Homeopathy (2017) 106, 47–54

http://dx.doi.org/10.1016/j.homp.2016.12.001, available online at http://www.sciencedirect.com

ORIGINAL PAPER

Number of succussion strokes affects effectiveness of ultra-high-diluted arsenic on *in vitro* wheat germination and polycrystalline structures obtained by droplet evaporation method

Lucietta Betti[1,*], Grazia Trebbi[1], Maria Olga Kokornaczyk[2], Daniele Nani[3], Maurizio Peruzzi[4], Giovanni Dinelli[1], Paolo Bellavite[5] and Maurizio Brizzi[6]

[1]*Department of Agricultural Sciences, University of Bologna, Italy*
[2]*Society for Cancer Research, Switzerland*
[3]*Italian National Health System, Lombardy District, "Azienda Sanitaria Locale" Milan, Italy*
[4]*Association for Sensitive Crystallization, Andalo Valtellino, Sondrio, Italy*
[5]*Department of Pathology and Diagnostics, University of Verona, Italy*
[6]*Department of Statistical Sciences, University of Bologna, Italy*

Objectives: **The aim of this study is to investigate whether the number of succussion strokes applied after each dilution step when preparing the homeopathic treatments influences the effectiveness of ultra-high-diluted (UHD) arsenic trioxide at the 45th decimal dilution/dynamization (As_2O_3 45x).**
Design: **Wheat seeds, previously stressed with ponderal As_2O_3, were treated with: As_2O_3 45x, H_2O 45x (dynamized control), or pure water (negative control). The succussion was done manually, and various succussion durations (numbers of strokes) were tested for each treatment. Treatment effectiveness was tested blind using the *in vitro* germination test and the droplet evaporation method (DEM). Data were processed by the Poisson test (germination test) and by two-way analysis of variance (DEM).**
Main outcome measures: **We evaluated both the *in vitro* germination rate, by counting the non-germinated seeds, and the complexity of polycrystalline structures (PCS) (local connected fractal dimension (LCFD)) obtained by evaporating leakage droplets from stressed seeds that had been watered with the different treatments.**
Results: **We observed a highly significant increase in germination rate when the number of strokes (N_S) was ≥32 for both As_2O_3 45x and H_2O 45x, and a significant increase in the LCFD of PCS for As_2O_3 45x when the N_S was ≥32 and for H_2O 45x when it was 70.**
Conclusions: **Both experimental approaches showed increased effectiveness for treatments prepared with a higher number of succussion strokes. These results indicate that succussion may have an important influence on treatment effectiveness, and so highlight the need for further research.** *Homeopathy* (2017) **106**, 47–54.

Keywords: Ultra high dilutions; Succussion number; Isopathic model; Wheat; Germination test; Droplet evaporation method; Crystalline structures

*Correspondence: Lucietta Betti, Viale Fanin 42, 40127 Bologna, Italy.
E-mail: lucietta.betti@unibo.it, grazia.trebbi@unibo.it, m.kokornaczyk@vfk.ch, daniele.nani@crs.lombardia.it, giovanni.dinelli@unibo.it, maurizio.brizzi@unibo.it
Received 14 December 2015; revised 9 November 2016; accepted 4 December 2016

Introduction

Homeopathic remedies are prepared through a 'dynamization' process of serial dilution and succussion (shaking). The succussion step can be done in a variety of ways (vertical strokes effected mechanically or by hand, vortexing, lemniscate-like movement, continuous flux).[1] Succussion can be regarded as a process that affects the physical state of the aqueous solution (a typical example of a dynamic unstable system), by transferring energy from the macroscopic (mechanical agitation) to the molecular scale. This process creates a turbulent regime where vortices continually appear and disappear, and dynamic superstructures may form from the molecular chaos of the liquid.[2–5] Succussion is therefore an operation whose intensity and duration must be carefully tuned. Some authors report[5–7] that the shock waves of succussion may increase gas dissolution, nanobubbles and material exchange between container and solvent. For this reason, it has been highlighted that research on dynamized treatments must use dynamized controls with equal preparation and storage time.[6,7] An unresolved question is what happens during a single succussion stroke: one hypothesized mechanism[8] is that each already-formed superstructure uses the added material (i.e. added water, or newly dissolved air, or contaminants from the container) to create more copies of itself, thereby allowing the active units to replicate. Hahnemann[9] himself pointed out that, "*by […] succussion […] the medicinal power of medicines may be increased almost to an infinite degree*". This assertion highlights the fundamental role that succussion might play in preparing a homeopathic remedy, and suggests the need to investigate this aspect more in depth.

In our previous works, we tested the effectiveness of ultra-high-diluted (UHD) treatments using a plant model based on *in vitro* wheat germination and growth. In particular, we showed that arsenic trioxide at the 45th decimal dilution/dynamization (As_2O_3 45x), applied to seeds previously stressed with sub-lethal doses of As_2O_3 (isopathic approach[10]), induced an increase in seed germination and seedling growth rate,[11–14] a decrease in experimental system variability,[15,16] and changes in seedling gene expression profiles.[17] We also investigated the As_2O_3 45x effectiveness using the droplet evaporation method (DEM), a technique that we had previously applied for quality analysis.[18] We found that the complexity of the polycrystalline structures (PCS) that form during evaporation of wheat seed leakage droplets was sensitive to UHDs.[19] As we reported in detail,[19] pattern formation during droplet evaporation is a complex process, dependent on the phase transitions and different flow dynamics that occur during evaporation.[20] Since in our experimental protocol the droplets were evaporated under identical ambient conditions, we hypothesized that any variations in the phase transitions – and hence the resultant variations in patterns – must depend solely on differences in the internal conditions of the droplets, i.e. in the composition of the liquid. This feature makes the DEM well-suited for studying the characteristics of homeopathic treatments.

The aim of the present study is to investigate whether the number of strokes (N_S) performed during each succussion step influences treatment effectiveness in the above-described isopathic model, by analyzing the wheat seed germination rate and the PCS complexity obtained by the DEM. The different treatment conditions were statistically compared with the control represented by unsuccussed water.

Materials and methods

Treatment preparation and blinding

The treatments considered were grouped into three classes:

- As 45x: As_2O_3 (Aldrich, St. Louis, MO, USA) at the 45th decimal dilution/succussion
- W 45x: pure water (p.A., Merck, Darmstadt, Germany) at the 45th decimal dilution/succussion, as a dynamized control
- C: pure water (p.A., Merck, Darmstadt, Germany), undiluted and non-succussed control.

As reported in our previous work,[13,15] As 45x and W 45x were obtained starting from As_2O_3 0.01 M aqueous solution and pure water, respectively. After each dilution step, succussion was done manually, and consisted of a series of sharp vertical strokes against a semi-hard surface. At each succussion step, a N_S was applied to polyethylene bottles (filled to 90% capacity) at a rate of 70 strokes/minute, with an oscillation amplitude of about 25 cm. In this step of the preparation we used plastic bottles to make it easier for sterility and because they were more available in our laboratory. In other experimental work plastic bottles were used effectively to prepare diluted and agitated homeopathic solutions.[21] The N_S values tested were 4, 8, 16, 20, 24, 28, 32, 40, 70, 100 for As 45x, and 4, 8, 16, 32, 40, 70, 100 for W 45x. Therefore, both the As 45x and W 45x classes were subdivided into multiple subclasses, differentiated by N_S. All the test samples were then poured into Pyrex bottles, letter-coded (blinded) by a person not involved in the experiments and stored at 5°C in the dark for at least three months. This period of time was chosen because in a previous experimentation we observed that treatments aged for less than 3 months did not showed a significant effect on wheat germination.[14]

Biological model

Two separate biological experimentations were carried out at the Association for Sensitive Crystallization (Nibbiano, PC, Italy): the first lasted from October 2009 to January 2010, and the second from November 2010 to May 2011. In the first experimentation we tested As 45x and W 45x treatments with N_S = 4, 8, 16, 32, 70. Since a preliminary statistical analysis showed that the significance level was reached with N_S = 32, in the second experimentation we studied a wider range of N_S values: 16, 20, 24, 28, 32, 40, 70, 100 for As 45x and 32, 40, 70, 100 for W 45x. We investigated the effects of the treatments on an *in vitro* germination model, using wheat seeds (*Triticum aestivum* L.) of the Pandas variety (Consorzio Agrario,

Pavia, Italy), previously stressed with 0.1% As_2O_3 aqueous solution, as already described[12–14]: we placed 36 seeds, selected for integrity, on sterilized sand in sterile plastic Petri dishes measuring 10 cm in diameter; each experimentation consisted of a series of 6 trials, each using 6 Petri dishes/treatment, so that the total number of Petri dishes tested per treatment was 36 per experimentation. At the beginning, 20 ml of treatment was pipetted into each dish. The dishes were randomly distributed inside a germination box mounted on an electrically-driven plate rotating at 90 rpm, at room temperature (20°C), in daylight and at a constantly high humidity rate (70% RH). This germination assay requires a rotation in order to ensure the homogeneous exposure of culture dishes to ambient conditions, namely light, humidity and oxygen. In the experimental cupboard all dishes have the same angular speed. After 96 h, the number X of non-germinated seeds in each dish was counted, and recorded as the outcome variable.

Droplet evaporation method

The experiment was carried out on April 2013 at the Department of Agricultural Sciences (University of Bologna), using treatments freshly prepared as described above. The experimental procedure, previously reported,[18] is briefly described here. Five seeds were weighed, rinsed in distilled water, placed in a test tube, and watered with the treatment (As 45x or W 45x at N_S = 8, 32, 70, or C) to reach a w/v proportion 1:20 (g/ml). The test tubes were shaken slightly by hand and left at room temperature. After 1 h, leakage drops were collected using a micropipette, placed on a clean microscope slide, and allowed to dry in a thermostat at 25°C under UV light (PHILIPS TL-D 18W BLB 1SL, Monza, Italy). The residues were then photographed under a dark field microscope (MT4300H, MEIJI Techno, Saitama, Japan) with a connected CMOS Camera (UK1175-C, EHD imaging GmbH, Damme, Germany), in QXGA (2048 × 1536) resolution, and with 100× magnification. The experiment was repeated three times to obtain a total of 105 droplet residues (7 treatments, 3 replicates, 5 droplet residues/replicate). The PCS were then evaluated for their local connected fractal dimension (LCFD) using the software Image J for microscopy 1.43 m.[22] The evaluation was performed on pattern images at 100× magnification. The LCFD was measured on images converted to binary using the installed fractal analysis plug-in FRAC-LAC 2.5.[23] This analysis was designed to avoid any experimenter bias.

Statistical analysis

When dealing with the seed germination model, the main outcome variable is the number X of non-germinated seeds in a Petri dish. We checked the goodness of fit of the Poisson distribution, as already suggested,[12,13] by the Kolmogorov Smirnov test.[24]

Having ascertained the goodness of fit, we made an overall comparison between the experiments using the multiple Poisson test, as previously described.[25]

After calculating the sample averages and standard deviations (SD), we compared treatment and control data by a pairwise Poisson test.[25]

To get an overall evaluation of the specific effect of As 45x vs. W 45x we considered a set of 11 experimental data including those already published.[12,13] Since the data were paired (As 45x and W 45x for each experiment), we decided to apply the Wilcoxon non-parametric test for paired data, as already reported.[26] The results have been furtherly analysed by using a non-parametric approach, based on binomial distribution.

When comparing the variability of As 45x, W 45x and C, we computed the SD of each class of treatment, dividing it into two complementary sources: variability 'within' and 'between' experiments, as already described.[16] When making variability comparisons between treatments (T) and control (C), we calculated the percentage difference (T/C −1)%. We applied the sign test to make an overall comparison between T and C by counting the number of positive and negative differences between the two classes. If one of the signs was significantly more frequent than the other, the null hypothesis of equal level of variability was rejected. The significance was evaluated using binomial probabilities.

LCFD data deriving from the DEM were processed by two-way analysis of variance; the separation of means was performed using Fisher's least significant difference test at a significance level of $p \leq 0.05$ (CoStat; version 6.002, CohortSoftware, Monterey, CA, USA).

Results and discussion

Germination model

Negative control experiments: The variability of the test system was evaluated in a preliminary set of independent and systematic negative control experiments using pure water as the only test substance. Considering that we had repeatedly checked the goodness of fit of the Poisson model for germination data,[10,14,27] we adopted this distribution to evaluate any differences between control groups in both the first and the second experimentation. We performed a multiple Poisson test for each experimentation, comparing a set of four trials, and found no significance whatsoever (first experimentation: w = 3.37; second experimentation: w = 4.74, with 3 degrees of freedom; the critical value is 7.81). This confirms that our experimental set-up is stable and should not generate false positive results.

Exploratory statistics and statistical inference: First of all, we applied some exploratory statistics to the germination data from both experimentations (Table 1): these clearly indicate that the average number of non-germinated seeds M (X) is globally higher in C. We note that, in the second experimentation, M (X) in the control group is higher than in the first experimentation, possibly owing to a slightly different effect of the stress procedure. Considering the class average of W 45x and As 45x we can see that, irrespective of N_S, there is in both experimentations a reduction in non-germinated seeds with respect to

Table 1 Germination tests: exploratory statistics on the number (X) of non-germinated wheat seeds and statistical inference by Poisson test (number of dishes per treatment = 36, number of seeds/Petri dish = 36)

Classes of treatment	N_s	*I experimentation*			*II experimentation*		
		M (X)	*SE*	*Signif. vs. C*	*M (X)*	*SE*	*Signif. vs. C*
C	0	6.08	0.52		7.22	0.68	
W 45x	4	5.75	0.42	ns	–	–	–
	8	5.97	0.49	ns	–	–	–
	16	5.33	0.44	ns	–	–	–
	32	4.61	0.51	**	6.22	0.57	*
	40	–	–	–	6.25	0.56	*
	70	4.53	0.52	**	5.39	0.66	***
	100	–	–	–	5.81	0.59	**
Class average		5.24 (−13.8%)	0.48 (−8.5%)	–	5.92 (−18.0%)	0.60 (−12.5%)	–
As 45x	4	5.58	0.54	ns	–	–	–
	8	6.44	0.54	ns	–	–	–
	16	5.61	0.46	ns	7.58	0.61	ns
	20	–	–	–	6.06	0.65	*
	24	–	–	–	6.22	0.72	*
	28	–	–	–	6.28	0.64	6.2%
	32	4.17	0.49	***	4.75	0.61	***
	40	–	–	–	5.08	0.57	***
	70	4.25	0.47	***	4.89	0.57	***
	100	–	–	–	5.11	0.59	***
Class average		5.21 (−14.3%)	0.50 (−3.8%)	–	5.75 (−20.4%)	0.62 (−8.8%)	–

C = undiluted, non-succussed water control; N_S = number of strokes; M(X) = average value of non-germinated seeds; SE = standard error; – = not tested; ns = not significant.
* = significant at $p < 0.05$; ** = significant at $p < 0.01$; *** = significant at $p < 0.001$.

C. In particular, W 45x induced an average reduction vs. C of 13.8% and 18.0% (1st and 2nd experimentation, respectively), while As 45x induced an average reduction of 14.3% and 20.4% (1st and 2nd experimentation, respectively). Moreover, when considering the class average, the standard error also shows a clearly visible decreasing trend against C, reflecting a decrease in variability. To make it easier to read the data, Figure 1 reports the average number of germinated seeds normalized against the corresponding C values (unsuccussed water) set equal to 100. Here we can clearly see that both the W 45x and As 45x treatments induced a stimulating effect on germination, with an increasing trend as N_S becomes larger. The two experiments showed the same trend of the effect of succussions, in both diluted water and arsenic. A global Poisson test was then applied simultaneously comparing all the treatments (11 and 13 for the 1st and 2nd experimentation, respectively). Since the outcome of this test was highly significant for both experimentations ($w = 44.15$ and $w = 47.77$, $p < 0.001$), we applied the pairwise Poisson test for comparing each treatment to the corresponding C. As shown in Table 1, in the first experimentation significant results appear when N_S is large (32, 70), for both classes of treatment. In the second experimentation, we

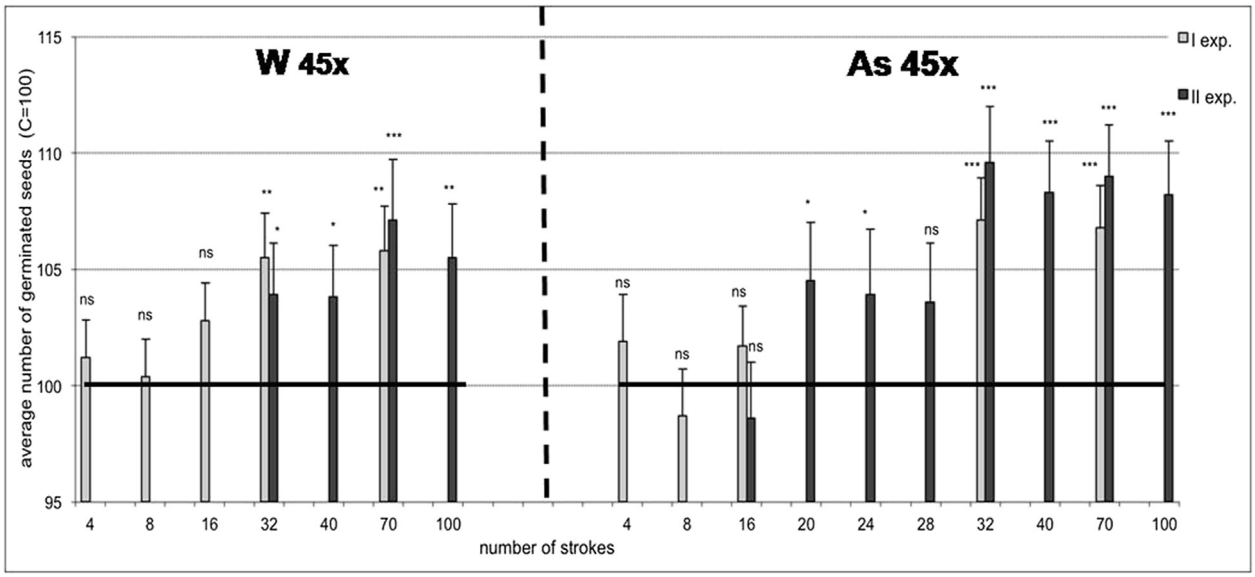

Figure 1 Average numbers of germinated seeds following W 45x and As 45x treatments prepared with different number of strokes in the two experimentations (control = 100).

Table 2 Overall comparison between W 45x and As 45x treatments throughout the experimentations at 20°C on *in vitro* wheat germination model

Reference	N_S	*M (X) W 45x*	*M (X) As 45x*	*Significance level*	*Effect on germination vs. W 45x*
Brizzi *et al.*[12]	70	3.78	3.25	*	Stimulating
Brizzi *et al.*[13]	70	4.90	4.50	ns	Stimulating
Present paper	4 (I exp.)	5.75	5.58	ns	Stimulating
	8 (I exp.)	5.97	6.44	ns	Inhibiting
	16 (I exp.)	5.33	5.61	ns	Inhibiting
	32 (I exp.)	4.61	4.17	ns	Stimulating
	32 (II exp.)	6.22	4.75	***	Stimulating
	40 (II exp.)	6.25	5.08	***	Stimulating
	70 (I exp.)	4.53	4.25	ns	Stimulating
	70 (II exp.)	5.39	4.89	ns	Stimulating
	100 (II exp.)	5.81	5.11	ns	Stimulating

N_S = number of strokes; M (X) = average value of non-germinated seeds; ns = not significant; * = significant at $p < 0.05$; *** = significant at $p < 0.001$.

confirmed for W 45x the same trend, with a significant increase (sharper for largest values) for $N_S = 32, 40, 70, 100$; when focusing on As 45x, we observed some significant results with $N_S = 20$ and 24 ($p < 0.05$), not completely confirmed at $N_S = 28$ ($p = 0.062$), while there was a strong increase in significance ($p < 0.001$) for the highest values of N_S (32, 40, 70, 100). These results seem in line with the mathematical model proposed by Anick,[8] according to which a minimum of 38 and 72 succussion strokes per cycle are needed for the centesimal and LM scale, respectively.

Comparison of As 45x vs. W 45x: To study the specific effects of As 45x vs. W 45x, we performed (Table 2) an overall comparison between these treatments through all our experimentations carried out in the same way, at 20°C with the *in vitro* wheat germination model. The table reports n = 11 experimental values, comparing the average value of non-germinated seeds M (X) for As 45x vs. W 45x class. The comparison reaches the significance level only three times, but the direction of the comparison is towards stimulation (decrease in the mean number of non-germinated seeds) 9 times out of 11. This result led us to consider the possibility of applying the Wilcoxon test to average values of M (X). The test statistic value is $w = 8.5$ ($p < 0.02$), confirming a highly significant specific effect of As 45x as compared to W 45x.

Moreover, if we consider that three times (out of 11) the result is significant, and that the probability θ of having a significant result due only to random effects is 0.05, the number X* of significant results out of 11 independent experiments follows, under the null hypothesis of no difference between As 45x and W 45x, a Binomial distribution with $n = 3$ and $\theta = 0.05$; the probability of having $X^* \geq 3$ is $p = 0.0152$, which can be considered as the p-value of this test. These results led us to reject the null hypothesis and to state that As 45x and W 45x are significantly different.

Analysis of variability: Finally, we investigated variability, split into its two essential components (within and between experiments), expressed in terms of SD (Table 3). We considered only N_S used in both classes of treatment (As 45x and W 45x). These classes showed a

Table 3 Analysis of variability: standard deviation (SD), standard deviation within experiments (SD_W) and between experiments (SD_B), percentage comparisons with respect to control (diff. %)

Classes of treatment	N_S	*I experimentation*			*II experimentation*		
		SD (Diff. %)	*SD_W (Diff. %)*	*SD_B (Diff. %)*	*SD (Diff. %)*	*SD_W (Diff. %)*	*SD_B (Diff. %)*
C	0	3.09	1.11	2.88	4.08	3.45	2.18
W 45x	4	2.53 **(−18.1)**	0.87 **(−21.6)**	2.38 **(−17.4)**	–	–	–
	8	2.91 **(−5.8)**	0.88 **(−20.7)**	2.77 (−3.8)	–	–	–
	16	2.64 **(−14.6)**	0.67 **(−39.6)**	2.55 **(−11.5)**	–	–	–
	32	3.06 (−1.0)	1.07 (−3.6)	2.86 (−0.7)	3.42 **(−16.2)**	3.25 **(−5.8)**	1.08 **(−50.5)**
	40	–	–	–	3.38 **(−17.2)**	3.21 **(−7.0)**	1.06 **(−51.4)**
	70	2.93 **(−5.2)**	1.10 (−0.9)	2.73 **(−5.2)**	3.95 (−3.2)	3.41 (−1.2)	1.98 **(−9.2)**
	100	–	–	–	3.53 **(−13.5)**	2.88 **(−16.5)**	2.05 **(−6.0)**
As 45x	4	3.24 (+4.9)	1.20 **(+8.1)**	3.01 (+4.5)	–	–	–
	8	3.25 **(+5.2)**	0.76 **(−31.5)**	3.16 **(+9.7)**	–	–	–
	16	2.79 **(−9.7)**	1.15 (+3.6)	2.54 **(−11.8)**	3.68 **(−9.8)**	1.78 **(−48.4)**	3.22 **(+47.7)**
	32	2.91 **(−5.8)**	1.33 **(+19.8)**	2.59 **(−10.1)**	3.67 **(−10.0)**	3.08 **(−10.7)**	2.00 **(−8.3)**
	40	–	–	–	3.41 **(−16.4)**	2.84 **(−17.7)**	1.89 **(−13.3)**
	70	2.84 **(−8.1)**	0.67 **(−39.6)**	2.76 (−4.2)	3.43 **(−15.9)**	2.72 **(−21.2)**	2.09 (−4.1)
	100	–	–	–	3.52 **(−13.7)**	2.89 **(−16.2)**	2.00 **(−8.3)**

C = undiluted, non-succussed water control; N_S = number of strokes; M (X) = average value of non-germinated seeds; – = not tested; negative percentage differences are in bold.

Table 4 Droplet evaporation method: exploratory statistics on local connected fractal dimension (LCFD) values of polycrystalline structures and statistical inference by ANOVA (number of patterns per treatment = 15)

Classes of treatment	N_S	*M (LCFD)*	*SE*	*Signif. vs. C*
C	0	0.672	0.063	
W 45x	8	0.792	0.070	ns
	32	0.797	0.055	ns
	70	0.880	0.056	*
Class average		0.823 (+22.47%)	0.060 (−9.58%)	–
As 45x	8	0.841	0.067	ns
	32	0.870	0.035	*
	70	0.935	0.031	*
Class average		0.882 (+31.25%)	0.044 (−29.63%)	–

C = undiluted, non-succussed water control; N_S = number of strokes; M (LCFD) = average value of local connected fractal dimension; SE = standard error; ns = not significant; * = significant at $p < 0.05$.

tendency towards a reduction in variability components when compared to C, taking into account that we discarded as immaterial any variation (positive or negative) of less than 5% of the C value: by this criterion, we considered 20 negative variations out of 27 for W 45x, and 17 out of 30 for As 45x (numbers in bold). No positive variation was detected for W 45x; whereas 5 positive variations were observed for As 45x. In both experimentations, we can see there is a larger number of negative differences (lower variability) when considering treatments (both W 45x and As 45x) prepared with N_S greater than 16. These results are in line with our previous findings[10,15,16] and are consistent with the decrease in variability observed in other plant models.[26,28]

Droplet evaporation method (DEM)

The PCS obtained through droplet evaporation of leakages from seeds watered with the tested treatments differed significantly in their LCFD values. As shown in Table 4, the LCFD of the PCS grew in proportion to the N_S applied at each succussion step in the preparation of the treatment: for $N_S = 8$ both As 45x and W 45x did not differ significantly from C; for $N_S = 32$ only As 45x significantly increased the LCFD of PCS vs. C; for $N_S = 70$ both treatments resulted in a significantly higher LCFD vs. C. The differences in pattern complexity can also be noticed visually; as shown in Figure 2, the PCS ranged from single spots (C), through to non-connected (As 45x and W 45x, $N_S = 8$) and connected (As 45x and W 45x, $N_S = 32$) cross-like structures, to complex and well-organized structures (As 45x and W 45x, $N_S = 70$). It is worth noting that

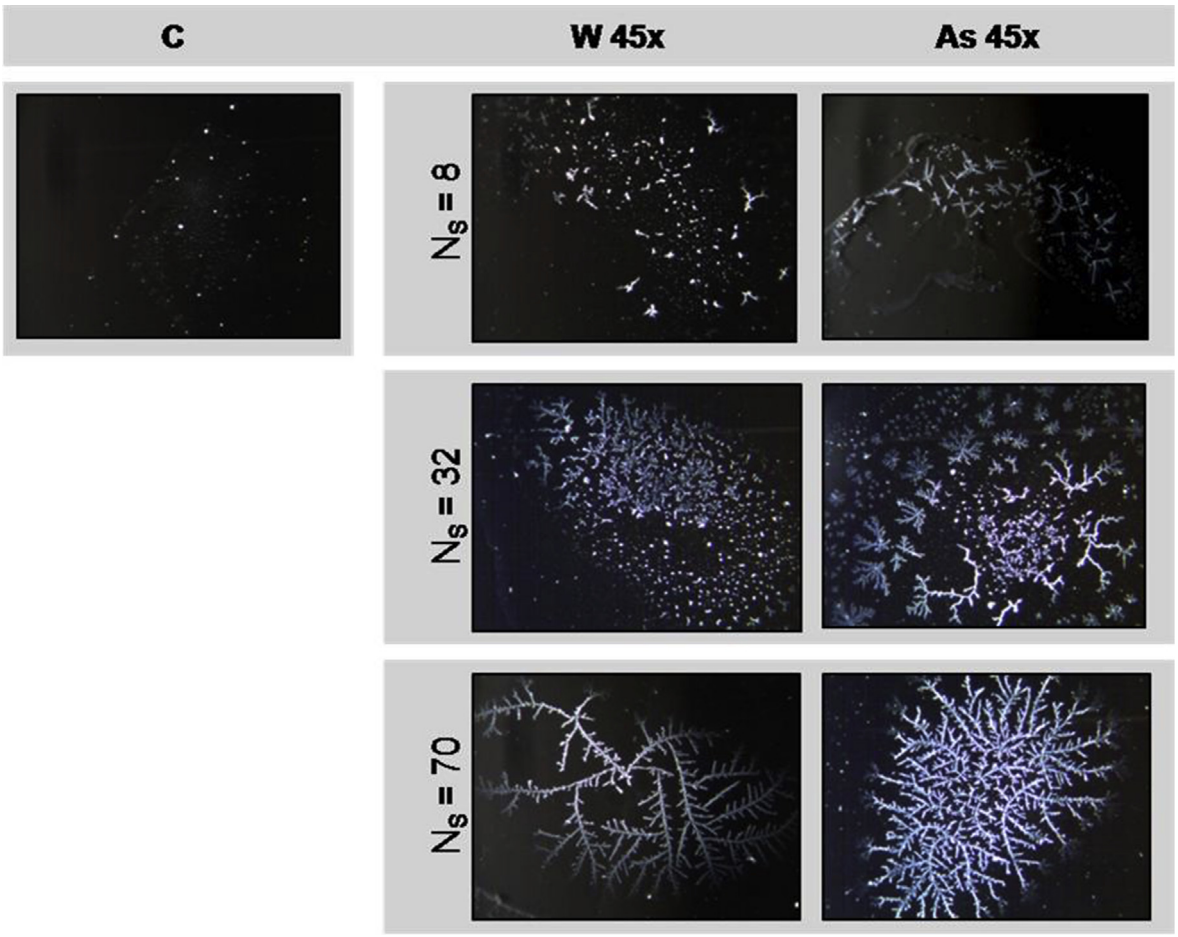

Figure 2 DEM pattern examples obtained from wheat seed leakages using undiluted, non-succussed water control (C), and W 45x and As 45x treatments prepared with a stroke number (Ns) = 8, 32, 70.

these DEM results agree with those of the germination tests: for both experimental approaches, no significant differences were found when $N_S = 8$, and the As 45x treatment showed a higher effectiveness when compared to W 45x. Moreover, a tendency towards a decrease in variability vs. C can be observed for the W 45x (SE −9.58%) and, in particular, for the As 45x (SE −29.63%) classes. The mean LCFD values correlated significantly with the numbers of germinated seed at r = 0.64. These results confirm the sensibility of PCS to UHDs, and hence the possibility of using the DEM as a new tool for testing UHD effectiveness.[19]

Conclusions

Our study suggests that a self-organization process of solutions by means of succussion is a crucial step in the preparation of homeopathic medicines, and reveals that N_S may strongly influence their effectiveness. In fact, in our experimental model low N_S (under 16) produced treatments whose effectiveness did not differ significantly from the control for both studied parameters (seed germination rate and fractal complexity of PCS). Moreover, the small but statistically significant effect of UHD arsenic, as compared to the dynamized control, seems to indicate that superstructures specific to the solute are more biologically active than those formed in absence of an initial mother tincture.

For what concerns the decreased variability in experimental outcomes following the application of homeopathic preparations vs. control, the present study confirms our previous findings[10,16] and shows that the same tendency, observed previously in germination tests, also appears in the DEM. Moreover, since the decrease in variability was more marked for treatments with a greater N_S, we can say that succussion influences not only the 'local effect' (germination increase) of the treatment, but also its 'equilibrating systemic effect' (variability decrease): the more homogeneous seed germination may reflect a trend toward a restored normal condition of the system after a homeopathic treatment, as reported also for other experimental models.[28]

Finally, the greater fractal complexity of PCS formed with treatments prepared with a higher N_S seems to support the hypothesis that, through dynamization, aqueous solutions may undergo a process of increasing physical structuring, similar to that of geometrically branched fractal images.[29–31]

This study demonstrates that succussion duration (in terms of N_S) may be an important factor in the treatment preparation; further research on this topic is needed to consider both a wider range of N_S (done by hand or mechanically) and other possible influencing factors (such as stroke speed, whether strokes are applied by impinging on a surface or in air, and different kinds of succussion movements).

The differences between succussed and not succussed samples can be also due to gasification during agitation, which may slightly change pH, electrical conductivity, and chemical equilibria. However, it is difficult to envisage that those chemical changes can be the only explanation, since the dynamization effects appeared to increase with time, even in closed vials and in the absence of further succussions.[14] Moreover, the small but statistically significant differences of As 45x as compared with W 45x indicate that a specific change related to the original substance dissolved is retained in the solution.[32]

The evidence on the variation of effectiveness of homeopathic remedies as a function of N_S raises the question of whether the observations reported here are relevant only to basic research or might also have implications for pharmaceutical industry. In our opinion, plant model systems (which are free of the placebo effect, and allow for a rigorous statistical approach) can represent an interesting biological sensor, complementary to clinical research, for testing the many hypotheses formulated to explain the effectiveness of homeopathic treatments, and in particular of UHDs, which are so widely criticised by conventional science.

Conflict of interest statement

All the Authors declare no financial/commercial conflict of interest.

Acknowledgements

The Authors thank Laboratoires Boiron, Italy, and in particular Dr. Silvia Nencioni and Dr. Luigi Marrari, for their technical assistance. The sponsors had no influence upon the design, conduct, evaluation and manuscript of this investigation.

Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.homp.2016.12.001.

References

1 Holandino C, Leal FD, de Oliveira Barcellos B, *et al*. Mechanical versus handmade succussions: a physical chemistry comparison. In: Bonamin LV (ed). *Signals and Images*. Springer Science+Business Media B.V., 2008, pp 37–48.
2 Torres JL. On the physical basis of succussion. *Homeopathy* 2002; **91**: 221–224.
3 Yinnon TA, Yinnon CA. Electric dipole aggregates in very diluted polar liquids: theory and experimental evidence. *Int J Mod Phys B* 2011; **25**: 3707–3743.
4 Yinnon TA, Elia V. Dynamics in perturbed very diluted aqueous solutions: theory and experimental evidence. *Int J Mod Phys B* 2013; **27**: 1350005 (35 pages).
5 Bellavite P, Marzotto M, Olioso D, Moratti E, Conforti A. High-dilution effects revisited. 1. Physicochemical aspects. *Homeopathy* 2014; **103**: 4–21.
6 Witt CM, Lüdtke R, Weißhuhn TER, Quint P, Willich SN. The role of trace elements in homeopathic preparations and the influence of container material, storage duration, and potentisation. *Forschende Komplementarmedizin* 2006; **13**: 15–21.
7 Demangeat JL. Gas nanobubbles and aqueous nanostructures: the crucial role of dynamization. *Homeopathy* 2015; **104**: 101–115.

8 Anick DJ. The octave potencies convention: a mathematical model of dilution and succussion. *Homeopathy* 2007; **96**: 202–208.
9 Hahnemann S. In: Dudgeon RE (ed). *Lectures on the Theory and Practice of Homeopathy*; 1853.
10 Betti L, Trebbi G, Zurla M, Nani D, Peruzzi M, Brizzi M. A review of three simple plant models and corresponding statistical tools for basic research in homeopathy. *ScientificWorldJournal* 2010; **10**: 2330–2347.
11 Betti L, Brizzi M, Nani D, Peruzzi M. Effect of high dilutions of *Arsenicum album* on wheat seedlings poisoned with the same substance. *Br Homeopath J* 1997; **86**: 86–89.
12 Brizzi M, Nani D, Peruzzi M, Betti L. Statistical analysis of the effect of high dilutions of arsenic in a large dataset from a wheat germination model. *Br Homeopath J* 2000; **89**: 63–67.
13 Brizzi M, Elia V, Trebbi G, Nani D, Peruzzi M, Betti L. The efficacy of ultramolecular aqueous dilutions on a wheat germination model as a function of heat and aging-time. *Evid Based Complement Alternat Med* 2011;11. ID 696298.
14 Betti L, Elia V, Napoli E, *et al*. Biological effects and physic-chemical properties of extremely diluted aqueous solutions as a function of aging-time. *Front Life Sci* 2012;10 http://dx.doi.org/10.1080/21553769.2011.638986.
15 Brizzi M, Lazzarato L, Nani D, Borghini F, Peruzzi M, Betti L. A biostatistical insight into the As_2O_3 high dilution effects on the rate and variability of wheat seedling growth. *Forschende Komplementarmedizin* 2005; **12**: 277–283.
16 Nani D, Brizzi M, Lazzarato L, Betti L. The role of variability in evaluating ultra high dilution effects: considerations based on plant model experiments. *Forschende Komplementarmedizin* 2007; **14**: 301–305.
17 Marotti I, Betti L, Bregola V, *et al*. Transcriptome profiling of wheat seedlings following treatment with ultrahigh diluted arsenic trioxide. *Evid Based Complement Alternat Med* 2014;15. ID 851263.
18 Kokornaczyk MO, Dinelli G, Marotti I, Benedettelli S, Nani D, Betti L. Self-organized crystallization patterns from evaporating droplets of common wheat grain leakages as a potential tool for quality analysis. *ScientificWorldJournal* 2011; **11**: 1712–1725.
19 Kokornaczyk MO, Trebbi G, Dinelli G, *et al*. Droplet evaporation method as a new potential approach for highlighting the effectiveness of ultra high dilutions. *Complement Ther Med* 2014; **22**: 333–340.
20 Deegan R. Pattern formation in drying drops. *Phys Rev E* 2000; **61**: 475–485.
21 Magnani P, Conforti A, Zanolin E, Marzotto M, Bellavite P. Dose-effect study of *Gelsemium sempervirens* in high dilutions on anxiety-related responses in mice. *Psychopharmacol (Berl)* 2010; **210**: 533–545.
22 Collins TJ. ImageJ for microscopy. *Biotechniques* 2007; **43**: 25–30.
23 Karperien. FracLac for ImageJ, version 2.5. http://rsb.info.nih.gov/ij/plugins/fraclac/FLHelp./Introduction.htm.
24 Stephens MA. EDF statistic for goodness-of-fit and some comparisons. *J Am Stat Assoc* 1974; **69**: 730–737.
25 Sachs L. *A handbook of techniques*. New York: Springer Verlag, 1984, pp 186–189.
26 Betti L, Lazzarato L, Trebbi G, *et al*. Effects of homeopathic arsenic on tobacco plant resistance to tobacco mosaic virus. Theoretical suggestions about system variability, based on a large experimental data set. *Homeopathy* 2003; **92**: 195–202.
27 Betti L, Brizzi M, Nani D, Peruzzi M. A pilot statistical study with homeopathic potencies of *Arsenicum album* in wheat germination as a simple model. *Br Homeopath J* 1994; **83**: 195–201.
28 Majewsky V, Scherr C, Arlt SP, *et al*. Reproducibility of effects of homeopathically potentised gibberellic acid on the growth of *Lemna gibba* L. in a randomised and blinded bioassay. *Homeopathy* 2014; **103**: 113–126.
29 Garner C, Hock N. Chaos theory and homeopathy. *Berlin J Res Homeopathy* 1991; **1**: 236–242.
30 Shepperd J. Chaos theory: implications for homeopathy. *J Am Inst Homeopathy* 1994; **87**: 22–29.
31 Bellavite P. Complexity science and homeopathy: a synthetic overview. *Homeopathy* 2003; **92**: 203–212.
32 Elia V, Ausanio G, Gentile F, Germano R, Napoli E, Niccoli M. Experimental evidence of stable water nanostructures in extremely dilute solutions, at standard pressure and temperature. *Homeopathy* 2014; **103**: 44–50.