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# Effect of Age on Apoptosis and Necrosis of Peripheral Blood Lymphocytes in Sample of Iraqi Type 2 Diabetes Patients

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**Abstract:** Type 2 diabetes (T2DM) is a metabolic disorder usually in adults, resulting from insulin insufficiency or function. Peripheral Blood Lymphocytes (PBLs) are the key components of the body's defenses which offer resistance against infective micro-organisms. Aging is an extremely intricate process related with declines in renewing potential plus adaptive reactions to stress. The aim of the study was to investigate the effect of age on apoptosis and necrosis of peripheral blood lymphocytes in a sample of T2DM Iraqi patients. Venous blood (4 ml) was collected in heparinized tubes under optimal condition and processed for peripheral blood lymphocytes separation by density-gradient centrifugation with lymphocyte separation medium. Apoptotic and necrotic lymphocytes were detected by using dual acridine orange / ethidium bromide staining and fluorescence microscopy, and the relationship between apoptosis, and necrosis with age of volunteers was analysis by using static methods. The morphological observations of lymphocytes from diabetic patients showed morphological features consistent with apoptosis more than control. Peripheral blood lymphocytes apoptosis and necrosis of T2DM patients showed significantly higher ($P<0.01$) when compared to control, whereas the peripheral blood lymphocytes (PBLs) apoptosis percentage reached to the maximum value (50.52 %), at age group more than 60 years of T2DM patients, however necrosis percentage reached to (21.8 %).It can be concluded that high blood glucose in poorly controlled diabetes disease is concomitant with increase rate of apoptosis and necrosis of PBLs which can be considered as a marker of severity for this disease.

**Keywords:** T2DM; PBLs; Apoptosis; Necrosis; Dual AO/EB staining; Iraq.

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## Introduction

Type 2 diabetes (T2DM) is a chronic complex disease triggered by the interaction of genetic and environmental factors, affecting over 425 million people worldwide (1). It's best typical sign is an elevation of glucose owing to inadequate insulin for the body requirements (2). In Iraq, T2DM affects approximately 1.4 million Iraqis, with prevalence ranging from 8.5 percent to 13.9 percent (3). T2DM transmuted from a slight disease of oldness to a grave reason of morbidity and death in children, adolescents, and young adults owing to increasing levels of fatness, physical lethargy, and high-energy diets high-energy diets people (4). Recent studies have shown that both humans and animal models can develop free radicals and oxidative stress as a result of DM-induced chronic hyperglycemia (5, 6). Inflammation is regarded as the common antecedent of T2DM. Additionally, an inflammatory process can cause T2DM-related complications in the kidneys, arteries, and eyes (7). Circulating white blood cell (WBC) count was an established

biomarker for inflammation (8), due to the pancreatic beta cells being damaged by this inflammation, there is insufficient insulin synthesis, which causes hyperglycemia. This is thought to lead to immune system malfunction, which prevents diabetic people from controlling the spread of invading microorganisms (9). Innate immune function was the primary topic of early studies on the regulation of inflammation in diabetes (10). However, recent studies indicate that the pathogenesis of T2DM may also be significantly influenced by the adaptive immune system, particularly lymphocytes(11). Lymphocytes are part of the adaptive immune response, which is essential for maintaining both cellular and humoral immunity, and whose growth needs cellular selection to eliminate potentially auto-reactive cells by apoptosis (12). Apoptosis is a protective mechanism which eliminates old, unusable, and damaged cells. It is an organized and controlled process and involves individual cells (13), necrosis instead, is frequently considered an inert response to devastating stress that is chemical or physical and hence uncontrolled and unexpected (14). Apoptosis of lymphocyte clones is essential for removing harmful lymphocytes from the body while retaining immune system development and function, so these cells are prudently regulated through the balance between cell birth and cell death (12). Lack regulation of immune cells apoptosis may participate to impair immune system and body exposed to the risk of various diseases and disorders (15). There is growing evidence that T2DM is correlated with T lymphocyte activation. Some studies demonstrated that the total lymphocytes apoptosis is significantly higher in T2DM as compared with healthy control because increased the oxidative stress in diabetics which induces lymphocyte apoptosis (16, 17). Aging is an extremely complex process involving regeneration potential decreases and stress-induced adaptive responses. Therefore, the principal aim of this study was to investigate the age effect on apoptosis and necrosis of PBLs in sample of T2DM Iraqi patients.

## Materials and methods

### Study design and setting

Current study was conducted at Institute for Genetic Engineering and Biotechnologies for Post-graduate Study/ University of Baghdad from 15th of November 2021 to 10th of June 2022. Four ml of venous blood sample was taken from 25 patients suffering from T2DM and 25 healthy volunteers as control group, with age ranged between 35-65 years. Samples were collected in heparinized tubes under strict laboratory sterile condition, and processed for PBLs separation immediately to count apoptotic and necrotic lymphocytes.

### Lymphocytes isolation

Isolation of human PBLs was carried out by density-gradient centrifugation with Lymphocyte Separation Medium (LSM) (Corning\ USA). The fresh anticoagulant-treated blood was diluted with an equal volume of freshly prepared phosphate buffer saline (pH-7.2). Then layered over LSM solution and centrifuged in cold centrifuge at 400 ×g for 30-40 minutes at 18–20 °C. The lymphocytes were discovered at the plasma-LSM interface along with other slowly settling particles (platelets and monocytes) due to their lower density. After being removed from

the interface, the lymphocytes underwent a series of brief washing procedures in a balanced salt solution until a pellet was formed to remove any platelets; LSM and plasma (13). The lymphocyte cells were re-suspended in PBS after the supernatant had been discarded.

**Detection of apoptosis and necrosis in lymphocytes**

The preparation AO/EB solution was done according to Kasibhatla *et al*. (18). Apoptotic and necrotic lymphocytes were detected by using dual AO/ EB staining and fluorescence microscopy. Intact cell membranes can be penetrated by acridine orange, which can also bind to nuclear DNA and exhibit brilliant green fluorescence at a 488 nm wavelength. Additionally, EB only penetrated the disrupted cell membrane, incorporated nuclear DNA, and clearly shows orange fluorescence at a wavelength of 515 nm (19). Just before microscopy and quantification, amount of 25 µl of lymphocytes suspension was incubated with 1 µl of AO/EB solution and gently mixed. Each sample was evaluated immediately by placed 10 µl of stained lymphocytes suspension onto a glass coverslip-covered microscope slide; at least 300 cells were then investigated under a fluorescence microscope with imaging system. Two filters were used for each captured picture: FITC for green color and Texas Red for red color. The two pictures were merged together to give the real state for each patients regarding apoptosis and necrosis.

**Statistical analysis**

To determine how various factors affected the study parameters, the Statistical Analysis System- SAS (20) program was employed.

**Results and discussion**

The mean age of T2DM patients was (55.72±1.17) years. The results can be explained that by aging the physical activity reduces and the fat mass increases and the accumulation of the fat in visceral area become more than in the other area this lead to an increase the resistance to insulin and thus enhancing the risk of T2DM (21). In general, there are significant age-related immunologic and physiological changes that complicate the manifestation, diagnosis, and therapy in the senior population, making elderly T2DM patients are more at risk for morbidity and death than younger patients (22). It was clearly from the results that patients at age fifty year and more had most susceptible to disease due to age; it is known that diabetes increases the risk of developing it with age for psychological, physical and environmental reasons (23). Furthermore there are different environmental and life habits factors that could be related to this subject include an increased walkability and proximity to green space are linked to a decreased risk of T2DM, while increased T2DM risk is associated with increased levels of environmental pollution and noise (24). This result was agreement with the results of Shehab (25) who studied (50) T2DM patients, and a control group of (50) people who appeared healthy and had normal fasting blood sugar to found the association of some cytokine genes polymorphisms with type2 diabetes mellitus incidence in Iraq, also with Farhan (26) when she found a significant association between age and T2DM in Iraqi diabetic patients when study the effect of blood sugar level on *TNFα* gene expression and relation with liver disorders. Another study by Wali (27) found a significant association

between age and T2DM in Iraqi patients when study the relationship between *adiponectin* Gene polymorphism and type 2 diabetes mellitus in sample of Iraqi patients, also Sasaki *et al*. (28) who showed that age was related to variances in the incidence of type 2 diabetes, there was a verified elevated risk of Type-2 diabetes in the Japanese population with various stages of pre-diabetes. These results disagreed with Jung *et al*. (29) who studied the association between T2DM and bone fractures, also with Shamikh (30) who showed that there was non-significant difference between T2DM patients and control in terms of BMI. The incidence of apoptosis in freshly obtained lymphocytes from diabetic patients was quantified using dual AO/EB staining; through the staining pattern, cells were identified. Viable lymphocytes were seemed consistently green as illustrated via blue arrow in figure (1) unlike early apoptotic cells, which appeared as brilliant patches which indicated for chromatin condensation as pointed by purple arrow, while the orange arrow showing membrane blebbing which regarded to be one of the signs of early apoptosis that are frequently linked. Late apoptotic cells had chromatin that was constricted or disrupted and had orange to red-stained nuclei as indicated by red arrow, as well as necrotic cells were showed evenly pigmented orange to red nucleus as pointed by yellow arrow in figure (1).

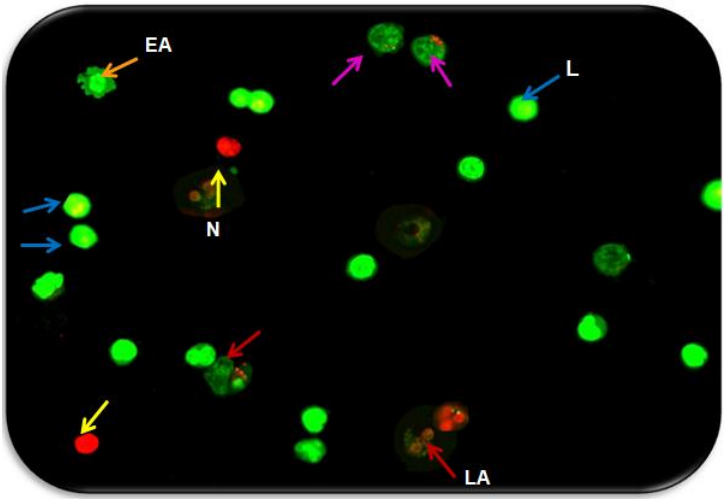


**Figure (1): Detection of AO\EB double stained lymphocytes by fluorescent microscope**

Blue arrow indicates live cell, purple arrow indicates early apoptotic cell, orange arrow showing blebbing membrane (one of the most typical signs of early apoptosis), red arrow indicates late apoptotic cell and yellow arrow indicates necrosis. The images were taken with a fluorescence microscope at 40×.

This pair of dyes is easily and frequently used to distinguish the difference between living, apoptotic, and necrotic cells (31). Furthermore, the morphological characteristics of apoptotic cells, which including membrane blebbing and damaged nuclei, were employed to determine if the cells were

apoptotic or whether (32). In current study, based on the nuclear morphology observed with the fluorescent AO/EB stains (nuclear collapse, chromatin condensation) of lymphocytes, an apoptotic cell's full profile was existent. The observations of these cells from diabetic patients were considerably different from the pattern observed in the control group, cells of diabetic patients exhibited morphological characteristics compatible with apoptosis more than healthy individuals as clarified in figure(2).

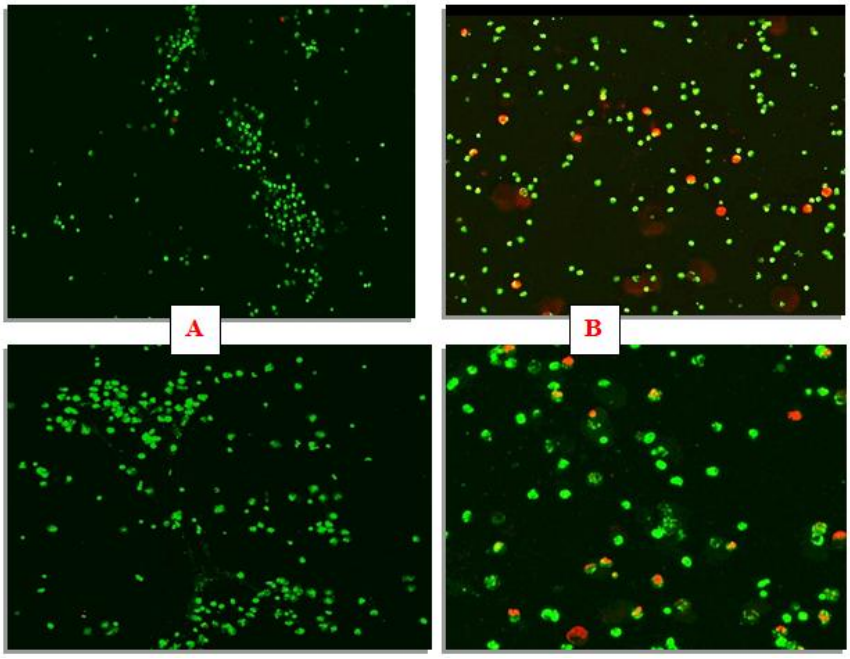


**Figure (2): Detection of a AO\EB double stained lymphocytes by fluorescent microscope (10X magnification). A: Healthy individual; B: Diabetic patient**

The present study observed an increase in the number of lymphocytes stained with red in diabetic patients in compared with control. Also the cells tend to appear as ambiguous nuclear outline, maybe as a result of nuclear and plasma membranes rupture. To support the idea that T2DM can cause lymphocytes to undergo cell death via apoptosis, at least 300 cells was examined in a fluorescence microscope with imaging system by using two filters for each captured picture: FITC for green color, and Texas Red for red color. The two pictures were merged together to give the real state for each individual regarding apoptosis and necrosis. The results of investigated the effect of age on apoptosis and necrosis of PBLs in T2DM patients revealed that the percentages of apoptosis and necrosis were higher in diabetic patients' lymphocytes when compared to control, especially at group of age more than 60, when the apoptosis reached to a maximum value (50.52 %) with significant difference ($P \leq 0.01$) as compared with the rest groups. Also, it was seen that T2DM enhanced necrosis in PBLs when the value reached to (20.3%) with significant difference ($P \leq 0.01$) at

group of age more than 60 when it was compared with other groups as illustrated in tables (1, 2).

**Table (1): Effect of T2DM and age on apoptosis of Peripheral Blood lymphocytes measured by fluorescence microscope.**

| Age group (years) | Number of Patients | Apoptosis Percentage | Number of control | Apoptosis Percentage | T-Test |
|---|---|---|---|---|---|
| **45-49** | 6 | 33.98 ±2.81 b | 10 | 12.63 ±1.26 b | 6.931 ** |
| **50-59** | 11 | 40.66 ±3.37 b | 9 | 15.02 ±1.58 ab | 8.046 ** |
| **60≥** | 8 | 50.52 ±3.95 a | 6 | 20.33 ±2.07 a | 6.859 ** |
| **Total** | 25 | -- | 25 | -- | -- |
| **P-value** | -- | 0.0028 ** | -- | 0.0077 ** | -- |
| Means have different letters in same column mean significantly different. ** (P≤0.01). | | | | | |

**Table (2): Effect of T2DM and age on necrosis of Peripheral Blood lymphocytes measured by fluorescence microscope.**

| Age group (years) | Number of Patients | Necrosis Percentage | Number of control | Necrosis Percentage | T-Test |
|---|---|---|---|---|---|
| **45-49** | 6 | 8.32 ±0.76 c | 10 | 5.83 ±0.33 b | 3.855 NS |
| **50-59** | 11 | 15.25 ±1.14 ab | 9 | 10.04 ±0.69 a | 4.763 * |
| **60≥** | 8 | 21.8 ±2.35 a | 6 | 11.64 ±0.78 a | 6.902 ** |
| **Total** | 25 | -- | 25 | -- | -- |
| **P- value** | -- | 0. 0001 ** | -- | 0.0097 ** | -- |
| Means have different letters in same column mean significantly different. * (P≤0.05), ** (P≤0.01). | | | | | |

Radically, Aging has been linked to elevated oxidative stress, changes in energy homeostasis, buildup of damaged proteins, as well as DNA lesions. Final results of all these disturbances include enhanced cell death and tissue atrophy (33, 34). In the periphery, lymphocyte counts are tightly maintained and, despite cyclical expansion during immunological responses, are quite steady in mature individuals. This is achieved by coordinating the synthesis of newly developed cells in the thymus and bone marrow with the expansion of peripheral lymphocytes and cell death (35, 36). Otton *et al*. (37) reported that the number of lymphocytes was reduced in diabetes individuals, which may have a significant impact on their weakened immune system and higher incidence of infections. The incidence of the apoptosis described here seems to be likely a clinical consequence of the cell count, enhanced lymphocyte apoptosis can cause immunosuppression (38). Arya and coworkers (17) demonstrated that the total T-cell apoptosis is significantly higher in T2DM with diabetic foot ulcer as compared with healthy control, also showed that increased oxidative stress in diabetics with chronic non healing wound induces lymphocyte apoptosis.

Current results came in accordance with earlier studies that reported an increased susceptibility of human lymphocytes to apoptosis induced by Fas or CD95 ligands (34, 39). In T2DM stress can induced pro-inflammatory cytokines: TNF- and IL-6, and the outcome of intricate processes resulting in apoptotic death (40). Mattisson and coworkers (41) showed that subjects with high levels of circulating markers of apoptotic cell death are more likely to develop diabetes. This

simply suggests that soluble apoptotic death receptors are markers of β-cell and vascular injury and potentially could be used as surrogate markers of therapeutic efficiency in risk factor interventions. In conclusion, the result of morphological detection of cell death caused by T2DM using AO/EB double staining revealed that diabetes states have a higher percentage of apoptotic and necrotic lymphocytes and this was increased with age progression; this could explain the immune system's dysfunction in elderly and poorly controlled diabetic patients.

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