

Effects of Extreme Dilutions of *Apis mellifica* Preparations on Gene Expression Profiles of Human Cells

Dose-Response:
An International Journal
January-March 2016:1-7
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DOI: 10.1177/1559325815626685
dos.sagepub.com



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Abstract

Gene expression analysis has been employed in the past to test the effects of high dilutions on cell systems. However, most of the previous studies were restricted to the investigation of few dilutions, making it difficult to explore underlying mechanisms of action. Using whole-genome transcriptomic analysis, we investigated the effects of a wide range of *Apis mellifica* dilutions on gene expression profiles of human cells. RWPE-1 cells, a nonneoplastic adult human epithelial prostate cell line, were exposed to *Apis mellifica* preparations (3C, 5C, 7C, 9C, 12C, 15C, and 30C) or to the reference solvent solutions for 24 hours; nonexposed cells were also checked for gene expression variations. Our results showed that even the most diluted solutions retained the ability to trigger significant variations in gene expression. Gene pathway analysis revealed consistent variations in gene expression induced by *Apis mellifica* when compared to nonexposed reference cells but not to reference solvent solutions. Since the effects of *Apis Mellifica* at extreme dilutions did not show dose-effect relationships, the biological or functional interpretation of these results remains uncertain.

Keywords

gene expression, *Apis mellifica*, extreme dilutions, homeopathy, microarrays

Background

In the course of a research program on the study of the low-dose pharmacology of chemicals and drugs, we investigated the effects of drugs at different dilutions on appropriate cellular systems by means of the DNA-array technique. Our goal was to obtain information through a experimentally reliable platform on the perturbation mechanisms of xenobiotics, with the secondary aim of devising unprecedented applications or optimizing the currently used ones. In this contribution, we report the results of a study concerning the effects of a range of concentrations of *Apis mellifica* on gene expression profiles using microarrays and the same cellular system (human prostate epithelial cells, RWPE) tested before by our group.¹⁻²

Bee venom stings produce persistent pain and inflammation under normal conditions, but few controlled reports in humans indicate that they can also exert anti-inflammatory and antinociceptive effects: in fact, a Chinese clinical trial showed beneficial effects of bee venom therapy relieving pain knee osteoarthritis.³ A beneficial clinical effect of combined bee-sting venom therapy and classical medications in the treatment of rheumatoid arthritis (RA) was also reported.⁴ More recently,

the therapeutic effect of bee venom on several immunological and neurological diseases was also described,⁵ and a randomized clinical trial demonstrated the efficacy of bee venom acupuncture in patients with chronic lower back pain.⁶ In homeopathy *Apis mellifica* is obtained as a hydroalcoholic extract of the whole bee assuming, on the basis of the law of similarities, it could exert anti-inflammatory or antiedema activity.

Although the present contribution is not aimed at validating any specific therapeutic philosophy, it is worth mentioning that the concept that modulation of gene expression could explain

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the effects of chemicals and drugs at extreme dilution has been present for a relatively long time in the scientific literature to support the claimed effects of some homeopathic preparations.^{7,8} However, until recently, the available methods were too imprecise or insensitive to test this hypothesis. Accordingly, an early article in this field, using as a testing method the ribonuclease protection assay and testing various homeopathic preparations (*Conium maculatum*, *Sabalserrulata*, *Thujaoccidentalis*, *Asterias*, *Phytolacca*, and *Carcinosin*) on prostate and breast cancer cells, showed no changes in messenger RNA levels of bax, bcl-2, bcl-x, caspase-1, caspase-2, caspase-3, Fas, or FasL.⁹ However, according to several recent reports, cells treated with diluted solutions differentially express a significant number of genes, when compared with reference control cells.¹⁰⁻¹⁶ A further relevant feature emerging from these studies is the fact that differential gene expression is maintained at high dilutions. A recent report by Khuda-Bukhsh and his research group indicated that 30C *Hydrastis canadensis* and 30C *Marsdenia condurango* triggered epigenetic modifications and alterations in microarray gene expression profiles of many genes associated with carcinogenesis in HeLa cells in vitro.¹⁷ Two reviews discussing these data recently appeared in the literature.^{18,19} It is obvious that all the above-mentioned studies can be helpful for elucidating the claimed effects of homeopathic preparations. Unfortunately, these investigations have employed a single dilution of the homeopathic preparation, and it is hard to infer from these data the underlying mechanisms of action.

We were among the first to apply a sensitive transcriptomic method based on microarray to the study of diluted solutions and demonstrated that copper(II) sulfate could affect gene expression at concentrations varying from 10^{-6} to 10^{-17} mol/L.¹ The most relevant feature of this work resides in the fact that for the first time it was shown that different gene expression profiles of the same cell were observed upon treatment of the same perturbing chemical at different dilutions. The pattern of gene expression observed suggested the existence of a hormetic mechanisms also described in our recent report on *Apis mellifica* (3C, 5C, and 7C).² The results of Bellavite and co-workers on 2C and 9C *Gelsemium sempervirens*²⁰ also seem to be consistent with a hormetic response at least for a subset of genes.¹⁹

These data indicate that such experimental approach may disclose the mechanisms involved in hormetic effects and are an attempt of shifting homeopathy into the field of low-dose pharmacology.^{19,21-31} This approach, which considers hormesis as an operational framework,³² has been criticized by several homeopaths³³⁻³⁷ according to whom the holistic approach of homeopathy as a healing system goes beyond the identification of specific mechanistic information.

On the basis of new evidence published in the literature, we investigated the effects of a range of concentrations of *Apis mellifica*, including the ultra-low (3C, 5C, 7C, 9C, 12C, 15C, and 30C), on gene expression profiles using microarrays and the same cellular system (human prostate epithelial cells, RWPE) tested before by our group.^{1,2} We also devised an

experimental set up that could control the variables possibly interfering with the interpretation of the results. Specific attention was addressed to discriminate between the effect of the solvents and the effect of the test solutions.

Methods

Preparation of *Apis Mellifica* Dilutions and Control Solutions

The homeopathic dilutions/dynamizations were performed using 30% ethanol for all dilution/succussion steps. A whole ethanol extract of *Apis mellifica* (mother tincture) was produced by Boiron Laboratoires (Lyon, France) according to the French Homeopathic Pharmacopoeia. The mother tincture underwent subsequent serial 100× dilutions up to 30C, performed in the Boiron Laboratories according to standard homeopathic procedures. In brief, the first centesimal (1C) dilution is obtained by dissolving 1 volume of mother tincture in 99 volumes of 30% ethanol in water and then subjecting it to vigorous shaking (succussion or “dynamization”). Subsequent C dilutions are prepared by repeating the same procedure. The control solutions (solvents) containing 30% ethanol were prepared in parallel with the drug dilutions, without adding *Apis mellifica* extracts, and were not dynamized. To better clarify this point, the wording EtOH 3C indicates that this solutions was obtained performing the same dilution steps used to obtain the corresponding *Apis mellifica* preparations. The amount of ethanol in the control solutions was the same as that for *Apis mellifica* preparations.

Solvent solutions or *Apis mellifica* preparations were added to the culture medium in a volume of 5 µL in a total incubation medium of 0.5 mL, obtaining a 0.3% final ETOH concentration, which did not have any cytotoxic effect.

Cell Cultures and Treatments

RWPE-1 cells,^{1,2} a nonneoplastic adult human epithelial prostatic cell line, were grown in keratinocyte serum-free medium containing 5 ng/mL epidermal growth factor and 25 mg/mL bovine pituitary extract and 1% penicillin/streptomycin solution at 37°C in a 5% CO₂ atmosphere, until confluence in 75 cm² flasks. The cells were originally immortalized with human papillomavirus and are phenotypically stable. Thereafter, confluent cells were splitted in 24-well culture flasks and allowed to reach 80% confluence. *Apis mellifica* dilutions 3C, 5C, 7C, 9C, 12C, 15 C, 30C or the reference water-ethanol solutions were added to the culture media for 24 hours, reaching a further final 1:100 dilution (final concentration of ethanol was 0.3% in all final incubation media). At the end of this period, cells were harvested with RLT lysing buffer (Qiagen, Milan, Italy) and stored at -20°C until analysis. To take into account the experimental variability, for each experimental point, we performed a microarray analysis of 3 independent biological replicates, each of which was obtained by pooling 2 additional biological replicates.

RNA Isolation

Total RNA was isolated using the RNeasy Mini kit Plus (Qiagen) according to the manufacturer's protocol. RNA concentration and purity were determined using a NanoPhotometer spectrophotometer (IMPLEN, Implen, Munchen, Germany). RNA integrity (RIN) was checked by using a 2100 Bioanalyzer and a RNA 6000 Nano LabChip kit (Agilent Technologies, Santa Clara, CA, USA). RNAs from the 2 technical replicates of each experimental point were combined.

Gene Expression Analysis

Total RNA sample of 100 ng was used. To produce Cy3-labeled complementary RNA (cRNA), the RNA samples were labeled using the Agilent Quick Amp Labeling Kit (Agilent Technologies) following the manufacturer's protocol. Yields of cRNA and the dye incorporation rate were measured with NanoPhotometer spectrophotometer (IMPLEN). RNA Spike-In kit Agilent Technologies was used as control to monitor and calibrate the linearity, sensitivity, and accuracy of the microarray workflow. The Cy3-labeled samples were hybridized to Agilent Human GE 8 × 60 K Oligo 60-mer microarrays in Agilent microarray chambers (G2534A) at 65°C for 18 hours. Finally, the microarrays were washed once with the Agilent Gene Expression wash buffer 1 for 1 minute at room temperature followed by a second wash with preheated Agilent Gene Expression wash buffer 2 (37°C) for 1 minute. After washing, fluorescent signal intensities were detected using the Agilent Scan Control 7.0 Software on an Agilent DNA Microarray Scanner, at a resolution of 2 μm. Data were acquired using Agilent Feature Extraction 9.5.3.1 software. Values for control spots and spots that did not meet the quality criteria were flagged. Quality criteria included a minimal spot size, a median–mean ratio of at least 0.9 for each spot, nonsaturated intensity, a signal well above background, and a minimal signal intensity. We set up a total of 45 hybridization, 3 for each experimental point. Data obtained from biological replicates were mediated, and 3 types of evaluations were performed:

1. Genes differentially expressed ($P < .05$) by comparing cells exposed to *Apis mellifica* preparations versus those exposed to the corresponding reference water–ethanol solutions (eg, Apis 3C vs ETOH 3C, etc).
2. Genes differentially expressed ($P < .05$) by comparing cells exposed to *Apis mellifica* preparations versus non-exposed (eg, Apis 3C vs RWPE).
3. Genes differentially expressed ($P < .05$) by comparing cells exposed to the water–ethanol solutions versus non-exposed (eg, ETOH 3C vs RWPE).

Pathway analysis was performed by means of GO-Elite software, version 1.2.5, an open source, freely available (http://www.genmapp.org/go_elite), using as input data the list of the differentially expressed genes from every comparisons, with a statistical significance less of .05.

Results

Gene Expression Analysis

Genes significantly modulated by each treatment were measured, and the number of gene modulated is reported as Venn diagrams (Figure 1). As expected, the final 0.3% ethanol solutions induced biological effects and modulated a number of genes (lower circles of the Venn diagrams in Figure 1). Although the analysis of the specific genes regulated by ethanol was not the target of this investigation, we discovered that ethanol changed the expression from a minimum of 198 to a maximum of 378 genes (lower circles of the Venn diagrams); this reflects the experimental variability of the system, since the incubation conditions were the same and ethanol concentration was constant in all samples. These analyses let us to discriminate the effects related to the solvent from those univocally exerted by *Apis mellifica* preparations.

According to the Venn diagrams (Figure 1), *Apis mellifica* modulated a number of genes “per se” at all dilutions, including the extreme ones. It is interesting to note that comparing the expression profiles of cells exposed to *Apis mellifica* with those of nonexposed cells, we identified gene variations not revealed by the comparison of cells exposed to *Apis mellifica* preparations versus those exposed to the water–ethanol solutions.

The left panel of Figure 2 shows the number of genes significantly modulated by *Apis mellifica* compared to the corresponding water–ethanol reference solutions; here, it is possible to observe clear effects at 5C, 7C, 9C, and 12 C, whereas the variations are smaller at 3C and 30C. Comparing the gene expression profiles of RWPE cells exposed to *Apis mellifica* to nonexposed cells, effects were observed at 7C, 12C, and 15C (Figure 2, right panel). No clear dose–response was apparent in these results.

We also performed a gene pathway analysis, which showed that several pathways were significantly and often recurrently modulated by *Apis mellifica* at various dilutions when compared to nonexposed RWPE cells (Table 1). On the contrary, the functional effects in pathway analysis emerging in the comparison of *Apis mellifica* preparations versus the corresponding water–ethanol reference solutions were few and inconsistent. The specific genes modulated are indicated in Table 1, but the biological relevance of such effects is difficult to assess without functional data obtained in the same system.

Discussion

We previously demonstrated that the measurement of gene expression is a very sensitive method for detecting the effects of extremely dilute solutions. We tested copper at concentrations varying from 10^{-6} to 10^{-17} mol/L and *Apis mellifica* preparations from 3C to 7C, showing responses that seemed to fit a hormetic model.^{1,2} The results of Bellavite and co-workers on 2C and 9C *Gelsemium sempervirens*²⁰ also seems to be consistent with a hormetic response.¹⁹ Other independent groups have conducted experiments that confirm gene

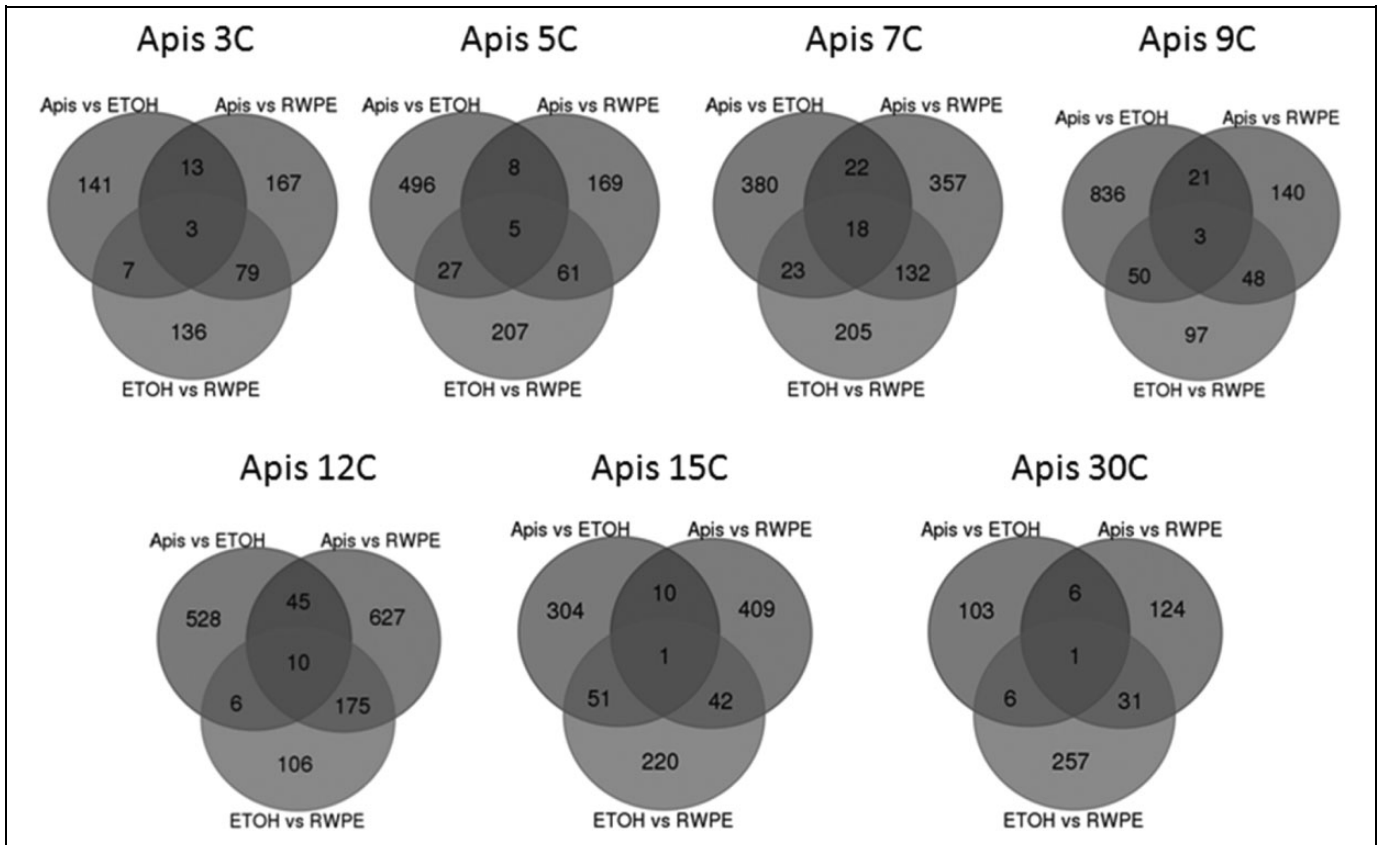


Figure 1. Venn diagrams showing the number of genes significantly modulated (up- or downregulated) by treatments with *Apis mellifica* preparations varying from 3C to 30C. The circle in the upper left of each Venn diagram shows the genes significantly modulated comparing cells treated with different *Apis mellifica* preparations versus RWPE (a nonneoplastic adult human epithelial prostate cell line) cells treated with ethanol–water solvents alone (Apis vs ETOH). The circle on the upper right shows the number of genes modulated by the *Apis mellifica* preparations versus RWPE nontreated cells (Apis vs RWPE). The lower circle shows the number of genes modulated by the ethanol:water solvents versus RWPE nontreated cells (ETOH vs RWPE). The intersections between circles show the degree of genes signals overlapping among different treatments.

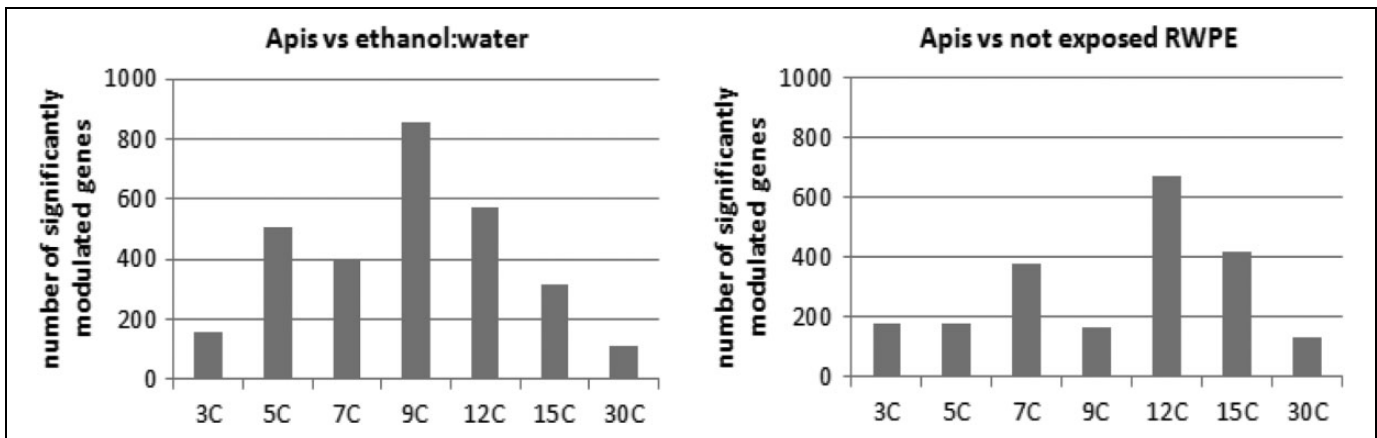


Figure 2. Left panel: Number of genes significantly up- or downregulated comparing RWPE (a nonneoplastic adult human epithelial prostate cell line) cells exposed to *Apis mellifica* preparations versus cells exposed to the reference ethanol:water solutions (Apis vs ethanol–water). Right panel: Number of genes significantly up-or downregulated comparing RWPE cells exposed to *Apis mellifica* preparations versus nonexposed reference cells (Apis vs not exposed RWPE).

Table 1. Pathways Significantly Enriched and Genes Modulated by *Apis mellifica* Preparations Versus NonExposed Reference Cells.

Pathways	Genes
Androgen receptor signaling pathway: WP138	Apis 3C FGF2 PIK3R1 ROCK2 SSH1 SSH3 Apis 5C AR CCNE1 PTK2 RLN1 RUNX2 EGFR MDM2 PIK3R1 Apis 7C CDC42 NCOR1 PIAS4 RAD9A BMF EGFR NCOR1 PIK3R1 ROCK2 Apis 12C BMF EGFR LIMK2 NCOR1 PIK3R1 PTEN ROCK2 Apis 15C BMF LIMK2 NCOR1 ROCK2
Aryl hydrocarbon receptor (AhR) signaling pathway: WP2100	Apis 5C CYP1B1 EGFR GSTA2 Apis 7C ALDH3A1 CYP1B1 EGFR GSTA2 Apis 12C ALDH3A1 CYP1B1 EGFR Apis 15C APOB CCL2 DIO1 GGT1
IL-1 signaling pathway: WP195	Apis 7C CCL2 MAP3K14 PIK3R1 Apis 12C CCL2 IL1R1 MAP3K14 NFKB1B PIK3R1
Insulin signaling: WP481	Apis 3C EGR1 INPPL1 PIK3R1 RAPGEF1 Apis 7C EGR1 MAP3K14 MINK1 PIK3R1 RAPGEF1 TSC2 Apis 9C EGR1 FLOT2 PIK3R1 RAPGEF1 Apis 12C EGR1 FLOT2 MAP3K14 MAP4K1 MINK1 PIK3R1 PTEN RAPGEF1 RRAD TSC2
Metapathway biotransformation: WP702	Apis 5C CHST5 CYP1B1 CYP2C9 GSTA5 HS6ST3 Apis 9C CHST5 CYP1B1 CYP26B1 HS6ST3
Oncostatin M signaling pathway: WP2374	Apis 3C CCL2 EGR1 PIK3R1 Apis 5C CCL2 EGR1 PIK3R1 Apis 9C CCL2 EGR1 PIK3R1
Regulation of actin cytoskeleton: WP51	Apis 3C FGF2 PIK3R1 ROCK2 SSH1 SSH3 Apis 5C ACTN1 EGFR PIK3R1 SSH1 WASF2 Apis 7C ARHGEF4 BAIAP2 EGFR FGFR3 PIK3R1 ROCK2 Apis 12C ACTN1 ARHGEF4 BAIAP2 CYFIP2 EGFR FGFR3 FGFR4 GIT1 MYL3 PIK3R1 ROCK2 SSH1 WASF2
Selenium micronutrient network: WP15	Apis 9C APOB CCL2 PTGS1 Apis 12C APOB CCL2 DIO1 GGT1 KMO NFKB2 Apis 15C APOB CCL2 DIO1 GGT1
Type II interferon signaling (IFNG): WP619	Apis 5C EIF2AK2 IFIT2 IRF1 Apis 9C IRF1 IRF9 PRKCD PTPN11 EIF2AK2 IFI6 IFIT Apis 12C EIF2AK2 IFI6 IFIT2 IRF1 Apis 15C EIF2AK2 IFI6 IFIT2 IRF1

modulation in cells incubated with homeopathic dilutions of different substances.^{10-17,38}

All these data indicate that gene expression can be used to detect effects of homeopathic preparations *in vitro*, but several studies on gene expression were restricted to the evaluation of the effects of mother tinctures and of a single dilution.¹³ In our experimental approach and the similar ones carried out by the Bellavite group, the changes in gene expression induced by a wide range of concentrations were instead measured.^{1,2,38}

The experiments conducted in the present article were designed to avoid possible interfering factors and specifically solvent effects. In fact, many homeopathic remedies contain ethanol, a chemical with powerful biological effects, including gene regulation. In our experiments, we could in fact detect gene expression variations in RWPE cells exposed at low concentrations of ethanol (0.3%).

We were able to discern specific effects related *per se* to *Apis mellifica* from those related to the solvent. Another point of our investigation was the study of dose–effect relationships, a crucial information in pharmacological research. Unfortunately, we did not find clear dose–effect responses, and the modulation of gene expression by *Apis mellifica* seemed non-linear, with major effects exerted by 9C and 12C dilutions.

Moreover, at ultra-low concentrations, we did not observe the hormetic response previously described for dilutions up to 7C.² Possibly, hormesis is more easy to observe at ultra-low dilutions with single chemicals than with complex preparations.

Pathway analysis revealed several significant gene pathway modulations at high dilutions of *Apis mellifica* (androgen receptor, aryl hydrocarbon receptor, interleukin 1, insulin, oncostatin M, type II interferon signaling, metapathway biotransformation, and Selenium Micronutrient network). Some specific genes showed consistent variations with different dilutions, although, as pointed out before, a clear dose-related pattern did not emerge. An interesting element of the results is that the pathways significantly modulated were observed in the comparison between *Apis mellifica*-treated and untreated cells, suggesting that these effects were due to the interaction between *Apis mellifica* and the vehicle.

Pharmacological responses with concentration up to 10⁻¹⁰ mol/L are explained using sound theoretical models, such as substrate–enzyme or drug–receptor interactions; growth factors and microelements have effects at lower concentrations (usually in the range 10⁻¹⁰/10⁻²⁰),³⁹ but dose–effect relationships are usually apparent. Responses at ultra-low dilutions are confirmed by the present data, and variations in gene expression

have been observed in various laboratories, using different substances and approaches.^{7-20,38} Several hypotheses have been formulated for explaining this behavior^{18,40}; we do not wish to comment on the hypotheses advanced so far to explain the puzzling behavior of diluted solutions.

Notably, in our experiments, serial dilutions/succussions were performed in *Apis mellifica* preparations but not in control solvents, creating possible bias due to compounds hypothetically released from the glass containers or from caps and seals. However, a mass spectrometry analysis of *Apis mellifica* preparations and reference ethanol–water solutions showed no relative differences, and similar traces of phthalates and tensioactives were found in all solutions examined (data not shown).

Conclusion

Our results demonstrate that extremely diluted *Apis mellifica* preparations retain effects on gene expression in human cells. Given the fact that no clear dose–effect relationships were observed in the ample concentration range used in our experiments, we do not feel like suggesting biological effects of such modulations without functional studies specifically designed to clarify this point.

Acknowledgments

We gratefully acknowledge a liberal grant support from the Laboratories Boiron 2, Avenue de l'Ouest Lyonnais-Messimy(France).

Authors' Contributions

All the authors planned the experiments, EB and CL designed, performed the experiments and analyzed the data. PD wrote the original version of the paper and revised the final version. All authors modified and approved the final draft.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

The author(s) received no financial support for the research, authorship, and/or publication of this article.

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