THE MECHANISM OF CELL DEATH INDUCED BY NECROTIC WAVE DURING ANTHRANILATE FLUORESCENCE IN *Caenorhabditis elegans*

A DISSERTATION SUBMITTED

BY

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FOR THE AWARD OF

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SREE CHITRA TIRUNAL INSTITUTE FOR MEDICAL SCIENCE AND TECHNOLOGY

THIRUVANANTHAPURAM - 695011

DECLARATION

I, **Cuckoo Teresa Jetto,** hereby declare that I had personally carried out the work depicted in the thesis entitled, "The mechanism of cell death induced by necrotic wave during anthranilate fluorescence in *Caenorhabditis elegans*" under the direct supervision of **Dr Anoopkumar Thekkuveetil, Scientist F and Head, Molecular medicine division**, Biomedical Technology wing, Sree Chitra Tirunal Institute for Medical Science and Technology, Thiruvananthapuram, Kerala, India.

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CERTIFICATE

This is to certify that the dissertation entitled "The mechanism of cell death induced by necrotic wave during anthranilate fluorescence in *Caenorhabditis elegans*" is a bonafide work done by **Ms. Cuckoo Teresa Jetto** in partial fulfillment for the degree of Master in Philosophy under my supervision and guidance at **Molecular Medicine Division**, Biomedical Technology wing, Sree Chitra Tirunal Institute for Medical Science and Technology, Thiruvananthapuram, Kerala, India.

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The Thesis entitled

"The mechanism of cell death induced by necrotic wave during anthranilate fluorescence in Caenorhabditis elegans"

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For the degree of

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INTRODUCTION

1.1 BACKGROUND

A cell is the basic structural and functional unit of all living organisms that can replicate autonomously and is often called as the building blocks of life. They can exist either as a functionally independent unit of life in the case of unicellular organism, or as a sub-unit in the case of multicellular organism, which is specialized to carry out particular functions in the organism. Cells are composed of various molecules such as DNA, RNA, proteins, lipids, glycans etc. that have definite biological functions and properties.

The cells in a multicellular organism are highly organized and the number of cells in an organism is tightly regulated. This regulation is brought about not only by controlling the rate of cell division in an organism, but also by controlling the rate of cell death. For example, in the case of developing neurons half to two-thirds of the neurons will die before the completion of development. Likewise large numbers of T- and B-lymphocytes also undergo developmental selection (Lockshin and Zakeri, 2007). Cell death can also be seen in almost all other developing organs such as the cornea and interdigital tissues of the limbs of amniotes that do not have webs.

Cell death in an organism can be classified as apoptotic, necrotic and autophagic. These classifications are on the basis of structural changes during cell death as well as involvement of nucleases or that of distinct classes of proteases, such as caspases, calpains, cathepsins etc.

1.1.1 Apoptosis:

Apoptosis is the major mechanism of regulated cell death that has been studied extensively during the past few decades (Nikoletopoulou et al., 2013). Apoptosis, otherwise known as programmed cell death, comes into play not only during cell damage or stress, but also in normal development of an organism and its morphogenesis.

The various morphological characteristics of apoptosis include rounding-up of the cell, reduction of cellular volume (pyknosis), condensation of chromatin, fragmentation of nucleus(karyorrhexis), little or no ultrastructural modifications of cytoplasmic organelles, plasma membrane blebbing and finally engulfment by resident phagocytes (Kroemer et al., 2009). Apoptosis could be triggered by two ways: one, by extrinsic stimuli through cell surface death receptors, like TNFα (tumor necrosis factor-α), TRAIL (TNF related apoptosis inducing ligand) receptors and Fas (CD95/APO1) and second, by intrinsic stimuli through the mitochondrial signaling pathway. (Adams, 2003). In both cases, there is activation of a distinct set of cysteine aspartyl proteases, called caspases, which results in permeabilization of mitochondrial membrane, chromatin condensation and fragmentation of DNA, which ultimately leads to the destruction of the cell (Green, 2005).

1.1.2. Necrosis

Necrosis is considered as one of the main caspase-independent cell death pathway and is morphologically very distinct from that of apoptosis. The morphological characteristics of necrotic cell death include gain in cell volume (oncosis), extensive swelling of cell and its

organelles, rupturing of plasma membrane and loss of intracellular contents which very well contrast with that of apoptosis (Kroemer and Martin, 2005).

Cells usually undergo necrosis when they are exposed to excessive stress and adverse environmental conditions like hypoxia or lack of essential nutrients (eg. ischemia during a heart attack or following stroke), elevated temperature, exposure to toxic chemicals and also excessive mechanical strain as in the case of trauma (Nicotera et al., 1999). Necrotic cell death can be also caused by various genetic abnormalities such as deleterious gene mutations in the case of neurodegenerative disorders. Since necrosis has been associated with extensive cell loss it is accompanied by various human pathological conditions like stroke, heart diseases and several neurodegenerative diseases such as Huntington's disease, Alzheimer's disease, epilepsy, Parkinson's disease and amyotrophic lateral sclerosis (Stefanis, 2005).

For a long time, necrosis has been regarded as an accidental and uncontrolled form of cell death, but now the scenario has changed. Lot of evidences are now accumulating which points out that the execution of necrotic cell death pathway maybe a finely regulated one which is controlled by a set of signal transduction pathways and catabolic mechanisms (Festjens et al., 2006). However, it is still not fully understood how various mediators, organelles and cellular processes interact with each other and bring about necrotic cell death. Major organelles that undergo alterations during this process are mitochondria(for example, uncoupling, production of reactive oxygen species, mitochondrial membrane permeabilization etc), lysosomes(for example lysosomal membrane permeabilization and ROS production by Fenton reactions), nucleus (for example,. hyperactivation ofPARP-1 and

concomitant hydrolysis of NAD+) etc. (Kroemer et al., 2009). Major biochemical changes involved are, lipid degradation (due to activation of phospholipases, sphingomyelinases and lipoxygenases), increase in the level of cytosolic concentration of calcium (Ca2+) that in turn result in mitochondrial activation and thereby the activation of non-caspase proteases like calpains and cathepsins (Nicotera and Melino, 2004). During several instances , a crucial role for the serine/threonine kinase RIP1 has also been established (Festjens et al., 2007).

Since necrosis has a role in many important human diseases like neurodegenerative diseases, stroke etc, understanding various biochemical events that occur during the cell death will give new insights to identify potential targets for effective pharmacological interventions.

1.1.3 Autophagy

A cellular process, which is involved in both major types of cell death i.e apoptosis and necrosis is autophagy. There are three types of autophagy: microautophagy, chaperone-mediated autophagy and macroautophagy (Klionsky and Emr, 2000). Macroautophagy is a self-cannibalization mechanism in which the cytoplasmic material and intracellular organelles are engulfed within double-membrane vesicles, called autophagosomes. Once the autophagosome are formed it will fuse with a lysosome to form autolysosome, where the material inside is degraded with the help of specific acidic hydrolases (Mizushima et al., 2002). Initially autophagy was considered to have cytoprotective function but now evidences show that it can also promote cell death during normal development process as well as during diseased conditions.

A low level of constitutive autophagy has also got an important housekeeping role in maintaining the normal turnover of proteins with longer life and whole organelles, which thereby are important for maintain the cells in a healthy state. This homeostatic role of autophagy is mainly very important in post-mitotic differentiated cells like cardiomyocytes and neurons.

Autophagy is activated in response to conditions like starvation, nutrient deprivation, hypoxia, growth factor depletion etc. As a consequence of this, there is an enhancement in the degradation of cytoplasmic components which in turn promotes the survival in the stressed condition. Excessive autophagy may turn out to be harmful (Baehrecke, 2005).

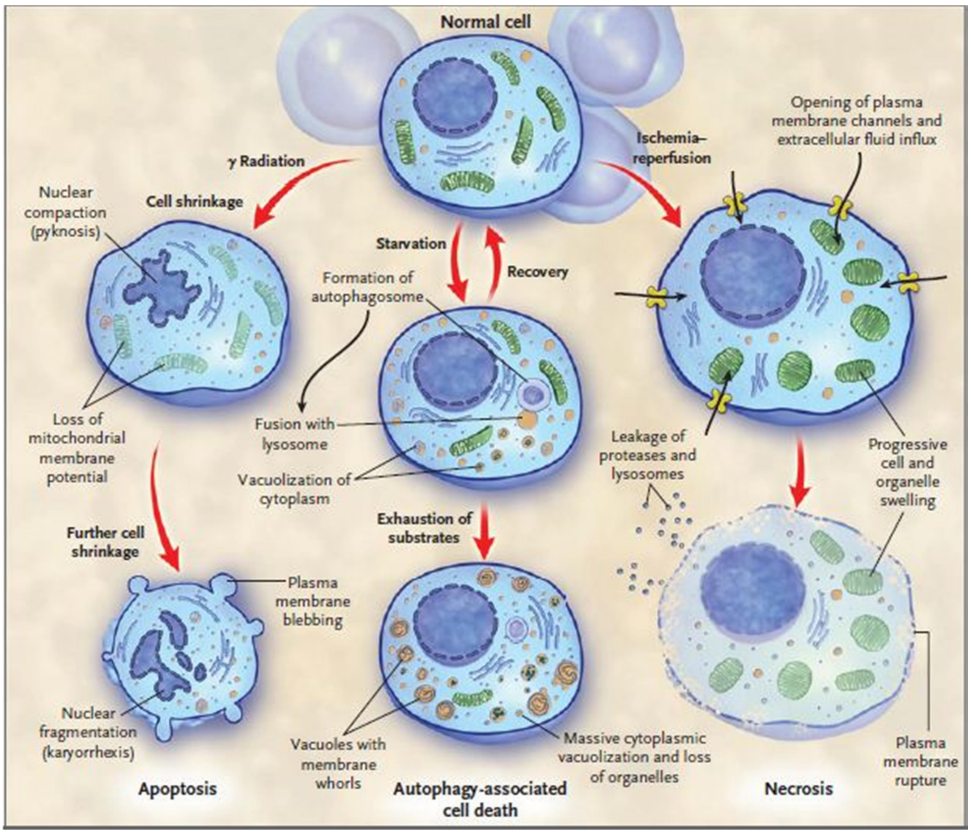


Fig. 1 Classification of cell death pathways.(Hotchkiss et al., 2009)

1.1.4 Cell death in *Caenorhabditis elegans*

As understanding the molecular mechanisms and biochemical pathways involved in cell death is important in studying various human pathological conditions, selection of a suitable model organism is a very crucial factor. Simple model organisms are now becoming increasingly important to study various aspects of biology including the molecular and biochemical mechanisms involved in cell death pathways. *Caenorhabditis elegans* is one of the most powerful model organism used in research and is an ideal model for studying cell death pathways. It is a transparent soil nematode with a relatively simple anatomy and short life cycle. The total number of cells in *C.elegans* is 959, which include 302 neurons thereby forming a simple nervous system.

C.elegans is found to be an excellent model to study the molecular and biochemical aspects of major cell death pathways and thereby can be used to investigate the mechanisms involved in human diseases related to cell death. During embryonic development and the L2 larval stage in *C.elegans* 131 cells in the organism undergo apoptosis (Kimble and Hirsh, 1979). They are derived mainly from neuronal lineages but they also include muscle cells, cells of the hypodermis and pharynx. In *C. elegans* about 50% of oocytes generated in the mature gonads are also destroyed through apoptotic pathway(Gumienny et al., 1999).This helps to maintain homeostasis in germline, thereby limiting the number of oocytes which compete for nutrients in the gonad. Apoptosis is also activated as part of the innate immunity response in the organism following the pathogenic invasion of microorganisms, and also in response to various DNA damaging agents ,toxic ionizing radiation and some specific cancer drugs(van Haaften et al., 2006).

Various studies in *C. elegans* have also provided novel insights on the physiological roles of autophagy in developmental processes as well as in tissue remodeling. In *C.elegans* autophagy is triggered as a response to various intracellular and extracellular stress conditions like starvation, accumulation of damaged cytoplasmic components etc. Dauer formation in *C.elegans* in response to stress conditions also requires upregulation of autophagy. Furthermore autophagy also has a role in the regulation of inhibitory synapse strength and thus, maintaining a balance between excitatory and inhibitory inputs in nematodes(Kroemer and Martin, 2005).Thus *C. elegans* is found to be a good model to investigate some poorly understood aspects of apoptosis, like caspase substrate specificity, the contribution of cell-corpse clearance to the execution of death etc. and importance of autophagy pathway.

In *C.elegans* several genetic as well as environmental insults like severe deprivation of oxygen and energy sources, exposure to toxins and elevated temperature trigger necrosis(Kourtis and Tavernarakis, 2007). Necrotic neuronal death in *C.elegans* can be triggered by different extrinsic and intrinsic signals. It is mainly studied via the expression of ion channels that bears a hyperactive mutation(Syntichaki and Tavernarakis, 2003). In *C.elegans* the most widely studied necrotic cell death is the one which is induced by hyperactive *deg-1*(d) (*deg*enerin) and *mec-4*(d) (*mec*hanosensory) both of which carries dominant mutations and causes necrosis in special neurons of *C. elegans*(Chalfie and Wolinsky, 1990).

Gain of function mutation in *deg-1* induce necrosis in a group of interneurons of the posterior touch sensory circuit whereas the gain of function mutations in *mec-4* induce necrosis in the six touch receptor neurons (Syntichaki and Tavernarakis, 2004). Both these genes have a property to induce cell degeneration when mutated to a hyperactive form and dying cells show the typical morphological characteristics of that of a necrotic cell death. Cell death is mainly caused by the ionic imbalance due to the mutated ion channels. This ionic imbalance followed by cell death which is induced by mutant degenerins show high resemblance of excitotoxicity in vertebrates. In excitotoxicity the presynaptic neuron membrane potential collapse due to energy depletion which results in the increased release of excitatory neurotransmitter glutamate into the synaptic cleft, which in-turn cause hyper-excitation and necrotic cell death of postsynaptic neurons. Excitotoxicity is one of the major mechanisms involved in neuronal death during stroke. Thus degenerin-induced neuronal death in *C. elegans* has been used as a model to study excitotoxicity and to investigate the molecular mechanisms of neurodegeneration(Kroemer and Martin, 2005).

In *C.elegans* necrotic cell death can also be triggered by various other conditions like constitutive activation of the GTP binding protein Gas, chemicals that inhibit respiratory chain (e.g., NaN3),hypoxic treatment, toxins, macromolecular damage due to radiation etc. These inducers are used in genetic and molecular studies so as to understand the key mechanisms involved in necrotic cell death (Kourtis and Tavernarakis, 2007).

Intestine is the largest somatic organ in *C.elegans*. During death, intestine undergoes a stereotyped process of self-destruction which involves an intra- and intercellular cascade of

cellular necrosis and the mechanisms that are involved is similar to those which are active in the propagation of cellular necrosis in mammals. An important feature of intestinal necrosis in *C elegans* is that it originates in the anterior int1cells. This implies that these cells have an unusual vulnerability to necrotic cell death and can be analogous to localized failure (e.g., in the heart or kidneys) in humans(Coburn et al., 2013).

In *C.elegans* the organismal death is accompanied by a burst of intense blue fluorescence, which is believed to be generated within intestinal cells by the calpain-cathepsin necrotic cell death pathway. Calcium found to be essential for organismal propagation of blue flurescence, probably via the innexin INX-16. The death fluorescence marks an anterior to posterior wave of intestinal cell death and is accompanied by cytosolic acidosis(Coburn et al., 2013). Inhibiting the components of necrotic pathway can bring about a delay in stress-induced death. The predictability of organismal death in *C. elegans* and the blue fluorescence of intestine as a marker for cell death make it an excellent model to study the pathways involved in cell death during organismal death.

1.2 REVIEW OF LITERATURE

Living cells show a remarkable diversity in its form as well as in its function. Cells from one type of tissue can be completely different from that of another tissue type. This type of difference can be seen even within cells of a tissue type in same genus of a specific organism. Despite this fact, cells of an organism exhibit some unifying characteristics as well. Every cell is essentially a collection of its functional parts called organelles suspended in a liquid medium called cytoplasm, which altogether enclosed in a semi permeable membrane called cell membrane. The presence and proportions of the organelles which is present inside cells will vary with different functions a cell has to perform.

As cells are active participants in the environment around them, they have to constantly adjust their structure as well as function to put up with the changing demands and extracellular stresses. So a living cell in an organism exists as a dynamic chemical system. For a cell to grow or to form a new cell, it has to take in free energy from the environment, and also from raw materials, to drive the synthetic reactions necessary for this process. This consumption of free energy is very vital for the life. So when it stops, a cell will decay towards the chemical equilibrium and will ultimately die.

1.2.1 CELL DEATH PATHWAYS

Cell death plays an important role in all multicellular organisms during their early development i.e. by sculpting the body parts as well as during their adult life by maintaining

cellular homeostasis. The human body is composed of approximately 10^{14} cells and billions of cells die every day in order to make functionality of the whole organism secured(Raff, 1992). Thus in an organism cell division balances the cell death thereby maintaining the supply of cells that can be mobilized readily when a need comes. Cell death further has a protective role in an organism by removing the cells that are damaged by various disease conditions, aging, genetic mutation, infection and exposure to different toxic agents.

As cell death is closely linked to tissue homeostasis the disruption of cell death is an important cause for numerous pathological conditions. Approximately half of all medical illnesses are caused either due to low or high level of cell death and in most of the cases there is no adequate therapy. Abnormalities in cell death regulation has an important role in various diseases like cancer, autoimmune syndromes, ischemia, AIDS, liver diseases, neurodegenerative disorders including Alzheimer's and Parkinson's disease (Fischer and Schulze-Osthoff, 2005). So considerable interest has now emerged in devising various therapeutic strategies that aims at modulating these cellular life-and-death decisions in an organism.

The three types of cell death mechanism in mammalian cells that is distinguished according to morphological criteria (Kroemer et al., 2009) are as follows.

Type I cell death, also called apoptosis is defined by characteristic changes in the morphology of nucleus, including condensation of chromatin and its fragmentation, overall shrinkage of cell, blebbing of the plasma membrane and formation of apoptotic bodies that contain the nuclear or cytoplasmic material.

Type II cell death, also called autophagic cell death is characterized by massive accumulation autophagosomes, a double-membrane containing vacuoles, which then fuse with lysosome vacuoles.

Type III cell death, also called as necrosis, is often defined in a negative manner, as a cell death mechanism that lacks the characteristics of both type I and type II cell death.

Distinction of different cell death modes have various important clinical implications when a new potential therapeutic targeting of cell death processes is considered. However, in some conditions these distinct cell modalities may only be present in the extremes and there are various examples in which cell death demonstrates a continuum of intermediate features, for instance in the case of both necrosis and apoptosis. Additionally, in some cases the cell death mechanism is determined by the type of the cell or the nature and duration of cellular injury. Therefore, in many situations the cell death in an organism may not occur as a clear-cut and paradigmatic form as expected.

1.2.1. a. APOPTOSIS

Apoptosis is an evolutionarily conserved cell death pathway in multicellular organisms, which help them to maintain tissue homeostasis during development and differentiation. It occurs in a well regulated sequence of morphological events. The term ‘apoptosis’ has been coined by Kerr *et al.* , to describe a cell death process with specific morphological characteristics(Kerr et al., 1972). The word ‘apoptosis’ comes from the ancient Greek, meaning ‘falling off ’ and it refers to the morphological aspect of the formation of apoptotic bodies during apoptosis. The morphologic features of apoptosis is caused mainly due to the

activation of a type of cysteine proteases called caspases, by either death receptor ligation or through the release of apoptotic mediators from the mitochondria (Taylor et al., 2008). Death by apoptosis is an energy requiring process, hence utilizes energy in the form of ATP.

As mentioned earlier, in nematodes, insects as well as in human cells, most of the morphological changes during apoptosis are caused due to the activation of an evolutionarily conserved and unique class of intracellular proteases called caspases. Apoptosis sets in due to various events like disintegration of the cellular infrastructure due to proteolytic digestion, which in turn leads to dissolution of cytoskeleton, metabolic dearrangement and fragmentation of the genome(Taylor et al., 2008). The different members of caspase family play an important role in apoptosis and are involved in the different phases of the pathway i.e. initiation phase, execution phase and the regulatory phase. Each caspase is maintained in an inactive zymogen form that consists of a prodomain which is followed by two subunits with the catalytic domain. Once activated caspases cleave around 400 substrates, that includes proteins involved in scaffolding of the cytoplasm, signal transduction, cell cycle-controlling and DNA replication and repair(Festjens et al., 2006) Caspases also destroy several proteins which is required for maintenance of the cytoskeletal architecture, e.g. intermediate filament cytokeratin-18.

The activation of caspases is initiated mainly by two mechanisms: one extrinsic death receptor pathway and second is the intrinsic mitochondrial pathway (Figure 2). Death receptor mediated apoptosis plays an important role in the maintenance of tissue homeostasis

whereas the mitochondrial pathway plays a major role in response of cell to extracellular cues and damages occurring inside the cell like DNA damage.

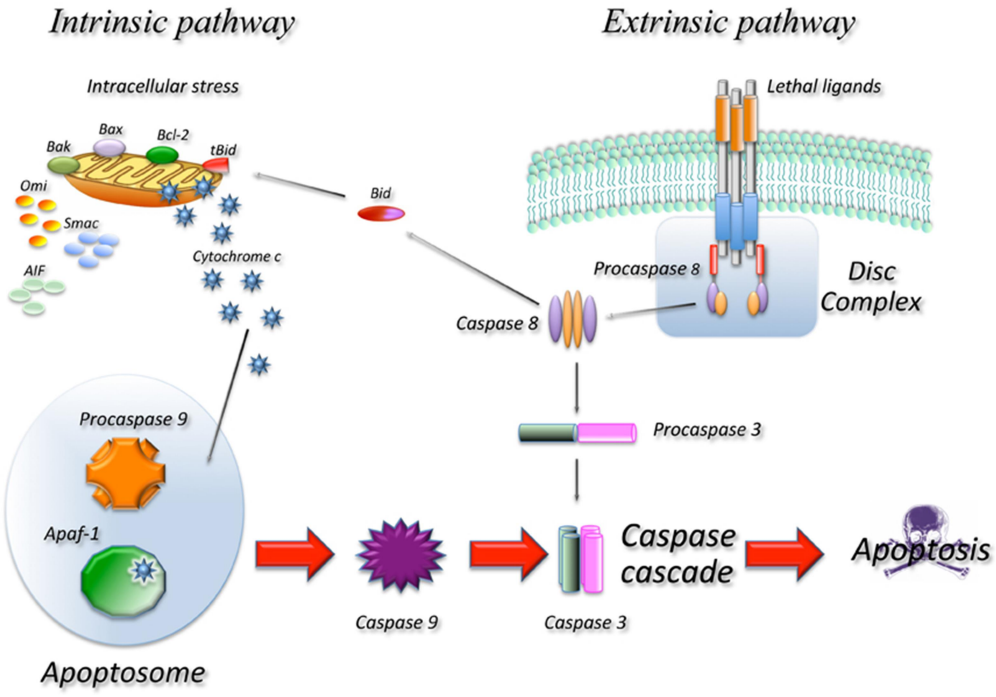


Fig. 2 Intrinsic and extrinsic pathways of apoptosis (Adapted from Favaloro et al., 2012)

Death receptors form a subgroup of the tumor necrosis factor (TNF) receptor superfamily which includes TNF-R1, CD95 (Fas/APO-1) and receptors which binds to the TNF-related apoptosis-inducing ligand (TRAIL). All death receptors have an intracellular motif, called death domain, which has an important role in transmitting the apoptotic signal. When a ligand binds to the receptors, they interact via their death domain with a corresponding protein motif in adapter proteins like FADD. These adaptor proteins in turn bind to prodomain of initiator caspase 8 forming death-inducing signaling complex (DISC). The

assembly of DISC is followed by activation of caspase8 which in turn activates other effector caspases and further proceeds to apoptosis (Debatin and Krammer, 2004), (Peter et al., 2007, p. 95).

Most cell death mainly proceeds via the mitochondrial pathway, which integrates various signals generated by a range of stressors including DNA damage, loss of adhesion, growth factor withdrawal etc (Green and Kroemer, 2004). The key event of this pathway is the release of cytochrome c from the mitochondrial intermembrane space into the cytosol in response to the various apoptosis stimuli. In apoptotic cells cytochrome c serves as a cofactor for the adapter protein Apaf-1. The binding of cytochrome c helps in the oligomerization of Apaf-1which then recruits the initiator caspase-9 for triggering the formation of the apoptosome and subsequently triggers the caspase cascade and apoptosis.

1.2.1.b. NECROSIS

Necrosis is one of the major caspase-independent cell death pathways which is morphologically different from that of apoptosis. The word necrosis is derived from the Greek term “necros,” meaning “dead” and it was traditionally regarded as the chaotic breakdown of the cell. Unlike apoptosis, necrotic cell death was thought to be a passive process that does not require any energy or synthesis of new proteins or specific regulatory mechanisms(Syntichaki and Tavernarakis, 2002). But recent findings in *Drosophila* and *C. elegans* bring a change in this simplistic view about necrotic cell death (Kourtis and Tavernarakis, 2007).

Initially the term *necrosis* was used to refer to a series of changes that accompany cell death that is largely resulting from the degradative action of enzymes on cells which are lethally injured. Since necrotic cells were unable to maintain the integrity of the cell membrane, their contents often leak out. The enzymes which are responsible for digestion of the cell are either derived from the lysosomes of the dying cells or from the lysosomes of leukocytes which are recruited to that area as part of the inflammatory reaction.

When observed through an electron microscopy, necrotic cells showed various characteristics like discontinuities in plasma membrane and organelle membranes, marked dilation of mitochondria along with the appearance of large amorphous densities, intracytoplasmic myelin figures, lysosome disruption and nuclear changes that ultimately results in nuclear dissolution.

Nuclear changes that occur during necrosis show one of three patterns all due to breakdown of DNA and chromatin in the cell. In first pattern the basophilia of the chromatin may fade i.e **karyolysis**, which most probably occur secondary to the activity of deoxyribonuclease (DNase). The second pattern is **pyknosis,** which is characterized by shrinkage of nucleus and increased basophilia. Here the DNA condenses into a solid shrunken mass. In the case third pattern, i.e. **karyorrhexis,** the fragmentation of the pyknotic nucleus takes place within 1 to 2 days, and the nucleus in a dead cell will disappear completely(Robbins and Kumar, 2010).

There are different morphologically distinct patterns of tissue necrosis, which are found in clinical practice and provide clues about the underlying cause of the necrotic cell death as described by Robbins and Cotran in the book Pathologic Basis of Disease. They are:

Coagulative necrosis is a form of tissue necrosis in which the basic architecture of the tissue is preserved for at least several days even after the death of the component cells. The affected tissues will form a firm texture. It is assumed that the injury denatures the structural proteins as well as the enzymes thereby blocking the proteolysis of the dead cells. As a result of this, the eosinophilic, anucleate cells may persist for days or weeks even after death. Ultimately, these necrotic cells are removed by phagocytosis by infiltrating leukocytes and by the action of lysosomal enzymes of the leukocytes on dead cells. Coagulative necrosis is important characteristic of **infarcts** (areas of ischemic necrosis) in all solid organs except that of brain.

Liquefactive necrosis is a type of necrosis seen in focal bacterial or occasionally in some fungal infections, because the microbes stimulate the accumulation of inflammatory cells and the enzymes of the infiltrating leukocytes will digest or "liquefy" the tissue. Hypoxic death of cells that occur within the central nervous system often evokes liquefactive necrosis. No matter what the pathological condition is in liquefactive necrosis, liquefaction completely digests the dead cells, which results in the transformation of the tissue into a liquid viscous mass.

Gangrenous necrosis is not a distinctive pattern of cell death, but still a commonly used term in clinical practice. This term is usually applied to limb, usually the lower leg that has lost the supply of blood and has undergone the coagulative necrosis process that involves

multiple tissue layers. When a bacterial infection is superimposed to this condition, coagulative necrosis gets modified by the liquefactive action of the bacteria and the leukocytes attracted to the site.

Caseous necrosis is most often observed in foci of tuberculous infection. The word "caseous" (cheese like) has derived due to the friable yellow-white appearance of the area where the necrotic cell death has occurred. In contrast to coagulative necrosis, the tissue architecture in caseous necrosis is completely distorted and cellular outlines cannot be discerned.

Fat necrosis, refers to focal areas of fat destruction that typically results from the release of activated pancreatic lipases into the pancreas and the peritoneal cavity. This usually occurs during the abdominal emergency condition known as acute pancreatitis. Histological examination of the fat necrosis show shadowy outlines of necrotic fat cells with calcium deposits in the basophilic region which is surrounded by an inflammatory reaction.

Fibrinoid necrosis is a special form of necrosis which is usually observed during immune reactions that involves blood vessels. The pattern of fibrinoid necrosis is seen prominently when complexes of antigens and antibodies are deposited along the walls of the arteries. These deposits together with fibrin results in a bright pink and amorphous appearance in H&E stains and is called the 'fibrinoid' structure from which the name fibrinoid necrosis came.

Thus understanding of the different morphological pattern of necrosis in various tissues helps in studying the reasons that caused it which in turn will help to understand the pathological condition associated with it.

For a long time necrosis has been described as a consequence of various physicochemical stress conditions like extreme heat, freeze thawing, osmotic stress, increased level of hydrogen peroxide etc. And the defining features of necrotic cell death included defective plasma and organelle membranes, enlargement of the cell and organelle size, severe depletion in the level of ATP, and finally marked inflammation due to the rupture of necrotic cell. In response to these severe stress conditions the cell death occurred quickly which created the notion that necrotic cell death is uncontrolled and accidental. But this view has been now changed as a result of various researches. Several data is now available to support both the programmed course and occurrence of necrosis. For e.g., necrosis can be seen during the development i.e., during the death of chrondrocytes thereby controlling the longitudinal growth of bones (Roach and Clarke, 2000) and also during adult tissue homeostasis as in the case of intestinal epithelial cells (Barkla and Gibson, 1999). It is also observed that necrosis can be triggered by the occupation of specific plasma membrane receptors by the action of their physiological ligands. This observation strongly implies the existence of specific signal transduction pathways which are connected for the induction of necrosis rather than for induction of other types of cell death. Furthermore it is observed that susceptibility to necrotic death can also be regulated by genetic and epigenetic factors. These data point towards the fact that necrosis can have a programmed course. But however, this does not imply that all necrotic pathways are regulated. But, a significant proportion of necrosis seems

to be regulated and play important roles in the pathogenesis of myocardial infarction, heart failure, stroke, neurodegenerative diseases etc. However more work is needed to understand the roles of necrosis in these diseases.

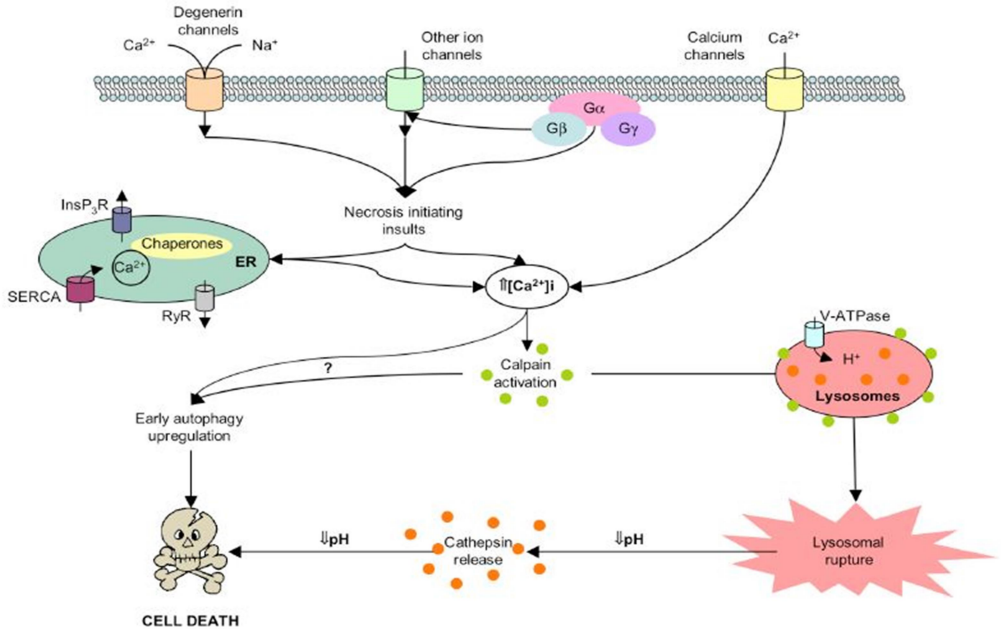


Fig.3 : Necrotic cell death pathway in invertebrates (Adapted from Samara and Tavernarakis, 2008)

1.2.1. c. AUTOPHAGY

The term "autophagy" is used to describe the various cellular process involved in degrading and recycling different proteins and organelles. Autophagy is classified into three different types; microautophagy, chaperone mediated autophagy and macroautophagy (Fig. 4) (Klionsky and Emr, 2000). Microautophagy is the process in which invagination of the lysosomal membrane initially which is then followed by the simultaneous internalization of cytoplasmic parts for degradation process within the lysosome. In chaperone-mediated autophagy, the soluble cytosolic proteins which carry the KFERQ motif are specifically

targeted into the lysosome, where rapid proteolysis of the cytosolic proteins occurs. This process requires the action of cytoplasmic and lysosomal chaperones and also the action of functional lysosomal membrane protein LAMP2.

In macroautophagy, which is one of the best studied autophagy pathway, cytoplasmic double-membrane vesicles called autophagosomes or autophagic vacuoles are formed first. This autophagosomes then engulf large portions of cytoplasm and transfer them to the lysosomes for degradation(Wang and Klionsky, 2003). The outer membrane of the autophagosome fuses with the lysosomal membrane and the remaining autophagic body is then released into the lumen of lysosome. The fusion with lysosomes is usually the last step of consecutive fusions that occur between autophagosomes and early endosomes. A critical prerequisite required for the fusion process is acidification of endocytic compartments. Macrophagy can be further divided into pexophagy, mitophagy and endoplasmic reticulum (ER) autophagy (ER-phagy) based on the specific organelles that undergo degradation i.e degradation of peroxisomes, mitochondria and endoplasmic reticulum respectively.

Autophagy has been studied extensively in yeast model. Through these studies identification of various genes that encodes proteins involved in autophagy (ATG proteins) was made possible. Major roles of ATG proteins include induction of autophagy, the formation, expansion and maturation of autophagosomes, and retrieval of autophagic proteins from mature autophagosomes. Fusion processes that occur during autophagy is *via* the t- and v-SNARE complexes, and also due to molecules like the Rab GTPases and components of the vacuolar protein-sorting (VPS) complex.

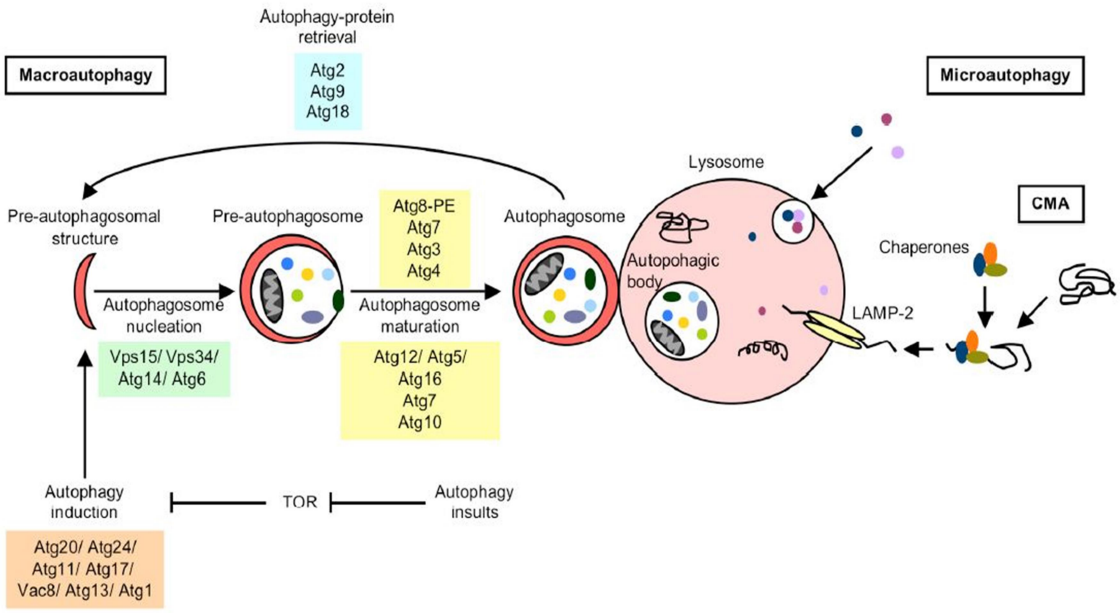


Fig.4 Different types of autophagy (Adapted from (Samara and Tavernarakis, 2008)

The autophagic pathway is a highly conserved mechanism and most yeast Atg genes involved in it has homologs in higher organisms. Several homologs of Atg genes have been identified in *C.elegans.* In addition to the sequence similarity, some indirect experimental evidences suggest the role of specific nematode genes in autophagy. An example for this is the *C. elegans* beclin BEC-1 can functionally substitute for Atg6 in yeast autophagy but it cannot substitute for the cytoplasm-to-vacuole targeting (CVT) pathway (Meléndez et al., 2003).

Autophagy pathway is also been linked to various other cell death pathways and the dual role of autophagy in cell survival as well as in cell death is being studied. Interest in autophagy is now increased remarkably as it plays an important role in human pathophysiology (Klionsky, 2007) .

1.2.2 CELL DEATH PATHWAYS IN *C.elegans*

In 1974, Sydney Brenner first introduced the transparent, soil nematode *Caenorhabditis elegans* as a model organism to the scientific community. Since then this nematode has been used extensively as a model system to study various processes in cellular biology. One of the most exciting field studied using this model organism, which led to the Nobel Prize in Physiology or Medicine in the year 2002, is programmed cell death. *C.elegans* is also used widely to study the mechanisms underlying the different cell death pathways other than programmed cell death.

1.2.2. a. APOPTOSIS IN *C.elegans*

The work of Horvitz and colleagues brought the first insights into the molecular mechanisms involved in the developmental cell death in *C. elegans* (Yuan and Kroemer, 2010). In *C. elegans*, apoptosis is a normal process that occurs during the growth and development of the organism. At the time of development 131 cells out of 1090 somatic cells in each hermaphrodite will undergo apoptosis. An interesting fact regarding apoptosis in *C.elegans* is that, the main effectors involved in apoptosis in the worm are conserved in mammalian organisms also.

In *C.elegans* the main apoptotic pathway starts with the activation of EGL-1 which is a BH3 only protein .Other proteins that have a role in promoting apoptosis in *C.elegans* include EFL-1,, LIN-35, LIN-37 , LIN-52,DPL-1, and MCD-1. Their proposed action in promoting apoptosis is through transcriptional regulation of *egl-1.*When EGL-1 is activated, it binds to CED-9,the only BCL-2-like proteinin *C. elegans*, and inhibits it action. This further nullifies

the inhibiting effect of CED-9 on CED-4, which is orthologous to human pro-apoptotic APAF-1. This in turn cause the formation of oligomeric complexes called apoptosomes thereby activating CED-3 which is a caspases that leads to the cell death (Lettre and Hengartner, 2006).

Activation of EGL-1 also has a role in germ cell apoptosis which is caused due to genotoxic stress. DNA damage activates CEP-1, which is the homolog of the mammalian apoptosis-inducer p53. CEP-1 act as a transcriptional activator of *egl-1*, which is one of the key players of apoptosis.

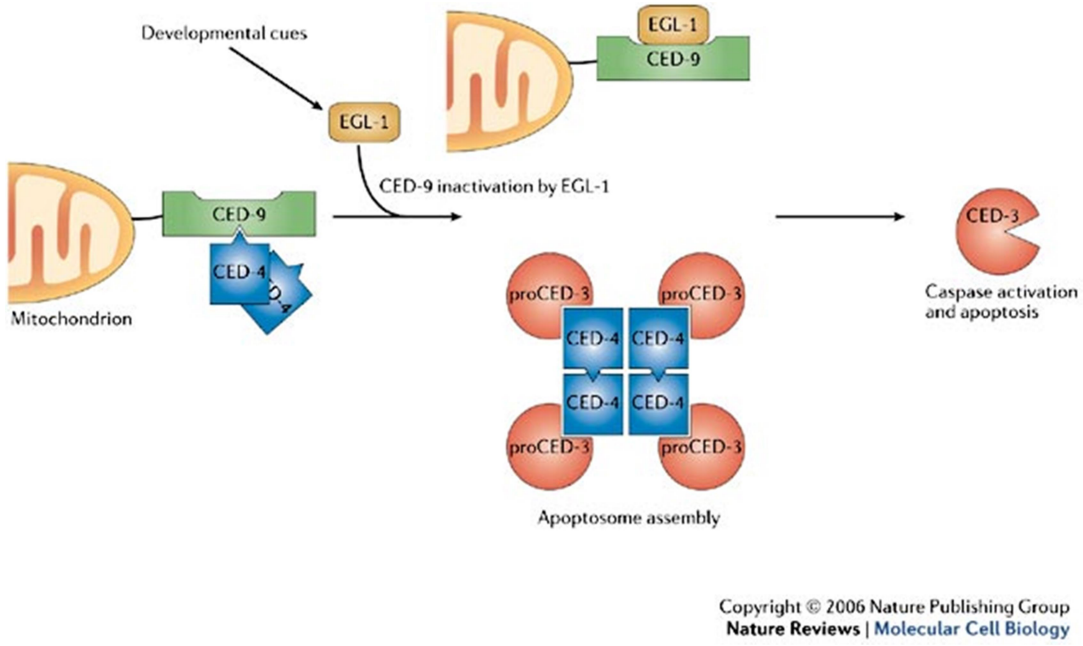


Fig.5. Molecular model of apoptosis activation in *C. elegans*. Adapted from **(Lettre and Hengartner, 2006)**

Once the apoptotic pathway is initiated it triggers the activation of neighboring cell pathways thereby leading to the engulfment and ultimately the degradation of apoptotic bodies inside phagolysosomes. The two separate molecular cascades involved in this process are: one that includes the proteins CED-7, CED-6 CED-1, and DYN-1, and the second which requires the function of MIG-2, UNC-73, CED-12, CED-5 and CED-2. Both these cascades converge to the small GTPaseCED-10, which further induce the rearrangements in the cytoskeleton there by promoting engulfment of cell (Yu et al., 2006).

Other genes which are involved in apoptotic pathway in *C. elegans* include *ced-8* and *nex-1*. *nex-1* is a homolog of the annexin I in mammals (Kinchen and Hengartner, 2005). The knockdown of *nex-1* by RNAi causes the persistence of cell corpses (Arur et al., 2003). *ced-8*codes for an integral membrane protein and its deficiency delays appearance of cell corpse as well its removal, suggesting its role in killing of cell or engulfment (Hoeppner et al., 2001).

In *C. elegans* apoptosis also plays an important role in innate immunity. For example in one study it was found that colonization of *Salmonella typhimurium* in *C. elegans* intestine leads to increase in the level of cell death in the worm, which is dependent on the well-characterized EGL- 1/CED-9/CED-4/CED-3 pathway.

The apoptotic program is highly conserved mechanism, and the core mechanisms that mediate apoptosis in higher organisms is similar to that in the nematode. Various mammalian orthologs have been identified for most apoptotic proteins involved in *C.elegans* apoptotic

pathway. Furthermore, there are specific mammalian apoptotic molecules which are functional in *C.elegans* for e.g. the human Bcl-2 can interact with the proapoptotic nematode BH3-only protein EGL-1 and thereby can reduce the developmental apoptosis. But compared to the apoptotic process in the nematode, the mammals have a more complex pathway with higher level of redundancy and sophisticated regulation. However, *C. elegans* is a good model to study the various aspects of apoptosis as well as the pathways involved in it.

1.2.2. b. AUTOPHAGY in *C.elegans*

Autophagic cell death pathway, which is a cellular process involved in degrading and recycling of proteins and organelles, is also been studied in *C. elegans*. In *C.elegans* the physiological function of autophagy is mainly associated with formation of dauer larva. (Riddle and Gorski, 2003). When unfavorable conditions like elevated temperature, scarcity of food etc comes the growth of L2 larvae will get arrested and they enter a dauer developmental state. Entering of the L2 larvae into this state is negatively regulated by the insulin-like signaling pathway. The important player of dauer morphogenesis in *C.elegans* is *Bec-1*, which is the *C. elegans* ortholog of the yeast and mammalian autophagy gene *Atg6/Vps60/beclin. bec-1* forms a complex with CED-9/Bcl-2 and also has a role in *C. elegans* apoptotic pathway. Deletion of *bec-1* triggers the CED-3/caspase-dependent cell death in the nematode. BEC-1 is also essential for the function of the class III PI3 kinase LET-512/ Vps34, which required for autophagy, membrane trafficking, and endocytosis (Roudier et al., 2005).

In addition to that, the orthologs of the yeast autophagy genes like *Atg1*, *Atg7*, *Atg8,* and *Atg10* are also involved in the process and down regulation of these genes will result in defect in dauer formation.

During starvation the muscarinic acetylcholine pathway signals induction of autophagy in pharyngeal muscles of *C. elegans*. When the muscarinic acetylcholine pathway is activated the pumping rates are enhanced and the energy required for this process is likely obtained through autophagy pathway. RNAi knockdown of *bec-1* decreases autophagy as well as pumping rates during starvation (Kang et al., 2007).

Autophagy also has a role in endocytosis in *C. elegans*. The regulation of formation of endosomal sorting complexes is done by *Ce*VPS-27, which is the ortholog of the yeast endosomal Vps27p. *Ce*VPS-27 has an important role in larval development and inactivation of the corresponding gene will lead to defects in formation of endosome, as well as accumulation of autophagosomes, suggestive of its role in autophagy (Roudier et al., 2005).

Recent findings in *C. elegans* indicate that autophagy also has a role in necrotic cell death pathway. In an *unc-51* deficient background, the process of necrotic cell death, which is triggered either by *mec-4(d)* or *deg-3(d)* or hypoxia is significantly suppressed. The same effect is also observed when other autophagy-related genes like *bec-1*, *lgg-1* and *atgr-18* is downregulated by RNAi. Additionally, increase of autophagosomes formation is also observed under the conditions like neurodegeneration. Furthermore calpain proteases and

autophagy also appear to act in the same pathway. These findings indicate the role of autophagy in the necrotic pathway (Samara et al., 2007).

1.2.2.c. NECROSIS IN *C.elegans*

In *C. elegans* necrosis can be triggered by several genetic and environmental insults. Among them mutations that result in the hyper-activation of degenerin (DEG) ion channels is considered as the most potent one .E.g, dominant, hyperactivating mutations in the *mec-4* gene. It encodes a subunit of a mechanosensitive ion channel expressed in the six touch receptor neurons and the mutations cause the death of these neurons in animals. Likewise, the gain-of function *u38* allele of the *deg-1* degenerin gene also causes late-onset degeneration of posterior interneurons(Kourtis and Tavernarakis, 2007).

Microscopic observations of dying *mec-4(d)* neurons show a series of morphological changes that show resemblance to experimentally induced excitotoxicity in rats. The cell death begins with the usual cellular distortion and accumulation of small wrapped membrane whorls occur near the plasma membrane. Further these membrane structures will combine and move towards the nucleus, *during which the swelling of the cell occurs* (Hall et al., 1997).

Aspartyl proteases involved in necrosis can be either cytoplasmic or lysosomal and they are released into the cytoplasm after the rupturing of lysosome. This in turn causes degradation of cellular components and ultimately leads to cell death.

Other paradigms of necrotic-like cell death have also been described in *C. elegans*. Absence of EGF-like-signaling in four precursor uterine cells that participates in the vulva-uterine connection prevents their differentiation and thereby causes their death by necrotic pathway .Furthermore, necrotic-like death has also been recently observed in post mitotic vulval cells, after their precursors (VPCs) has been exposed to radiation . Similar to vertebrates, adverse environmental conditions also induce necrosis in *C.elegans*. For example, hypoxia condition will reduce the animal viability and will induce necrotic-like cell morphology in several tissues like the pharynx, gonads and body wall muscles (Weidhaas et al., 2006).

Since studying the mechanisms underlying necrotic cell death is highly relevant to human health, using *C.elegans* as a potential model to study the necrotic pathway can be considered as a promising solution.

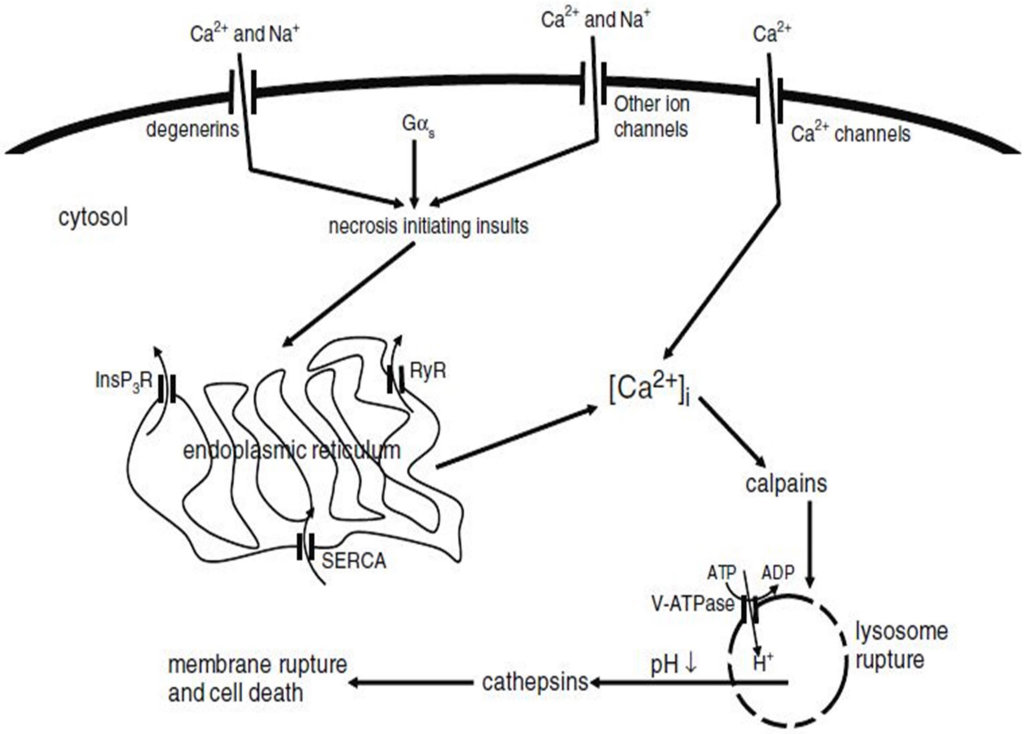


Fig.6 Mechanism of necrosis in *C.elegans* ((Kroemer and Martin, 2005)

1.2.3 DEATH FLUORESCENCE IN *C.elegans*

1.2.3.1 *C. elegans* intestine

In *C. elegans*, the intestine is one of the major organs that comprises roughly about one third of the total somatic mass. It is produced as a clone of cells that descend from a single cell called E blastomere, of the eight-cell embryo. The intestine is composed of 20 large epithelial cells that are mostly positioned as bilaterally symmetric pairs. They are large, cuboidal cells, with distinct apical, lateral and basal regions. These cells contain large nuclei with a prominent nucleolus, extensive rough endoplasmic reticulum (RER), many mitochondria, ribosomes, and a large collection of membrane-bound vesicles as well as vacuoles. This cells

which are bilaterally arranged in pairs, will form a long tube around a lumen and each of these cell pairs combine to form an intestinal ring (II-IX int rings). But the anterior most intestinal ring (int ring I) is an exception to this and it is composed of four cells. Initially the intestine fills the entire body cavity behind the pharynx, but later as the animal ages it becomes deflected to allow the outgrowth of the gonad within the body cavity (Vogel and Hedgecock, 2001).

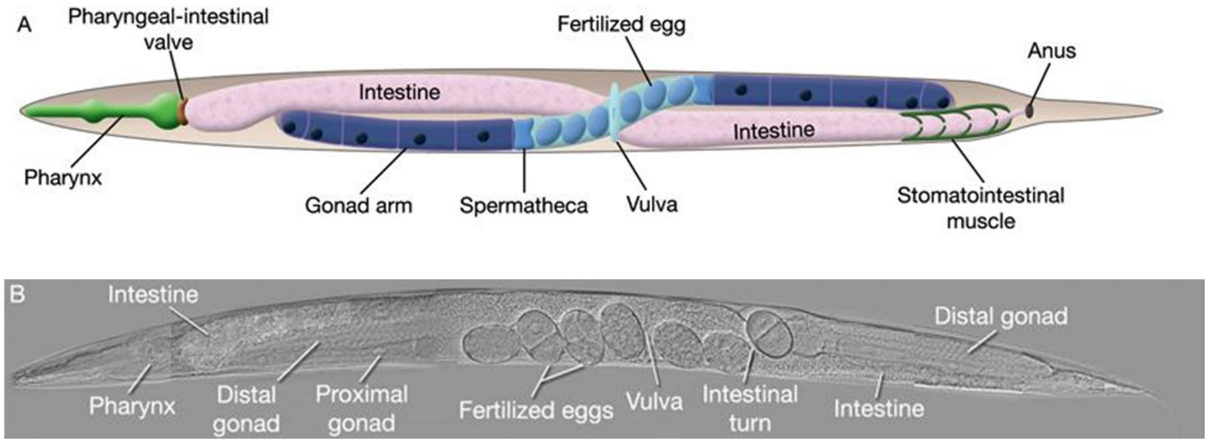


Fig.7 C. elegans intestine(Wood et al., 1996)

The major functions of intestine include food digestion, assimilation of nutrients, synthesis and storage of various macromolecules, initiation of innate immune response, nurturing of germ cells by producing yolk etc. Recent studies show that intestine can also functions as a cold temperature sensor. The intestinal cells exhibit a robust increase in calcium level in response to cooling. But this calcium response is greatly reduced in trpa-1 mutant worms, thereby showing the importance of TRPA-1 in cold-reception in intestine as well as in cold-dependent lifespan extension. In addition to all these, the intestine of *C.elegans* is now

emerging as a powerful experimental system which can be used to study various biological processes like vesicular trafficking, biochemical clocks, aging and stress responses.

.1.2.3.2. Gut granules in *C. elegans* and death fluorescence

Gut granules are prominent organelles in *C. elegans* that are seen numerously in the intestinal cells of the nematode. A remarkable feature of these gut granules is that they emit a blue fluorescence when they are exposed to ultraviolet light (Klass, 1977). *Gut granules are considered as lysosome like organelles due to their acidic interior*, capacity for endocytosis as well as the presence of the fluorescent material, similar to that of pigment containing melanosomes in mammals and eye pigment granules in Drosophila (Raposo and Marks, 2007) .

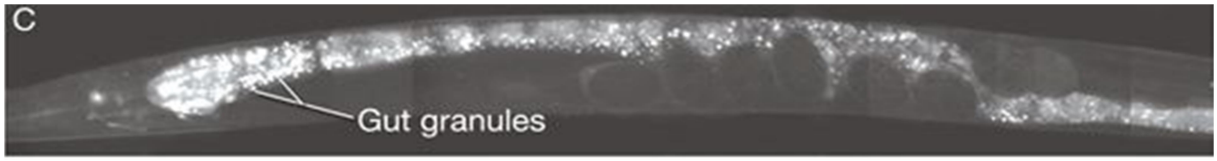


Fig .8 Gut granules in *C. elegans* (Adapted from (Wood et al., 1996)

According to recent chemical analysis, the fluorescent substance present inside the gut granules is recognized as kynurenine pathway product, anthranilic acid (AA) glucosyl ester. This chemical identification was confirmed by comparing the wild-type worms with glo-1 mutants, that lack these gut granules (Coburn et al., 2013).

The intensity of blue fluorescence starts to increase as the worm gets older. But as the worm dies, they emit a sudden burst of blue anthranilic acid fluorescence .This is called death fluorescence and it typically occurs as an anterior to posterior wave that courses along the intestine. It is not only seen in older worm that undergo normal death process but also in young worms that are subjected to lethal injury. Death fluorescence is a peculiar phenomenon seen in the nematode because it shows the passage of death through the semi-transparent body of the worm as an increased burst of blue fluorescence. Death fluorescence in *C. elegans* is promoted by the calpain–cathepsin necrotic cell death pathway (Coburn et al., 2013). In this cascade, the levels of the intracellular Ca2+ increase, thereby activating the Ca2+-dependent calpains. This further cause the lysis of lysosomes thereby leading to cytosolic acidosis and the release of destructive lysosomal cathepsin proteases .Mutational attenuation of this cascade usually causes reduction of the death fluorescence in the worms. Furthermore, it was also observed that the intercellular propagation of the death fluorescence is dependent upon the innexin gap junction INX-16, showing the role of calcium signaling in death fluorescence. Thus the anthranilic acid death fluorescence in *C.elegans* can be used as a natural, endogenous marker of organismal death and also can be used to study the mechanisms underlying cell death pathway.

1.3 HYPOTHESIS

C. elegans undergo predictable anthranilate fluorescence before death. This gives an opportunity to analyze the cellular changes within the organism during organismal death. It has been argued that during the process of the organismal death, the cells undergo necrotic changes. However it is not clear about the time frame required for the necrotic changes to set in. Since *C.elegans* has a fluorescence signal before the animal dies, we hypothesized that it could be possible to measure the time kinetics of early necrotic changes in the model system.

1.4 OBJECTIVES

1. Standardization of organismal death in *C.elegans* and to measure the time kinetics of anthranilate fluorescence.
2. Measure changes in cellular level, especially the early nuclear changes during organismal death.
3. Measure the time kinetics of karyolysis, the early necrotic initiation.
4. Identify whether alterations in biochemical requirements of necrosis affect the cellular changes and delay organismal death.

CHAPTER 2

MATERIALS AND METHODS

2.1 Worm maintenance and strains

C.elegans strains used for the study were maintained under standard conditions. All the strains were grown at 20°C, unless otherwise mentioned, on Nematode Growth Medium (NGM) plates seeded with the E.coli strain OP50, which is an auxotropic mutant, as the food source.

Strains used:

The wild type strain used for the study was N2(Bristol).

The following mutant strains were used for the study :

1. EG144 [inx-16(ox144)I] – This strain is an innexin-16 mutant. Innexin is an invertebrate gap junction protein which is necessary for calcium transmission by creating calcium channels between adjacent intestinal cells.

2. MR142 [cdc-25.1(rr31)I; rrIs1] – cdc-25.1 is a cell cycle phosphatase and rr31, is a fully dominant gain-of-function mutation in the cdc-25.1, that sensitizes the intestinal lineage to an extra cell division.

Mutant strain MR142 was maintained at 25°C. All the strains were obtained from *Caenorhabditis* Genetics Centre (CGC), University of Minnesotta. All experiments were performed on worms in their adult stage unless it is mentioned otherwise.

2.2 *C. elegans* killing assays

2.2.1 Heat shock

The worms were transferred to an agar pad (4%) on a glass slide. Worms were anesthetized in 25mM sodium azide (Himedia laboratories, India). Heat shock was given to the anesthetized worms by placing a flame heated wire on the agar immediately adjacent to the worm usually near the head of the worm. After giving heat shock, 15µl of M9 buffer was added and a coverslip was placed over the worms. The sides of the coverslip were sealed using petroleum jelly to prevent dehydration. Worms were subjected to time lapse imaging using inverted microscope Olympus IX51 equipped with Rolera-XR CCD camera (Q Imaging) and image acquisition software NIS Elements-Advanced Research (NIKON). N2 and EG144 worms were observed under ultraviolet light at λex/λem 330-385/420 and MR142 was observed under blue light at λex/λem 460-490/520.

2.2.2 1% acetic acid (pH -3)

The worms were transferred to an agar pad (4%) on a glass slide. 15µl of 1% acetic acid in distilled water (Merck-India-Ltd.) was added to the worms. To prevent dehydration, coverslip was placed over the worms and the sides were sealed with petroleum jelly. Worms were then subjected to time lapse imaging. Worms N2 and EG144 were observed under ultraviolet light at λex/λem 330-385/420 and MR142 was observed under both blue light at λex/λem 460-490/520 and ultraviolet light at λex/λem 330-385/420.

2.2.3 Control

The worms were transferred to an agar pad (4%) on a glass slide. 15µl of M9 buffer was added to the worms. A coverslip was placed over the worms and the sides were coated with petroleum jelly to prevent dehydration. Worms were then subjected to time lapse imaging. N2 worms were observed under ultraviolet light at λex/λem 330-385/420 and MR142 worms were observed under blue light at λex/λem 460-490/520.

2.3 Induction of prolonged hypoxia

Prolonged hypoxia condition was induced in the mutant strain MR142 by treating the nematode with 0.5M sodium azide (Himedia Laboratories, India) for 30 min at 20°C. After incubation the worms were treated with 1% acetic acid (Merck (India) Ltd.) and subjected to time lapse imaging for 1 hour. The worm MR142 was observed under blue light at λex/λem 460-490/520. After imaging worms were subjected to DAPI staining.

2.4 Alkalization assays

The mutant strain MR142 was treated with 5mM ammonium chloride (Merck India Ltd.) and imaging was done under blue light at λex/λem 460-490/520.

2.4.1 Ammonium chloride treatment

NGM plates with OP50 were flooded with 5mM ammonium chloride. The worms were then transferred to the plates and incubated for 1 hour. After incubation the ammonium chloride solution was removed and the worms were washed using M9 buffer. The worms were then

transferred to 100mM sodium azide and incubated for 5 min. The anesthetic was removed after 5min and the worms were treated with 15µl 1% acetic acid and subjected to time lapse imaging. After imaging the worms were subjected to DAPI staining.

2.5 Actinomycin D treatment

In order to check whether the nucleolysis require transcription of new genes, MR142 strain was treated with actinomycin D which is a transcription blocker. NGM plates with OP50 were exposed to UV radiation for 10 min. Actinomycin D (Himedia Laboratories, India) was spread on the top of OP50 plates to a final concentration of 0.1µg/ml of agar volume and the plates were allowed to dry. Worms were transferred into the plates containing actinomycin D and incubated for 2 hours at growth temperature. After incubation, the worms were transferred to 100mM sodium azide and incubated for 5 min. The anesthetic was removed and the worms were treated with 1% acetic acid and subjected to time lapse imaging for 1 hour. The worm MR142 was observed under blue light at λex/λem 460-490/520. After imaging the worms were subjected to DAPI staining.

2.6 Cycloheximide treatment

In order to check whether translation requirement is essential for nucleolysis, the worms were treated with the cycloheximide which is potent and specific inhibitor of protein synthesis (Kourtis and Tavernarakis, 2009). NGM plates with OP50 were exposed to UV radiation for 10 min. Cycloheximide (Sigma-Aldrich, Germany) solution was spread on the OP50 plates to a final concentration of 500µg/ml of the agar volume and the plates were allowed to dry. Worms were transferred into the plates containing cycloheximide and incubated for 2 hours

at growth temperature. After incubation the worms were transferred to 100mM sodium azide and incubated for 5 min. The anesthetic was removed and the worms were treated with 1% acetic acid and subjected to time lapse imaging for 1 hour. The worm MR142 was observed under blue light at λex/λem 460-490/520. After imaging the worms were subjected to DAPI staining.

2.7 Time lapse imaging

The worms were subjected to time lapse imaging after the various treatments to study the characteristics of cell death. Worms were photographed in every 15 sec for 30 min - 1 hour depending on the parameters of the experiment. The images were taken using the inverted microscope Olympus IX51 equipped with a Rolera-XR CCD camera (Q Imaging) and image acquisition software NIS Elements-Advanced Research (NIKON) under the 4X and 10 X objectives as per the requirement of the experiment.

2.8 Time lapse imaging in bright field for studying changes in nuclear structure

The worms were subjected to time lapse imaging under bright field so as to study the changes in the nuclear structure and cellular architecture. Photographs were taken in every 15 sec for 30 min - 1 hour depending on the parameters of the experiment. The images were taken using the inverted microscope Olympus IX51 equipped with a Rolera-XR CCD camera (Q Imaging) and image acquisition software NIS Elements-Advanced Research (NIKON) under the 4X and 10 X objectives as per the requirement of the experiment.

2.9 Nuclear staining in *C.elegans*

2.9.1 Poly L Lysine Coating

One ml of 0.1mg/ml of Poly L Lysine hydro bromide solution was added on to the surface of the glass slides. The slides were then incubated at room temperature for 15 min. The solution was then removed and the slides were then allowed to dry at room temperature for 2 hours.

2.9.2 DAPI Staining

DAPI staining was performed to stain the nucleus of the worms after treatment with 1% acetic acid. 4 time points were selected - 5 min, 10 min, 30 min and 1 hour. Worms treated with M9 buffer alone were stained to see the pattern in controls. DAPI staining was also done to stain the nucleus of worms after giving heat shock to the worms.

Prior to DAPI staining the worms were subjected to freeze cracking to break the cuticle and fixed using methanol. Briefly, a drop of distilled water was placed on the middle of a sticky polylysine slide. Few worms were transferred into the water drop. After transferring, another polylysine coated slide was placed over the worms. The slide was then immediately transferred to -80^{o}C and incubated for 30 min. After incubation the slides were cracked by swiftly pulling apart the two slides. The worms were then fixed by treating it with methanol (100%) and giving incubation for 2 min on ice. The fixed worms were then stained using DAPI (4',6-diamidino-2-phenylindole) which is a fluorescent, light sensitive stain that binds strongly to A-T rich region in DNA. For staining, the worms were incubated in DAPI for 5 min in dark. After incubation the worms were observed under UV light with λex/λem 330-385/420 and images were taken.

2.9.3 Propidium Iodide (PI) staining

PI is a fluorescent, light sensitive stain which intercalates between the bases in nucleic acids and was used to differentiate between apoptotic, necrotic and live cells. The worms were transferred to an agar pad (4%) on a glass slide. 15µl of solution containing equal volume of 2% acetic acid and PI (100µg/ml) was added to the worms. A coverslip was placed over the worms and the sides were coated with petroleum jelly to prevent dehydration. Images were taken at different time point in bright field; blue light (λex/λem 460-490/520) and green light (λex/λem 510-550/590).Worms were also subjected to time lapse imaging. Both N2 and MR142 were stained using PI. Worms treated only with PI in the absence of acetic acid was taken as control.

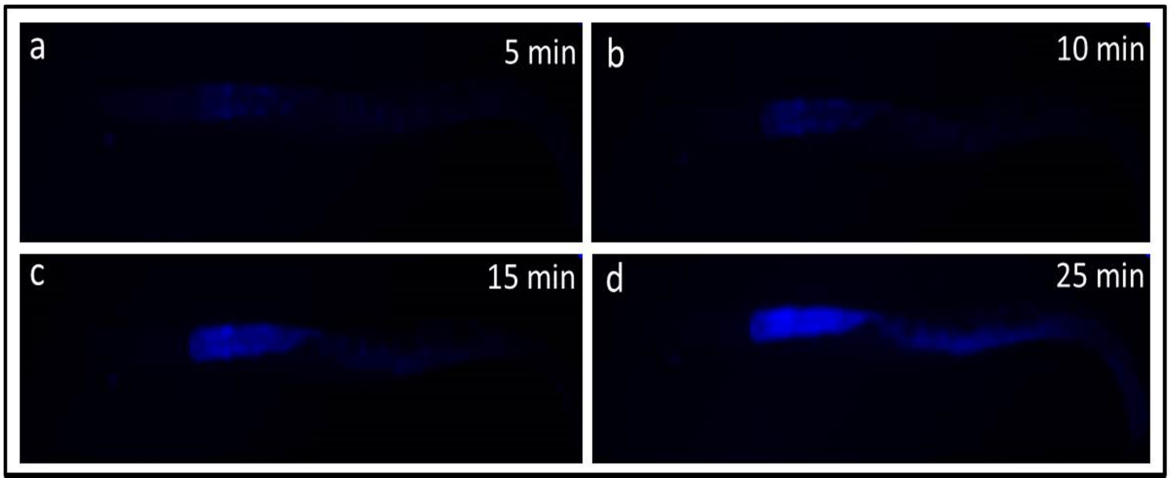
CHAPTER 3

RESULTS

3.1 DEATH FLUORESCENCE (DF) IN *C.elegans*

It has been reported that the intestinal gut granules in *C.elegans* emits a bright blue fluorescence when they are exposed to UV light with maximal intensity λex/λem 340/430 nm (Gerstbrein et al., 2005). During death, the worms exhibit a sudden burst of intense blue anthranilic acid fluorescence. We confirmed this observation in N2 worms. We used the following killing assays to verify this, which included heat shock and 1% acetic acid (pH3) (Figure 9A, B). All the conditions developed DF in the digestive tract of the worms with minor variations with respect to time of initiation. Acetic acid treatments were the most productive assay among all (Figure 9B). As a control, worms were exposed to UV alone to confirm that death fluorescence development is specific to the treatment conditions (Figure 9 C).

A.



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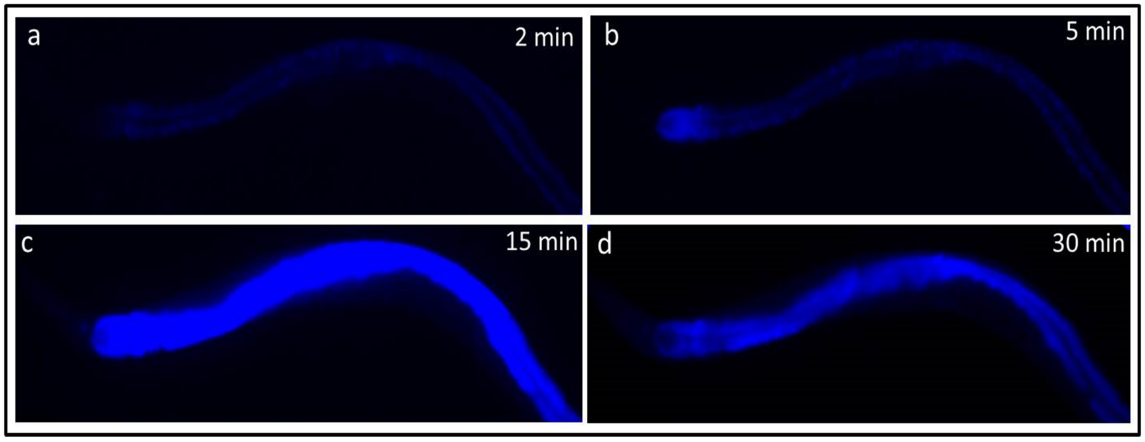


C.

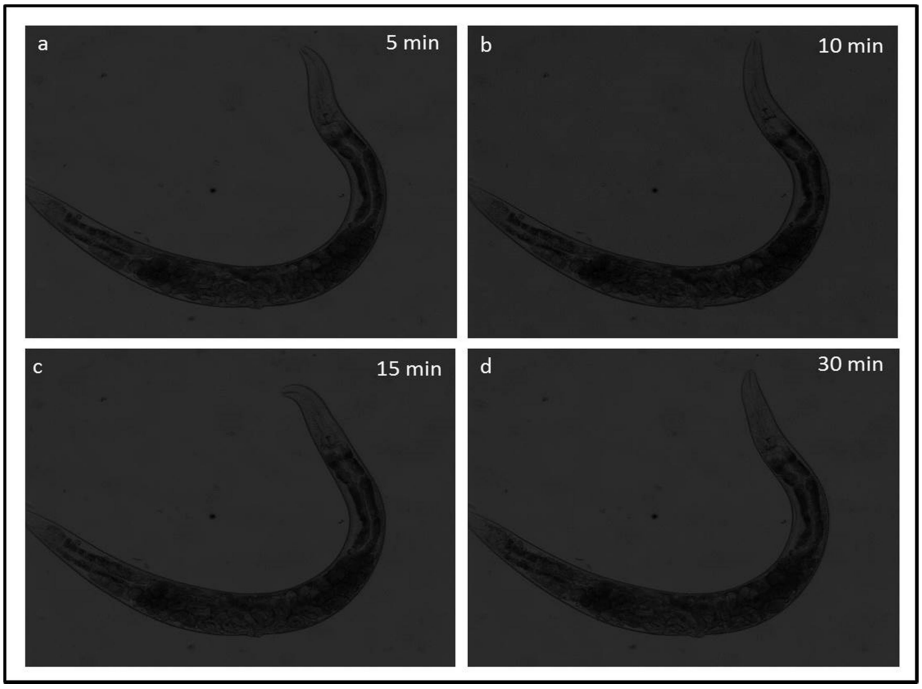


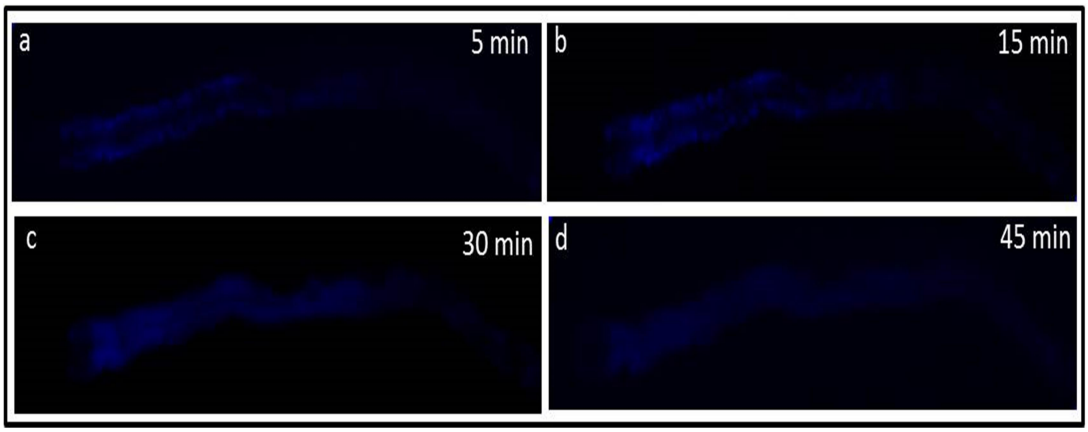
Figure 9: Death fluorescence under various death assays in N2 worms. A. Heat shock, B. 1% Acetic acid. C. worms exposed to UV light alone without any treatment. The images were taken in time lapse mode. These are representative images of a series of observations (N=4 for acetic acid and N=3 for heat shock)

3.2 Calcium is essential for development of DF

Calcium is reported to be an essential requirement for the development of death fluorescence. (Coburn et al., 2013) We made use of the strain EG144, a calcium channel mutant, to confirm this observation by using it for the death assay (Figure 10A and B).

No or very faint death fluorescence were observed in this strain, confirming that the calcium is an essential requirement for anthranilate DF.

A.



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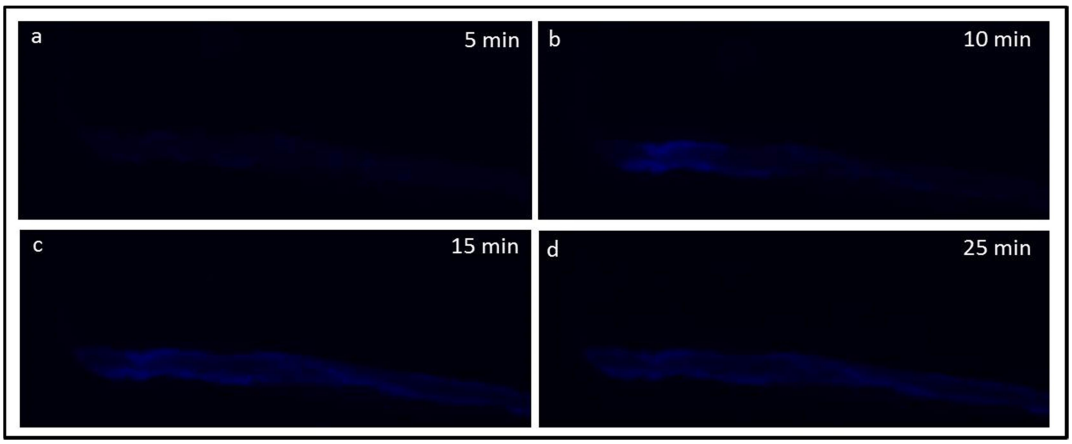
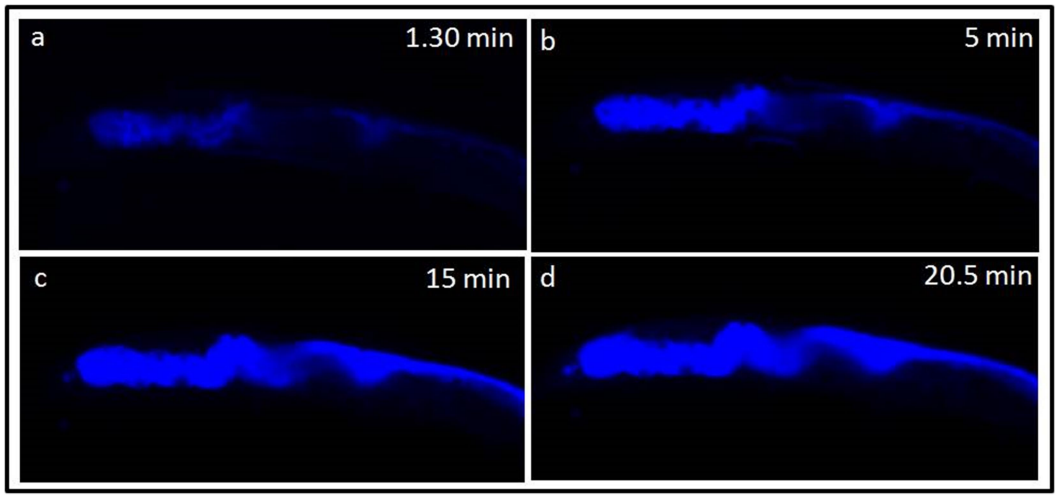


Figure 10: Death fluorescence under various death assays in EG144. A. Heat shock, B. 1% Acetic acid. The images were taken in time lapse mode. These are representative images of a series of observations (N= 3 for acetic acid and heat shock).

3.3 Death fluorescence is followed by nucleolysis, the early stage of necrosis, in *C. elegans.*

It has been argued that the organismal death is due to necrosis. (Coburn et al., 2013) To verify this, we used MR142 strain, which is a mutant transgenic strain having the digestive tract nucleus labeled in green fluorescence. These worms were assayed for DF and observed for the associated changes in digestive system at the cellular level.

A.



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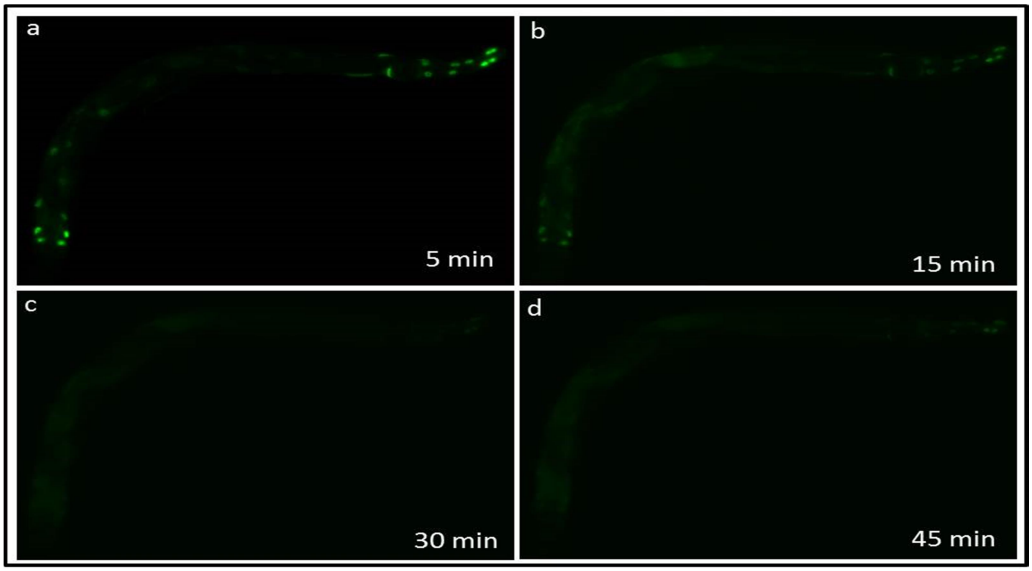
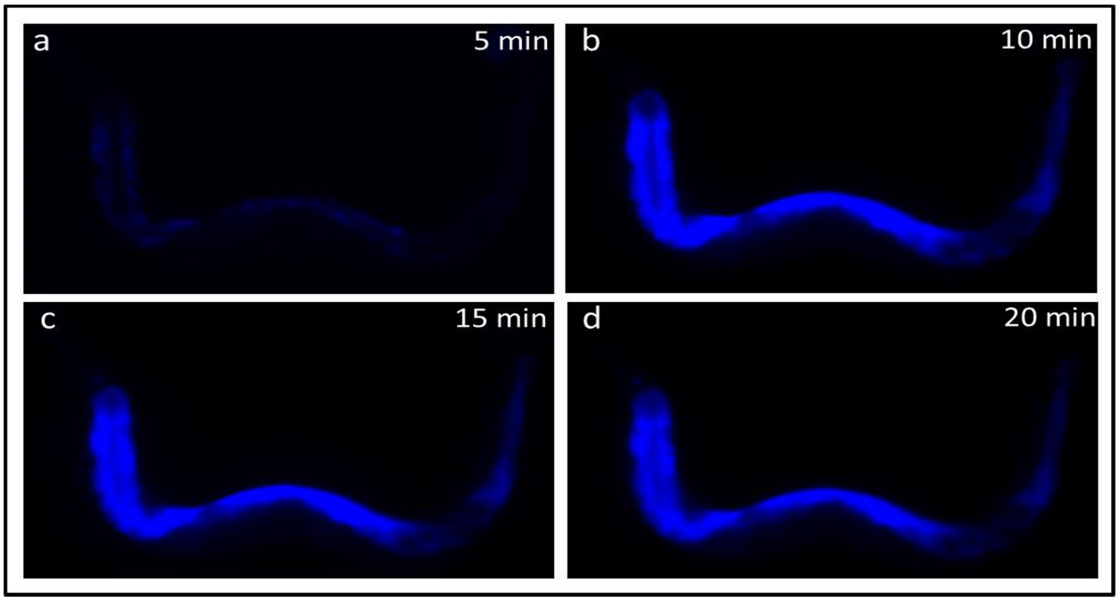
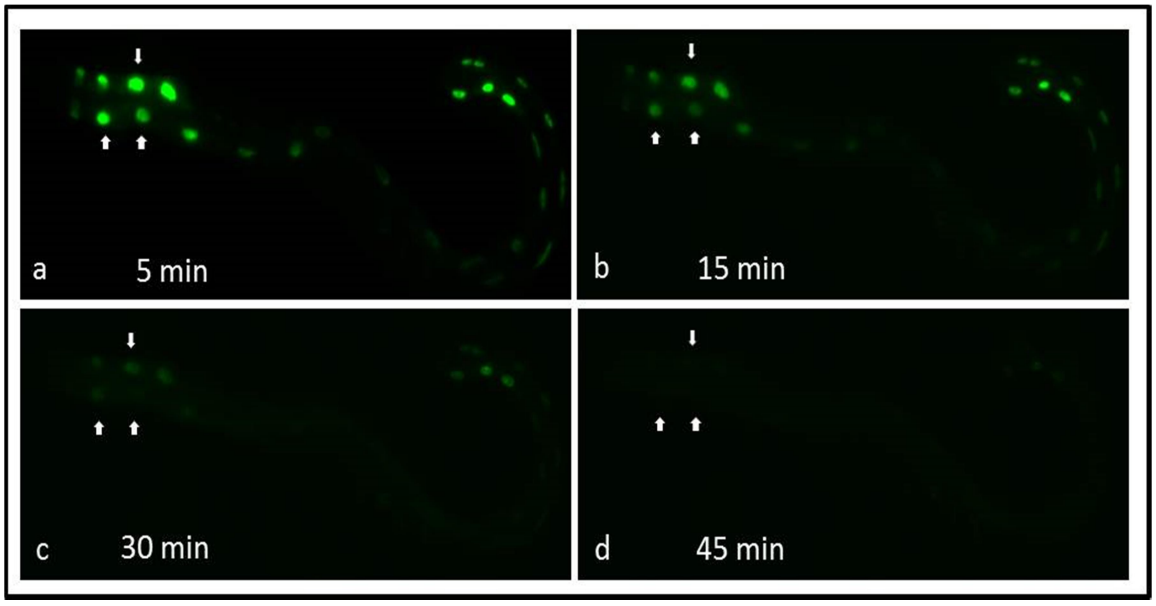


C.



D.



E.

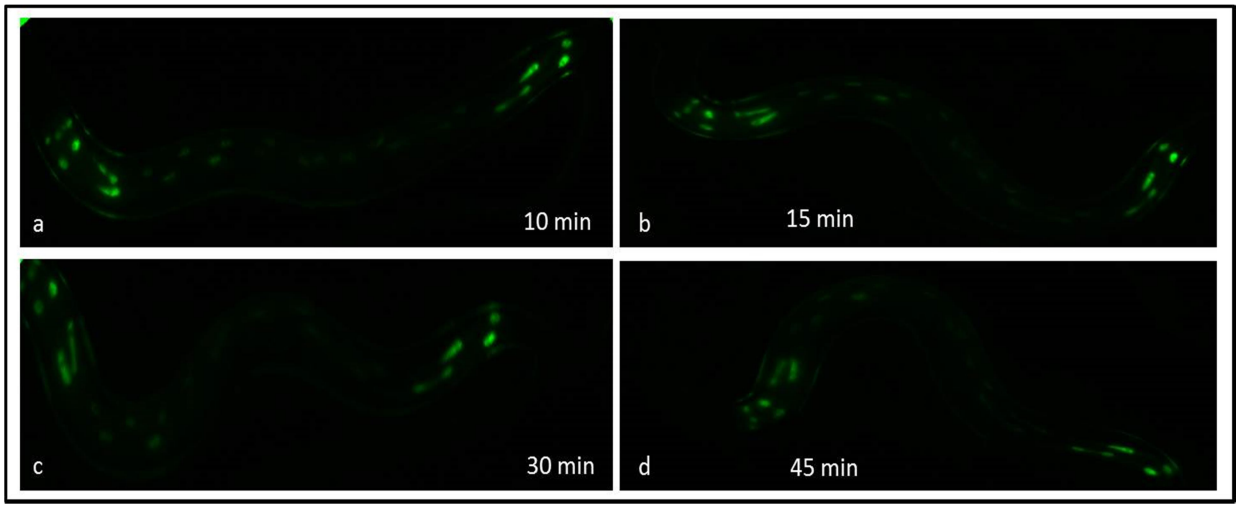


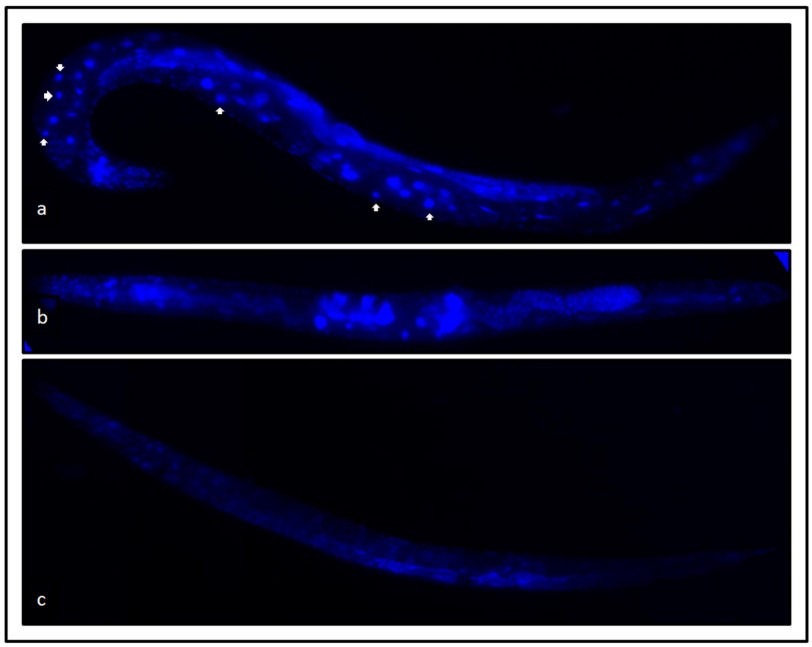
Figure 11. DF and cellular changes in MR142 strain. A and B, Heat shock treated worms with DF and GFP expression in nucleus respectively. C and D, shows 1% acetic acid treated worms with DF and GFP expression in nucleus. E, control MR142 worms with GFP expression from nucleus without any treatment(M9 buffer). The intensity of green fluorescence is indicative of alterations in the nucleus of intestinal cells. N=3 to 5 independent experiment for each conditions. All images are from time lapse recordings.

In worms that were exposed to heat shock, the DF started within ~12 min, from the middle region and spread throughout the body within 18 min (Figure 11A). We also observed a reduction in the intensity of green fluorescence from the nucleus, indicating karyolysis. The first nucleus disappeared at ~14 min and complete nuclear disappearance occurred within 45 min (Figure 11B). In 1% acetic acid treated worms, DF started within time range 3.30-4.58min and complete spreading of the DF was seen within 8.30-13.13 min (Figure 11C). Karyolysis was observed in 1% acetic acid treated worms also (Figure 11D). Under these conditions the first nucleus disappearance was seen from 13.30 - 24.31 min and complete disappearance was seen within 39.15 - 97.05 min. The data suggests that the karyolysis followed the DF and the first disappearing nucleuses where the initiation positions of DF. Some nucleuses, for example in the tail region, took more time to disappear. To rule out that

the observed reduction in GFP in nucleus is not caused by photobleaching, worms were exposed to blue light for one hour. We did not observe any changes in the levels of nuclear GFP expression (Figure 11E).

In order to confirm that the reduction in green fluorescence in the nucleus is due to karyolysis; the worms were fixed after death assay and stained with DAPI, a fluorescent dye which binds to the AT rich region in DNA. The results showed significant reduction in nuclear staining in these worms after the treatment of heat shock or 1% acetic acid (Figure 12 A and B).

A.



B.

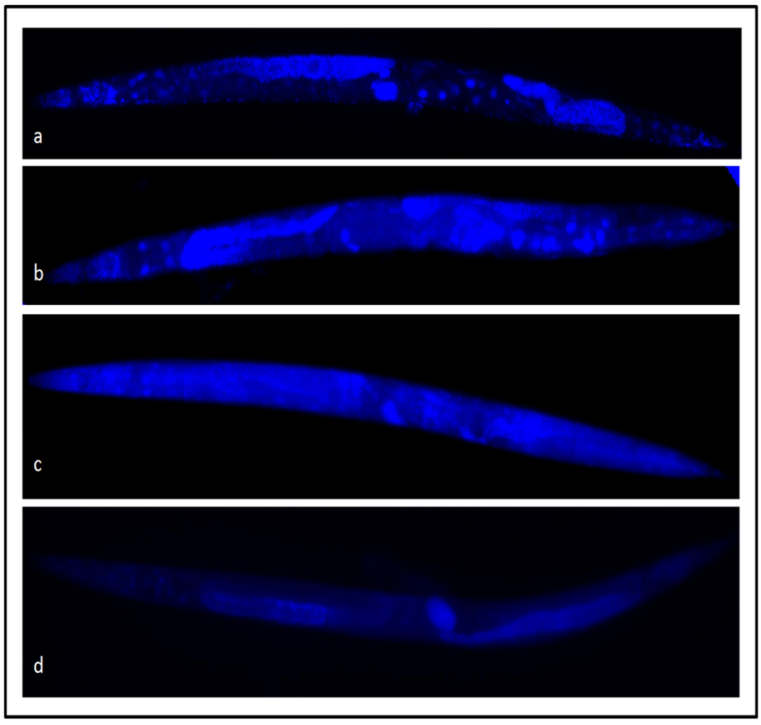


Fig. 12 DAPI staining of strain MR142 after heat shock and 1% acetic acid treatment. 12 A. DAPI staining after heat shock. a. control (arrowhead shows nucleus), b. image after giving heat shock and c, 10 min after heat shock. B. DAPI staining after 1% acetic acid treatment. a, b, c and d are 5min, 10 min, 30min and1 hour treatment with 1% acetic acid, respectively. To observe the pattern of control refer image 12 A.

3.4 Karyolysis did not alter the cell structures in *C. elegans*

Time lapse bright field imaging of the strain MR142 was performed to study the changes in morphology as well as cellular architecture of the worms treated with acetic acid. Structural changes were confined to nucleus and no major cellular architecture changes were observed (Figure 13). To validate this observation, measurements were made from the images and the ratio of diameter of the worm to diameter of the digestive tract was calculated. The distance between nucleuses was also calculated. These measurements did not show any statistically significant variation between different time points for each worm thereby confirming that there are minimal changes to the morphology and cellular architecture (Figure 14). Since the

worms are still in the early stages of necrotic pathway, it is quite possible that changes to the cellular architecture may not be obvious yet.

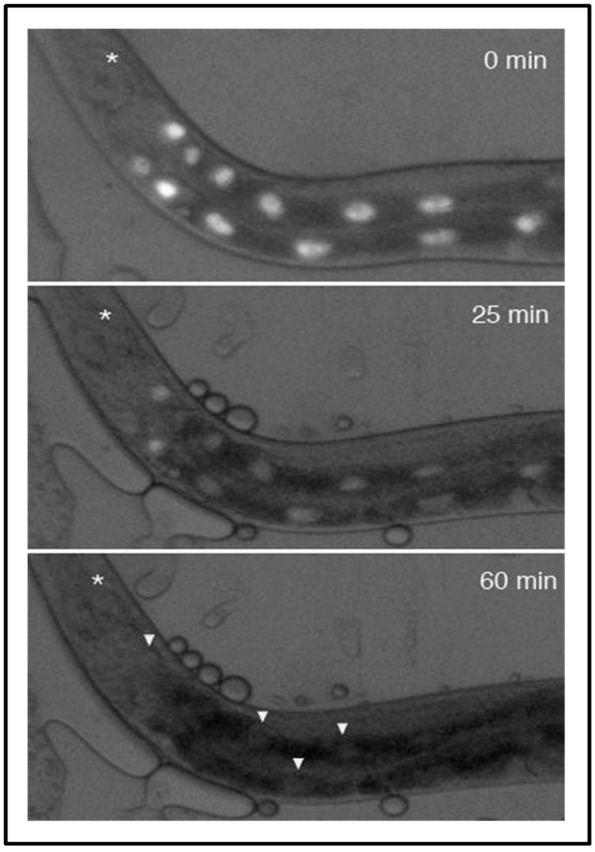


Figure 13: MR142 strain exposed to 1% acetic acid showing nuclear disappearance within 60 min. Nuclear membrane seems intact(arrow heads). The cytoplasm appear darker probably due the phenomenon of death fluorescence (DF); however the complete nuclear disappearance lags behind the DF approximately more than 30 minutes to one hour. * indicates the pharynx, arrow heads mark the original nuclear position. Images were taken at 15 sec interval for 1 hour. Images were further processed using Aperture image processing software (Apple Inc.) for enhancing clarity. All the images were adjusted to same exposure, black points and brightness.

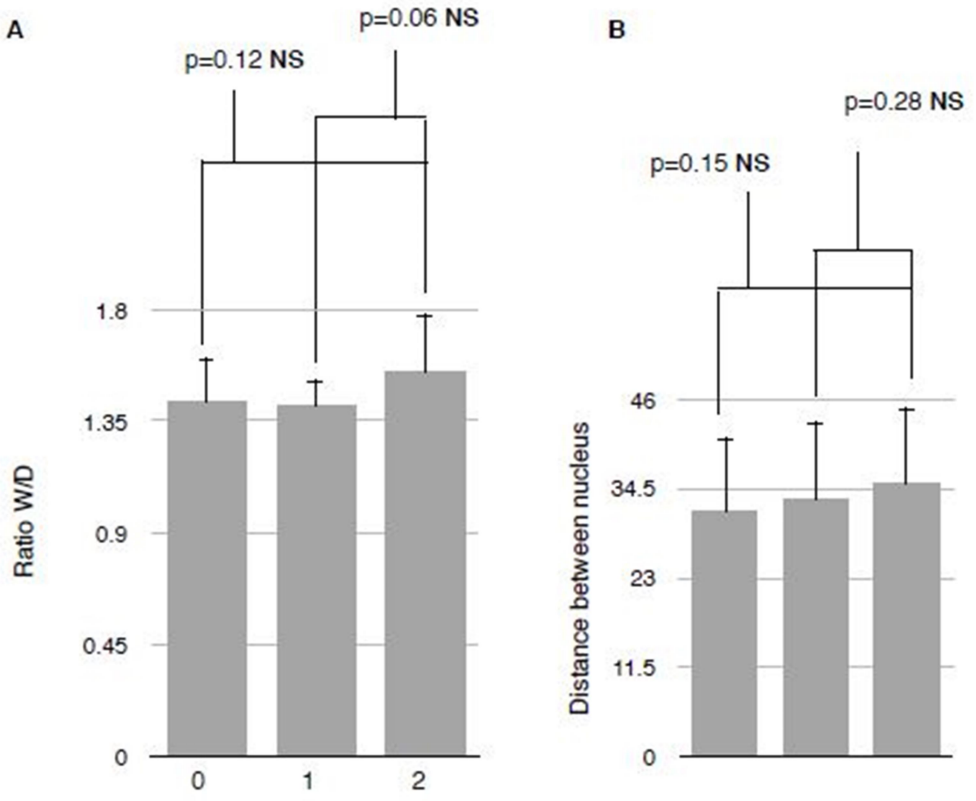


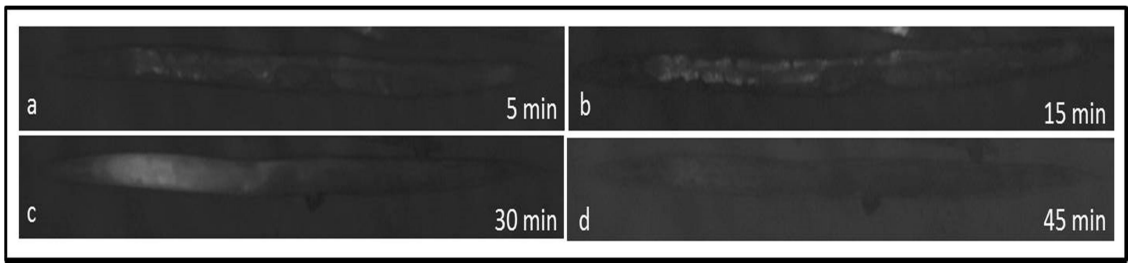
Figure 14: Negligible changes in cellular architecture of digestive tract in worms (MR142 strain) after treatment with 1% acetic acid. A. Structural changes in digestive tract was calculated using diameter of worm (W) vs the diameter of the digestive tract (D) in the anterior part of the middle body at three points. B. Distance between the nucleus. C - MR132 worms immediately after acetic acid treatment; 1- stage at which worms started showing nuclear disappearance (~20 to 30 minutes) and 2- worms after complete disappearance of nucleus (>60 minutes). Measurements were made using ImageJ software (V1.48). Images were adjusted using Aperture photo editing software to clearly visualise the cell boundaries and position of the nucleus. From each worm 3-5 values were calculated at each stage. N=3; worm were 3-4 days old adults. Error values are standard deviation. P values were calculated using student T-test (unpaired, one tail). NS - not significant.

3.5 Alterations in necrosis conditions did not alter the organismal death or karyolysis

3.5.1. Hypoxia

Prolonged hypoxia is a condition in which there is very low availability of oxygen and it is often seen in disease conditions like ischemia and stroke. Prolonged hypoxia induces necrotic cell death in *C. elegans*. In this study prolonged hypoxia was induced in *C.elegans* using sodium azide (Artal-Sanz et al., 2006) and effect of acetic acid was studied on that to check whether the nucleolysis occur faster compared to acetic acid treatment alone. Complete nucleolysis occurred (Figure 15A) within the time range of 14.10-50.10 min. Death fluorescence started within 9.25-18.10 min and fully spread within the time range of 12.55-28.00 min. This time frame was comparable to the worms treated with acetic acid alone. DAPI staining confirmed karyolysis (Figure 15B)

A.



B.

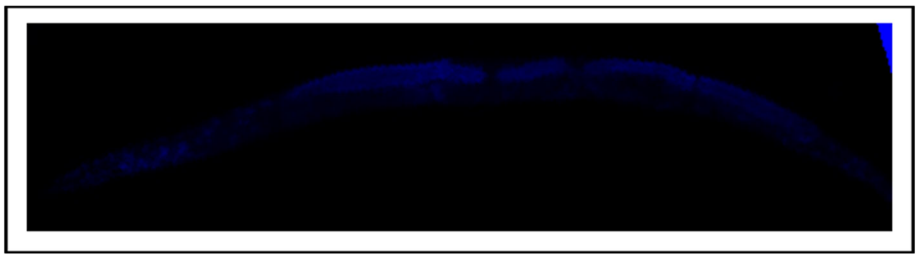


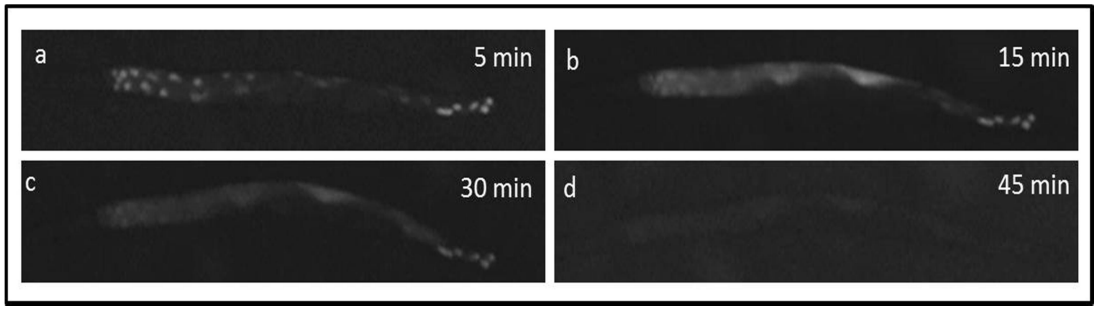
Figure 15. MR142 worms show both DF and nucleolysis when preconditioned with hypoxia. 1% acetic acid was used to induce death. A, low magnification images (4X)in time lapse mode. B. DAPI staining of treated worm. Representative image of more than 3 independent experiments. To observe the pattern of control refer figure 12 A.

3.5.2 Alkalization

Alkalization of endosomal and lysosomal compartments has protective effect on the organism against necrotic cell death. MR 142 worms were treated with alkalization agents like ammonium chloride and effect of acetic acid induced organismal death was assayed.

Complete nucleolysis was observed (Figure 16A) within the time range 23.05-36.10min and death fluorescence started within 5.10-11.35 min and completely spread within the time range of 9.20-16.20 min. This suggests that alkalization could not prevent the necrosis pathway. DAPI staining showed karyolysis (Figure 16B).

A



B.

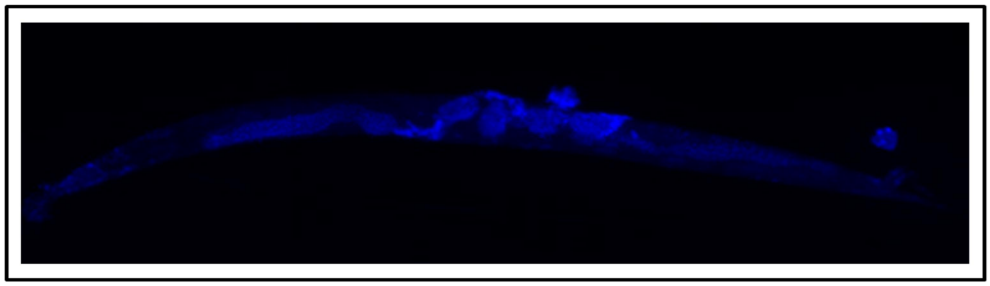
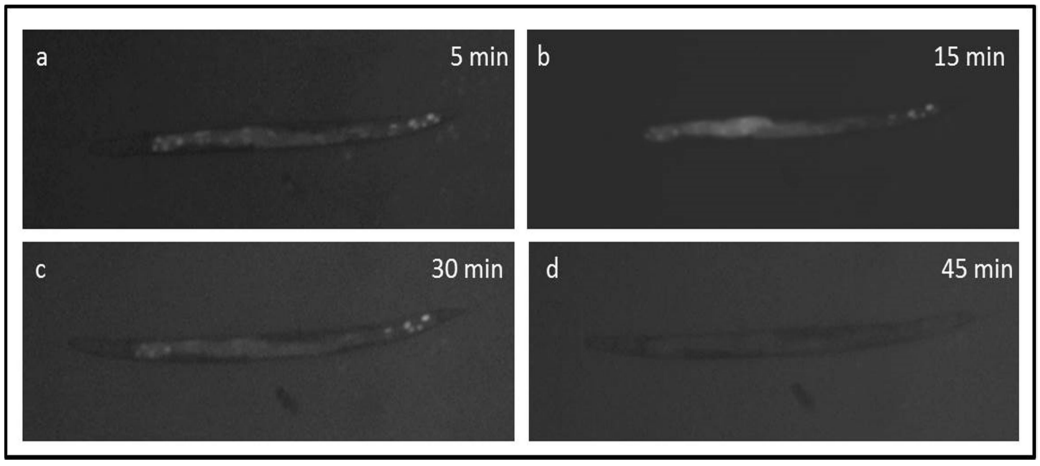


Figure 16. MR142 worms show both DF and nucleolysis when preconditioned with alkalization. 1% acetic acid was used to induce death. A. low magnification images(4X) taken in time lapse mode. B. DAPI staining. Representative image of more than 3 independent experiments of ammonium chloride treated worms. To observe the pattern of control refer figure 12 A.

3.5.3 c. Actinomycin D

Since karyolysis require nucleases, MR142 were treated with actinomycin D which is a potent transcription blocker to see whether that could alter the time kinetics of the nuclear disappearance and organismal death. Complete nucleolysis was observed (Figure 17A) within the time range of 31.15-47.05 min. Death fluorescence started within 4.05-11.00 min and fully spread within 9.35-29.50 min. This confirms that nucleolysis does not require newly synthesized nucleases. DAPI staining confirmed the karyolysis (Figure 17B).

A.



B.

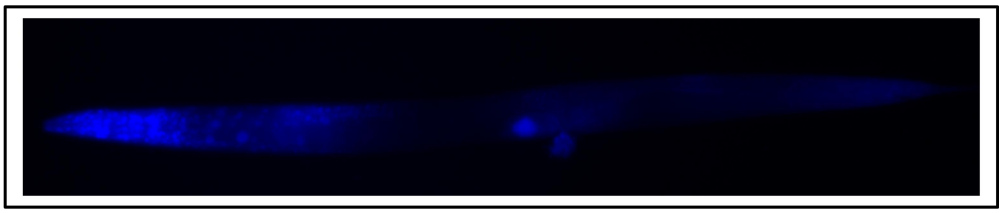
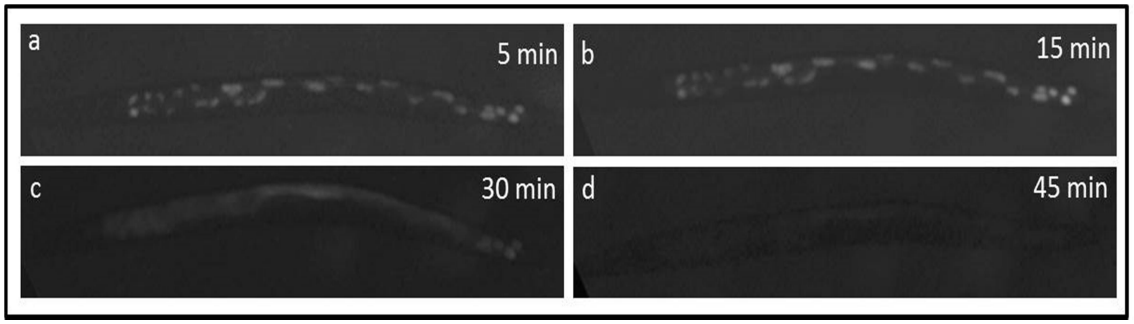


Figure 17. MR142 worms show both DF and nucleolysis when preconditioned with actnomycin D. 1% acetic acid was used to induce death. A, low magnification images (4X) taken in time lapse mode. B. DAPI staining of treated worm. Representative image of more than 3 independent experiments. of worms after actinomycin D treatment. To observe the pattern of control refer figure 12 A

3.5.4 Cycloheximide

To further test whether the nucleolysis require new transcription of nucleases, MR142 worms were treated with cycloheximide, a potent translational blocker. The data showed that complete nucleolysis occurred (Figure 18A) within the time range of 25.30-53.00 min. Death fluorescence started within 4.00-25.00 min and fully spread within10.00-33.00 min. This data further confirmed that the nuclear degradation was initiated with the existing nucleases in the cell. DAPI staining confirmed the karyolysis (Figure 18B).

A.



B.

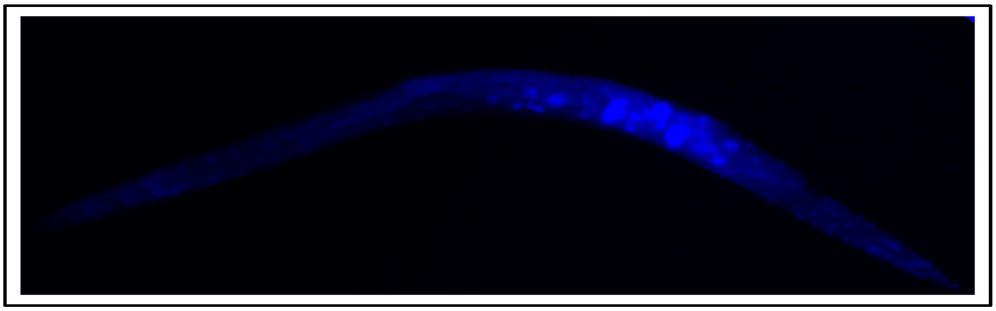


Figure 18. MR142 worms show both DF and nucleolysis when preconditioned with cyclohexamide. 1% acetic acid was used to induce death. A, low magnification images (4X) taken in time lapse mode. B. DAPI staining of treated worm Representative image of more than 3 independent experiments of worms after cycloheximide treatment. To observe the pattern of control refer figure 12 A.

3.6 Comparison of time kinetics of death fluorescence and complete nuclear disappearance of various treatments that alter necrosis.

A comparison between the time kinetics of death fluorescence and total nuclear disappearance of various treatments done to alter necrosis was attempted. It showed that in all treatments, the nuclear disappearance or karyolysis follows the death fluorescence. Translation block did show a significant delay in death fluorescence compared to that of control. However, this delay did not change the average timing of karyolysis though individual worms showed a wide range of variations in this timing compared to that of control. Since anthranilate pathway involves a series of enzyme kinetics, it could be possible that a protein in that pathway, probably, glucosyl esteration of anthranilate is regulated at the final stage. Sodium azide treatment also delayed the onset of death fluorescence in a significant way, suggesting need of oxygen to initiate the reaction. However these pathways did not affect the karyolysis in a significant manner, though the time delay in karyolysis after

death fluorescence showed a reduction (Fig.19). The reasons for these variations need to be further investigated.

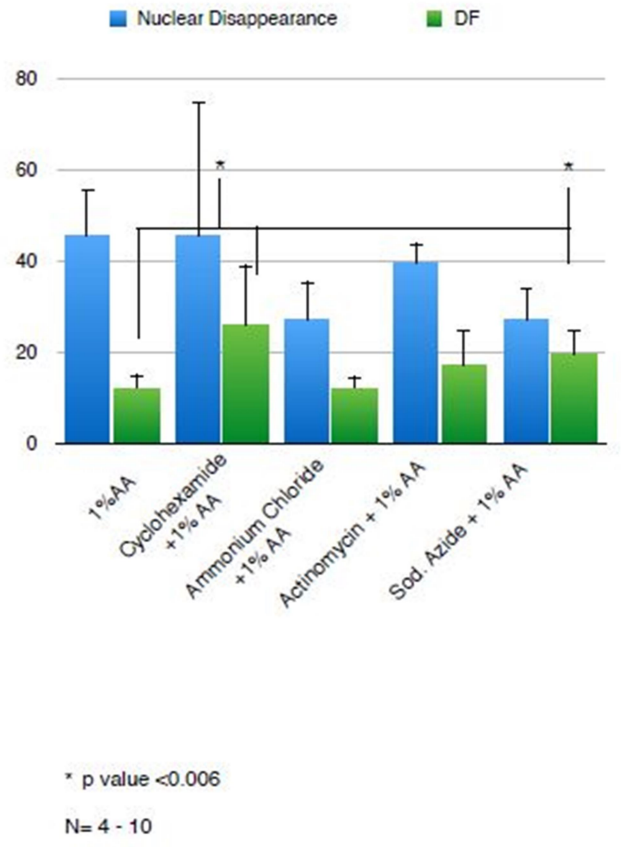


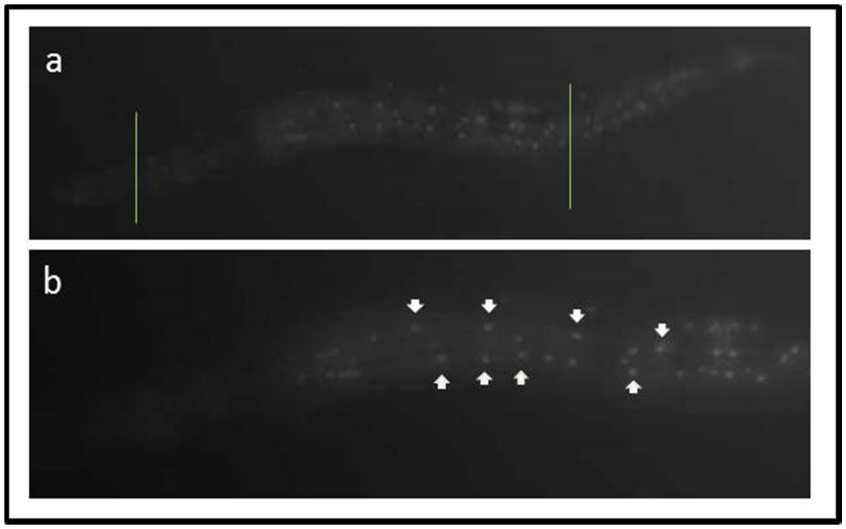
Figure19. Comparison of time kinetics of death fluorescene and complete nuclear disappearance of various treatments for altering necrosis.

3.7 PI staining

PI is a fluorescent stain that is used to differentiate between apoptotic, necrotic and live cells. PI is impermeable to cell membrane so live cell will not be stained with PI but as the cell dies the membrane integrity will be lost and thus allow PI to stain the nucleus. We attempted to test whether we can observe PI staining in the nucleus of worms as they undergo organismal

death. The worms were treated with acetic acid in the presence of PI and it was observed that the nucleus of the worm got stained within 5-7 minutes(Figure 20A) suggesting that the cells started undergoing necrotic process even before the initiation of DF. The worms without the acetic acid treatment but incubated with PI, was taken as the control (Figure 20B)

A.



B.

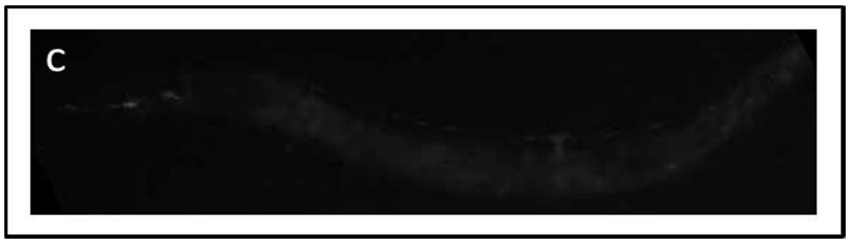


Figure 20. PI staining after acetic acid treatment. A. a.MR142 worms show PI staining during 1% acetic acid induced death. b. enlarged image of the treated worm with arrows showing the stained nucleus. B. Control worms without treated with acetic acid, in the presence of PI. Representative image of more than 3 independent experiments.

CHAPTER 4

DISCUSSION

Necrosis is initiated in C. elegans during organismal death due to several environmental insults. Often the cell death during necrosis undergoes visible cellular distortion and accumulation of small membrane whorls near plasma membrane. These changes have been documented in dying *mec-4(d)* neurons in C. elegans(Driscoll and Chalfie, 1991). Swelling of the cell is one of the characteristic changes during necrosis and has been reported in C. elegans also. However, in our study it has been found that no cell volume changes occur in the worm during death and the only visible necrosis marker is nuclear disappearance.

Recent studies reveal that during necrosis, there is fusion and gathering of intracellular organelles including lysosome around a *swollen nucleus*(Artal-Sanz et al., 2006). A central event that occurs in the necrotic process is the *elevation of cytoplasmic Ca2+ level* (Xu et al., 2001). The elevation is caused due to the influx of calcium from the extracellular space through voltage-gated channels and Ca2+/Na+ exchangers, and efflux from intracellular stores, mainly the endoplasmic reticulum and mitochondria. The potential mediators of Ca2+ are ER-channels, like the ryanodine receptors (RyR) and the inositol-1,4,5-triphosphate receptors (Ins(1,4,5)P3R). The dysfunction of these channels as well as the elimination of calcium-binding ER-chaperones like calnexin and calreticulin gives resistance to necrosis. Execution of necrotic cell death requires the activity of calcium-dependent calpain protease and aspartyl protease.

To carry out necrosis in nematodes the calpains CLP-1 and TRA-3 are required upstream of cathepsins ASP-3 and -4(Syntichaki and Tavernarakis, 2002). We tried to manipulate these pathways indirectly through Ca2+ channel mutants and through alkalization, but no significant delay was observed. It is tempting to suggest that there could be an alternate pathway for karyolysis and could be independent of necrosis. Our results also showed that PI staining was picking up in worms which are not yet dead, indicating a strong nucleolysis signal was initiated in dying cells. The DF may not have a significant correlation with karyolysis as worms lacking Ca2+, which could not release anthranilic acid, were also undergoing similar changes. Besides the intactness of cellular structure, intact nuclear membrane was also observed in these worms lacking nucleus.

Karyolysis has been observed in ischemic heart tissue and has been suggested as a necrotic change. Moreover, the nucleolysis is not dependent on excessive synthesis of nucleases. This could be the reason for less delay in initiation of karyolysis in these cells. The predictability of organismal death in C. elegans makes it a very good model to study death associated changes in cells. Besides it has an internal fluorescence marker, DF, to show that the cell death has been initiated. Both these parameters help in following the cellular changes in a time kinetics manner. To our knowledge this is the first study, which could document these cellular changes in a living animal system.

It would be interesting to see the downstream changes in the cell after the necrotic pathway sets in. Since all cell death pathways have nuclear disintegration as one of the marker, it is essential to understand and decode the underlying molecular mechanism.

CHAPTER 5

SUMMARY

The study was initiated to understand the cellular changes during anthranilate fluorescence and organismal death in C. *elegans*. We could be able to document a series of interesting changes in the animal including karyolysis. The kinetics of these changes indicate that the process is highly orchestrated and once initiated it has a dominos effect.

We could induce organismal death both by heat shock and 1% acetic acid in a predictive manner. Using various mutants we have shown that the karyolysis follow the pattern of death fluorescence since the cells, which first showed the nuclear disappearance, coincided with the cells which first showed death fluorescence. However, these changes in nucleus are very subtle without affecting any major cellular architecture. Karyolysis seems to be independent of Ca2+ and lysosomal changes. Altering both these pathways did not alter the time kinetics of karyolysis. This cellular insult does not depend on new transcription or translation and could orchestrate the event within a short period of time. Propidium iodide (PI) staining showed a time dependent staining pattern in worms as soon as the DF was initiated. The worms were still alive when PI started showing staining in the organism.

It would be interesting to see the downstream changes in the cell after the necrotic pathway sets in. Since all cell death pathways have nuclear disintegration as one of the marker, it is essential to understand and decode the underlying molecular mechanism.

CHAPTER 6

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ANNEXURE

1. Nematode Growth Medium (NGM)

For 100 ml

NaCl	0.3 g
Agar	1.7 g
Peptone	0.25 g
Distilled water	97.5 ml

Cover it and autoclave for 50 min. Cool the flask in 55□C for 15 min in water bath. Then add:

1 M $CaCl_2$	0.1 ml
5mg/ml cholesterol	0.1 ml
1 M $MgSO_4$	0.1 ml
1 M KPO_4 buffer	2.5 ml

Mix well and pour into petriplates and allow to dry.

2. M9 buffer

For 1 litre

KH_2PO_4	3 g
Na_2HPO_4	6 g
NaCl	5 g
1M $MgSO_4$	1 ml

Mix well and make up the solution to 1 litre and sterilize by autoclaving.

3.100X Poly L Lysine Stock Solution

Poly L Lysine hydrobromide	10 mg
Sterile distilled water	10 ml

Stored at -20°C