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ORIGINAL PAPER

A morphometric and molecular study of the apoptosis observed on tadpoles' tail explants under the exposition of triiodothyronine in different homeopathic dilutions

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Background: **As a therapeutic system, homeopathy is supported by: i) similitude and experimentation in healthy individuals, ii) potentization. A challenge for researchers consists in looking for signals in water (or vehicle) to explain the storage of information in extremely high dilutions and the transfer of such information to the living systems. Anuran amphibian metamorphosis is controlled by thyroid hormones (TH), including the resorption of the tadpole tail. Apoptosis is a genetically regulated form of cell death that can be triggered by various extracellular and intracellular stimuli resulting in coordinated activation of a family of cysteine proteases called caspases.**
Methods: **This study was blind and randomized. It performed in three stages: I) the identification of the most effective T3 homeopathic dilution to induce apoptotic reactions in *Rana (Lithobates) catesbeianus* tadpole tail explants stimulated by T3 in substantial, II) study of different controls and III) detection in explants under the action of the most effective dilution of T3, as established in Stage I.**
Results: **There was no statistically significant difference between tail macroscopic dimensions between the groups. T3 10cH decreased the expression of caspase 3/7 mRNA, in explants treated with T3 20 nM.**
Conclusion: **The present experiment is in agreement with the hypothesis that T3, at a 10cH homeopathic dilution, changes the metamorphosis molecular network.** *Homeopathy* (2016) **105**, 250–256.

Keywords: Homeopathy; Apoptosis; Metamorphosis; Triiodothyronine; RT-PCR; Caspases

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Introduction

As a therapeutic system, homeopathy is classically supported by: I) similitude and experimentation in healthy individuals, II) potentization. The principle of similars states that patients with particular signs and symptoms can be cured if a medicine, that produces the same signs and symptoms in a healthy individual, is given. Potentization is the process of manufacturing homeopathic medicines, involving: i) a trituration of a substance in lactose and/or a stepwise dilution of it in a diluent medium (water, ethanol) and ii) at each dilution, the vials are successed (vigorous repeated cycles of shaking via hand or standardized mechanical arm pounding against a flat surface to create mechanical shocks).

A stimulating challenge for homeopathic researchers consists in looking for the existence of signals in water (or vehicle), which are able to explain the storage of information in high dilutions, even beyond Avogadro−Loschmidt limit, and the transfer of this information to the living systems.[1]

Metamorphosis in amphibian and insects is a dramatic example of a late developmental switch, resulting in the reprogramming of morphological and biochemical characteristics of virtually every postembryonic and larval tissue. The entire process of anuran amphibian metamorphosis is under the control of the thyroid hormones (TH) thyroxine (T4) and triiodothyronine (T3). One of the more dramatic effects of T3 and T4 in metamorphosis is to induce the complete regression of the tadpole tail. The dependence of this resorption upon the local action of the TH has been clearly established. The isolated *Xenopus laevis* tadpole tails which were maintained in vitro in simple chemically defined medium will undergo significant resorption in the presence of very low doses of T3. Apoptotic pathways mediate tadpole tail resorption.[2,3]

Initially, necrosis was the only known way for cells to die. In 1972, Kerr *et al.* challenged this concept, defining apoptosis as a novel form of cell death, which was substantially different from necrosis, with regards to morphological features as well as most biochemical processes. While necrosis is considered an accidental form of cell death, often triggered by external factors or disease, leading to membrane rupture and associated inflammatory responses, apoptosis is a genetically regulated form of cell death that plays an important role in eliminating infected, damaged, and other unwanted cells from the body. Apoptosis can be triggered by various extracellular and intracellular stimuli that result in coordinated activation of a family of cysteine proteases called caspases.[4–6]

The aim of this study is the identification of morphometric and molecular changes, generated by homeopathic dilution of T3, on tadpoles' tail explants apoptosis induced by the action of molecular T3.

Experimental procedures

Animals and staging

The experiment was performed with *Rana (Lithobates) catesbeianus* tadpoles, at Gosner stage 35−37,[7] in the early hind limb development. Frogs were supplied by Aquaculture Research Center, Fisheries Institute, Brazil. They were maintained in tanks of aerated water with potassium permanganate 2.37×10^{-6} M, 4000 UI/mL of penicillin G sodium and 4000 mg/mL of streptomycin sulfate for 24 h before caudectomy.

It is important to know that the tadpoles were not sacrificed after the caudectomy, they were returned to the frog farm, where the metamorphosis was completed.

The care and treatment of the animals used in this study were in accordance with the Ethics Committee of the School of Medicine, University of São Paulo (protocol number 062/12).

Tail organ culture

The animals were immobilized by chilling them in ice water for 15 min. The tails were treated with a solution of 10,000 UI/mL of penicillin, 10,000 mg/mL of streptomycin and 25 mg/mL of amphotericin. Then around 2.5 cm tail tips were aseptically removed and rinsed in 4 individual flasks containing: phosphate buffered saline (PBS), 70% ethanol and two subsequent successive immersions in PBS. Afterwards, the explants were placed into individual 25 cm^2 tissue culture flasks (Costar-USA) with 20 mL of Leibovitz medium with glutamine 60% (Sigma L4386) and 10% Antibiotic-Antimycotic Solution (Sigma A5955).[3]

Test solutions preparation

The stock solution was obtained by dissolving 3,3′,5 triiodo-L-thyronine sodium salt (Sigma T 6397) in NaOH 40 mM; the T3 concentration was $5 \cdot 10^{-4}$ M, maintained in the dark at 2°C and diluted in the culture medium. When used, the final molecular concentration of T3 (acting directly on the explants) was $10 \cdot 10^{-9}$ M and $20 \cdot 10^{-9}$ M. Plastic pipettes and medium bottles were used, since the hormone adsorbs strongly to non-siliconized glass. The homeopathic dilution solutions were prepared according to Brazilian Homeopathic Pharmacopoeia[8]: to obtain the 1cH we added 1 part of T3 $5 \cdot 10^{-4}$ M (T3 dissolved in NaOH 40 mM) in 99 parts of NaOH 40 mM. To 2cH and 3cH we added 1 part of the previous dilution in 99 parts of NaOH 40 mM and to 4cH until 100cH we dissolved 1 part of the previous dilution in 99 parts of ethanol 70%, always successing the mixture with 100 manual horizontal shakes at each dilution step in sterilized plastic flasks. The control solution was unsuccussed ethanol 70%.

Experimental model

The work was performed in three stages:

- Stage I: the identification of the best effective T3 homeopathic potency, which induces apoptotic reactions on explants under the molecular action of T3,
- Stage II: the study of different homeopathic controls and,
- Stage III: the apoptosis detection, based on molecular method, in the remaining explants under the action of the most effective T3 homeopathic potency established in Stage I.