Correlations Between Oxidative Stress and Apoptosis During Anuran Metamorphosis

VASILE SIRBU[1], ANNAMARIA PALLAG[2], ANA HONIGES[3], VLADIMIR POROCH[4]*, SABINA IOANA COJOCARU[1]

[1] Alexandru Ioan Cuza University of Iasi, Faculty of Biology, 20A,Carol I Blvd., 700505 Iasi, Romania

[2] University of Oradea, Faculty of Medicine and Pharmacy, Pharmacy Department, 29 Nicolae Jiga Str., 410028 Oradea, Romania

[3] Vasile Goldis Western University of Arad, 94-96, Revolutiei Blvd., 310025 Arad, Romania

[4] Grigore T Popa Univeristy of Medicine and Pharmacy Iasi, 16 Universitatii Str., 700115 Iasi, Romania

The living organisms can trigger the defense mechanisms against free radicals, by synthesizing different antioxidant enzymes. The present study is focused on establishing some correlation between oxidative stress and the structural changes in cell death at the intestinal larval epithelium level during anuran metamorphosis. Cell death in such conditions may be regarded as the result of an interaction activity in which takes place apoptosis, autophagy, and necrosis, the cell choosing one or more. The amphibian metamorphosis is a complex process, divided into three major periods: prometamorphosis, premetamorphosis and climax. The process ensures the passage of the organism from aquatic to terrestrial life, with dramatic changes in the morphology and structure of some organs. In the climax stages of metamorphosis, a variety of free radicals are produced, starting a numerous cellular oxidation reactions.

Keywords: oxidative stress, apoptosis, autophagy, necrosis, anuran amphibians, metamorphosis.

The metamorphosis of anuran amphibians represents a study model for different biological mechanisms such as the programmed apoptosis [1]. During the developement, apoptosis occurs normally as a proces to maintain normal cells populations in tissue [2]. This process is related to the morphogenesis [3] and is controlled by the thyroid hormones triiodothyronine and tetraiodothyronine, which in the climax stages induce cellular oxidative stress that lead to larval structures removal and their replacing with juvenile structures that will also function in the adult individuals.

The amphibian metamorphosis is a complex process, divided into three major periods: prometamorphosis, premetamorphosis and climax. The process ensures the passage of the organism from aquatic to terrestrial life, with dramatic changes in the morphology and structure of some organs [4-6].

The larval intestine is one of the organs undergoing profound structural alterations in metamorphosis. In these structural reshuffle, the larval epithelium, equipped with an enzymatic set corresponding to microfage nutrition is replaced with juvenile epithelium, equipped with an enzymatic set intended for macrophage, aquatic and terrestrial feeding [7-18].

A big variety of free radicals are produced and they will start a big number of cellular oxidation reactions. The living organisms can trigger the defense mechanisms against free radicals, by synthesizing different antiradicalic enzymes [19-23].

Reactive oxigen species, oxidize biomolecules as proteins, lipids, carbohidrates, DNA and impair normal cellular functions. A shift in balaced between oxidant and antioxidant balance in favour of oxidants, results in oxidative stress. Antioxidant defence system comprise many enzimes known as antioxidant enzymes (superoxide dismutaza, catalase, glutathione peroxidase).

The oxidative stress manifests extra and intracellular. Intracellular it acts on the cytoskeleton and on the membranes of different cellular organelles (nucleus, mitochondria, lysosoms), by degrading them [24]. The present study analyses the correlation between the oxidative stress and cells death as a result of oxidative stress, in the complex phenomenon of programmed cell death at *Rana temporaria temporaria* (L.1758) during metamorphosis.

Experimental part

Material and methods

The biological material used in this study is represented by *Rana temporaria temporaria* (L.1758) pontes collected from nature. These were brought in the laboratory, were they hatch and were observed during larval development. They were maintained in 10 liters pots which contained dechlorinated tap water. The water temperature was between 18 - 22°C.

The metamorphosis stages were established using Taylor si Kollros tables [25]. For each stage, seven individuals were sacrificed. The microdisection was performed on ice, under the binocular magnifying glass, the digestive tube exposed in Sörensen phosphate buffer and photographed.

For histological observations, parts of the medium intestine were fixed in glutaraldehyde and osmium tetraoxide, then included in epoxy resins. The semifine sections were stained with toluidine blue. For electron microscopy the contrast was made with uranyl acetate. A part of the larval intestine was ice sections, colored with acridine orange and photographed on a fluorescence microscope.

Superoxide dismutase (SOD - E.C. 1.15.1.1) activity was measured according to the method of Winterbourn et al. (1975) [26]. Enzyme activity was determined thanks to the capacity of this enzyme to inhibit Nitrobluetetrazolium reduction by superoxide anions generated after riboflavin photoreduction. The decrease of the absorbance was read at 560 nm using a Shimadzu UV-Vis 1700 spectrophotometer (Japan).

Catalase (CAT - E.C. 1.11.1.6) activity was determined using the method of Sinha (Sinha, 1972) [27]. The method

*email:vlader2000@yahoo.com; sabina_18ro@yahoo.com

is based on the colorimetric determination (at λ = 570 nm) of chromic acetate obtained through reduction of potassium dichromate in acid medium by the hydrogen peroxide that remains after enzyme inactivation.

Peroxidase (POX - E.C. 1.11.1.X) activity was measured according to the method of Gudkova and Degtiari (1968) [28]. POX catalyzes the reaction between o-dianisidine and hydrogen peroxide with the formation of a colored product with an absorption maximum at 540 nm.

The total soluble protein content, expressed as mg/mL, was determined using Bradford's method with bovine serum albumin as standard [29-32]. All the investigations were made in triplicate. The results are expressed as means ± standard deviations.

Results and discussions

The structural and ultrastructural features of apoptotic cells were observed using optical and electron microscopy techniques. In the early stages of climax the deformation and condensation of the cells are observed. In this process the cell loses it's cytoplasm volume, becomes densely, the cell organells density increases and pyknosis defines condensation to different degrees of the nuclear material.

The histological sections of apoptotic structures, present punctual areas that comprise a single cell, or stretched areas, in the same tissue. In the optical microscope, the apoptotic cells appear rounded, eosinophilic structures, with a pyknotic nucleus. In electron microscopy, pyknosis is observed by condensing DNA beside the nuclear membrane, in the form of more or less bulky masses, with more or less regular layout. The cell membrane escapes "bubling", including within the region of cytoplasmic fragments, integral cellular organelles, nucleus fragments. Apoptotic bodies are formed and can be found in neighboring cells, as a result of heterophage phenomenon in macrophages, or as in the case of amphibian metamorphosis, in the intestine lumen, for the most part of the larval epithelial tissue.

Apoptosis in *Rana temporaria temporaria* (L. 1785) begins at the XVIII stage, begining the period called climax. In the larval digestive tube, we followed the structures in the small intestine, especially the intestinal epithelium. The larval epithelium consists of columnar enterocytes, among which are mucous cells [10]. Enterocytes are endowed with an enzymatic charge specific of the larval feedind. They are destroyed and replaced with the juvenile epithelium resulting from the action of the existing cells in the underlying *cellular nests* [33].

As a result of action of the free radicals, destruction of cells by destroying the cytoskeleton tackes place. Cell lysis occurs in the larval epithelium, at the beginning of the climax stages, in small areas, which grow in the advanced climax stages and comprise the entire structure. At the level of enterocytes, we observed the changes in the nucleus in relation with the antioxidant enzyme activities. The reactive oxygen species produce oxidative changes in the cell DNA [33]. In the early stages of metamorphosis climax, a condensation of nuclear material is produced and the nuclei appear pyknotics in photon microscopy (fig. 1a). They lose their elongated oval shape and become irregular. Inside nucleus, near the nuclear membrane, the concentration of chromatin in the form of heterochromatin is observed (fig. 1b). This type of structure has been described in apoptosis in other animal cells under the name of *chess board table*. In the next stage XXI, the cell lysis of the epithelium becomes generalized, cells lose cellular boundaries by disrupting cell membranes.

In the destructive cellular material we can see the apoptotic bodies (fig. 1c). Apoptotic bodies are membrane-bound formations with a non-uniform content in which nuclear material spherical shape and other cellular debris are observed (fig. 1d).

The number of apoptotic bodies and their volume increases in the advanced stages of metamorphosis XXII-XXIII (fig. 2).

Between stages XXIII-XXIV, the larval epithelium is completely destroyed. Because the larva does not feed at climax stages, a part of the destructured material is consumed by autophagy, but most of which is eliminated in the intestine lumen (fig. 3).

Figures 4, 5 and 6, show the variation of superoxide dismutase, catalase and peroxidase specific activities during the stages XVI-XXV of anuran metamorphosis. The activity of the three types of enzymes is profoundly connected, superoxide dismutase (SOD), hydrogen peroxidase (POX) and catalase (CAT). The role of superoxide dismutase in living cells is the dismutation of the superoxide radicals (O_2^-) into molecular oxygen (O_2)

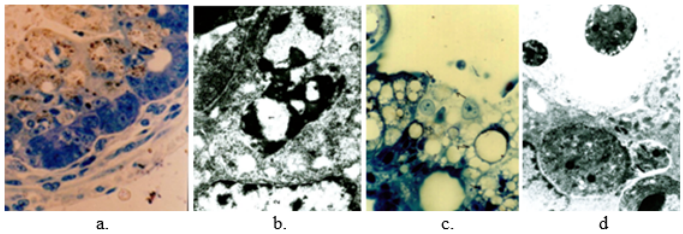

Fig. 1. Middle intestine, *Rana temporaria temporaria*, stage XX and XXI: a - cross section, toluidine blue, om.40×; b - chess board table,nucleus, em. 20000×; c - toluidine blue, apoptotic bodyes, om. 60×; d - apoptotic bodyes, cellular lysis, em 20000×

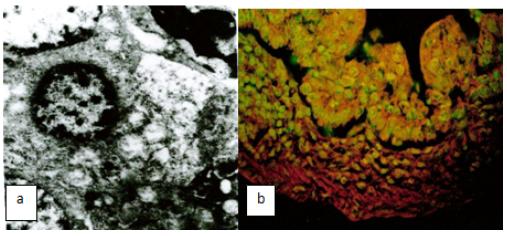

Fig. 2. Middle intestine, *Rana temporaria temporaria:* a - stage XXII, autosomal vacuoles, em 25000×, b - stage XXIII, nuclear ADN, acridin orange, dark field fluorescence microscopy, 40×

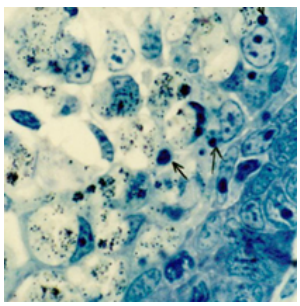

Fig. 3. Middle intestine, *Rana temporaria temporaria*, stage XXI, toluidine blue, arrows apoptotic bodys, mo.60×

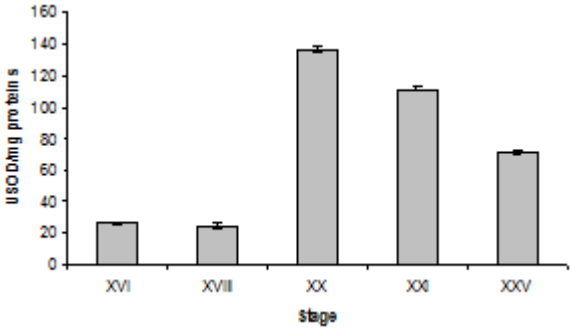

Fig. 4. Variation of SOD activity during *Rana temporaria temporaria* metamorphosis

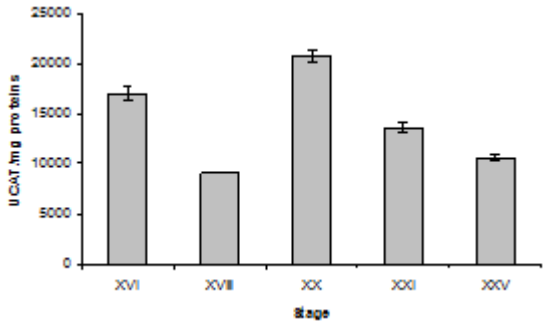

Fig. 5. Variation of CAT activity during *Rana temporaria temporaria* metamorphosis

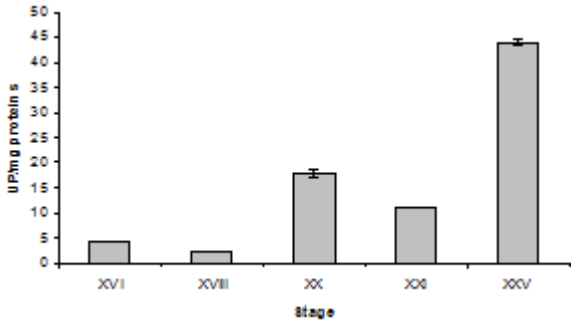

Fig. 6. Variation of POX activity during *Rana temporaria temporaria* metamorphosis

and hydrogen peroxide (H_2O_2). Hydrogen peroxide is then degraded by catalase and different peroxidases.

As our study showed, in the stages XVI-XVII, the activity of the antioxidant enzymes is decreasing. Only catalase registers an increased activity, which may suggest an accumulation of hydrogen peroxide in the cells, in the stages before climax.

The stage XX is marked by a significant increase of all three enzyme activities. SOD activity increases approximately seven times comparing to the previous stage analyzed, while CAT activity is doubled and POX activity is four times more increased. This response is correlated to the climax of the metamorphosis which is accompanied by an overproduction of free radicals. The intestinal epithelium cells respond to the reactive oxygen species accumulation by increasing the antioxidant defense mechanisms.

After stage XX, both SOD and CAT activities decrease. This response is correlated with the accumulation of apoptotic bodies, as discussed above. The total destruction of the larval epithelium is related to an intense accumulation of free radicals and a decrease in the protection mechanisms. Therefore, the tissues are characterized by an imbalance between the free radicals production and the antioxidant defense, so an increased oxidative stress is installed.

The significant increase in POX activity in the last stage may be related to the increase of the blood lipid levels, evidenced by other authors [34]. The accumulation of different types of free radicals may lead to lipid peroxidation which is accompanied by the stimulation of glutathione peroxidase activity [33-35-41].

Conclusions

In *Rana temporaria temporaria* preclimax stages are marked by a decreased level of antioxidant enzyme activities. In the climax stages of metamorphosis of the anuran amphibians, a variety of free radicals are produced. They will start a big number of cellular oxidation reactions. The living organisms can trigger the defense mechanisms against free radicals, by synthesizing different antioxidant enzymes. The activities of antioxidant enzymes significantly increase during stage XX, as a result of cellular defense mechanisms. The last stages of climax are characterized by a decrease of antioxidant protection, which may be correlated to the overproduction of free radicals and the accumulation of apoptotic bodies. As a result of the oxidative stress, in the climax stages deformation and condensation of the cell is observed. In this process, the cell loses cytoplasm volume, becomes densely, the cell organells density increases and pyknosis defines condensation to different degrees of nuclear material. At the cellular level autolitic vacuoles and apoptotic bodies are observed.

Cell death in such conditions may be regarded as the result of interaction activity between apoptosis, necrosis and autophagy mechanisms, the cell choosing one or more for its existence.

References

1.TATA, J.R., Biochem., **81**, no. 4, 1999, p. 359.
2.ELMORE, S., Toxicol. Pathol., **35**, no. 4, 2007, p. 495.
3.DAVID, L.V., ATANLEY, J.K., Cell, **96**,1999, p. 245.
4.ISCHIZUYA-OKA, A., SHUICHI, U., Cell Tissue Res., **286**, no.2, 1996, p. 467.
5.MENON, J., ROZMAN, R., Comp. Biochem. Physiol. C. Toxicol. Pharmacol., **145,** no. 4, 2007, p. 625.
6.HOURDRY, J., DAUCA, M., Int. Rev. Citol. Supplm., 5, 1977, p. 337.
7.FOX, H., Amphibian Morphogenesis, Humana Press, Clifton New Jersey, 1984.
8.SIRBU, V., Anal. Biol., Tom XLVI, 2000, p. 143.
9.SIRBU, V., J. Med. Biochem., **2**, no. 3, 1998, p. 273.
10.ISCHIZUYA-OKA, A., Microsc. Res. Tech., **34**, 1996, p. 228.
11.COHEN, J.J., Adv. Immunol., **50**, 1991, p. 55.
12.HELLQUIST, B., SVENSSON, I., BRUNK, T., Redox Rep., **3**, 1997, p. 65.
13.FOYET, H., HRITCU, L., CIOBICA, A., STEFAN, M., KAMTCHOUING, P., COJOCARU, D., J. of Ethnopharmacol., **133**, no. 2, 2011, p. 773.
14.CIOBICA, A., HRITCU, L., NASTASA, V., PADURARIU, M., BILD, W., J. Med. Biochem., **30**, no. 2, 2011, p. 109.
15.CIOBICA, A., BILD, V., HRITCU, L., PADURARIU, M., BILD, W., Central European Journal of Med., **6**, no. 3, 2011, pp. 331-340.
16.DOBRIN, R., CIOBICA, A., TOADER, E., POROCH, V., Rev. Chim..(Bucharest), **67**, no. 9, 2016, p. 1778.
17.ARCAN, O., CIOBICA, A., BILD, W., HRITCU, L., COJOCARU, D., J Med Biochem, **32**, no. 1, 2013, p. 52.
18.ARCAN, O., BILD, W., CIOBICA, A., SERBAN, D., ANTON, E., PETRARIU, F., TIMOFTE, D., NASTASA, V., Rom Biotech Let, **19**, no. 5, 2014, p. 9763.
19.MASAYASU, I., EISUKE, F. S., MANABU, N., KEIIEKE, H., AKIHIKO, K., KOZO, U., Redox Report, **9**, no. 5, 2004, p. 237.
20.CANTEMIR, A., ALEXA, A., CIOBICA, A., TOADER, E., BALMUS, I., COJOCARU, S.I., CHISELITA, D., COSTIN, D., Rev.Chim.(Bucharest), **67**, no. 9, 2016, p. 1725.
21.ALEXA, A.I., CANTEMIR, A., ANTIOCH, I., BALMUS, I.M., COJOCARU, S., GARDIKIOTIS, R., LUCA, A., FILIP, M.A. ABABEI, D.C., ZAMFIR, C.L., Rev.Chim.(Bucharest), **68**, no. 2, 2017, p. 350.
22.CANTEMIR, A., ALEXA, A., CIOBICA, A., BALMUS, I., ANTIOCH, I., STOICA, B., CHISELITA, D., COSTIN, D., Rev.Chim.(Bucharest), **67**, no. 8, 2016, p. 1538.
23.HONCERIU, C., CIOBICA, A., STOICA, B., CHIRAZI, M., PADURARIU, M., Rev.Chim. (Bucharest), **67**, no. 11, 2016, p. 2246.
24.PADURARIU, M., ANTIOCH, I., CIOBICA, A., LEFTER, R., Rev.Chim.(Bucharest), **68**, no. 8, 2017, p. 1879.
25.GAGAN, C., BISWARANJAN, P., JAGNESWAR, D., Scientifica, 2016.
26.TAYLOR, A. C., KOLLROS, J. J., Anat. Rec., **94,** no. 23, 1946, p. 7.
27.WINTERBOURN, C., Hawkins, R., BRIAN, M., CARELL, R., J. Lab. Clin. Med., **85**, 1975, p. 337.
28.SINHA, A.K., Anal. Biochem., **47**, 1972, p. 389.

29.GUDKOVA, L.V., DEGTIARI, R.G., Metod opredelenija aktivnosti peroxidazî. În kn.: Fermentî v mediþine, piºcevoi promîºlennosti i v sel skom hoziastve. Naukova dumka, Kiev, 1968.
30.BRADFORD, M.M., Anal. Biochem., **72**, 1976, p. 248.
31.COJOCARU, D., TOMA, O., COJOCARU, S.I., CIORNEA, E., Practicum de biochimia proteinelor ºi acizilor nucleici, Tehnopress, Iaºi, 2009.
32.COJOCARU, D. Enzimologie practica, Tehnopress, Iaºi, 2009.
33.SIRBU, V., PALLAG, A. M., HONIGES, A., COJOCARU, S. I., Rev.Chim. (Bucharest), **68**, no. 2, 2017, p. 390.
34.ISHIZUYA-OKA, A., SHIMOZAWA, A., J. Morph., **213**, 1992, p. 185.
35.ICHU, A., HAN, J., BORCHERS, H., LESPERANCE, M., HELBING, C., Dev. Biol., **14**, no. 5, 2014, p.1.
36.NOUE, M., SATO, E.F., NISHIKAWA, M., HIRAMOTO, K., KASHIWAGI, A., UTSUMI, K., Redox Report, **9**, no.5, 2004, p. 238.
37.ACHITEI, D., CIOBICA, A., BALAN, G., GOLOGAN, E., STANCIU, C., STEFANESCU, G., Digestive Diseases and Sciences, **58,** no**.** 5, 2013, p. 1244.
38.BEREA, G., BALAN, G.G., SANDRU, V., SIRBU, P.D., Rev. Chim. (Bucharest), **68,** no. 6, 2017, p. 1341.
39.SAAD, A.H., AZIZ, A.A., YEHIA, I., EL-GHAREEB, A.W., ISMAIL, H., Australian Journal of Basic and Applied Sciences, **3**, no. 4, 2009, p. 4644.
40.GONCALVES, A.M., SANTOS, L.J., SANTANA, C.C, COLOSIO R.R., PIZAURO, M.J., Copeia, **103**, no. 3, 2015, p.634.
41.TRIPATHY A., Int. J. Curr. Res. Biosci. Plant Biol., **3,** no. 10, 2016, p. 79.

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