

## ORIGINAL PAPER

# Homeopathic treatments modify inflammation but not behavioral response to influenza antigen challenge in BALB/c mice



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**Background:** Influenza affects thousands of people worldwide every year, motivating the development of new therapies. In this work, the effects of two homeopathic preparations (influenza biotherapies and thymulin) were chosen following two different rationales: isotherapy and endo-isotherapy models. The homeopathic effects were evaluated individually considering the inflammatory and behavioral responses against influenza virus antigen were studied in BALB/c mice.

**Methods:** Male adult mice were treated orally and blindly for 21 days with highly diluted influenza virus or with thymulin, and were divided in two sets of experiments. The first series of experiments aimed to describe their behavior, using an open field (OF) device. In the second series, mice were challenged subcutaneously with influenza hemagglutinin antigen (7 µg/200 µl) at day 21. At day 42, behavior and inflammation response were evaluated.

**Results:** No behavioral changes were seen in OF tests at any time point after treatments. Flow cytometry and morphometry revealed significant changes in T and B cell balance after influenza antigen challenge, varying according to treatment.

**Conclusion:** The results show that both homeopathic treatments induced subtle changes in acquired immune anti-viral response regulation. A deeper understanding of the mechanism could elucidate their possible use in influenza epidemiological situations.

*Homeopathy* (2016) 105, 257–264.

**Keywords:** Homeopathy; Influenza virus; Influenza hemagglutinin antigen; Behavior; Thymulin; Immunology

## Introduction

Influenza A virus has been responsible for successive pandemics around the world, leading to more deaths than

natural disasters, mainly due to the continuous changes in its antigenic structure.<sup>1–5</sup> The continuous impact of influenza virus on the human population has motivated the development of new approaches less sensitive to these variations, including homeopathy,<sup>6,7</sup> with special focus on biotherapies, that are medicines prepared from etiologic agents following homeopathic procedures (ultradilution and succussion).

In previous *in vitro* studies made by our group, intact influenza biotherapy 30DH was able to change cellular

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Received 10 December 2014; revised 23 March 2016; accepted 5 April 2016

and biochemical aspects of MDCK and J774G8 cell lines with an increase in TNF- $\alpha$  production by these macrophages cells.<sup>6,12</sup> On the other hand, thymulin, a nine-aminoacid thymic hormone which modulates several endocrine and immunological effects, has also shown important immunomodulation effects on birds and mice challenged with bacteria and protozoa, even when prepared as 5CH homeopathic dilutions,<sup>8–11</sup> but there is no experimental description about thymulin 5CH effects in viral disease. Thus, we could compare two different approaches to influenza induced body changes: (a) classical isotherapy (influenza biotherapy) and (b) homeopathic preparations of an endogenous immune-modulator peptide (endo-isotherapy with thymulin). The motivation to the present study was to compare the putative effects on inflammation and behavior of both homeopathic preparations, in order to reveal some critical aspects of their clinical potential to influenza treatment.

## Materials and methods

### Animals

Male 21 days old BALB/c mice were kept in *quarantine* in micro-isolators (Technoplast<sup>®</sup>) during 7 days, until the beginning of the experiment. After this adaptation, the animals were randomly divided in groups, receiving sterile water and commercial food *ad libitum*. The animals were kept under controlled environmental conditions (temperature: 20–24°C; humidity: 50–65%; light/dark cycle: 12/12 h; air cycle: 75 changes/hour) throughout all the experimental period. Water and food consumption and body weight were checked three times a week. Their handling was done exclusively by authorized people throughout the experimental period.

### Ethics

The protocol was approved by the Ethics Committee on Animal Use (CEUA), at the Federal University of Rio de Janeiro, under reference numbers: DFBCICB 037 (Experiment 1) and DFBCICB 040 (Experiment 2). All animal procedures were done according to EU Directive 2010/63/EU for animal experiments.

### Randomization

The animals were randomly distributed in boxes and weighed. Before starting the tests, the average of weight of each box was evaluated and did not show statistically significant differences (ANOVA-one way) in any of the two experimental phases.

### Homeopathic solution preparation

The homeopathic solutions used in this study were prepared according to the Brazilian Homeopathic Pharmacopoeia,<sup>13</sup> using a mechanical automatic device (Autic<sup>®</sup>) for succussion. The protocol was designed to compare two different rationales: the effects of classical isotherapy and those of endogenous molecules isotherapy (endo-isotherapy). The chosen dilutions for both, H3N2 biotherapy

and thymulin (decimal or centesimal, respectively), were selected according to previous experimental results.<sup>9,13</sup>

The biotherapies were prepared using purified H3N2 influenza virus sample (A/AICHI/2/68), at 10,240 HAU/25  $\mu$ l as a starting point, following a standardized protocol established by Siqueira et al.<sup>12</sup> Briefly, we previously detected that the use of water as solvent preserve the virus particle. On the other hand, when ethanol was used as solvent, the virus particles were lysate. In the present work, we compared the efficacy of both medicines, considering the use of both solvents: water (called as intact H3N2) and ethanol (called as inactivated H3N2). Both solvents and protocols are usually used in current homeopathic pharmaceutical practice.

Two variations of biotherapy were prepared: a) the first using 70% alcohol as solvent in all decimal dilution steps, originating the 30DH H3N2 inactivated virus biotherapy; and b) the second using sterile distilled water as solvent from 1st to the 10th or 28th decimal dilution and 70% alcohol in the last two steps, to prepare the 12 and 30DH dilutions, respectively. The use of water as the initial solvent kept the virus particles intact, originating the H3N2 intact virus biotherapy. The control samples corresponded to water, whose preparation followed the same dilutions and procedures except for the addition of influenza virus (water 30DH).

Thymulin 5CH was prepared using a thymulin 4CH matrix (Boiron<sup>®</sup>), which was prepared from the synthetic zinc-free peptide, whose purity was 98.66%. This matrix was diluted at a centesimal basis (1:100) in 30% alcohol and mechanically succussed (Autic<sup>®</sup>) to obtain the 5CH homeopathic dilution, whose estimated concentration is  $4 \times 10^{-13}$  M, a hundred fold less concentrated than the physiological effective level.

All treatments were offered to mice into the drinking water by free access, according to.<sup>8–10</sup> The test solutions were diluted 1:100 in 250 ml of drinking water, in such a way that the final alcohol concentration was 0.3%, low enough not to produce behavioral changes in mice.

### Experimental groups and study design

All procedures were done in blind. Thus, medicines flasks were labeled with a code, whose identification was kept under the responsibility of a laboratory staff not involved with any experimental procedure. The codes were broken only after statistical analysis.

*Set one:* In this experimental set, 62 animals were used and the behavioral parameters and the influence of the starting point of the biotherapy (intact and inactivated H3N2 influenza virus) were evaluated. The experimental groups were distributed as follows (N = 14 mice per group):

Group A – Animals treated with Thymulin 5CH (N = 14).

Group B – Animals treated with water 30DH (N = 14).

Group C – Animals treated with H3N2 inactivated virus 30DH (n = 14).

Group D – Animals treated with H3N2 intact virus 30DH (n = 14).

Group E – Untreated animals (control; n = 6).

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