Role of mitochondria transmembrane potential in lymphocyte apoptosis

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Summary:

Background: Mitochondria play an important role in the regulation of physiological type of cell death (Apoptosis) this type of cell death can be stimulated by two major pathways: external (Fas-Fas ligand interaction) and internal mitochondrial pathway which require disruption of mitochondrial transmembrane potential ($\Delta\Psi_m$) which leads to opening of mitochondrial channels that lead to release of cytochrome –C which would execute apoptotic process.

Patients and Methods: The study included 32 normal subjects; 2 ml of venous blood were aspirated from each of them and processed for peripheral blood lymphocytes separation (PBL)

Then lymphocyte apoptosis was studied before and after exposing (PBL) to hyperthermia by these three methods to compare which of them can detect earliest apoptotic changes: Cell count and viability, morphological changes (by acredine orange stain) and the changes in mitochondrial membrane potential by Mitolight Apoptosis Detection Kit.

Results: Our results showed that the percentage of lymphocyte apoptosis detected by mitolight was significantly higher than those detected by other two methods.

Conclusion: This study shows that mitochondrial changes can detect earlier apoptotic process than morphological features detected by DNA stains.

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Introduction:

Apoptosis is a physiological type of cell death that occurs in all multicellular organisms as a part of normal development; deletion of un-needed cells and tissues, regulation of growth and cell number, and elimination of abnormal and potentially dangerous cells. It occurs in response to a variety of physiological and pathophysiological stimuli, like formation of the hand during embryogenesis, the peeling of burned skin and the regression of uterus to normal size after delivery (1).

Apoptosis can be stimulated by two major pathways:

1. Extrinsic pathway:

a- The binding of death inducing ligands (Fas-Fas ligands interaction) to cell surface receptors (2).

CD95/ Fas /APO-1 are a cell surface receptor of the tumor necrosis factor (TNF) family. Other members of this family include TNF receptors TNF-R1 and TNF-R2, CD30, CD40 and APO-2. (3)Binding of CD95 by its ligand usually results in apoptosis of CD95 bearing cell. The cytoplasmic domain of CD95 bears a motif termed the death domain " DD" that upon ligation of CD95 ligand, allows it to bind the death domain of cytoplasmic protein FADD (Fas associated death domain). FADD has a carboxy-terminal death domain, and a death effecter domain "DED" at its N terminus that allows it to interact with the cysteine protease FLICE (FADD-like ICE "Interleukin Converting Enzyme") in this way, ligation of CD95 can lead to activation of the cysteine aspartic acid proteases (Caspase) that are the common effectors of apoptosis (4).

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There are two types of caspases: The initiator caspases: such as caspase 8 or caspase 10. These caspases, upon activation, can activate other caspases in a cascade. This cascade eventually leads to the activation of the second type of caspases. 5.

The effecter caspases: such as caspase 3 and caspase 6. These caspases are responsible for the cleavage of the key cellular proteins like endonuclease. (5)

b- Induction of apoptosis by cytotoxic T-lymphocytes by granzyme B which is a serine protease that directly activates the target cell caspases. The latter occurs when T-cells recognize damaged or virus-infected cells and initiate apoptosis in order to prevent damaged cells from becoming neoplastic or virus-infected cells from spreading the infection (6).The intrinsic pathway (mitochondrial pathway):

Mitochondria play an important role in the regulation of cell death. For example, anti-apoptotic members of the Bcl-2 (B cell lymphoma gene) family of proteins, such as Bcl-2 and Bcl-X_L, are located in the outer mitochondrial membrane and act to promote cell survival. Many of the pro-apoptotic members of the Bcl-2 family, such as Bad and Bax also mediate their effects through the mitochondria, either by interacting with Bcl-2 and Bcl-X_L, or through direct interactions with the mitochondrial membrane. Mitochondria have the ability to promote apoptosis through release of cytochrome C, which together with Apaf-1 (Apoptosis protease- activating factor-1) and ATP forms a complex with pro-caspase 9, leading to activation of caspase 9 and the caspase cascade (7, 8) The release of cytochrome C from mitochondria is regulated through Bax and Bcl-2, since Bax, and other Bcl-2 proteins, show structural similarities with pore-forming proteins. It has therefore been suggested that Bax can form a

transmembrane pore across the outer mitochondrial membrane, leading to loss of membrane potential and efflux of cytochrome C as illustrated in figure (1)

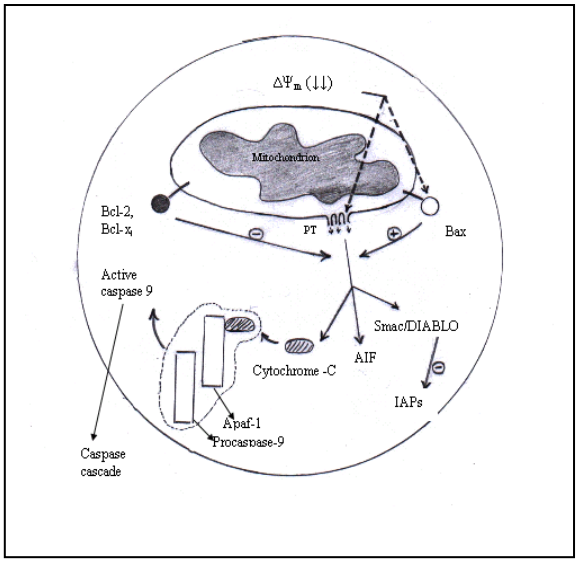

Figure (1)

Fig 1. Mitochondrial pathway of apoptosis

$\Delta\Psi_m$ mitochondrial transmembrane potential.

PT Permeability Transitional pores.

AIF Apoptosis Inducing Factor.

Smac/Diablo Second mitochondrial derived activator of caspase/ Diablo: Direct IAP- binding protein with low iso-electric point

IAPs Inhibitors of Apoptotic Protein.

Apaf-1 Apoptosis protease- activating factor-1.

Leakage of mitochondrial cytochrome C through the permeability transition pore system resulted in an irreversible commitment to cytoplasmic and nuclear apoptosis. Cytochrome C acts as the mitochondrial factor which confers this effect. (9).

There is accumulated evidence that such a ($\Delta\Psi_m$) breakdown is an invariant feature of early apoptosis (10). The mitochondrial transmembrane potential ($\Delta\Psi_m$) results from the asymmetric distribution of protons and ions on both sides of the inner mitochondrial membrane, giving rise to a chemical (pH) and an electric gradient that are indispensable for the mitochondrion to fulfill its essential metabolic functions (11).

The cells that undergo apoptosis exhibit a decrease in $\Delta\Psi_m$ that precedes nuclear signs of apoptosis.

This change in $\Delta\Psi_m$ leads to an immediate shutdown of mitochondrial biogenesis. Accordingly, both the transcription of the mitochondrial genome and the synthesis of mitochondrial proteins are perturbed early during the apoptotic process. Another consequence of $\Delta\Psi_m$ disruption is the uncoupling of the oxidative phosphorylation and the generation of superoxide anions by the uncoupled respiratory chain. (12).

Subjects and methods:

The study included 32 normal subjects, from each of them two milliliters of venous blood were aspirated, which was processed for peripheral blood lymphocytes separation (PBL)

Peripheral blood Lymphocytes (PBL) separation:

2 ml of anticoagulated fresh blood in EDTA or heparin tube, diluted 1:1 with a balanced salt solution, phosphate buffered saline (PBS pH 7.2).

2 ml of the diluted blood was layered carefully over 4 ml of lymphocyte separation medium (Ficoll) in a10 ml siliconised glass centrifuge tube, Centrifuge for 30 minutes at 3100 rpm in cold centrifuge (24 ċ).After centrifugation, the lymphocytes will form a white puffy coat at the interface of the blood plasma and the separation media .Aspirate the layer of lymphocytes with a clean Pasteur pipette, Transfer the lymphocytes to another siliconized glass tube containing PBS at least 3 times the volume of lymphocytes. Suspend evenly, Centrifuge the lymphocyte suspension at 2500 rpm for 10-15 minutes. Discard the supernatant .repeat this step 3 times for washing the lymphocytes. Resuspend the cells pellet with 0.5 ml of PBS by gentle drawing of lymphocytes in and out of the Pasteur pipette. This represents the lymphocytes suspension (13, 14).

Cell suspension then was exposed to hyperthermia by putting cell suspension in water bath heated to 40˚ C for 15 minutes to induce lymphocyte apoptosis (15, 16).

then all the following test were made at the same time to compare between apoptosis of lymphocyte before and after hyperthermia by these three methods to compare which of them can detect earliest apoptotic changes.

1. Cell count and viability:

The number and viability of lymphocytes in the lymphocytes suspension were detected by using Haemocytometer counting chamber, the results were expressed as cell/mm (3,17).

Trypan blue exclusion test (0.4 gm Trypan blue was dissolved in 100ml PBS) was done to asses cell viability. The principle of this test is that the viable cells exclude Trypan blue dye (i.e. does not be stained); while the dead cells accept the dye (stained blue). (17).

2. Cellular morphology:

Fresh slide smear was prepared from lymphocyte suspension. The smear was allowed to dry at room temperature, fixed for 30 minutes. Then slides were stained with DNA-binding fluorescent dye (acredine orange) and examined by fluorescent microscope. According to the Procedure of Vacca(18).The morphological characteristics of lymphocyte apoptosis were assessed according to the method of Willingham(19).The morphological changes related to apoptotic process as stated by (Collins et al, 1997) (20). These changes include: Cell shrinkage and membrane blebbing.Chromatin condensation and fragmentation of nuclear material and DNA fragmentation.

3. Detection of mitochondrial pathway of apoptosis by Mitolight Apoptosis Detection Kit:

Mitolight Apoptosis Detection Kit is a mitochondrial dye that stains mitochondria in living cells in a membrane-dependant fashion so it acts as a mitochondrial activity marker. In healthy cells with normal membrane potential ($\Delta\Psi_m$) the dye with its cataionic lipophilic structure, accumulates and aggregates in the mitochondria giving off a bright red fluorescence. In apoptotic cells with altered mitochondrial membrane potential, the dye in its monomeric form stays in the cytoplasm, fluorescing green providing a ready discrimination between pre-apoptotic which exhibit a low membrane potential and non apoptotic cells with normal membrane potential.

Chemical structure:
5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine chloride, $C_{25}H_{27}CL_3N4$, M wt. 561.5, ε (MeOH)=190,000.
Mitolight™ preparation (prepare only enough for the number of samples to be analyzed immediately): Thaw the Mitolight reagent and 10X incubation buffer. Mix well. For each sample to be analyzed, dilute 1 µL of Mitolight with 900 µL of deionized water. Mix the solution well. Immediately, add 100µL of 10X incubation buffer. Mix well.

Incubation of cells with Mitolight reagent:
Count cells and pellet $1X10^6$ cells. Resuspend cells in 1 ml of the prediluted Mitolight solution. Incubate at 37Ċ in a 5% CO_2 incubator for 15-20 minutes. Centrifuge for 2 minutes in a micro centrifuge (2000 rpm) and discard supernatant. Resuspend in 1 ml of 1X incubation buffer (dilute 100 µl of 10X incubation buffer with 900µl of deionized water).Centrifuge for 2 minutes in a micro centrifuge (2000 rpm) then discard the supernatant leaving only few micro liters with the resultant pellet and mix well.
Detection by fluorescence microscopy:
Place the cell suspension on a glass slide. Cover the cells with a glass cover slip. Observe cells immediately under a fluorescence microscope using a band-pass filter. Mitolight that has aggregated in the mitochondria of healthy cells fluoresces red. In apoptotic cells, monomers aggregate in the cytoplasm, and fluoresces green.

Results:
Peripheral blood lymphocyte apoptosis were assessed pre and post exposure to hyperthermia in water bath for 15 minutes, this table shows percentage of PBL apoptosis after staining with acredine orange.

Table (1): Comparison between the percentage of apoptosis of peripheral blood lymphocytes before and after exposure to hyperthermia (by acredine orange stain).

parameter	mean % ± SD	P-value
Apoptosis of PBL before Hyperthermia	4.1 ± 1.8	0.00001
apoptosis of PBL after	13.9 ± 2.06	

*P value is significant at 0.05

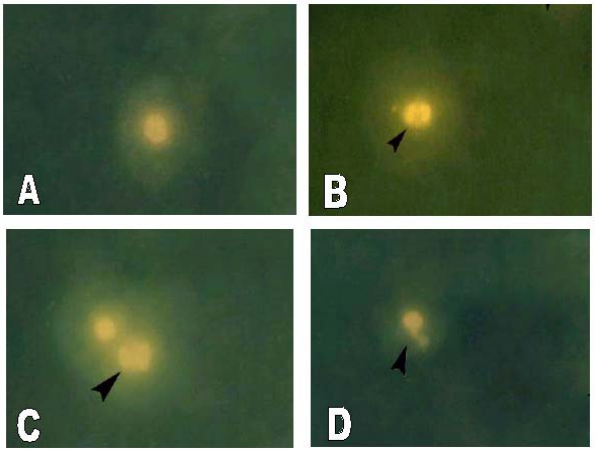

Figure (2)
Peripheral blood lymphocytes stained by acredine orange (before and after hyperthermia) 400X
A: Normal lymphocyte.
B: Apoptotic lymphocyte showing division of the nucleus into two parts.
C: Apoptotic lymphocyte showing division of the nucleus into three parts.
D: Apoptotic lymphocyte showing apoptotic body formation.

Detection of peripheral blood lymphocytes apoptosis by Mitolight kit: the percentage of apoptosis of peripheral blood lymphocytes detected by the Mitolight kit after treatment with hyperthermia was significantly more than that detected before hyperthermia (18.1 ± 2.9), (7.8 ± 1.7) respectively, with P value less than 0.0001 as compared by paired T test as illustrated in table (2). The morphological features of viable lymphocytes and apoptotic lymphocytes stained by Mitolight kit are shown in figures (3) and (4) respectively.

Table (2): Comparison between the percentage of apoptosis of peripheral blood lymphocytes before and after exposure to hyperthermia (by Mito Light kit).

parameter	mean % ± SD	P value
Apoptosis of PBL Before hyperthermia	7.8 ± 1.7	0.0001
Apoptosis of PBL after hyperthermia	18.1 ± 2.9	

* P value is significant at 0.05.

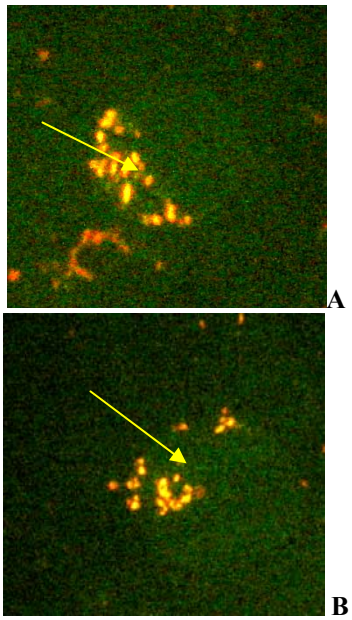

Figure (3)
A, B: Viable peripheral blood lymphocytes stained by Mitolight kit, showing the aggregation of fluorescent dye inside the mitochondria giving rise to red-orange fluorescence. (400X)

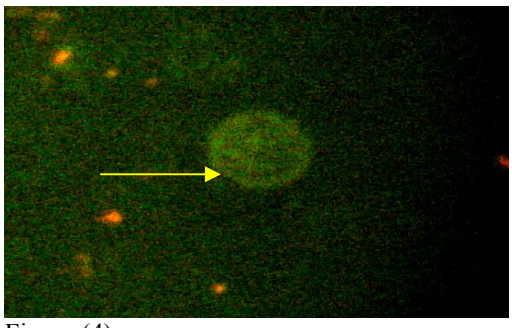

Figure (4)
Apoptotic peripheral blood lymphocyte, stained by Mitolight kit, showing the aggregation of fluorescent dye in the cytoplasm outside the mitochondria, giving rise to green fluorescence, (400X).

Then comparison between table1 and 2 shows that although detection of apoptosis was done at the same time but the % of lymphocyte apoptosis was greater in both conditions pre and post hyperthermia, when lymphocyte apoptosis was detected by mitolight (4.1 ± 1.8. 13.9 ± 2.06) (7.8 ± 1.7, 18.1 ± 2.9) pre and post exposure to hyperthermia detected by acridene orange and mitolight respectively and the difference was statistically significant (P<0.001)

Discussion:

The mitochondrial transmembrane potential ($\Delta\Psi_m$) results from asymmetric distribution of proton and ions on both sides of the inner mitochondrial membrane (21). The inner side of the inner mitochondrial membrane is negatively charged. So cataionic lipophilic flurochromes such as mitolight can distribute to the mitochondrial matrix following the Nernst equation .In healthy cells with normal membrane potential ($\Delta\Psi_m$) the dye accumulates and aggregates in the mitochondria giving off a bright red fluorescence. In apoptotic cells with altered mitochondrial membrane potential, the dye in its monomeric form stays in the cytoplasm, fluorescing green providing a ready discrimination between pre-apoptotic which exhibit a low membrane potential and non apoptotic cells with normal membrane potential. In this study we found that the number of cell detected by mitolight (that detect apoptotic and pre apoptotic cell) which also detect the mitochondrial changes was greater than those detected by acriden orange (which stain the nucleus), which detect the nuclear changes, this means that mitochondrial changes preceded nuclear changes in this type of apoptosis which was induced by hyperthermia. In this study we induce apoptosis by hyperthermia as in our previous study we found that hyperthermia induce apoptosis by generation of oxidative stress which acts on mitochondrial pathway(15,16).This means that mitochondrial pathway (as detected by mitolight) can induce earlier apoptosis than nuclear changes.

Conclusion:

changes in mitochondrial membrane potential ($\Delta\Psi_m$) can be detected earlier than nuclear changes especially if we induce apoptosis by stimuli that stimulate mitochondrial pathway of lymphocyte apoptosis.

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