Homeopathy (2011) 100, 220–227

doi:10.1016/j.homp.2011.05.007, available online at http://www.sciencedirect.com

ORIGINAL PAPER

Ultra High Dilution of triiodothyronine modifies cellular apoptosis in *Rana catesbeiana* tadpole tail *in vitro*

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Background: **Ultra High Dilutions (UHD) are diluted beyond the Avogadro limit with dynamization (dilution with succussion). The process of anuran amphibian metamorphosis is controlled by thyroid hormones, including the resorption of the tadpole tail.**
Methods: **A randomized and blinded study was performed to investigate the influence of triiodothyronine (T3) $5 \cdot 10^{-24}$ M (10cH) on apoptosis induced by T3 100 nM in *Rana catesbeiana* tadpoles' tail tips, *in vitro*. Explants were randomized to three groups: control: no T3 in pharmacological or UHD dose; test: T3 100 nM and challenged with T3 10cH (UHD); positive control: T3 100 nM, treated with unsuccussed ethanol. The apoptotic index and the area of explants of test and control groups at the first and final day of the experiment were compared by *t*-test.**
Results: **There was no difference in tail tip area between test and control groups, but a significantly higher ($p < 0.01$) index of apoptosis in explants of the test group.**
Conclusion: **This data suggest that T3 10cH modifies the effect of T3 at pharmacological dose, opening new perspectives for further studies and investigation of the dose–effect curve.** *Homeopathy* (2011) **100**, 220–227.

Keywords: Ultra High Dilution; Triiodothyronine; Apoptosis; *Rana catesbeiana*; Organ culture; Metamorphosis; Endo-isopathy; Homeopathy

Introduction

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 since isolated *Xenopus laevis* tadpole tails maintained *in vitro* in simple chemically defined medium will undergo significant resorption in the presence of very low doses of T3.[1] Tadpole tail resorption is mediated by apoptotic pathways.[2]

Ultra High Dilutions (UHD) are solutions, diluted beyond the Avogadro limits, with dilution and succussion. They may elicit effects on living beings, which may be suppressive or stimulant at multiple levels, including the cellular level.

In the 1990s, Endler *et al.* adapted an amphibian model to study the sensitivity of *Rana temporaria* to UHD of T4 during metamorphosis. The action of substances so highly diluted that no original molecule is present yet which still

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Received 14 June 2010; revised 27 April 2011; accepted 6 May 2011

have biological effects is thought to be related to molecular bio-information transduced *via* water.[3,4] In 2003, based on this work, we published the results of experiments with UHD of thyroid gland showing decreased rate of metamorphosis in *Rana catesbeiana*.[5] This study reproduced Endler's experimental results, and reproducibility is one of the most serious problems with UHD research.

In this work, the hypothesis is that the test dilution (T3 in UHD) modifies the effect of T3, at pharmacological dose, in inducing apoptosis in tadpole tails *in vitro*.

Experimental procedures

Animals and staging

The experiment was performed with *R. catesbeiana* tadpoles, at the beginning of metamorphic climax, i.e., before the forelimbs' exteriorization. Frogs were supplied by Aquaculture Research Center, Fishery 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 μg/mL of streptomycin sulfate for 24 h before caudectomy. 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 099/04).

Tail organ culture

The animals were immobilized by chilling them in ice water for 15 min. The tails were disinfected with a solution of 10,000 UI/mL of penicillin, 10,000 μg/mL of streptomycin and 25 μg/mL of amphotericin. Then 2 cm tail tips were aseptically removed and rinsed in 4 individual flasks containing phosphate buffered saline (PBS), followed by a 5 s immersion in 70% ethanol and two subsequent successive 10-s immersions in PBS. After, the explants were placed into individual 75 cm^2 tissue culture flasks (Costar-USA) with 50 mL of Leibovitz medium with glutamine 60% (Sigma L4386) and 10% Antibiotic–Antimycotic Solution (Sigma A5955).

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, maintained in the dark at 2°C, and diluted in the culture medium when used. According to Sigma Product Information, NaOH is indicated to solubilize or reconstitute T3. Plastic pipettes and medium bottles were used, since the hormone adsorbs strongly to non-siliconized glass. The final pharmacological concentration of T3 at the medium culture, in both control and test group, was 100 nM.[2]

The UHD test solution was prepared according to Brazilian Homeopathic Pharmacopoeia[6]: 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 10cH we dissolved 1 part of the previous dilution in 99 parts of ethanol 70%, always succussing the mixture with 100 manual horizontal shakes at each dilution step in sterilized plastic flasks. The UHD concentration of T3, added to the medium culture, was $5 \cdot 10^{-24}$ M, corresponding to the 10th Hahnemannian Centesimal Dilution (10cH). The control solution was unsuccussed ethanol 70%.

Experimental model

Three groups of 20 explants each were studied:

Negative control: 20 explants + 50 mL of culture medium. Without T3 action, nor at pharmacological neither at UHD dose.
Test: 20 explants + 50 mL of culture medium + 1 mL $5 \cdot 10^{-6}$ M T3 + 150 μL T3 10cH.
Positive control: 20 explants + 50 mL of culture medium + 1 mL $5 \cdot 10^{-6}$ M T3 + 150 μL control solution (unsuccussed vehicle).

The explants were randomly allocated to tissue culture flasks and the treatments were blinded: a person external to the study, coded as flask A and B the two solutions: 1) T3 10cH and 2) control. They were identified only at the end of the study. Subsequently the explants were transferred to an incubator at 25°. The culture medium with the additives was changed every 48 h, for 4 days. All manipulations were carried out under sterile conditions.

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®[7] and UTHSCSA Image Tool®.[8]

Histological procedures

The organ-culture tails were fixed in a Bodian Solution[9] for 6 h at room temperature and then dehydrated with a series of increasing ethanol concentrations. Then, they were classically processed for embedding into paraffin and then serially sectioned at 4 μm thickness.

Apoptosis detection

In situ hybridization was used to identify apoptosis of the remaining explants, *via* nuclear expression by deoxynucleotidyl transferase (TdT) end labeling (TUNEL).[10,11] This method involves the addition of deoxyuridine triphosphate (dUTP) labeled with fluorescein to the ends of the DNA fragments by the catalytic action of TdT. Paraffin-wax embedded sections were layered on to glass slides. The tissue sections were dewaxed with xylene and rehydrated with graded dilutions of ethanol in water. The slides were then washed four times with double distilled water for 2 min and immersed in TdT buffer (Boehringer Mannheim). The sections were then covered with TdT and fluorescein-labeled dUTP in TdT buffer, and the samples were incubated in a humid atmosphere at 37°C for 60 min. For negative controls, TdT was eliminated from the reaction mixture. The sections were then incubated with an antibody specific for fluorescein conjugated to peroxidase. The staining was observed with a substrate system, in which nuclei with DNA

fragmentation stained brown. The reaction was terminated by washing the sections twice in PBS. The nuclei without DNA fragmentation stained blue as a result of counterstaining with hematoxylin.

Morphometry

The apoptosis index was determined in the remaining explants. At ×400 magnification an eyepiece systematic point-sampling grid with 100 points and 50 lines was used to count the fraction of points overlying nuclei positively-stained structures.[12] We averaged this over ten microscopic fields to obtain the test and control apoptosis index as a percentage of staining nuclei.

Interobserver comparisons were performed in 20% of the slides by two observers. The coefficient of variation for the interobserver error regarding cell count was <5%.

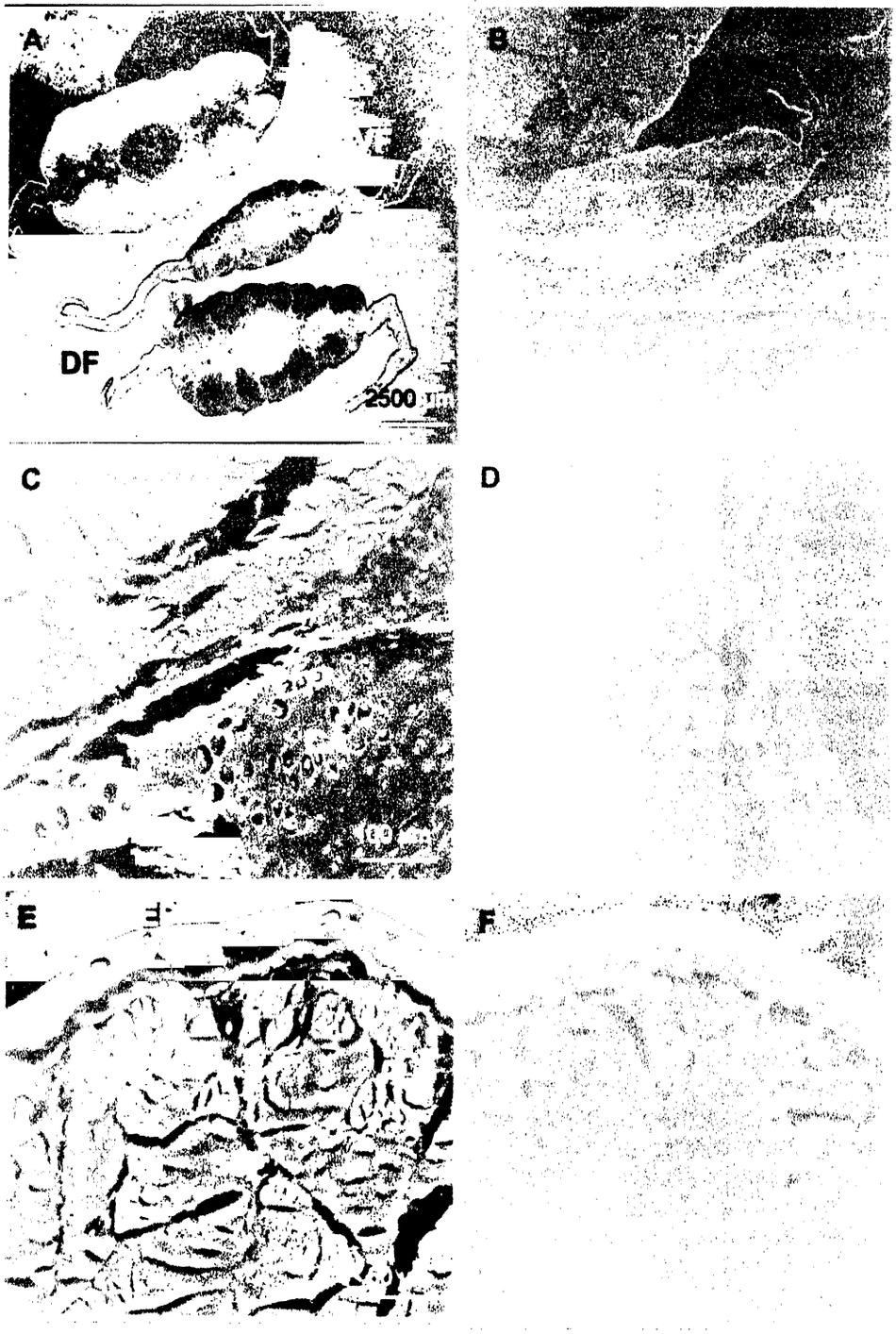

Figure 1 Histology of the tadpole's tail tips of Group A, cultured for 96 h without T3 pharmacological action, stained by H&E (A, C, E) and *in situ* hybridization for TUNEL + apoptosis (B, D, F). Panoramic view of the explants with normal histoarchitecture and preservation of dorsal and ventral fins is well identified (A, B), as well as intact nuclei in epidermis (E), skeletal muscle (C, E), cartilage (C) and notochord (A). Physiologic apoptosis is identified in nuclei from epidermis and skeletal muscle cells (D, F).

Statistical analysis

The normality of the data (Kolmogorov–Smirnov test with Lilliefors' correction) and the homogeneity of variances (Levene median test) were tested. If both conditions were satisfied, the analysis of variance of tadpole tail tips means between the two groups (test and positive control) was carried out by Students *t*-test. Otherwise, the Fisher test was used. The significance level was set at 5%. Parametric data were expressed as mean ± SD. The data were analyzed using SPSS for Windows release 18.0.

Results

The explants' histological features, without T3 pharmacological action, cultured for 96 h from negative control stained by hematoxylin and eosin (H&E) (A, C, E) and *in situ*

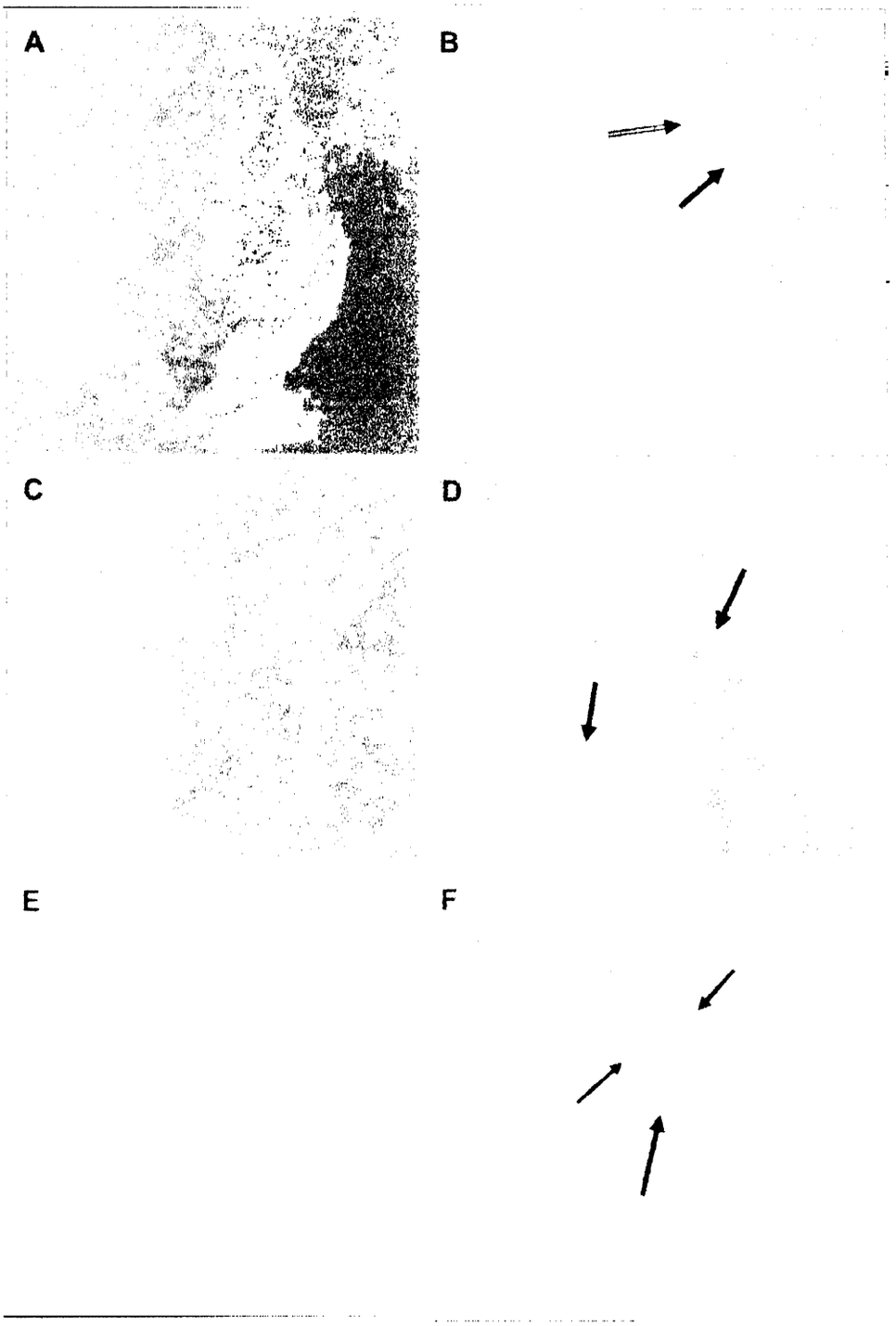

Figure 2 Histological representation of tadpole's tail tips of Group B (test) and Group C (control), cultured for 96 h under the action of T3 100 nM, stained by H&E (A, C, E) and *in situ* hybridization for TUNEL + apoptosis (B, D, F). Panoramic view of the explants with reduction of the cellular components (A) and correspondent apoptosis (arrows) (B). High magnification showing the involution of the skeletal muscle (C), and correspondent apoptosis (D). Detail of the epidermis distortion (E) and correspondent apoptosis (F).

hybridization for TUNEL + apoptosis (B, D, F) are shown in Figure 1. Normal histoarchitecture with preservation of dorsal and ventral fins is identified (A, B), as well as intact nuclei in epidermis (E), skeletal muscle (C, E), cartilage (C) and notochord (A). Physiologic apoptosis is identified in nuclei from epidermis and skeletal muscle cells (D, F).

The explants' histological features, with T3 pharmacological action, cultured for 96 h from test and positive control stained by hematoxylin and eosin (A, C, E) and *in situ* hybridization for TUNEL + apoptosis (B, D, F) showed no significant differences and are shown in Figure 2. In both groups, the remaining explants show distortion of the histoarchitecture and prominent shortening with total elimination of dorsal and ventral fins (A, B). Numerous apoptotic nuclei from epidermis (B) and skeletal muscle (D, F), as well as notochord (F), are identified in the middle of the explants' disorganized tissue upon the concentration of 100 nM of T3. The mean area measurements and apoptosis index of the remaining explants from positive control and test groups are shown in Table 1.

After 96 h of tissue culture, the mean of initial and final area (1.05 *vs.* 0.98 cm^2) and apoptotic index of the explants from negative control were minimally different and for this reason are not included in Table 1. However, the mean initial and final explants' area between test and positive control groups were respectively 1.09 *vs.* 0.22 cm^2 and 1.00 *vs.* 0.24 cm^2, with a mean reduction of 0.87 cm^2 and 0.76 cm^2, this is not statistically significance ($p > 0.05$). In contrast, apoptosis index was significantly higher in test than in positive control 11.7 *vs.* 7.9 ($p < 0.01$).

Discussion

In the present study, we established a protocol model to culture *R. catesbeiana* tadpoles' tail tips *in vitro*. These explants were distributed in three groups: negative control without T3, at pharmacological or UHD dose; test: T3 100 nM and treated with T3 10cH (UHD); control: T3 100 nM and treated with ethanol 70% unsuccussed. After 96 h of tissue culture, tadpoles' tail tips from negative control without pharmacological T3 stimulus showed no significant resorption by apoptosis. However explants of test and control groups lost a significant portion of their total area, and more in the test group. The explants, of test and control groups, lost an average 80% *vs.* 76% respectively. We did not find a statistically significant difference in macroscopic area of the tadpoles' tail tips between test and control groups, but a statistically significantly high index of apoptosis in histology was found in explants of test group ($p < 0.01$). The data suggest that T3 10cH may modulate the effect of T3 at pharmacological dose.

Under the action of T3 100 nM, the reduction of area of the explants in the test group was greater than in the positive control group, but this difference did not achieve statistical significance, perhaps because the apoptotic index is more sensitive for detecting the effect of UHD than macroscopic measurement. It is important to note that UHD T3 (10cH) did not replace T3 in pharmacological concentrations, instead, it exerted a modulatory effect. Such effects have been detected in previous studies.[13–18]

Our results are in line with those of others researches[2,19,20] that have demonstrated the effect of pharmacological dose of TH in reducing the area of tadpoles tails *in vitro*, and that this resorption is mediated by apoptotic pathways.[2]

In pilot experiments, all the explants treated with pharmacological doses of T3 showed muscle cell devoid of nuclei but without a change in area, after a week of tissue culture (Figure 3). According to Prof. Jamshed R. Tata's experience (personal communication), *R. catesbeiana* tadpole tails used in the experiments were difficult to maintain in organ culture. He was not surprised that we noticed muscle tissue without nuclei. This occurred because of the volume of the explants, with consequent problem of insufficient diffusion of nutrients and oxygen, resulting in necrosis. To solve this problem we used around 2 cm from the posterior tail extremity.

Another important finding from our experiment model, is that L-15 Medium (Leibovitz) was originally formulated for use in carbon dioxide (CO_2) free systems. In fact L-15 medium may be used in CO_2 systems, nevertheless when the explants were transferred to a CO_2 incubator, the pH of the medium dropped from 7.4 to 6.2 in 24 h, and T3 is not viable at acid pH.

We used unsuccussed diluent as a control and this is the basis of a possible criticism of our experiments. "*Controls are the basis of the evaluation of an effect. In classical research based on molecule action, the control is the solvent used to prepare the product. Informative models require several controls. The basic one is the unsuccussed solvent. Researchers reported activity of the succussed solvent when the model was adapted to the evaluation of the endogenous molecule high dilutions*".[21] Our control solution was unsuccussed in order to avoid nonspecific effects of succussed vehicle.[16,18,21–23]

The cellular level actions of TH are highly conserved from frogs to humans. Although its transport across the cell membrane and interactions with cytoplasmic proteins

Table 1 The area (cm^2) and the apoptosis index of *Rana catesbeiana* tadpoles' tail tips under the stimuli of T3 100 nM, exposed to two different treatments: T3 10cH (test) and control solution

	Initial area (cm^2)		*Final area (cm^2)*		*Reduction (cm^2)*		*Apoptosis index N° X/1000 points*	
	Test	*Control*	*Test*	*Control*	*Test*	*Control*	*Test*	*Control*
N° explants	19	20	19	20	19	20	19	20
Mean	1.09	1.00	0.22	0.24	0.87	0.76	11.7*	7.90
SEM	0.04	0.04	0.04	0.02	0.19	0.15	2.08	1.28

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

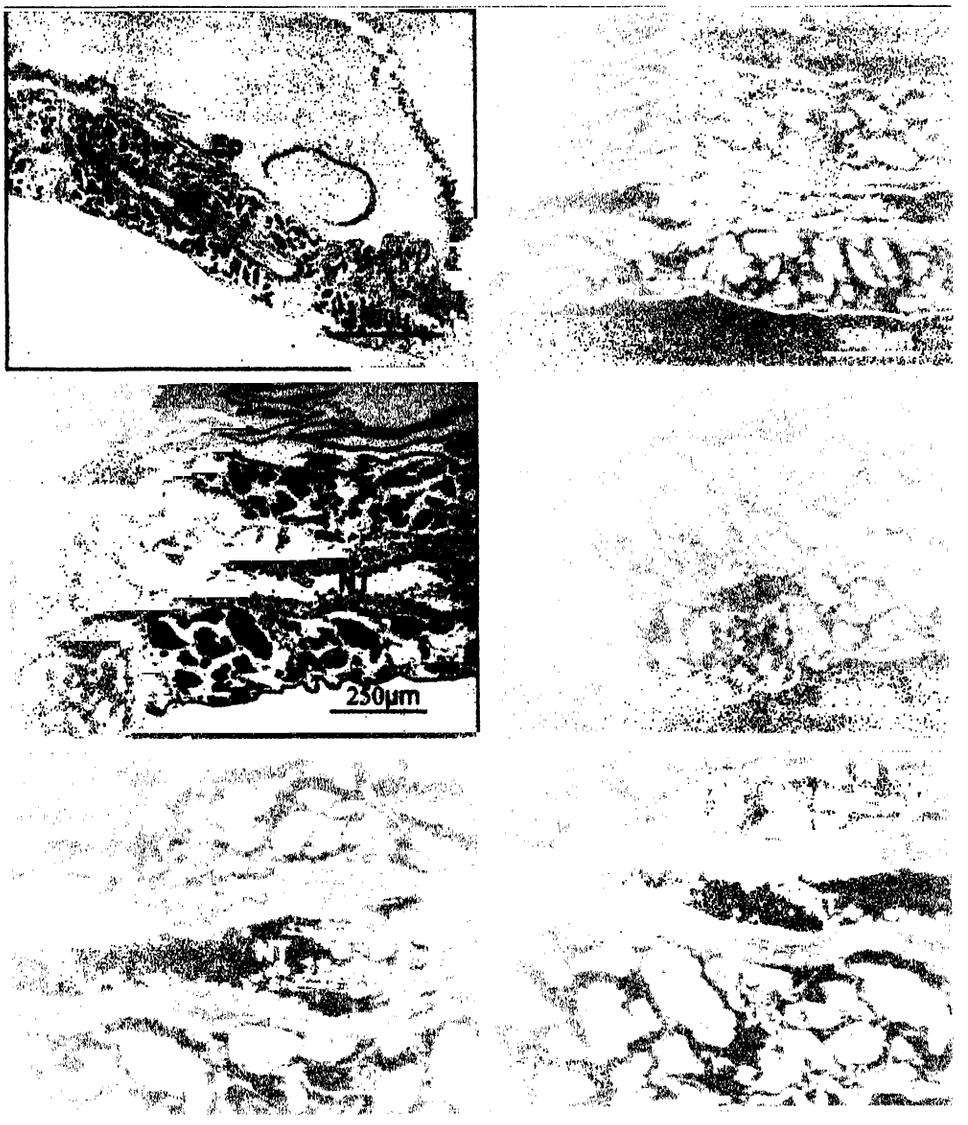

Figure 3 Illustrative panel of the tadpole's tail tips of one of the first pilot projects, showing after a week of tissue culture, muscle cell devoid of nuclei without difference in their length. H&E. Epidermis (Ep), notochord (NT), skeletal muscle (SM).

determines the dynamics of TH distribution within the target cell, the mechanisms involving TH binding to specific nuclear thyroid hormone receptors (TRs) are the crucial step that initiates the molecular and biochemical process leading to physiological response to the hormone.[24] TRα and TRβ are the major TR isoforms that posses dual functions as transcription repressors and activators in the absence and presence of ligand (primarily T3), respectively. TRα is present in the premetamorphic tadpole tail whereas TRβ levels are extremely low until TH induction, suggesting that TRα is critical for the competence to respond to TH while TRβ is required for establishment of metamorphic genetic program. Based on this information we choose the metamorphosis stage for this experiment, i.e., the tadpoles before the forelimbs' exteriorization, when there is high level of TRβ and, thus, the tissue is primed to respond to pharmacological dose of T3. How important these receptors – or the balance between them – is for the effect of UHD of T3 is still unknown and deserves further investigation.

Apoptosis is regulated directly or indirectly by number of death signal-controlling genes, many of which are localized in the nuclear, outer mitochondrial and endoplasmic reticulum membranes, regulating the electrochemical homeostasis of the organelles. The actual executioners are proteolysis occurring during apoptosis, whose targets are factors involved in the repair, fragmentation and duplication of DNA, in RNA splicing, in the maintenance of cytoskeletal integrity, and in cell division.[25] In future we intend to study the expression of Fas (CD 95), caspase-8, P 53, Bax etc., in explants treated pharmacological doses of T3 may be modified by the T3 UHD action.

There are many causes of cell injury and cell death, among others, hypoxia and nutritional imbalances. The Extracellular Matrix (EM) and, particularly, the basement membrane (BM) provide a substratum where cells can adhere, migrate, proliferate and directly influence the form and function of cells. The BM surrounds skeletal muscle fibers and is know to be critical to muscle fiber structure and function. In recent years there has been great progress in the identification and

characterization of the two sarcolemma protein complexes that connect cytoskeleton to the BM in skeletal muscle: integrins and the dystrophin-glycoprotein complex (DGC). DGC binds laminin and has similar roles in skeletal muscle as the integrin transmembrane linkage system. Integrin-mediated adhesion regulates apoptosis in a variety of cells, including skeletal muscle.[26]

In our experiment we noticed that the muscle cells of the explants, stained by the two ways (TUNEL and H&E), had no nuclei, unlike cells like epidermis and notochord. This confirms Sachs *et al.*[27] conclusions: 1) the first cells to respond to apoptosis in tadpole tail is the skeletal muscle cell, a non-dividing, a permanent cell, probably because the destruction of the BM, 2) for apoptosis to be induced in muscle cells *in vitro*, epidermal cells must be present.

UHD are not easy to study because: multiple dilutions must be used because the dilution/response curve is non-linear. There is often a temporal component. Adequate statistical power and replication are also necessary. These features often lead researchers to investigate less resource-intensive and more readily definable phenomena.[28]

Our next challenge will be the repetition of the experiment with a more physiological concentration of T3, around 5 nM[1,29] and with others dynamizations of T3. According to Bonamin *et al.*[30] "*Perhaps, the most enigmatic feature regarding the properties of ultra-dilutions is the non-linearity of their effects. In several studies employing* in vivo *and* ex vivo *models, especially involving iso-endopathy, an oscillatory potency-effect curve has appeared. The first observations were initially considered as artifacts, but the repetition of this pattern in different studies involving completely different experimental models, in times and places equally different, points out to the existence of a property intrinsic to* dynamized systems."

Conclusion

We developed an UHD model to study the influence of T3 10cH in modifying the apoptosis of *R. catesbeiana* tadpole tail, *in vitro*. In this model, T3 $5 \cdot 10^{-24}$ M (10cH), added to the medium containing the explants treated with T3 100 nM, induced a significant ($p < 0.01$) index of apoptosis. This is in line with the conclusions of other related research with UHD. The contribution of our new experimental model lies in its ability to demonstrate the biological effect of high dilutions, their specific features, and to investigate the pathophysiological processes, signaling and cell adaptation involved.

Acknowledgements

This study was supported by the Foundation for the Support of Research of the State of São Paulo [FAPESP – protocol number 2005/56551-9] and Brazilian Council for Scientific and Technological Development [CNPq]. We are particularly grateful to Prof. Jamshed R. Tata for helpful discussions and suggestions, and to the City University of São Paulo.

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