SUMMARY

In vitro studies represent a reliable model to investigate the action of several homeopathic medicines or even high diluted/succussed substances on cell function and much evidence concerning a critical reappraisal about basic research in homeopathy has appeared. Human granulocytes are a good promising cellular model due to their involvement in inflammatory and immune response and to their rather easy experimental handling. Most of previously published results are related to the effect of low potency remedies on neutrophil function (Sulphur 6x, Bryonia 4CH e 9CH, Ferrum Phosphoricum 4CH and 9CH, Phosphorus 15x, and so on), and involved indefferently medicines made up of organic acids, or minerals or plant extracts (Podophyllum compositum). Both activation and inhibition on superoxide production and in vitro adhesion were observed with these remedies in different experimental conditions. Neutrophil has been used for a long time as a good cellular model to examine commercial remedies as low as 10CH potency, while basophil is still currently used to investigate the effects of very highly diluted and succussed compounds (16CH and over). Many reports have shown that highly diluted (over the Avogadro’s limit i.e. upper than the 12th centesimal dilution) and succussed histamine, inhibit cellular function, sometimes in a fashion comparable to the ponderal molar dilutions. Some evidence from our laboratory has shown that histamine 13CH and histamine 14CH inhibit basophil function as respect to the same diluted/succussed preparation of water alone. In this report we summarize the main evidence concerning these studies.

KEYWORDS

Neutrophil - Basophil - Basic research in homeopathy - In vitro studies - Highly diluted compounds

In vitro studies represented a model to investigate the action of homeopathic remedies and highly diluted substances; actually, basic research of cellular response to highly diluted-dynamized solutions is slowly accumulating new evidence, especially due to the existence of conflicting reports. Critical evaluations of basic homeopathic research have been recently reviewed. Main advantages deriving from in vitro models are related to their intrinsic reliability due to protocol standardization and to a relatively low time-consuming approach, especially when they are compared to a dose-effect evaluation on the whole organism. Actually, a specific cell function induced and/or regulated by soluble factors can be controlled by automated devices, metrologic algorithms and ongoing checking. In this perspective, a strategic role is played by the proper choice of the assay modeling. The use of human leukocytes was preferred by several authors because of three reasons: 1) they can be easily extracted from a simple blood withdrawal, 2) they
are involved in immunity and inflammation; 3) they possess functional parameters that can be analysed by well established in vitro protocols.

Almost all reports are concerned mainly with neutrophils or basophils, maybe because neutrophils are largely represented in the whole peripheral blood and hence they are easy to extract and because basophils are related to a high cell sensitivity pattern, due to their involvement in hypersensitivity reactions. In this paper we reviewed the results obtained with these cells in homeopathic basic research.

**Neutrophils**

Most of the studies of the possible regulatory effects of homeopathic dilutions on inflammation involved phagocytic cells and in particular the polymorphonuclear leukocytes (neutrophil granulocytes): in this context, the tested substances were those used by homeopaths in situations of acute inflammation.

Early research on phagocytes dates back to sixties with reports about oxidase granule reactions to homeopathic remedies by Seitchek in the XXV Homeopathic Congress in Amsterdam4 and by Douch at the Triennial Congress of the International Homeopathic League, London in 19655. Douch studied the effect of Aristolochia clematis, a plant extract which was studied also by others6-9, and for which there are evidences about high toxicity and inhibitory function towards leukocytes of this plant extracts. Different authors studied the effect of plant extracts or low potencies of inorganic acids such as the inhibitory effect of Ecballium elaterium juice on neutrophil oxidative burst10 and recent evidence has reported that this Cucurbitaceae seeds contain a strong serine-protease inhibitor11.

Poitevin and coll. reported the effect of Belladonna and Ferrum phosphoricum by showing that 5CH and 9CH potencies inhibited the production of free oxygen radicals (measured as chemiluminescence, i.e. release of light generated during metabolic reactions) by granulocytes stimulated by opsonized zymosan12. The inhibition was highly significant and as much as 30-40%, approximately the same as that obtained using 10 μM of dexamethasone and 0.1 mM of indomethacin. The same authors pointed out a considerable difference in individual sensitivity to these drugs, a problem previously highlighted by others who have investigated the effect of Belladonna, Hepar sulfur, Pyrogenium, Silicea and Staphylococcinum on chemotaxis and obtained conflicting results13.

Among plants another Cucurbitaceae family member has tested on neutrophil function: it has been reported (in a preliminary communication) that Bryonia 4CH and 9CH had a stimulatory effect on the oxidative metabolism of polymorphonuclear leukocytes, which may be both direct and indirect (increasing the response to chemotactic peptides)14.

Our own group investigated the effects of homeopathic medicines on inflammatory cells and has obtained significant results particularly in relation to low potencies. In a first study we explored the possible direct effect of homeopathic medicines on cell systems by evaluating their in vitro effects on the oxidative metabolism of cultured neutrophils activated by formylated peptides15. These results, based on the analysis of multiple potencies of a large series of compounds, can be summarized as follows: a) Manganum phosphoricum 6x and 8x, Magnesium phosphoricum 6x and 8x, Sulphur 6x, Acidum citricum 3x; Acidum succinicum 3x and 4x showed highly reproducible inhibitory effects on our in vitro assay system; b) Acidum fumaricum and Acidum malicum (both at a dilution of 4x) showed a slightly potentiating effect on oxidative metabolism; c) during the course of various experiments, Phosphorus
and Magnesium phosphoricum often showed inhibitory effects even at very high potencies (over than 15x), but these did not always appear at the same dilutions, thus making it difficult to analyse them statistically; however, by pooling all of the data concerning the effects of high Phosphorus dilutions (up to 30x), it has been possible to identify a small (10-15%) but statistically significant inhibition of cell activation. These results suggest that the majority of the tested remedies interfere with subtle cell regulatory mechanisms known to be based on ion exchanges, phosphorylation processes and reduced oxidation. In normal cell physiology, phosphorus, sulphur, magnesium, manganese, calcium and other elements play a major role in such mechanisms, and so it is particularly interesting that some homeopathic medicines can act at these levels of control.

Human blood neutrophilic granulocytes (neutrophils) treated with low potencies (4x) of a homeopathic drug extract (Podophyllum peltatum) had specific stimulating effects on their metabolic responses: an enhanced oxidative response to a subsequent challenge with bacterial formyl-peptides. This priming effect was related to superoxide anion (O$_2^-$) release (respiratory burst), and was quantitatively similar to the priming of the effect of TNF-$\gamma$. The phenomenon was observed with a homoeopathic preparation containing, among other things, podophyllum extract (Podophyllum compositum) and a 4x homeopathic potency of Podophyllum (the final concentration of the active principle was about 0.025 $\mu$g/ml), whereas no enhancement of O$_2^-$ release was caused by homoeopathic Podophyllum 12x or the other components contained in the complex homeopathic preparation. Purified podophyllotoxin caused the same effect at doses of 0.1-10 $\mu$g/ml, whereas doses of more than 100 $\mu$g/ml inhibited the respiratory burst so that pure toxin showed a typical bi-phasic dose-response curve. Similar effects were obtained with purified colchicine (1-1000 $\mu$g/ml), a microtubule-disrupting agent. Furthermore, both homoeopathic podophyllum-derived compounds and pure podophyllotoxin inhibited cell adhesion to the serum-coated surface of culture microplates. These results show that low potencies of a homoeopathic drug extract have stimulant-specific effects on the activation of neutrophil metabolism. Interest in this drug also comes from the fact that much higher doses of podophyllotoxin are used by conventional pharmacology to inhibit cell proliferation and appear to be efficacious against condilomata of the skin. Priming doses are those contained in the homeopathic preparation, toxic doses are those contained in the allopathic preparation.

**Basophils**

Since the beginning of in vitro research, the best studied laboratory model in which the phenomena of similarity and of high dilution effects have been most widely investigated is the biology of human basophils. Early evidence reported the effect of highly diluted/dynamized substances (IgE, histamine) or homeopathic remedies (Lung histaminum, Apis mellifera) on cellular degranulation. Intracellular granule disease triggered by the interaction with IgE-receptor or by other agonists represents a direct manifestation of cellular activation and many attempts to assess the dynamics of degranulation event and to understand its biological mechanism have been made. Cellular activation involves changes in membrane ion fluxes (particularly calcium ions), changes in cell membrane electrical polarity, and other mechanisms that eventually lead to exocytosis and the release of mediators, such as the cytokines IL-3 or IL-13, histamine, PAF and LTC$_4$. It is well known that one of the main mediators is histamine, which is produced by the decarboxylation of histidine, stored in granules of basophils and mast cells, and released only a few seconds after activation.

Histamine acts as a downstream regulator by interacting with H$_2$-receptors and thus抑制ing basophil release of mediators and degranulation.
Initially the effects of highly diluted/dynamized substances on basophil activation were evaluated by observing the degranulation with optical microscopy but metachromatic false positive results could not be prevented at all and they might influence significantly the resulting outcome of the in vitro evaluations. Moreover, when weak stimuli or very low agonist doses are used the activation of basophils without true degranulation (i.e. without histamine release) has been observed under many circumstances.

Progress in the experimental setting concerning the human basophil activation arose from independent immunology research groups endeavoured to build up an analytical tool to diagnose allergy affections. Degranulation tests have been replaced by flow cytometry. Evidence was reported that highly diluted histamine is able to affect CD63 and CD203c up-regulation in fMLP and anti-IgE stimulated basophils.

These markers are expressed on the membrane of activated basophils: the CD63, a tetraspanin, known also as lysosome-associated membrane protein (LAMP-3), is very low expressed in resting (non activated) basophils and up-regulated following stimulation. The protein is stored inside the intracellular granules, so its membrane displacement can be related to degranulation. CD203c, known as ENPP-3, is an ectoenzyme, constitively expressed also by resting cells and up-regulated following stimulation. CD203c is highly specific of basophilic cells and could be used also as a phenotyping marker.

The highest effects are observed with 15CH or 16CH highly diluted/dynamized histamine on CD203c expression when basophils were triggered with relatively low doses of anti-human IgE ranging from 0,5-1,0 μg/ml, and these data have shown a correlation with degranulation assay by alcyan blue staining. The inhibition pattern throughout the high dilution interval spanning from 2CH to 40CH has shown either an inhibitory effects on basophil activation involving all the centesimal dilutions ranging from 14CH to 18CH or punctual effects related only to certain potencies.

Multicentre analysis have been performed to assess these results but not all the researchers were able to confirm them by applying the same experimental setting: effects might be related to various experimental variables, and both inhibitory and stimulatory effects were reported.

Recently, also our own research group has undertaken a study project aiming at investigating the effect of highly diluted/dynamized histamine aqueous solutions on the same experimental model. Early results, albeit preliminary in nature, seem to confirm the existence of the phenomenon.
Figure 1 - Effect of highly diluted histamine solutions on human basophil triggered with anti-IgE.

Values refer to the ratio % ± SD between CD203c fluorescence of basophils treated for 10 min at 37°C with serial centesimal succussed dilutions of histamine (5 separate triplicate experiments) and CD203c fluorescence of basophils treated at the same condition but with serial centesimal succussed dilutions of pure water as control (2 separate triplicate experiments). Statistical analysis was performed by applying the Kolmogorov-Smirnov non parametric test (*** < 0,0001; * < 0,05).

Figure 1 reports the ratio % of the effect of various diluted/dynamized histamine preparations vs the same serially dilutions prepared only with pure H2O (no histamine). CD203c membrane expression was evaluated by the mean fluorescence intensity (MFI) and MFI related to activated basophils pre-treated with diluted/dynamized preparations of simple water (without histamine) were reported as basal 100% (control). The hahnemannian potencies 2CH, 13CH and 14CH showed a statistically significant inhibition of CD203c membrane expression following anti-IgE activation, when compared to the control. Significativity was evaluated by a Kolmogorov-Smirnov non parametric test.

Future perspectives

The overall bulk of evidence suggests that in vitro investigation about highly diluted/dynamized compounds on granulocyte function could throw a light on the complexity of biological systems responding to homeopathic aqueous preparations, an issue that cannot be related any longer to a placebo explanation. In this context research about water structure and behaviour should focus a possible suggestive hypothesis able to explain the mechanism by which high diluted substances act on cell function. However many questions are still to be faced statistical evaluation has to be definitely assessed, and several data have to be confirmed. Few evidence reported, for instance, the role of succussion-dynamization on water high diluted compounds or the role of the physico-chemical features of diluted water. A promising discussion arena could arise from
molecular biology of cell signaling systems, intracellular and vicinal-water (including its interaction with biological structures)\textsuperscript{4} and physical properties of highly diluted/dynamized water.

An interdisciplinary approach should be preferred in order to comprehend how and why homeopathy works. Such an attitude allows clinical studies to draw new suggestions from basic research and hence to confirm the role of homeopathy inside medical sciences.

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