

Apoptosis - A Distinctive Form of Cell Death as Biochemical, Molecular and Morphological Changes

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Abstract: Apoptosis is the genetically regulated form of cell death that permits the safe disposal of cells at the point in time when they have fulfilled their intended biological function. It is a vitally important process during normal development and the adult life of many living organisms by which the cytotoxic drugs kill tumor cells. Apoptosis or programmed cell death is of great importance in normal development, homeostasis and pathogenic processes. Apoptosis is characterized by a set of morphologic changes which include detachment of cells from their surroundings, shrinking of the cytoplasm with relative conservation of organelles, condensation of chromatin and fragmentation of the cell and nucleus into well contained fragments called apoptotic bodies. It can be considered as the process by which cells undergo as form of non-necrotic cellular suicide that permits the safe disposal of cells at the point in time when they have fulfilled their intended biological function. Apoptotic bodies arising in tissues are quickly ingested by nearby cells and degraded within their lysosomes, so there is no associated inflammation. Apoptosis involves compaction and margination of nuclear chromatin, condensation of cytoplasm and convolution of nuclear and cell outlines. At the later stage, the nuclear fragments and protuberances that form on the cell surface separate to produce apoptotic bodies which are phagocytosed by nearby cells degraded within lysosomes.

Keywords: Apoptosis, Xenobiotic, Biomarker, Phagocytosis, necrosis

1. Introduction

Apoptosis is a regulated form of cell death that can be induced or blocked by the groups of specific stimuli. Although it is a programmed process it can be induced by various stressors. Apoptosis is an active process frequently requiring new protein synthesis (Leist et al., 1995). An apoptotic index of 2 apoptotic cells per thousand over 4 d would result in a loss of about 19 percent of all cells. Therefore, it seems likely that apoptosis is a significant factor in the remodeling of the developing lung (Scavo et al., 1998). Apoptotic bodies are taken up by macrophages or neighboring cells, presumably allowing the salvage of cell components and avoidance of an inflammatory response. Apoptosis is a distinctive form of cell death whose function is the deletion of cells in normal development, embryogenesis, immune function and tissue growth but which can also be induced by pathological stimuli (Robbins et al., 1994). Professor James Carmack of the Greek department, University of Aberdeen in 1972 suggested the term apoptosis. Apoptosis is characterized by shrinkage of cells and the earliest recognized morphologic changes are condensation and segregation of the nuclear chromatin that become malignant against nuclear envelope and condensation of cytoplasm. The organelles although relatively normal are tightly packed. Condensation of cytoplasm is accompanied by the convolution of the nuclear and the cell outlines and this is followed by breaking up of the nucleus into discrete fragments that are surrounded by a double layered envelope and by the budding of the cell as a whole to produce membrane bounded apoptotic bodies. The size and composition of the latter vary considerably. Many contain several nuclear fragments whereas others lack a nuclear component. The rapid phagocytosis of apoptotic bodies before they lyse is of critical importance in preventing inflammation and injury in the tissues in which they are formed especially when the process is occurring under physiological conditions (Kerr et al., 1994). German scientist Karl Vogt was first to describe the principle of apoptosis in

1842. In 1885, anatomist Walther Flemming delivered a more precise description of the process of programmed cell death. While studying tissues using electron microscopy, John Foxton Ross Kerr at University of Queensland was able to distinguish apoptosis from traumatic cell death (Kerr, 1965). Kerr had initially used the term programmed cell necrosis, but in the article, the process of natural cell death was called *apoptosis*. Kerr, Wyllie and Currie credited James Cormack, a professor of Greek language at University of Aberdeen, with suggesting the term apoptosis.

2. Biochemical Changes of Apoptosis

It is now established that the apoptotic machinery is constitutively expressed and that survival signals suppress its activation cascade. The sequence of events involved in this apoptotic commitment appears to begin from the cytoplasm and gradually spread to the plasma membrane and nucleus. The earliest events known to occur at the onset of apoptosis is the loss of mitochondrial membrane potential, leakage of mitochondrial contents leading to decrease in intracellular pH and vacuolation (Gottlieb et al., 1996). Chromatin condensation is accompanied by cleavage of the DNA at the linker regions between nucleosomes leading to the formation of fragments that are multiples of units comprising 180-200 basepairs. These fragments are detected readily by agarose gel electrophoresis, a characteristic ladder being evident when Ethidium bromide stained gels are viewed in UV light (Kerr et al., 1994). It has been found that internucleosomal cleavage is preceded by cleavage of DNA into 300 or 50 kilobase pair fragments in cells undergoing apoptosis (Oberhammer et al., 1993). Mitochondrial DNA does not appear to be cleaved. It has been proposed that the cleavage of nuclear DNA at an early stage of the process may serve a protective function in preventing the transfer of potentially active genetic material to nearby cells when apoptotic bodies are phagocytosed (Arends et al., 1990).

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Apoptosis features high molecular-mass DNA fragmentation, abnormal chromosome condensation, a reduced membrane electric potential in mitochondria, formation of apoptotic bodies and a shift in membrane phospholipids so that anionic phosphatidylserine, which is normally located in the normal plasma membrane, become exposed to the cell membrane (Hale et al.,1996).Cell proteins involved in apoptosis include the DNA repair associated p53,Bcl-2 and its homologs (Bax,Bad,Bcl -X_L ,Bcl-X_s) the transcription factors Myc and NF κ B and numerous others(Osborne 1996 and Nagata 1997).Although the apoptotic process is heterogeneous depending upon the cell type and cycle status ,stimuli and intracellular ATP levels, there are generally recognized cellular and morphological hallmark. The most notable biochemical determinants of apoptosis and necrosis include the following: dissipation of mitochondrial transmembrane potential ,production of reactive oxygen species ,loss of ATP, levels of calcium and thiol antioxidants(McConkey 1998); and sensitivity of capsases to oxidative inactivation (Hampton and Orrenius 1997;Lemaire et al.1998).Apoptosis was evaluated by —DNA ladder” detection in agarose electrophoresis and Tdt-mediated dUTP nick end-labelling (TUNEL) assay.

3. Major Pathways of Apoptosis

The cellular signaling pathways involved in controlling the apoptosis remain poorly defined and little is known about the biochemical mechanisms underlying the dramatic changes that accompany cell death .In mammalian cells different pathways of apoptosis converge on a single mechanism of cell killing and bcl2 functions at a late common stage of cell death(Although not the only one).

- 1) **Irradiation:** Radiation is known to induce DNA damage.Ionizing radiation when given in small to moderate doses greatly enhance apoptosis. If DNA is damaged expression of p53 increases which arrests the cell cycle at G1 to allow extra time for repair .If repair fails p53 may trigger deletion of the cell by apoptosis.
- 2) **Activation by Fas/TNF:** A major pathway that triggers apoptosis in mammalian cells was discovered via the properties of Fas receptors. An antibody directed against Fas kills cells that express the protein on their surface.The reason is that the antibody-Fas rection activates which triggers a pathways of apoptosis. Fas is a cell surface receptor related to the tumour necrosis factor (TNF) receptor. Its ligand is a transmembrane protein related to TNF. There is a family of receptors related to the TNF which includes several kind of receptors found on T lymphocytes.Among the numbers of this family both Fas and TNF receptors can activate apoptosis. This pathway is triggered by a cell-cell interaction in which the ligand on one cell surface interacts with the receptors on one surface of the other cell. Both ligand and receptor have death domains .Members of the interleukin-1 β converting enzyme (ICE) family of proteases are Important downstream components of the pathway. The Fas receptor is distributed in T cells, virus infected cells, cancer cells etc. and respond to apoptotic signals by activating capsase 8.

- 3) **Attack by cytotoxic lymbocytes:** Another apoptotic pathway is triggered by cytotoxic T lymbocytes which kill target cells by a process that involve the release of granules containing serine proteases and other lytic components. One such component is perforin , which can make holes in the target cell membranes and under some conditions can kill target cell. The serine proteases in the granules are called granzymes. In the presence of perforin granzyme B can induce many of the features of apoptosis, including fragmentation of DNA.
- 4) **Withdrawal of essential growthfactors:** A number of growthfactors stimulate cell proliferation. Following are the sequence of events that characterizes normal cell proliferation.
 - a) Binding of growth factors to its specific receptor on cell membrane.
 - b) Transient and limited activation of growth factor receptor which in turn activates several signal transducing proteins on the inner leaflet of the plasma membrane.
 - c) Transmission of transduced signal across the cytosol to the nucleus via the second messengers.
 - d) Induction and activation of nuclear regulatory factors that initiates DNA transcription and ultimately cell division. Absence or withdrawal of essential growth factors may lead to lead to disruption of cell division by disturbing the above mechanism of cell proliferation.
- 5) **Glucocorticoids:**Increased levels of glucocorticoid induce apoptosis of thymocytes and a similar effect is observed with many lymphocytic leukemias and malignant lymphomas (Kerr et al,1994).

4. Spontaneous Occurrence of Apoptosis in Tumours

Apoptosis occurs spontaneously in malignant tumors to certain extend. The factors responsible for spontaneous occurrence of apoptosis in tumours are diverse. TNF alpha has been shown to induce apoptosis in vitro. So some of the apoptosis observed in tumours in vivo may be attributable to the release of this cytokine by infiltrating macrophages (Bellomo et al,1992.,Wright et al,1992). Extent of apoptosis often can be enhanced in tumors by well established treatment modalities such as irradiation, cytotoxic chemotherapy, hyperthermia etc.

- 1) **Induction of apoptosis by radiation:** Radiations in small to moderate doses induce DNA damage resulting in an increased expression of p53 protein and thus arrests the cell cycle at G1 to allow extra time for repair .If repair fails, p53 may trigger deletion of the cell by apoptosis.
- 2) **Induction of apoptosis by cancer chemotherapeutic agents:** A variety of anticancer drugs have been shown to induce apoptosis in tumours. Anticancer agents mediate their therapeutic effect by triggering apoptosis.Thus enhanced apoptosis is responsible for tumour regression by chemotherapy.
- 3) **Induction of apoptosis by mild hyperthermia:** In susceptible tissues , heating to 43⁰C for 30 minutes induces extensive apoptosis whereas heating to temperatures of 46⁰C and greater for similar periods produces necrosis.

5. Tumour Metastasis

Metastasis is the process by which tumours are spread from the primary organ, in which they arise, to the other parts of the body. To grow beyond a small sphere, tumour cells must be able to invade across a barrier called the basement membrane and into the local tissue. At this point they also must be able to produce factors that recruit new blood vessel formation (angiogenesis), which promotes the expansion of the tumour to a large size. The tumour cells that enter the new tumour blood vessels will circulate in the blood stream until they are either killed, trapped in a capillary bed in another organ or pass across the blood vessel wall (extravasate) into the tissue in a distant organ. Only a small percentage of the cells that reach the blood stream ever form metastatic colonies (as well as 1 in 100,000). In many cases the first metastasis detected in cancer patients consist of tumor cells that have spread to regional (local) lymph nodes. The changes from the normal or biological processes that indicate the risk, presence or the future behavior of the malignancy indicated as tumour malignancy. The most important predictors of survival in invasive cancer are tumour size, presence or absence of axillary lymph node metastases and histological features such as histological grade or nuclear grade. Other factors include patient's age, the growth rate of tumour, measured in terms of S phase of fraction, DNA ploidy, the occurrence of oncogene amplification, mutation of tumor suppressor genes etc (Czuba et al., 2002).

DNA cleavage is believed to be essential for the irreversible completion of apoptosis. During apoptotic cell death endonuclease activation results in the formation of high molecular weight DNA fragments and internucleosomal cleavage, sufficient to allow chromatin to collapse (Walker and Sikorska 1997). The rate of apoptosis is dictated by a balance between intracellular factors that either facilitate or delay cell death. The smaller DNA fragments are separated by size using agarose gel electrophoresis in the presence of constant electrical field, leading to ladder formation. The higher molecular weight fragments associated with early DNA cleavage may be separated by pulse field electrophoresis (Spector et al., 1997). The toxic induced (eg. mercury, DDT etc.) laddering pattern has been extensively described in a variety of mammalian cell types (Rossi et al., 1997, Renvoize et al., 1998, Tebourbi et al., 1998). It is important to note that not all cell systems undergoing apoptosis produce endonucleolytic cleavage and DNA laddering (Leist and Nicotera, 1997). Studies on one single compound like DDT sometimes wrongly gives negative results while multiple chemical exposure reveals adduct formation (Lebailly et al., 1998) and cancer initiation (Sellers, 1997). Their ability to survive stress is usually the result of possession and selective expression of certain novel genes. Information and regulation of such genes is an important pre-requisite for devising future strategies aimed at building stress tolerant bacteria and crop plants (Bohert and Jensen 1996).

6. Molecular, Cellular and Morphological Hallmarks of Apoptosis

Stages	Events
Early	Protease activation (eg. caspases) in the nucleus and cytoplasm; disruption of mitochondrial transmembrane potential
Intermediate	Calcium flux; actin cleavage; loss of intercellular junctions and surface extensions; membrane asymmetry (eg. phosphatidylserine externalization); loss of cellular potassium and water; intracellular acidification
Late	Nuclear chromatin coalescence; endonuclease activation; DNA fragmentation; membrane bound apoptotic body formation

(Leonard, 1999)

Apoptosis can be triggered by agents that can penetrate the cell directly, and modulate the apoptotic cascade in the absence of specific cell surface receptors. Examples of such agents include heat shock/stress factors (Thompson, 1995), free radicals (Buttke, 1994), Ultraviolet radiation (Yamada, 1988), numerous drugs. Drug related hepatotoxicity is an important cause of morbidity and mortality and indeed is the most common reason for withdrawing new drugs and synthetic peptides (Schwall et al, 1993), toxins (Sachs, 1993), and potent lymphocyte enzymes (granzymes) (Hayes et al, 1989). Other mechanisms are dependant on expression of appropriate cell surface receptors. Drugs and other exogenous compounds may affect the liver in various ways. Chemical agents that used in laboratories and industries, natural chemicals and herbal remedies can induce hepatotoxicity (Bhaskar, 2016b). The hepatotoxicity of therapeutic agents and pharmaceutical chemicals has become an area of intense research interest (Bhaskar, 2015). In the case of severe toxicity the patient may develop liver failure. Cytotoxic injury resembles acute hepatitis and is characterized by damage to the hepatocytes with prominent elevation of amino transferase (Bhaskar, 2012). Idiosyncratic reaction is attributable to pharmacogenetic differences between individuals (genetic polymorphism in the metabolism of compounds). In the case of severe toxicity the patient may develop liver failure (Bhaskar, 2016a).

Subsequently, numerous mechanisms of induction of genetic instability have been identified :-

- 1) Template DNA: exogenous genotoxic 'adducts' (see above); depurination, deamination and oxidation; alkylation.
- 2) Nucleotides: imbalanced concentrations; inappropriate analogues and enzymes normally degrading these.
- 3) Error-prone DNA polymerases (including proofreading and MMR) and accessory proteins.
- 4) Presence of DNA polymerases which synthesize across abnormal templates ('translesional synthesis') so that the usual arrest of synthesis does not occur (Kunkel et al., 2000)
- 5) Strand misalignments.
- 6) Abnormalities of DNA repair enzymes.
- 7) Abnormalities of cell cycle checkpoints (Cahill, et al. 1998)

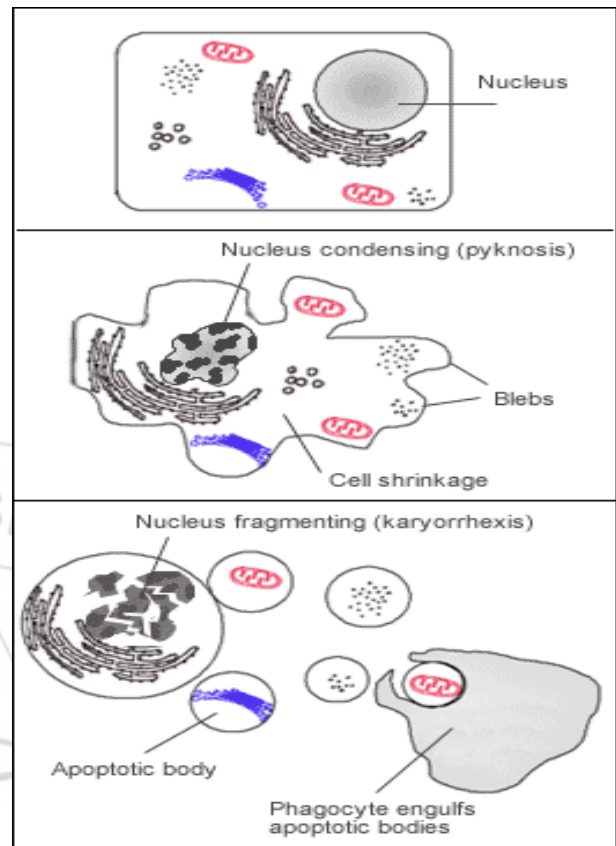
- 8) Mechanisms affecting chromosomal integrity and ploidy (Miyagawa et al ;1998.,Jones.,1999).
- 9) Epigenetic mechanisms, including DNA methylation (Jones .,1999).
- 10) Telomerase dysfunction
- 11) Other mechanisms.

Not all carcinogens are mutagens, and many mutagens are not carcinogens. Among related chemicals, small changes of structure can markedly influence carcinogenic potency. Many tumours are genetically unstable, but some, especially benign types, rarely exhibit progression or show other evidence of genetic instability. Cells of particular tumor types exhibit identifiable particular sets of phenotypic abnormalities (e.g. rapid growth, uniform nuclei, little cytoplasm and occasionally production of adrenocorticotrophic hormone by anaplastic small-celled carcinoma of the bronchus). A possible mechanism, which might explain these phenomena is carcinogen-induced reduction of fidelity of replication of DNA polymerase complexes during S phase of normal tissue stem cells. A single hit by a reactive agent (chemical or physical) on one of the major enzyme-sites (synthesis, proofreading, mismatch repair—MMR) could cause multiple sequence abnormalities in the length of DNA synthesized by one DNA polymerase complex. Because this length of DNA (half a replication bubble) averages 15 000–150 000 nucleotides, the affected DNA could include two or more significant genomic elements (genes, especially for tumour suppression, regulatory loci and other elements). The particular mutant elements in the affected DNA could then determine the set of phenotypic abnormalities exhibited by a resulting tumour. Non-genotoxic carcinogenicity, non-carcinogenic mutagenicity, structure-dependent chemical carcinogenicity and the phenomenon of sets of phenotypic abnormalities could thus be accommodated. In experimental studies, the hallmark pattern of mutation caused by this mechanism would be multiple mainly point mutations clustered within the length of half a replication bubble. Such a hallmark pattern of mutation might be detectable in carcinogen-treated cell cultures by the use of cycle-synchronized cultures, single cell sub-culturing, restriction (endonuclease) fragment length analysis of the clones and nucleotide sequencing of abnormal bands for localization in the human genome.

In human tumour cells, the hallmark pattern of mutations may be demonstrable in genetically stable human tumours, but might well be lost or obscured by secondary mutations in genetically unstable tumors. Among different cases of the same type of human tumour, the clustered point mutations might be tumor-type specific in their location in the genome, but vary case-to-case in the precise points mutated in the cluster region. New assays for assessing the carcinogenic potential of environmental and synthetic substances for human and animal populations may result. The hypothesis is not put forward to the exclusion of some established mechanisms of carcinogenesis for particular human tumors: for example, the two-hit mutational hypothesis for retinoblastoma, the multiple sequential mutational hypothesis for UV-induced lesions of the epidermis, and the possibility of adduct-induced frameshift mutations by some

chemical carcinogens for experimental tumors (Bignold, 2004).

7. Activation Mechanisms



8. Control of the Apoptotic Mechanisms

The initiation of apoptosis is tightly regulated by activation mechanisms, because once apoptosis has begun, it inevitably leads to the death of the cell. The two best-understood activation mechanisms are of the intrinsic pathway (also called the mitochondrial pathway) and the extrinsic pathway. The *intrinsic pathway* is activated by intracellular signals generated when cells are stressed and depends on the release of proteins from the intermembrane space of mitochondria. The *extrinsic pathway* is activated by extracellular ligands binding to cell-surface death receptors, which leads to the formation of the death-inducing signaling complex (DISC). A cell initiates intracellular apoptotic signaling in response to a stress, which may bring about cell suicide. The binding of nuclear receptors by glucocorticoids, heat, radiation, nutrient deprivation, viral infection, hypoxia and increased intracellular calcium concentration, for example, by damage to the membrane, can all trigger the release of intracellular apoptotic signals by a damaged cell. A number of cellular components, such as poly ADP ribose polymerase, may also help regulate apoptosis. Before the actual process of cell death is precipitated by enzymes, apoptotic signals must cause regulatory proteins to initiate the apoptosis pathway. This step allows those signals to cause cell death, or the process to be stopped, should the cell no longer need to die. An extrinsic pathway for initiation identified in several toxin studies is an increase in calcium concentration within a cell caused by drug activity, which

also can cause apoptosis via a calcium binding protease calpain

9. Methods for distinguishing apoptotic from necrotic (necroptotic) cells

In order to perform analysis of apoptotic versus necrotic (necroptotic) cells, one can do analysis of morphology by time-lapse microscopy, flow fluorocytometry, and transmission electron microscopy. There are also various biochemical techniques for analysis of cell surface markers (phosphatidylserine exposure versus cell permeability by flow fluorocytometry), cellular markers such as DNA fragmentation (flow fluorocytometry), caspase activation, Bid cleavage, and cytochrome c release (Western blotting). It is important to know how primary and secondary necrotic cells can be distinguished by analysis of supernatant for caspases, HMGB1, and release of cytokeratin 18. However, no distinct surface or biochemical markers of necrotic cell death have been identified yet, and only negative markers are available. These include absence of apoptotic parameters (caspase activation, cytochrome c release, and oligonucleosomal DNA fragmentation) and differential kinetics of cell death markers (phosphatidylserine exposure and cell membrane permeabilization). A selection of techniques that can be used to distinguish apoptosis from necroptotic cells (Krysko *et al*, 2008., Krysko *et al*, 2008).

Differential features and significance of necrosis and apoptosis
Morphological features
Loss of membrane integrity - Membrane blebbing, but no loss of integrity Aggregation of chromatin at the nuclear membrane Begins with swelling of cytoplasm and mitochondria - Begins with shrinking of cytoplasm and condensation of nucleus Ends with total cell lysis - Ends with fragmentation of cell into smaller bodies No vesicle formation, complete lysis - Formation of membrane bound vesicles (apoptotic bodies) Disintegration (swelling) of organelles - Mitochondria become leaky due to pore formation involving proteins of the bcl-2 family.
Biochemical features
Loss of regulation of ion homeostasis No energy requirement (passive process, also occurs at 4°C) Tightly regulated process involving activation and enzymatic steps Energy (ATP)-dependent (active process, does not occur at 4°C) Random digestion of DNA (smear of DNA after agarose gel electrophoresis) Non-random mono- and oligonucleosomal length fragmentation of DNA (Ladder pattern after agarose gel electrophoresis) Postlytic DNA fragmentation (= late event of death) - Prelytic DNA fragmentation Release of various factors (cytochrome C, AIF) into cytoplasm by mitochondria Activation of caspase cascade Alterations in membrane asymmetry (i.e., translocation of phosphatidylserine from the cytoplasmic to the extracellular side of the membrane)
Physiological significance

Affects groups of contiguous cells
 Evoked by non-physiological disturbances (complement attack, lytic viruses, hypothermia, hypoxia, is chemical ,metabolic poisons)
 Phagocytosis by macrophages
 Significant inflammatory response
 Affects individual cells
 Induced by physiological stimuli (lack of growthfactors , changes in hormonal environment)
 Phagocytosis by adjacent cells or macrophages
 No inflammatory response

10. Conclusion

Apoptosis is not merely a physiological mode of cell death as it can be induced in a pathological manner by environmental chemical toxicants, depending upon chemical species, exposure level and duration, receptor sites and energy supply of the cell. Apoptosis refers to the constitutively expressed elements of signalling pathways that control the execution of cell death which are present in essentially all cells. Apoptotic death is characterized by cellular changes such as cytoplasm shrinkage, chromatin condensation, and plasma membrane asymmetry. This form of cell suicide is appealing as a general biomarker of response in that it is expressed in multiple cell systems (eg. immunoneuronal, hepatal, intestinal, dermal, reproductive), is conserved phylogenetically (eg. fish, rodents, birds, sheep, amphibians, round worms, plants, humans), is modulated by environmentally relevant levels of chemical contaminants and indicates a state of stress of the organism. The liver undergoes dramatic changes in structure and function during development. The developmental changes that occur in the liver determine the rate and metabolic pathways used in the disposition of drugs and other xenobiotic (Bhaskar 2015). Apoptosis has been reported to be a self protective phenomenon of organisms in the face of complex pathophysiological conditions during sepsis, allowing the body to down regulate the intense inflammatory response caused by necrosis. However most studies have suggested that extensive apoptosis does harm to general physiological functions of tissues or organs. Apoptosis is a sensitive early indicator of acute and chronic chemical stress, loss of cell function and structure and organismal health (Leonard *et al*, 1999). Apoptotic cells could be detected and visualized by fluorescent emission to detect DNA fragmentation which is the biochemical characteristic of apoptosis.

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