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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.

Stage I: Six groups of 15 explants each were studied:

- Negative control: explants in culture medium (without T3 action, nor at molecular neither at homeopathic dose).
- Positive control: explants in culture medium + T3 10 nM + 150 μL Ethanol 70% (unsuccussed vehicle).
- Group 10cH: explants in culture medium + T3 10 nM + 150 μL T3 10cH.
- Group 30cH: explants in culture medium + T3 10 nM + 150 μL T3 30cH.
- Group 60cH: explants in culture medium + T3 10 nM + 150 μL T3 60cH
- Group 100cH: explants in culture medium + T3 10 nM + 150 μL T3 100cH.

Subsequently the explants were transferred to an incubator at 25°C. The culture medium with the additives was changed every 48 h. All manipulations were carried out under sterile conditions. The explants were randomly allocated to tissue culture flasks and the treatments were blinded: a person external to the study coded as flask A, B, C, etc. the different solutions. They were identified only at the end of the study.

Stage II: Five groups of 17 explants each were studied:

- Negative control: explants in culture medium.
- Positive control: explants in culture medium + T3 20 nM + 150 μL Ethanol 70%.
- Group 10cH: explants in culture medium + T3 20 nM + 150 μL T3 10cH (T3 diluted and succussed).
- Group T3 $5 \cdot 10^{-24}$ M: explants in culture medium + T3 20 nM + 150 μL T3 $5 \cdot 10^{-24}$ M (T3 diluted but unsuccussed).
- Group Ethanol 10cH: explants in culture medium + T3 20 nM + 150 μL Ethanol 70% 10cH (solvent succussed without T3).

Subsequently, it was carried out the same procedure of Stage I.

Tail measurement

The explants were photographed at the beginning and at the end of the experiment. The digital photographs were analyzed with ImageJ Launcher software®[9] and UTHSCSA Image Tool®.[10]

Apoptosis detection based on molecular method

Stage III: For analysis of caspase-3 and caspase-7 mRNA expression, total RNA was isolated from remaining explants for reverse transcriptase/polymerase chain reaction (RT-PCR) analysis. Total RNA was isolated from explants using Trizol (Invitrogen) reagent following the Invitrogen protocol.

SYBR® Green qPCR. Specific primer sets of oligonucleotides were elaborated by Invitrogen. We used the following primers: (i) Caspase-3 forward sequence (5′ to 3′):CTT TAT TCA GGC ATG CAG AGG, reverse sequence (5′ to 3′):CTG AAT GCC ACA TAA CCT TGG, (ii) Caspase-7 forward sequence (5′ to 3′):TGT GTA TTC GGA AAG GGA CAG, reverse sequence (5′ to 3′):TGT CAC TGT GGT CGT TCT TTG (iii) Beta Actin Forward Sequence (5′ to 3′):TTC ACC ACC ACA GCA GAA AG, Reverse Sequence (5′ to 3′):TTC CGA TGG TGA TGA CTT GA.[11]

All primers were diluted to a concentration of 10 μM and 1 μl was used in a total volume of 20 μl for the qPCR reactions. Power SYBR® Green PCR Master Mix from Applied Biosystems by Life.

The PCR conditions used were as follows: 48°C for 30 min; 95°C for 10 min, followed by 60 repeat cycles of 95°C for 15 s; 60°C for 1 min. A dissociation stage of 95°C for 15 s, 60°C for 15 s and 95°C for 15 s was added. All samples were run in triplicate.

Quantitative real-time PCR analysis. Data generated from the qPCR reactions were analyzed using the $2^{-\Delta\Delta CT}$ method.[12] Tissue expression values were normalized to Beta Actin. For these two studies, all the CT values for the experiment were averaged and used to calculate individual sample ΔCT. The average ΔCT values of the control group were used to calculate the $\Delta\Delta$CT values for individual samples.

Statistical analysis

Data shown are mean $\pm$ SD and are representative of at least two separate experiments. Differences were analyzed using ANOVA followed by *post hoc* analysis using Bonferroni test for comparison between any two groups. Statistical significance was set at p value less than or equal to 0.05. All graphs were generated using the GraphPad Prism 6.0C program (GraphPad Software, San Diego, CA).

Results

The Stage I experiment was performed to identify the best effective T3 homeopathic potency, which induces apoptotic reactions on explants under the molecular action of T3 10 nM. After 7 days of tissue culture, the mean initial and final explants' length between the 6 groups are shown in table below.

According to Table 1 data, the negative and 10cH groups had a reduction tail length lower than other four groups; however, we did not find a statistically significant difference in macroscopic length of the tadpoles' tail between these six groups.

In Stage II of the experiment, we studied the action of different controls on the tadpole tail apoptosis. After 5 days of tissue culture, the mean initial and final explants' length between the 6 groups are shown in table below.

Table 2 data indicate that negative group had an A.M.I. lower than other four groups ($p < 0.01$). This result was expected, since this group did not have the molecular action of T3 20 nM. The others 4 groups did not have statistical difference in A.M.I.

Looking at Figure 1 we can see three examples of significant decrease in the explants length.

In Stage III of the experiment, our aim was the identification of apoptotic changes induced by T3 in high potency on the apoptosis of tadpole tails, utilizing an apoptosis index detection based on molecular method,

Table 1 Stage I: The length (cm) of *Rana (Lithobates) catesbeianus* tadpoles' tail under the molecular stimuli of T3 10 nM, exposed to different T3 homeopathic dilution

	N° lexplants	*Initial mean length (cm)*	*Final mean length (cm)*	*Mean reduction (cm)*	*Mean reduction (%)*	*Standard deviation*
Negative control	14	2.37	1.98	0.39	16.5	0.13
Positive control	14	2.12	1.61	0.51	24.1	0.12
Group 10cH	12	2.18	1.79	0.39	17.9	0.20
Group 30cH	14	2.28	1.75	0.53	23.2	0.23
Group 60cH	14	2.3	1.74	0.56	24.3	0.10
Group 100cH	14	2.14	1.63	0.51	23.8	0.16

namely, real-time PCR using SYBR Green methodology. For this, we selected 5 explants of Stage II, each one of the following groups: negative, positive and 10cH group. This choice was based on the reduction of tadpole's tails length nearest the average, i.e., with minimum standard deviation. Table 3 below summarizes these data.

A quantitative analysis of caspase-3/7 mRNA expression assessed by RT-PCR, using SYBR Green methodology, is summarized in Figure 2 below. We found a decreased mRNA expression of caspase-3 (Figure 2A) and caspase-7 (Figure 2B) in tadpoles' tail tips of T3 10cH group when compared with positive group.

Discussion

In Stage I, our objective was to identify the most effective T3 homeopathic potency, which modifies apoptotic reactions on explants under the molecular action of T3 10 nM.

In Table 1, data indicate that when initial mean tail length was set to 100%, mean tail reduction was 16.5% in the untreated (negative) control group and 24.1% in the positive control group treated with molecular T3 10 nM. This difference was expected, T3 being the well-known physiological stimulus of tail reduction. Tail reduction in the group treated with T3 10 nM plus T3 10cH was 17.9%, i.e. in the range of untreated control rather than in the range of the positive control. Groups treated with T3 10 nM plus T3 30cH, 60cH and 100cH ranged between 23.2 and 24.3%, i.e. similar to positive control. Differences were not statistically significant ($p > 0.05$), probably due to the high standard deviation. This result was interesting, because it means that T3, at molecular concentration of 10 nM, did not induce statistically significant apoptosis, at least at morphometric method, on the explants from all groups.

In Guedes *et al.* 2004 and 2011,[2,3] they worked with T3 at 10cH obtaining positive results. Based on data presented here and on previous studies, it can be concluded that the best homeopathic action, specifically in tadpole tail apoptosis, is obtained with T3 at 10cH potency.

In Stage II we studied the effects, of different homeopathic controls, on explants treated with molecular T3 20 nM. Table 2 reveals that mean tail reduction was 18.0% for untreated control and 35.1% for positive control ($p < 0.01$) as could be expected. Tail reduction in the test group (T3 10 nM plus T3 10cH) (37.3%) was not different to positive control or to further control groups.

Metamorphosis has been divided into three stages: premetamorphosis (a period in which growth occurs with little change in form of the animal), prometamorphosis (a period of differential hind leg growth and continued body growth but at reduced rate) and climax (starting with forelegs emergence followed by rapid changes in mouth and tail, resulting in a small frog).[13]

The remaining apoptosis found on explants of negative group, represented by a length reduction of 18%, is interpreted to mean that these tissues had been exposed to a sufficient physiological level of TH, until the time of caudectomy, to program them for some resorption.[14,15] In the tadpoles' tail, muscles begin to undergo apoptosis at prometamorphosis.[16]

In Stage III, based on molecular method, we performed the apoptosis detection on the remaining explants under the action of the most effective T3 homeopathic potency established in Stage I and in previous studies.

Guedes *et al.* (2011)[3] hypothesized that the tadpole's tail macroscopic measurement could not be sensitive enough to detect the T3 effect on high dilution, since the authors obtained a statistically significant apoptotic index by Immunohistochemical method (in situ hybridization for TUNEL), on explants with no statistically significant length reduction.[3]

Table 2 Stage II: The length (cm) of *Rana (Lithobates) catesbeianus* tadpoles' tail under the molecular stimuli of T3 20 nM, exposed to different T3 homeopathic controls

	N° explants	*Initial mean length (cm)*	*Final mean length (cm)*	*Mean reduction (cm)*	*Mean reduction (%)*	*Standard deviation*
Negative control	15	2.5	2.05	0.45	18.0	0.16
Positive control	14	2.39	1.55	0.84*	35.1	0.17
Group 10cH	17	2.36	1.48	0.88*	37.3	0.18
Group T3 5.10^{-24} M	16	2.37	1.49	0.88*	37.1	0.17
Group Ethanol 100cH	16	2.6	1.82	0.78*	30.0	0.17

*Significantly different from negative control ($p < 0.01$).

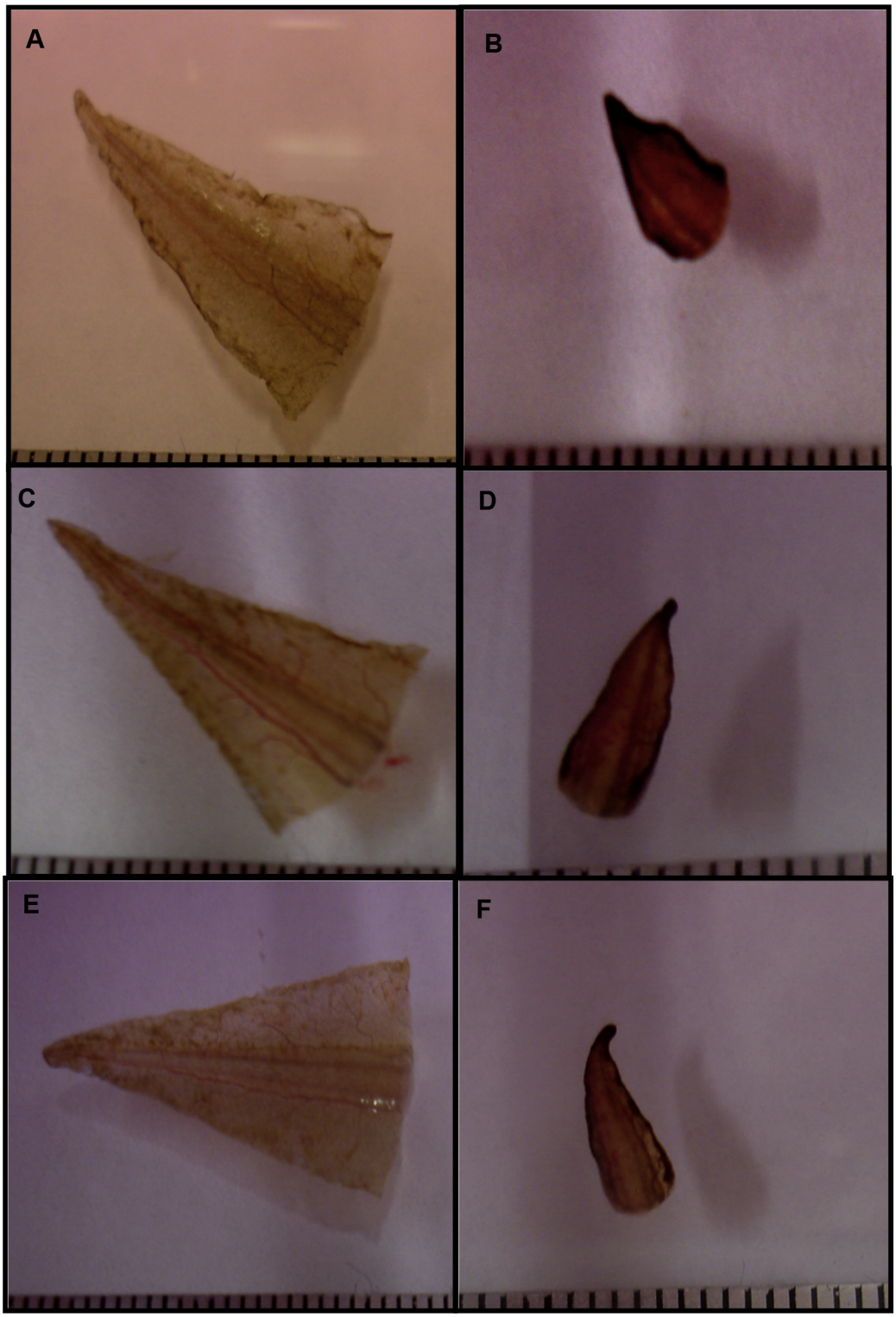

Figure 1 Three examples of explants length reduction. Initial length (A, C, E) and final length (B, D, F).

At the cellular level, T3 controls cell metabolism, proliferation, and commitment to differentiation or apoptosis. The diverse effects of TH suggest the existence of tissue- and developmental stage-specific control of gene expression by TH to coordinate different transformations in various organs. At more advanced metamorphosis stage, more tadpoles' tissues are prepared to respond to TH.

One of the biggest challenges in metamorphosis experiments is the tadpoles' selection with exactly the same stage of metamorphosis. With the intent of minimizing this problem, we choose the remaining explants, of Stage II, with around the same average reduction based on morphometric method, i.e., explants with similar response to T3. It can be seen in Table 3 that from each of the negative and the positive controls as well as from the test group, those 5 explants the mean reduction tail length of which was closest to average were analyzed for caspase-3 and caspase-7 mRNA.

Table 3 The length (cm) of *Rana (Lithobates) catesbeianus* tadpoles' tail under the molecular stimuli of T3 20 nM, selected to SYBR Green PGR

	N° explants	*Initial mean l ength (cm)*	*Final mean length (cm)*	*Mean reduction (cm)*	*Mean reduction (%)*	*Standard deviation*
Negative control	5	2.51	2.06	0.45	17.9	0.09
Positive control	5	2.53	1.63	0.90	35.6	0.09
Group 10cH	5	2.34	1.49	0.85	36.3	0.1

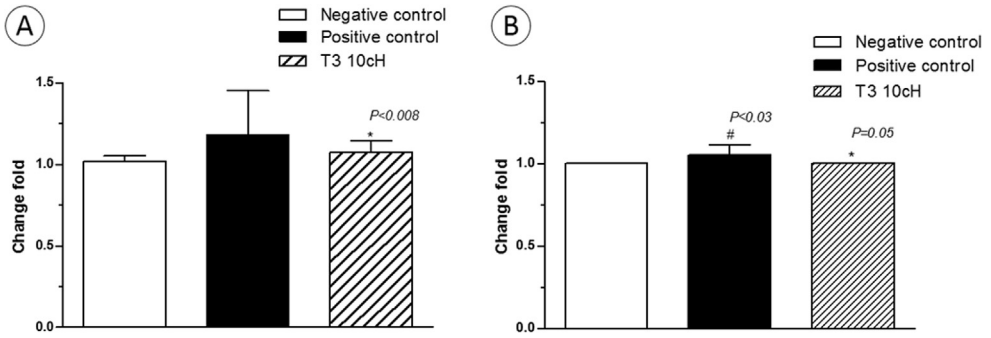

Figure 2 Molecular apoptosis detection. **A** – Expression of caspase-3 mRNA, of the remaining tadpoles' tail explants, after treatment with T3 10cH. **P* < 0.008 compared with positive control. **B** – Expression of caspase-7 mRNA, of the remaining tadpoles' tail explants, after treatment with T3 10cH. #*P* < 0.03 compared with negative control; **P* = 0.05 compared with positive control.

The data of Figure 2A indicates that the expression of caspase-3 mRNA, of the remaining tadpoles' tail explants of T3 10cH group is smaller than its expression on the positive group. The same is observed in Figure 2B, showing that the expression of caspase-7 mRNA, of the explants of T3 10cH group is smaller than its expression on the positive group. When positive control was set to 100%, caspase 3 expression was 92.3%, i.e. 7.7 percent points below control ($p < 0.01$), and caspase 7 expression was 94.0%, i.e. 6 percent points below control ($p = 0.05$). In both graphics we conclude that T3 10cH decreased the expression of caspase 3/7 mRNA, when compared with positive control.

In the present study, the inhibitory effect of T3 10CH on the expression of caspase-3 and caspase-7 corroborates previous studies about the modulatory action of homeopathic hormone preparations.[2,3,17–20]

Bellavite *et al.* 2015 documented around 35 studies, published in peer-reviewed literature, related with the ability of highly diluted compounds to modulate gene expression in human/animal cells and unicellular organisms.[21] Bishayee *et al.*, 2013[22] advocated a working hypothesis claiming that the working principle of the homeopathy remedy should be based upon a switch on/off mechanism of gene expression.[22]

It is known that vast areas of genomic DNA include many 'non-coding' segments. Many different proteins and DNA sequences have to come together in choreographed succession to form and rearrange the nucleoprotein complexes necessary for directing the precise cut and splice operations involved. The question is how these molecules, with very specific functions, can find one another and join up to do their job just at the right time and place. One hypothesis that may be considered is electromagnetic signaling and resonance.[23] Jacques Benveniste, in the same way, proposed that a ligand molecule emits an electromagnetic signal with a frequency identical to the receptor's molecules that causes them to co-resonate and activate the same intracellular responses.[24]

Conclusions

Based on the present data, it can be concluded that T3 10cH is able to impair the expression of caspase 3 and 7 mRNA induced by T3 20 nM *in vitro*, using a frog tail explant model. This finding explains, at a molecular level, several reproducible results obtained in vivo since 1994, in which the treatment of tadpoles with homeopathic potencies of TH was able to delay the metamorphosis process.[2,3,18–20]

The present experiment is in agreement with the hypothesis that T3, at a 10cH homeopathic dilution, would be able to change the metamorphosis molecular network.

Conflicts of interest

The authors declare no conflict of interest.

Acknowledgments

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