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ORIGINAL PAPER

Exploring the effects of homeopathic *Apis mellifica* preparations on human gene expression profiles

Elisabetta Bigagli[1,*], Cristina Luceri[1], Simonetta Bernardini[2], Andrea Dei[3], Angelica Filippini[1] and Piero Dolara[1]

[1]*Department of Neurofarba, Section of Pharmacology and Toxicology, University of Florence, Viale Pieraccini 6, 50139 Florence, Italy*
[2]*SIOMI Research Unit, Via Orti Oricellari 26, 50123 Florence, Italy*
[3]*Department of Chemistry, University of Florence, Via della Lastruccia, 3, 50019 Sesto F.no, Florence, Italy*

Background: **Diluted preparations obtained from *Apis mellifica* are reported in the homeopathic literature to have anti-inflammatory activity. The present study was designed to explore the effects on global gene expression profiles of human cells by means of microarrays, using *Apis mellifica* mother tincture (TM) and its 3C, 5C, 7C dynamized dilutions; the technique employed allowed us to study the changes in gene expression at concentrations much lower than those associated with pharmacological responses.**
Methods: **An RWPE-1 cell line (human immortalized prostate epithelial cells) was used to study the effects on global gene expression by transcriptomic analysis.**
Results: ***Apis mellifica* TM and its 3C, 5C, 7C dynamized dilutions modulated hundreds of genes; using cluster analysis we observed groups of genes up- or down-regulated with similar expression profiles among treatments; other genes showed opposite regulation profiles at low and high dilutions of *Apis mellifica*, suggesting a hormetic response. In particular, genes involved in cytokine expression, inflammatory processes, anti-oxidative responses and proteasome degradation were differentially, and sometimes divergently expressed by the TM or by *Apis mellifica* 3C, 5C and 7C dilutions. We confirmed these data by RT-PCR analyses on 5 selected candidate genes (IL1β, CD46, ATF1, UBE2Q2 and MT1X).**
Conclusions: ***Apis mellifica* TM modifies gene expression in human cells and has inhibitory effects on regulatory processes of inflammation; in addition, extremely diluted dynamized dilutions (3C, 5C and 7C) still exert significant effects on genes involved in inflammation and oxidative stress.** *Homeopathy* (2014) **103**, 127–132.

Keywords: *Apis mellifica*; Homeopathic dilutions; Gene expression; Inflammation

Introduction

The use of bee products for medicinal purposes has been described in many antique medical books by Hippocrates (460–370 BC), Aristotle (384–332 BC) and Galen (130–200 AD), just to mention the most famous sources. In the Middle Ages, bee venom was used as a medical remedy to relieve pain and to treat inflammatory diseases such as arthritis and rheumatism[1–3] and is now a common homeopathic remedy. In fact one of the cardinal principles of homeopathic medicine is the 'law of similarities', according to which patients can be treated by administering substances which cause symptoms similar to those presented by the patient himself, when tested in healthy subjects.[11]

Bee stings produce persistent pain and swelling under normal conditions and a few controlled reports in humans indicate that bee venom can exert systemic anti-inflammatory and antinociceptive effects. A Chinese

*Correspondence: Elisabetta Bigagli, Department of Neurofarba, Section of Pharmacology and Toxicology, University of Florence, Viale Pieraccini 6, 50139 Florence, Italy.
E-mail: elisabetta.bigagli@unifi.it
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clinical trial recently showed beneficial effects of bee venom therapy in knee osteoarthritis[4]; in combination with conventional drugs bee venom therapy was also reported to cure the symptoms and inhibit the relapse of rheumatoid arthritis.[5] Bee venom has antinociceptive activity and reduces inflammation in collagen-induced arthritis in rodents[6–8]; suppresses the NF-kappaB pathway through interaction with the p50 subunit[6]; reduces visceral pain through spinal alpha2-adrenergic activity in mice[9]; inhibits beta-tyrosine phosphorylation of PDGF receptors and modulates downstream intracellular signal transduction.[10]

Dose-related inverse responses, defined as 'hormetic', using the terminology proposed by Edward Calabrese,[17] could be a mechanism underlying the inversion of the effect of a xenobiotic compound at low dilution, recalling the traditional '*similia principle*' of homeopathy. However, even the most sensitive technologies used in modern pharmacology cannot detect signals below a certain threshold. We tried, with success, to overcome this difficulty using genome-wide gene expression analysis on cellular systems *in vitro*. We previously demonstrated that solutions of copper sulfate in concentrations ranging from 10^{-6} to 10^{-17} M modulated many genes, some dose-dependently; many of these genes belong to gene families with important functions in the cell.[12]

Based on these premises, we studied the effects of *Apis mellifica preparations*, using a human cell culture to evaluate global gene expression with the microarray technique, a method which can detect integrated cell responses at extremely diluted concentrations.[12]

Materials and methods

Apis mellifica mother tincture (TM) was prepared by the Boiron Laboratories, Lyon, France, according to Pharmacopoeia procedures in 65% ethanol. Dynamized dilutions (3C, 5C, 7C) were prepared in 30% ethanol by Boiron Laboratories (Lyon, France). The reference 65% and 30% ethanol solutions were prepared at the University of Florence using dynamized water obtained from Boiron Laboratories.

Cell line and treatments

We used RWPE-1 cells, an immortalized human normal prostate epithelial cell line obtained from ATCC (Manassas, VA, USA) for all the experiments. The cells were grown in keratinocyte-SFM medium (Gibco, Milan, Italy), in T-25 plastic culture flasks at 37°C in a 5% CO_2 atmosphere, until 60% of confluence and were then exposed to the *Apis mellifica* solutions or to the reference ethanol solutions for 24 h. Each test solution was added to the culture medium in a volume of 50 μl in a total incubation medium of 5 ml, obtaining a final dilution of 1:100. Each treatment was replicated 5 times, obtaining 5 biological replicates for each experimental point.

Cells were then harvested using the lysis buffer provided by the RNAasy Mini kit Plus (Qiagen, Milan, Italy) and stored at −20°C.

The cytotoxicity of each *Apis mellifica* dilution was preliminarily estimated using the neutral red uptake inhibition assay; none of the tested solutions of *Apis mellifica* induced discernible cytotoxicity.

Transcriptomic analysis

Total RNA was extracted using the RNeasy Mini kit Plus (Qiagen, Milan, Italy). Gene expression analysis was performed using the Agilent Whole Human Genome microarray, containing 44K spots, corresponding to 41,000 human genes and transcripts; for each experimental point we prepared pools of RNAs obtained by mixing equal amount of RNA from the 5 biological replicates.

The labeling and hybridization steps were carried out according to the Agilent protocol (Two-Color Microarray-Based Gene Expression Analysis version 5.7), using a two-color microarray protocol in which *Apis mellifica*-treated cells were contrasted with cells exposed to the corresponding ethanol solution (65% ethanol for the TM and 30% ethanol for the homeopathic *Apis* dilutions). The labeled samples were hybridized to Agilent Human GE 4x44K v2 Oligo 60-mer microarrays, in Agilent microarray chambers (G2534A) at 65°C for 18 h.

The images were scanned using a Genepix 4000B microarray scanner at 5-μm resolution (Axon Instruments, Foster City, CA, USA). Image analysis and initial quality control were performed using Agilent Feature Extraction Software v9.5. 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 for both channels, a signal well above background and a minimal signal intensity for at least one channel.

Microarray data were used for hierarchical unsupervised cluster analysis (Eisen software, freely available at http://rana.stanford.edu) to compare and correlate the expression profiles. Pathway analysis to evaluate the biological effects of *Apis mellifica* was performed using GO-elite version 1.2 beta, an open-source, freely available application (http://www.genmapp.org/go_elite).

RT-PCR

The results obtained from the microarray analysis were confirmed by semi-quantitative RT-PCR by selecting the following 5 candidate genes from those most differentially expressed by comparing TM and homeopathic dilution-treated cells: interleukin1beta (IL1β), CD46 complement regulatory protein (CD46), activating transcription factor1 (ATF1), ubiquitin-conjugating enzyme E2Q (putative) 2 (UBE2Q2) and metallothionein 1X (MT1X).

About 100 ng of total RNA were reverse-transcribed using 100 units of SuperScript™ II Reverse Transcriptase (Life Technologies, Milan, Italy) and 1× random examers (Roche Diagnostics, Monza, Italy). Each gene was co-amplified with GAPDH as an internal standard. PCRs were carried out using 2 μl of cDNA in a 25 μl total volume containing 1× PCR buffer, 1.5 mM $MgCl_2$, 0.5 mM dNTPs, 8 ng/μl of primer, 0.1 ng/μl of GAPDH primers

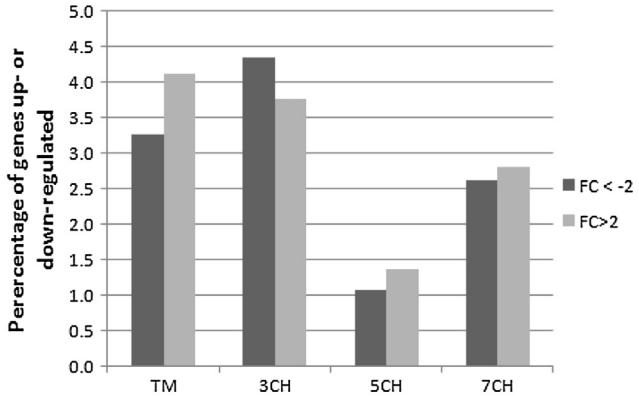
and 1.25 units of Taq polymerase (Sigma−Aldrich, Milan, Italy). The primers used were, for IL1β: 5′-GGA CAAGCTGAGGAAGATGC-3′ and 5′-TCTTTCAA CACGCAGGACAG-3′ (360 bp); for CD46: 5′-GTCCCTGCAAATGGGACTTA-3′ and 5′-TGGAAA TCGACATTTGACCA-3′ (363 bp); for ATF1: 5′-GA AGATACACGGGGCAGAAA-3′ and 5′-CCACAGT TTGTGGCAGAGAA-3′ (364 bp); for UBE2Q2: 5′-CCTCCATTTGTTCGAGTGGT-3′ and 5′-GCTTT TGTCTGCATGTTGGA-3′ (355 bp); for MT1X: 5′-AC CACGCTTTTCATCTGTCC-3′ and 5′-AGGAGCAG CAGCTCTTCTTG-3′ (177 bp); for GAPDH: 5′-CCC TCAAGGGCATCCTGGGCT-3′ and 5′-GCAGGGACT CCCCAGCAGTGA-3′ (275 bp). The PCR conditions were: 95°C for 7 min and 35 cycles at 95°C for 30 s, 60°C (55°C for ASP90AA1) for 30 s and 72°C for 55 s and a final extension at 72°C for 5 min. The PCR products were separated on 1.8% agarose gel and visualized by ethidium bromide staining. Gel images were captured by a digital photocamera and the intensity of the bands analyzed with the Quantity-One software (Bio-Rad, Segrate, Milan, Italy).

Results

Figure 1 shows the percentage of differentially expressed genes induced by the treatments with *Apis mellifica* relative to controls treated with the same type and amount of solvent (ethanol:dynamized water). It is interesting to note that the number of genes modulated by the TM and the 3C dilution was similar; this finding suggests that the 3C dilution behaved similarly to the concentrated solution, while treatments with lower dilutions (5C and 7C) modulated a smaller number of genes.

Hierarchical cluster analysis, performed on gene expression data which passed the quality control step and with quantitative data present in all experiments, discriminated between the expression profile of cells treated with *Apis mellifica* TM and that of the cells exposed to 3C, 5C and 7C solutions. One can observe that some genes showed similar expression profile, being up- and down-regulated in cells treated with TM and diluted *Apis mellifica*, solutions, including the most diluted one (7C). It is worth noting that clusters of genes also emerged (indicated by the arrows in Figure 2) with different and even opposed profiles in cells treated with TM or with 3C, 5C and 7C solutions.

We identified several pathways modulated by *Apis mellifica* by functional analysis. Among the most interesting were proteasome degradation and Keap1-Nrf2 which were up-regulated while TNFa/NfkB signaling pathways were down-regulated in cells treated only with *Apis mellifica* TM. The apoptotic process was down-regulated only by the most diluted solutions of *Apis mellifica* (5C and 7C). IL-5 and IL-6 and Wnt signaling pathways were down-



	FC<-2	FC>2	number of genes passing the quality control step
TM	391	495	11985
3CH	558	483	12828
5CH	132	168	12352
7CH	328	352	12530

Figure 1 Percentage of genes with fold change above or below a conventional cut-off value of 2, after comparing cells exposed to *Apis mellifica* solutions with cells exposed to the corresponding vehicle. The table below reports the number of genes found up or down-regulated in each comparison.

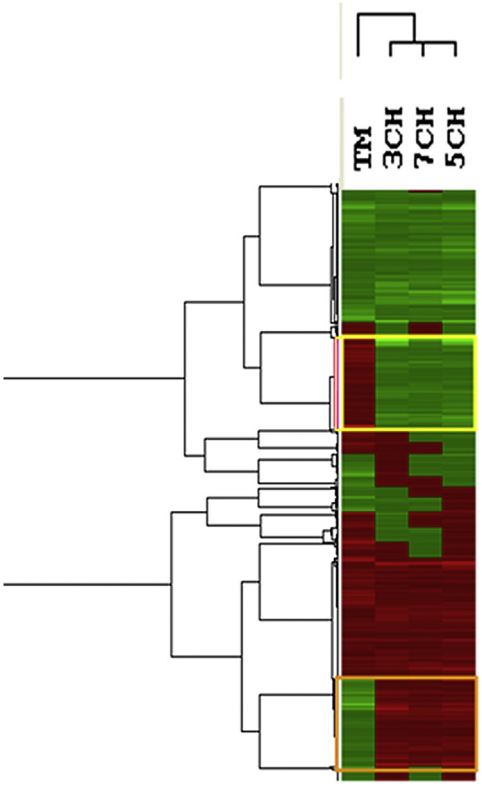


Figure 2 Hierarchical cluster of the gene expression profiles of cells exposed to *Apis mellifica* (TM) and 3C, 5C and 7C dilutions in comparison to the corresponding vehicle. Columns represent each experiment, rows represent each single gene analyzed; red represents up-regulation; green indicates down-regulation. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

regulated by *Apis mellifica* 3C and 5C. Among the group of genes significantly modulated by all *Apis mellifica* dilutions (3C, 5C and 7C) we noticed up-regulated genes of Rho-GTPase family and down-regulated genes of the major histocompatibility complex and of the tubulin gene family. The most diluted solutions (5C and 7C) up-regulated interferon regulatory factors and integrin receptors, which instead were not modulated by the TM or by the lowest *Apis mellifica* dilution (3C). *Apis mellifica* TM up-regulated genes of pro-inflammatory cytokines such as IL1β and IL1α and HMGO family box, but down-regulated IL18 and chemokine CX3CL as well as genes encoding for collagen subunits. These families of genes were not regulated in cells exposed to 3C, 5C and 7C solutions.

We confirmed data obtained with microarrays by RT-PCR, selecting 5 candidate genes among those observed as the most up- (highlighted as red (in the web version)) or down-regulated (highlighted as green (in the web version)). Microarray results on the expression of all five genes were confirmed by RT-PCR. It is interesting to note that IL1β, a potent pro-inflammatory cytokine, was up-regulated by the TM and down-regulated by all *Apis mellifica* dilutions (Figure 3).

Discussion

In recent decades many laboratories, some of which are not currently involved in homeopathic research, have attempted to study the effects of extremely diluted concentrations of drugs or natural products. Unfortunately, most methods employed even in modern pharmacological sciences, although sensitive, cannot detect signals below certain threshold concentrations.

We have tried to overcome this difficulty by using genome-wide gene expression analysis. These technologies offer the possibility of analyzing global patterns of gene variation and functional cell changes at concentrations lower than classical pharmacological and toxicological responses.

Using microarray analysis we previously demonstrated that cells exposed to copper sulfate at concentrations varying from 10^{-6} to 10^{-17} M alter gene expression even at the most diluted concentreations.[12] The advantage of using xenobiotic preparations in these studies rests on the possibility of obtaining signatures of specific effects at extreme dilutions; in fact, studying substances naturally present in cells is difficult since signals are often small and can be confused with experimental noise.

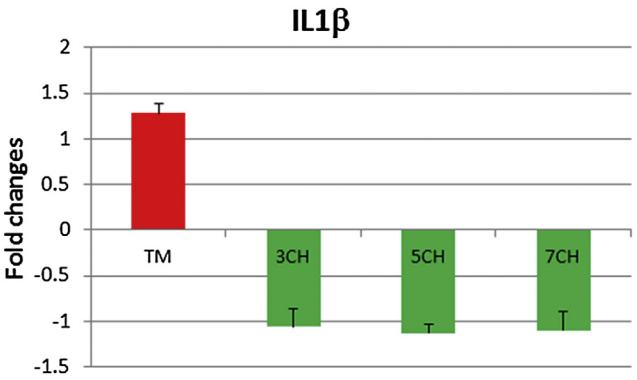


Figure 3 RT-PCR results relative to the IL1β expression in cells exposed to *Apis mellifica* tincture and 3C, 5C and 7C preparations, for 24 h. Results are expressed as fold changes compared to cells exposed to the corresponding vehicle. Means ± SE.

The present study was designed to test extreme dilutions of *Apis mellifica* on gene expression. According to the law of similarities[11] and the specific literature, these preparations can have anti-inflammatory or anti-edema activity. In fact, 9C *Apis mellifica* has been reported to inhibit basophil degranulation induced by anti-IgE antibody[13]; dilutions of *Apis mellifica*, obtained from the whole bee (7C or 10^{-14} and 9C or 10^{-18}) have been shown to reduce UV-induced erythema in guinea pigs[14]; D4 *Apis mellifica* administered orally or by subplantar injections in rats has a slight inhibitory effect on edema (this effect was not confirmed in a second set of experiments).[15] Recently, Yang and coworkers[16] observed that bee venom prevented the inflammation-induced death of motor neurons and alleviated mitochondrial disruption in a transgenic mice model of amyotrophic lateral sclerosis.

In this context our experiments are intriguing and potentially relevant. We demonstrate that there is a marked variation in gene expression due to the venom TM solution. This was not surprising, since an ethanol bee extract contains fractions of the bee venom, which has known pharmacological and toxicological effects.

The interesting and novel observation is that extremely diluted solutions of *Apis mellifica* (up to 10^{-16}) still have effects on gene expression. The key finding of our experiments rests on the observation that, according to the laws of hormesis, groups of genes are regulated in opposite directions (up or down, respectively) by the TM and by extremely diluted solutions. This is often the case in homeopathy and can be explained as 'hormetic' responses, whereby hormesis is a biphasic dose—response curve in which stimulatory effects are observed with low doses and the reverse is observed after high dosage.[16] These stimulatory responses are often difficult to detect and regularly overlooked by the mainstream scientific community.

Using the highly sensitive technique of microarray gene expression analysis, we were able to demonstrate the hormetic principle as described by Edward Calabrese, by which diluted concentrations often exhibit opposite effects of concentrated ones.[17]

Apis mellifica TM exerted several effects on gene expression; the most interesting being related to oxidative stress and inflammation. These effects were complex: *Apis mellifica* TM down-regulated TNFα receptors as well as their associated factor (TRAF-3), suggesting an anti-inflammatory effect. It also down-regulated the CX3CL gene, encoding for a chemo-attractant pro-inflammatory chemokine with a likely role in the pathogenesis of arthritis.[18] On the contrary, we observed an up-regulation of the genes of IL1α and IL1β (well known pro-inflammatory cytokines) and of High-Mobility Group Box (HMGO), a free radical damage-associated molecule

released from injured and necrotic cells[19]; the gene of IL18 was also down-regulated. Free radicals decrease IL-18 secretion,[20] while its levels are significantly elevated in patients with rheumatic arthritis.[21]

The activation of an anti-oxidative response is also supported by the induction of the Keap-Nrf2 pathway. The transcription factor Nrf2 has been identified as the master regulator of several hundreds of genes involved in the anti-oxidant defense response.[22] It is worth highlighting that *Apis mellifica* TM also down-regulated several genes encoding for collagen, an important structural protein which is likely an active player in inflammatory processes.[23]

The key result of our experiments is the demonstration that 3C, 5C and 7C *Apis mellifica* dilutions exerted complex effects on gene expression on their own: in fact, only they, and not the TM, up-regulated members of the Rho-GTPase family of genes, which control phagocytosis, cell polarity, proliferation, survival, gene transcription, microtubule dynamics and vesicular transport and are critical coordinators of inflammatory responses.[24] Moreover, only 3C, 5C and 7C *Apis mellifica* down-regulated genes of the major histocompatibility complex, induced by interferon and modulated in the presence of cytokines, hormones and other inflammatory agents. It should be highlighted that the most diluted solutions of *Apis mellifica* (5C and 7C) were able to retain effects on gene expression by up-regulating IRF-2 genes which are critical suppressors of IFN-α/β signals[25] and $\beta 2$ integrin receptors, known for regulating adhesion and phagocytosis and the resolution of inflammation.[26]

In conclusion, our results are consistent with the few previous documented anti-inflammatory activities of *Apis mellifica* preparations and present evidence supporting the statement that substances at homeopathic dilutions may have biological effects.

The key finding of our experiments rests on the observation that, according to the hormetic phenomenon, groups of genes were regulated in opposite directions (up or down, respectively) by the TM and by extremely diluted solutions. It is worth emphasizing that *Apis mellifica* 3C, 5C and 7C homeopathic dilutions were able to modulate the expression of many genes, including some linked to inflammatory processes, offering a possible mechanistic explanation for the anti-inflammatory activity of these preparations *in vivo*.

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