Conference presentation

Searching for the mechanisms involved in *Antimonium crudum* action on macrophage – *Leishmania* interaction *in vitro*

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Abstract

In previous studies [1,2] we showed that treatment of mice with *Antimonium crudum* (*Ant-c*) 30cH was able to significantly reduce monocyte migration to the infection site after injection of *Leishmania (L) amazonensis* into the subcutaneous tissue, resulting in clinical improvement. Follow up was performed with an *in vitro* model, which showed that treatment of co-cultures of RAW 264.7 macrophages and parasites with *Ant-c* 30cH inhibited two parasite-induced CCL2 peaks 48 and 120 hours after infection together with early inhibition of lysosome activity. These findings explained the results previously obtained *in vivo*. In turn, treatment with *Ant-c* 200cH resulted in an early and transitory peak of cell spreading at 48 hours. The coherence between the *in vivo* and *in vitro* results indicates that this is a good model to study more thoroughly the mechanisms of action of homeopathic medicines, being the first step to establish correlations between the biological effects and the physical and chemical features of *Ant-c* 30cH and 200cH.

In the present study, the same experimental model was replicated, through comparison of vehicle (30% cereal alcohol), *Ant-c* 200cH, *Zincum metallicum* (*Zinc*) 200cH and *Arsenicum album* (*Ars*) 200cH, to confirm the specificity of *Ant-c* effects. In addition, *Ant-c* 200cH was ultra-centrifuged, and only the superficial phase was applied to the culture medium. This procedure intended to separate the heavier particles from the lighter ones suspended in the homeopathic medicine. The physical-chemical profile of the medicines was assessed. Solid contaminants (microparticles) in the suspension were analyzed. Conductivity was assessed through measurement of the electron current induced by a micro-amperimeter (Ryodoraku®) connected to 2 clean electrodes immersed in the samples, prepared immediately before the analysis, diluted in pure water (MilliQ, Millipore®) and filtered in 22-µ filter (Millipore®). Pure water was used as control. The device was calibrated immediately before measurements. The microparticle profile was assessed with a scanning electronic microscope - SEM (JEOL JSM 6510®) coupled to an energy dispersive spectroscopy (EDS) system to identify the nature of the elements present in each particle. The size and the number of particles were analyzed from the images generated by electronic microscopy with an automatic image analysis system (Metamorph®). For this purpose, all materials used was cleansed through immersion in pure acetone and subjected to 30-minute sonication before insertion into the microscope to avoid secondary contamination. The samples of medicines were subjected to ultra-centrifugation (10000rpm for 60 minutes) to induce particle sedimentation in the bottom of microtubes. 10 microliters of each sample were collected from the bottom of tubes and placed on a copper stub and kept in a closed recipient until the material was fully dry. The samples were directly analyzed with the microscope. Metallization was not necessary, because the

analyzed particles had metallic nature.

The biological effects of *Ant-c* 200cH reproduced the previous ones: spreading and phagocytosis index were significantly higher in the co-cultures treated with *Ant-c* 200cH compared to vehicle and other, non-specific treatments (*Ars* 200cH and *Zinc* 200cH) ($p=0.05$). However, these results were not exhibited by centrifuged *Ant-c* 200cH. Analysis of the supernatant after 48-hour incubation revealed increase of the GM-CSF content only in cultures treated with *Ant-c* 200cH and centrifuged-*Ant-c* 200cH. No change was observed in the cytokine profile in the cultures treated with *Ars* 200cH or *Zinc* 200cH.

Morphological analysis of *Ant-c* samples on SEM showed that the microparticles in *Ant-c* 30cH were smaller compared to *Ant-c* 6cH, most of them having half-moon shape. Curiously, agglomerates of particles were detected in *Ant-c* 200cH. Contaminant particles suspended in pure water contained Pb, Zn, Ca, Na, Au, Hg, Nb and Si, therefore, not related to any specific biological effect of *Ant-c*. P was identified only in *Ant-c* 30cH (6.51%) and *Ant-c* 200cH (13.56%). This wide-range profile of different microparticles did not change after centrifugation, which indicates that the weight of these particles is not conditioned by the nature of their component elements.

Conductivity was lower in the vehicle (30% alcohol) compared to *Ant-c* 6, 30 and 200cH ($p=0.0001$); the conductivity of *Ant-c* 200cH was the highest ($p=0.008$). Also *Ars* 200cH exhibited higher conductivity ($p=0.001$) compared to the vehicle.

Taken together, these data suggest that the biological effect of *Ant-c* 200cH on macrophage spreading and phagocytosis might be partially related to the size of the microparticles found in suspension. However, specific effects relative to cytokine production did not depend on microparticle size or content. The changes in conductivity changes exhibited correlation with presence of some elements, such as P, but not with any biological effect.

To summarize, the results point to the relevance of eventual false-positive effects relative to phagocytosis in macrophages treated with homeopathic medicines *in vitro*, due to the interference of larger sized microparticles. They also points to the specificity of GM-CSF expression after 48-hours of co-culture exposure to *Ant-c* 200cH, centrifuged or not, which suggests it was independent from microparticle content and conductivity. The physical-chemical features of homeopathic medicines related to their specific biological effects are still unknown. Additional studies are needed in this regard.

Keywords: Conductivity, scanning electron microscopy, energy dispersive spectroscopy, *Antimonium crudum*, macrophages, *Leishmania amazonensis*

References

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