

# Dose response of high dilutions in gene expression and the concept of hormesis

Human and Experimental Toxicology  
2017, Vol. 36(8) 876–877  
© The Author(s) 2016  
Reprints and permission:  
sagepub.co.uk/journalsPermissions.nav  
DOI: 10.1177/0960327116665677  
journals.sagepub.com/home/het



Salvatore Chirumbolo

I read with particular interest the recent article by Bigagli et al. on the latest issue of dose response, where the authors showed the effect of homeopathic dilutions of *Apis mellifica* on the gene expression of human RWPE-1 cells (a human prostate cell line containing human papilloma virus sequences). This is an human papilloma virus (HPV) immortalized adult human epithelial prostate cell line.<sup>1</sup> The authors assessed that genome microarray is a highly sensitive approach to verify the hormetic effect of very low concentrations of agonists and reported several references in the literature.<sup>1</sup> Effects of water/ethanol (EtOH) high diluted materials, made following the French Homeopathic Pharmacopoeia, were thoroughly discussed elsewhere.<sup>2–4</sup>

Although recent studies were performed in the field, particularly from Bellavite's group at the University of Verona, Italy,<sup>2–4</sup> the article by Bigagli et al. deserves some further comment, yet.

The authors concluded that extremely diluted *A. mellifica* preparations retained a biological effect on gene expression, although they did not forward any speculative explanation of the reported evidence, contrarily to others.<sup>4</sup> This biological effect should be associated with *Apis* venom components but, while describing the preparation of their dilutions, the authors did not perform either any analytical chemistry of *Apis* components or mention any effort to assay if testing solutions were contaminants and endotoxin free, as any dilution was prepared with the same solvent concentration. The authors assessed that some contaminants were checked, yet no biological traces appeared to be monitored. They considered high dilutions as “nanomaterials,” despite the fact that homeopathic preparations should theoretically contain no molecule of the starting concentrated material. In this sense, high dilutions undergoing contamination from adventitious adsorption of bacteria-derived ubiquitous endotoxins, pyrogens or lipopolysaccharide, or

even polyaromatic hydrocarbons, a cross-contamination possibly occurring in solvents used for the study, should retain practically only contaminants as bioactive material.<sup>5,6</sup> Furthermore, many physical and chemical properties of these materials present in dilutions used for the experimental settings may alter dramatically biological systems at the nanoscopic scale, and furthermore the use of nanotechnological systems such as gene microarray requires a skillful and careful assessment of unexpected toxicities, contaminants, and biological interactions to prevent the possible experimental bias.<sup>7</sup> This issue may be yet misunderstood. Homeopathic dilutions cannot be considered a “nanopharmacology” issue at all, as many homeopathic preparations are unidentifiable about their actual molar concentration of substances, they often involve a water/alcohol solvent and possess features that overwhelm the Avogadro's constant-related physical chemistry. Yet, contaminants and specific reactants are brought on the experimental setting by solvents, which were never diluted during the serially diluting process. In this circumstance, substances present in solvents increase their relative concentrations with respect to possible active principles that yet undergo a serially diluting process, then enhancing the bioactivity of these solvent-bearing cross-contaminants at the nanoscopic level. It would be very interesting to know whether the authors used the same preparations for different separate experiments and how many. The presence of

---

Department of Medicine, University of Verona, Verona, Italy

**Corresponding author:**

Salvatore Chirumbolo, Department of Medicine, University of Verona, LURM Est Policlinico GB Rossi, Piazzale AL Scuro 10 37134 Verona, Italy.

Email: salvatore.chirumbolo@univr.it

organic contaminants in high dilutions used for tests, if ascertained, may generate a bias in the interpretation of results.<sup>1</sup> Chemical analyses of dilutions should verify the hypothesis of which *Apis* component is active within the water/EtOH systems. This fact, if verified, may shed a very interesting light on the hormetic effect of some *Apis* venom components of RWPE-1 gene expression.

*A. mellifica*, likewise other complex mixtures used in this research field, contains a panoply of different molecules, for which no chemical analysis and molar concentration were performed and described in the article.<sup>1</sup> Interestingly, the authors did not report any significant effect with *Apis* 3C, which should represent the highest concentration of *Apis* bioactive components in this research study.<sup>1</sup> Therefore, many biased issues are present.

The authors reported that the Venn diagram EtOH versus RWPE shows a highly different number of genes, from 97 (*Apis* 9C) to 257 (*Apis* 30C), apparently without a reason, the evidence probably due to intra-series variability, although no sound statistics was accomplished.<sup>1</sup> Dilutions of EtOH, according to the same process used with *Apis* dilutions, should report a decreasing number of genes, thus following the dilution series. It should be stated that EtOH sham dilutions were prepared in a final 0.3% EtOH, starting from a 30% EtOH, therefore they were not comparable to *Apis* dilution in water-EtOH solvent, that is, the concentration of EtOH in the control EtOH does not decrease serially with the dilution step, as a relevant quantity of EtOH is always present in the testing system, due to the solvent added.<sup>1</sup> This may explain why some values in the bottom Venn diagrams are approximately the same in the series. Furthermore, as authors' data did not clarify whether dilutions were made either into water or not, to better highlight the effect of EtOH, a water versus RWPE Venn diagram should be needed.

From a simple chemical point of view, as expressed genes are collectively gathered indifferently as either inhibited or stimulated, one should expect a decrease in the whole expressed genes following dilutions and then a significant increase within the lowest concentration range, for example, from 9C or 12C onwards. Furthermore, one should expect that, in some of them, dose response should perform a U-shaped curve, while plots resembled a normally distributed curve, suggesting likewise the effect of chance.<sup>1</sup> Yet, a hormetic effect of gene expression may be very difficult to demonstrate. In this respect, the authors should correlate their graphs with rigorous statistical calculations. Furthermore, the effect of EtOH cannot be dismissed, despite the many crossed controls versus sham dilutions, particularly at

the 5C dilution.<sup>1,2</sup> This bias seems to be evident when comparing each panel in the article results and one should expect that the effect of *Apis* is conserved in both the reported plots for dilutions 5C and 9C, while they disappeared (particularly for 9C) by withdrawing EtOH from the assay, while 12C remains unchanged.<sup>1</sup>

The authors showed therefore the overall distribution of up- or downregulated genes and reported some bias. If *Apis* 9C is the only cause of the significant expression of genes, once removed the effect of EtOH, this effect should be observed also in the control *Apis* versus non *Apis*. Main unexplained discrepancies are shown in 5C and 9C and the relative graph, which was plotted based on changes of individual genes over different dilutions, could be more meaningful.

In conclusion, the evidence reported by Bigagli et al. on the effects of homeopathic dilutions of *Apis* raised some criticism because (a) *Apis* was not molecularly and chemically characterized; (b) dilutions may contain microbial or chemical contaminants, working at the nanoscopic level; (c) EtOH was not completely subtracted from statistics as a potential confounder; and (d) the relationship between gene up- or downregulation did not involve further important controls to prevent bias from the solvents.

While this group of studies appear highly appealing in the dose-response field, a sound and reliable bias and confounders checking has a major role and cannot never be disappointed.

## References

1. Bigagli E, Luceri C, Dei A, et al. Effects of extreme dilutions of *Apis mellifica* preparations on gene expression profiles of human cells. *Dose Response* 2016; 14(1): 1559325815626685. doi: 10.1177/1559325815626685.
2. Chirumbolo S. High diluted molecules and gene expression. *Front Pharmacol* 2014; 5: 183.
3. Marzotto M, Olioso D and Bellavite P. Gene expression and highly diluted molecules. *Front Pharmacol* 2014; 5: 237.
4. Olioso D, Marzotto M, Moratti E, et al. Effects of *Gelsemium sempervirens* L. on pathway-focused gene expression profiling in neuronal cells. *J Ethnopharmacol* 2014; 153(2): 535–539.
5. Jones CF and Grainger DW. In vitro assessments of nanomaterial toxicity. *Adv Drug Deliv Rev* 2009; 61(6): 438–456.
6. Chirumbolo S. On gelsemium and complementary and alternative medicine (CAM) in anxiety and experimental neurology. *Neurol Therapy* 2015; 4(1): 1–10.
7. Aillon KL, Xie Y, El-Gendy N, et al. Effects of nanomaterial physicochemical properties on in vivo toxicity. *Adv Drug Deliv Rev* 2009; 61(6): 457–466.