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# Cell Deletion by Apoptosis During Regression of Renal Hyperplasia

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***Regression of renal hyperplasia after withdrawal of the mitogenic stimulus induced by a single injection of lead nitrate was studied in male Wistar rats. Lead nitrate administration (10 μmol/100 g body weight) resulted in a ninefold increase in the incorporation of labeled thymidine into renal DNA and in an enhancement in the mitotic index; these changes were accompanied by an increase in the organ weight and DNA content that reached a maximum at 2 days. Regression of the renal hyperplasia was observed as early as 3 days after treatment and was completed within 2 weeks. Although lytic necrosis was not responsible for cell loss, the elimination of the excess renal cells took the form of apoptosis. This distinctive mode of cell death, which has been implicated in the involution of hyperplasia in other tissues and organs, was characterized by the occurrence of intracellular and extracellular membrane-bounded eosinophilic globules that often contained nuclear fragments. It affected mainly cells of the proximal tubules, and it was not detected once the kidney had regressed to its original mass. These results support the hypothesis that apoptosis is involved in the regulation of organ size. (Am J Pathol 1989, 135:657–662)***

The size of many organs and tissues is believed to be under the control of growth factors or hormones, with rapid decrease in cell number occurring after withdrawal or addition of the relevant trophic substances.[1] In these circumstances, the type of cell deletion that is commonly observed is a particular mode of cell death, basically different from lytic cell necrosis and defined as apoptosis.[2,3] Apoptosis involves scattered individual cells and can be recognized under the light microscope by the appearance of acidophilic globules (apoptotic bodies), which may contain nuclear fragments; apoptotic bodies may be observed in the extracellular space, but a large number have also been found within the cytoplasm of intact cells as a consequence of their phagocytosis by adjacent cells, where they undergo lysosomal digestion. Of interest, the inflammatory response that always accompanies extensive necrosis is not commonly associated with apoptosis.

Apoptosis has been implicated in a variety of circumstances involving tissue regression such as when atrophy of adrenal cortex and prostate after trophic hormone withdrawal occurs,[4,5] when atrophy of the pancreas after ligation of its duct occurs,[6] during normal regression of corpus luteum,[7] when liver atrophy induced by mild ischemia occurs,[8] and when renal tubular atrophy occurs after experimental hydronephrosis.[9] In addition, apoptosis has been implicated as the mechanism responsible for reducing cell number during the regression of parenchymal hyperplasia, which follows the withdrawal of liver mitogens such as cyproterone acetate and lead nitrate,[10,11] for the involution of biliary duct hyperplasia produced by ligation of the bile duct or by administration of α-naphtyl isothiocyanate (ANIT),[12] and for the regression of hyperplasia of the pancreas after removal of a diet containing a trypsin inhibitor.[13]

The kidney, like the liver, is an organ that possesses a very slow mitotic capacity, but it can be stimulated to grow under a variety of circumstances.[14–17] Of various proliferative stimuli, lead acetate and lead nitrate have been shown to exert a strong mitogenic effect on rat renal cells in the absence of any obvious sign of cell damage.[16–18] Therefore, it was of interest to investigate the fate of the excess cells generated by the mitogenic stimulus induced by lead nitrate.

In the present study, light microscopic analysis of the kidney at various times after lead nitrate administration revealed the presence of many apoptotic bodies during the regression of renal hyperplasia; no apoptotic bodies were observed before the mitogenic event or at a time

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when the kidney had regained its original size. These findings support the concept that apoptosis plays an important role in reducing cell numbers after chemical-induced organ hyperplasia.

## Materials and Methods

### Animals

Male Wistar rats (Charles River Laboratories, Milan, Italy) weighing 200 to 220 g were fed a semisynthetic diet (Ditta Piccioni, Brescia, Italy), and water *ad libitum*. Ether anesthesia was used during treatment of the animals and when rats were killed.

### Treatment of the Animals

Lead nitrate (Carlo Erba, Milan, Italy) dissolved in distilled water was injected intravenously at a dose of 10 μmol/100 g body weight. Controls received distilled water only. The animals were killed at 1, 2, 3, and 15 days after treatment.

### Determination of Renal DNA Content and DNA Synthesis

After treatment with lead nitrate, rats were injected intraperitoneally with 30 μCi/100 g body weight of [$^3$H]-thymidine (New England Nuclear, Boston, MA, specific activity 20 Ci/mmol) at 24, 30, 36, 48, and 60 hours, and were killed 12 hours after the last injection.

After killing, the tissue samples were homogenized in 6 volumes of 0.075 M NaCl-0.025 M EDTA, pH 7.6, and precipitated in ice-cold 1N perchloric acid (PCA). The pellet was washed three times with ice-cold 0.5N PCA and extracted with 0.5N PCA at 70 C for 1 hour. Suitable aliquots of the supernatant were used for measurement of radioactivity in a Beckman LS 1801 liquid scintillation spectophotometer using Biofluor (New England Nuclear) as solvent.

Total renal DNA content was quantitatively assayed by Burton's diphenylamine method.[19]

### Histologic Examination

After removal of the kidneys, small portions were immediately fixed in 10% formalin and embedded in paraffin; sections were then processed and stained with hematoxylin and eosin (H&E); some sections were also stained with the periodic-acid Schiff (PAS) technique, and with Feulgen's technique.

For autoradiographic studies, deparaffinized sections were dipped in NTB 2 nuclear emulsion (Eastman Kodak, Rochester, NY), exposed for 3 weeks at 4 C, developed in Kodak D-19 developer, and fixed, washed, and stained with H&E.

**Table 1.** *Effect of a Single Dose of Lead Nitrate on [$^3$H]-Thymidine Incorporation Into Renal DNA*

| Treatment* | [$^3$H]-Thymidine incorporation (cpm/μg DNA) |
|---|---|
| Controls | 12 ± 1† |
| Lead nitrate | 103 ± 8 |

* Lead nitrate was injected intravenously at a dose of 10 μmol/100 g body wt. At 24, 30, 36, 48, and 60 hours after lead administration, [$^3$H]-Thymidine (sp. act. 20 Ci/mmole) was injected intraperitoneally at a dose of 30 μCi/100 g body weight. The rats were killed 72 hours after treatment with lead nitrate.

† Values are the mean ± SE of five rats.

### Quantitation of Apoptotic Bodies and Mitotic Figures

The number of cells undergoing apoptosis or mitosis was counted in 30 to 50 high power microscope fields by using paraffin sections of the renal cortex of control and treated kidneys. The number of apoptotic bodies and mitoses was expressed as number per 1000 nuclei. Only apoptotic bodies containing nuclear fragments were recorded.

## Results

A single administration of lead nitrate caused a marked stimulation of kidney DNA synthesis as shown by the ninefold increase in the extent of labeled thymidine incorporated into DNA (Table 1); these results agree with those of previous reports on the mitogenic effect of lead salts on rat kidney.[16,18] Cells stimulated to proliferate by lead treatment were present in proximal as well as distal tubules, as shown by autoradiographic analysis (Figure 1). The enhancement in DNA synthesis was accompanied by an increase in renal weight and total renal DNA content, with the maximum increase occurring at 2 days after treatment (+25% and +35% respectively) (see Table 2). Regression of the kidney accompanied by a loss of excess DNA started as early as 3 days after treatment.

Histologic examination of kidney sections of rats killed at various intervals during the first 24 hours after treatment did not show any significant change. Tubular cells appeared normal, and no necrosis or exfoliation of tubular epithelial cells was detected.

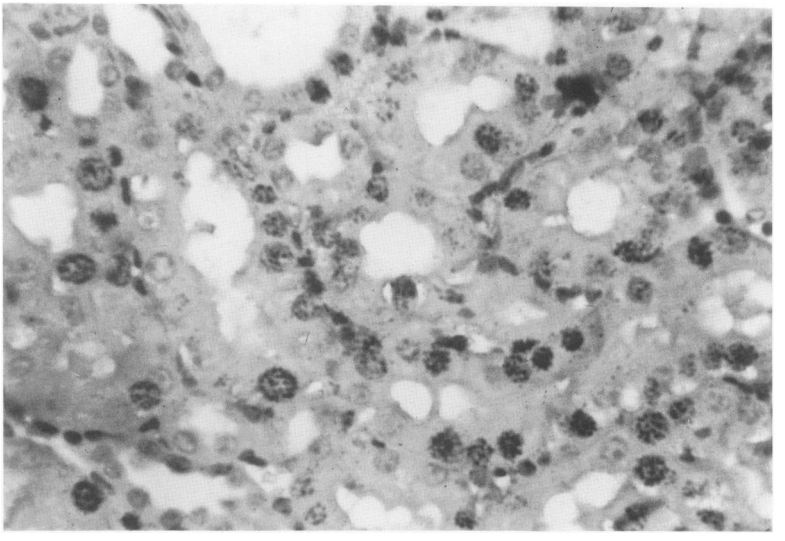

**Figure 1.** *[$^3$H]-labeled cells in the kidney of a rat killed 72 hours after a single dose of 10 μmol/100g body weight of lead nitrate (H&E, original magnification ×250).*

At 48 hours, a marked increase in the mitotic index was observed in the tubular epithelium, particularly in the deeper layers of the cortex (Table 3). Occasionally, scattered epithelial cells with condensed cytoplasm and marginated nuclear chromatin were observed in the tubular epithelium. At this point, rare membrane-bounded vesicles showing the typical aspect of apoptotic bodies were also detected.

At 72 hours, a decrease in mitotic activity was observed; on the other hand, concomitantly with a partial loss of DNA, a striking increase in the number of cells undergoing apoptosis (10.8 apoptotic bodies/1000 nuclei *versus* 0.04/1000 of control kidney) was observed in the tubular epithelium. Apoptotic bodies were scattered (one per tubule), or appeared as clusters (Figure 2). In some tubules, different aspects of apoptosis were observed, including cell shrinkage with loss of contact with tubular adjacent cells (Figure 3), cell fragmentation with apoptotic bodies shed in the tubular lumen, and phagocytosis of cell fragments, with or without nuclear remnants, by normal adjacent cells (Figure 4). Although apoptotic bodies were present in all the 72-hour samples, differences were found not only in their incidence, but in their localization; in fact, although in some animals most apoptotic bodies were in the tubular lumen, in others, apoptotic bodies were detected mainly within the cytoplasm of intact tubular cells.

After the 3rd day, the number of apoptotic bodies fell rapidly, and no differences with the controls were observed 15 days after treatment, a point when the renal mass and total DNA content had regressed to normal values. No exudative inflammation or necrosis was found at any time on examination. Glomeruli appeared unaffected.

**Table 2.** *Effect of Lead Nitrate on Kidney Growth and Renal DNA Content*

| Treatment* | Relative liver weight (mg/100 g body weight) | DNA content (mg/100 g body weight) |
|---|---|---|
| Controls | 0.404 ± 0.01 | 1.05 ± 0.03 |
| Lead nitrate | | |
| Day 1 | 0.465 ± 0.02† | 1.18 ± 0.06 |
| Day 2 | 0.507 ± 0.01§ | 1.42 ± 0.07‡ |
| Day 3 | 0.499 ± 0.01§ | 1.30 ± 0.04‡ |
| Day 15 | 0.410 ± 0.02 | 1.15 ± 0.04 |

* Lead nitrate was injected intravenously at a dose of 10 μmol/100 g body weight. Controls received distilled water only. Values are means ± SE of five to six animals per group.
† Significantly different from control, $P < 0.025$.
‡ Significantly different from control, $P < 0.005$.
§ Significantly different from control, $P < 0.001$.

## Discussion

The present study found that renal hyperplasia, which follows the administration of lead nitrate, rapidly undergoes

**Table 3.** *Incidence of Renal Mitoses and Apoptotic Bodies at Different Times after Lead Nitrate Administration*

| Treatment* | Mitoses/ 1000 nuclei | Apoptotic bodies/ 1000 nuclei |
|---|---|---|
| Controls | 0.02 ± 0.01 | 0.04 ± 0.02 |
| Lead nitrate | | |
| Day 1 | 0.02 ± 0.01 | 0.03 ± 0.02 |
| Day 2 | 13.41 ± 3.43† | 0.91 ± 0.15‡ |
| Day 3 | 1.26 ± 0.51§ | 10.82 ± 4.57§ |
| Day 15 | 0.08 ± 0.05 | 0.13 ± 0.10 |

* After lead administration, the animals were killed at the times shown. Only apoptotic bodies with nuclear material were recorded. For each animal 30 to 50 microscope fields were scored. Seven to 9 rats per group were used. Results are means ± SE.
† Significantly different from control, $P < 0.001$.
‡ Significantly different from control, $P < 0.025$.
§ Significantly different from control, $P < 0.010$.

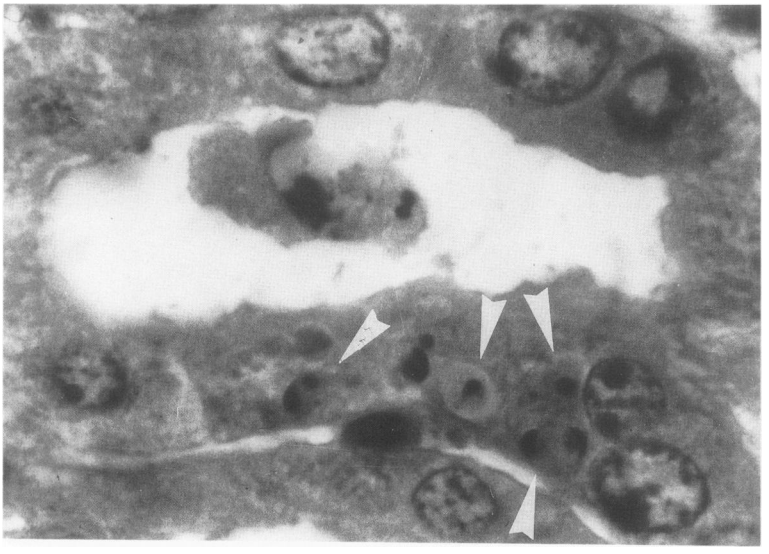

**Figure 2.** *Cell deletion by apoptosis in the tubular epithelium. Clusters of apoptotic bodies (arrows) containing nuclear fragments are shown. An apoptotic body is also present in the tubular lumen (H&E, original magnification × 1000).*

involution; this study also revealed that the involution is the result of the occurrence of cell deletion by apoptosis.

This particular type of cell death can be recognized by specific morphologic changes, such as the occurrence of membrane-bounded cytoplasmic vesicles (apoptotic bodies) that often contain nuclear fragments, which frequently can be found inside adjacent cells where they undergo lysosomal digestion; in addition, apoptosis is often observed in the complete absence of the inflammatory response that usually accompanies necrosis.[1-3]

In our study, several morphologic features of cells dying by apoptosis were detected, despite that the "life-span" of identifiable apoptotic bodies is considered to be very short[1]; thus, dead or dying epithelial cells shed into the lumens of unaffected tubules or lying within the cytoplasm of normal tubular cells were frequently seen during the regression of lead-induced renal hyperplasia, similar to that observed by others during renal tubular atrophy in rat experimental hydronephrosis.[9]

The occurrence of apoptosis during the involution of renal hyperplasia observed in the present study supports the concept that apoptosis is a form of cell death that plays an important regulatory role in many organisms and tissues: for example, it accounts for cell deletion during normal embryogenesis and metamorphosis,[20-22] it is involved in the shrinkage of adult endocrine-dependent tissues after withdrawal of trophic hormones,[4,5] and it has been shown to contribute to cell loss in neoplasms induced by radiation and cytotoxic drugs.[23,24] As far as the carcinogenic process is concerned, apoptosis has also been found to occur in very early hepatic preneoplastic lesions induced by chemical carcinogens[10,25]; it is interesting to note that apoptotic bodies were found more frequently in preneoplastic lesions than in the surrounding normal liver.

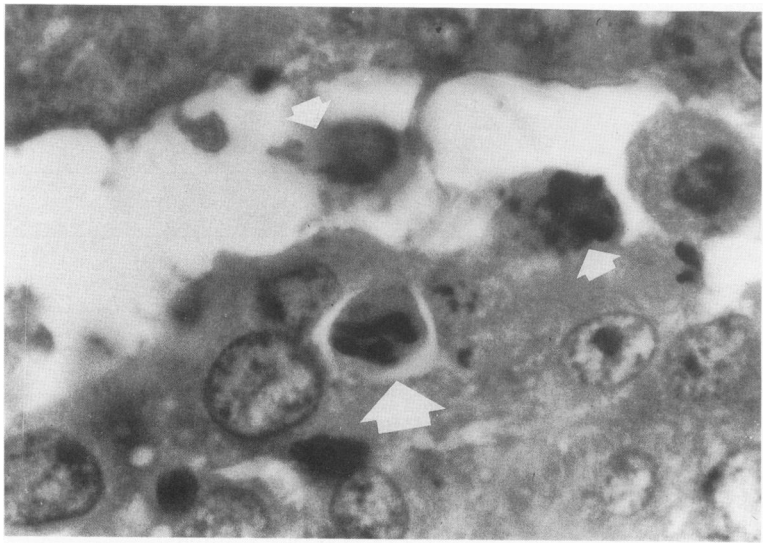

**Figure 3.** *Cell deletion by apoptosis in the tubular epithelium. Proximal tubular epithelial cell (large arrow) undergoing early apoptosis and featuring condensation and nuclear fragmentation. Other apoptotic bodies shed in the lumen are also present (small arrows) (H&E, original magnification × 1000).*

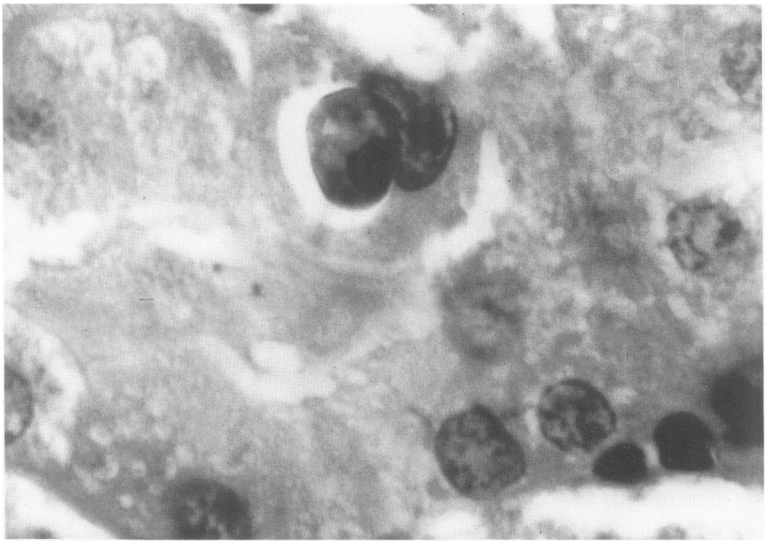

Figure 4. *An intracellular apoptotic body with residual nuclear material is shown (H&E, original magnification × 1000).*

Recently, apoptosis has also been proposed as actively participating in the regression of liver hyperplasia after withdrawal of two hepatic mitogens, cyproterone acetate[10] and lead nitrate.[11] In these studies, the peak incidence of apoptotic bodies occurred within 2 to 5 days after cessation of administration of the mitogen. Of interest, during the involution of lead nitrate-induced liver hyperplasia, no elevation in levels of serum glutamate pyruvate transaminase was observed[11]; this finding suggests that, despite an extensive cell loss (50% of liver DNA was eliminated in 12 days), leakage of such an enzyme may not occur when hepatocyte death takes the form of apoptosis. The possibility of discriminating hepatic necrosis from apoptosis on the basis of the levels of serum transaminases is currently under investigation.

The active role played by apoptosis during regression of hyperplasia was confirmed in other experimental models.[12,13] In these studies also deletion of the excess cells was effected by apoptosis without any sign of inflammation or scarring.

Our results, which showed that cell death by apoptosis occurs during the regression of lead nitrate-induced renal hyperplasia, agree with the aforementioned findings supporting the hypothesis that apoptosis plays an important role in maintaining the homeostasis by regulating cell numbers in tissues and organs.

That apoptotic bodies did not occur until the renal cells had completed their replicative cycle strongly suggests that the deletion of cells observed under our experimental conditions is probably not the consequence of lead-induced cell damage; in fact, cell death such as that induced by administration of nephrotoxins is the cause and not the consequence of cell proliferation.

In conclusion, the present study indicates that cell death by apoptosis plays a crucial role in the regression of renal hyperplasia, and supports the concept that this type of cell death may be part of a regulatory mechanism induced to restore the normal size of the organ.

## References

1. Walker NI, Harmon BV, Gobè GC, Kerr JFR: Patterns of cell death. Methods and Achievements in Experimental Pathology, vol. 13. Edited by G Jasmin. Basel, Karger 1988, pp 18–54
2. Kerr JFR, Wyllie AH, Currie AR: Apoptosis: A basic biological phenomenon with wide-ranging implications in tissue kinetics. Br J Cancer 1972, 26:239–257
3. Wyllie AH, Kerr JFR, Currie AR: Cell death: The significance of apoptosis. Int Rev Cytol 1980, 68:251–306
4. Wyllie AH, Kerr JFR, Currie AR: Cell death in the normal neonatal rat adrenal cortex. J Pathol 1973, 111:255–261
5. Kerr JFR, Searle J: Deletion of cells by apoptosis during castration-induced involution of the rat prostate. Virchows Arch [B] 1973, 13:87–102
6. Walker NI: Ultrastructure of the rat pancreas after experimental duct ligation. I. The role of apoptosis and intraepithelial macrophages in acinar cell deletion. Am J Pathol 1987, 126: 439–451
7. Azmi TI, O'Shea JD: Mechanism of deletion of endothelial cells during regression of the corpus luteum. Lab Invest 1984, 51:206–217
8. Kerr JFR: Shrinkage necrosis: A distinct mode of cellular death. J Pathol 1971, 105:13–20
9. Gobè GC, Axelsen RA: Genesis of renal tubular atrophy in experimental hydronephrosis in the rat: Role of apoptosis. Lab Invest 1987, 56:273–281
10. Bursch W, Lauer B, Timmermann-Trosiener I, Barthel G, Schuppler J, Schulte-Hermann R: Controlled death (apoptosis) of normal and putative preneoplastic cells in rat liver following withdrawal of tumor promoters. Carcinogenesis 1984, 5: 453–458
11. Columbano A, Ledda-Columbano GM, Coni P, Faa G, Liguori C, Santacruz G, Pani P: Occurrence of cell death

(apoptosis) during the involution of liver hyperplasia. Lab Invest 1985, 52:670–675
12. Bhathal PS, Gall JAM: Deletion of hyperplastic biliary epithelial cells by apoptosis following removal of the proliferative stimulus. Liver 1985, 5:311–325
13. Oates PS, Morgan RGH, Light AM: Cell death (apoptosis) during pancreatic involution after raw soya flour feeding in the rat. Am J Physiol 1986, 250:G9–G14
14. Taylor DM, Threlfall G, Buck AT: Stimulation of renal growth in the rat by folic acid. Nature 1966, 212:472–474
15. Malamud D, Malt RA: Stimulation of cell proliferation in mouse kidney by isoproterenol. Lab Invest 1971, 24:140–143
16. Choie DD, Richter GW: Cell proliferation in rat kidney induced by lead acetate and effects of uninephrectomy on the proliferation. Am J Pathol 1972, 66:265–275
17. Ledda-Columbano GM, Columbano A, Coni P, Curto M, Faa G, Pani P: Cell proliferation in rat kidney induced by 1,2-dibromoethane. Toxicol Lett 1987, 37:85–90
18. Ledda-Columbano GM, Columbano A, Dessi S, Coni P, Chiodino C, Faa G, Pani P: Hexose monophosphate shunt and cholesterogenesis in lead-induced kidney hyperplasia. Chem Biol Interact 1987, 62:209–215
19. Burton K: A study of the conditions and mechanisms of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. Biochem J 1956, 62:315–323
20. Bellairs R: Cell death in chick embryos as studied by electron microscopy. J Anat 1961, 95:54–60
21. Goldsmith M: The anatomy of cell death (abstr). J Cell Biol 1966, 31:41
22. Decker RS: Influence of thyroid hormones on neuronal death and differentiation in larval Rana Pipiens. Dev Biol 1976, 49: 101–118
23. Searle J, Lawson TA, Abbott PJ, Harmon B, Kerr JFR: An electron-microscope study of the mode of cell death induced by cancer-chemotherapeutic agents in populations of proliferating normal and neoplastic cells. J Pathol 1975, 116:129–138
24. Potten CS: Extreme sensitivity of some intestinal crypt cells to X- and $\gamma$-irradiation. Nature 1977, 269:518–521
25. Columbano A, Ledda-Columbano GM, Rao PM, Rajalakshmi S, Sarma DSR: Occurrence of cell death (apoptosis) in preneoplastic and neoplastic liver cells: A sequential study. Am J Pathol 1984, 116:441–446