

REVIEW

Hormetic effects of extremely diluted solutions on gene expression



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This paper summarizes the results of investigations showing how molecular biological tools, such as DNA-microarrays, can provide useful suggestions about the behaviour of human organisms treated with microamounts of drugs or homeopathic medicines. The results reviewed here suggest firstly that the action of drugs is not quenched by ultra-high dilution and proceeds through modulation of gene expressions. The efficacy of drug solutions seems to be maintained in ultra-highly diluted preparations, a fact which constitutes a challenge to the dogma of quantization of matter.

The second and more important result is that the different gene expression profiles of cell systems treated with the same drugs at different dilutions suggest the existence of hormetic mechanisms. The gene expression profiles of cells treated with copper(II) sulfate, *Gelsemium sempervirens* and *Apis mellifica*, are characterized by the same common denominator of the concentration-dependent inversion of gene expression, which can justify at a molecular level the concept of *simile* adopted in homeopathy.

The main conclusion we draw from these results is that these procedures provide new kinds of information and a tool for disclosing the mechanisms involved in hormetic effects. The application of these effects to modern medicine may allow researchers to conceive unprecedented therapeutic applications or to optimize the currently used ones in the framework of a low-dose pharmacology based on a reliable experimental platform. *Homeopathy* (2015) 104, 116–122.

Key words: Gene expression; Hormesis; Microarray; Homeopathy; Low-dose pharmacology

Introduction

The term hormesis means the phenomenon of dose–response relationships in which something (such as a heavy metal or ionizing radiation) that produces a given biological effect at moderate to high doses may produce an opposite effect at low doses. This term briefly defines the behavioral relationships between living systems and their surrounding world. In a general sense hormesis means that a living organism experiences an advantageous and

favorable biological response once slightly perturbed, the perturbation being an environmental stress. Since the same stressor agent may induce harmful consequences at high doses, the theory of hormesis maintains that the reaction of a living organism to an external perturbation can qualitatively differ, according to the intensity of the perturbation.^{1,2} This enantiodromic response is related to the nature of the living organism. The number of depicted examples suggests that this behavior represents a general biological law whose roots can be found in the autopoietic character of the living organism, as defined by Maturana and Varela,³ which in turn can be described in terms of negative entropy, information theory, non-equilibrium thermodynamics, Lamarckian evolution and biological plasticity. In this sense this concept constitutes a basic pillar for defining the complex paradigm involved in the interaction of any living system with environmental

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molecular events. The enormous implications of this achievement have been reported and discussed in detail by Edward Calabrese and his co-workers in the recent past, and in this issue of *Homeopathy*.^{4–9} This work constitutes a Keplerian revolution of toxicology and environmental sciences.

Even though hormesis should be considered a theme of the ontogeny and phylogeny of any living being, it still is not appropriately considered for its biomedical implications. This is not a novelty since it is common practice in human society for one group to try to deny access to reward to another group, if the latter invalidates the power prerequisites of the former.

Biomedicine achieved its undeniably enormous success by assuming that the complexity of phenomenology can be considered as the result of the overlap of many simple, individual events, which can be intrinsically described in terms of a linear relationship $y = kx$, the classical cause-effect quantitative relationship of Galilean philosophy. The classical approach to biomedicine presumes the existence of a direct correlation between the dose of drug and its therapeutic effect, expressed by the well known sigmoidal dose–response curve which plots the response vs. the logarithm of drug concentration. This hypothesis is valid in a limited dose range and is currently adopted in biomedical pharmacology. However, when the magnitude of the stressor (dose of drug) is diminished, a qualitatively inverted response is observed and the dose–response curve is generally better described as J-shaped. This feature, which illustrates the hormetic behavior of a perturbed biological system, can provide support to all therapeutic methodologies involving low doses of drugs or stressors and the *simile* as pharmacological philosophy.

Evidence of hormesis experimentally refutes traditional pharmacology by showing the interplay of related biological events of a living organism once perturbed by a drug.^{10–13} Modern pharmacology now accepts experimental evidence by often assuming hormesis as an example of the traditional paradigm. However, it is important to emphasize that there is a big difference between the traditional view and the hormesis-based one. The difference has been outlined in the second half of the past century, starting with Warren Weaver more than 60 years ago.¹⁴ The response of a living organism to a stressor must be considered an emergent property of a nonlinear network and cannot be interpreted with the rough approximation of a linear relationship, which is operative in a set of independent events.

Pharmacologists have difficulty with the concept of a nonlinear network because they hardly realize it in its own whole scientific perspective. In particular it is not easy to elicit an unambiguous interpretation when the observed effects are controlled by a large number of parameters and depend on the physiological state of the organism. Notwithstanding these difficulties, this interplay cannot be denied, since both useful and harmful consequences in medical care can occur.

Hormesis can support and justify some aspects of therapeutic methodologies such as homeopathy,^{15–23} which are

sometimes based on speculative claims. This feature has undoubtedly contributed in the past to academic pharmacology to understating hormetic phenomenology. The relevance of the hormetic concept has also been opposed by those who, like homeopaths, should have found in it the keystone of their therapeutic tenets.^{24–28} As observed by Bellavite,²⁶ the ‘holistic’ approach of homeopathy as a healing system goes far beyond the identification of specific information. Using similar arguments, our proposal²⁰ of using hormesis to shift homeopathy into the framework of rational low-dose pharmacology has been strongly criticized.²⁸

Our approach, which does not necessarily fit with any holistic consideration, is based only on experimental evidence regarding the determination of gene expression profiles by assuming that they provide a reliable experimental platform for showing the dose-dependent effects of diluted drugs in living organisms. In other words, we wish to answer the question put by Jonas and Ives “*Should we explore the clinical utility of the hormesis?*”^{29,30} The present article summarizes the results of investigations showing how the use of molecular biological tools, such as DNA-microarrays, can provide useful suggestions about the behavior of human organisms once treated with different microamounts of drugs or homeopathic remedies. We are convinced that this procedure is of fundamental importance in identifying the main pathways of interaction between living organisms and perturbing drugs. We then discuss the potential impact that these studies on the future of pharmacology.

DNA-microarrays as a tool for measuring gene expression profiles

The properties of macroscopic matter are related to the properties of its microscopic units. This is in agreement with the statement that the whole is nothing but the sum of its parts. The problem is what ‘the sum of parts’ means. Basic research in pharmacology is carried out according to the belief that the interactions of a molecule with organism units follow simple rules, though often the application of these rules is complicated. But this in principle is relatively unimportant since it is always possible that in the future tools may be developed which could solve these complications.

Bearing this in mind, pharmacology describes its own perspective in terms of ontological or sometimes epistemological reductionism. Indeed the real problem is to conceptualize an external perturbation–response pattern in a network approach, even though the perturbation modifies the local environment of individual units according to the expectations mentioned above. In this framework the measure of gene expression profiles provides an important tool for understanding the very heart of the network system. This measurement is usually carried out by using DNA-microarray technology.

The power of this technique lies mainly in observing a change of gene expression pattern in patients with the

same disease. A second powerful application relies on identifying the drug properties by following the change of DNA gene expression.³¹ The technique exploits the fact that only some of the 20,000–25,000 genes constituting a genome produce mRNA, this active fraction varying as environmental conditions change. The technique therefore is particularly suitable for investigating the response of the genomic system to different amounts of a perturbing agent, e.g. a drug in different concentrations. It is important to stress that the technique offers the possibility of determining the activity of many genes at the same time, and can compare the activity of genes before and after the cell has been treated with a drug.

Traditional physical chemistry laboratory methods work in a significantly more limited range of concentrations (>than picomolar) than the DNA microarray technique. Although the technique has some intrinsic limitations³¹ due to the fact it provides too many answers at once (difficult to analyse) or too complex to interpret, or often not quantitative enough, it provides a very effective tool for learning about the reactivity of a biological system in limiting conditions. For example, the results we obtained³² in investigating how copper(II) ion concentrations affect the gene expression profiles of human prostate epithelial cell lines (RWPE-1). This choice was determined by the fact that these cells can grow in the absence of serum which constitutes an undesirable source of Cu ions.

Treated RWPE-1 cells were exposed for 24 h to copper(II) sulfate at concentrations varying from 10^{-6} to 10^{-17} M, untreated reference cells were exposed to the same volume of water. The copper solutions were not succussed as is usual in homeopathic preparations, only mechanically stirred. Relative gene expression differences between copper-treated and control cells were examined using microarray technology on a platform containing 44K spots, corresponding to 41,000 human genes and transcripts, permitting a complete investigation of gene expression profiles. Microarray data demonstrated that the addition of Cu to the medium modulated gene expression at all concentrations, including the most dilute. The percentage of significantly up- or down-regulated genes was not correlated in a simple fashion with the amount of Cu added to the medium, although higher concentrations of Cu had a tendency to show bigger effects.

We identified a group of 156 down-regulated genes (Cluster 1) and a second group of 249 up-regulated genes (Cluster 2) at all Cu concentrations compared to control. The most interesting result we observed was a third cluster (Cluster 3) of genes with opposite behavior at high and low Cu concentrations, suggesting that hormetic mechanisms were operative.

In Cluster 3 we found 164 up-regulated genes in cells treated with 10^{-6} – 10^{-9} M Cu, and down-regulated by Cu at a lower concentration (Cluster 3a). This group included genes codifying for an ATPase transporting protein, members of the collagen family, histone proteins, heat shock proteins, metallothioneins and metal-regulatory transcription factors. A second group of 244 genes was down-regulated in cells treated with

10^{-6} – 10^{-13} M Cu and up-regulated at lower doses (Cluster 3b). Among these genes we noted an ATPase, a lysyl oxidase (LOX), and a metal ion transporter. Finally, a third group of 233 genes were up-regulated up to 10^{-14} M Cu and down-regulated from 10^{-15} to 10^{-17} M Cu (Cluster 3c). Among them, we noted a cytochrome c oxidase gene coding for histone proteins, a heat shock protein and ribosomal protein solute carriers, ubiquitin conjugating enzymes and metallothioneins.

Reverse-transcription-PCR (RT-PCR) analysis was performed on 5 biological replicates for each Cu concentration tested, with the aim of evaluating the variability of the experimental system and of controlling the quantitative variation observed with the microarray experiments. For this analysis we selected four genes among those showing opposite behavior at different Cu concentrations (HIST1H1D, HIST1H4B, HSP90AA1, HSPA8). The results were consistent with those obtained with the microarray technique.

In conclusion, this study shows that the technique allowed study of the changes in gene expression at concentrations much lower than those associated with pharmacological responses. This investigation emphasizes that hormetic effects operate in well defined ranges of concentration. Finally and unexpectedly, we observed a modulation of gene expression even at extremely low copper concentrations. This is possibly the result of very low concentrations of free copper(II) inside the cells and suggests that even extremely low doses of copper may have biological effects. In the next section we will summarize the results of investigations aimed at determining the gene expression profiles of cells treated with diluted homeopathic remedies.

Homeopathic medicine studies

Some studies have been carried out using the microarray technique with the aim of supporting the claim that ultra-high diluted homeopathic remedies induce biological effects which thus affect the gene expression profiles. This hypothesis of Khuda-Bukhsh^{33,34} has been confuted by some studies of Thangapazham, Jonas *et al.*^{35,36} However this hypothesis seems to be consistent with the findings of several studies. Unfortunately these investigations used a single dilution of the homeopathic preparation and it is not possible to demonstrate the existence of hormetic effects. For example, de Oliveira *et al.*³⁷ investigated the effects of Canova, a complex homeopathic medication (*Aconitum* 11D, *Thuja* 19D, *Bryonia* 18D, *Arsenicum* 19D and *Lachesis* 18D), on cytokine production and gene expression in murine macrophages. It was found that the Canova-treated samples differentially expressed 147 genes and a decrease in IL-2 and IL-4 production when compared to those treated with placebo. These genes are mainly involved in transcription/translation, cell structure and dynamics, immune response, cytoprotection, enzymatic process, and receptors/ligands.

Recently Khuda-Bukhsh and coworkers have shown³⁸ that the gene expression profiles of HeLa cells (an established epigenetic model of HPV18 positive cell line)

treated with *Condurango* 30c and *Hydrastis canadensis* 30c show distinctly different expression patterns of over 100 genes when compared to control. The authors suggest that this result demonstrates that both drugs and placebo differ in their ability to trigger gene responses. Finally Preethi *et al.*³⁹ investigated the effects of different homeopathic preparations (*Ruta* 200c, *Carcinosinum* 200c, *Hydrastis* 200c, *Thuja* 200c) on Dalton's lymphoma tumor cells. By using both RT-PCR and microarray, the apoptosis and expression of specific genes were measured. It was found that these ultra-highly diluted preparations exhibited cytotoxic activity higher than the mother tincture, although the authors stress that the ultra diluted solutions and the mother tinctures have qualitatively similar activity.

Two recent studies carried out by using a homeopathic medication at different concentrations provide clear evidence of the existence of hormetic effects. The studies concern *Gelsemium sempervirens* and *Apis mellifica*. Traditionally, *Gelsemium* is described as a remedy for a variety of anxiety-like psychological and behavioral symptoms. The main active principles of the plant are believed to be gelsemine and other strychnine-like alkaloids. The operative mechanism of this remedy is largely unknown, although some hypotheses about its pharmacodynamics have been formulated. This plant may possess anticancer and immunomodulation properties.

One intriguing study about the mechanism of this plant has been recently reported by Bellavite and coworkers^{40,41} using the microarray technique. This study shows that when human SH-SY5Y neuroblastoma cells are exposed for 24 h to a series of test dilutions of *Gelsemium* (2c, 3c, 4c, 5c, 9c, and 30c) and their transcriptome is compared by microarray to that of cells treated with control vehicle solutions, the expressions of a large number of genes are modulated. Here it is worth mentioning that the gelsemine content of the above test solutions formally ranges from 10^{-9} M (the 2c solution) to 10^{-65} M (the 30c one). Although the analysis of the results is complex, the authors stress that the expression of 56 genes is significantly changed (49 down-regulated and 7 up-regulated).

Most down-regulated genes are involved in G-protein coupled receptor signaling, calcium homeostasis, inflammatory response, and neuropeptide receptors. This trend, which appears beginning with the 2c dilution, was maintained following exposure to more diluted solutions, though the observed differences were rather small. On the basis of their results, the authors suggest that the anxiolytic and analgesic properties of *Gelsemium* can be attributed to the negative modulation of some neuronal excitatory signaling pathways. Although surprisingly not stressed by the authors, we wish to mention that the comparison between the gene expression profiles of cells treated with different test solutions are consistent with the existence of hormetic mechanisms.

Since the time of Hippocrates, bee venom has been used as a medical remedy to relieve pain and to treat inflammatory diseases such as arthritis and rheumatism. This substance (*Apis mellifica*) is currently used in homeopathy for the same purpose. Along the lines previously shown

in the study concerning copper(II) solutions, we tested extreme dilutions of the homeopathic remedy *Apis mellifica* on the gene expression of RPEW-1 cells.⁴² According to the literature, these preparations were expected to show anti-inflammatory or anti-edema activity. Treated RWPE-1 cells were exposed to 1:100 water-diluted *Apis mellifica* mother tincture (Boiron, 65% ethanol) and its 3c, 5c, 7c dynamized dilutions (30% ethanol) for 24 h. The untreated reference cells were exposed to the same volume of ethanol-diluted dynamized water solutions.

We found that all the solutions we used in this study including the 7c, which corresponds to about a 10^{-16} M concentration, modulated a number of genes. Hierarchical cluster analysis showed that some genes exhibited similar expression profiles for all the dilutions we used, thus exhibiting concentration-independent activity. It is also worth mentioning that gene clusters also emerged with different and even opposite profiles in cells treated with the *Apis mellifica* mother tincture or with 3c, 5c and 7c solutions. Again this behavior strongly suggests that hormetic mechanisms are operative. The genes involved in cytokine expression, inflammatory processes, antioxidative responses and proteasome degradation were differentially, and sometimes divergently expressed by the *Apis mellifica* mother tincture or by *Apis mellifica* 3c, 5c and 7c dilutions. IL1b, a potent pro-inflammatory cytokine, was up-regulated by the *Apis mellifica* mother tincture and down-regulated by all *Apis mellifica* dilutions. These data were confirmed by RT-PCR analyses on 5 selected candidate genes (IL1b, CD46, ATF1, UBE2Q2 and MT1X).

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 these dilutions and not *Apis mellifica* mother tincture up-regulated members of the Rho-GTPase gene family, which controls several processes (phagocytosis, cell polarity, proliferation, survival, gene transcription, microtubule dynamics, vesicular transport) which are critical to inflammatory responses. It should also be emphasized that only these dilutions down-regulated genes of the major histocompatibility complex, induced by interferon and modulated in the presence of cytokines, hormones and other inflammatory agents. The most dilute solutions of *Apis mellifica* (5c and 7c) retained effects on gene expression by up-regulating IRF-2 genes which are critical suppressors of IFN-a/b signals and b2 integrin receptors, known for regulating adhesion and phagocytosis and the resolution of inflammation.

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

Discussion

Recent advances in technology have made it possible to document gene expression profiles of suitable groups of

cells. It must be realized that the experiment is only a tool and does not constitute the essence of all our possible knowledge. The results reviewed here suggest firstly that the action of drugs is not quenched by ultra-high dilution and proceeds through modulation of gene expression. This statement supports the hypothesis Khuda-Bukhsh made in 1997³³ and in our opinion erroneously confuted by Thangapazham *et al.*³⁵ In addition the efficacy of a drug solution seems to be maintained for ultra-highly diluted preparations, a fact which constitutes a challenge to the dogma of quantization of matter. We do not wish to comment on the several different hypotheses which have been formulated to explain this puzzling behavior.^{43,44}

The second and more important consideration in our opinion is that the different gene expression profiles of cell systems treated with the same drugs at different dilutions suggest the existence of hormetic mechanisms. The gene expression profiles of cells treated with copper(II) sulphate, *Gelsemium sempervirens* and *Apis mellifica*, are characterized by the same common denominator of the concentration-dependent inversion of gene expression, which can justify at a molecular level the concept of *simile* adopted in homeopathy.⁴⁵ This consideration seems both premature and reductive. Discussion on this point can be found in references 22 and 46.^{22,46} The main conclusion we can draw from these results is that these procedures provide new kinds of information and a tool for investigating the mechanisms involved in hormetic effects. According to Lushchak,⁴⁷ an evaluation of the dynamics of hormetic effects may provide a solid basis for pharmacokinetic investigations and application of hormetic approaches to modern medicine. This may allow researchers to conceive unprecedented therapeutic applications or to optimize the currently used ones.

It is important to stress again that the above reported observations are a direct consequence of scientific developments in the fields of thermodynamics, biology and information science which occurred in the second half of the twentieth century. The great achievement of scientists in this period was to completely define the living organism and its development and to merge a variety of phenomena, such as aging, hormesis, biological plasticity, material and cognitive stress effects, stress induced premature senescence (SIPS) and so on into one general concept.^{48–53} Life extension as a result of caloric restriction and the negative effects deriving from disuse both at a cardiovascular and nervous system level become tenets of a theorem which had been perceived by humans long ago but had never been clearly expressed. The basic problem remains quantification of the statement because it is not yet possible to foresee why and how cell alteration caused by interactions of an organism with the environment or merely by spontaneous decay of molecules due to aging could provoke the end of life process. Likely the answer is that at present we have not yet understood either the reason for the presence in the cell or the reason for that special structure we observe in different living organisms.

These observations could represent the crucial pillar upon which therapeutic medicine is based. Many therapeutic models can be found in such a general framework, even if they are observed from a completely different perspective. We leave the work of specifying distinctions and emphasizing disapproval, dissension and disagreement to experts in the respective models.⁵⁴ We merely emphasize the inadequacies of these paradigm. What we have summarized here suggests the possibility of a less aggressive therapeutic model entailing a review of the principles of the art of healing. The therapeutic acceptance of the hormesis concept would require a complete review of pharmacological science.

According to this concept, the progressive dilution of a drug, causes opposite effects at low concentrations. At the end of World War II it was realized that inadequate dose of penicillin resulted in worsening of pneumonia because low doses of penicillin selected resistant bacteria. More recently, numerous cases have shown cancerous cells to be induced by anti-tumor drugs.⁵⁵ On the basis of these and other observations, further study of hormesis can prompt research into a new category of drugs which different pharmacokinetic features.

Microdose pharmacology could become a conceptual revolution of extraordinary importance for future medical developments. In conclusion, a new perspective on the nature of the organism entails a new vision of medical therapeutics. By using the non-linear hormesis curve of dose–response relationship, the physician can assess if the diagnostic moment and clinical observation suggest the prescription of an inhibitory or stimulus-based treatment is more appropriate, thus conceiving an integrated pharmacology for Integrated Medicine. This consideration holds, even leaving aside any *prius posterior* argument, as suggested by a principle of logical causality.

Conflict of interest

The Authors declare that they have no conflict of interest. The University of Florence and INSTM have established a scientific collaboration (a not for profit agreement) with Laboratoires Boiron, from which A.D. does not receive any direct benefit. S. B. is President of SIOMI (Società Italiana di Omeopatia e Medicina Integrata) which is a non-profit scientific society and does not receive too any direct benefit.

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