Requiem for Avogadro: recent advances from laboratory research

Prof. Paolo Bellavite MD

With collaboration
Marta Marzotto PhD and Clara Bonafini PhD

Department of Medicine, University of Verona, Italy
Introduction: about the title

“Requiem aeternam dona eis, Domine, et lux perpetua luceat eis.”

(Grant them eternal rest, O Lord, and may perpetual light shine on them)

St Stephen’s cathedral 31 october 2014
Introduction: about the title

“Requiem aeternam dona eis, Domine, et lux perpetua luceat eis.”

(Grant them eternal rest, O Lord, and may perpetual light shine on them)

The title I chose is a tribute to Avogadro – an Italian scientist – but especially to this wonderful city, that I consider the cultural capital of Europe.

The last time I came to Vienna was a couple of years ago, following the kind invitation from Michael Frass. After my lecture to medical students I had the opportunity to attend a performance of the Requiem Mass by Mozart in the cathedral.

So, this is the right place to connect Mozart requiem and a famous Italian scientist whose name is often linked to homeopathic issues.

My general intention is to demonstrate how and why Avogadro was a great scientist but now he can rest in peace, without his work being used to attack homeopathy.
Requiem for Avogadro: recent advances from laboratory research

- 1) Avogadro’s PRINCIPLE
- 2) Avogadro-Loschmidt’s NUMBER
- 3) What EXPERIENCE tells us

© P. Bellavite, Università di Verona
Declarations of the Italian most famous and influential pharmacologist at «Ballarò» 26 May 2016

- «Why they don’t contain anything?»
- «Because it depends on the preparation procedure:
  - «1 cubic centimeter of that substance…»
  - «You shake, for magical reasons…»
- «You then take another cubic centimeter and put in 99 ccs of water and so on…»
- «At the end, chemistry tells us that according to the principle of Avogadro, there is no longer even a molecule of that product»
Who was Avogadro?

- Amedeo Avogadro graduated in law at the age of 31.
- Soon after, he dedicated himself to physics and mathematics.
- In 1811, he published an article which contains his major contribution to physics.
- He worked as professor at Turin University
- He married Felicita Mazzé and had six children
- His hypothesis was rejected by contemporary scientists and was only recognized after his death.
Avogadro's PRINCIPLE

“Equal volumes of all gases, at the same temperature and pressure, have the same number of molecules”.

\[ \frac{V}{n} = k \]

It is important to note that: THIS PRINCIPLE CONCERNED ONLY GASES. MOREOVER, THAT HE FORMULATED A PHYSICS HYPOTHESIS BUT DIDN’T CALCULATE THE NUMBER OF MOLECULES.
Avogadro’s principle and Homeopathy

A. Avogadro (1776 – 1856)

(1)

AVOGADRO’S «PRINCIPLE» IS CORRECT
BUT IT HAS NOTHING TO DO WITH HOMEOPATHIC DRUGS

...Requiescat in pace
Requiem for Avogadro: recent advances from laboratory research

1) Avogadro’s PRINCIPLE
2) Avogadro-Loschmidt’s NUMBER
3) What EXPERIENCE tells us
Avogadro-Loschmidt’s NUMBER

The number of molecules in one Mole of substance (es. 12 grams of Carbon) was established in 1865 by Loschmidt and confirmed in 1909 by Perrin.

It is approximately $6.022 \times 10^{23}$

This number – or «constant» - was then named after Avogadro and Loschmidt in their honor.

(Aincidentally, Josef Loschmidt was an Austrian high school teacher who later became a professor at the University of Vienna!)

Possibly in Italy only the name of Avogadro is known, but to be honest we must name also Josef Loschmidt!
Avogadro-Loschmidt’s number and Homeopathy

A. Avogadro
(1776 – 1856)

C.F.S. Hahnemann
(1755-1843)

If 1 Mole of pure and concentrated substance
= 6.022×10^{23} molecules

Then, a dilution beyond 10^{24} times (24 X or 12CH) = «Zero molecules»

Since many MT are not pure and concentrated substances but often contain low doses (10^{-3}, 10^{-4} Mol) of active principles:

✓ 10^{-20} Mol/L (≈ potency of 20 X or 10 CH)

corresponds to the molecular limit
The idea of extreme dilution has become a good opportunity to mock homeopathy. A major example of these vitriolic attacks is Wikipedia, as in this quotation by a so-called “Homeopathy Tutorial” (sic) for university students.
For example, a 30C preparation (most preferred by Hahnemann) is a dilution equal to $1:10^{60}$, is equivalent to one molecule diluted in a sphere the size of the orbit of Neptune!

© P. Bellavite, Università di Verona
Wikipedia is extremely biased against homeopathy, reporting only negative results ignoring positive ones and recent advances in basic research.

Homeopathy is defined as a “pseudoscience”.

Well, if homeopathy is a “pseudoscience”, it means that Wikipedia is a “pseudoencyclopedia”!
A POPULAR HOMEOPATHIC 6X

✓ Arnica extract (MT) contains 0.36 grams/L of sesquiterpene active principles
  → → 6x potency = 3.6 x 10^{-7} grams/L
  = 3.6 x 10^{-10} grams/ml

✓ Since sesquiterpenes MW = 340 Da,
  = about 10^{24} molecules/340 grams
  = about 2.9 x 10^{21} molecules/gram

✓ = 2.9 x 10^{21} molecules/gram x (3.6 x 10^{-10} grams/ml) = 10^{12} molecules/ml

✓ Assuming that a minimum of 1 ml 6X is used in 500 tablets, we have a minimum of 2x10^{9} (2 BILLION) molecules in a tablet
A POPULAR HOMEOPATHIC 6X

- Contains at least 2 BILLIONS active molecules in a tablet

- THE LIMIT OF AVOGADRO-LOSCHMIDT HAS NOTHING TO DO WITH HOMEOPATHIC LOW POTENCIES

- FURTHERMORE, HOMEODRUGS ARE NOT ONLY DILUTED BUT ALSO TRITURATED AND SUCCUSSED
HOEWEVER: ARE HOMEOPATHIC DRUGS SIMPLY DILUTED MOLECULES?

“No one really understands water. It’s embarrassing to admit it, but the stuff that covers two-thirds of our planet is still a mystery.”


I can’t enter into this problem of water physics, there is no time and it’s too difficult. In connection with our problems, however, it is important to state that pure water does not exist: On the contrary, evidence is emerging that water is heterogeneous liquid at the nanoscale level: This heterogeneity can be represented according three major concepts, or working models as in the next slide.
AND... WATER IS NOT ONLY "FRESH WATER"

Hydrogen-bonded chratrates (clusters)

Coherence domains (CD)
Superradiance

Nanoparticles, nanobubbles, aggregates

http://www.ncbi.nlm.nih.gov/pubmed/24439452
Homeopathic drugs are not «pure» solutions

Zeta-potential:
\[-25.54 \pm 9.50 \text{ mV}\]

Nanoparticle spectrum of \textit{Arnica m.} 1c used in experiments with macrophages
(Marzotto et al. PlosONE 2016)
Avogadro-Loschmidt number and Homeopathy

A. Avogadro (1776 – 1856)

(2)

AVOGADRO-LOSCHMIDT’S NUMBER IS CORRECT

BUT:

1) IT HAS NOTHING TO DO WITH LOW-POTENCY HOMEODRUGS

2) IT ASSUMES HOMOGENEOUS WATER «SOLUTIONS», BUT THIS IS NOT THE CASE

…Requiescat in pace
“Whenever a theory appears to you as the only possible one, take this as a sign that you have neither understood the theory nor the problem which it was intended to solve.”

K. Popper, *Objective Knowledge: An Evolutionary Approach* (1972)
Requiem for Avogadro: recent advances from laboratory research

- 1) Avogadro’s PRINCIPLE
- 2) Avogadro-Loschmidt’s NUMBER
- 3) What EXPERIENCE tells us
“How small, in other words, must be the dose of each individual medicine, to effect the best cure?”

“This is not the work of theoretical speculation. (...) Pure experiment, careful observation of the sensitiveness of each patient, and accurate experience can alone determine this in each individual case”

Organon, par. 278
Results of models of basic research submitted to replication (98 studies until 2015)

Replications of fundamental research models in ultra high dilutions 1994 and 2015
update on a bibliometric study
Results of models of basic research submitted to replication (98 studies until 2015)

- Opposite result
- No effect
- Positive replication

External replications
Multicentre
Internal replications

“The null-hypothesis (dominance of zero results) should be rejected.”
“We encourage further replications of studies in order to learn more about the model systems used.”
Macrophages have a central role in inflammatory processes, immunity and even in wound healing in all tissues.

In our laboratory we cultivate human cell lines that can be considered as good models for this type of research since they are very stable and can be differentiated in various types. For example if we add interleukin4 to these cells, they differentiate into a type that is called macrophage of “wound-healing” type.
Central role of the Macrophage in wound healing

Hemostasis
- Stop bleeding

Inflammation
- Inflammatory mediators
  - Coagulation, Platelets, Fibrinopeptides
  - Cytokines, Neutrophils, Macrophages, Fibronectin

Regeneration
- Endothelial/epithelial proliferation
- Collagen production, scarring, maturation

- Microvessels, Fibroblasts, Collagen, Keratinocytes

© P. Bellavite, Università di Verona
Macrophages treatment with *Arnica m.* and gene expression (Verona group 2015-2016)

- **Day 1**: Seeding of THP-1 cells
- **Day 2**: PMA addition
- **Day 3**: IL-4 addition
  - **Normal Mac**
  - **IL-4 Mac**
- **Day 4**: Arnica 2c, 3c, 5c, 9c, 15c or placebo
  - 2h or 24h
  - RNA extraction (24h)

- **c-DNA synthesis**
- **Real-time PCR**
- **Whole transcriptome sequencing**

- Homeopathy published May 2016
- PlosONE in Press
Differential gene expression analysis with high throughput technologies

- Small changes in the gene regulation networks have a relevant impact in the cell

**Phenotype**
- Metabolic activity

**Environmental inputs**
- Small changes in the gene regulation networks have a relevant impact in the cell

**Differential gene expression**
- Conditions

- Quantification of the RNA expression levels of all the genes (microarray, RNA-seq)
- Compare to control
- High sensitivity and many replicates

**RNAs**
- Quantification of the RNA expression levels of all the genes (microarray, RNA-seq)
- Compare to control
- High sensitivity and many replicates

**expr. level**
- Gene expression levels

**Genes**
- Differential gene expression analysis
- Conditions

© P. Bellavite, Università di Verona
Arnica m. effects on Macrophages (Olioso et al., RT-PCR study)

This is the main result of the study with RT-PCR that was published in Homeopathy.

One of the most striking and unexpected effects of Arnica m. on our macrophages was the increase of chemokines of the CXC family (CXCL1, CXCL2, IL8). The most consistent findings concerned the CXCL1 gene whose expression was increased in all conditions. This finding suggests a new property of this plant in wound healing processes. Cytokines and chemokines promote inflammation, angiogenesis, facilitate the passage of leukocytes from circulation into the tissue, and contribute to regulation of the events involved in the healing process.
Effect of *Arnica m. 2c* on gene expression by human macrophages (Marzotto et al., RNA-seq study)

- fibronectin ($p < 0.001$)
- fibrillin ($p < 0.05$)
- heparan sulfate proteoglycan ($p < 0.05$)

http://journals.plos.org/plosone/article?id=10.1371/journal.pone.0166340
Fibronectin is the major protein that organizes the connective tissue during the first phase of wound healing and inflammation.

- Fibronectin has the ability to **specifically** bind a large number of molecules.
- Cellular interactions with fibronectin lead to **bidirectional crosstalk** that directs **cell functions** such as:
  - Proliferation
  - Cell migration
  - Survival and growth

**Kinetics of protein production in connective tissue during wound healing**

http://www.slideshare.net/fullscreen/smileycty/wound-healin-gs/1

**Immunofluorescence image of cells in culture**

http://www.bme.rochester.edu/research/fibronectin-matrix-remodeling.html
Effect of *Arnica m.* increasing dilutions on IL-4-polarized human macrophages (pool of 5 experiments)

- **3c**: $10^{-10}$ Mol/L sesquiterpene
- **5c**: $10^{-14}$ Mol/L sesquiterpene
- **9c**: $10^{-22}$ Mol/L sesquiterpene
- **15c**: $10^{-34}$ Mol/L sesquiterpene
The responses of cells to Arnica at high dilutions were in the same direction of those observed with 2c. Upregulated genes remained upregulated and downregulated remained downregulated, with few exceptions. This trend of the group of genes was always statistically significant and can’t be due to chance. If the observed changes were due to chance or to noise in the cell treatments, the genes would have responded in a random way, that is some in up and other in down direction, without any order and any distinction between the two groups. I am proud to say that this paper has just been published the great Scientific Journal PlosONE, that is not a homeopathic journal.
Number of genes significantly up- or downregulated comparing cells exposed to *Apis mellifica* preparations versus cells exposed to the reference ethanol:water solutions (Apis vs ethanol–water).
AVOGADRO-LOSCHMIDT’S NUMBER IS THEORETICALLY CORRECT BUT CAN’T BE USED TO EXPLAIN HOMEOPATHIC PHENOMENA.

IN FACT, EXPERIMENTAL EVIDENCE SHOWS THAT THIS LIMIT IS OVERCOME BY HOMEOPATHIC DRUGS!

"Eppur si muove!" (and yet it moves!)

Galileo Galilei
(1564–1642)
"The game of science is, in principle, without end."

K. Popper, Ch. 2 "On the Problem of a Theory of Scientific Method", Section XI: Methodological Rules as Conventions
Auf Wiedersehen!

Department of Medicine
Functional Genomics Lab

University of Verona
Laboratoires Boiron

Marta Marzotto
Clara Bonafini
Paolo Bellavite
Main references