Journal of Integrative Medicine xxx (2018) xxx–xxx

Contents lists available at ScienceDirect

Journal of Integrative Medicine

journal homepage: www.jcimjournal.com/jim
www.journals.elsevier.com/journal-of-integrative-medicine

Commentary

Arnica montana experimental studies: Confounders and biases?

Bellavite Paolo, Marzotto Marta *, Bonafini Clara

Department of Medicine, University of Verona, Strada Le Grazie 8, Verona 37134, Italy

ARTICLE INFO

Article history:
Received 5 July 2017
Accepted 27 November 2017
Available online xxxx

Keywords:
Arnica montana
Gene expression
Ethanol
Statistics

ABSTRACT

Arnica montana is a popular traditional remedy widely used in complementary and alternative medicine, in part for its wound-healing properties. The authors recently showed that this plant extract and several of its homeopathic dilutions are able to modify the expression of a series of genes involved in inflammation and connective tissue regeneration. Their studies opened a debate, including criticisms to the "errors" in the methods used and the "confounders and biases". Here the authors show that the criticisms raised on methodology and statistics are not consistent and cannot be considered pertinent. The present comment also updates and reviews information concerning the action of *A. montana* dilutions in human macrophage cells while summarizing the major experimental advances reported on this interesting medicinal plant.

Please cite this article as: Bellavite P, Marzotto M, Bonafini C. *Arnica montana* experimental studies: Confounders and biases?. *J Integr Med.* 2018; xx(x): xxx–xxx

1. Introduction

Experimental homeopathy is a field that has attracted the interest, curiosity and doubts of the scientific world for years. Research at the laboratory level on homeopathy investigates various areas, from physical–chemical studies on highly diluted medicines to biological studies with *in vitro* and *in vivo* models, and the results support evidence of an effect that is not merely placebo [1–5]. All the experiments conducted to verify the activity of the homeopathic preparations require scrupulous settings that make possible precise measures of even small events with high reproducibility and discrimination against any systematic or random errors. Careful design of controlled experiments with adequate biological replicates is important, especially given the current lack of a unique physicochemical description of high dilutions or of the factors that may affect the transmission of information at the biological level.

Our research team has been engaged in homeopathic research for a long time. Together with other research groups worldwide, we have made theoretical contributions to homeopathic concepts such as the "simile" mechanism of action [6–9] and to experimental studies in animals [10,11] and in cell lines (neurons and macrophages) [12–15]. Today, the availability of modern, high-throughput laboratory technologies, based on the study of gene expression, has enabled us to demonstrate a biological basis, both cellular and molecular, for the medicinal action, completing certain pieces of a very complex mosaic. The ability of highly diluted compounds to modulate gene expression in human/animal cells and unicellular organisms has been reported previously by a number of authors [9,16–24]. Ribonucleic acid (RNA)-seq technique, in particular, is a reliable and well established procedure, both for preparing libraries of transcripts and for bioinformatic analysis and statistics. In addition, collaboration among researchers with advanced technical expertise and skill in setting up biological studies with high dilution medicines ensures reliable results. This appears to be a good direction for future homeopathic experimental research [25,26].

Starting from year 2011, our publications have been the subject of several critical annotations by Dr. Salvatore Chirumbolo [27–30], and a summary of our technical responses was reported immediately afterward [31]. More recently, we used real-time polymerase chain reaction to investigate the effects of *Arnica montana* on gene expression of the THP-1 myelomonocytic cell line, differentiated by phorbol-myristate acetate and interleukin-4 in the wound-healing phenotype [32]. These findings also drew critical commentary by Chirumbolo and Bjørklund [33], based on some recalculations and extrapolations from the values of standard errors of the mean. Our reply, accepted by the journal *Frontiers in Immunology*, showed that those recalculations were wrong [15].

A subsequent series of our studies on the effects of *A. montana*, based on RNA-seq methodology, were published by PlosOne in

* Corresponding author.
E-mail address: marta.marzotto@univr.it (M. Marzotto).

https://doi.org/10.1016/j.joim.2018.01.001
2095-4964/

November 2016 [14]. This article was the subject of a recent commentary by Chirumbolo and Bjørklund [34], published in the *Journal of Integrative Medicine*.

Such debate has enabled us to define in more detail the experimental methods applied, providing a useful tool for experimental homeopathic research.

2. *A. montana* drug composition

Chirumbolo and Bj∅rklund [34] criticize the molar estimation of sesquiterpene lactones in *A. montana* dilutions. We do not agree with this remark. In actual fact, in our study, *A. montana* mother tincture is chemically characterized in accordance with the European Pharmacopoeia Guidelines [35], which conventionally measure the quality of *A. montana* preparation in terms of the content of sesquiterpene lactones, considered to be the active ingredients of reference, even if a complex variety of chemical ester variants and other chemical compounds could be present as a function of the cultivar, the part of the plant and the timing and methods of harvesting. Sesquiterpene lactones are determined by liquid chromatography and expressed as dihydrohelenalin tiglate. It follows that estimating the average molar weight of sesquiterpene lactone esters as 340 g/mol [36] is adequate for converting chromatographic quantification into molar concentration. Note that we had already published a rebuttal reaffirming the validity of our calculation of standard sesquiterpene lactones in our samples [15] in response to a previous letter from Chirumbolo and Bjørklund [33].

A major argument of Chirumbolo and Bjørklund [34] is that they think we used 51.1 mmol/L ethanol in our assay system, a dose which they consider to be too high and toxic for cells. However, the two authors made an error in their calculations: as is clearly stated in the methods of our paper, all the dilutions tested in cells (2 c and higher) were prepared in 0.3% ethanol and then further diluted 1/10 in culture medium. Since ethanol in final cell culture was 0.03%, and the molar mass of ethanol is 46.07 g/mol, with a density of 0.7893 g/cm^3, this corresponds to 5.1 mmol/L and not 51.1 mmol/L.

Taking into consideration the presence of factors that may alter cell viability is a mandatory step, especially in *in vitro* experiments. It was precisely for this reason that we chose to dilute both the medicinal product and its vehicle (control), originally hydroalcoholic solutions at 30% v/v ethanol, as a large part of homeopathic medicinal products, a total of 1000 times the concentration present in cell cultures. This operation guarantees the non-toxicity of the ethanol, whether in the *verum* or placebo, as shown by suitable cell viability assays [13–15]. The residual ethanol (0.03%) could have an effect on the background activity of the cells, but it would be identical in the medicine and in the control. Consequently, all considerations about the purported confounding effects on gene expression and cell viability ascribed to ethanol in control and in *A. montana*-treated samples should be considered to be wrong and misleading, since they refer to ethanol doses ten times higher than the actual ones. Note that a refutation of Chirumbolo's erroneous opinions on the toxicity of ethanol in homeopathic medicines has already been published [12].

Throughout the text, Chirumbolo and Bjørklund [34] suggested a possible biasing effect on the control samples due to a supposed difference in the handling of the *A. montana* dilutions and of the control vehicle (the preparations and the treated samples). This is an important point in the design of experiments with highly diluted medicine, giving us the opportunity to emphasize how these controls were properly performed in our experiments. Actually, as described by Marzotto et al. [14], the control solutions (solvents) in all the reported experiments were prepared under the same procedure as the drug dilutions, just without the plant extract, as indicated. All the procedural handling was conducted in parallel, including filtering of the first 1c solution of the *A. montana* or of the ethanol vehicle (the same batch of the *verum*, provided by the pharmaceutical manufacturer). The time of conservation was exactly the same. Control and *A. montana* samples were subjected to matching experimental steps, from the cell cultures to RNA-seq and bioinformatic pipeline.

3. Clarification of protocol and statistics

Chirumbolo et al. [34] hint at purported "biases" due to the pooling of RNA samples. Actually, the pooling of equal amounts of carefully quantized RNAs from replicated experiments in the same cell line—as we did in our experiments—is a conventional procedure for evaluating the presence of general trends in gene expression, in cases where the number of test samples must be minimized. As confirmation of the reliability of the results from the pooled samples, in our RNA-seq study (not DNA-microarray), we analyzed the gene expression values of *A. montana* 2 c and of the control-treated samples, both as five separate samples and as a pool, and the values matched very well, as reported in Fig. 5 and Table S1 [14]. Moreover, in a recent paper [37], it was reported that *A. montana* 2c up-regulated the same gene set both in THP-1 cells activated with lipopolysaccharide 10 ng/mL and in cells in a resting condition. These data confirmed that such differentially expressed genes (DEGs) are the true target of *A. montana* 2 c and excluded the possibility of control biases. In any case, the significant genes differentially expressed upon *A. montana* 2 c treatment, as reported by Marzotto et al. [14], were derived from a statistical analysis of five independent experiments and not from pooled samples. We calculated the *P* values of DEGs with *A. montana* 2 c by DESeq2, one of the most modern and rigorous statistical methods, specifically designed and applied to RNA-seq dataset experiments [38]. The few genes that emerged from our analysis as targets of *A. montana* action on human macrophages have an elevated biological significance and internal coherence; moreover, increased fibronectin secretion was observed at the protein level as well. Therefore, the criticism of Chirumbolo and Bjørklund [34] regarding the statistical approach we used to infer the significance of DEGs, claiming that a greater number of replicated samples was necessary and a more rigorous test should have been used, is unwarranted.

The paper cited [34] also criticized the statistical test used to compare expression trends of the set of 20 DEGs after treatment with higher *A. montana* dilutions (2 c, 3 c, 5 c, 9 c, 15 c) and the control. The authors wrongly declared that the criticized paper used only the Friedman test (defined as not effective), while Marzotto et al. [14] applied the Friedman test (as a non-parametric analysis of variance), followed by the Wilcoxon-signed rank test (as a paired comparison post-hoc test). Chirumbolo and Bjørklund [34] purport to recalculate *P* values with the Wilcoxon–Mann–Whitney and Kolmogorov–Smirnov tests, concluding with the contention that most of our results are false and not meaningful. This re-analysis is erroneous for a series of reasons:

(1) The Wilcoxon–Mann–Whitney and Kolmogorov–Smirnov tests are not appropriate for this analysis, because those tests, unlike the Wilcoxon-signed rank test, must be applied to independent samples, but the geneset expression profiles of treated and control samples found in Tables S1 and S2 are absolutely matched or dependent samples. This means that, in their own statistical tests, Chirumbolo and Bjørklund [34] compared expression profiles without any matching of genes between the treated and the control, i.e., the

Please cite this article in press as: Bellavite P et al. *Arnica montana* experimental studies: Confounders and biases? J Integr Med. (2018), https://doi.org/10.1016/j.joim.2018.01.001