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ORIGINAL ARTICLE

# Evidence of apoptosis in some cell types due to pentachlorophenol (PCP) in *Heteropneustes fossilis*

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**Abstract** The study aimed to clarify the role of apoptosis in pentachlorophenol (PCP) induced testicular, ovarian and renal cell genotoxicity of *Heteropneustes fossilis*. It was further intended to find the target germ cell type and assess the cellular and nuclear damage. Treatment of PCP was used for multiduration on the germinal tissues and they were processed to detect structural changes by light and electron microscopic evaluation and kidney cells for subsequent detection of DNA fragmentation by agarose gel electrophoresis. Findings suggest functional and morphological changes in the tissues are due to apoptosis, as evidenced by some biochemical and cytological signs. Histological observation on germinal epithelium reveals cell suicidal symptoms such as vacuolization, liquefied regions in the cytoplasm of oocytes, margination of nuclei, clumping of chromatin, and compaction of cytoplasmic organelle. Biochemical manifestation concurrent to this, is; cleavage of kidney cell DNA into low molecular weight fragments confirming apoptosis. Subsequently, it is further cleaved into nucleosome size fragments or its multiples. Ultra-structural histopathology and DNA studies conclusively lead to the PCP induced apoptosis in the exposed cell types. Results further support the usefulness of this assay in the related studies and its feasibility in generating a base line data.


## 1. Introduction

Apoptosis is a major form of cell death, useful in development, atrophy, immunity and toxic cell killing (Cohen et al., 1992; Arends and Wyllie, 1991; Ellis et al., 1991). Chromatin condensation and cell shrinkage are morphological characteristics of induced apoptosis (Weber et al., 2002; Berntssen et al., 2001; Toomey et al., 2001) and biochemically best represented by the cleavage of DNA resolved by gel electrophoresis (Solov'yan et al., 1992). As a result, it is fastly recognized as a good parameter of assessing the genotoxic insult. Not surprisingly therefore, it has found favor in many studies involving environmental toxins (Dong et al., 2009; Michaowicz and Sicińska, 2009). At the molecular level, three patterns of DNA degradation are recognized to indicate the level of injury: single strand nicks (Gorczyca et al., 1992), large DNA fragmentation of 50–200 kb and nucleosome size fragments of 180–200 bp (Higuchi,

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2003). One or combination of these may occur during apoptosis in a single cell.

There is an increasing emphasis now on this area using chlorophenolic compounds (Bradberry et al., 2000; Seiler, 1991) as the convincing proof of genetic damage. The mutagenicity of PCP and related derivatives is well-documented not only in prokaryotes (DeMarini et al., 1990; Nishimura and Oshima, 1983) but also in some other mutation system (Jansson and Jansson, 1986). Other effects of apoptosis have been documented, that include, inhibition of growth, RNA and ribosome synthesis (Ehrlich, 1990). Many carcinogenic effects are equally significantly reported during the course of similar studies in mammals (Nodera et al., 2001). Aquatic organisms though largely remained neglected. Present study is the extension of our previous observations taken to define the genotoxic effects of PCP with respect to cell death. Earlier studies targeted histopathological changes (Ateeq et al., 1999), chromosomal damages (Ali and Ahmad, 1998) and micronucleus appearance and their evaluation (Farah et al., 2003; Ahmad et al., 2002; Ateeq et al., 2002). An extension to this is to ascertain whether or not apoptosis is involved in PCP induced testicular, ovarian and renal cells, if so, to identify the target germ cell type. The extent and nature of cellular and nuclear damage by PCP were evaluated by histological examination using light and electron microscopy and DNA cleavage pattern. The studies are significant in order to generate a base line data in stressed fishes in apoptotic conditions and to make a significant comparison with other organisms to gain an sight into PCP induced genotoxicity as a whole.

## 2. Materials and methods

### 2.1. Specimen and treatment

*Heteropneustes fossilis* weighing 40–50 g and 15–20 cm long were acclimatized and employed in experiments. The specimen received a single dose of 0.4 ppm of PCP in alcoholic water as whole body immersion to variable exposures, 48, 72 and 96 h ($n = 10$). The dose was standardized earlier (Ateeq et al., 2002). Solvent control ($n = 10$) received 0.1% alcoholic water immersion for 96 h and normal control ($n = 10$) created for the same duration in plain water. The germinal tissues were processed to detect structural changes by light and electron microscopic evaluation and kidney cells were assessed for DNA fragmentation by agarose gel electrophoresis.

### 2.2. Light microscopic preparation

After exposure, a small piece of the ovary and testis was excised, fixed in Bouin's fixative (24 h), dehydrated in ethanol, cleaned in xylene and embedded in paraffin wax. Sectioning of the desired tissue was serially done at 5 μm, followed by staining in haematoxylin–eosin. Hundred sections per specimen were studied and representative displays having structural changes were photographed (Automatic photomicrography equipment, Mirax Labrec).

### 2.3. Electron microscopic preparation

Standard methods of Culling et al. (1985) were followed. Cubes of 2–3 $mm^3$ from infused tissues were immersed in 2.5% glutaraldehyde (4 °C for 6 h), post fixed in 1% osmium tetroxide (4 °C for 4 h), dehydrated in acetone (15 min) and embedded in epoxy resins. Ultra thin sections (50–60 nm) were stained with uranyl acetate-lead citrate, examined and photographed for ultrastructural changes by TEM (EM 300: Philips, Holland).

### 2.4. DNA extraction

The protocol of Sambrook and Russel (2001) was adopted for these studies. From the frozen kidney, 200 mg of tissues from control and exposed specimen was minced on dry ice and digested with proteinase K (100 μg/ml for 90 min) to lyse the cells. The suspension was incubated (50 °C for 3 h), swirled periodically and gradually cooled to room temperature. An equal volume of salt saturated (SS) phenol equilibrated with 0.5 M Tris HCl (pH = 8.0) was added and the two phases were mixed gently (10 min) and centrifuged at 20,000*g* (10 min). The supernatant (aqueous phase) was saved and 2 ml of chloroform and 2 ml of SS-phenol were added (15 min), and subjected to RNase-A treatment (10 ng/ml for 60 min). The step was repeated twice to isolate RNA free DNA. The aqueous phases were pooled and distributed in fresh centrifuge tubes, 0.2 volumes of ethanol was added (at room temperature) and swirled until the solute was mixed thoroughly. The precipitated DNA was removed from ethanol, washed twice with 70% ethanol and centrifuged at 5000*g* (5 min). To the pellet, 1 ml of TE (pH = 8.0) was added to completely dissolve the DNA.

### 2.5. Electrophoretic separation of DNA

For the detection of DNA pattern the Strauss method was adopted (Strauss, 1994). Agarose gel (0.6%) was prepared and casted. The 1/10th volume of 10× tracking dye (bromophenol blue) was added to the DNA and samples were loaded. Hind III DNA fragment (Sigma Chemicals) was used as a marker to estimate the molecular weight of DNA fragment. A steady voltage of 40–50 volts (5–6 h) was applied, till the dye front reached near the positive end. The DNA was stained by addition of ethidium bromide (0.5 μg/ml). The molecular weights of resolved DNA fragment were estimated by comparing it with the marker using a gel base documentation system equipped videography thermal printer (Ultraviolet Product: England).

## 3. Results

### 3.1. Histopathological abnormalities of gonads

Structural changes in testis and ovaries are displayed by light microscopy and electron microscopy as shown in Figs. 1 and 2. The affected testes were generally found in recrudescence stage. Interstitial cells were frequently involuted and aggregated near the intertubular junction (1A). Leydig cells conceived hypertrophy with moderate to complete pycnosis (1B). This is unlike normal cells, which showed the signs of activity. The primary spermatogonia, endoplasmic reticulum, mitochondria, ribosomes – all normally distributed initially (1C), were found having sertoli cells with a protrusion on the lumen

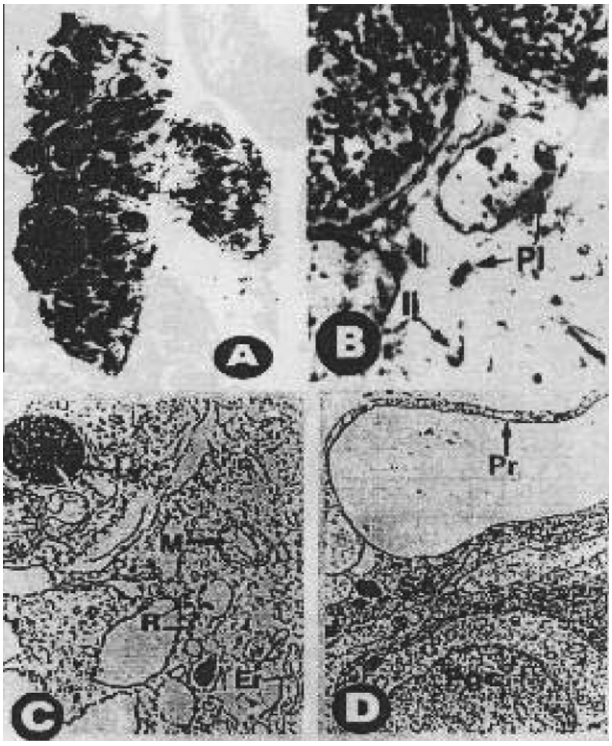


**Figure 1** Light (A and B) and electron (C and D) micrographs showing apoptotic characteristics in PCP exposed testicular cells of *Heteropneustes fossilis*; (A) recrudescence stage of whole testis X 250; (B) involuted interstitial cells [li] and pycnotic Leydig cells [PI] X 675; (C) normal distribution of endoplasmic reticulum [Er], free ribosomes[R], lysosomes [Ly] and mitochondria [M] X 34, 000; (D) primordial germ cell [Pgc] with attached Sertoli cell [Sc] showing protrusion [Pr] X 5,800.

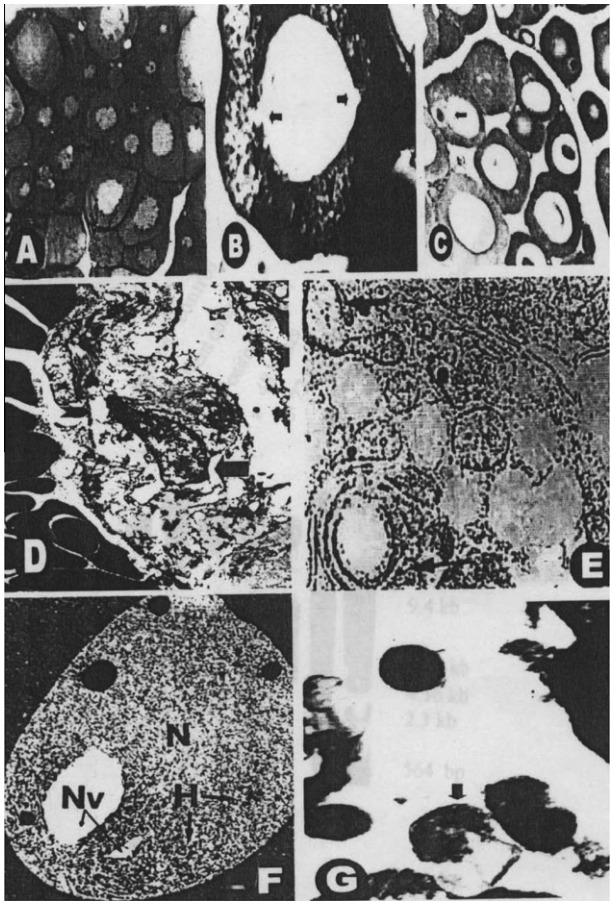


**Figure 2** Light (A–C and G) and electron (D–F) micrographs showing apoptotic properties in PCP exposed ovarian cells of *Heteropneustes fossils*; (A) different stages of oocytes showing vacuolation of cytoplasm (Nv) in immature oocytes X 450; (B) appearance of liquefied region (arrowhead) in the cytoplasm of hypertrophied granulose layer (arrow)X 675; (C) uniformly condensed chromatin abutting on the nuclear envelope (arrow) of oocyte X 450; (D) crater like depression of nucleus (arrowhead) along with vacuole (arrow) X 15,000; (E) attenuated mitochondria (arrow) indicating the characteristic of biogenesis X 17, 500; (F) prominent irregular nucleus (N) showing patches of heterochromatin (H) with large vacuolation (Nv) X 17,500; (G) apoptotic bodies showing fragmented epithelial cells with dark masses of chromatin surrounded by clear haloes (arrow) X 675.

of the seminiferous tubules in exposed forms (1D). The cytological evaluation shows a significant increase of structural abnormalities irrespective of the time of exposure in PCP.

Cellular modification of female germinal cells was equally affected. Vacuolization of cytoplasm in immature oocytes and the appearance of liquefied regions in the cytoplasm and hypertrophied granulosa layer were evident, indicated by differential staining marking the initial symptoms of apoptosis (2-A,B). Thereafter, deleterious effects extended to the oocytes wall where disintegration of cortical alveoli and yolk globules became markedly apparent and the cell transiently adopted a deeply convoluted outline. The signs of oocyte cytoplasm undergoing degeneration were evident by contraction of cytoplasm.

Concurrently, nuclear changes were observed in capillary endothelium. The sharply circumscribed masses of uniformly condensed chromatin abutting on the nuclear envelop become apparent (2D). Besides, a crater like depression along with a vacuole in the middle (2C), and in some cases, vacuolated nuclei was also marked by blebbing.

The morphological progression of apoptosis on mitochondria was ultrastructurally noteworthy. Enlarged and packed mitochondria with tubular cristae were frequently observed during the course of the study. Majority of mitochondria were attenuated indicating the characteristic process of biogenesis (2E). The critical signs of apoptosis were equally noticed in the nuclei of oocytes and epithelial cells. The oocyte nuclei became irregular with dense clumps of heterochromatin masses aligned across the inner nuclear membrane (2F). The epithelial cells appeared fragmented in an irregular fashion showing a portion of cytoplasm with dark masses of altered nuclear chromatin coalesced making apoptotic bodies. These were surrounded by clear haloes (2G).

### *3.2. Electrophoretic pattern of kidney DNA*

DNA profiles of standard marker (Lane 7), normal (Lane 1), solvent and positive control (Lanes 2 and 3), along with the time dependent exposures of PCP (Lanes 4–6) are illustrated in Fig. 3. The DNA laddering was apparent at all durations.

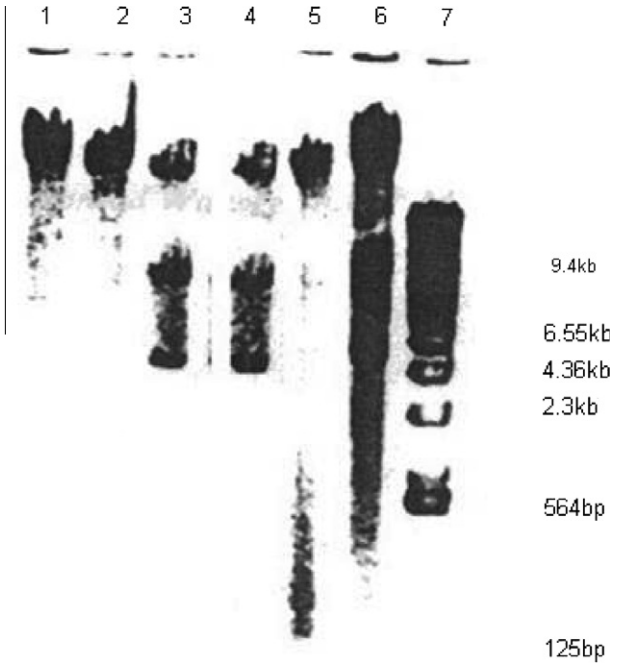


**Figure 3** Electrophorogram of low-molecular weight DNA isolated from renal cells of PCP exposed *Heteropneustes fossilis*. The number at the right indicates molecular weight of the standard size; lanes 1, 2, 3 and 7 correspond to normal, solvent control, positive control and standard λ DNA/Hind III marker; and lanes 4–6 illustrate multiple exposures in 48, 72, and 96 h.

The induction of DNA cleavage in renal cells, appeared to have two components: the endonuclease cleavage into high molecular weight fragments of approximately more than 50 kb followed by subsequent cleavage into fragments of 180–200 bp or multiples thereof (400–800 bp). Since, DNA fragments of low molecular weight are beyond the resolving power of simple agarose gel electrophoresis, the pattern gave the appearance of a general smear (Lane 6). However, characteristic laddering was seen in Lanes 4 and 5. Interestingly, the small amount of fragmentation observed in control (lane 1) was apparently the result of spontaneous apoptosis normally observed.

## 4. Discussion

The apoptotic bodies which are the result of a breakup of nuclear material into several membrane bound bodies have been a regular feature of apoptosis (Save et al., 2001). Nuclear fragments vary in size, and the number of apoptotic bodies produced from dying cells has shown to be related to the size of the cell (Wyllie et al., 1980). These apoptotic bodies are typically phagocytosed by the neighbouring cells without releasing their contents into the intracellular matrix explaining no inflammatory response (Samali et al., 1996). An explanation of this change could be due to the formation of vesicles that fuse with plasma membrane and void their contents extracellularly (Morris et al., 1984).

Even as the morphological progression of apoptosis is readily seen, little is known about the insight of this process in germ cells. Earlier studies on phenolic herbicides also revealed little by way of mechanism (Osterloh et al., 1983), but the studies by distinctive morphology of apoptosis register an exponential increase in testicular, ovarian and renal cell cytotoxicity (Fischer and Dietrich, 2000; Ishikawa and Kitamura, 2000). As for the relative sensitivity of cells towards apoptosis in dividing spermatocytes, is concerned it was shown to be the most sensitive; spermatogonia were also appeared highly vulnerable (Matsui et al., 1993). Cellular changes of apoptosis have been demonstrated in several *in vitro* cell lines (Jie et al., 2004) as also in other cell types (Eleouet et al., 2001).

The formation of low molecular weight fragments of DNA coinciding with early morphological features of apoptosis is a critical event seen presently in PCP induced apoptosis. The study confirms the earlier observations showing fragments normally precede the appearance of a DNA ladder derived from large fragments (Cohen et al., 1992; Wyllie et al., 1980). The internucleosomal DNA fragments frequently demonstrated in apoptosis is regarded as a hallmark of apoptosis in our observations also. Hence, the initial stage of apoptosis when single strands of DNA are nicked to the fragmentation of 5–200 kb size is followed by nucleosomal fragments usually. One or more nuclear endonucleases are critical to this pattern, since isolated nuclei can also be induced to undergo the same pattern (MacDonald and Lees, 1990; Fernandes and Cotter, 1993).

The results confirm the pattern, basic to the understanding mechanism of apoptosis, with particular emphasis on the recognition of early biochemical events in DNA degradation. The study facilitates in identifying apoptotic cells at an early stage of commitment with a reasonable degree of success. Results from tissues other than the germ cells and renal cells, as well as from other animals apart from *H. fossilis* will be needed to examine any broader application of these observations.

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