Original article

Antioxidant enzymes functions in intestinal remodeling in metamorphosing tadpoles *Xenopus laevis*

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Abstract

Towards understanding the role of oxidative stress in intestinal remodeling, we investigated the role of two antioxidant enzymes catalase and superoxide dismutase which safeguard cells from reactive oxygen species, during metamorphosis in tadpoles, *Xenopus laevis*. Preceding the beginning of remodeling, catalase and superoxide dismutase (SOD) both are up regulated, but SOD is increased eight times more than catalase. High SOD activity leading to formation of hydrogen peroxide corresponds with apoptosis during the same period. SOD positive cells, most likely apoptotic cells, are seen sloughed off in the lumen of the intestine. Catalase activity was also localized using diaminobenzidine for ultrastructural studies. Just before the remodeling begins, large number of peroxisomes ranging in size from 0.25 to 0.5 µm is seen in apical region of the intestinal cells which were positive for catalase activity. Large number of autophagic vesicles is seen in cells of intestine undergoing cell death. These data suggest a regulatory functional role of antioxidant enzymes in morphogenetic processes occurring in the intestine during its remodeling.

Keywords: oxidative stress, intestinal remodeling, catalase, superoxide dismutase.

1 Introduction

During amphibian metamorphosis, the intestinal epithelium dramatically changes from the larval to adult epithelium because of apoptosis of larval cells (ISHIZUYA-OKA and SHIMOZAWA, 1992) and proliferation and differentiation of larva-to-adult cells (YOSHIZATO, 1989). Tadpole intestine is comprised mainly of a single layer of larval epithelial cells which form a fold, the typhlosole, in the anterior part of the small intestine (MARSHALL and DIXON, 1978) that disappears as metamorphosis progresses. Concurrently, the connective tissue and muscles develop extensively, facilitating morphogenesis of the adult intestine (SHI and ISHIZUYA-OKA, 1996). Interestingly, the apoptosis (programmed cell death – PCD) observed in tail as well as intestine during metamorphosis is thyroid hormone dependent and thyroxin induces apoptosis in regressing tail by stimulating mitochondrial generation of reactive oxygen species -ROS (HANADA et al., 1997; KASHIWAGI, HANADA, YABUKI et al., 1999; INOUE, SATO, NISHIKAWA et al., 2004). The most abundant morphological forms of PCD in developing animals are apoptosis and cell death with autophagy (CLARKE, 1990); apoptotic cells die in isolation and require a phagocyte for removal and degradation, autophagic cell death occurs in groups of cells and require lysosomal machinery (BAEHRECKE, 2002).

Peroxisomes and microperoxisomes are characterized by the presence of H_2O_2 generating oxidases and by an H_2O_2 destroying catalase enzyme (BOCK, KRAMER and PAVELKA, 1983). Dauca, Calvert, Menard et al. (1982) have reported a developmental pattern of peroxisomes in liver and kidney and catalase activity in anuran tadpoles. These authors have suggested a possible of role of peroxisomes in adaptation to terrestrial habitat, mode of excretion as well as mitochondrial function in *Rana catesbeiana* tadpoles. No such changes were observed in liver and kidney of *Xenopus laevis* tadpoles as they remain aquatic and ammonotelic.

It has been well documented that ROS play critical roles in induction of apoptosis. A definite role of the ROS and antioxidants is also well established in various cellular processes such as development, differentiation, regeneration and apoptosis (ALLEN, 1991; MAHAPATRA, MOHANTY-HEJMADI and CHAINY, 2001, 2002). Superoxide dismutase (SOD) catalyzes the dismutation of highly reactive superoxide anions to molecular oxygen and hydrogen peroxide. Catalase catalyzes the conversion of H_2O_2 into molecular oxygen and water. It is reported that ROS and peroxisomal enzymes play an important role in tail regression (KASHIWAGI, HANADA, YABUKI et al., 1999). However, peroxisomes and ROS have received little attention during intestinal remodeling. Menon and Rozman (2007) have shown that the cellular environment in the intestine becomes progressively more oxidizing during its remodeling and depletion in antioxidants such as catalase and SOD probably sensitizes cells of these organs to oxidant stressors. However, the above studies were focused on biochemical assay and gene expression for these antioxidants. In the present study, we performed western blot analysis for catalase and SOD and the spatio-temporal distribution of both the enzymes using immunohistochemistry (IHC). We also carried out catalase enzyme activity with DAB reaction to understand the functional role of enzyme *in vivo* using light as well as electron microscopy.

2 Materials and Methods

2.1 Tadpoles

Xenopus laevis tadpoles, purchased from *Xenopus I*, Ann Arbor Michigan were maintained in the laboratory

in the aquaria and fed on the diet obtained from the same company. Water in the aquaria was changed every alternate day. Intestinal remodeling occurs between stage 60 and 63 (NIEUWKOOP and FABER, 1967).

2.2 Chemicals

For immunohistochemistry, catalase primary monoclonal antibody (mouse) and FITC conjugated secondary antibody (goat anti mouse) were obtained from Sigma/Aldrich. Primary antibody against SOD (goat polyclonal) and rhodamine conjugated secondary antibody (rabbit anti goat) were obtained from Santa Cruz Biotechnology, Inc. For western blot analysis, alkaline phosphatase conjugated secondary antibodies and protein standards were obtained from Biorad Precision. For catalase cytochemistry, diaminobenzidine, 3 – amino – 1, 2, 4 triazole (inhibitor of catalase) were obtained from Sigma/Aldrich.

2.3 Gel Electrophoresis and Western Blotting

Sample Preparation: Intestine pieces from *Xenopus laevis* tadpoles at the specified stages were homogenized in 5X volume of SDS PAGE sample buffer with dithiothreitol to which 1 mM phenylmethyl sulfonyl fluoride (PMSF) was also added as an enzyme inhibitor.

Electrophoresis: Eight percent discontinuous polyacrylamide gels were run for the studies with the Laemlli buffer system (LAEMMLI, 1970) in Biorad minigel apparati. The samples were applied, along with Biorad Precision Plus standards for molecular weight determination. Gels were transferred (see below) or stained with Zoion Fast Stain.

Transfer (Electroblotting): The electroblots were conducted by a modification of the Towbin procedure (TOWBIN, STAEHLIN and GORDON, 1979), using a Biorad minigel transfer apparatus and Immobilon membrane.

Western Blotting: Following an initial one hour blocking step in 5% bovine serum albumin (BSA) in tris buffered saline (Tween-20 included), western blots were conducted using the primary antibodies described above. The antibody was diluted 1/100 (anti-SOD) or 1/2000 (anti-catalase) in blocking buffer to which 0.1% aprotinin was added as a preservative. The primary incubation was conducted at room temperature overnight. The secondary incubation used alkaline phophatase conjugated secondary antibodies (Biorad). The blots were reacted with the color developing substrates BCIP (5-bromo-4-chloro-3-indolyl phosphate) and NBT (nitroblue tetrazolium) in bicarbonate buffer as described (MENON, GARDNER and VAIL, 2000). The blots were then rinsed in water and dried.

2.4 Immunohistochemistry for Catalase and SOD

Tadpoles at different stages (55, 58, 60, 62 and 66) were anesthetized in MS 222 and portions of the intestine (at the junction where bile and pancreatic duct opens) were dissected, luminal contents were flushed out with phosphate buffered saline and fixed in 4% formalin, processed for paraffin sections for immunohistochemistry for catalase and SOD according to the method of Ibabe, Grabenbaiere, Baumgart et al. (2003). Sections were deparaffinized and hydrated through a series of alcohol ending in water. Antigen was retrieved by treating sections three times in citrate buffer (0.01 M, pH 6.0) in a household conventional microwave (740 W) followed by 20 minutes cooling without changing the buffer. Slides were washed in Phosphate buffer saline (PBS), and incubated in 3% H_2O_2 dissolved in methanol for blocking endogenous peroxidase. Non specific binding sites were blocked with 0.1% rabbit serum by incubating for 30 minutes at 37 °C. Sections were then incubated overnight at 4 °C with monoclonal anti-catalase antibody (1:200) and polyclonal anti-SOD (1:100) diluted in the blocking buffer followed by incubating with secondary antibody (anti catalase- FITC conjugated and for anti SOD- Rhodamine conjugated diluted 1:50) for 30 minutes at room temperature. Sections were washed with PBS for 5 minutes each, washed with distilled water for 10 minutes, mounted and visualized under Zeiss LSM 510 META Confocal microscope.

2.5 Immunohistochemistry density count

Florescence intensity of catalase and SOD was measured in three different sections (from different animals for each enzyme) from ten different areas of the same section using Adobe Photoshop CS2. Images were not digitally modified to change the color and/or intensity. Intensity was calculated as the average of all the readings (0 being the lowest and 255 being the highest brightness). Data was normalized by making readings of background intensity from the same sections and subtracted from intensity observed for the enzyme. This data for both the enzymes was analyzed for

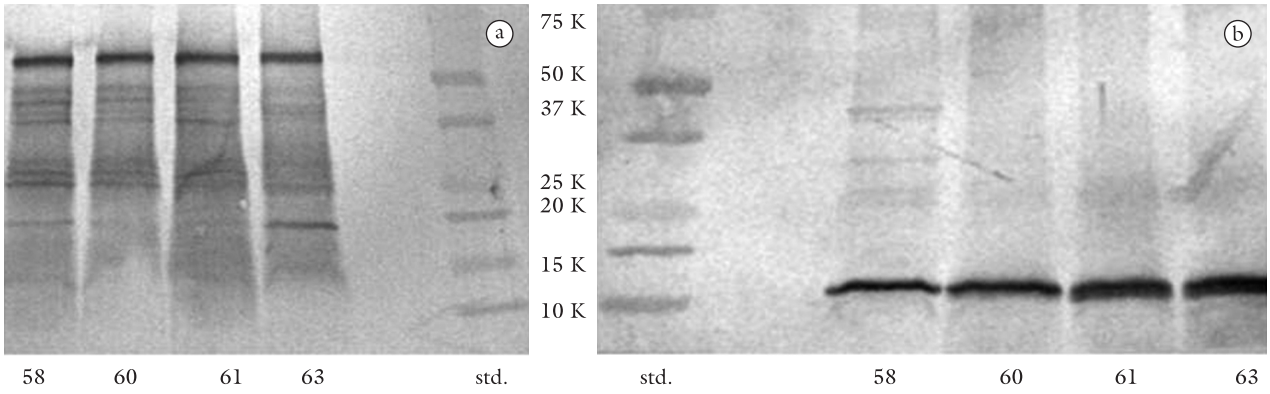

Figure 1. a) Catalase expression at 56 k; and b) SOD expression at 14 k.

t-test and plotted in a bar graph using Microsoft excel. *t*-test for both the enzymes at all the stages (stages, 58, 60, 62 and 63) were compared to stage 55 (before the remodeling begins).

2.6 Cytochemistry for Catalase

Intestine pieces were fixed in 2.5% glutaraldehyde for 2 – 2 1/2 hours at 4 °C and washed with cold 0.1M cacodylate buffer (pH 7.4) containing 5% sucrose for 24 hours in cold. Samples were incubated for 30 minutes at 37 °C in a medium containing, 0.03-0.05% H_2O_2, 1 mg.mL^{-1} DAB, 0.005 M KCN in 0.05 M propandiol buffer at pH 9.5 for 2 hours at 37 °C according to the method of Beard, Moser, Sapirsten et al. (1986). Control tissue samples were incubated with inhibitor of catalase (3 – amino – 1, 2, 4 triazole at 0.05 M). Tissue samples were washed and post fixed in 2% Osmium tetroxide in buffer for 1 hour at room temperature, dehydrated and embedded for routine electron microscopy. Thin sections were cut on Reichert Microtome and unstained sections were visualized under Zeiss EM 10.

3 Results

3.1 Western blot

Catalase expression at 56 K was uniform at all the stages (Figure 1a). Although it exists as a dimer *in vivo*, it migrates as monomer under conditions used. SOD expression at 14 K

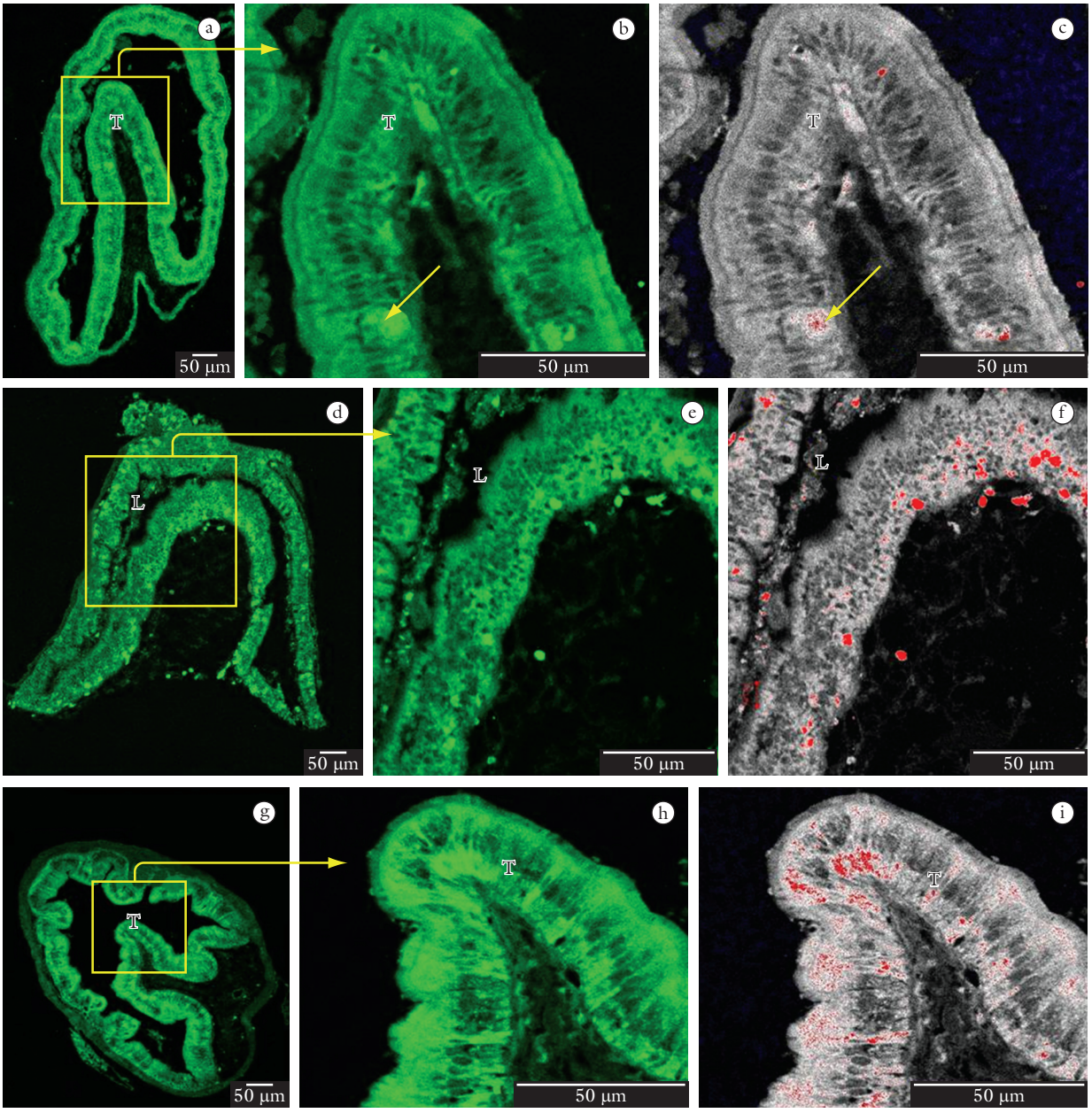

Figure 2. Catalase: Cross section of the intestine showing catalase activity in typhlosole and mucosal epithelium at stage 55 (a, b, c). Note few hot spots (in red). At stage 58 (d, e, f) and 60 (g, h, i) more hot areas are seen. Scale bar 50 µm. L – lumen; T – typhlosole.

was found to be increasing (stage 61) as metamorphosis progressed (Figure 1b).

3.2 IHC for Catalase and SOD

At stage 55, very few catalase positive cells are seen in mucosal epithelium and typhlosole (Figure 2a, b, c) but by stage 58 and 60 more catalase positive cells are discernible (Figure 2d, e, f, g, h, i). At stage 58, SOD positive cells were seen only in lumen (Figure 3a, b), and there was overlapping of both the enzymes (Figure 3c). At stage 60, the time period when intestinal remodeling begins, strong expression for SOD was seen in cells in the lumen of the intestine (Figure 3d) but neither enzyme was detected at stage 62 (Figure 3e, f, g, h). At stage 63, (completion of intestinal remodeling) catalase positive cells were seen in mucosa and lamina propria underlying the mucosa (Figure 3i, j, k) but no SOD expression was seen (Figure 3l, m).

3.3 Density count (Figure 4)

When density count for both the enzymes at stages 58 through 63 was compared to stage 55, catalase was found to be significantly different only at stages 58 ($p < 0.05$) but SOD was significantly different at all the stages ($p < 0.05$). It showed that just before remodeling begins (stage 58), both the enzymes are up regulated compared to stage 55; however increase in SOD was 8 times compared to the slight increase accounting for higher level of H_2O_2. High SOD activity was maintained at stage 60, but there was no significant change in catalase at the same stage. Thereafter both the enzymes showed a decreased density count reaching more or less the same value at stage 63 (Figure 4).

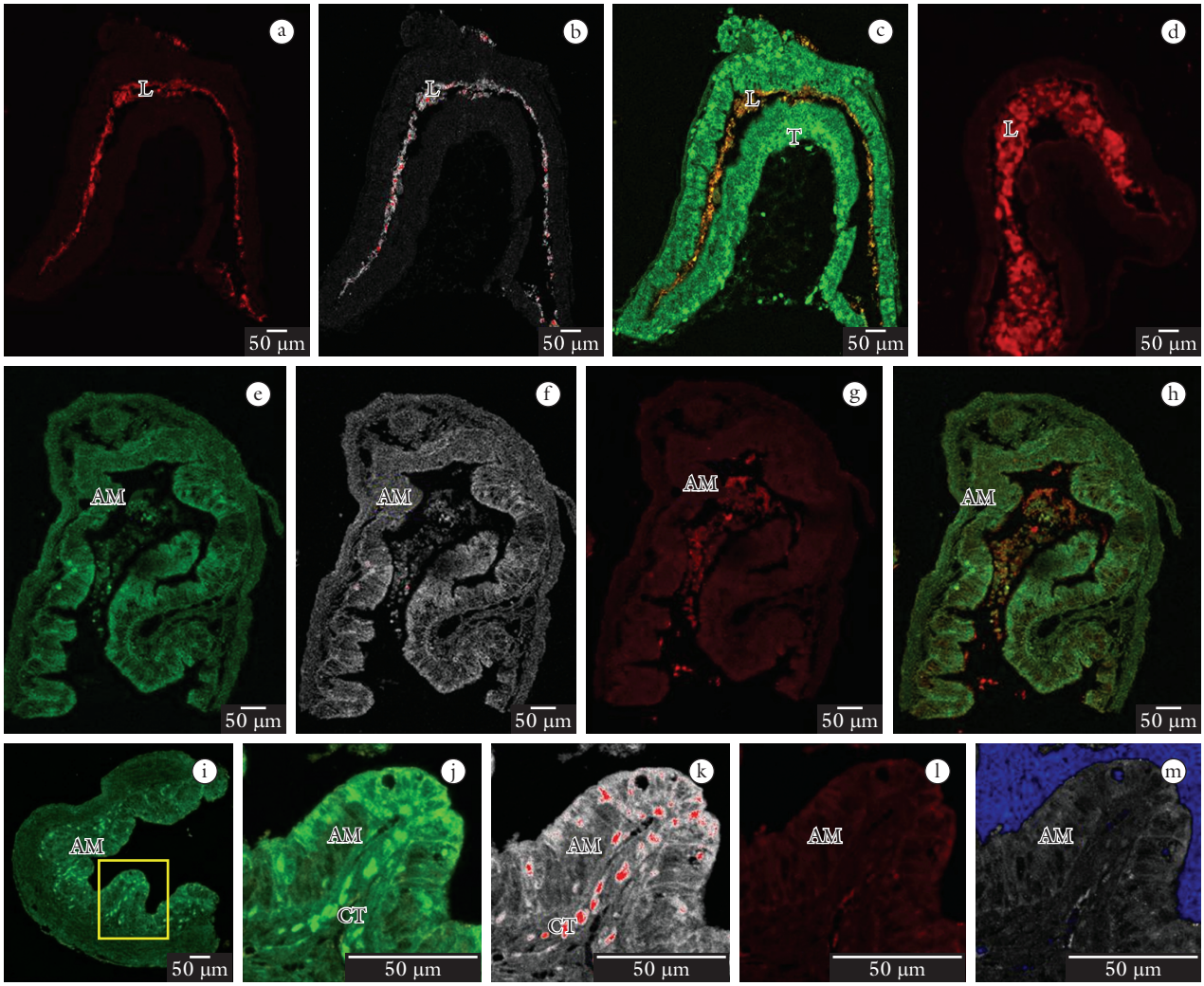

Figure 3. Doubleimmunostaining for catalase and SOD: At stage 55, SOD expression was completely absent (not shown) but at stage 58, SOD is expressed (a) and there is an overlapping of both the enzymes in lumen (represented by orange color (c)). Number of SOD positive cells increased considerably in lumen at stage 60 (d). At stage 62, very low expression of both the enzymes is noted (e, f, g, h). At stage 63 (completion of remodeling), catalase positive cells are dispersed in mucosa as well as underlying connective tissue layer (i, j, k) but SOD is completely absent (l, m). Scale bar 50 µm. AM- adult mucosa; CT – connective tissue; L – lumen; T – typhlosole.

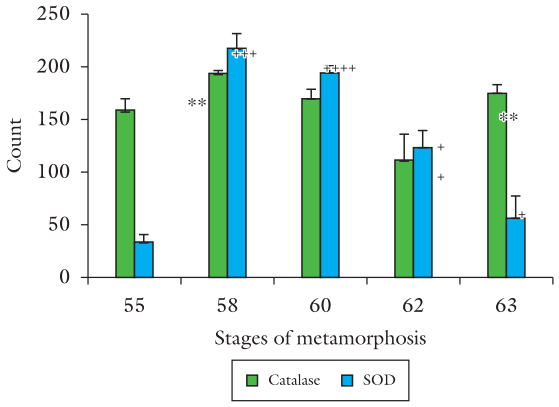

Figure 4. Immunohistochemistry density count for catalase and SOD revealed that at stage 55, catalase was higher than SOD. At stage 58, highest expression of both the enzymes was detected and was statistically significant from stage 55. At stage 60, high SOD expression was maintained and at stage 62, there was down regulation of both the enzymes. At the end of remodeling period, density count for both the enzymes were similar to one observed before the remodeling began. Catalase: **p < 0.05, ***p < 0.05, For stage 60, p = 0.09, p > 0.05. SOD: $^{+}p < 0.05$, $^{++}p < 0.05$, $^{+++}p < 0.05$, $^{++++}p < 0.05$.

3.4 Catalase cytochemistry (DAB reaction product) Light microscopy

At stage 58, no DAB reaction was observed in typhlosole (Figure 5a solid arrow) but intense activity of the enzyme was noted in mucosal epithelium destine to give rise to adult mucosa (Figure 5a). Enzyme activity was granular (Figure 5b, arrow) compatible with peroxisomal localization. Control tissue (stage 58) where enzyme inhibitor was added, did not show any activity for catalase (Figure 5c), though some DAB positive cells were seen. At stage 63 (end of intestinal remodeling), intense enzyme activity was noted in mucosal epithelium (Figure 5d).

3.5 Catalase cytochemistry (Ultrastructure)

At stage 58, large numbers of autophagic vesicles positive for DAB reaction are seen in mucosa destined to give rise to adult mucosa but in typhlosole, these vesicles did not stain for DAB (Figure 5e). Large numbers of peroxisomes are found in apical portion of the cell and have a diameter ranging from 0.25 to 0.50 µm (Figure 5f Inset). In control section, no DAB reaction was noted (Figure 5g).

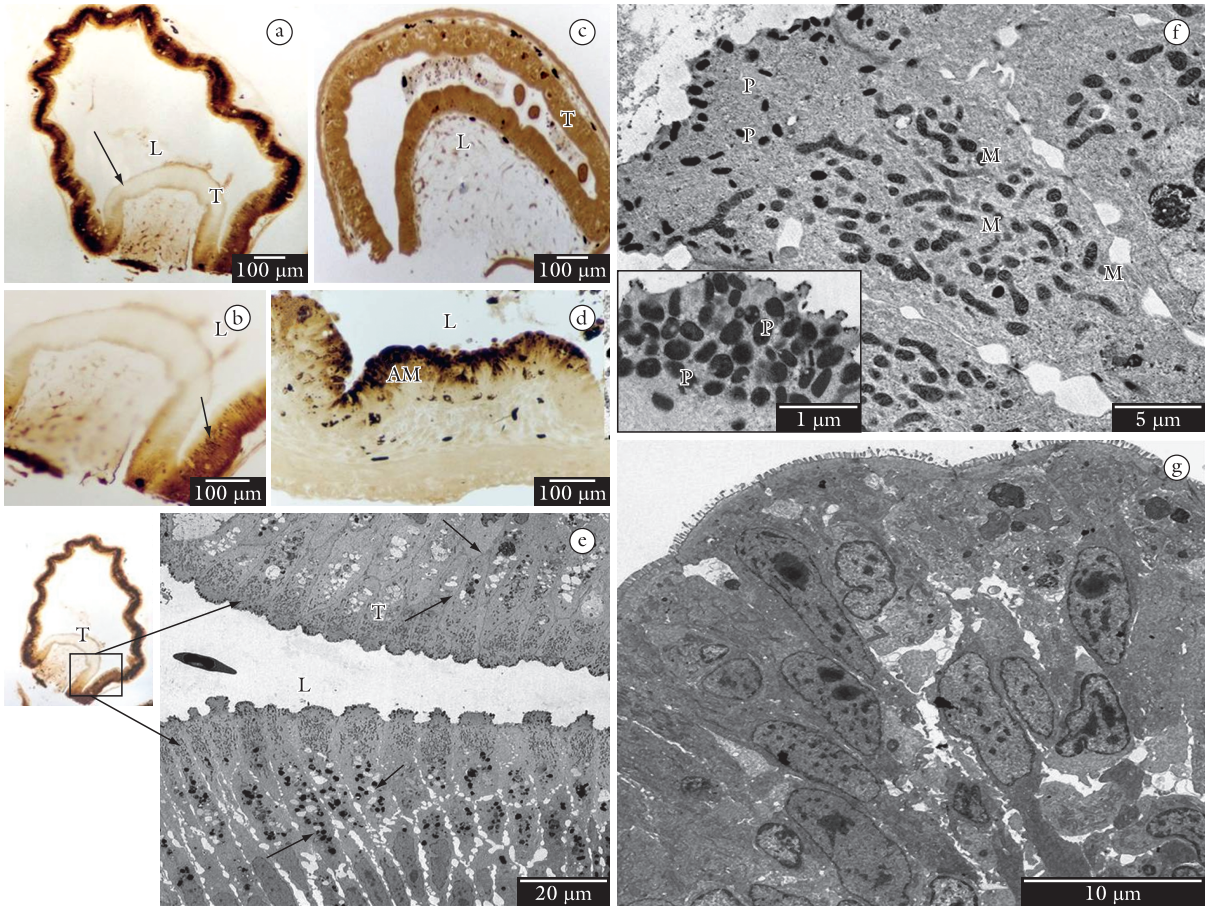

Figure 5. Catalase cytochemistry (DAB reaction product) - light microscopy: At stage 58, mucosal epithelium (destined to give rise to adult mucosa) shows strong activity of catalase enzyme (a, b), which was absent in typhlosole. Control (stage 58) tissue where inhibitor of catalase was added in incubation medium does not show any activity (c). However, some DAB positive cells such as macrophages are seen. Strong enzyme reaction product is seen at stage 63 (d). (Scale bar 100 µm for figures a, b, c, d). Electron Microscopy: At stage 58, large number of autophagic vacuoles in typhlosole, are devoid of DAB reaction whereas these vesicles in mucosa destined to give rise to adult one, show strong DAB reaction is (e). At stage 58, note presence of peroxisomes in apical portion of the mucosa (f) showing strong DAB reaction (f, Inset) whereas control does not show DAB reaction in peroxisomes (g). L – lumen; M – mitochondria; P – peroxisomes; T – typhlosole.

4 Conclusions

Our previous studies (MENON and ROZMAN, 2007) have shown that the gene expression as well as activity of catalase measured biochemically decreases during the critical period of intestinal remodeling (stages 60, 62) suggesting that cytotoxic agent H_2O_2 might be involved in apoptosis of larval cells in intestine and atrophying tail. Present results on immunohistochemistry and density count for catalase are in conformity with the above observations. A period of apoptosis during intestinal remodeling is known to precede cell proliferation whereas cell proliferation is very low before climax but gradually increases throughout the epithelium in late metamorphosis after the villi have been formed (SCHREIBER, CAI and BROWN, 2005). Hence, high catalase activity observed in specific cells of typhlosole as well as mucosa destined to give rise to adult mucosa during initial period of intestinal remodeling may provide protection to larva-to-adult cells from apoptotic machinery that comes into play during this period. However, during the critical period of remodeling, catalase activity seems to be reduced.

Ishizuya-Oka and Shimozawa (1992) reported that remodeling of the intestine involves separate nests of adult stem cells that proliferate and differentiate to replace the dying larval epithelium. However, recently Schreiber et al., (2005) reported that larval epithelial cells rather than a subpopulation of stem cells are the progenitors of the adult epithelium. If this is true, our present findings on widespread and diffuse populations of catalase positive cells in mucosa destined to transform into adult mucosal epithelium as well as typhlosole – a larval organ support the contention that this antioxidant is protecting these larval cells from oxidative assault.

Unlike immunohistochemistry observations which revealed presence of catalase protein in a specific cell types of typhlosole, DAB reaction did not show any activity of the enzyme. However, strong enzyme activity was found to be present in mucosa destined to give rise to adult mucosa, probably providing protection to larva-to-adult cells. While the enzymatic operation for H_2O_2 removal disappearing in typhlosole, but being active in mucosa (destined to give rise to adult mucosa), it seems plausible that ROS may function in removal of the larval structure typhlosole - but at the same time provide protection to larva-to-adult cells in mucosa from the oxidative assault. The period of apoptosis (stage 61/62) corresponds with high SOD activity as evident from western blot analysis as well as IHC leading to formation of superoxide that might be responsible for cell death. Increase in SOD expression is proportionately higher than catalase, allowing H_2O_2 to be used as a stressor for cell death. Apoptotic bodies from dying larval cells at least in part are phagocytosed by macrophages which are eventually extruded into the lumen while still retaining apoptotic bodies (DAMJANOVSKI, AMINO, UEDA et al., 2000; ISHIZUYA and SHIMOZAWA, 1992). Hence it is not surprising that the expression of SOD was found to be present in the sloughed cells in the lumen of the intestine. At the completion of remodeling, no catalase activity is observed in mucosa but it is confined to specific cells in underlying connective tissue, whereas SOD is completely absent. Biogenesis of peroxisomes and the developmental pattern of their enzymes have been reviewed during embryonic and post embryonic development of amphibians and higher vertebrates (KELLER, CABLE, BOUHTOURY et al., 1993). Presently, at the ultrastructural level, large number of DAB positive peroxisomes is seen in apical part of mucosal epithelial cells. During spontaneous as well as thyroid hormone induced metamorphosis, the frequency and dimensions of peroxisomes increase in liver, kidney and intestinal epithelial cells of tadpoles *Rana catesbeiana* and *Alytes obstrtricans* tadpoles (DAUCA, CALVERT, MENARD et al., 1983). The number of peroxisomes as well as catalase activity increase in liver and kidney of tadpoles *Rana catesbeiana* but not in *Xenopus laevis* and these differences in the behavior of peroxisomes are possibly related to the change in mode of excretion in *Rana catesbeiana* which become ureotelic (DAUCA, CALVERT, MENARD et al., 1982). However, here we report the role of peroxisomes and related enzymes in creating oxidative stress partly responsible for apoptosis of larval cells in intestine.

As for removal of larval cells including typhlosole, it probably represents a type of autophagy involving group of cells that need to be removed which is caused by increased ROS (YU, WAN, DUTTA et al., 2006). Characteristics of autophagy involve vacuolar proteolysis which serves to degrade cell and plays a part of the phagocyte lysosome during apoptosis (BAEHRECKE, 2002). Large number of autophagic vesicles in the typhlosole of the intestine probably is a part of autophagy occurring in this organ. In conclusion, during the critical period of remodeling of intestine, much higher SOD activity compared to catalase indicates presence of high levels of free radicals which might be responsible for generating oxidative stress leading to cell death. Presence of catalase in specific cell type may provide protection to larva to adult cells from oxidative assault.

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