Apoptosis in Pulmonary Tuberculosis
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Abstract:
The aim of this work is to detect the prevalence of isonizide(INH) and rifampicin(RIF) resistant Mycobacterium tuberculosis(M.TB)clinical isolates among the newly diagnosed pulmonary tuberculous patients and to assess the value of apoptosis as a laboratory technique for early diagnosis or follow up of pulmonary tuberculosis in comparison to the conventional identification methods (Z.N stain and culture on L.J medium).This study was carried out on 96 cases suspected of having pulmonary tuberculosis based on full history, clinical and radiological examination. They had never received antituberculosis treatment. Three successive morning sputum samples were collected from each patient and subjected to bacteriological study which includes: microscopical examination of Z.N stained sputum smears before and after concentration and decontamination by NALC-NaOH method, culture on L.J media and identification of isolates by biochemical reactions.Antimicrobial sensitivity tests were done for M.TB isolates to two antituberculous drugs: INH and RIF using the conventional proportion method on middle brook 7H10 agar.Blood samples from 14 cases harboring sensitive strains to INH and RIF from the newly diagnosed pulmonary tuberculous cases{classified into 4 mild and 10 advanced cases} and 10 control subjects were collected before and after antituberculosis treatment for assessment of lymphocyte apoptosis(by Giemsa & acridine orange stains and agarose gel electrophoresis) and detection of T cells activity by measuring IL-2 level of T-cell culture supernatant using ELISA method. Thirty six (37.5%) cases out of 96 showed mycobacterial growth on L.J medium, 20(55%) of them were positive by direct Z.N stain and 26(72%) were positive by Z.N stain after concentration and decontamination.By biochemical reactions identification 32 (88.8%) cases out of 36 mycobacterial isolates were M.TB and 4(11.2%) were mycobacteria other than T.B (MOTT). The primary drug resistance of M TB isolates for INH and RIF was 46.7%; 21.8%for INH,18.7%for RIF and 6.2% were MDR-TB isolates. Before treatment, apoptosis was significantly increased in tuberculous patients sensitive to antituberculous drugs(58.2±17.1 and 51.4±16.7 by Giemsa and acridine orange stains respectively) than control subjects(25.9±4.0 and 20.6±5.2 by Giemsa and acridine orange stains) respectively.)
stains respectively), while IL-2 level was significantly decreased in those patients \( (60.4 \pm 18.0) \) than controls \( (175.7 \pm 27.9) \). After treatment, the mean percentage of apoptotic lymphocytes in tuberculous patients by Giemsa and acridine orange stains were 27.3 \( \pm 6.1 \) and 25.0 \( \pm 7.7 \) respectively and the mean level of IL-2 was 163.71 \( \pm 20.55 \). In mild tuberculous group, the mean percentages of apoptotic lymphocytes were 33.5 \( \pm 2.4 \) and 28.3 \( \pm 2.4 \) by Giemsa and acridine orange stains respectively before treatment versus 24.0 \( \pm 6.1 \) and 17.5 \( \pm 5.9 \) after treatment and IL-2 level was 80.9 \( \pm 20.1 \) before treatment versus 173.8 \( \pm 13.8 \) after treatment. In advanced group, the mean percentages of apoptotic lymphocytes were 68.1 \( \pm 6.4 \) and 60.7 \( \pm 7.8 \) by Giemsa and acridine orange stains respectively before treatment versus 28.6 \( \pm 5.8 \) and 32.0 \( \pm 2.7 \) after treatment and the mean level of IL-2 was 52.5 \( \pm 9.8 \) before treatment versus 159.7 \( \pm 22.0 \) after treatment. As regards to lymphocyte apoptosis and IL-2 there is a significant statistical difference between the control and mild tuberculous groups before treatment and insignificant statistical difference between them after treatment. There is a significant statistical difference as regards lymphocyte apoptosis and a highly significant statistical difference in IL-2 level in mild group before and after treatment. There are highly significant statistical differences between the control and advanced tuberculous groups as regards to apoptosis and IL-2 level before treatment. After treatment, there is a significant statistical difference between both groups as regards apoptosis and insignificant statistical difference between them as regards IL-2 level. There are highly significant statistical values in lymphocyte apoptosis and IL-2 level of advanced group before and after treatment. In the advanced group, the percentage of apoptotic lymphocytes was significantly higher than in mild group. Morphological assessment of apoptosis was confirmed by agarose gel electrophoresis; DNA fragmentation was increased in patient's lymphocytes compared with that in controls before treatment and decreased by about 50% when restudied after treatment. In conclusion: the incidence of primary drug resistance in our locality is high, apoptosis as a simple and rapid test can be used for early diagnosis or follow up tuberculosis especially negative smear cases in addition to culture and other tests. Further follow up may be needed to established the relevance of apoptosis in the pathogenesis of tuberculosis.

**Introduction:**

Tuberculosis has reemerged in several industrialized countries. The cause of the global resurgence can be ascribed to the changes in the dynamics of the age-old battle between \( M. TB \) and the human host. Human immunodeficiency virus (HIV) infection is not the only
contributing influence; Socioeconomic poverty, homelessness, overcrowding and malnutrition have historically been associated with tuberculous infection\textsuperscript{(1\&2)}. The problem is compounded by the rising incidence of drug resistance and particularly the emergence of multidrug-resistant \textit{TB} (MDR--\textit{TB}). More than 50 million people may be already infected with MDR--\textit{TB} which is associated with a mortality of 50\% \textsuperscript{(3)}. Protective immunity against \textit{M.TB} essentially depends on cell mediated immunity, there are several types of cells involved in the expression of cell mediated reactions of which T-lymphocytes and macrophages are the most important\textsuperscript{(4)}. T cell function in protection against disease through activation of antimicrobial activities in macrophages by T-cell cytokines such as interferony (IFN $\gamma$), a major macrophage activating cytokines, tumour necrosis factor$\alpha$ (TNF-$\alpha$) and interleukin 2 (IL-2), and other Th1 cytokines\textsuperscript{(5)}. During active tuberculosis, exposure of T cells to \textit{M.TB} at sites of active \textit{M.TB} replication may lead to T cell activation and prime \textit{M.TB} responsive T cell to die through apoptotic mechanisms. Loss of \textit{M.TB} reactive T cells throughout apoptosis might play a role in limiting the cellular response to this infection\textsuperscript{(6\&7)}.

\textbf{Aim of the Work}

The aim of this work is to detect the prevalence of INH and RIF resistant \textit{Mycobacterium tuberculosis}(\textit{M.TB}) clinical isolates among the newly diagnosed pulmonary tuberculous patients and to assess the value of apoptosis as a laboratory technique for early diagnosis or follow up of pulmonary tuberculous cases in comparison to the conventional identification methods(Z-N stain and culture on L- J medium).

\textbf{Subjects And Methods:}

\textbf{I. Patients group:}

This study was carried out on 96 cases of suspected pulmonary tuberculosis; 70 males and 26 females, with age ranging from 14-63 years. They were selected from patients attending the Chest Outpatient Clinic of Benha University Hospital. They did not receive antituberculous drugs before. Ten healthy subjects were chosen as a control group.

\textbf{The patients were subjected to:}

- Full history taking including age, sex, demographic data, family and past history of similar condition and intake of antituberculous drugs.
- Clinical examination.
- Radiological examination: plain x-ray (A.P and lateral views).
Bacteriological study: three successive morning sputum samples were collected from each patient and subjected to:

- Microscopic examination of Z-N stained sputum smears before and after concentration and decontamination by NALC-NAOH method.
- Culture on L-J media.
- Identification of the isolates.
- Antimicrobial susceptibility to INH and RIF were detected using the conventional proportion method on Middlebrook 7H10 agar.

Assessment of lymphocyte apoptosis:

- Blood samples from 14 newly diagnosed pulmonary tuberculous cases harboring sensitive strains to INH and RIF and 10 control subjects were collected for assessment of lymphocyte apoptosis (by Giemsa & acridine orange stains and agarose gel electrophoresis) and detection of T cells activity by measuring IL-2 level of T-cell M.TB culture supernatant using ELISA method. Using the standardized radiographic criteria the patients were classified into: mild (4 patients had minimal disease) and advanced groups (10 patients had advanced disease). The cases with concomitant debilitating diseases such as diabetes mellitus, individuals being treated with immunosuppressive drugs, persons ≥ 50 years old and cases with drug resistant TB were excluded.

Lymphocyte separation was carried out according to Boyem. From each patient and control subject viable lymphocyte cells (2×10^6/ml) suspended in RPMI 1640 were cultured with M.TB susceptible isolates (infection ratio 10:1 M.TB to target cell) in tissue culture plate, then it was incubated in CO₂ incubator (5%) at 37°C for 48h. After that, the supernatants were collected and stored at -70°C till used for IL-2 determination. On the other hand, the cells were collected for apoptosis assay.

I. Morphological assessment of apoptosis using different stains:

1. Giemsa stain: The lymphocytes were removed from each culture (selected and control groups), smeared on slides and fixed with methanol for at least 15min. The slides were stained by Giemsa stain and examined by light microscope using the oil immersion lens. The cells were examined for apoptotic changes as decreased size, chromatin aggregation, cytoplasmic vacuolation, condensed and fragmented nuclei. The percentage of apoptotic cells was calculated (the number of apoptotic cells in 100 cells/slide).
- Fig(1) shows normal lymphocytes stained by Giemsa stain from control subjects.
- Fig(2) shows apoptotic lymphocytes stained by Giemsa stain from advanced tuberculous case before treatment.
- Fig(3) shows apoptotic lymphocytes stained by Giemsa stain from advanced tuberculous case after treatment.

2-Acridine orange stain\(^{(12)}\): One drop of lymphocyte suspension (selected and control groups) was added to one drop of acridine orange (50μg/ml in phosphate buffer solution) and mixed gently on glass slides. The lymphocytes were washed with phosphate buffer solution and examined by fluorescence microscope (1000x). Lymphocytes exhibiting bright fluorescent condensed chromatin located at the nuclear membrane and those with fragmented nuclei were interpreted as apoptotic cells and expressed as a percentage of the total cell number. Viable lymphocytes appeared with green diffusely stained intact nuclei.

- Fig(4): shows normal lymphocytes stained by acridine orange stain and examined by fluorescence microscope from control subject.
- Fig(5): shows lymphocytes stained by acridine orange stain from advanced tuberculous case before treatment.
- Fig(6): shows lymphocytes stained by acridine orange stain from advanced tuberculous case after treatment.

II. Assessment of lymphocyte DNA fragmentation by agarose gel electrophoresis(fig7):

- DNA isolation: was carried out according to: (Gentra, USA)
- Agarose gel electrophoresis of DNA was carried out according to Sambrook et al.\(^{(13)}\)

III- Detection of T cells activity by measuring IL-2 level of M.TB&T cell culture supernatant using the enzyme linked immunosorbent assay (ELISA) (Diaclone research, France).

All selected patients were treated with standard short-course antituberculous chemotherapy consisting of 2 months of daily isoniazid (5mg/kgm), rifampicin (10mg/kgm), ethambutol (15-25mg/kgm) and pyrazinamide (15-30mg/kgm) (intensive phase of treatment), followed by 4 months of daily INH and RIF (consolidation phase of treatment). These patients were restudied for apoptosis and detection of T cells activity by measuring of IL-2 level after completion of antituberculous treatment.
Statistical Analysis

Statistical analysis was done by using Statistical Package for Social Science (SPSS) version ten. Qualitative data was presented as number and percentage. Chi Square ($\chi^2$) and Fisher’s exact test (FET) were used for comparison between groups, as appropriate. Quantitative data was presented as mean ± SD (standard deviation). Student $t$-test was used for comparison between means of two groups. Paired $t$ test was used before and after comparison. Insignificant value: $P > 0.05$, Significance value: $P < 0.05$ and highly significant value: $P < 0.001$.

RESULTS

This study have been conducted on 96 patients with presenting symptoms suggestive of pulmonary tuberculosis. There is no history of previous receiving specific treatment for tuberculosis. The age of patients under study ranged from 14 to 63 years. They were 11 cases in age group 14-20 years, 55 cases in age group 21-40 years and 30 cases in age group 41-63 years. Sixty seven(69.7%) cases out of 96 were males and 29(30.3%) were females. Sputum specimens were collected from patients for detection of tubercle bacilli using Z-N stain and cultivation on L.J medium. Out of 96 sputum samples 36(37%) were positive on culture on L.J medium, 20(55%) of them were positive by direct Z.N stain and 26(72%) were positive by Z.N staining after processing. Thirty two(88.8%) isolates out of 36 positive mycobacterial cases were biochemically typed as $M. TB$ whereas the other 4(11.2%) isolates were MOTT. In tuberculous group: two (6.3%) out of 32 patients were within the 14-20 years group, 21 (65.6%) within 21-40 group and 9 (28.1%) within 41-63 years group. Twenty four (75%) out of 32 tuberculous patients were males and 8 (25%) were females.

- The antituberculous susceptibility testing of 32 $M. TB$ isolates revealed that 17 (53.2%) isolates were sensitive to INH and RIF whereas 15 (46.7%) isolates were resistant to one or both of them. Resistance pattern of 15 $M. TB$ isolates to INH & RIF showed that 13 isolates were single drug resistant distributed as follows: 7 (21.8%) isolates were resistant to INH and 6 isolates (18.7%) were resistant to RIF. Two(6.2%) isolates were Multidrug resistant (resistant to both INH and RIF).

Results of apoptosis:

Table(1) shows the comparison between 10 control subjects and 14 tuberculous cases sensitive to antituberculous drugs (3 susceptible cases were excluded) regarding the assessment of $M. TB$ induced lymphocytes apoptosis and IL-2 level in T-cell & $M. TB$ culture.
The mean percentage of apoptotic lymphocytes for control subjects was 25.9±4.0 and 20.6±5.2 by Giemsa and acridine orange stains respectively and the mean level of IL-2 for control group was 175.7 ± 27.9. Before treatment of susceptible cases, the mean percentage of apoptotic lymphocytes by Giemsa and acridine orange stains were 58.2 ±17.1 and 51.4±16.7 respectively and the mean level of IL-2 was 60.4±18.02. After treatment the mean percentage of apoptotic lymphocytes by Giemsa and acridine orange stains were 27.3±6.1 and 25.0±7.7 respectively and the mean level of IL-2 in susceptible cases was 163.7±20.55. There are highly significant statistical differences regarding lymphocyte apoptosis and IL-2 levels between the control group and 14 tuberculous cases sensitive to antituberculous drugs before treatment and insignificant statistical differences between both groups after treatment.

In this study 14 tuberculous cases sensitive to antituberculous drugs were classified according to the severity of disease, into mild group (4 patients had minimal lesions) and advanced group (10 patients had advanced lesions). In mild group, the mean percentages of apoptotic lymphocytes by Giemsa and acridine orange stains were 33.5 ± 2.4 and 28.3 ± 2.4 respectively before treatment while their mean percentages after treatment were 24.0 ± 6.1 and 17.5 ± 5.9 respectively. The mean level of IL-2 before treatment was 80.9 ± 20.1 but after treatment it was 173.8 ± 13.8. As regards to lymphocyte apoptosis and IL-2 there is a significant statistical difference between the control and mild tuberculous groups before treatment and insignificant statistical difference between them after treatment, there is a significant statistical difference in lymphocyte apoptosis and a highly significant statistical difference in IL-2 level in mild group before and after treatment (table 2).

In advanced tuberculous group, the mean percentage of apoptotic lymphocytes detected by Giemsa stain was 68.1 ± 6.4 before treatment and 28.6 ± 5.8 after treatment, by acridine orange stain it was 60.7 ± 7.8 before treatment and 32.0 ± 2.7 after treatment. The IL-2 level was 52.5 ± 9.8 before treatment and 159.7 ± 22.0 after treatment. There are highly significant statistical differences between the control and advanced tuberculous groups as regards to apoptosis and IL-2 level before treatment. After treatment, there is a significant statistical difference between both groups as regards apoptosis and insignificant statistical difference between them as regards IL-2 level. There are highly significant statistical values in lymphocyte apoptosis and IL-2 level of advanced group before and after treatment (table 3).
Morphological assessment of apoptosis by Giemsa and acridine orange stains was confirmed in 14 tuberculous cases by agarose gel electrophoresis DNA fragmentation with characteristic pattern of internucleosomal ladder. There was an increase of DNA fragmentation (an indicative of apoptosis) in tuberculous patient's lymphocytes compared with that of control before treatment. DNA fragmentation in the internucleosomal pattern reduced by about 50% when restudied after treatment.

Table(1): Mean percentages of apoptotic lymphocytes (by Giemsa and acridine orange stains) and interleukin-2 level for control group and 14 tuberculous cases sensitive to antituberculous drugs.

<table>
<thead>
<tr>
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<th>Control (N = 10)</th>
<th>Cases (N = 14)</th>
<th>Significance test</th>
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<tr>
<td><strong>Lymphocyte apoptosis:</strong></td>
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<tr>
<td><strong>Giemsa</strong></td>
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<td>Before treatment</td>
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<tr>
<td>Mean ± SD</td>
<td>25.9 ± 4.04</td>
<td>58.2 ± 17.1</td>
<td>6.81</td>
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<td>After treatment</td>
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<td>27.28 ± 6.06</td>
<td>0.69</td>
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<td><strong>Acridine orange</strong></td>
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<tr>
<td>Before treatment</td>
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<tr>
<td>Mean ± SD</td>
<td>20.6 ± 5.19</td>
<td>51.42 ± 16.71</td>
<td>5.65</td>
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<td>After treatment</td>
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<td>25.0 ± 7.71</td>
<td>1.43</td>
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<td><strong>IL-2 Level</strong></td>
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<td>Before treatment</td>
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<tr>
<td>Mean ± SD</td>
<td>175.7 ± 27.9</td>
<td>60.35 ± 18.02</td>
<td>12.33</td>
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<td>After treatment</td>
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<td>163.71 ± 20.55</td>
<td>1.21</td>
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HS* : highly significant
NS** : non significant
Table (2): Mean percentages of apoptotic lymphocytes (by Giemsa and acridine orange stains) and IL-2 level in control and mild tuberculous groups before and after treatment.

<table>
<thead>
<tr>
<th></th>
<th>Control (N=10) Mean ± SD</th>
<th>Mild group (N:4) Mean ± SD</th>
<th>Significance test</th>
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<tr>
<td>Lymphocyte apoptosis:</td>
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<tr>
<td>Giemsa</td>
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<tr>
<td>Before treatment</td>
<td>25.9 ± 4.04</td>
<td>33.5 ± 2.35</td>
<td>3.48</td>
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<td>After treatment</td>
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<td>24.5 ± 6.055</td>
<td>0.005 &lt; S*</td>
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<tr>
<td>Acridine orange</td>
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<tr>
<td>Before treatment</td>
<td>20.6 ± 5.19</td>
<td>28.25 ± 2.36</td>
<td>2.78</td>
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<tr>
<td>After treatment</td>
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<td>17.5 ± 5.97</td>
<td>0.017 &lt; S</td>
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<td>IL-2 level:</td>
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<tr>
<td>Before treatment</td>
<td>175.7 ± 27.9</td>
<td>80.9 ± 20.1</td>
<td>7.17</td>
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<td>After treatment</td>
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<td>173.75±13.7</td>
<td>&lt;0.001 &lt; HS</td>
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</table>

S*: Significant

$P_1$ is for comparison between control group and mild tuberculous group before treatment.

$P_2$ is for comparison between control group and mild tuberculous group after treatment.

$P_3$ is for comparison of mild tuberculous group before and after treatment.

Table (3): Mean percentages of apoptotic lymphocytes and IL-2 level in control and advanced tuberculous groups before and after treatment.

<table>
<thead>
<tr>
<th></th>
<th>Control (N:10) Mean ± SD</th>
<th>Advanced group (N:10) Mean ± SD</th>
<th>Significance test</th>
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<tr>
<td>Lymphocyte apoptosis:</td>
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<td>Giemsa</td>
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<tr>
<td>Before treatment</td>
<td>25.9 ± 4.04</td>
<td>68.1 ± 6.38</td>
<td>17.75 &lt; 0.001 HS</td>
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<tr>
<td>After treatment</td>
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<td>28.6 ± 5.83</td>
<td>2.01 &lt; 0.001 S</td>
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<tr>
<td>Acridine orange</td>
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<tr>
<td>Before treatment</td>
<td>20.6 ± 5.19</td>
<td>60.7 ± 7.84</td>
<td>13.84 &lt; 0.001 HS</td>
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<tr>
<td>After treatment</td>
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<td>32.0 ± 2.71</td>
<td>2.7 &lt; 0.001 S</td>
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<td>IL-2 level:</td>
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<tr>
<td>Before treatment</td>
<td>175.7 ± 27.9</td>
<td>52.5 ± 9.78</td>
<td>13.17 &lt; 0.001 HS</td>
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<tr>
<td>After treatment</td>
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<td>159.7±22.00</td>
<td>1.02 &lt; 0.001 NS</td>
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</table>

$P_1$ is for comparison between the control group and advanced tuberculous group before treatment.

$P_2$ is for comparison between the control group and advanced tuberculous group after treatment.

$P_3$ is for comparison of advanced tuberculous group before and after treatment.
Fig(1) shows normal lymphocytes stained by Giemsa stain from control subjects.
Fig(2) shows apoptotic lymphocytes stained by Giemsa stain from advanced tuberculous case before treatment.

Morphological changes of apoptosis in lymphocytes:
  a- Cell shrinkage and decrease cell volume.
  b- Eccentric nucleus.
  c- Vaculated cytoplasm.
  d- Budding cells.
  e- Apoptotic bodies.
Fig(3) shows apoptotic lymphocytes stained by Giemsa stain from advanced tuberculous case after treatment. Little number of apoptotic bodies show that apoptosis decreased after treatment (arrows refer to apoptotic bodies).
Fig(4): shows normal lymphocytes stained by acridine orange stain and examined by fluorescent microscope from control subject.
Fig(5): Apoptotic lymphocytes stained by acridine orange stain from advanced tuberculous case before treatment.

Apoptotic cells have bright fluorescent, condensed nuclei and many apoptotic bodies are seen.
Fig(6): Apoptotic lymphocytes stained by acridine orange stain from advanced tuberculous case after treatment.

Little number of apoptotic bodies show that apoptosis decreased after treatment.
**Fig 7:** Assessment of lymphocyte DNA fragmentation by agarose gel electrophoresis

**Lanes 1 and 2** show DNA electrophoresis of apoptotic lymphocytes from advanced tuberculous cases before treatment demonstrating multiple DNA fragmentation with more prominent banding pattern.

**Lanes 3 and 4** show DNA electrophoresis of apoptotic lymphocytes from advanced tuberculous cases after treatment demonstrating a very faint apoptotic ladder.

Lanes 5 and 6 show DNA electrophoresis of lymphocytes from control subject showing no bands.

**DISCUSSION**

Tuberculosis has been a leading cause of death in the world for centuries. *M. TB* is not only one of the oldest microbial threats to human health; it is also one of the most formidable, with an estimated one-third of the world population infected\(^{14}\). The severity of this global medical problem has worsened in recent years due to emergence of multidrug resistant *M. TB* strains and to its association with HIV infection\(^{15}\).

This study shows insignificant statistical difference between positive and negative tuberculous cases regarding the age; in positive tuberculous cases, the highest percentage of
tuberculosis 65.6% was in age group 21-40 years followed by 28.1% in age group 41-63 years then 6.3% in age group 14-20 years. This agrees with Abd El-Maksoud who found that 59.5% of tuberculous patients in his study were within 21-40 years age group and Bayomi results were found that the higher prevalence of tuberculosis infection (34.4%) was among the age group 21-30 years followed by 21.9% among the age group 31-40 years. Also, Zaghloul reported that 23.5% of tuberculous patients in his study were within the age group 20-30 years followed by 17.7% within the age group 30-40 years. These findings explained by Dunlap etal. who reported that tuberculosis is high among young adults 20-40 years as they are the productive sector of community and usually exposed to physical and mental overstrain. In this study there is insignificant statistical difference regarding the sex distribution between positive and negative tuberculous cases. In tuberculous group, 24 cases (75%) were males and 8 (25%) were females. Similar results were reported by Zaghloul who found that 76.4% of tuberculous patients in his study were males and 23.5% were females. The higher incidence of infection in males may be due to occupational hazards, smoking and stress of work.

The mycobacterial biochemical reactions revealed 32 (88.8%) M. TB and 4 (11.2%) MOTT isolates, These results agree with that of El-Gazar etal., who isolated 6 (12.8%) MOTT isolates out of 47 mycobacterial cultures. Somoskovi etal., isolated 5 (4.3%) MOTT isolates out of 117 mycobacterial cultures, while Ibrahim did not isolate any MOTT strains out of 231 mycobacterial cultures.

Studying the antituberculous sensitivity of 32 M.TB isolates to INH and RIF revealed that the primary drug resistance was 46.7%. The resistance to INH was 21.8%, 18.7% to RIF and 6.2% were MDR-TB isolates. Abd El-Maksoud found that the overall primary drug resistance was 41.2%. Also, Caviedes etal., showed primary drug resistance rates of 25% and 18% for INH and RIF respectively. These results are in agreement with that of the present study. Higher results were reported by Hazbon etal., who reported resistance rates of 38.9% to INH, 22.1% to RIF and 21.1% were MDR-TB isolates, while Cohn etal., reported primary resistance rates 16.9% for INH, 3% for RIF and Pablos-Mendez etal. reported 7.3% primary resistance to INH, 1.8% to RIF and 1.4% were MDR-TB isolates. The difference in drug susceptibility testing method used, quality control and laboratory proficiency variability may lead to the variation in results. Also, the exclusion of patients who had any previous antituberculosis chemotherapy from the study is related to this variation in results.
In this work apoptosis was examined in tuberculous patients harboring sensitive strains to INH & RIF (14 cases) and control subjects. Both of Giemsa and acridine orange stains revealed a high significant increase in lymphocyte apoptosis among the studied tuberculous patients before treatment than control subjects; the mean percentage of apoptotic lymphocytes were 58.2 ± 17.1 and 51.4 ± 16.7 by Giemsa and acridine orange stains respectively for tuberculous cases while they were 25.9 ± 4.0 by Giemsa and 20.6 ± 5.2 by acridine orange stains for control subjects. There was a high significant decrease in IL-2 level in tuberculous patients before treatment as compared with that in controls. In tuberculous patients, the mean level for IL-2 before treatment was 60.4 ± 18.02 while it was 175.7 ± 27.9 in control subjects. Similar results were reported by Barnes et al., and Hirsch et al.,(29,30) who found that lymphocyte apoptosis increased in tuberculous patients compared to control subjects. They observed an inverse correlation between increased T cell apoptosis and suppressed IL-2 level. Hertoghe et al.,(31) also reported that T cell apoptosis increased in patients with newly diagnosed tuberculosis as compared with control subjects.

The correlation between the severity of disease and degree of apoptosis showed that before treatment apoptosis in mild tuberculous group was significantly lower (33.5 ± 2.3 and 28.25 ± 2.36 by Giemsa and acridine orange stains respectively) than that in advanced tuberculous group (68.1 ± 6.4 by Giemsa and 60.7 ± 7.8 by acridine orange stains). This finding coincides with that reported by Hirsch et al.,(10) who found a correlation between radiographic severity of disease and apoptosis. They suggested that during tuberculosis both enhanced T cell apoptosis and T cell hyporesponsiveness occur as a consequence of a complex mechanism that involves excessive immune activation, production of deactivating cytokines and inflammation.

Alterations in apoptosis and IL-2 level after successful chemotherapy for M.T.B was examined; in mild tuberculous group, apoptosis was significantly higher before treatment than after treatment(33.5± 2.4 and 28.3±2.4 by Giemsa and acridine orange stains respectively before treatment versus 24.0±6.1 by Giemsa and 17.5±5.9 by acridine orange stains after treatment)and IL-2 level was significantly increased after treatment(80.9±20.1 before treatment versus 173.8±13.8 after treatment). In advanced group, after treatment apoptosis was significantly decreased(68.1±6.4 and 60.7±7.8 by Giemsa and acridine orange stains respectively before treatment versus 28.6±5.8 by Giemsa and 32.0±2.7 by acridine orange stains after treatment) and the level of IL-2 was significantly
increased (52.5±9.8 before treatment versus 159.7±22.0 after treatment). Compared with healthy control, apoptosis and IL-2 level in mild tuberculous group were significantly different before treatment but they showed insignificant statistical difference after treatment. While the comparison between healthy controls and advanced tuberculous group regarding apoptosis showed that the significant difference was still present between them after treatment. Conversely, IL-2 level in advanced group after treatment showed insignificant statistical difference compared with that of control subjects. This is supported by Hirsch et al., and Wilsher et al.,\(^{(10,32)}\) results; they reported that despite significant reduction of apoptosis in advanced tuberculous patients after treatment, the apoptotic T cells still differed significantly from that of control subjects. Also, they mentioned that IL-2 level in advanced cases increased after treatment to a level comparable to that produced in control subjects.

In this study, apoptosis was also confirmed by agarose gel electrophoresis and the evidence of chromatin fragmentation in the form of ladder was observed in lymphocytes from tuberculous patients cultured in vitro. Fragmented DNA in the internucleosomal pattern reduced to about 50% when restudied after treatment. These findings are in agreement with that of Hirsh et al.,\(^{(10)}\); they concluded that T cell apoptosis in patients with advanced tuberculosis decreased by about 50% after 6 months of treatment. In parallel with reduction in \(M.\ TB\) induced apoptosis, the level of IL-2 was significantly increased after treatment. They provided preliminary evidence of a relationship between low T cell responses, as evidenced by depressed IL-2 level, and T cell apoptosis.

The resurgence of tuberculosis and increase in the resistance of \(M.\ TB\) to antituberculous agents have focused attention on the need for simple and rapid means that help to diagnose tuberculosis. The rapid diagnosis of \(M.\ TB\) and accurate antituberculous susceptibility tests are essential for treatment of infected patients and to control the spread of the disease. Apoptosis as a test for T cell function and responsiveness can be used for early diagnosis or follow up of pulmonary tuberculosis besides Z.N., sputum culture and other investigations. It helps in diagnosis of pulmonary negative stained smears tuberculosis. It is evident also from the results of the present study that the incidence of primary drug resistance in our locality is high and tuberculosis control is not performed well in Egypt. Further studies are needed to evaluate these programmes. The high prevalence of RIF and INH resistance stress the needs for limitation of their use for treatment of tuberculous patients only and not to be prescribed as broad spectrum antibacterial agents.
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