, Haji T, Maruyama M, Katsuyama N, Uchida S, Hozawa A, et al. Cortical areas related to performance of WAIS Digit Symbol Test: a functional imaging study. Neurosci Lett 2009;463:1–5.
Yassin MM, Abu Mourad TA, Safi JM. Knowledge, attitude, practice, and toxicity symptoms associated with pesticide use among farm workers in the Gaza Strip. Occup Environ Med 2002;59:387–93.
Zaim M, Jambulingam P. In: Diseases DoCoNT, editor. Global insecticide use for vector-borne disease control 3rd ed. Geneva: World Health Organization; 2007.