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Effect of acetylhomocysteine thiolactone on nucleolar cytology and lipofuscinogenesis in electric lobe neurons

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Summary. Treatment with acetylhomocysteine thiolactone significantly reduces the cellular level of lipofuscin in neurons of the electric lobe of *Torpedo marmorata*. At the same time, this drug produces a 45% decrease in nucleolar volume in these neurons, reflecting decreased cellular synthetic activity.
Key words. Lipofuscin; nucleolus; nucleolar vacuole; acetylhomocysteine thiolactone; cythiolone.

Lipofuscin, or age pigment, is known to accumulate in the cytoplasm of many cell types in the course of aging [1, 2] and also as a result of environmental stress [3]. Lipofuscin accumulation has generally been regarded as an irreversible build-up of membrane peroxidation products which the post-mitotic cell cannot discard, in the form of inert lysosomal residual bodies [4]. Therefore, the discovery that the drug acetylhomocysteine thiolactone (also known as cythiolone and abbreviated here as CYT) is capable of bringing about a reduction of cellular lipofuscin levels both in vivo [5] and in vitro [6] is of particular interest. Since CYT acts as a free radical scavenger and activates the enzyme superoxide dismutase, this could explain its role in inhibiting lipofuscin formation [7].

The purpose of the present study is to further examine CYT for its effect on the cytology of the cell, particularly the cytology of the nucleolus. Since the size and activity of the nucleolus are well-established markers of ribosome formation and protein synthetic activity of the cell [8, 9] an alteration in nucleolar size produced by CYT could provide new insights into its cellular mode of action. Neurons of the electric lobe of *Torpedo marmorata* were selected for study because of the extensive studies on lipofuscin formation already carried out on these cells [7, 10, 11].

Materials and methods. Animals. Six adult *Torpedo marmorata* collected from the Bay of Naples were used in this study. Their age was estimated to be 1–2 years, based on growth and size parameters established by Aloj Totaro et al. [11]. They were maintained in the laboratory in tanks and fed for 30 days using methods described by Aloj Totaro and Pisanti [10].

Drug treatment. Three animals received daily i.m. injections of acetylhomocysteine thiolactone (cythiolone produced by Roussel-Maestretti, Milano, Italy) at a dosage of 8 mg/kg b.wt for a 30-day period. The remaining three animals received placebo injections for the same 30-day period.

Fixation and preparation of tissue. Prior to sacrifice, the animals were anesthetized by a 15-min immersion in 0.015% MS222 (Sandoz) dissolved in seawater. The aorta was then injected and the animals were perfused with an osmotically balanced buffer as described by Aloj Totaro et al. [7]. Following removal of the blood, perfusion with the fixative, 3.5% glutaraldehyde in a suitable buffer, was initiated [10]. The electric lobes were dissected out and embedded for electron microscopy using standard procedures [10]. Sections were then cut 2-µm-thick on an ultramicrotome, stained with methylene blue and mounted in immersion oil.

Microscopy and measurement of nucleolar diameter. The sections were observed using a Zeiss microscope at ×1600 magnification. An ocular micrometer was placed in the ocular lens, and calibrated using a 1/100 mm Leitz stage micrometer. One ocular micrometer unit was equal to 0.66 µm. The diameter of 30 nucleoli in each animal was measured using the ocular scale. Nucleoli were also scored for the presence or absence of a prominent nucleolar vacuole. Measurements were made only on those cells where the nucleolus appeared to be complete, not in those cells where only a small fragment of the nucleolus was present. Mean nucleolar diameter was first calculated in ocular micrometer units and then converted to µm. Nucleolar volume in μm^3 was calculated using the formula to determine the volume of a sphere, $V = 4/3\,\pi\,r^3$. Determination of the percentage of cytoplasmic area covered by lipofuscin granules had been established by means of electron microscopy in an earlier study [5]. Student's t-test was used to compare nucleolar diameter measurements of the control vs the drug-treated group.

Results. In the table, we present a summary of the cytological parameters measured in neurons of the electric lobe of *Torpedo marmorata*. Measurements from control animals are compared with those from CYT-treated animals. It can be seen that the mean nucleolar diameter of the control animals at 6.81 ± 0.13 µm is significantly larger ($p < 0.001$) than that of the CYT group at 5.58 ± 0.09 µm. When nucleolar volume is calculated, the actual difference in size between the nucleoli of the control and of the CYT-treated animals be-

Cytological parameters are compared in electric lobe neurons of *Torpedo marmorata* for control animals and for cythiolone-treated animals

	Control	Cythiolone	% Decrease with cythiolone
Nucleolar diameter in μm ± SEM	6.81 ± 0.13	5.58 ± 0.09*	18%
Nucleolar volume in μm³	166	91	45%
% of nucleoli with vacuole	77%	48%	38%
% cytoplasmic area covered by lipofuscin	2.52 ± 0.3	1.94 ± 0.6**	23%

* Significantly different from control at $p < 0.001$. ** Significantly different from control at $p < 0.01$.

comes even more apparent. The nucleolar volume of the control animals is 166 μm³, while the nucleolar volume of the CYT-treated animals is 91 μm³, that is 45% smaller. Another cytological parameter noted was the presence or absence of a prominent nucleolar vacuole. In the control group, 77% of the nucleoli had a prominent vacuole. In contrast, only 48% of the nucleoli in the CYT treated animals displayed a nucleolar vacuole. In the table we also present data on the percentage of cytoplasmic area occupied by lipofuscin in electric lobe neurons of control vs CYT-treated animals established in earlier study [5]. It can be seen that CYT treatment reduced cytoplasmic lipofuscin by 23%. We conclude that CYT has a significant effect both on nuclear and on cytoplasmic structures. It concomitantly produces a 23% decrease in the percentage of cytoplasmic area covered by lipofuscin and a 45% decrease in average nucleolar volume.

Discussion. Interest in the drug acetylhomocysteine thiolactone or cythiolone (CYT) used in this study has focused on its ability to reduce lipofuscin build-up in cells both in vivo [5] and in vitro [6]. In seeking explanations as to how CYT acts to reduce lipofuscin level in the cell, investigators have concentrated on its role as a free radical scavenger [7]. By reducing the level of intracellular peroxidation, CYT could reduce the build-up of lipid peroxidation products which contribute to the formation of lipofuscin.

The results presented here show that cythiolone may affect lipofuscin level by another mechanism as well; by slowing cell synthetic activity. The results reported here show that cythiolone has a pronounced effect on cytological parameters associated with RNA and protein synthesis. In the nucleolus of the neurons studied here CYT produced a significant decrease in nucleolar size. It is well established that cells with large nucleoli are particularly active in protein synthesis [8, 9]. Nucleoli increase in size when cells are stimulated to maximal synthetic activity, and decrease in size when synthetic activity is reduced. This correlation of nucleolar size with nucleolar activity has been extensively documented [12, 13]. The fact that CYT treatment produces a 48% decrease in nucleolar volume suggests that nucleolar synthetic activity is reduced by this drug.

CYT also produces a marked decrease in the percentage of nucleoli with a nucleolar vacuole. The nucleolar vacuole is understood to be the site where pre-ribosomal particles are assembled [14]. When RNA processing is impaired by drugs, incomplete rRNA precursors have been described within the vacuole [15]. The appearance of a nucleolar vacuole is correlated with the time of onset of active RNA synthesis in both plants [16] and animals [17]. In mammalian embryos, the appearance of the nucleolar vacuole is correlated with the onset of hnRNA and rRNA synthesis [18]. This evidence shows that the presence of a nucleolar vacuole is correlated with activation of RNA synthesis. The fact that treatment with CYT reduces the number of nucleoli with vacuoles again suggests that CYT interferes with nucleolar synthetic activity.

Cells with higher levels of synthetic or metabolic activity not only tend to have large nucleoli, they also tend to have elevated levels of lipofuscin. Sohal and Wolfe [2] point out that functionally active cells tend to accumulate more lipofuscin than inactive ones. Friede [19, 20] has demonstrated that nerve cells with high levels of oxidative enzymes have elevated levels of lipofuscin. The lower level of lipofuscin and the reduced nucleoli observed in electric lobe neurons of CYT-treated animals both suggest that CYT inhibits cellular synthetic activity. We suggest that CYT reduces lipofuscin build-up not only by its known free radical scavenger activity, but also by producing an overall slowing of cell synthetic activity.

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