Malondialdehyde and Lipofuscin, Biomarkers of Oxidative Stress in *Rana temporaria temporaria* During Metamorphosis

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Anuran amphibians metamorphosis represents a model for the study of different processes, including tissue remodeling. One of the body's systems whose morphological and structural properties are deeply transformed during metamorphosis is the digestive tract. The only structure in the digestive tract which is destroyed and then replaced with a new tissue (histolysis and histogenesis) under the action of oxidative stress and hormones, is the intestinal epithelium. Oxidative stress may be related to the production of malondialdehyde and the accumulation of lipofuscin. The two substances can be considered biomarkers of this process.

Keywords: malondialdehyde, lipofuscin, oxidative stress, metamorphosis, anuran amphibians

Metamorphosis is a model for the study of different processes [1-2]. The transition from the aquatic larval stage to the adult life (land and aquatic) is associated with a number of major changes in the larval organs [3 - 6]. The tissue degeneration through the action of free radicals represents a very complex system. Their combined activity during the metamorphic climax, leads to the larval structures remodeling.

One of the body's systems whose morphological and structural properties are profoundly changed during metamorphosis is the digestive tract.

Larval digestive tube has an anterior intestine, between mouth and hepatopancreatic opening, a middle intestine between hepatopancreatic channel and hindgut ampoule and a posterior intestine between the ampoule and the cloaca [7]. The larval gut (pre- and prometamorphic) is an approximately equal diameter tube, twisted in spiral with a dilated terminal region. Its structure from the inside to the outside contains a simple epithelium, followed by a mesenchyme and two smooth muscle layers, one internal (circular) and one external (longitudinal).

The middle intestine size grows between the stages I - XVIII and becomes structured into coils, with ascending and descending direction. The number of coils is independent of the length of the intestine and it is constant at the same species. Between the stages XIX - XXIV, the middle intestine shortens, it's posterior region becomes shorter with a higher speed. As a result, the stomach reaches the normal anatomic species position and the coils of intestine change [8].

The only structure of the digestive tract that passes through histolysis and histogenesis under the action of oxidative stress is the intestinal epithelium. The epithelium is of columnar type, with basal nucleus [9 - 12].

Among other things, oxidative stress leads to production of malondialdehyde and lipofuscin. Malondialdehyde is an important indicator of lipid peroxidation in biological materials [13-15]. Lipid peroxides resulting from polyunsaturated fatty acids are unstable, therefore decompose in a number of reactive carbonyl compounds, such as malondialdehyde [16 - 17]. The two substances can be considered biomarkers of this process.

Experimental part

Eggs pontes of *Rana temporaria temporaria L.* used in this study were collected from nature. These were brought to the laboratory where they hatched and the larval development was observed. The larvae were grown in 10 liter pots, with dechlorinated tap water. The water temperature varied between 18 - 22°C.

The metamorphic stages development was established according the tables of Taylor and Kollros [18]. For each stage, seven larvae were analyzed. The microdissection was done on ice, using a binocular microscope and the digestive tube was displayed in PBS and photographed.

For the histological observations, the midgut was fixed in glutaraldehyde and OsO_4 and then included in epoxin resin. Semi-fine sections were stained with toluidine blue and photographed using an Olympus microscope.

The amount of malondialdehyde was quantified using the Dobrian method, modified by Artenie et al., [17]. At high temperature and acidic *p*H, the malondialdehyde resulting from lipid peroxides decomposition reacts to 2-thiobarbituric acid (TBA) leading to a pink MDA-TBA_2 adduct that absorbs strongly at 532 nm.

The tissue was homogenized on ice in the presence of a 0.025 M Tris-HCl buffer (*p*H 7.4), containing 0.175 M KCl. The homogenate was centrifuged for 15 minutes at 3000 rpm and 4°C. 200 µL of tissue lysate were added to the reaction mix (1 mL of trichloroacetic acid at 50%, 0.9 mL of Tris–HCl buffer, *p*H 7.4 and 1 mL of thiobarbituric acid 0.73%). After 20 min of incubation (100°C), the samples were centrifuged at 3000 rpm for 10 min and the supernatant absorbance was read at 532 nm. The signal was read against an MDA standard curve and the results were expressed as nmol/mg protein (fig. 1).

The total soluble protein content was evaluated using Bradford's method. The assay is based on the observation that the absorbance maximum for an acidic solution of coomassie brilliant blue shifts from 465 nm to 595 nm when binding to protein occurs. The signal was read against a calibration curve using ovalbumine as standard (fig. 2) [19].

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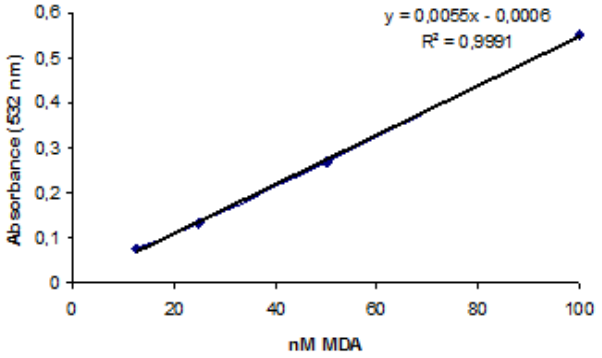

Fig.1. Standard MDA curve

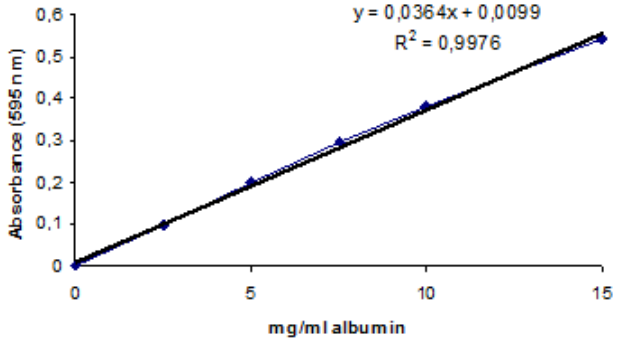

Fig. 2. Protein standard curve

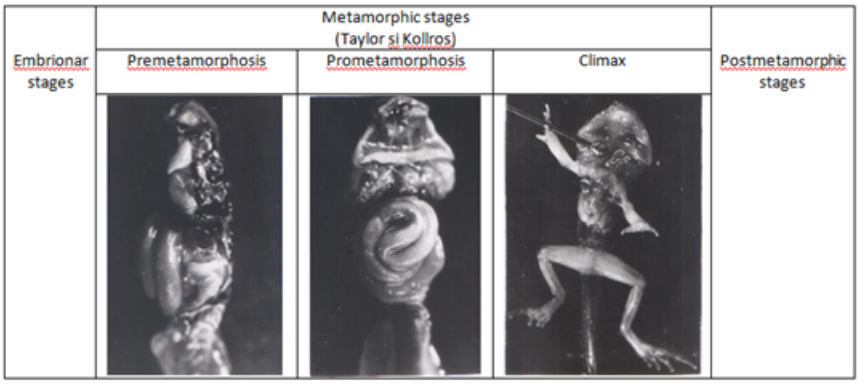

Fig. 3. Metamorphic stages, digestive tract of *Rana temporaria temporaria L.*

Results and discussions

In anuran amphibians, during the climax of the metamorphosis stages, the larval intestine and in particular, the middle intestine average length is reduced by 80% (fig. 3) [8]. Besides this phenomenon, a narrowing of it's lumen was observed.

As previously shown, the malondialdehyde values in premetamorphic stages are small [20]. This behavior is confirmed by the data obtained in this study. An increase of these values during the pre and prometamorphic stages is due to the increase of the number of larval epithelial cell populations, because of the increase in the average length of the intestine.

Starting with the first stage of the climax, malondialdehyde values increase significantly. The increase is due to the free radicals from the larval epithelium and the resulting lipid peroxides decomposing with the formation of malondialdehyde. The larval epithelium is destroyed and it will be replaced by a definitive epithelium. Scientific data indicate two main mechanisms by which this phenomenon happens, either by apoptosis (generally accepted hypothesis) or lysosomal-mediated autophagy [21].

The increase in the amount of malondialdehyde during metamorphosis climax is highlighted in figure 4. The maximum concentration of this compound is recorded in the stage XX.

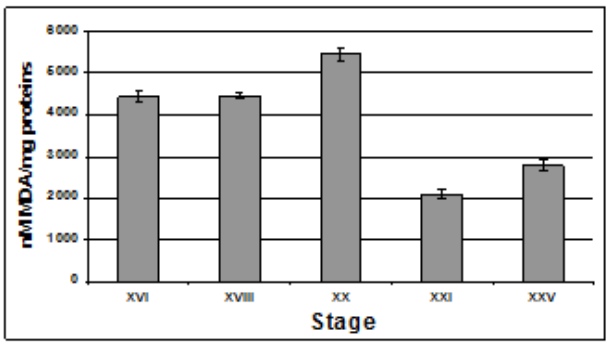

Fig.4. Variation of MDA levels in the middle intestine of *Rana temporaria temporaria L.* during the metamorphosis stages

The last investigated stages are characterized by a decrease of MDA concentration. This phenomenon could be explained by a stimulation of the antioxidant mechanisms in order to protect the newly formed tissue.

At the climax of metamorphosis, the only structure of the intestine that degrades totally and it is replaced is the larval epithelium. The larval epithelium is monostratified, consisting of high prismatic cells and mucous *goblet* cells. It is replaced by an epithelium that functions at juvenile and adult, by the action of the cells that develop in the underlying *cellular islands*. On the cross-sections through the midgut, they appear intensely colored, arranged between the base membrane and the larval epithelium. Their sizes are different, depending on the stage of metamorphosis.

The larval gut epithelium undergoes apoptosis, whereas in the underlying *cells islands*, DNA synthesis rate increases. During postmetamorphic stages, DNA synthesis rate decreases, being identified only in the cells of the intestinal crypts [22]. The mechanism seems to be controlled by the action of thyroid hormone T3 which induces the extracellular matrix remodeling with the intervention of stromelysin-3, a matrix metalloproteinase [23]. In the previous stages of the metamorphic climax, studies have shown an increase in the number of macrophages, but only at the level of the apoptotic epithelium. These cells are designed to absorb the resulting apoptotic bodies, thus the larval epithelium will be quickly removed [24].

In parallel with the increasingly higher amount of lipid peroxides formed in the larval gut epithelium, it has been proved that the level of blood lipids increases. They represent a source of energy for the tissue remodeling processes and are involved in specific inflammatory responses [25].

Another product related to lipid peroxidation is lipofuscin. Lipofuscin is present as a result of the larval epithelium degeneration, starting to the early stages of metamorphosis. The lipofuscin presence is visible through the middle intestine sections during all pre and prometamorphic stages. It appears in the form of small spherical granules, brown to black, arranged in particular at the apical pole of the cells belonging to the intestinal larval epithelium in the stage XI (fig. 5 - 6).

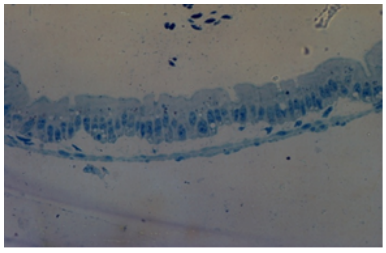

Fig. 5. *Rana temporaria temporaria L.*, stage XI, middle intestine, cross section, toluidine blue, om., x10

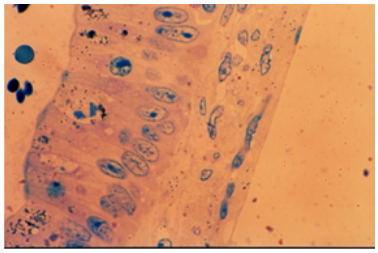

Fig. 6. *Rana temporaria temporaria L.*, stage XV, middle intestine, cross section, toluidine blue, om., x20

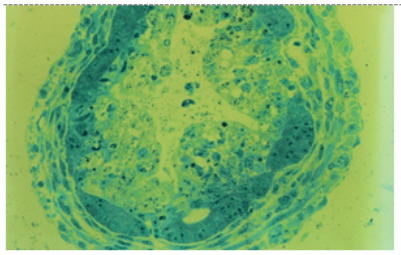

Fig. 7. *Rana temporaria temporaria L.*, stage XVIII, middle intestine, cross section, toluidine blue, om., x20

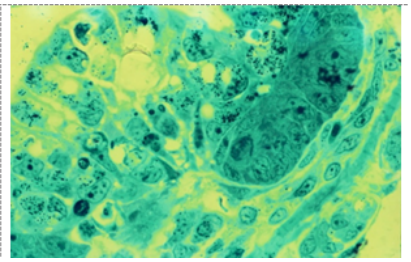

Fig. 8. *Rana temporaria temporaria L.*, stage XIX, middle intestine, cross section, toluidine blue, om., x40

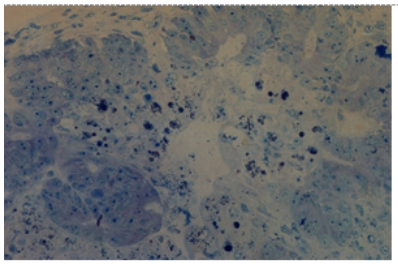

Fig. 9. *Rana temporaria temporaria L.,* stage XX, middle intestine, cross section, toluidine blue, om., x40

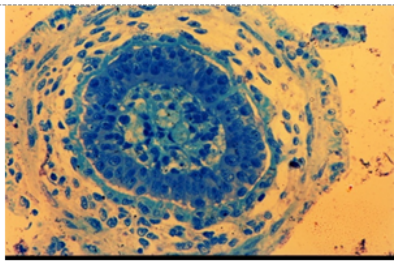

Fig. 10. *Rana temporaria temporaria L.,* stage XXV, middle intestine, cross section, toluidine blue, om., x20

At the beginning of the climax, stages XVIII-XX, the explosive action of the free radicals produces massive accumulations of lipofuscin. In these stages, the intestinal wall is composed of cellular debris from the larval epithelium, apoptotic bodies and macrophages migrated from the underlying connective tissue, plus the *cells islands* that will develop into the intestinal juvenile epithelium [26], (fig. 7 - 8).

Larval epithelial cells debris are pushed to the lumen of the intestine by the newly developed cells (from the *cellular islands*) which unite and form the juvenile intestinal epithelium. A portion of the material is phagocyted by the healthy neighbor cells [27 - 28]. On sections, lipofuscin is abundant, forming masses of irregular shape, large and brown to black in colour (fig. 9 - 10).

Conclusions

Lipid peroxidation, evidenced by the concentration of malondialdehyde in the intestinal structures of *Rana temporaria temporaria L.* during metamorphosis, is an indicator of profound structural and ultrastructural changes. The values of lipid peroxidation concentrations before the climax are increased, which leads to the destruction of the larval structures of the intestine, particularly the epithelium. The regeneration of the intestinal epithelium in the postclimax stages decrease lipid peroxidation and restores tissue defense capacity.

As a result of the apoptosis and the histolytic processes under the action of various free radicals, the larval epithelium is degraded to malondialdehyde and lipofuscin. The two substances can be considered biomarkers of this process in larval intestine, during anuran metamorphosis.

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