British Homeopathic Journal (2000) 89, 63–67

www.nature.com/bhj

ORIGINAL PAPER

Statistical analysis of the effect of high dilutions of arsenic in a large dataset from a wheat germination model

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This paper describes the statistical analysis of a series of experiments using a simple biological model (wheat germination *in vitro*), where a large number of wheat seeds were treated with homeopathic potencies of Arsenic trioxide. Some potencies, such as As_2O_3 40x, 42x and 45x, have repeatedly shown a significant stimulating effect on germination compared to controls, whereas As_2O_3 35x has a significant inhibiting effect. In some experiments the seeds were stressed before the experiment with a sublethal dose of the same substance. We performed a statistical analysis, both for stressed and non-stressed seed groups, using Poisson distribution as a suitable model for representing the number of non-germinated seeds in a standard experiment with 33 seeds in the same Petri dish. Finally, we have considered the most repeated potencies (30x and 45x), computing the sample odds ratio (OR) and a 95% confidence interval (CI) for the population OR. Our results show significant reproducible effects of some As_2O_3 decimal potencies, particularly As_2O_3 45x. In stressed seeds, even decimal potencies of water seem to give significant results compared to control, whereas high dilutions of As_2O_3 without potentization never show significant effects. *British Homeopathic Journal* (2000) **89,** 63–67.

Keywords: plant models; *in-vitro* germination; arsenic trioxide; Poisson distribution; odds ratio

Introduction

Recently a series of reviews and meta-analyses have appeared in the scientific literature, examining whether homeopathic treatments have any greater effect than placebo.[1–4] While some authors demonstrate that experimental results are not compatible with the hypothesis that the clinical effects of homeopathy are completely due to placebo, in other studies the final conclusion is the contrary.[5–8] The effectiveness of homeopathy is still an open question and it seems of primary importance to develop high quality studies based on randomization, reproducibility and standardizability.[3,9–11]

Structured statistical analysis is of great importance; however, a large data sample is required. When performing clinical trials, it is seldom possible to reach this goal, because of experimental and ethical difficulties. Some major studies have been done on the effects of ultra high dilution in plant models, as reviewed by Pongratz and Endler,[12] who also made a critical review of the reliability of the classical wheat germination model.[13] Unfortunately, only a small number of such experiments are supported by a satisfactory statistical analysis.

We have tried to contribute to the problem of the 'scientific basis' of homeopathy by performing a series of experiments based on such a simple model.[14,15] A pilot study had shown that some potencies of Arsenicum album (As_2O_3) have a statistically significant effect on wheat germination *in vitro*.[16] The differences observed between treatment groups could not be attributed to intrinsic seed variability alone. It

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Received 21 April 1999; revised 20 October 1999; accepted 11 January 2000

should be pointed out that, using such a simple model, it is possible to collect a great deal of data in a reasonably short time, making possible a powerful statistical analysis.

The main aim of the present study is to test the reproducibility of our previous results and to make a comparison between all the experiments we have performed. We have used different statistical methods: the Poisson test, as proposed in our pilot study, the global Poisson comparison test and finally the odds ratio (OR), following the guidelines of Linde's meta-analysis.[3,16] We stressed some of the seeds with sublethal doses of arsenic to reduce germination, and treated them with potencies, in order to establish whether there is amplification of the homeopathic treatment effect. Some potencies were tested both against analogously prepared distilled water and against arsenic dilutions without potentization, to study 'dilution' and 'succussion' effects separately.[17]

Our research group is multidisciplinary, including a biologist, a statistician, a physician and a laboratory technician, in order to take account of different aspects of the research. Lucietta Betti determined the main biological features of the experiment, Maurizio Brizzi chose and applied the statistical tools, Daniele Nani developed the homeopathic aspect of this study, while Maurizio Peruzzi performed the experiments.

Materials and methods

Trial design

We carried out two distinct series of experiments in 1993/94 and 1995/96, with the same experimental conditions. In these experiments, we used MEC variety wheat seeds (*Triticum durum* L.), selected for integrity, which were located in Petri dishes, randomly distributed on a rotating plate in a glass germination box, kept at room temperature (20°C) in daylight and a high level of humidity (the same environmental conditions of our pilot study).[16]

A number of decimal potencies (between 23x and 45x) of Arsenic trioxide (As_2O_3: BDH Chemicals) were freshly made for each experiment as described by Betti and colleagues.[16] We designate a standard experiment a set of 33 seeds, located in the same Petri dish, under the same treatment. Using a blind protocol, we counted, every 12 hours starting at 48, the number of non-germinated seeds in each standard experiment, stopping at 96 hours.

The seeds were treated in several ways, which may be classified essentially in four classes:

(a) H_2O (**C**, control group)
(b) H_2O, potentized (**WP**)
(c) As_2O_3, highly diluted without succussion (**AD**)
(d) As_2O_3, highly diluted and potentized (**AP**)

In both series of experiments, some of the seeds were previously stressed with a ponderal dose of As_2O_3 (5 μM) for 30 minutes, followed by a 60 minutes rinse. We distinguished between the results obtained with stressed and non-stressed seeds. The total number of observed seeds, as well as the number of standard trials for each class and experiment are shown in Table 1.

Table 1 Number of seeds and standard experiments

	Number of seeds				
	C	***WP***	***AD***	***AP***	***Total***
Non-stressed					
1993/94	1782	4752	4752	4752	16038
1995/96	1584	3168	1584	4752	11088
Overall	3366	7920	6336	9504	27126
Stressed					
1993/94	1122	2376	2376	2376	8250
1995/96	792	1584	792	3168	6336
Overall	1914	3960	3168	5544	14586
Overall total	5280	11880	9504	15048	41712
	Number of standard experiments				
	C	**WP**	**AD**	**AP**	**Total**
Non-stressed					
1993/94	54	144	144	144	486
1995/96	48	96	48	144	336
Overall	102	240	192	288	822
Stressed					
1993/94	34	72	72	72	250
1995/96	24	48	24	96	192
Overall	58	120	96	168	442
Overall total	160	360	288	456	1264

C = Control group (H_2O);
WP = H_2O, potentized;
AD = As_2O_3, highly diluted without succussion;
AP = As_2O_3, highly diluted and potentized.

Statistical analysis

We have used two different methods of statistical evaluation of our results. As in previous work, we have considered the main variable X to be the number of non-germinated seeds observed after 96 hours in a standard trial of 33 seeds, which is well-fitted by a Poisson function giving the following probabilities:[16]

$$P(X = x) = \frac{\lambda^x}{x!} e^{-\lambda}; \qquad x = 0, 1, 2,$$

where λ is the characterizing parameter of the distribution, which is the mean and the variance at the same time point, and may be estimated by the sample average.

In order to make a comparison between k values ($k > 2$) of the Poisson parameter λ, we have used the test $\hat{\chi}^2$ proposed in Sachs.[18] The null hypothesis is the equality of all the λ's; let n_i be the sample size of the i-th treatment and $\hat{\chi}_i$ the corresponding sample mean, being $\bar{\chi}$ the overall sample mean. The test statistic is

$$\hat{\chi}^2 = \sum_{i=1}^{k} Z_i^2, \text{ where } z_i = \begin{cases} 2\left(\sqrt{n_i\bar{x}_i + 1} - \sqrt{n_i\bar{x}}\right) & if\, \bar{x}_i \le \bar{x} \\ 2\left(\sqrt{n_i\bar{x}_i} - \sqrt{n_i\bar{x}}\right) & if\, \bar{x}_i > \bar{x} \end{cases}$$

The statistic $\hat{\chi}^2$ follows, under the null hypothesis, a χ^2 distribution with k degrees of freedom.

We then applied an exact Poisson test. Under the null hypothesis that a treatment T_i has no effect on germination, the total number of non-germinated seeds in n standard trials, for the i-th treatment group, follows a Poisson probability with parameter $n\lambda_C$, where λ_C is the average number of non-germinated seeds in a standard trial of non-treated (control) seeds. Therefore, it is possible to find the significance of the experimental result for each treatment group by calculating the exact Poisson probabilities. Since we have multiple comparisons, we applied a Bonferroni correction. Following Linde's work, we have also computed the odds ratio (OR).[3] We constructed a 2×2 contingency table with two experimental situations (here, treatment and control) and two possible outcomes (here, germinated and non-germinated seeds), as shown in Table 2.

The OR is constructed as follows:

$$OR = \frac{A/B}{C/D} = \frac{A \cdot D}{B \cdot C}$$

When OR = 1, the treatment has no effect compared to control; when OR > 1, treated seeds have a higher germination rate; when OR < 1, they have a lower germination rate. Usually the OR value is corrected for continuity (we denote the corrected odds ratio with OR*) by adding $\frac{1}{2}$ to each number of observations, as follows:

$$OR^* = \frac{(A + 1/2) \cdot (D + 1/2)}{(B + 1/2) \cdot (C + 1/2)}$$

Since the natural logarithm of OR*, when sample size is larger, follows approximately a normal (Gaussian) distribution, we can also build confidence intervals (CI) for the OR, using the methodology described in Agresti.[19] We have defined 95% CI for all the treatments involving 30x and 45x potencies, which were repeated in several experiments. We have considered also the first experiment (1992/93), described by Betti and colleagues[16] but with no reference to OR values.

Results and Discussion

Table 3 shows the global percentage of germinated seeds for each experiment and class of treatment.

The global results are consistent, in spite of the fact that the two experiments were performed two years apart; however, we have studied the results separately.

Table 2 2×2 Contingency table

	Germinated	*Non-germinated*	*Total*
Treated	A	B	A + B
Control	C	D	C + D
Total	A + C	B + D	N

Table 3 Global percentage of germinated seeds

	C	*WP*	*AD*	*AP*	*Total*
Non-stressed					
1993/94	95.3%	95.4%	95.0%	94.6%	95.0%
1995/96	93.9%	95.0%	93.6%	94.7%	94.4%
Stressed					
1993/94	84.8%	89.1%	85.7%	86.1%	86.6%
1995/96	80.0%	86.5%	81.4%	88.5%	86.0%

C = Control group (H_2O);
WP = H_2O, potentized;
AD = As_2O_3, highly diluted without succussion;
AP = As_2O_3, highly diluted and potentized.

We have then tried to compare simultaneously all the treatments of the same class (e.g. **AP**, potentized arsenic, see Trial design section). With this aim, we have supposed that the number of non-germinated seeds approximately follows a Poisson distribution. We had already tested this;[16] however, we can regard the number of non-germinated seeds in a standard trial as a binomial experiment with n quite large ($n = 33$) and P small (about $P = 0.05$ for non-stressed seeds and $P = 0.10$ to $P = 0.20$ for stressed seeds).

It is well known that a binomial distribution converges to a Poisson distribution as n increases and P decreases. Kolmogorov distance (i.e. the maximum distance between the cumulative distribution functions) between binomial and Poisson is < 0.03 for stressed seeds and < 0.01 for non-stressed seeds. Applying this hypothesis, we have applied the $\hat{\chi}^2$ test statistic to each class of treatments, distinguishing between non-stressed and stressed seeds. The null hypothesis is that all the Poisson distributions involved have the same parameter λ. Observed values and corresponding significance are shown in Table 4.

In Table 4 the **AP** class (As_2O_3, highly diluted and potentized) always shows significant differences

Table 4 Global test of comparison of Poisson parameters

Group	*Year*	$\hat{\chi}^2$	*Degrees of freedom*[a]	*P*
Non-stressed				
C + WP	1993/94	2.703	5	N.S.
	1995/96	12.698	5	< 5%*
C + AD	1993/94	0.636	5	N.S.
	1995/96	0.095	3	N.S.
C + AP	1993/94	23.140	5	< 0.1%***
	1995/96	65.734	7	< 0.1%***
Stressed				
C + WP	1993/94	10.952	3	< 5%*
	1995/96	13.363	3	< 0.5%**
C + AD	1993/94	1.264	3	N.S.
	1995/96	0.330	2	N.S.
C + AP	1993/94	29.741	3	< 0.1%***
	1995/96	35.059	5	< 0.1%***

N.S. = not significant; *, *P*-value is between 1% and 5%; **, the significance level is between 0.1% and 1%; ***, the significance level is < 0.1%.
[a]Note: The number of degrees of freedom is equal to $k - 1$, where k is the number of different treatments compared.
C = Control group (H_2O);
WP = H_2O, potentized;
AD = As_2O_3, highly diluted without succussion;
AP = As_2O_3, highly diluted and potentized.

between treatments; the **WP** class (H_2O, potentized) does so only with stressed seeds, whereas the **AD** class (As_2O_3, highly diluted) never shows any significant difference.

Then we considered the comparison between each experimental treatment group of seeds and the control. The null hypothesis is that the Poisson parameter λ is the same for both groups (treatment and control). The sample average and corresponding significance are indicated in Table 5.

In Tables 4 and 5, note the following:

- As_2O_3 45x always has a highly significant stimulating effect, both with non-stressed and stressed seeds. The same is true for As_2O_3 40x and As_2O_3 42x which were tested only in the last series of experiments;
- As_2O_3 35x always shows significant inhibiting effect with non-stressed seeds (it was not tested for stressed seeds);
- As_2O_3 30x shows some inhibiting effect in the 1993/94 experiment, and highly significant stimulating effect in 1995/96;
- Potentized H_2O (i.e. **WP** class of treatments) has generally shown a stimulating effect only when the seeds are stressed; only once (H_2O 45x in 1995/96) such effect was observed with non-stressed seeds;
- Highly diluted As_2O_3 without succussion (i.e. **AD** class of treatments) never shows any effect, stimulating or inhibiting.

Finally, we focused our analysis on 30x and 45x potencies (and corresponding dilutions), which were the most repeated in our experiments. Starting with our first series of experiments (1992/93), we have computed the OR (and the corrected ratio OR*), showing the boundaries of a 95% CI, as reported in Table 6. We have pointed out the treatments in which the lower boundary OR_{min} was >1 meaning that all the interval lies on the right of $x=1$ in a real line representation.

The analysis based on the odds ratio gives the same results as the Poisson test: it confirms the stimulating effect of As_2O_3 45x (with non-stressed and stressed seeds) and potentised water 30x and 45x (only on stressed seeds). Diluted As_2O_3 without potentisation always gives OR values very near to one, confirming the above mentioned absence of effects in such a class of treatments.

Table 5 Poisson test for the number of non-germinated seeds: overall results

Non-stressed seeds				
	1993/94		*1995/96*	
Treatment	*Average*	*Signif.%*	*Average*	*Signif.%*
H_2O	1.537	–	2.000	–
H_2O 23x	1.528	N.S.	–	–
H_2O 27x	–	–	2.375	N.S.
H_2O 30x	1.806	N.S.	1.458	N.S.
H_2O 35x	1.305	N.S.	–	–
H_2O 42x	–	–	1.708	N.S.
H_2O 45x	1.444	N.S.	1.125	0.28**(st)
As_2O_3 10^{-23}	1.750	N.S.	–	–
As_2O_3 10^{-30}	1.556	N.S.	2.125	N.S.
As_2O_3 10^{-35}	1.667	N.S.	–	–
As_2O_3 10^{-45}	1.528	N.S.	2.083	N.S.
As_2O_3 23x	2.111	1.36* (in)	–	–
As_2O_3 27x	–	–	3.125	< 0.10*** (in)
As_2O_3 30x	1.972	N.S.	1.083	0.23** (st)
As_2O_3 35x	2.111	1.36* (in)	3.083	0.11*** (in)
As_2O_3 40x	–	–	1.000	< 0.10*** (st)
As_2O_3 42x	–	–	1.125	0.42** (st)
As_2O_3 45x	0.944	0.56** (st)	1.125	0.42** (st)
Stressed seeds				
	1993/94		*1995/96*	
Treatment	*Average*	*Signif. %*	*Average*	*Signif.%*
H_2O	5.029	–	6.583	–
H_2O 30x	3.444	< 0.10*** (st)	4.208	< 0.10*** (st)
H_2O 42x	–	–	4.708	< 0.10*** (st)
H_2O 45x	3.778	< 0.10*** (st)	–	–
As_2O_3 10^{-30}	4.972	N.S.	–	–
As_2O_3 10^{-45}	4.472	N.S.	6.125	N.S.
As_2O_3 30x	5.944	1.52* (in)	3.375	< 0.10*** (st)
As_2O_3 40x	–	–	3.792	< 0.10*** (st)
As_2O_3 42x	–	–	4.670	< 0.10*** (st)
As_2O_3 45x	3.250	< 0.10*** (st)	3.375	< 0.10*** (st)

N.S. = not significant; (st) = stimulating, (in) = inhibiting; **P*-value between 1% and 5%; ***P*-value between 0.1% and 1%; ****P*-value below 0.1%.

Table 6 Odds ratio and confidence intervals

				Confidence Interval 95%	
Treatment	*Year*	*OR*	*OR**	*OR_{min}*	*OR_{max}*
Non-stressed					
H_2O 30x	1992/93	1.386	1.383	0.993	1.927
	1993/94	0.844	0.843	0.605	1.175
	1995/96	1.395	1.383	0.932	2.053
H_2O 45x	1993/94	1.067	1.064	0.747	1.514
	1995/96	**1.828**	**1.805**	**1.171**	**2.781**
As_2O_3 10^{-30}	1992/93	1.223	1.222	0.886	1.685
	1993/94	0.988	0.985	0.697	1.392
	1995/96	0.937	0.933	0.658	1.324
As_2O_3 10^{-45}	1993/94	0.988	0.985	0.697	1.392
	1995/96	0.957	0.953	0.671	1.355
As_2O_3 30x	1992/93	1.363	1.351	0.894	2.041
	1993/94	0.769	0.768	0.555	1.062
	1995/96	**1.901**	**1.875**	**1.209**	**2.908**
As_2O_3 45x	1992/93	**1.761**	**1.737**	**1.108**	**2.725**
	1993/94	**1.658**	**1.644**	**1.098**	**2.462**
	1995/96	**2.369**	**2.326**	**1.446**	**3.742**
Stressed					
H_2O 30x	1993/94	**1.543**	**1.541**	**1.204**	**1.972**
	1995/96	**1.705**	**1.702**	**1.297**	**2.233**
H_2O 45x	1993/94	**1.391**	**1.390**	**1.092**	**1.769**
As_2O_3 10^{-30}	1993/94	1.014	1.014	0.808	1.272
As_2O_3 10^{-45}	1993/94	1.147	1.147	0.909	1.447
	1995/96	1.093	1.093	0.852	1.403
As_2O_3 30x	1993/94	0.818	0.819	0.657	1.020
	1995/96	**2.188**	**2.181**	**1.635**	**2.908**
As_2O_3 45x	1993/94	**1.646**	**1.644**	**1.280**	**2.111**
	1995/96	**2.188**	**2.181**	**1.635**	**2.908**

OR = odds ratio; OR* = corrected odds ratio; OR_{min} = lower boundary of 95% interval; OR_{max} = upper boundary of 95% interval.
The values are evidenced in **bold** characters when the lower 95% boundary is greater than one.

Conclusion

Summarising the results presented in this study, the consistency of the different statistical analyses, as well as the possiblity to reproduce most of our experimental results in different years, is notable, giving a possible answer to the question posed by Reilly *et al*,[20] concerning reproducibility in homeopathy.

Among the decimal potencies tested in our experiments, As_2O_3 45x shows the most relevant effect, being highly significant against control even with non-stressed seeds. Similar results have also been obtained with As_2O_3 40x and As_2O_3 42x; these potencies were tested only in the last series of experiments. On the other hand, As_2O_3 35x repeatedly showed an inhibiting effect, but it was not tested in stressed seeds. Finally, As_2O_3 30x shows contradictory effects, being sometimes stimulating and sometimes inhibiting. This behaviour recalls Kolisko's studies[13] which showed an undulating dose–effect curve, as has the work Harisch and Dittmann.[17]

Another crucial point is the importance of succussion when preparing homeopathic treatments: the **WP** class often shows significant results, while the **AD** class does not. In any case, the interaction of succussion and high dilution gives the most relevant results, which seems to put in evidence the existence of a real efficacy of homeopathic treatments, at least inside our simple model.

This topic deserves further work, and we hope that the experimental design described in our study, followed by a rigorous statistical analysis, could be the methodological kernel of widely extended multicentre research.

Acknowledgements

We thank the Deutsche Antroposophische Gesellschaft and Micaela Glöckler of the Medizinische Sektion by the Goetheanum, Dornach (Switzerland), for the support which allowed us to complete this study.

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