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Human Exposure to Wastewater-Derived Pharmaceuticals in Fresh Produce: A Randomized Controlled Trial Focusing on Carbamazepine

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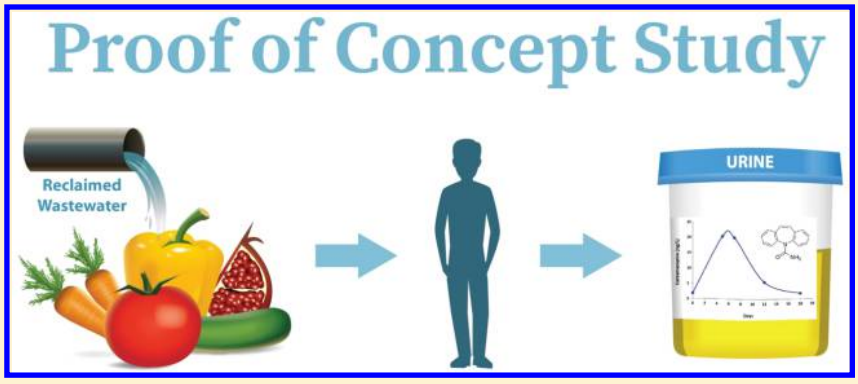
Supporting Information



ABSTRACT: Fresh water scarcity has led to increased use of reclaimed wastewater as an alternative and reliable source for crop irrigation. Beyond microbiological safety, concerns have been raised regarding contamination of reclaimed wastewater by xenobiotics including pharmaceuticals. This study focuses on carbamazepine, an anticonvulsant drug which is ubiquitously detected in reclaimed wastewater, highly persistent in soil, and taken up by crops. In a randomized controlled trial we demonstrate that healthy individuals consuming reclaimed wastewater-irrigated produce excreted carbamazepine and its metabolites in their urine, while subjects consuming fresh water-irrigated produce excreted undetectable or significantly lower levels of carbamazepine. We also report that the carbamazepine metabolite pattern at this low exposure level differed from that observed at therapeutic doses. This "proof of concept" study demonstrates that human exposure to xenobiotics occurs through ingestion of reclaimed wastewater-irrigated produce, providing real world data which could guide risk assessments and policy designed to ensure the safe use of wastewater for crop irrigation.

INTRODUCTION

As a result of population pressure, urbanization, climate change, and rising demand for food, fresh water for agricultural purposes has become an increasingly scarce resource. Thus, arid and semiarid regions across the globe have become progressively dependent on reclaimed wastewater for crop irrigation.[1−3] For example, reclaimed wastewater makes up 50% of the total irrigation-water used in Israel, 17% in Spain and only 6% in California. Recently, California has announced aims to increase reclaimed wastewater use for crop irrigation due to a prolonged drought.[4] Beyond water conservation, irrigation with reclaimed wastewater provides organic matter and nutrients to the agro-environment. However, safety concerns regarding the use of reclaimed wastewater for crop irrigation have also been raised.[1,3] Historically the focus of safety guidelines has been on transmission of wastewater-derived pathogens. More recently the contamination of ecosystems and arable land with organic pollutants such as endocrine-disrupting compounds, active pharmaceutical compounds and other synthetic compounds, and their eventual penetration to the food chain, have become emerging environmental and human health concerns.[5−7]

Carbamazepine (5*H*-dibenzo[*b*,*f*]azepine-5-carboxamide) is a tricyclic orally administered anticonvulsant, approved for treatment of partial and generalized types of epilepsy and trigeminal neuralgia.[8] In vivo, carbamazepine is almost completely metabolized, mainly to 10,11-epoxy-carbamazepine (EP-CBZ), an active metabolite, which is in turn metabolized

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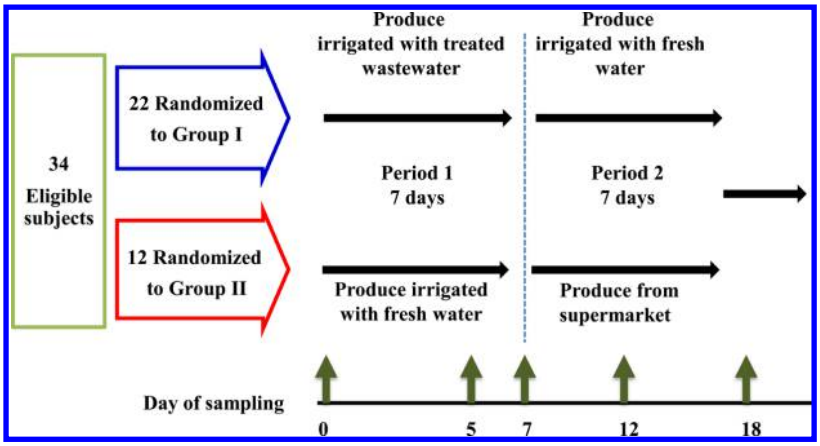

Figure 1. Study schema: following randomization (Day 0) Group I received reclaimed wastewater-irrigated produce for 7 days, followed by fresh water-irrigated produce. Group II received fresh water-irrigated produce for 7 days followed by produce bought from the supermarket. Arrows signify days of urine sampling and questionnaire collection.

by epoxide hydrolase to 10,11-dihydrocarbamazepine (DiOH–CBZ), the major (32%) metabolite detected in urine.[9] Conjugated carbamazepine (carbamazepine-*N*-glucuronide) is also commonly (11%) detected in urine.[9] Less than 3% of the parent compound is excreted in urine,[10] yet urine levels appear to accurately reflect free plasma carbamazepine concentrations.[11] Carbamazepine is detected in reclaimed wastewater in many countries and bodies of water receiving effluents.[9,12−14] Sources of carbamazepine in reclaimed wastewater include excretion by patients, discarded medications or pharmaceutical industry effluents. Carbamazepine is highly persistent in soils[15,16] and more importantly has been shown to be taken up and accumulate in a variety of crops.[6,17−20]

To date, in vivo exposure of humans to pharmaceuticals via consumption of reclaimed wastewater-irrigated produce has not been assessed. Moreover, uptake in crops has been studied mainly under controlled experimental conditions rather than in commercially available produce. In this study we aimed to determine whether carbamazepine is present in commercially available fresh produce, and to what extent carbamazepine and its metabolites are detectable in urine of human volunteers consuming commercially available produce grown in soils irrigated with reclaimed wastewater, compared to produce from other irrigation sources.

EXPERIMENTAL SECTION

Study Design. In September 2014, we performed a randomized, single blind, crossover study on human volunteers. Potential participants were recruited via posters placed in public areas of two academic campuses of the Hebrew University or via word of mouth. We excluded individuals who were therapeutically exposed to carbamazepine, pregnant women, and those adhering to strictly vegetarian, vegan or organic diets. We excluded vegans and vegetarians for whom fresh produce is a major component of their diets in order to decrease potential systematic differences in exposure compared to the general population. As well, their dietary needs may have required consumption of produce beyond that provided in the trial. After excluding two pregnant women and two individuals who were not available for the entire duration of the study, the final study population included 34 healthy volunteers (20 women and 14 men, aged 18−63 years) who were randomized and completed the study protocol.

Randomization and Blinding. Enrolled subjects were randomized in a 2:1 ratio to Group I or Group II using simple stratified randomization (in each of the two recruiting centers), using a random number table. Our original aim was to perform within-subject comparisons in a simple crossover design over two exposure periods: fresh water produce followed by reclaimed wastewater produce vs reclaimed wastewater produce followed by fresh water produce. However, in the second time period we were unable to secure adequate sources of reclaimed wastewater produce and thus decided to expose the participants to produce purchased in the supermarket.

Group I received produce irrigated with reclaimed wastewater followed by fresh water-irrigated produce; Group II received fresh water-irrigated produce followed by produce purchased from a local supermarket where sources of irrigation were unknown, but were likely to include some produce irrigated with reclaimed wastewater. The trial schema is shown in Figure 1. Participants were blinded as to the source of water for irrigation of the food packages provided. Characteristics of the two groups were balanced in terms of mean age, sex, body mass index, smoking, and exposure to medications (Supporting Information (SI) Table S1), and all recruited volunteers completed the study protocol and are included in the results.

Study Procedures. After receiving written and verbal information, participants signed informed consent forms and filled in baseline questionnaires which included demographic variables, questions concerning height and weight, current smoking status, and medications ingested. They then filled out a complete food frequency questionnaire. Baseline random urine samples were collected on day 0, then again on days 5, 7, 12, 18 in 100 mL plastic containers. All urine samples were tested for creatinine as well as carbamazepine and metabolite concentrations by personnel blinded to the exposure. On days 5, 7, 12, 18 study subjects completed a short food frequency questionnaire focusing on fresh fruit and vegetable consumption in the 3 days prior to sampling. Participants were encouraged to eat all or most of the food provided, but were not restricted from using other produce. They were given 1.5 L of bottled water, tested and found free of carbamazepine or its metabolites, for each day of the study. Food packages were provided twice during the experiment, on days 0 and 7, each package containing two heads of lettuce, 14−16 cucumbers, 14−16 tomatoes, carrots and peppers, and a variety of other produce. The packages were similar in all exposure periods with

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minor variations in the quantity and types of vegetables and fruit provided. Produce was obtained from (i) licensed growers irrigating with reclaimed wastewater, (ii) an organic produce supplier who ensured that the crops were irrigated in fresh water, and (iii) from a supermarket selling produce from farms distributed throughout the country, for which irrigation sources were unknown. Fresh produce was collected 1 day before it was supplied to the volunteers, and all food supplied in the trial was commercially available. The study protocol was approved by the institutional review board of Hadassah-Hebrew University Medical Center, and registered in www.clinicaltrials.gov (NCT02101801).

Statistical Methods. The differences in carbamazepine levels in all produce types were compared among the three sources using the Kruskal–Wallis test. We compared baseline demographic and medical parameters in the two exposure groups using χ^2 and Fisher exact tests. We assessed mean and median levels of carbamazepine and its metabolites at baseline, at peak, and at day 18. Since carbamazepine and metabolite levels were not normally distributed based on results of the Shapiro-Wilk test, we used nonparametric tests to assess between-group differences. Area under the curve for urinary carbamazepine was calculated using numeric integration computed from the measurements at five time points, employing the trapezoidal rule, and inserting area under the curve values into the Mann–Whitney U test to assess between-group differences. Within-groups difference between baseline and peak levels, and baseline and end-of study differences, were assessed using paired Wilcoxon tests. For statistical analysis, levels below the LOD were assigned a value of 0 and those between LOD and the LOQ were assigned a value of LOQ/2. Linear regression was used to assess the association between lettuce consumption (a vegetable with high levels of carbamazepine content) in period 1 with carbamazepine and metabolite levels in each group. The Ethics Committee approved the recruitment of up to 40 volunteers; eventually only 34 were recruited. The resulting sample size provided 98% power to detect a mean difference of 15 ng/L of carbamazepine between Group 1 and 2 on day 7 assuming standard deviations of 10 ng/L carbamazepine in each group, and $\alpha = 0.05$.

Analyses of Carbamazepine and Metabolites in Urine and Fresh Produce. Urine samples (10 mL) were frozen using liquid nitrogen and then freeze-dried. Acetonitrile (3 mL) was added to the freeze-dried urine and the liquid phase was cleaned-up by Strata X weak cation-exchange and Strata X weak anion-exchange cartridges (Phenomenex Inc., CA). Before use, the cartridges were washed with 2 mL of MeOH and preconditioned with 2 mL of acetonitrile/buffer (75/25 v/v). The eluent was evaporated to dryness and reconstituted in 100 μL of mobile phase (80/20 water/acetonitrile acidified with 1% of acetic acid), spiked with labeled internal standards and analyzed by Agilent 1200 Rapid Resolution LC system coupled to an Agilent 6410 triple quadruple mass spectrometer (Agilent Technologies Inc., Santa Clara, CA) with an electrospray ionization (ESI) in positive mode. The following parameters were used for the mass spectrometer: capillary voltage 4,000 V; drying gas (nitrogen) temperature and flow 350 °C and 10 L/min, respectively; nebulizer pressure 35 psi; nitrogen (99.999%) was used as a collision gas. LOQs for carbamazepine, EP-CBZ and DiOH-CBZ in urine samples were 3.6, 3.1, and 7.2 ng/L, respectively. LODs for carbamazepine, EP-CBZ and DiOH-CBZ in urine samples were 1.2, 2.4, and 1.0 ng/L, respectively. A detailed description of the method is reported in Fedorova et al.[21]

All produce was frozen and freeze-dried, ground to a fine and homogenized powder, and extracted using an accelerated solvent extractor (ASE350, Dionex, Sunnyvale, CA). Ground plant materials (1 g) were placed in 10 mL extraction cells on top of 1 g of Florisil (Mg_2O_4Si, Alfa Aesar, Ward Hill, MA) and covered with an extra 1 g of Florisil. Glass-fiber filters (27 mm) were placed at the bottom of the cells. The packed cells were extracted in two static cycles (5 min) with 100% methanol at 80 °C under a constant pressure of 10.34 MPa. All extracts were evaporated to dryness and reconstituted in 990 μL acetonitrile and 0.1% acetic acid in water (20/80). Then samples were spiked with 10 μL of a mixture of stable isotope labeled internal standards in acetonitrile, sonicated (37 kHz, 10 min), centrifuged (17 000*g*, 20 min) and filtered (0.22 μm PTFE). Quantification was conducted using LC/MS as listed above. LOQs for carbamazepine, EP-CBZ and DiOH-CBZ were 0.021, 0.015, and 0.025 ng/g fresh weight. Recovery values for CBZ, EP-CBZ, and DiOH-CBZ of different plants are listed in SI Figure S1. Water content of the produce is listed in SI Table S2.

RESULTS AND DISCUSSION

This "proof of concept" study aimed to provide real world data about human exposure to xenobiotics through ingestion of crops irrigated with reclaimed wastewater. Carbamazepine was chosen as a model compound for environmentally persistent xenobiotics. Carbamazepine is ubiquitous in reclaimed wastewater and is known to be taken up by crops; thus it is not surprising that carbamazepine and its metabolites were detected in all reclaimed wastewater-irrigated crops (Figure 2). Statistically significant differences in the fresh weight concentrations of carbamazepine in the produce from the three different sources were found for cucumbers ($p = 0.004$), lettuce ($p = 0.036$), parsley ($p = 0.004$), peppers ($p = 0.036$), and tomato ($p = 0.007$), whereas for carrots the difference showed borderline significance ($p = 0.054$). The highest carbamazepine levels were detected in leafy vegetables, especially lettuce and parsley. In most cases the parent compound was detected at higher levels than the metabolites EP-CBZ or DiOH-CBZ. However, carbamazepine made up less than 50% of the total carbamazepine species in the produce, similar to previous findings[6,17] suggesting significant metabolism of carbamazepine by plants. The ratio of carbamazepine to metabolites appeared to be lower in fruits as compared to vegetables (SI Figure S2). With the exception of parsley and coriander, none of the fresh water-irrigated produce contained carbamazepine and/or its metabolites at quantifiable levels (Figure 2). The supermarket-purchased produce (unknown source of irrigation) exhibited quantifiable levels of carbamazepine and its metabolites in four of the seven analyzed items. Lettuce exhibited significantly lower concentrations than observed in produce irrigated with reclaimed wastewater, and carbamazepine was not detected in peppers, coriander, or parsley. For cucumbers, carrots, and tomatoes the observed concentration of carbamazepine was similar in the supermarket-purchased produce and the produce irrigated with reclaimed wastewater. This suggests that some of the produce supplied by the supermarket on the day of sampling had originated from growers using reclaimed wastewater.

At baseline (i.e. on day 0 of the experiment), carbamazepine was undetectable in the urine of 13 (38.2%) individuals; 12

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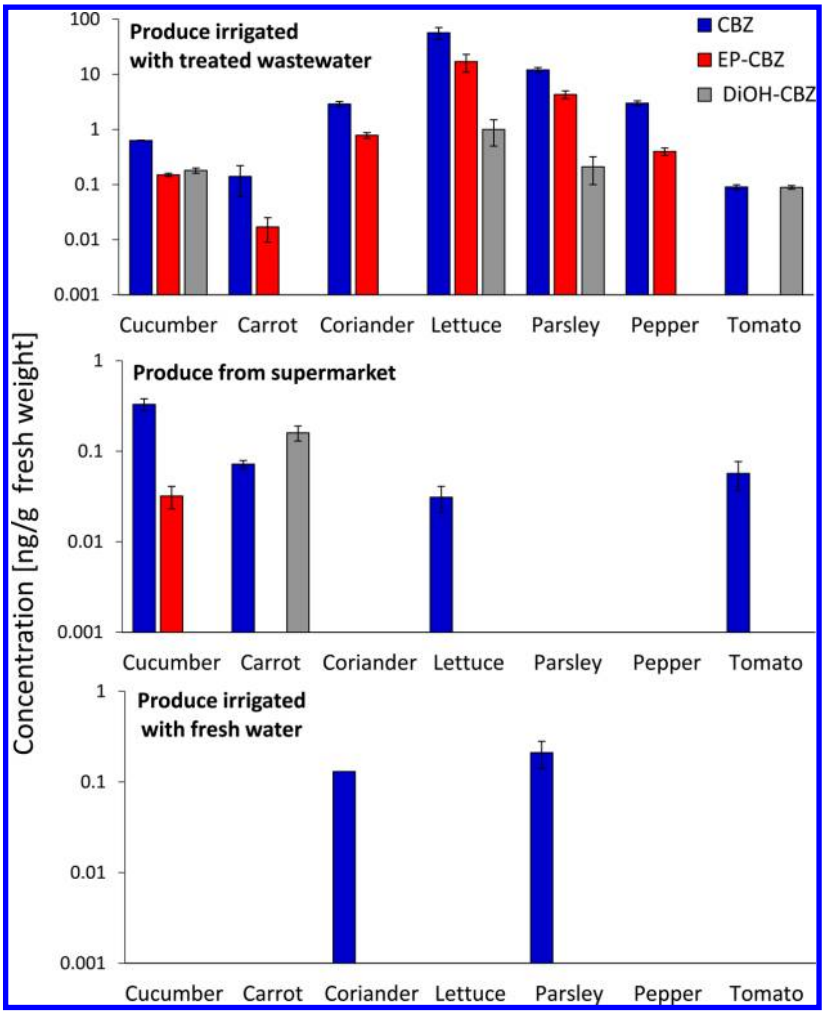

Figure 2. Concentrations of carbamazepine (CBZ) and its metabolites (EP-CBZ and DiOH-CBZ) in comparable produce provided to study participants by source: produce irrigated with reclaimed wastewater (top chart); supermarket, unknown irrigation source (middle chart); and fresh water-irrigated produce (lower chart).

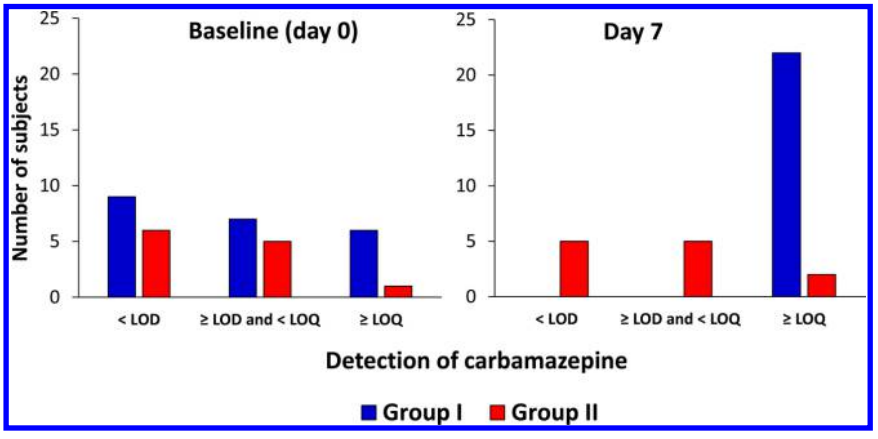

Figure 3. Numbers of participants in Group I (N = 22, produce irrigated with reclaimed wastewater) and Group II (N = 12, fresh water-irrigated produce) with urine carbamazepine levels below LOD, between LOD and LOQ, and at or above, on days 0 and 7.

(35.3%) individuals exhibited carbamazepine levels above LOD but below the LOQ (3.6 ng/L), and 9 (26.5%) subjects exhibited urine carbamazepine at concentration higher than LOQ (Figure 3, left), with no statistically significant differences between Groups I and II (p = 0.38). We queried whether the substantial interindividual variability in baseline levels of carbamazepine arose from prestudy patterns of fruit and vegetable consumption. Based on dietary questionnaires, we found that individuals with urine carbamazepine levels ≥ LOQ reported a higher frequency of consumption of cucumbers and tomatoes (Figure 4), which are staple items in the Israeli diet. Among those with levels > LOQ, 60% reported at least daily consumption of tomatoes and cucumbers, as compared to only

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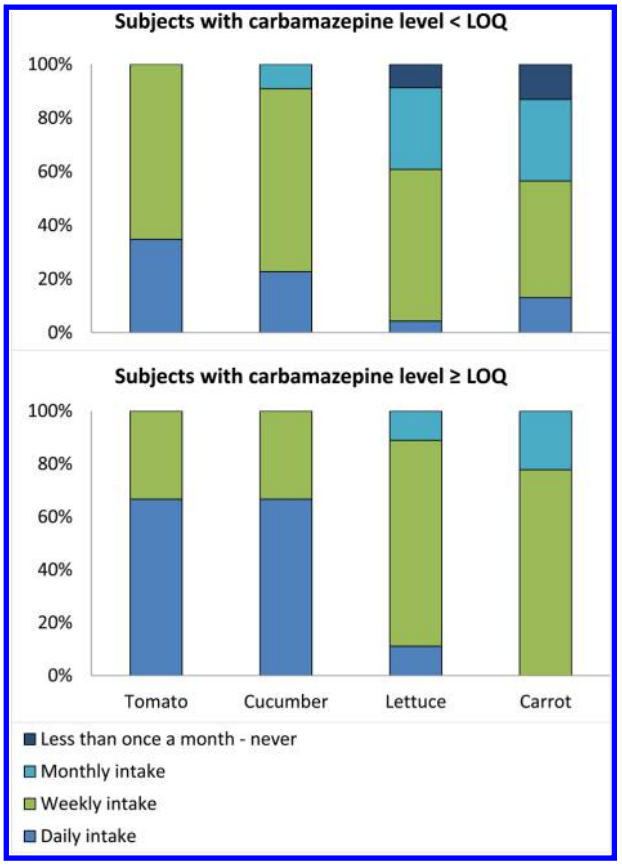
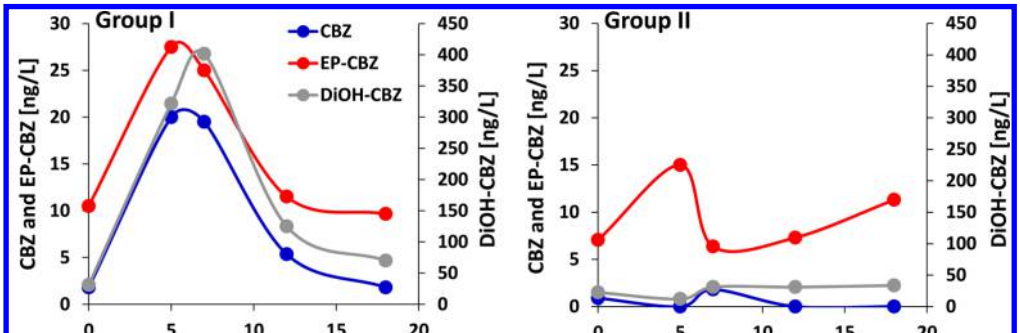


Figure 4. Association between baseline urinary carbamazepine levels and prestudy frequency of vegetable consumption in the entire study population (N = 34). Top chart represents individuals with baseline levels < LOQ, bottom chart represents individuals with baseline levels ≥ LOQ.

30% and 20%, respectively, among those with baseline values below LOQ (p < 0.05).

Following exposure to reclaimed wastewater-irrigated produce (Group I), there was a clear increase in both urinary carbamazepine and metabolite concentrations. In fact, following 7 days of consuming reclaimed wastewater-irrigated produce all 22 members of Group I (i.e., the exposed group) exhibited quantifiable levels (i.e., > LOQ) of carbamazepine; in members of Group II (i.e., control group) the distribution remained unchanged (Figure 3, right; p < 0.001). These levels returned to baseline by day 18, following 7 days of exposure to fresh water-irrigated produce. In contrast, in the control group (Group II) following exposure to fresh water-irrigated produce, carbamazepine levels did not rise appreciably and did not differ from those measured after consumption of supermarket produce. Area under the curve analysis revealed significant differences (p < 0.0001) between Groups I and II in carbamazepine excretion over the entire period. Results were unchanged after normalizing carbamazepine and metabolite levels to urine creatinine values. Lettuce consumption within each group was not correlated with peak carbamazepine and metabolite levels (r = 0.018, p = 0.94, and r = 0.131, p = 0.69, for Groups I and II, respectively) presumably since the variability in consumption was low, given that trial participants were instructed to eat all or most of the provided produce.

The median urinary levels of carbamazepine, EP-CBZ and DiOH-CBZ during the whole experiment are shown in Figure 5 (urine concentrations for each study subject are presented in SI Figure S3). The concentration of DiOH-CBZ was highest among the metabolites in all urine samples, an order of magnitude higher than that of the parent compound, carbamazepine, or the EP-CBZ metabolite (Figure 5) as expected from previous work.[22,23] EP-CBZ is quickly metabolized in the liver to DiOH-CBZ via epoxy hydrolase.[22] The dominance of DiOH-CBZ in urine corresponds to reports on carbamazepine metabolism in people taking therapeutic



Group I						←Days→		Group II				
0	5	7	12	18				0	5	7	12	18
2.0	20.0	20.0	5.4	1.8	CBZ	Median	CBZ	0.9	0.0	1.8	0.0	0.0
3.7	34.7	34.6	14.7	5.2		IQR		1.8	1.8	1.8	1.8	1.8
11.0	28.0	25.0	12.0	10.0	EP-CBZ	Median	EP-CBZ	7.1	15.0	6.4	7.3	11.3
16.4	38.5	46.0	11.5	26.6		IQR		8.4	37.3	13.5	10.9	23.1
31.0	322.0	402.0	125.0	70.0	DiOH-CBZ	Median	DiOH-CBZ	23.0	12.0	31.0	31.0	34.0
54.0	514.0	863.0	246.0	92.0		IQR		41.0	28.1	41.8	39.5	81.8

Figure 5. Median urine concentration (ng/L) interquartile ranges at each sampling time point of carbamazepine (CBZ, blue symbols), EP-CBZ (orange symbols), and DiOH-CBZ (gray symbols, right y-axis scale) for subjects in Group I and Group II.

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doses.[23] In our experiment the origin of some of the excreted metabolites might have been from direct exposure to these compounds, as they were present in the ingested produce (Figure 2, SI Figure S2), and not merely due to metabolism of carbamazepine. Interestingly, the conjugated form of carbamazepine (carbamazepine *N*-glucuronide) was undetected in the urine samples following consumption of produce irrigated with reclaimed wastewater. In contrast, in patients chronically receiving carbamazepine in therapeutic doses the metabolite profile includes carbamazepine *N*-glucuronide as one of the most abundant metabolites of carbamazepine excrete in the urine.[9,23] Uridine diphosphate glucuronosyltransferase is responsible for the *N*-glucuronidation in the liver.[24] Our findings of an altered metabolic profile may be due to the lack of autoinduction of enzymatic activity at very low exposures, or due to the actual ingestion of metabolites in produce.

Randomized trials to investigate effects of environmental exposures via food are rare, but provide direct experimental evidence for variation in chemical exposures based on different food sources. A short-term randomized trial in adult humans showed an 89% reduction in organophosphate levels after 7 days of exposure to an "organic" diet.[25] Less striking reductions were noted for children after 7 days of an organic diet.[26] Other observational studies have reported at link between pesticide exposure and food consumption; Berman et al.[27] reported that individuals with high fruit consumption exhibited high level of dialkyl phosphates in their urine. Similarly, higher levels of metabolite of chlorpyrifos were found in children with high consumption of vegetables.[28] Associations between dietary factors and urinary concentrations of organophosphate and pyrethroid metabolites were also reported for the general population in Canada.[29] These findings suggest that exposure to pollutants originating in the agro-environment among humans consuming large quantities of fresh fruits and vegetables is to be expected. Thus, it is not surprising that in our study higher baseline consumption of fresh fruits and vegetables resulted in higher exposure to carbamazepine.

Limitations of this study include the small and selective sample. Nevertheless, power was sufficient to demonstrate substantial between-group differences in carbamazepine excretion. The short exposure duration precludes inference on long-term effects. Strengths include the randomized design and blinding of subjects to exposure, complete follow-up, and blinded assessment of urinary carbamazepine and metabolite levels. Ingestion of produce other than that provided during the study period is possible but would have biased the results toward the null.

■ ENVIRONMENTAL AND HUMAN HEALTH IMPLICATIONS

To the best of our knowledge, our study is the first to report carbamazepine contamination of commercially available fruits and vegetables and the first to assess unintentional human pharmaceutical exposure as a function of irrigation source of this produce. Given the widespread and growing use of reclaimed wastewater for irrigation, and the ubiquity of carbamazepine in treated effluents worldwide, the potential for unwitting exposure of consumers to contaminants via this route (reclaimed wastewater → soil → plant → human) is real. In this small randomized study among healthy individuals we provide "proof of concept" that irrigation with reclaimed wastewater can lead to low-level contamination of fresh produce, and results in unintentional human exposure to active pharmaceuticals. Furthermore, we found that carbamazepine metabolites, some of which are equipotent to the parent compound[30] are both consumed and excreted following exposure to crops irrigated with reclaimed wastewater, and their clinical effects at these levels are currently unknown.

Investigators have debated the potential consequences of exposure to wastewater-derived pollutants.[4,6,31−34] Risk estimates for humans ingesting food-borne pharmaceuticals have ranged from "de minimis"[34] to a substantial risk to children from minimum consumption,[4] however these assessments were based on simulations or measurements in vegetables grown in controlled conditions, not on actual measurements in human biological samples. Peak urinary levels of carbamazepine in this study were 4 orders of magnitude lower than the level expected after a single ingestion of 400 mg of carbamazepine,[11] thus are unlikely to have clinical effects under most circumstances in adults. However, certain individuals exposed to carbamazepine, especially Asians bearing HLA-B1502 alleles,[35] are prone to severe hypersensitivity reactions which are not necessarily dose-related. As for pregnant women, carbamazepine is considered potentially teratogenic, especially at high doses,[36] although chronic exposure at ng/L levels has never been assessed. The potential effects on children, the elderly, or vegetarians/vegans with high levels of fresh produce consumption are unknown.

From the public health standpoint two aspects of this study are reassuring. First, although participants reported eating more fresh produce during the study than they would normally consume, end-of-study carbamazepine levels returned to baseline values. Second, somewhat lower levels of the drug were detected in supermarket produce, presumably since it originates from a variety of farms and irrigation sources, thus "diluting" the effect of potential contaminants. Assessments of the potential for human risk versus benefit associated with reclaimed wastewater-irrigated produce should take into account the patterns demonstrated in this trial.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.est.5b06256.

Recovery values for the analytes for different plants (Figure S1); concentrations of carbamazepine and metabolites in produce samples irrigated with reclaimed wastewater (Figure S2); urinary carbamazepine levels in all participants throughout the trial (Figure S3); study population characteristics (Table S1); and water content in produce (Table S2) (PDF)

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Notes

The authors declare no competing financial interest.

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