VARIATIONS OF ATP CONTENT IN V79 CELLS TREATED WITH CRUDE TOXINS OF Aequorea aequorea (CNIDARIA: HYDROZOA) AND Rhizostoma pulmo (CNIDARIA: SCYPHOZOA). A PRELIMINARY STUDY

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INTRODUCTION

Cnidarian toxins are stored mostly in small capsules (nematocysts) located in tentacles and elsewhere in the body; they are important for the ecology of aquatic environments and also for their impact on some human activities and health, in particular where species lethal for humans live. Though studies about toxic properties of Cnidaria started in the early twentieth century, leading to the formulation of anaphylaxis (1,2), an input to the researches on toxic Mediterranean species was given, only during the late '70s, with the occurrence of jellyfish blooms.

The toxicological studies concerning Mediterranean Cnidaria led to toxin characterization in some species, but the utilization of cell cultures to assay toxicity of Mediterranean Cnidaria was developed only recently. It's well known that in cells ATP is a key-molecule in production and utilization of energy; therefore substances causing alterations of ATP levels can play an important role in cell metabolism and affect several cell functions and activities; furthermore, "in vitro" ATP is an important cell metabolic and energetic parameter providing evidence of cell metabolism. Since some studies have emphasized the evident cytotoxic and cytolytic properties of venom from both planktonic (3-6) and benthic (5,7-11) Mediterranean Cnidaria, it is a matter of concern to investigate the role played by toxins in affecting energetic metabolism of injured cells, by evaluating ATP levels after exposure to growing doses and for different times. Therefore, the aim of this preliminary study is to evaluate the variations of ATP levels on V79 cells "in vitro" by crude extracts obtained from the jellyfish

Aequorea aequorea (Hydrozoa) and Rhizostoma pulmo (Scyphozoa).

MATERIALS AND METHODS

Specimen sampling - Jellyfish were collected in the Ligurian Sea in front of Savona and Camogli (Genova) and maintained at -20°C until utilization.

Crude toxin extraction - Tentacles of A.aequorea and oral arms of R.pulmo were treated as reported (12,13). Nematocysts were counted by hemocytometer (Thoma); the resulting number (N/ml) was taken as an index of toxicity, considering that nematocysts contain the bulk of toxin. Nematocyst discharge was induced by freezing-thawing cycles (-80°C/ +37°C) and sonication in ice-bath, taking care to break off treatment every 30 sec to avoid suspension heating. This method caused discharge of around 70-80% nematocysts. Finally, suspensions containing ruptured nematocysts and disjointed tissues were filtered on plankton nets (200 to 20 μm).

Cell cultures - Fibroblasts of the continuous cell line V79 (NIH, Coriell Inst. for Medical Res., Camden, NJ), originally obtained from Chinese Hamster lung, were cultured in DMEM medium (Biochrom, Berlin) supplemented with 10% FCS (Technogenetics, Cassina de' Pecchi Milan), l-glutamine and antibiotics (penicillin 100 U/ml; streptomycin 100 μg/ml) (Biochrom, Berlin), and maintained in CO_2 atmosphere (5%) in a humidified incubator at 37°C.

Cytotoxicity tests and ATP analysis - In order to evaluate the activity of crude toxin of A.aequorea and R.pulmo on cell ATP pattern, extracts were tested on $1x10^6$ V79 cells seeded on Petri dishes using two doses: dose A 150,000 N/ml, dose B 15,000 N/ml. To exclude a possible influence of pH shifting in medium caused by toxin addition, the same doses were also added to medium samples and pH measured. After contact times ranging from 15 to 180 min for toxin of A.aequorea and, in two separate experiments, from 15 to 60 min and from 75 to 120 min for toxin of R.pulmo, cells were trypsinized; 1 ml of suspension was used for ATP extraction in 20 ml of boiling TRIS-EDTA (0.1 M, pH = 7.75) for 10 minutes (14). ATP samples were stored at -20°C till analysis. Light emission during the

reaction of ATP with luciferine/luciferase enzyme was measured using a LKB 1250 luminometer; results were expressed in mV and subsequently converted in ATP (10^{-7}mM/ml) using the following analytical conditions: analyzed sample: 0.05 ml, TRIS dilution: 0.3 ml, internal ATP standard concentration: 10^{-7}M, ATP standard volume: 0.025 ml. The data were reported as percent of controls. The statistical analysis was carried out by ANOVA.

RESULTS

Table 1 reports pH variations caused by toxin in culture medium; pH shifting was scarce, therefore its role in affecting cultured cells was excluded.

Tab. 1 - Shifting from normal values of medium pH caused by crude toxin of Aequorea aequorea and Rhizostoma pulmo.

Species	Dose	pH shifting
Aequorea aequorea	A	0.14
	B	0.03
Rhizostoma pulmo	A	0.07
	B	-0.01

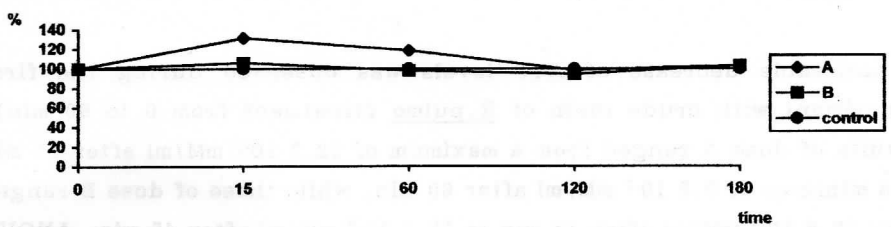

Fig. 1 - Variations in time of ATP levels in V79 cells treated with crude toxin of Aequorea aequorea, expressed as percent of control values.

In figure 1 results of experiments carried out with crude toxin of A.aequorea are reported. For both doses a slight but constant increase

of cell ATP levels can be noted, with highest values for dose A, whose range varies from 28.6 10^{-7} mM/ml, after 15 min treatment, to 41.2 10^{-7} mM/ml after 180 min; for dose B recorded values range from 23.3 10^{-7} mM/ml after 15 min to 38.9 10^{-7} mM/ml after 180 min. Significant differences between doses were not emphasized, while differences between treatment times were significant (tab. 2).

Tab. 2 - ATP content as a function of treatment time and dose of crude toxin extracted from Aequorea aequorea (ANOVA). df: degrees of freedom.

Source	df	sum of squares	mean square	F-test	P value
time (A)	3	476.843	158.948	15.326	0.0002
dose (B)	2	50.029	25.014	2.412	0.1317
AB	6	56.024	9.337	0.9	0.5254
error	12	124.451	10.371		

Tab. 3 - ATP content as a function of time (15 to 60 min) and dose of crude toxin extracted from Rhizostoma pulmo (ANOVA). df: degrees of freedom.

Source	df	sum of squares	mean square	F-test	P value
time (A)	3	550.024	183.34	130.451	0.0001
dose (B)	2	395.872	197.936	32.876	0.0001
AB	6	16.412	2.735	0.454	0.8287
error	12	72.249	6.021		

A noticeable decrease of ATP levels was observed during the first experiment with crude toxin of R.pulmo (treatment from 0 to 60 min); results of dose A ranged from a maximum of 22.7 10^{-7} mM/ml after 15 min to a minimum of 9.5 10^{-7} mM/ml after 60 min, while those of dose B ranged from 26.6 10^{-7} mM/ml after 15 min to 13.1 10^{-7} mM/ml after 45 min. ANOVA showed significant differences between times and between doses; therefore, in spite of the internal variability, an evident ATP depletion after toxin treatment was verified (tab. 3).

During more prolonged treatments, dose A from R.pulmo caused total depletion of cell ATP beginning from 115 min; with dose B low values,

reaching a minimum of 5.41 10^{-7} mM/ml after 120 min, were obtained. Also for this trial ANOVA showed significant differences between times and between doses (tab. 4).

Tab. 4 - ATP content as a function of time (75 to 120 min) and dose of crude toxin extracted from Rhizostoma pulmo (ANOVA). df: degrees of freedom.

Source	df	sum of squares	mean square	F-test	P value
time(A)	3	368.472	122.824	48.93	0.0001
dose (B)	2	1080.468	540.234	215.215	0.0001
AB	6	28.229	4.705	1.874	0.1669
error	12	30.123	2.51		

In figure 2 results of experiments have been reported expressing data of ATP of treated cells as percent of control values; a consistent ATP decrease, depending on treatment time and dose, can be noted.

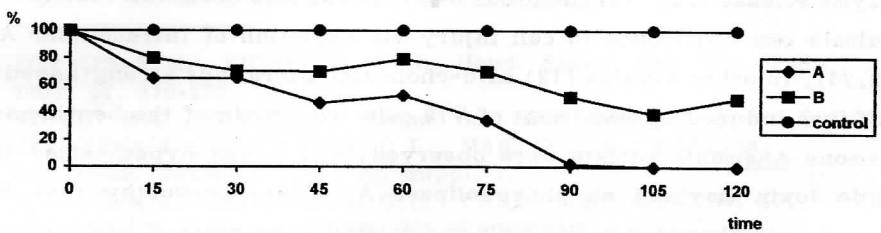

Fig. 2 - Variations in time of ATP levels in V79 cells treated with crude toxin of Rhizostoma pulmo, expressed as percent of control values.

DISCUSSION

The importance of ATP in biological systems is well known; for this reason, ATP analysis allows to evaluate the conditions of different cell types. For example, the analysis of cell ATP level is important to evaluate growth of bacteria and algae in natural environments (15). It was also reported that the occurrence of toxic substances can cause ATP depletion in marine bivalves (16), in bacteria and in algae (17), and that alkaloids can cause ATP depletion in rat alveolar cells (18).

Data here reported show that toxins of studied Cnidaria seem to produce different effects on cultured V79 cells. Indeed, treatment with extracts from Aequorea aequorea induced ATP level increase during 3 hours after inoculum: otherwise, toxin of Rhizostoma pulmo caused marked and constant ATP decrease. As variations of extracellular pH can affect cell ATP levels, pH shiftings of medium after addition of toxins were also recorded, showing that the shifting from normal values was very scarce; therefore, presumably pH was not the cause of recorded ATP changes. It was reported (19,20) that ATP depletion causes intracellular accumulation of metabolites and to anomalies in lipid metabolism by activation of endogenous phospholipases. In this connection, it's also known that some nematocyst toxins can interact with membrane phospholipids of V79 cells affecting and increasing the permeability of plasmalemma (9). As water uptake and breaking of external and intracellular membranes causes cell damage and lysis, also generation of toxic oxygen radicals and activation of Ca-dependent phospholipases occur as consequence of lysosomal enzyme release (21). On the other hand, it was also observed that oxygen radicals can contribute to cell injury via depletion of intracellular ATP (22,23). In other studies (12) mito-chondrial alterations strengthened by Ca^{2+} ions induced by treatment of V79 cells with toxin of the benthic sea-anemone Anemonia sulcata were observed, and it was hypothesized that crude toxin may act as phospholipase A. It is noteworthy that ATP increase was observed in V79 cells and related to decrease of mitochondrial GSH (24). On the other hand, it was reported that V79 cells seem to have an adequate metabolic reserve allowing them to maintain normal ATP concentrations for several hours after treatment with DNA intercalators (25). On the whole, the interpretation of obtained data is not easy; if the toxin of R.pulmo caused marked decrease of ATP levels, as was generally recorded in toxicity studies (26,27); nevertheless, using toxin of A.aequorea a constant ATP increase was recorded. Therefore, it can be hypothesized that toxin of A.aequorea block ATP consumption processes while production continues; otherwise, the toxic stress or compounds contained in the extract can stimulate ATP production also if utilization

is unaffected. To date, these hypotheses are not quite explainable and need further studies.

The toxicity of Cnidaria exerts a noticeable influence on some human activities, such as fishery and bathing, and on public health. As toxins of Mediterranean Cnidaria are located in nematocysts and in tissues, in this study the influence of crude toxins (nematocyst and surrounding tissue venom) extracted from the jellyfish Aequorea aequorea and Rhizostoma pulmo on ATP content of cultured V79 cells was assessed. Using the crude toxin of A.aequorea an increase of ATP levels in treated cells was noted; highest values (41.2 10^{-7} mM/ml after 180 min treatment) were reached using the highest dose. Otherwise, a generalized decrease of ATP levels was observed treating cells with crude toxin of R.pulmo; recorded values showed the complete depletion of cell ATP at 115 min treatment with the highest dose. A statistical significance was recorded between treatment times and between doses using crude toxin of R.pulmo, and only between treatment times for A.aequorea.

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