Mechanisms of cell death in eukaryotic microorganism: An in-depth overview

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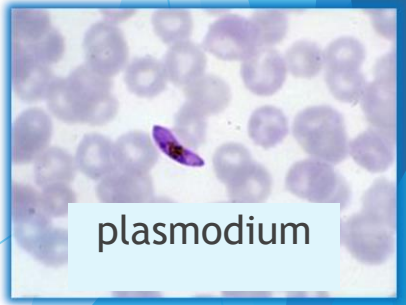
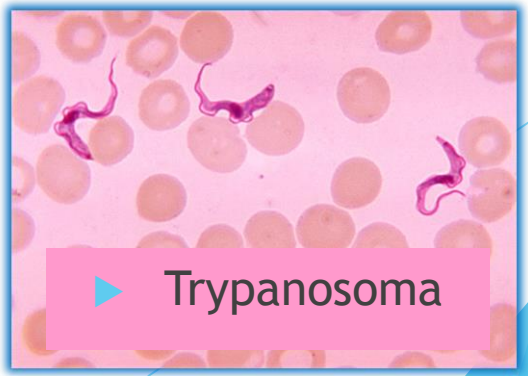
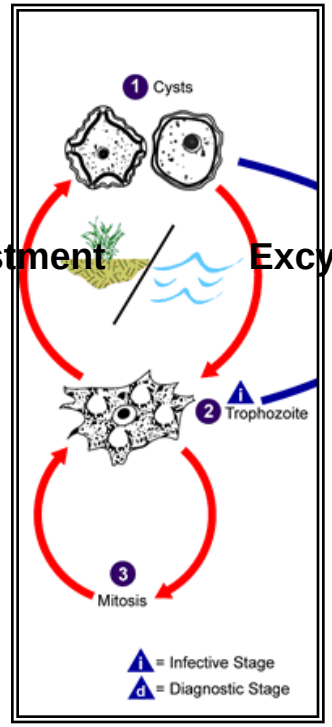
- Eukaryotic microorganisms- shared similar characteristics with mammalian cells,
  - typical plasma membrane,
  - organelles,
  - life cycle progression
  - death pathway mechanisms,
- single living cell- life cycle is shorter than mammalian cells.
- Pathogenic species of eukaryotic microorganism such as *Leishmania*, *Plasmodium*, *Trypanosome*, *Toxoplasma* have been studied for many years for their potential antimicrobial therapy.
- The mode of drug action and cell death - crucial as treatment for this disease wouldn't harm the host.

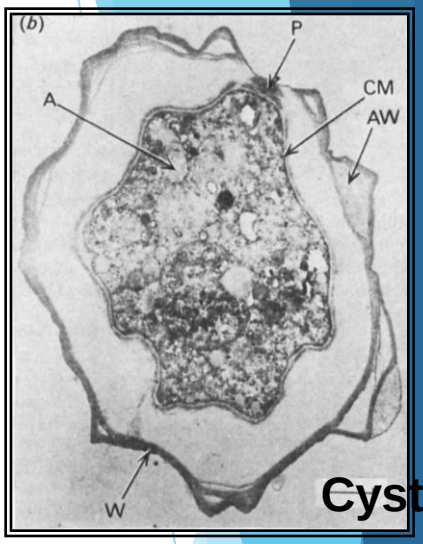




Acanthamoeba

- Abundant in all type of habitat which support growth for bacteria, its major source of nutrient.
- Free living and opportunistic protozoa





Cyst


Trophozoite

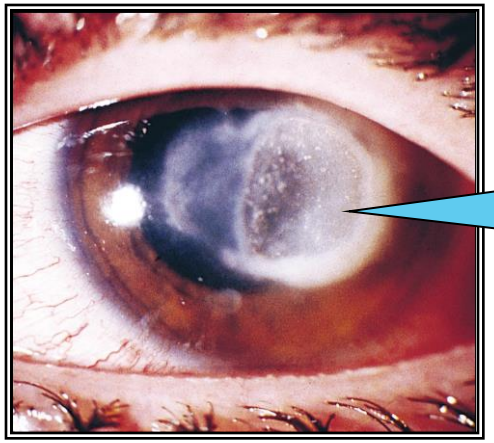
Infections inflicted in man by *Acanthamoeba*

- Systemic infection (brain, skin, CNS)
- Ocular infection - *Acanthamoeba* keratitis (AK)

"... 85 % of AK cases is from contact lens wearers.." (Murdoch et al., 1998)

".....Often misdiagnosed as bacteria, fungi keratitis..." (Yun et al., 2006)

".....Delayed treatment can cause blindness...." (Khan, 2003)



Formation of ring infiltration after 5 months infected by *Acanthamoeba*

Current treatment for *Acanthamoeba* keratitis

- Chlorhexidine gluconate (CHX) and Polyhexamethelene biguanide (PHMB), effective in killing *Acanthamoeba* spp. (Khan, 2003)
- CHX

  "…. more effective than PHMB…" (Noble, 2002)

  "….has been recommended for many *Acanthamoeba* keratitis treatment…" (Hay et al., 1994)

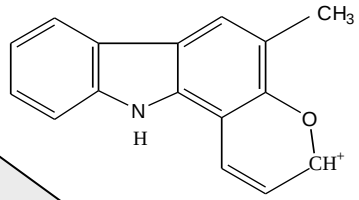
  "…. induced membrane disruption in bacteria and yeast…." (Russel and Hugo, 1988)

  "…. able to irritate to the eye, ulcerative keratitis, swollen cornea nuclei, alteration to the cell membrane and cytolysis… " (Masamichi et al., 2005)

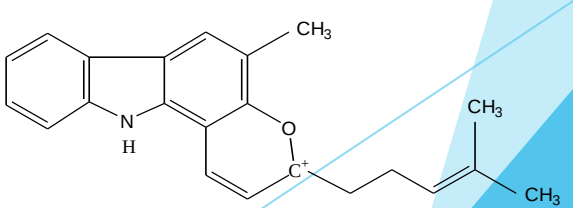
- Alternative treatment – surgical excision of the infected cornea
- Search for drug with low toxicity is essential!
- **Apoptosis and autophagic cell death mechanisms - the safest way for the diseased cell or parasite should die.**

Murraya koenigii (L) Spreng.

GIRINIMBINE (GR)



MAHANIMBINE (MH)



The information obtained from Prof. Dr. Aspollah Sukari , UPM

Cytotoxicity in drug discovery

- *Acanthamoeba* - Euchromatin, mitochondria and cell membrane
- Cytotoxicity and mode of cell death determination is important in cancerous cell
- The important of cytotoxicity assay-

  -to determine the cell killing property of chemical compound or mediator cell (Doris et al., 1988), confirmed that the drugs can be used or not
- IC_{50} value- allows the comparison of chemical necessary to inhibit any measurable parameter (cell proliferation, protein synthesis, DNA synthesis etc.)
- Membrane integrity, chromatin pattern, DNA cleavage
- Cell death : Apoptosis (Programmed cell death) and Necrosis
- Cytotoxicity in this study -Drug search for pathogenic amoeba:
- This study focused only on cytoxicity and genotoxicity effects of the MH, GR and CHX on trophozoite of *Acanthamoeba* spp.

- Cultivation and maintenance of the test Organisms
- Determination of *Acanthamoeba* Population Doubling Time (Freshney et al. 1987)
- Preparation of MH, GR and CHX stock solution
- Cell viability and IC_{50} values determination by Eosin Dye Technique Assay (Wright et al., 1988) after treatment with MH and GR.
- Observation on Morphology of *Acanthamoeba* spp. after treatment with MH and GR.
  - By Light Microscopy
  - By SEM
  - By TEM

METHODOLOGY

Results and Discussion

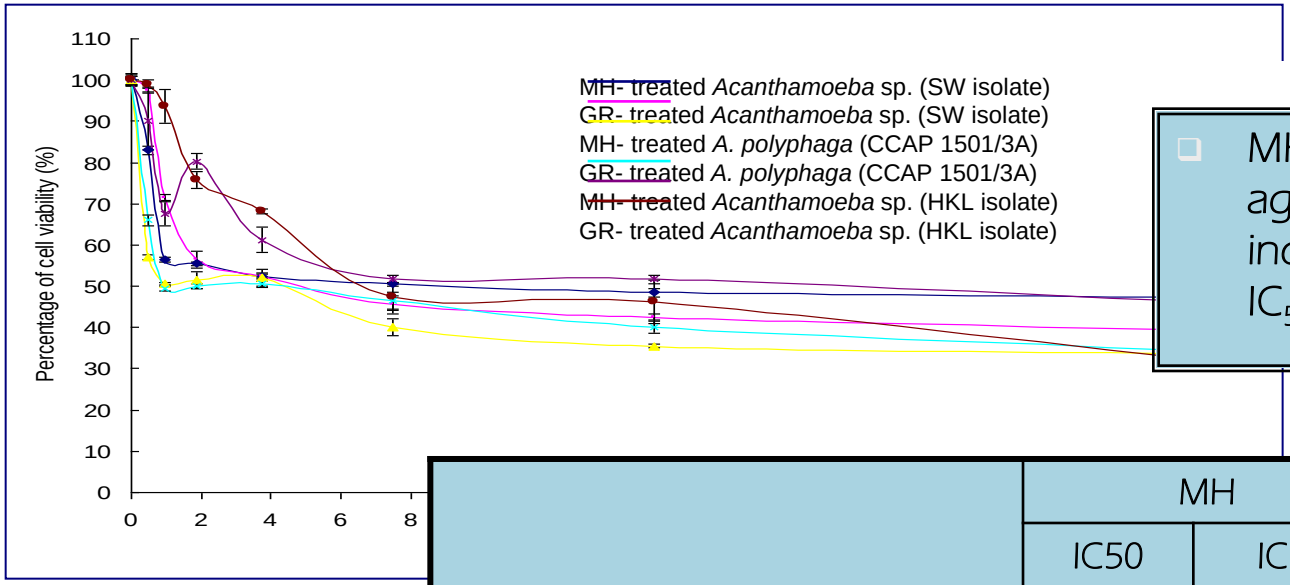
MATERIALS AND METHODS

Test organisms and cultivation

No.	Species	Isolate	Source	Pathogenicity
1	*Acanthamoeba castellanii*	IMR	Unknown	Pathogen
2	*Acanthamoeba polyphaga* (Puschkarew) Volkonsky 1931	CCAP 1501 /3A	Freshwater, Winsconsin USA	Potential pathogen
3	*Acanthamoeba* sp.	HKL	Corneal scrapping from a keratitis patient (Hospital Kuala Lumpur)	Pathogen
4	*Acanthamoeba* sp.	SW	Freshwater, Setiu Wetland	Not known

(Sub-cultured every 3 to 5 days and were maintained in 30 °C incubator)

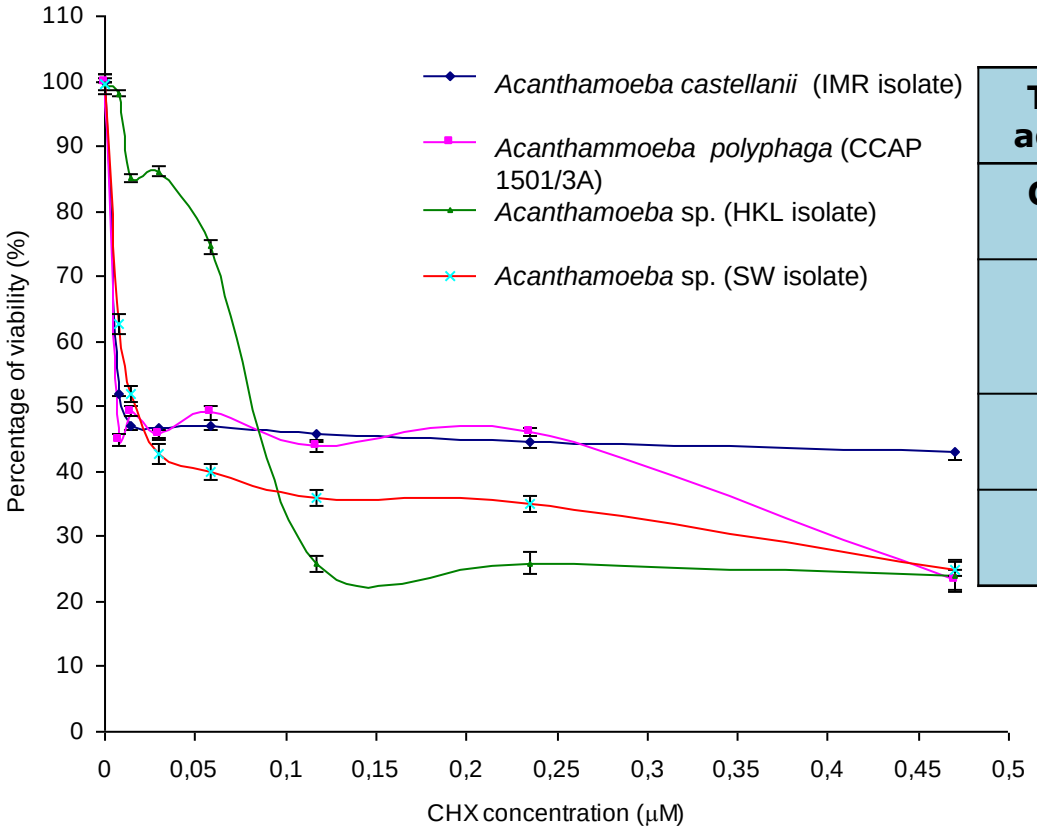
Fifty percent Inhibition Concentration (IC_{50}) Values for MH and GR Determined by Eosin Dye Technique Assay



- MH and GR have the potential against *Acanthamoeba* spp. indicated by active range of IC_{50} values

	MH		GR	
	IC50 (µg/mL)	IC50 (µM)	IC50 (µg/mL)	IC50 (µM)
A. castellanii (IMR isolate)	1.18	3.5	3.04	11.1
A. polyphaga (CCAP 1501/3A)	1.00	3.0	2.00	7.6
Acanthamoeba sp. (HKL isolate)	20.6	62.2	6.8	25.8
Acanthamoeba sp. (SW isolate)	7.2	21.7	4.2	15.9

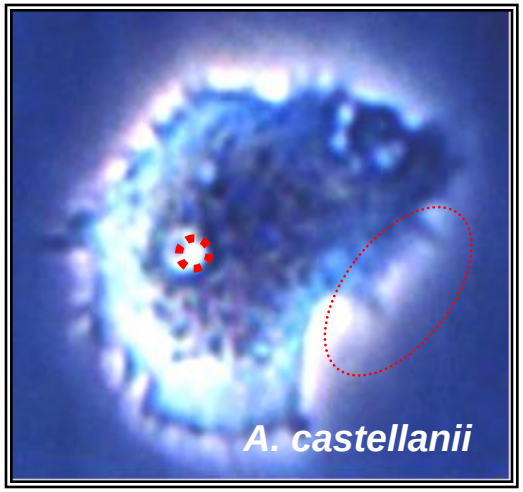
Fifty percent Inhibition Concentration (IC_{50}) Values for CHX Determined by Eosin Dye Technique Assay

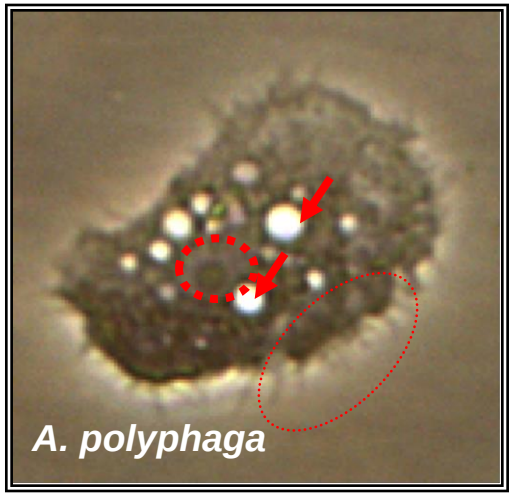


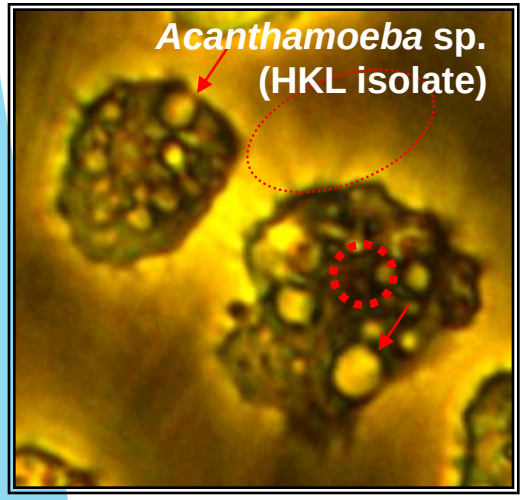
Test agent	*Acanthamoeba* spp.	IC_{50} (μM)
CHX	***Acanthamoeba castellanii*** **(IMR isolate)**	**0.055**
	Acanthamoeba polyphaga **(CCAP 1501/3A)**	**0.094**
	***Acanthamoeba* sp.** **(HKL isolate)**	**0.968**
	***Acanthamoeba* sp.** **(SW isolate)**	**0.34**

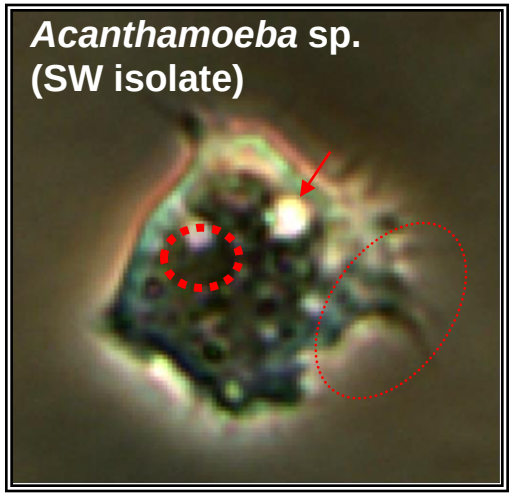
Light Microscopy Observation.

Untreated *Acanthamoeba* spp.



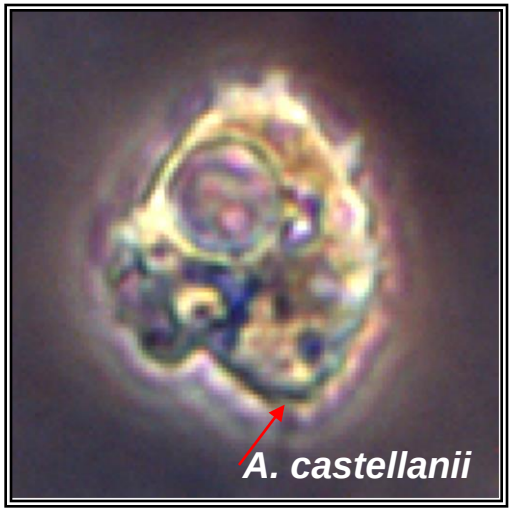


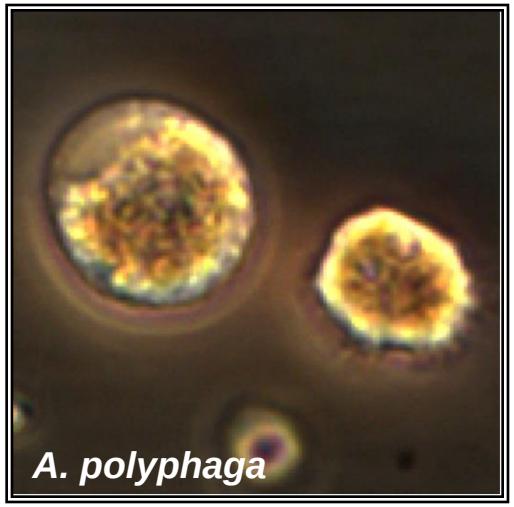


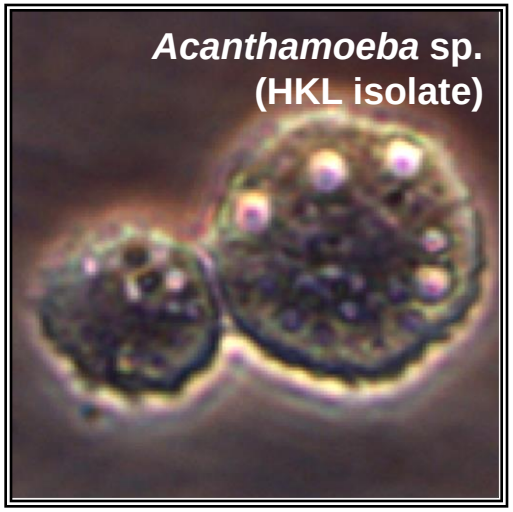


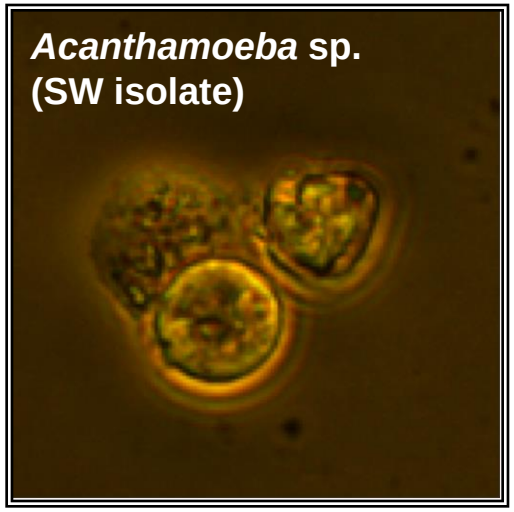
- Asymmetrical flat shape of amoeba cells
- Prominent nucleus, contractile vacuoles & acanthapodia

GR-treated *Acanthamoeba* spp.



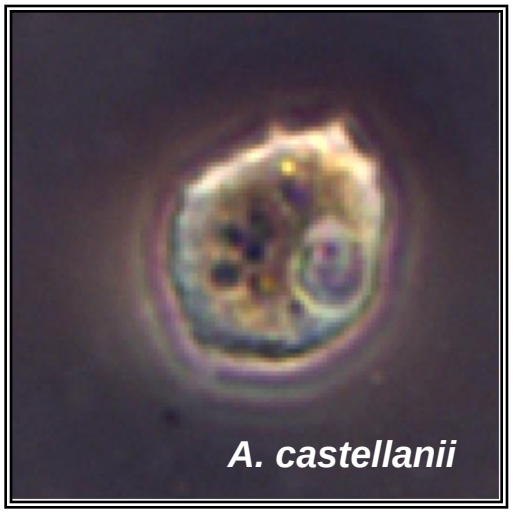


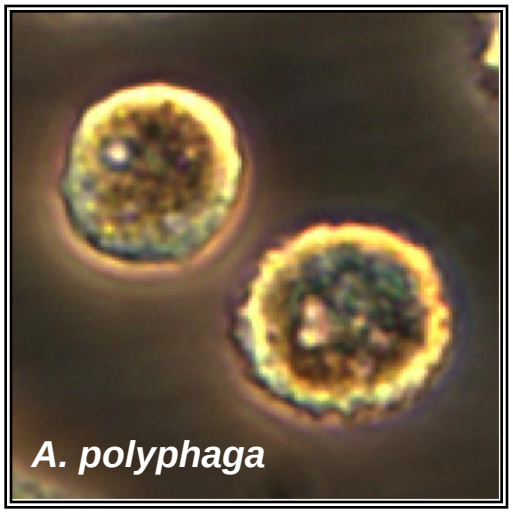


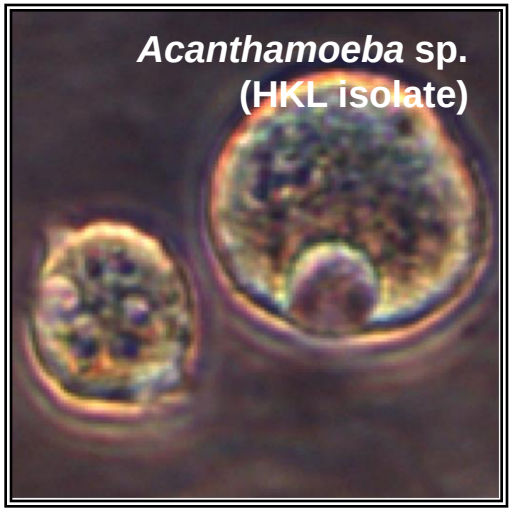


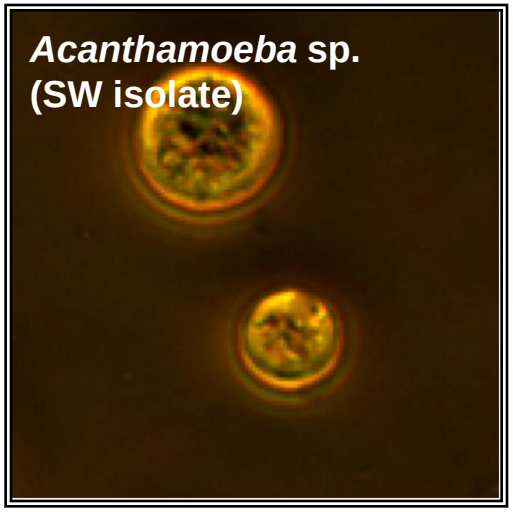
- Rounded shape of *Acanthamoeba* cells
- Shortened of acanthapodia structure
- Begin to encyst

MH-treated *Acanthamoeba* spp.



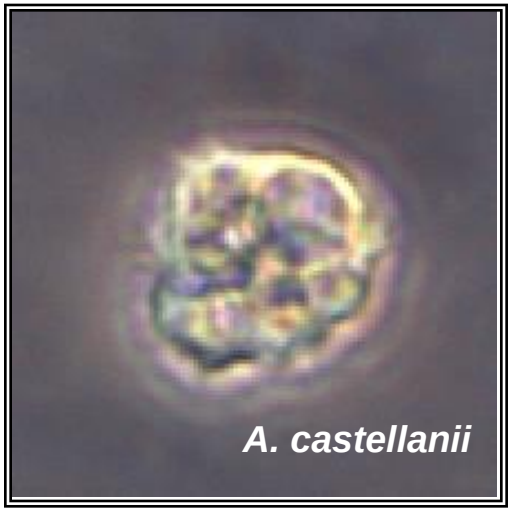


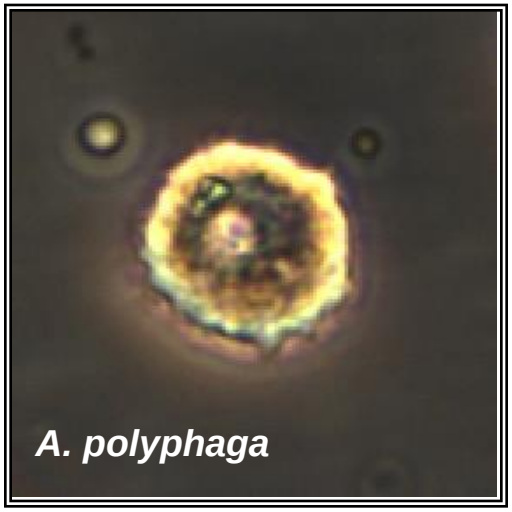


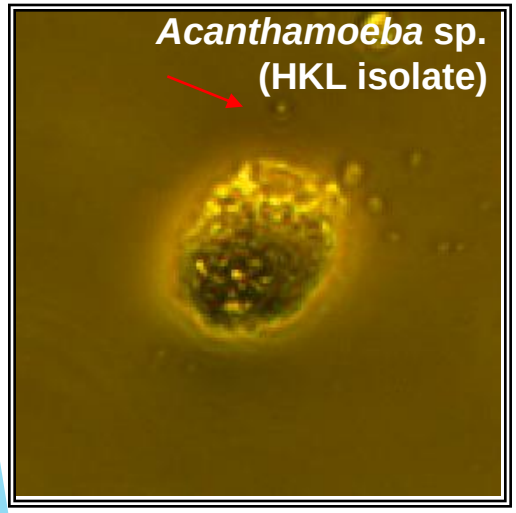


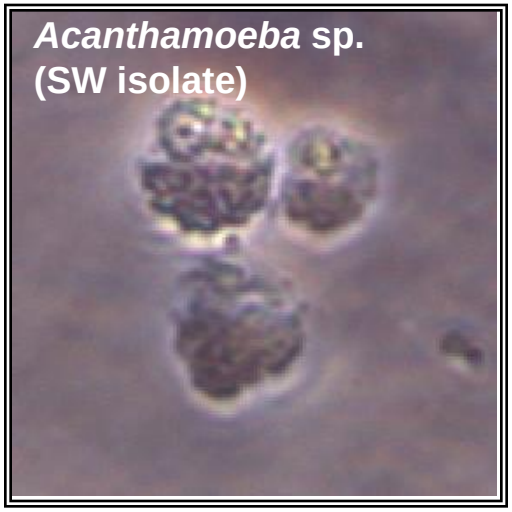
- Rounded shape of *Acanthamoeba* cells
- Shortened of acanthapodia structure
- Begin to encyst

CHX-treated *Acanthamoeba* spp.









- Rounded shape of *Acanthamoeba* cells
- Shortened of acanthapodia structure
- Begin to encyst

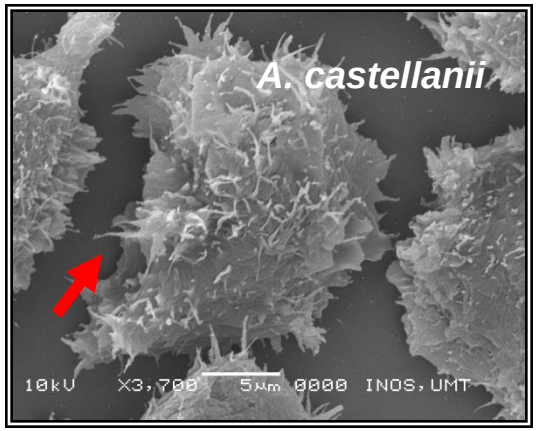
The effects of MH, GR and CHX on *Acanthamoeba* spp. size

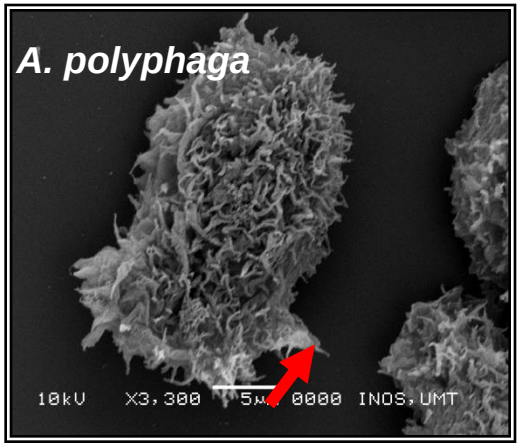
Acanthamoeba isolates	Treatment	Size	
		Length ± S.D (µm) (n=30)	Width ± S.D (µm) (n=30)
Acanthamoeba castellanii (IMR isolate)	Control	23.55 ± 4.36	13.75 ± 2.50
	MH	17.07 ± 3.69*	13.81 ± 3.42
	GR	14.39 ± 3.61*	13.20 ± 3.42
	CHX	15.96 ± 2.36*	13.00 ± 3.13
Acanthamoeba polyphaga (CCAP 1501/3A)	Control	25.62 ± 5.80	16.23 ± 4.26
	MH	20.16 ± 3.17*	12.36 ± 3.48*
	GR	23.25 ± 5.84	16.23 ± 5.12
	CHX	19.82 ± 3.60*	12.65 ± 6.52*
Acanthamoeba sp. (HKL isolate)	Control	23.82 ± 5.24	16.98 ± 4.67
	MH	17.92 ± 3.23*	13.29 ± 2.84*
	GR	18.32 ± 3.20*	14.19 ± 3.15*
	CHX	19.54 ± 3.86*	14.56 ± 3.88*
Acanthamoeba sp. (SW isolate)	Control	23.21 ± 3.09	15.78 ± 3.87
	MH	21.51 ± 3.75	19.32 ± 3.84
	GR	17.78 ± 2.47*	15.02 ± 2.16
	CHX	16.95 ± 3.11*	14.37 ± 3.91

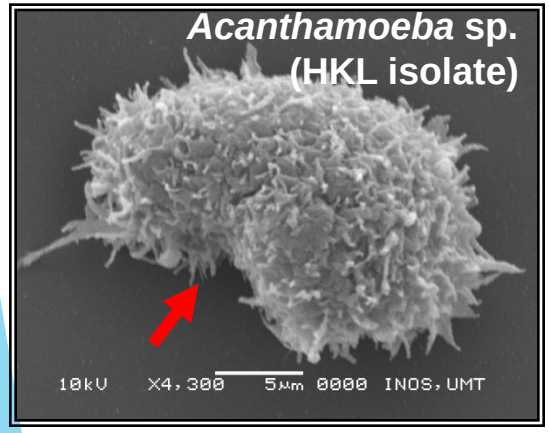
Significant reduction of cell size compared to untreated *Acanthamoeba* ($P<0.001$)

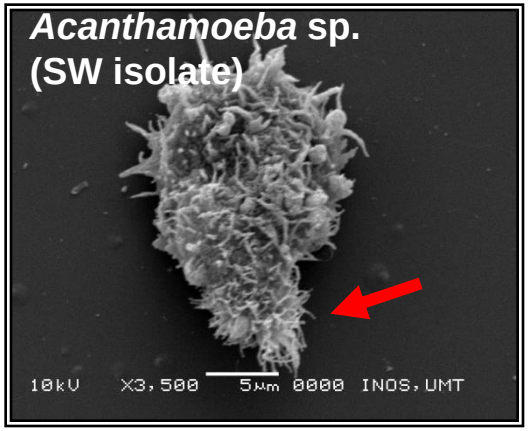
- Implies a significant loss of cytoplasmic constituents
- Dense cytoplasms observed- dehydration

SEM Observation





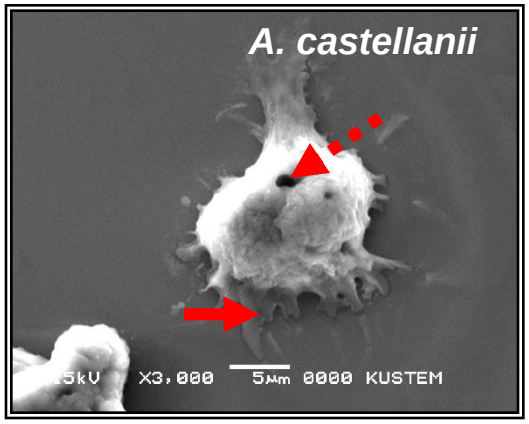


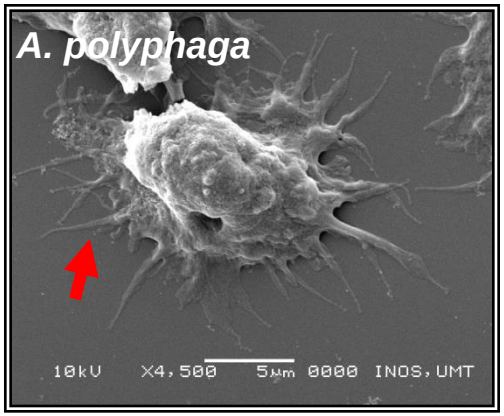


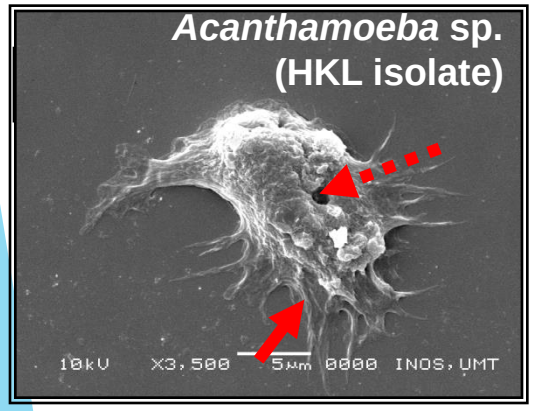
Untreated *Acanthamoeba* spp.

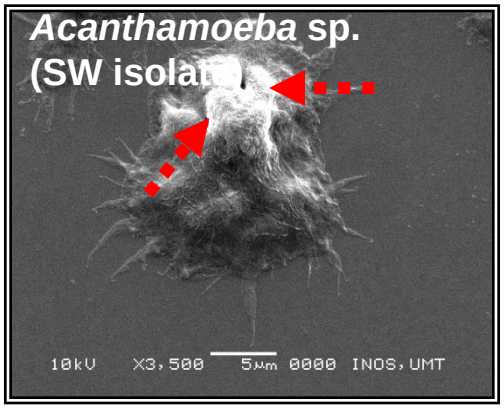
- Irregular *Acanthamoeba* shape
- Numerous acanthapodia (spine-like structure) on cell surface

MH-treated *Acanthamoeba* spp.



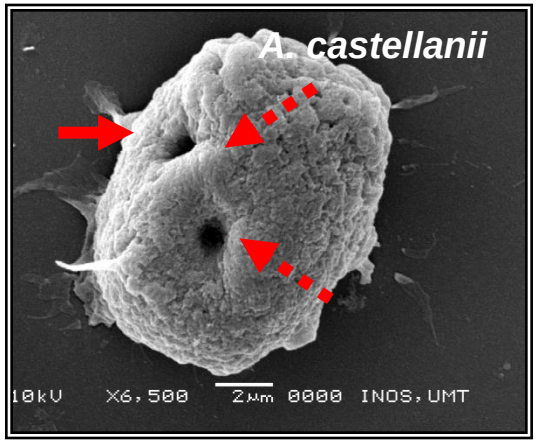


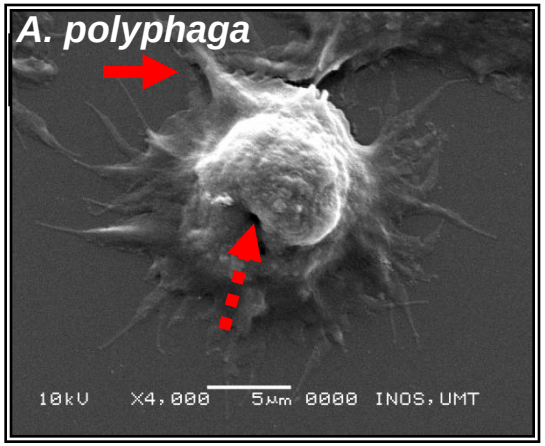


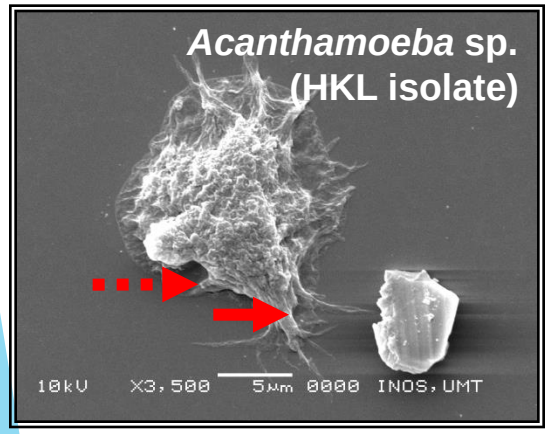


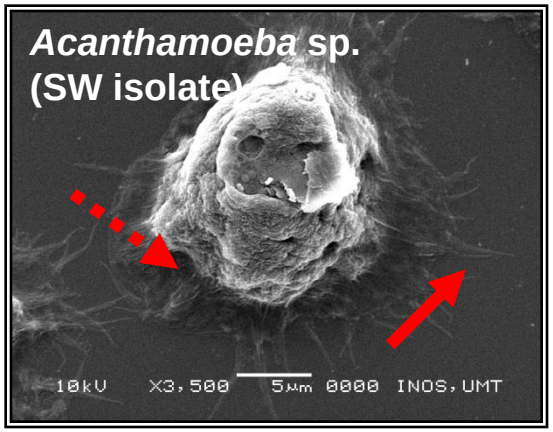
- Loss of spine-like appearance (acanthapodia)
- Uneven cell surface
- Cystic shape of *Acanthamoeba* cell
- Sunken food cup
- Elongated acanthapodia attached to the substratum

GR-treated *Acanthamoeba* spp.









- Loss of spine-like appearance (acanthapodia)
- Uneven cell surface
- Cystic shape of *Acanthamoeba* cell
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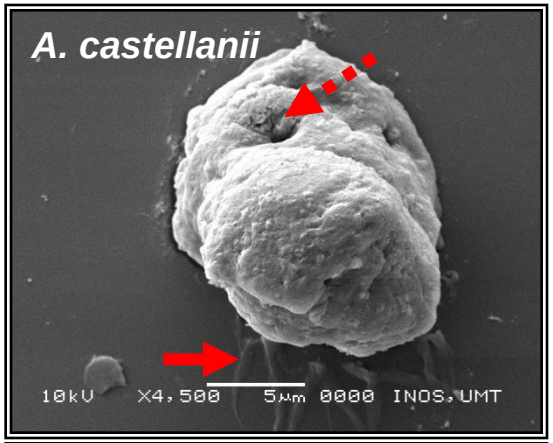
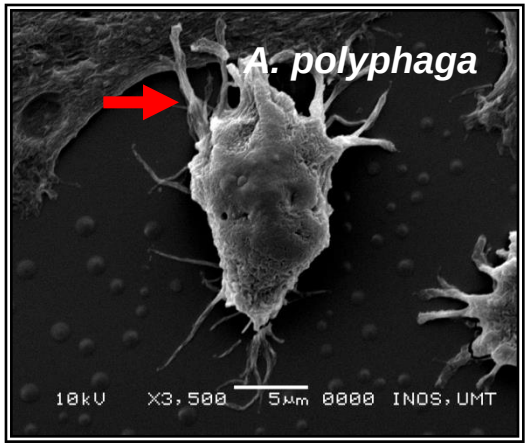
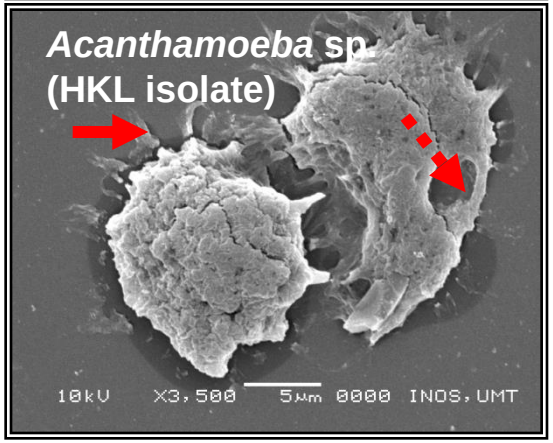
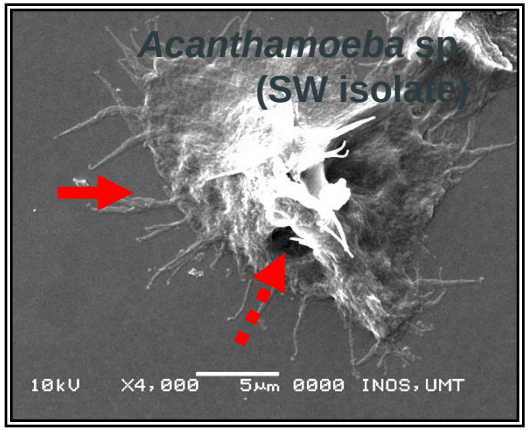
- Elongated acanthapodia attached to the substratum

CHX-treated *Acanthamoeba* spp.









- Typical acanthapodia still occurred after treatment with CHX on the cell surface
- Wrinkle cell surface
- Cystic shape of *Acanthamoeba* cell
- Sunken food cup
- Loss of acanthapodia
- Abnormal appearance of CHX-treated *Acanthapodia* (compressed).

- The amoebae were undergo encystment process
- Loss of acanthapodia and pseudopodia structure, rounded shape of amoebae

  "..unable to bind to the host cells which leads to phagocytosis (engulfing host cells).." (Khan, 2003)
- Cyst are survival forms of Acanthamoeba under harsh conditions.
- Cystic shape cells- reduce surface area for absorption

  "....cyst are non-infective stage of *Acanthamoeba.."*

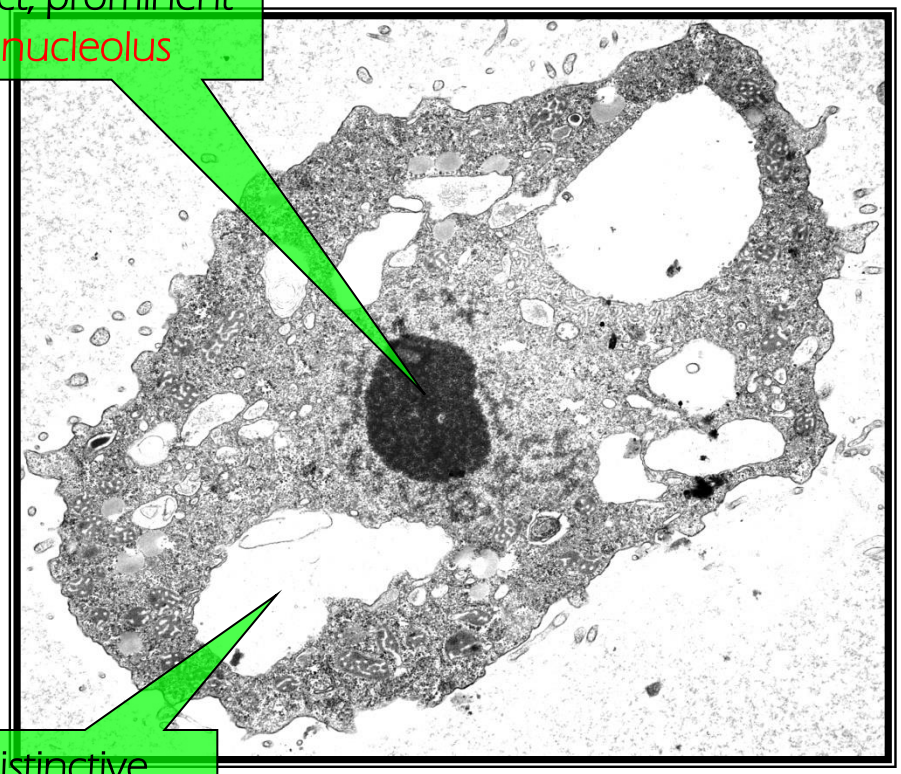
  (Dudley et al., 2005, Garate et al., 2006)

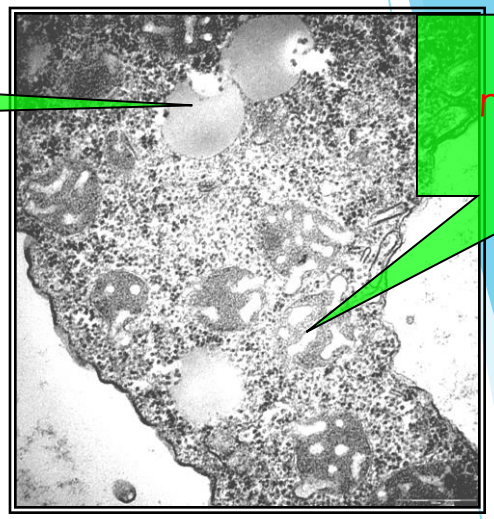
TEM Observation

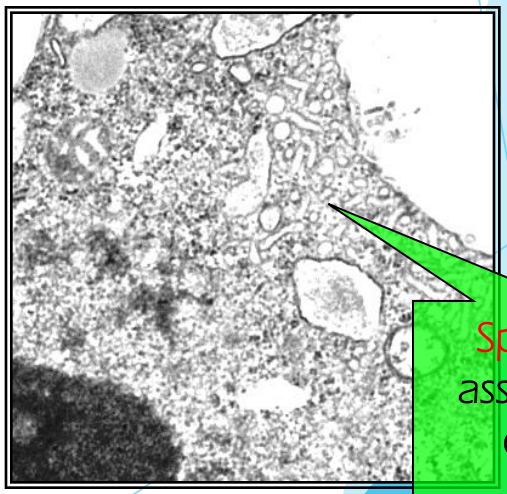
Rounded shape of lipid droplets

Distinctive mitochondria cristae

Intact, prominent nucleolus





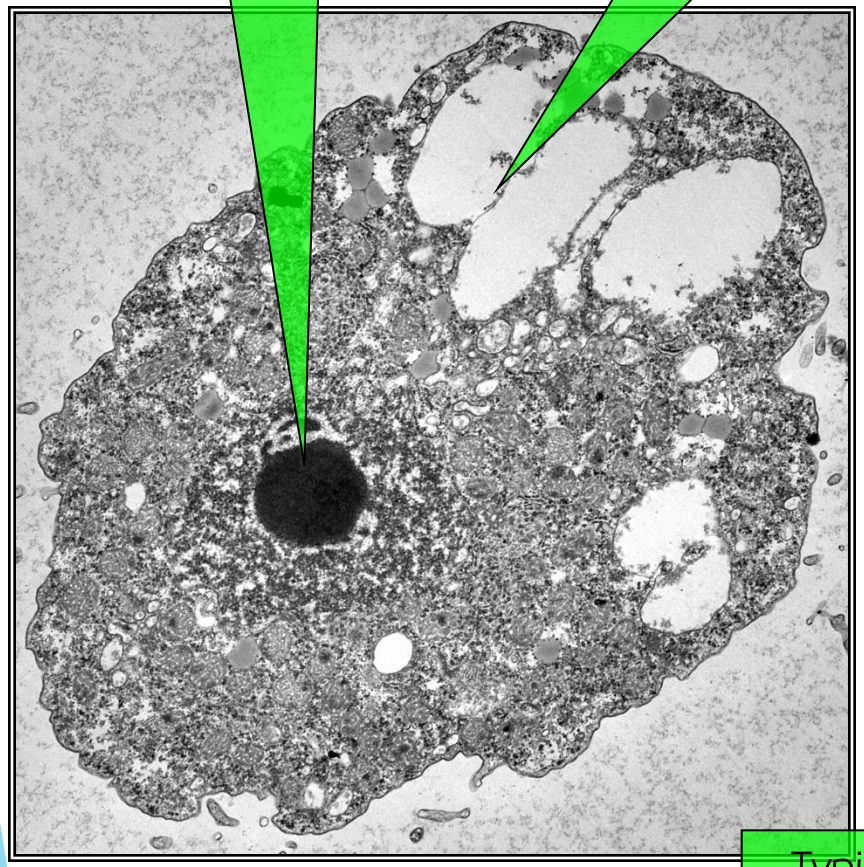


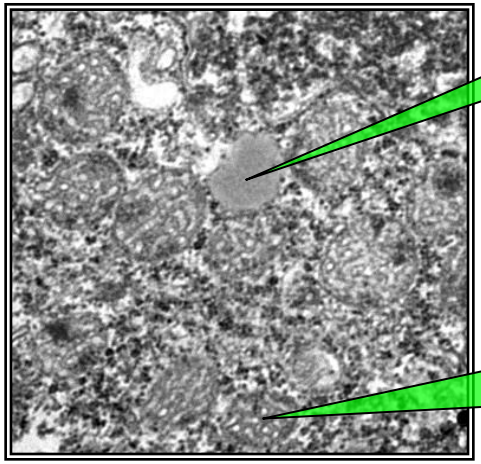
Spongisomes associated with contractile vacuoles

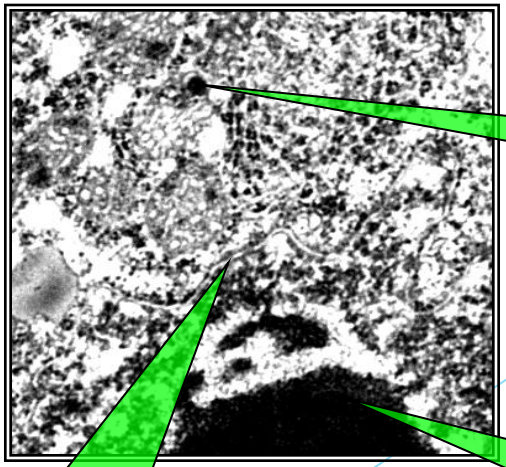
Distinctive contractile vacuoles

Untreated *A. castellanii* (IMR Isolate)

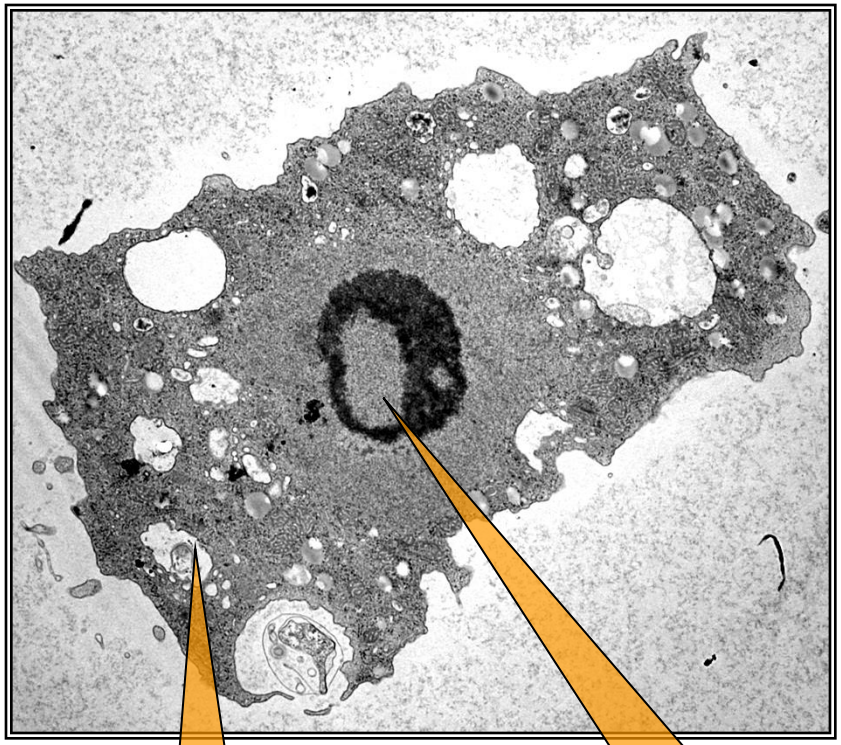
Untreated *A. polyphaga* (CCAP 1501/3A)

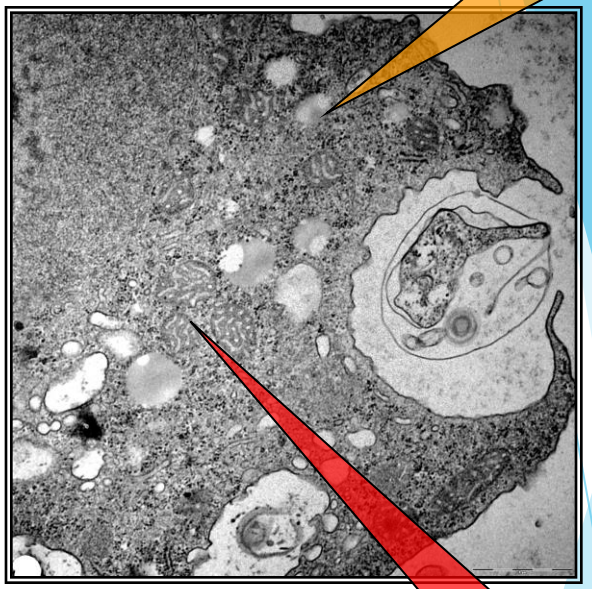






MH-treated *A. polyphaga* (CCAP 1501/3A)





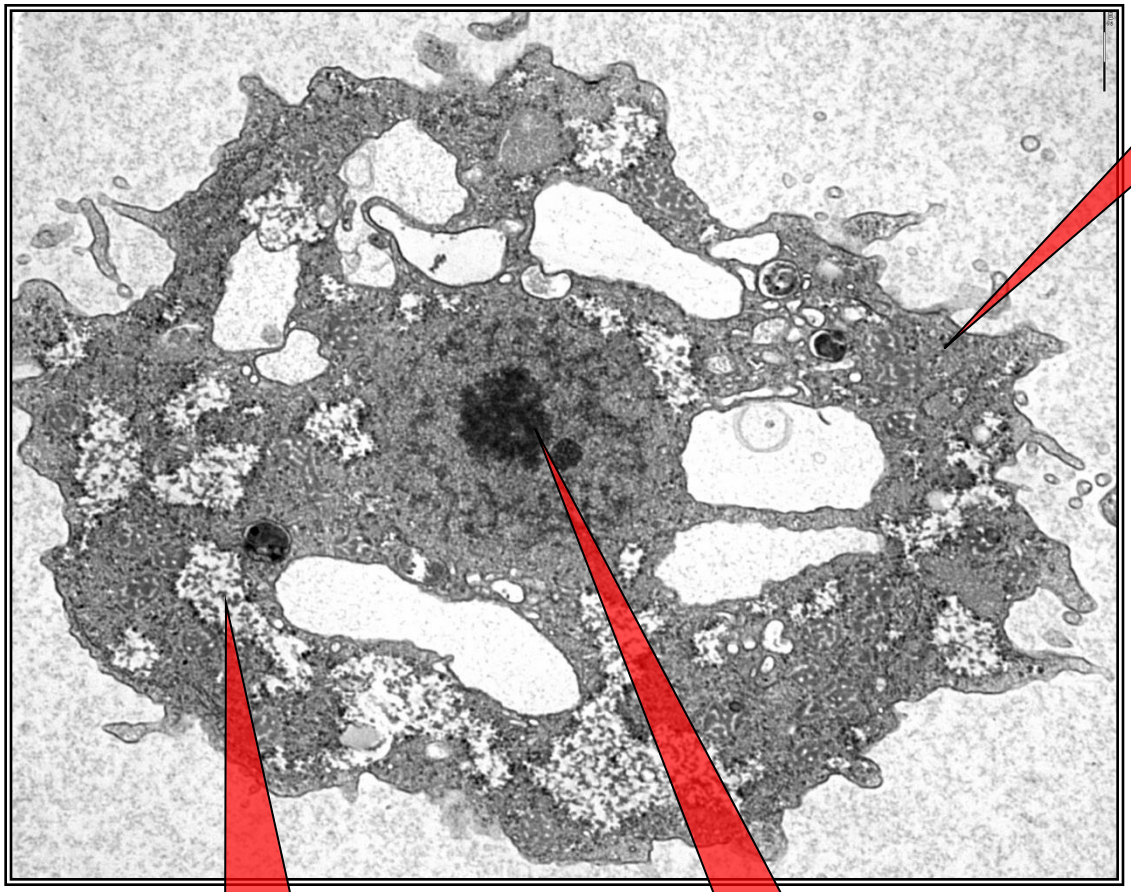
Decreasing volume of lipid droplets

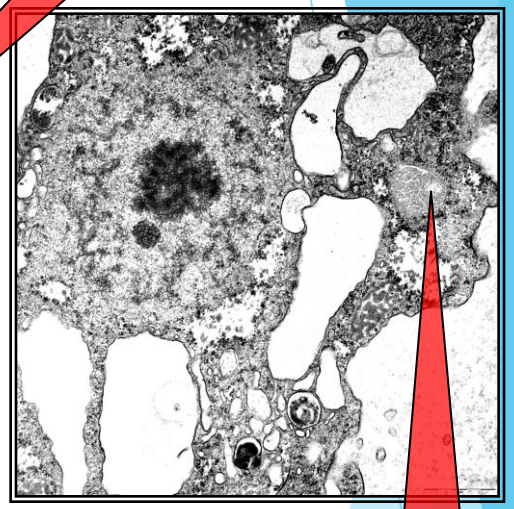
Abnormal mitochondria cristae formation

Formation of lacuna in the nucleolus

Activity in digestive vacuoles

MH-treated *Acanthamoeba* sp. (HKL isolate)





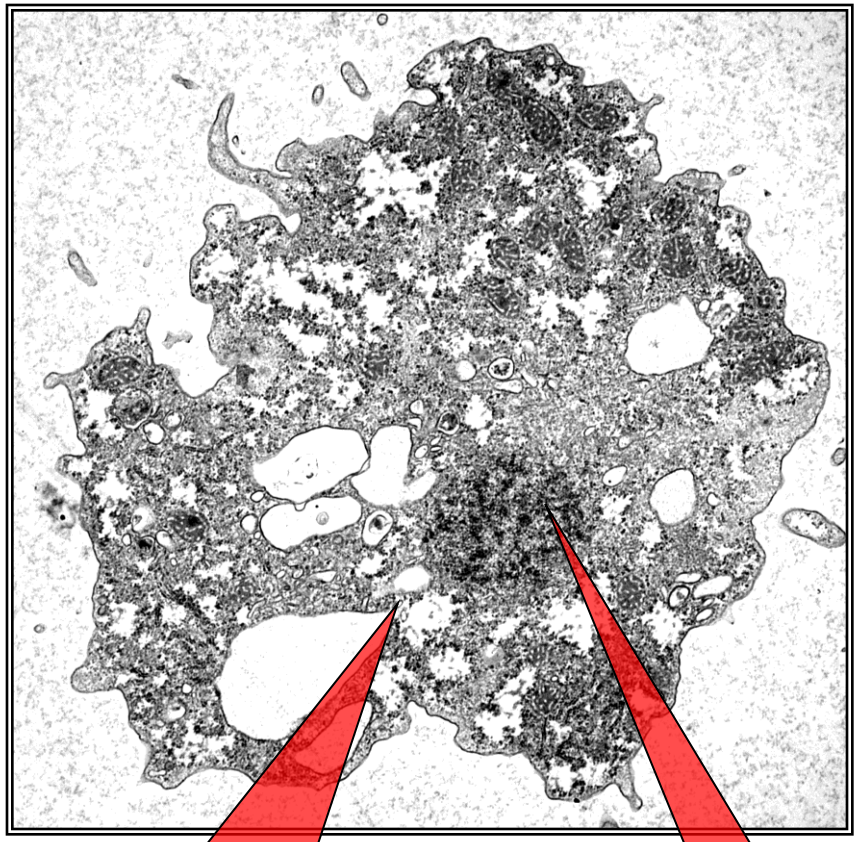
Abnormal shape of mitochondria

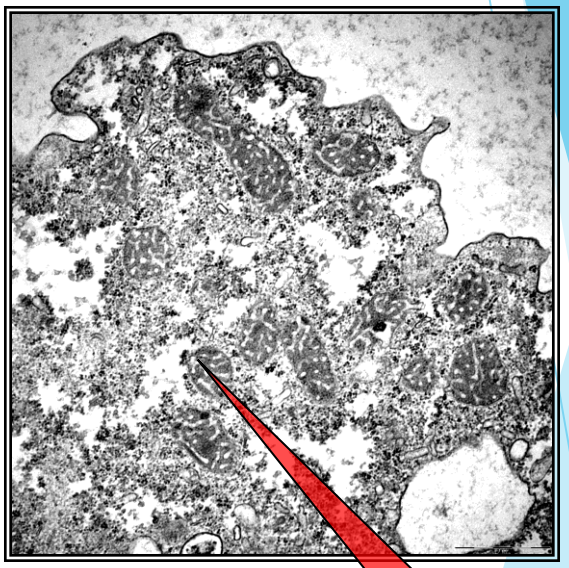
Abnormal shape of mitochondria

Depositions of cytoplasm

Pyknotic nuclelous

MH-treated *Acanthamoeba* sp. (SW isolate)



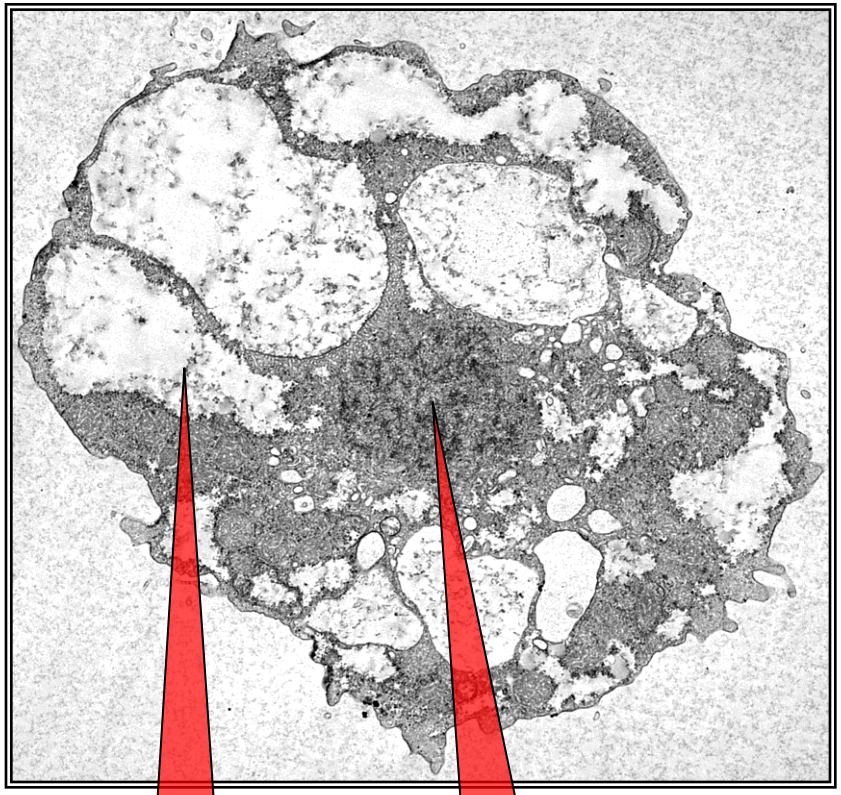


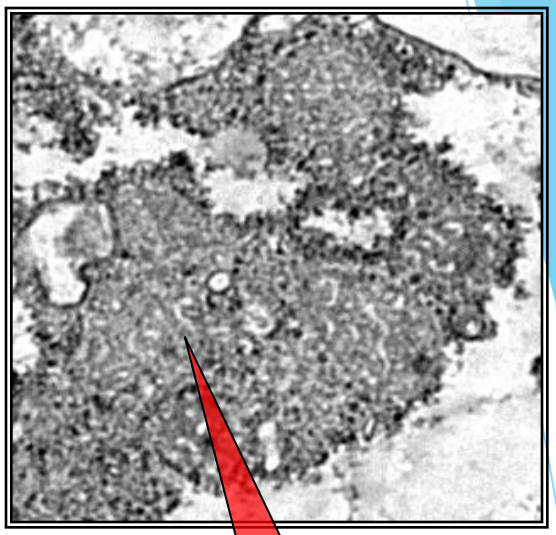
Depositions of cytoplasmic constituents

Degradation of nucleus

Abnormal shape of mitochondria

CHX-treated *A. castellanii* (IMR isolate)



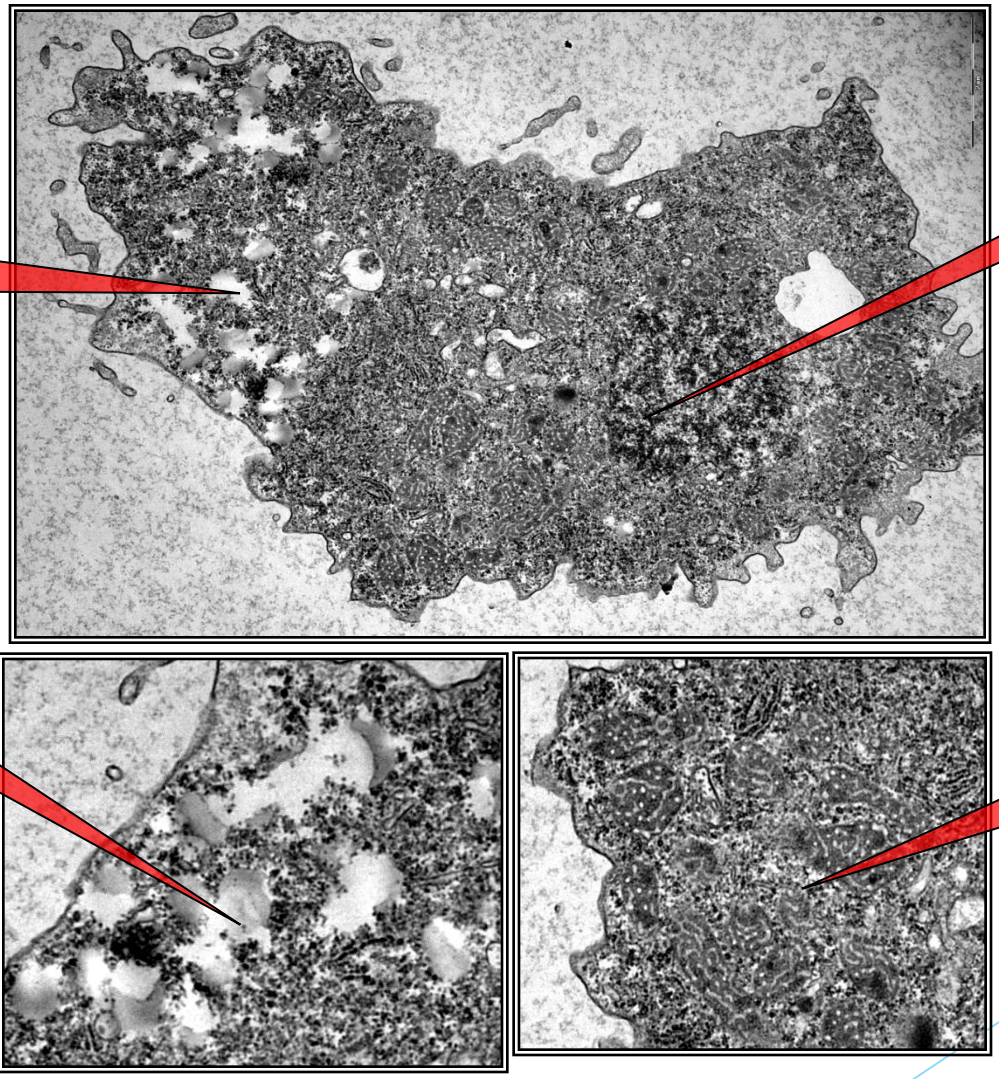


Depositions of cytoplasm

Degradation of nucleus

Disintegration of mitochondria cristae

CHX-treated *A. polyphaga* (CCAP 1501/3A)



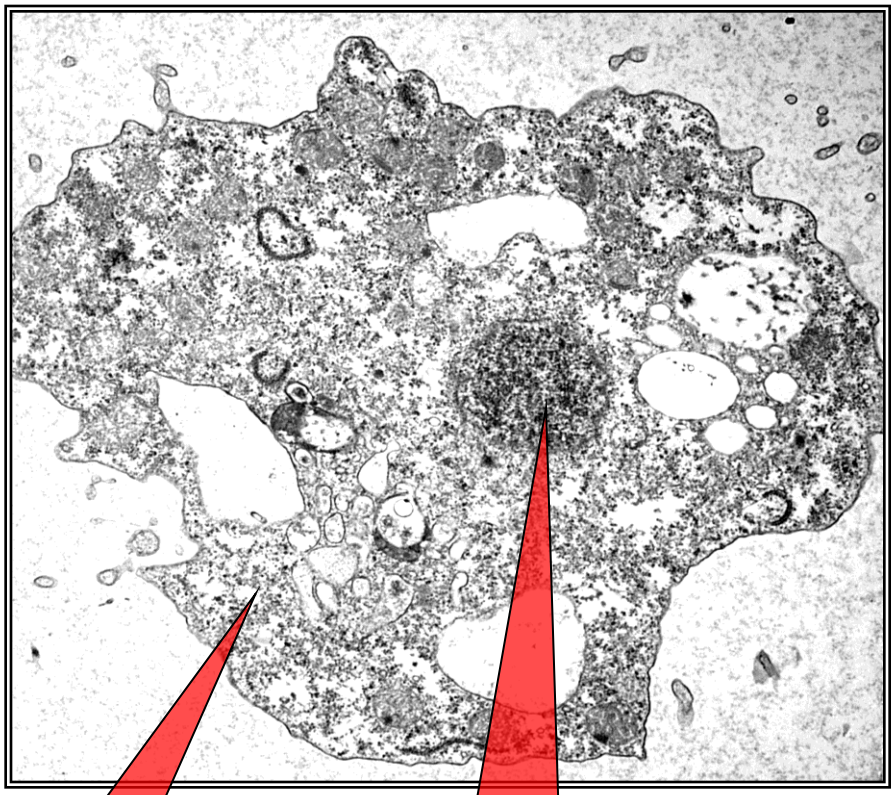
Depositions of cytoplasm

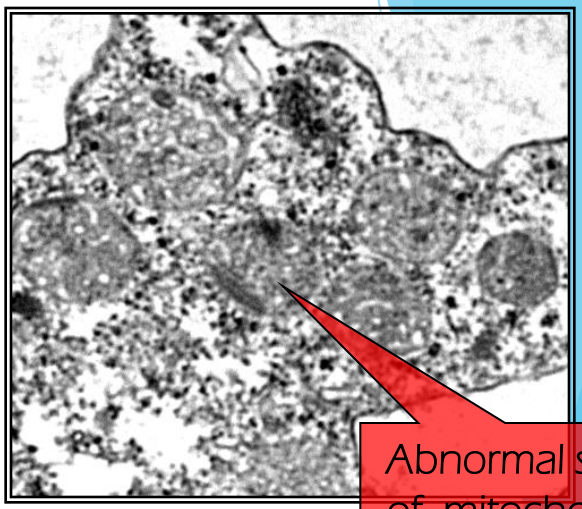
Degradation of nucleus

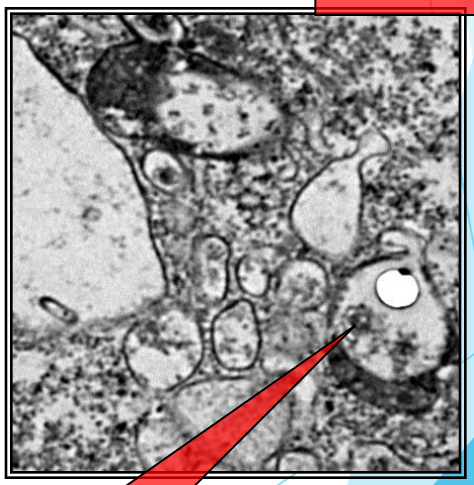
Ruptured lipid droplets

Abnormal shape of mitochondria

CHX-treated *Acanthamoeba* sp.
(SW isolate)







Depositions of cytoplasm

Degradation of nucleus

Abnormal shape of mitochondria

Crescent shape mitochondria

- Fragmented and condensed chromatin after treatment with MH and GR – similar type of chromatin condensation in apoptotic intestinal parasite, *Blastocyst hominis* (Tan and Nasirudin., 2005)
- Chromatin condensation pattern different from mammalian cells – activation of different protease
- Pyknotic nucleus – volume of nucleus of a damaged cell became decreased & darker due to the condensation of nuclear chromatin.
- Alteration to the mitochondria cristae – pathway of mitochondria damage, attempt to maintain the energy generating in mitochondria of amoeba cell
- Mitochondria adjacent to the lipid droplets (LD)- reported in higher eukaryotic cells, suggested lipid metabolism by mitochondria after treatment with MH, GR and CHX
- Ruptured LD – metabolized in the encystment process
- Autophagy – damaged organelle surrounded by endoplasmic reticulum

CONCLUSION…

MH and GR having potent anti amoebic activity against all species of Acanthamoeba used in this study.

Evidences

"……By Light Microscopy and SEM, the alterations and changes observed were mainly on the surface of cell membrane and acanthapodia structure…"

"…..TEM revealed the changes occurred at *Acanthamoeba* ultrastructural level. The noticeable changes were on the nuclear structure, mitochondria, lipid droplets and endoplasmic reticulum complex….."

MODE OF CELL DEATH DETERMINATION IN *ACANTHAMOEBA* SPP. TREATED WITH MAHANIMBINE AND GIRINIMBINE

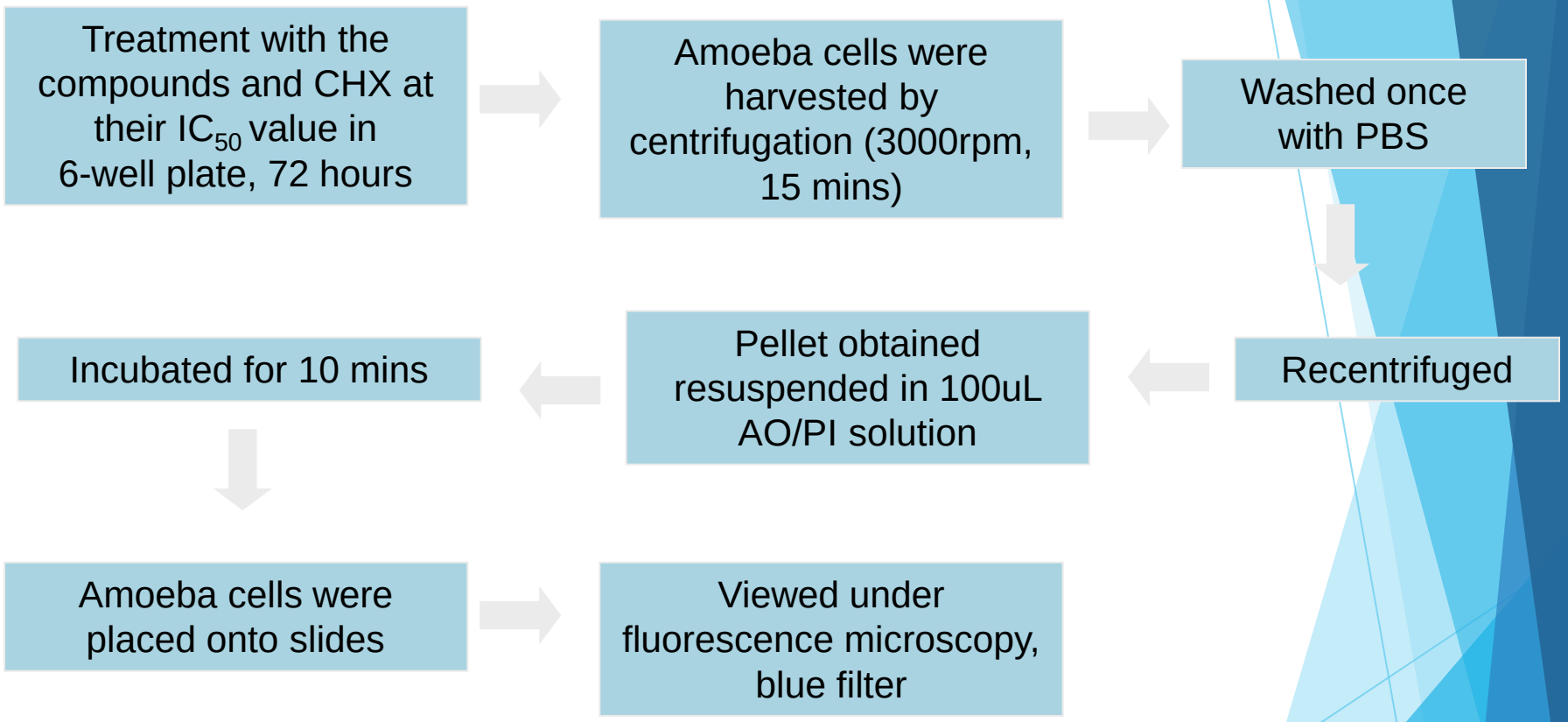
Objectives

- Cell viability determination in amoeba cell by AO/PI staining method.
- Fragmentation of DNA (apoptosis) in amoeba cell by DNA laddering assay.
- Mode of cell death determination (by apoptosis)-

>potent mechanism to remove parasitized cell from host (Bruchhaus et al., 2007).

Results and discussion

Fluorescence Microscopy by AO/PI Staining



DNA Laddering Assay by Agarose Gel Electrophoresis

Treatment with the compounds and CHX at their IC_{50} value for 72 hours

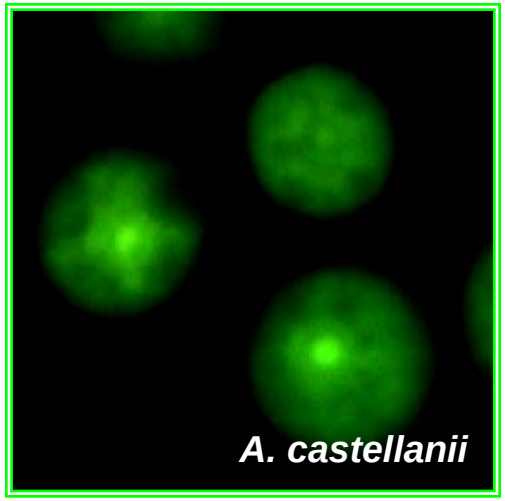
Amoeba cells were harvested by centrifugation (3000rpm, 15 mins)

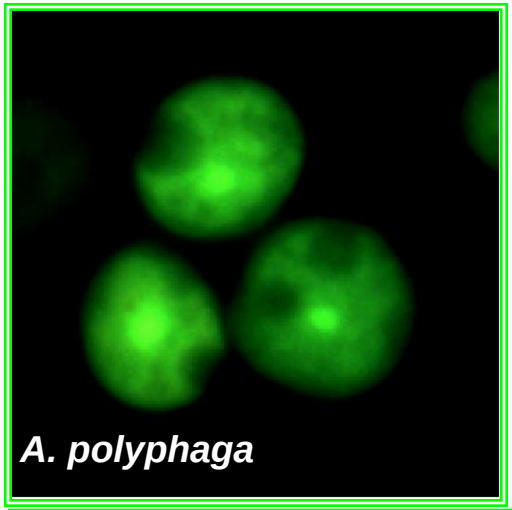
The DNA was extracted using DNeasy Kit (Qiagen)

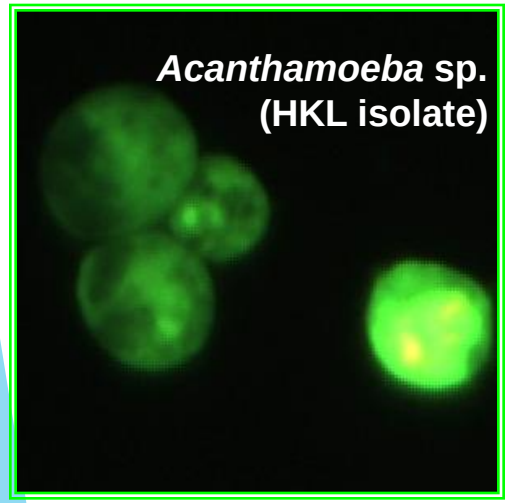
The integrity of DNA was confirmed (1.2% Agarose, 20 Volts)

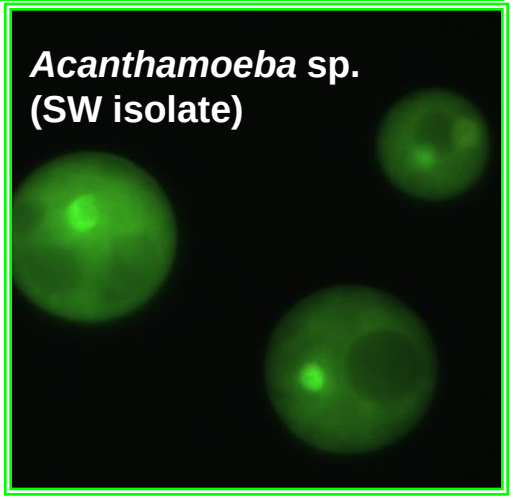

RESULTS
AND
DISCUSSION

Evaluation of Mode of Cell Death in Individual *Acanthamoeba* Cell Using AO/PI Staining







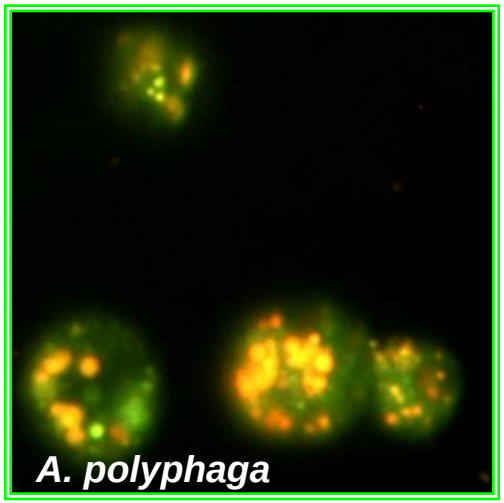


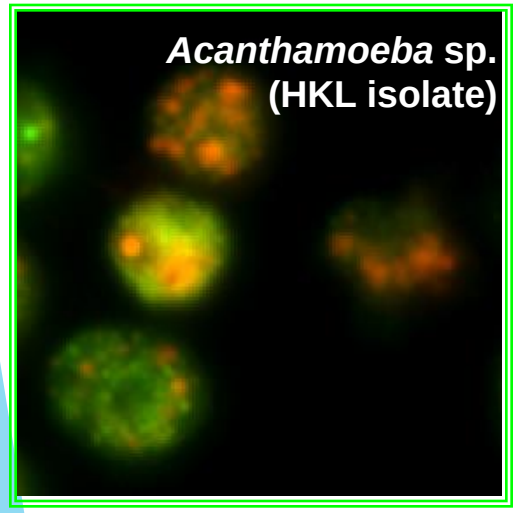
Untreated Acanthamoeba spp.

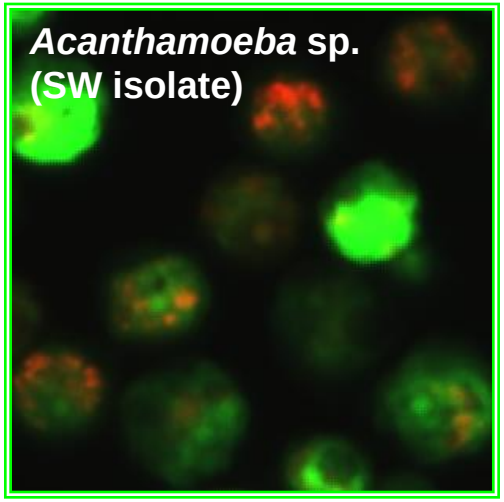
Green fluoresce *Acanthamoeba* cytoplasma, nucleus

- viable *Acanthamoeba* cells
- intact membrane
- intact nucleus

MH-treated *Acanthamoeba* spp.





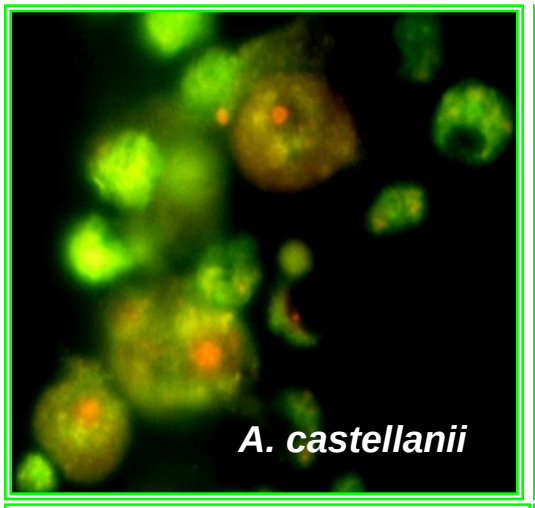


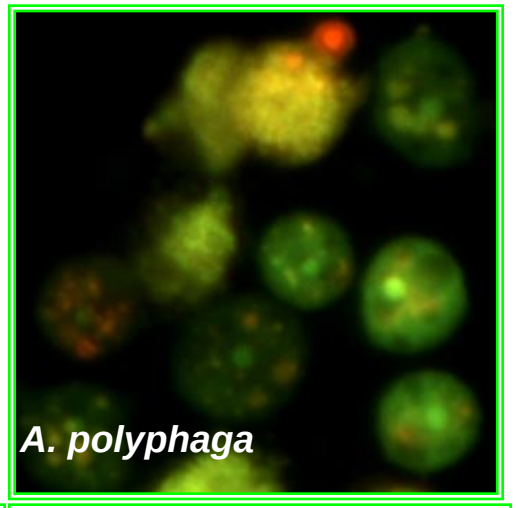
Denaturation and condensation of nuclear structure

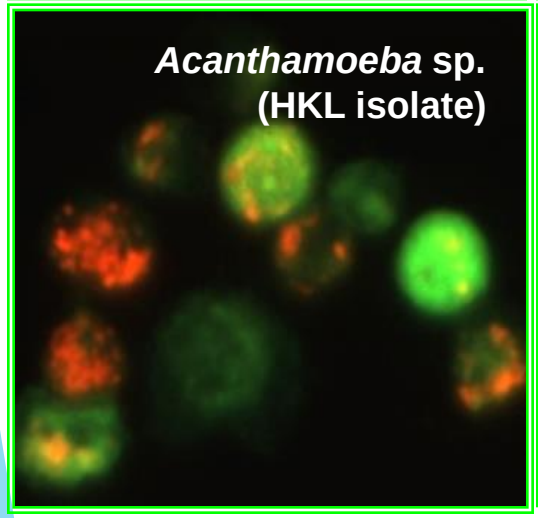
Evidences:

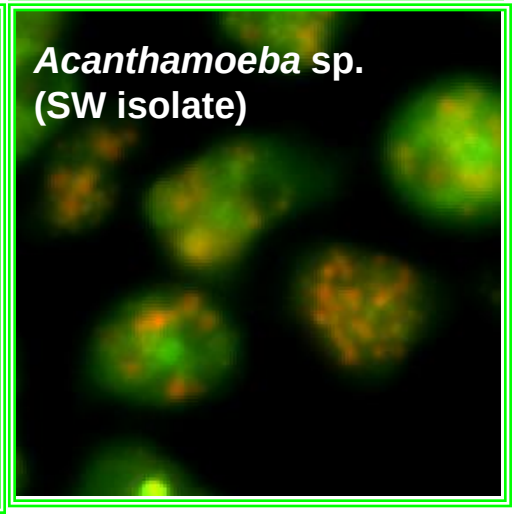
- Yellow-orange precipitates
  -ssb of DNA
- Green precipitates- dsb of DNA
  (based on Darzynkewicz et al., 1983)
- Yellow-orange granules
  -active lysosomes during autophagy
  (based on Darzynkiewicz et al.,)

GR-treated *Acanthamoeba* spp.





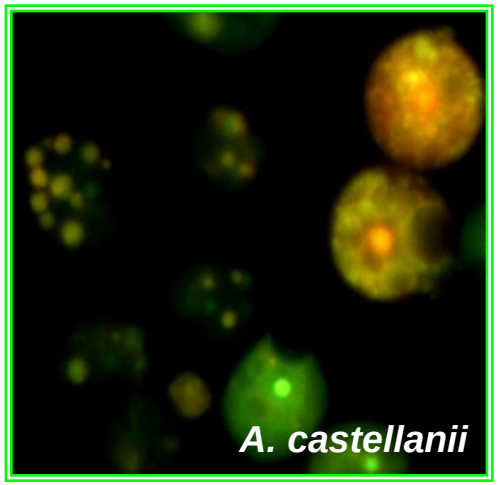


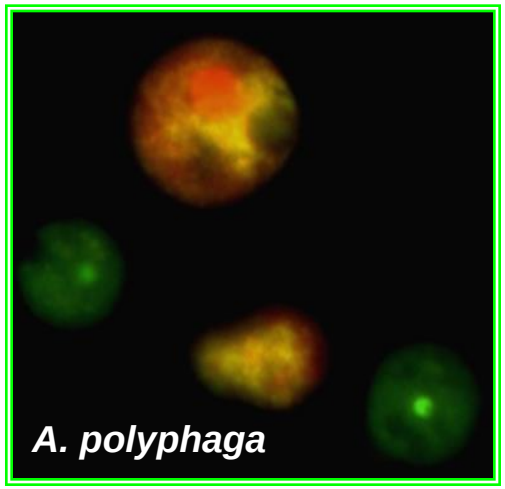


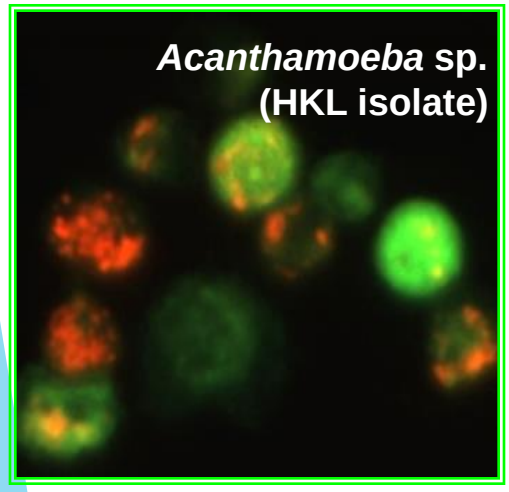
Denaturation and condensation of nucleus structure

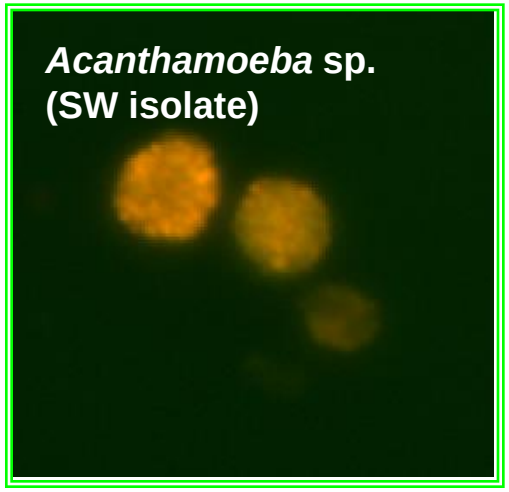
- Yellow-orange precipitates
  -ssb of DNA
- Green precipitates
  -dsb of DNA
- Yellow-orange granules
  -active lysosomes during autophagy
- Yellow-orange amoeba cells
  -membrane leakage

CHX-treated *Acanthamoeba* spp.









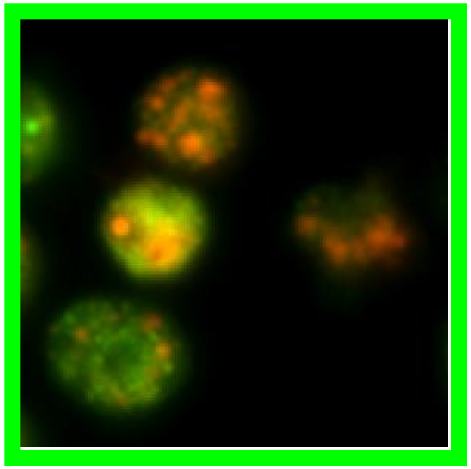
- Yellow-orange amoeba cells
  -membrane leakage
- Yellow-orange precipitates
  -ssb of DNA (apoptosis)
- Green precipitates
  -dsb of DNA (apoptosis)
- Yellow-orange granules
  -active lysosomes during autophagy

Green and red precipitates:

Single and Double strand of Fragmented DNA

Green precipitates :

Double strand break of DNA



- AO: Binds to dA & dG moieties of DNA
- Intercalate between the DNA base pairing
- No H^+ (intact hydrogen bond), no acidification and AO fluoresce green

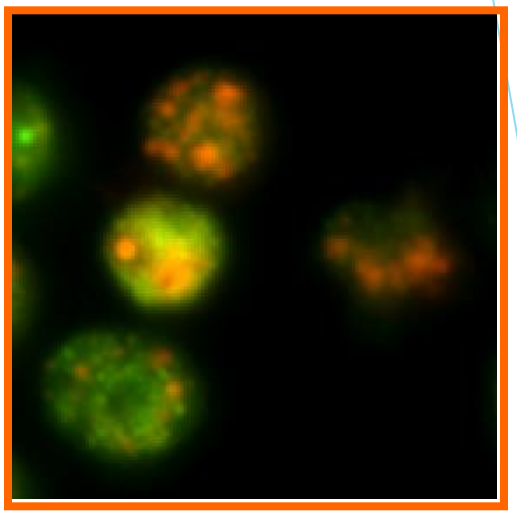
Green and red precipitates:

Single and Double strand of Fragmented DNA

Red DNA precipitates:

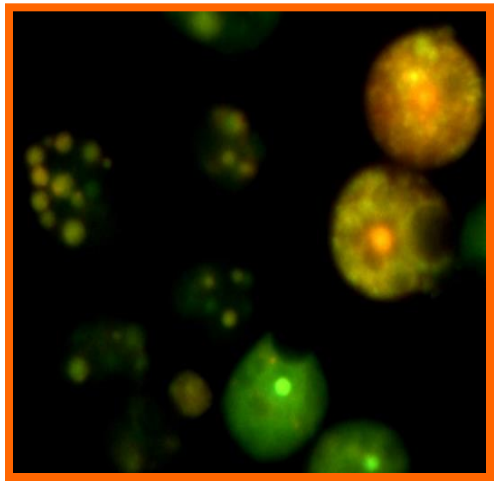
Single strand

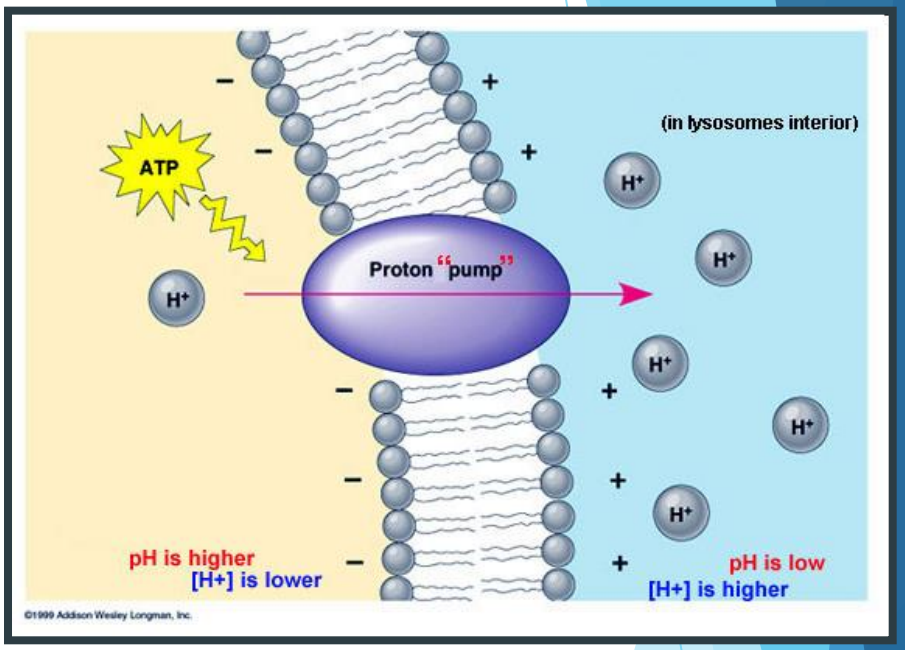
break of DNA



- Proton (H^+) due to the breakage of the H bond between the DNA strand
- Acidification to the DNA moieties
- AO fluoresce red

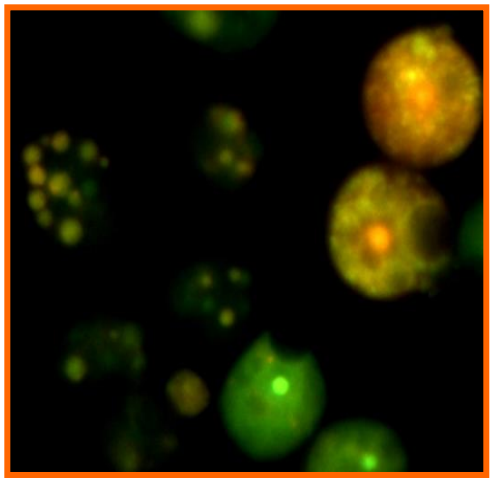
Yellow-orange vesicular structure: Protonated Lysosomes

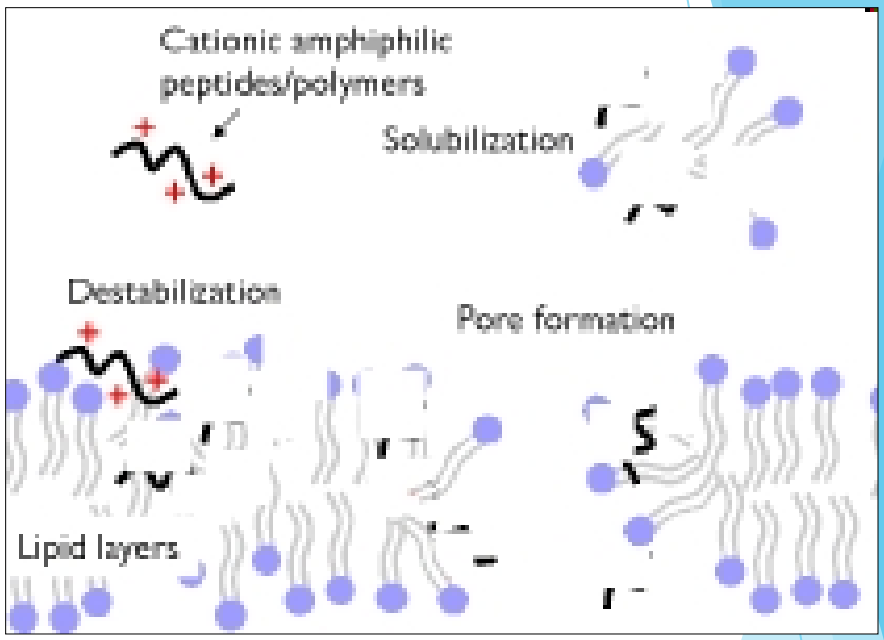




- Inside lysosome : pH ~ 4.5-5.0
- Cytosol : pH ~ 7.0-7.4
- AO:
  - Accumulate in lysosome
  - Quenching of its fluorescence correlated with ΔpH across the lysosomal membrane
  - Sensitive to the acidification (H^+)
  - Support the existence of lysosomal H^+ pump in amoeba lysosomes

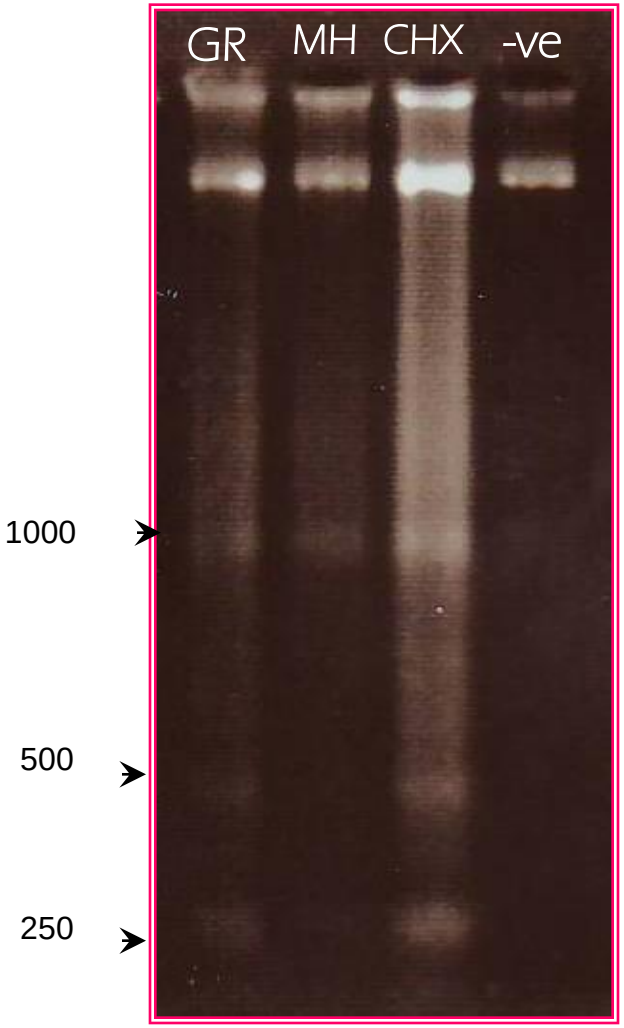
Yellow-orange Amoeba Cells:
Disrupted Amoeba Cell Membrane





Propidium Iodide
- charged molecule, excluded from a cell's lipid membrane
- passes freely through the permeabilized membrane of dead and dying cells (Stork and Li., 2006)
- reaction with Zn^{2+} (high in dying or dead cells) (Frederickson et al., 2005)

Analysis of DNA Fragmentation in *Acanthamoeba* spp.



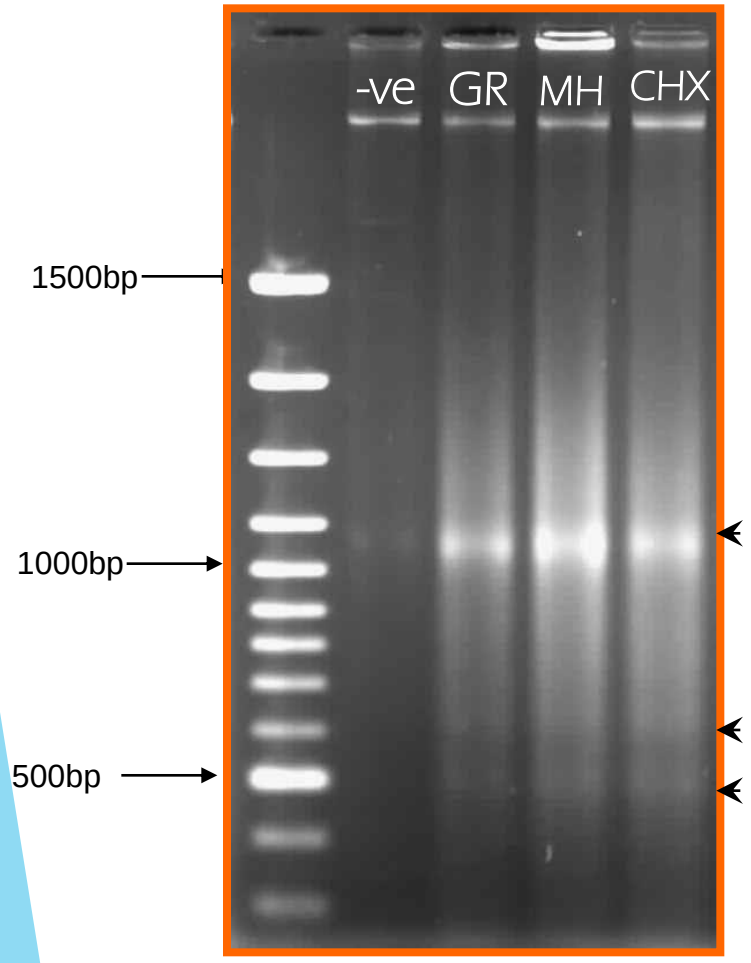
----1500bp

----1000bp

---- 500bp

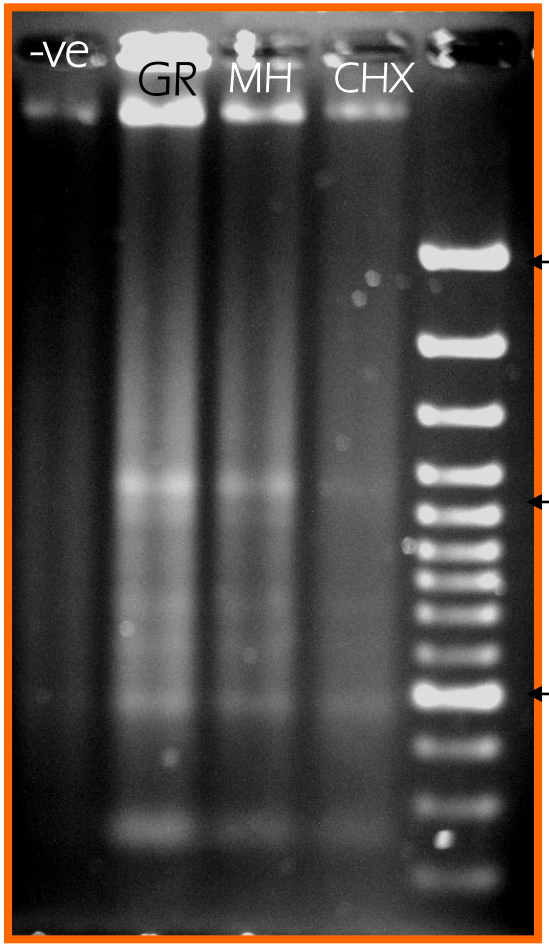
DNA fragmentation in *A. castellanii* (IMR isolate)

- DNA fragmentation in treated *Acanthamoeba* (apoptosis)-arrow head
- Smeary pattern and fragmentation of DNA in CHX-treated *Acanthamoeba* (necrosis)
- Intact DNA of untreated *Acanthamoeba*



DNA fragmentation in *A. polyphaga* (CCAP 1501/3A)

- DNA fragmentation in treated *Acanthamoeba* (apoptosis)
- Smeary pattern and fragmentation of DNA in – combination of apoptosis and necrosis *Acanthamoeba* cells after treatment
- Intact DNA of untreated *Acanthamoeba*



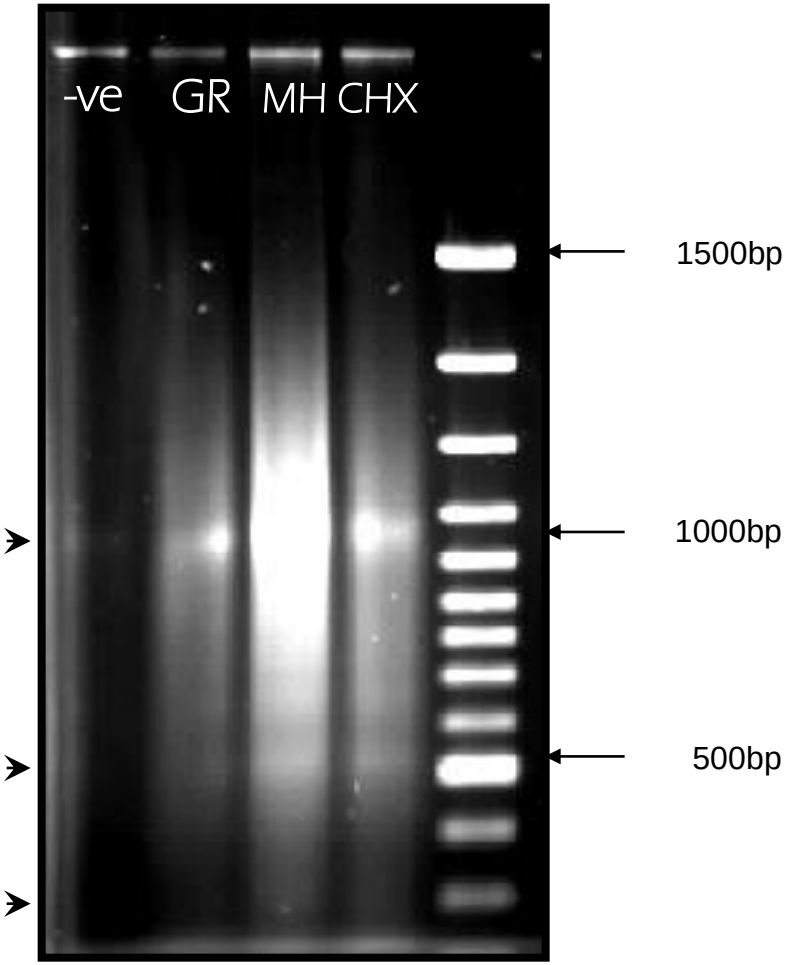
1500bp

1000bp

500bp

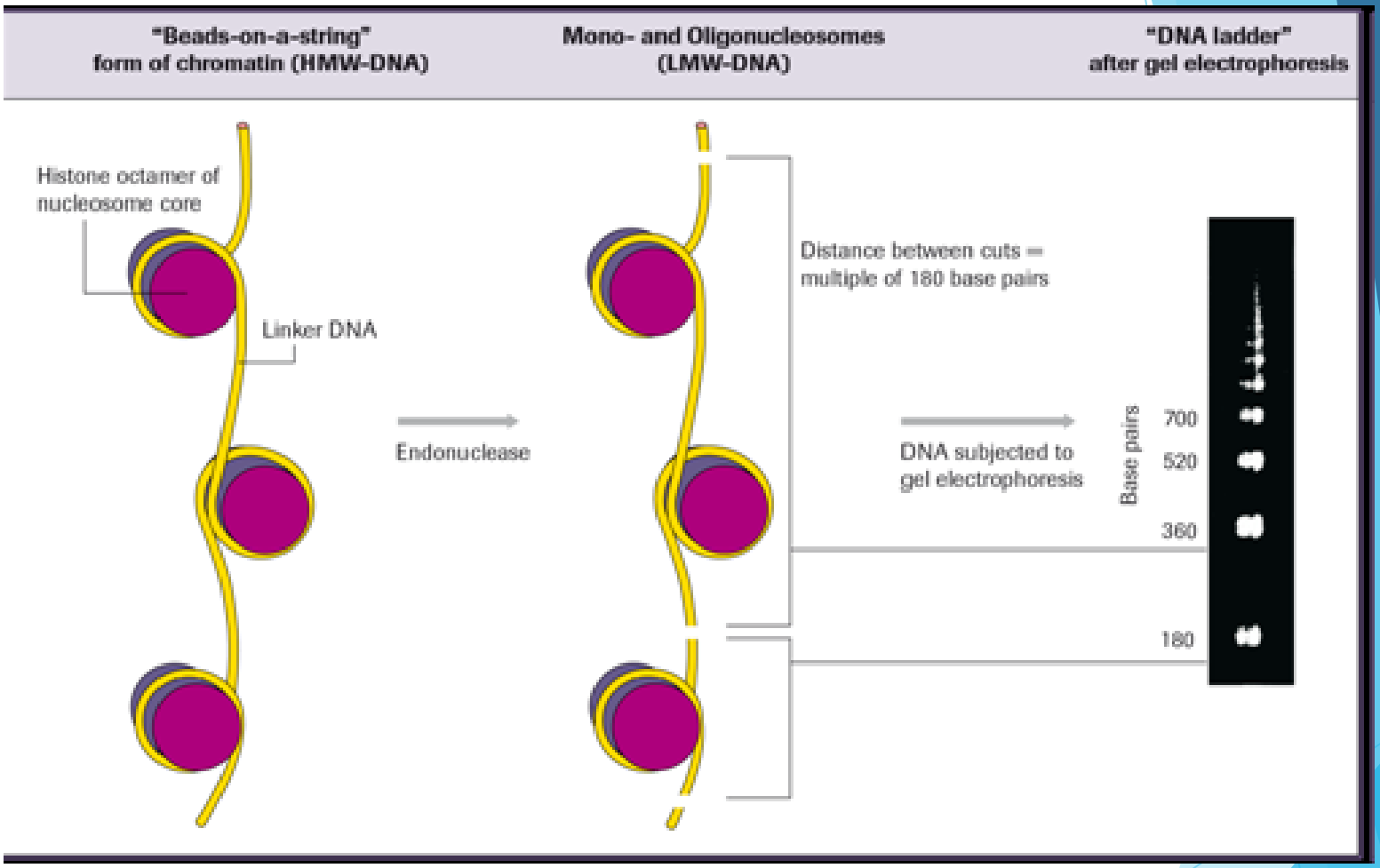
DNA fragmentation in *Acanthamoeba* sp. (HKL isolate)

- DNA fragmentation in treated *Acanthamoeba* (apoptosis)
- Smeary pattern and fragmentation of DNA in – combination of apoptosis and necrosis *Acanthamoeba* cells after treatment
- Intact DNA of untreated *Acanthamoeba*



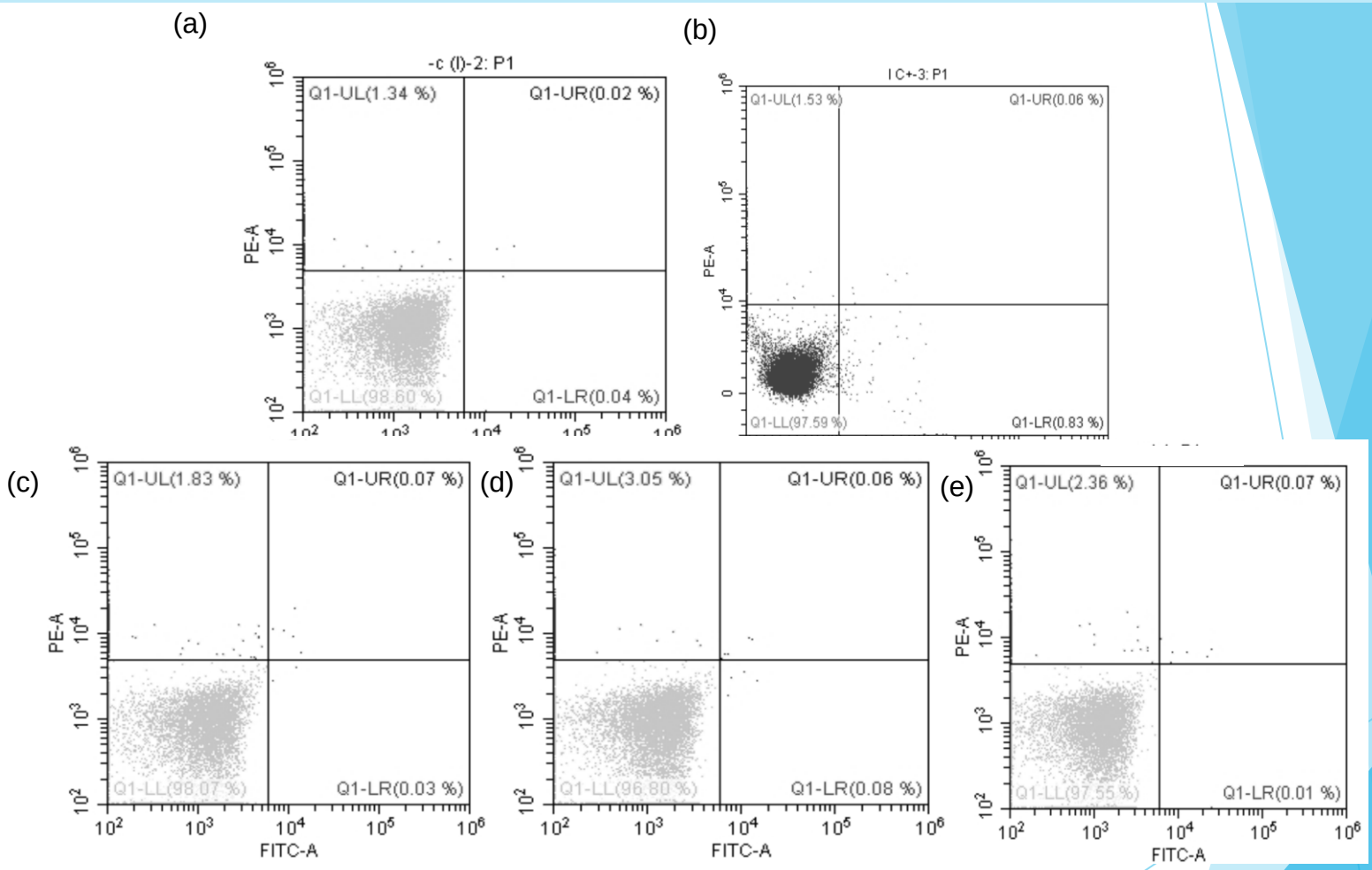
DNA fragmentation in *Acanthamoeba* sp. (SW isolate)

- DNA fragmentation in treated *Acanthamoeba* (apoptosis)
- Smeary pattern and fragmentation of DNA in – combination of apoptosis and necrosis *Acanthamoeba* cells after treatment
- Intact DNA of untreated *Acanthamoeba*



Endogenous Nuclease Enzyme

Confirmation of apoptosis and necrosis by Annexin V-FITC after 24 hours treatment confirm the absent of apoptosis event at early stage of *Acanthamoeba* cell death



The apoptosis was measured by Annexin V-FITC and flow cytometry analysis (a) untreated cells as negative control with 0.06% of apoptosis (b) chloramphenicol-treated cells 0.89% of apoptosis (c) EO_3.Pr.Pic treated cells with 0.10% of apoptosis (d) $EO_3(Nd)H_2O$.Pic treated cells with 0.14% of apoptosis (e) and EO_3.Gd.Pic treated cells with 0.08% of apoptosis.

APOPTOSIS

Chloramphenicol-treated cells - 0.89% of apoptosis
EO_3.Pr.Pic treated cells - 0.10% of apoptosis
EO_3(Nd)H_2O.Pic treated cells - 0.14% of apoptosis
EO_3.Gd.Pic treated cells - 0.08% of apoptosis.

Apoptosis
- Indicated externalization of PS.
- Annexin V-FITC bind to PS.

Autophagy – lanthanide complexes treated cells

- Intercellular cell death
- No externalization of PS occurred.
- Annexin V-FITC unable to detect due to no exposure of PS.

CONCLUSION....

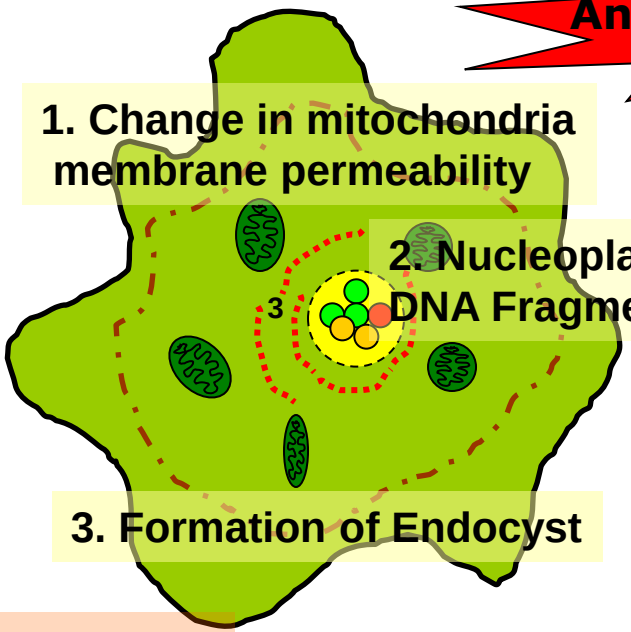
- *Necrosis and programmed cell death occurred in Acanthamoeba cells after treatment with MH and GR*
- *Fragmentation of DNA, typical apoptosis criteria were also observed in amoeba cells after treatment with both compounds*

Evidences

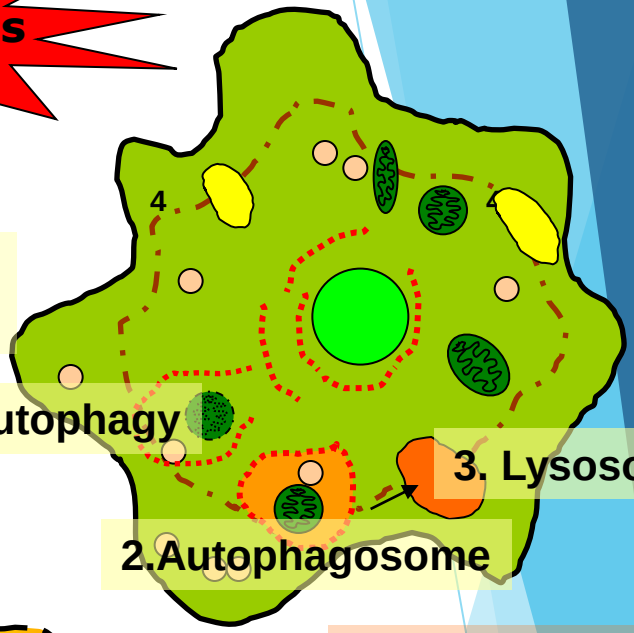
- Necrosis and programmed cell death (autophagy and apoptosis)were recognized and quantified in a population of amoebae when double fluorochromes staining AO/PI, an express, fast, easy, sensitive and reproducible method were utilized.
- DNA fragmentation after treatment with MH and GR, a characteristics feature of apoptosis in *Acanthamoeba* cells were confirmed by DNA laddering assay in agarose gel.
- MH and GR exhibited similar ladder pattern (250 internucleosomal cleavage) in all treated *Acanthamoeabae*
- Staining by AO/PI also indicated that autophagic type of cell death also occurred in Acanthamoeba cells after treatment with both compounds.

- MH and GR exhibited very low IC_{50} values on isolates of *Acanthamoeba* used in this study
- Significant changes on treated *Acanthamoeba* observed by LM, SEM, TEM.
- Modification in SEM technique.
- Type of *Acanthamoeba* cell after exposed to the compounds were confirmed by combination of AO and PI (apoptosis, autophagic cell death and necrosis)
- Activation of lysosomal activity in treated *Acanthamoeba* observed by AO/PI staining
- DNA laddering confirmed the apoptosis process in treated *Acanthamoeba* cells
- Genus *Acanthamoeba* , model for mitochondriate eukaryotic cells
- No similar death cascade has been reported in *Acanthamoeba* to mammalian cells. Metacaspase in *Acanthamoeba* (Trzyna et al., 2008) - Arg/Lys specific protein

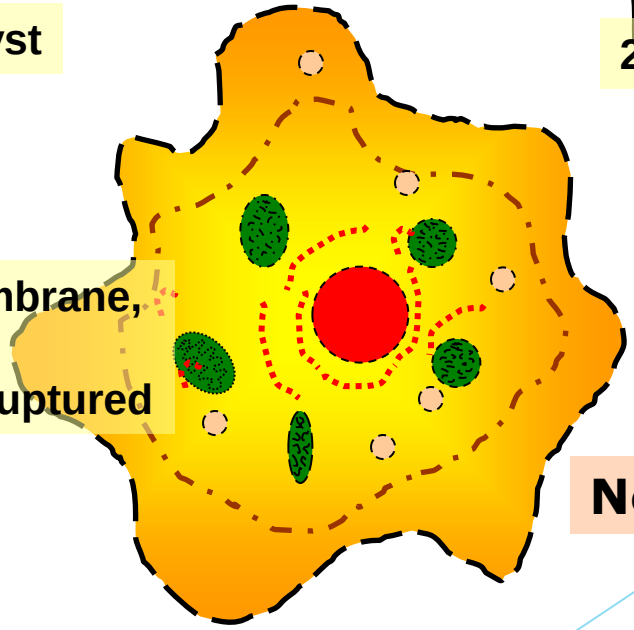
Anti-amoebic agents



Apoptosis



Autophagic cell death



Necrosis

The End

&

Thank You