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Induction of Apoptosis in Peripheral Blood Mononuclear Cells by Anti-Fas Monoclonal Antibodies in Rheumatoid Arthritis Patients With or Without Uveitis

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Abstract: Rheumatoid arthritis (RA) is a systemic inflammatory autoimmune disease associated with a number of articular and extra-articular organ manifestations. The imbalance between cell proliferation and apoptosis has been incriminated in the pathogenesis of autoimmunity. Thus, the objectives of this study were: to evaluate the apoptosis process of peripheral blood mononuclear cells (PBMNCs) in RA patients and RA patients with or without uveitis that may contributes to the inflammation in RA, to test the susceptibility of PBMNCs to the *in vitro* induction of apoptosis with anti-Fas monoclonal antibodies (mAb) and the possibility of using it as an apoptosis therapy to modulate the inflammatory process. PBMNCs were isolated and cultured in the presence or absence of anti-Fas (mAb). Evaluation of apoptosis was determined by measuring the (%) of cell viability and the levels of apoptosis (DNA and histones components of the nucleosomes) as indicated by levels of absorbance in the supernatant of cultured cells by ELISA before and after induction of apoptosis. Results of this work revealed that before induction of apoptosis with anti-Fas (mAb) the mean % of cell viability of all the studied groups was high, however, highly significant reductions ($P < 0.001$) in cell viability were found in groups ($P < 0.001$) after induction of apoptosis and the reduction was more pronounced in RA patients with uveitis ($P < 0.001$). On the other hand, anti-Fas (mAb) induced significant elevations in the mean values of apoptosis levels (absorbance) in all RA patients ($P < 0.01$) and RA with uveitis ($P < 0.05$). Additionally, negative correlations were detected between % of cell viability and apoptosis levels, and between % of cell viability and inflammatory markers (DAS, CRP, Anti-CCp and ANA). In conclusion, insufficient apoptosis of PBMNCs of RA patients, RA patients with and without uveitis may play an important role in RA disease and these cells can be induced to undergo apoptosis *in vitro* by anti-Fas (mAb). The study points out to the possibility of using anti-Fas (mAb) as an apoptosis induction therapy to modulate the inflammatory process.

Key words:

INTRODUCTION

Rheumatoid arthritis (RA) is a chronic inflammatory disorder presumably of autoimmune origin principally producing a progressively crippling polyarthritis. It reflects abnormal proliferation and/or persistence of inflammatory cells in synovial fluid. Several different cell types and their mediators are involved in the tissue destructive inflammation, e.g. T cells, B cells, monocyte/macrophages, and proinflammatory cytokines such as tumor necrosis factor (TNF-α) and interleukin-1β (IL-1 β) (Weyand; 2000).

The early stage of the disease is characterized by synovial hyperplasia, angiogenesis and mononuclear cell infiltrate which lead to the destruction of the bone and cartilage of joints. Hyperplasia encompass both macrophage-like synovial cells and fibroblast-like synovial cells that exhibit pre-neoplastic characteristic with expression of proto-oncogenes (Tak and Bresnihan; 2000). In the later stage, imbalance between cell proliferation and apoptosis results in reduction in synovial cells proliferation and replacement by connective tissues (Pope; 2002).

Disrupted apoptosis of the different immune cells has been incriminated in the pathogenesis of autoimmunity. Such process is involved in RA in two locations. The first and the one extensively studied is the apoptotic process taking place in the affected joints. Herein, there were many evidences suggesting defective T and B cell apoptosis in rheumatoid synovium. The second location is the peripheral blood mononuclear cells (PBMNC) which play an important role in the perpetuation of the autoimmune process in RA (Mohamed et.al; 2008).

Rheumatoid arthritis is a systemic inflammatory disease associated with a number of extra-articular organ manifestations such as pericarditis, pleuritis, ocular manifestations, major cutaneous vasculitis, Felty's syndrome, neuropathy, glomerulonephritis, and other types of vasculitis (Sahatçiu-Meka *et al*; 2010). The association of ocular lesions with rheumatoid arthritis has long been recognized (Chan and Li; 1998). The well documented ocular lesions of this disease are keratoconjunctivitis sicca, anterior uveitis, episcleritis, scleritis, scleromalacia perforans and massive granuloma of sclera. Failure to recognize the significance of this

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association by Rheumatologist, may result in acute loss of vision with blindness in some diseases when irreversible damage to the eye has occurred (Goronzy and Weyand; 2009).

Although several receptor and/or biochemical pathways are known to regulate cellular apoptosis, the Fas (CD95)–Fas ligand (FasL, CD178) death receptor pathway has received the most attention in inflammatory arthritis, particularly RA (Wang *et al*; 2000). Apoptotic cells are uncommonly observed in RA tissues in vivo, but synoviocytes, synovial T cells and macrophages have often been observed to express high levels of Fas and/or FasL, and are highly susceptible to Fas/FasL induced apoptosis *in vitro* (Peng; 2006). Increased intrasynovial and/or serum sFas appears to compete with mFas and prevent apoptosis of synoviocytes. In addition, in some studies, invading T cells have been found to be defective in FasL expression, which could account for ineffective clearance of activated Fas-expressing cells (Cantwell *et al*; 1997). The induction of apoptosis has proposed as a potential therapeutic approach (Baier *et al*; 2003).

In addition, lymphocyte apoptosis via Fas-FasL interactions has been shown to play a key role in maintaining immune privilege in the eye and inhibiting inflammation. However, absence of either FasL from the eye tissues, or Fas from the lymphocytes, results in a destructive inflammatory response like inflammatory uveitis (Chan and Li; 1998, Marrack and Kappler; 2004). Investigations of the role of apoptosis in uveitis and modulation of the Fas pathway have been conducted which may provide therapeutic benefits (Clewes *et al*; 2005). Indeed, it is interesting to note that few direct human RA studies have been performed to demonstrate the pathogenic and/or therapeutic relevance of the Fas-FasL pathway *in vivo* (Smith *et al*; 2001 and Catrina *et al*; 2005).).

The objectives of this study were to evaluate the apoptosis process of PBMNCs in RA patients and RA patients with or without uveitis that may contributes to the inflammation in RA disease, to test the susceptibility of PBMNCs to the *in vitro* induction of apoptosis with anti-Fas mAb and the possibility of using it as an apoptosis therapy to modulate the inflammatory process.

Subjects and Methods:

Subjects:

The study involved 20 adult patients, diagnosed clinicaly, and confirmed by laboratory investigations, and radiologicaly to have chronic RA (grade II; moderate or III; severe) according to the American Rheumatism Association criteria of RA attending the outpatient clinic of Rheumatology at Bolak El-Dakror General Hospital. They were 17 females and 3 males and their ages ranged from 27-80 years. They were maintained on 7.5 mg methotrexate / week and concomitant non steroidal antiinflammatory oral drugs (NSAIDS) and local application to the eyes in patients with severe uveitis.

All patients were subjected to I) clinical history regarding disease duration, extent of joint and eye envolvement and medications, (II) Clinical examination of the joints using disease activity score (DAS) for assessement of disease activity and of the eye using Slit Lamp examination of the anterior and posterior chambers respectively. The DAS combines several aspects of disease activity into a single outcome, (III) Laboratory investigations: erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), rheumatoid factor (RF), antinuclear autoantibody (ANA) and anti-cyclic citrullinated peptides (anti-CCp). (IV) X-ray examination of the joints for bone destruction and deformity. Patients with other diseases which may induce joint inflammation were excluded. Patients were classified into 2 groups according to the involvement of uveitis: group I included 10 RA patients associated with uveitis and group II included 10 RA patients without uveitis. Twenty age and sex matched healthy volunteers were included as controls (group III).

Sample:

Five milliliter of peripheral blood samples were obtained from all patients and controls using vacutainer tubes containing EDTA.

Methods:

Isolation of Peripheral blood Mononuclear cells:

Mononuclear cells (PBMCs) were isolated from anti-coagulated peripheral blood samples of patients and controls using Ficoll-Hypaque density gradient centrifugation according to *(Perper et al; 1977 & Boyum, 1974)*. PBMC pellets were suspended at $1X10^6$ cells/ml in complete RPMI-1640 tissue culture medium supplemented with 200 µg/ml penicillin, 1 µg/ml streptomycin, 0.1 mg/ ml gentamicin 2.5 mg / ml amphotericin B, 10 % heat-inactivated fetal calf serum (Sigma, St Louis, MO) and 2 mmol/L L-glutamine (GIBCO, Grand Island, NY).

In vitro induction of Apoptosis in PBMNCs (Hoa et al; 1996):

Two aliquots of 500 µl of $1X10^6$ /ml PBMCs of each sample were prepared. One aliquot was treated with 1µg/ml of Anti-Fas monoclonal antibodies (mAb) (Sigma Aldrich, USA) and the second was left untreated (culture medium only) and was used as a negative control tube.

All tubes were incubated at 5% CO2 humid atmosphere, 37°C, for 24 hours. Both anti-Fas (mAb) treated and untreated cells were centrifuged at 1800 rpm for 10 min. and the culture supernatants were collected and stored at -70°C until assessed for apoptosis and cells were assessed for viability.

Evaluation of Apoptosis:

Apoptosis levels before and after apoptosis induction by anti-Fas mAb were evaluated in 20 ul of cell culture supernatant equivalent to a cell of 5 x 10^4 cell/ml. Apoptosis was detected by two different methods: counting of cell viability and measuring the absorbance values of oligonucleosomes produced by endonucleases involved in the apoptotic pathway.

1- Calculation of Cell Viability (Paul, 1968):

The effect of apoptosis induction on PBMCs was evaluated by counting the number viable cells using the trypan blue exclusion test and the percent of viable cells was determined relative to the total number of cells. The non-viable cells take the blue stain:

$$\% \text{ of viable cells} = \frac{100 - \text{number of dead cells} \times 100}{\text{Total number of cells}}$$

In addition, the % of change was calculated as follows:

$$\% \text{ of change} = \frac{\text{value before treatment} - \text{value after treatment}}{\text{Value before treatment}} \times 100$$

2- Quantitative Determination of DNA and Histones Components of the Nucleosomes in Cell Culture Supernatants:

Apoptosis was assessed by specific determination of mono- and oligonucleosome in the cytoplasmic fraction of cell lysates using a quantitative sandwich-enzyme-immunoassay; ELISA (Roche Diagnostics) utilizing mouse monoclonal antibodies directed against DNA and histones components of the nucleosomes. Dead cells of the samples will cause certain absorbance value (*Ab*) that may vary depending on the amount of dead cells and released of mono- and oligonucleosomes into the cytoplasm of apoptotic cells.

Breifly, 20 ul of cell culture supernatants before and after induction of apoptosis and positive control (DNA-histone-complex) were transfered to the streptavidin coated plate followed by addition of 80 ul of immuneoreagents (anti-histone biotin + anti-DNA peroxidase conjugate), incubated on a shaker for 2 hours at room temperature. The anti-histone bound to the histone component of the nucleosomes and simultaneously fixed the immunocomplex to the streptavidin coated plate via biotin. Additionally, anti-DNA peroxidase reacts with DNA component of the nucleosomes. Then, ABTS substrate followed by stop solution were added. The test was performed following the manifactiorer's instructions.

Absorbance of samples of patients groups and controls before and after induction of apoptosis were compared and the % of change was calculated by the previous equation.

Statistical Analysis:

Data was analyzed by Microsoft Office 2003 (excel) and Statistical Package for Social Science (SPSS) version 16. Parametric data was expressed as mean ± SD, and non parametric data was expressed as number and percentage of the total. Comparing the mean ± SD of 2 groups was done using paired and unpaired student's t test. Measuring the mutual correspondence between two values was done using the Spearman correlation coefficient. P value > 0.05 is considered non-significant, P value < 0.05 is considered significant, P value < 0.01 is considered highly significant.

Results:

Clinical and Laboratory Data of the Studied Groups:

The study was carried out on 20 adult RA patients; They were 17 females and 3 males, the mean±SD of age was (49.05±14.31) years ranging from 21-80 years. The frequency of RA increases with age. RA is more common in females than in males. The mean of disease duration of RA was (6.80 ±4.07) years, ranging from 2-15 years. Ten patients have associated unilateral or bilateral uveitis (group I), and the other 10 patients without uveitis (group II). At the time of examination the mean duration of arthritis was significantly higher in patients with uveitis (9.00±4.83) as compared to those without uveitis (4.60±3.31) years, (p<0.05).

The extent of joint envolvement and activity of the disease revealed DAS score (mean±SD) in the two groups of, (9.00±3.30) and (5.80±3.46) respectively, with a significant higher in group I (P<0.05). Plain X ray for the affected joints showed erosion and bone destruction of joints and vertebrae in severe cases.

The mean±SD of ESR, CRP, RF, anti-CCp and ANA were (42.40±16.11), (19.26±10.83), (18.32±18.87), (52.23±9.27) and (24.17±11.16) respectively in group I and they were (38.50±15.62), (16.49±8.74), (24.25±21.20), (38.61± 21.30) and (24.64±8.48) respectively in group II. Twenty healthy controls (group III)

matched age (mean 49.30±12.82) years and sex (8/12) ratio were included. The characteristics of the studied groups are shown in Table 1.

Laboratory investigations of group I and group II RA patients were significantly higher than normal controls and in RA with uveitis than those without uveitis (P<0.01).

Percentage (%) of PBMN Cell Viability in all Rheumatoid Arthritis Patients, RA Patients with Uveitis, RA without Uveiti and Normal Controls Before and After Apoptosis Induction by Anti-Fas mAb:

The mean % of cell viability of all RA patients, RA patients with uveitis, RA patients without uveitis and normal controls were (95.35 ±1.27), (94.40± 0.84), (96.30± 0.82) and (97.45± 0.94) respectively before induction of apoptosis. After induction of apopotosis the mean % of cell viability was reduced to (90.90±1.86), (89.50±1.58), (92.30± 0.67) in RA patients respectively and (95.40±0.94) in normal controls. Highly significant reductions (P <0.001) in the mean % of cell viability were found between groups (P<0.001) and the reduction was more pronounced in RA patients with uveitis (P <0.001) (Figure 1a & b).

The % change (reduction) in cell viability of PBMNCs induced by Anti-Fas mAb in all RA patients, RA patients with uveitis, RA without uveitis and controls was (-4.91±1.14), (-5.49±1.32) ,(-4.33±0.51), and (-2.15± 0.24) respectively. Highly significant differences in the % change of cell viability was noticed after induction of apoptosis in all patient groups (P< 0.01) which was more pronounced in RA patients with uveitis, (Figure 3 a & b).

Apoptosis Levels as Determined by Absorbance in the Supernatant of Cultured PBMNCs:

The mean values of apoptosis levels as detected by absorbance of all RA patients, RA patients with uveitis, RA patients without uveitis and normal controls were (0.21± 0.24), (0.34± 0.28), (0.08 ± 0.06) and (0.10± 0.07) respectively before induction of apoptosis. However, after induction of apopotosis the mean values of apoptosis levels was increased to (0.42± 0.52), (0.69± 0.62), (0.14± 0.11) in RA patients respectively and (0.12 ± 0.08) in controls. Highly significant (P<0.01) and significant (P<0.05) elevations in all RA patients and RA with uveitis were detected. While, insignificant difference was detected between RA without uveitis and normal controls (P**>0.05**), (Figure 2 a & b).

The % change in apoptosis levels (absorbance) of PBMNCs supernatant induced by anti-Fas mAb in all RA, RA with uveitis, RA without uveitis and controls was (41.91±26.63), (43.08±29.46), (40.74±25.03) and (20.69±19.43) respectively. Significant changes were revealed in all RA patients and RA with uveitis as compared with controls (P<0.05), insignificant difference (P>0.05) was revealed between RA with uveitis and RA without Uveitis (Figure 3 c & d).

Table 1: Clinical and laboratory data of the studied groups.

Parameter	Group I (RA with uveitis) N=10	Group II (RA without uveitis) N=10	Group III (Normal controls) N=20	P value
Age (years)				
range	21-80	27-67	21- 70.00	
mean±SD	48.8±17.42	49.30±11.20	49.30±12.82	>0.05
Sex (♂/♀)	2/8	1/9	8/12	>0.05
Duration of RA (years),				
range	3-15	2-12		< 0.05
mean±SD	9.00±4.83	4.60±3.31		
Active joint count, DAS	9.00±3.30	5.80±3.46		< 0.05
ESR (mm/first hour),	42.40±16.11	38.50±15.62	12.89±6.18	< 0.01
CRP (mg/dl),	19.26±10.83	16.49±8.74	5.64±4.65	< 0.01
RF (IU/ml),	18.32±18.87	24.25±21.20	8.04±3.13	< 0.01
Anti-CCP	52.23±9.27	38.61± 21.30	16.26±5.38	< 0.01
ANA	24.17±11.16	24.64±8.48	8.80±4.19	< 0.01

* CRP = C-reactive protein (normal value 0–5 mg/l); ESR = erythrocyte sedimentation rate (normal value <14 mm/1 st hour); RF, rheumatoid factor (normal value 0–14 IU/ml); Anti-CCP = anti-cyclic citrullinated peptides (normal value <40 IU/ml); ANA= antinuclear autoantibody (normal value <10 IU/ml)

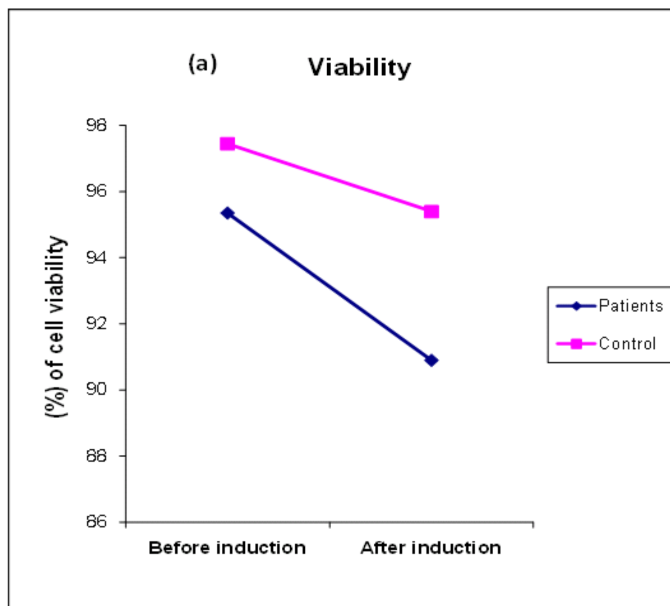

Fig. 1: (a) Percentage of cell viability of PBMNCs induced by anti-Fas mAb in all RA patients (n=20) and controls. Highly significant reduction in the % of cell viability was noticed after induction of apoptosis in patient groups as compared with controls (P<0.01).

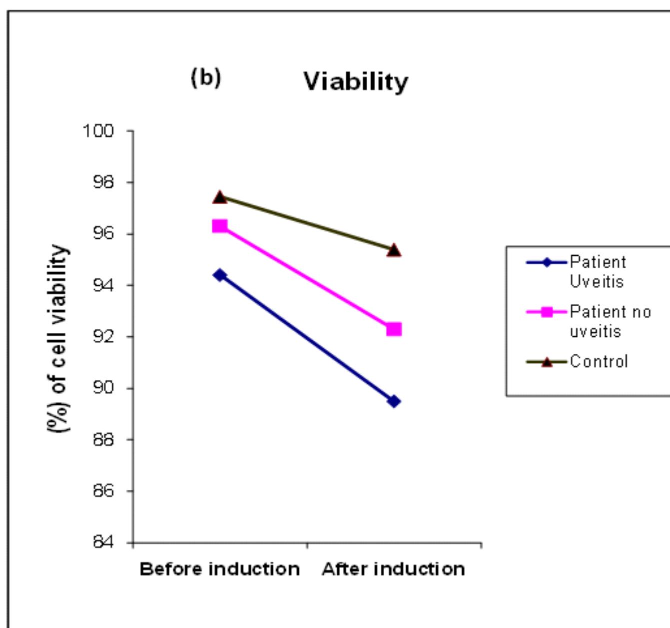

Fig. 1: (b) Percentage of cell viability of PBMNCs induced by anti-Fas mAb in RA with uveitis, RA without uveitis (n=10) and controls. Highly significant reduction in the % of cell viability was noticed after induction of apoptosis in patient groups as compared with controls (P<0.01) which was more pronounced in RA patients with uveitis.

Correlation Study Revealed That:

In all RA Patients:

A negative correlation was detected between apoptosis levels (absorbance) and % of cell viability before induction of apoptosis (r = -0.506, P<0.05) and after induction of apoptosis (r = -0.621, P<0.01) since as apoptosis level increases, the viability of PBMNCs decreases. There are negative correlations between cell

viability and DAS (r = -0.563, P<0.01), between cell viability and disease duration (r= -0.482, P<0.05) and Anti-CCp (r = -0.452, P<0.05).

In RA with Uveitis:

Positive correlations were detected between apoptosis levels (absorbance) and CRP (r = 0.713, P<0.01) and DAS (r = 0.646, P<0.05), negative correlations between cell viability after induction of apoptosis and age (r = -0.642, P<0.05), between cell viability before and after induction of apoptosis and DAS (r = -0.703, P<0.01) and (r = -0.719, P<0.05) respectively, between cell viability before and after induction of apoptosis and Anti-CCp (r = -0.757, P<0.05), (r = -0.877, P<0.01) respectively, between cell viability before induction of apoptosis and ANA (r = -0.714, P<0.05).

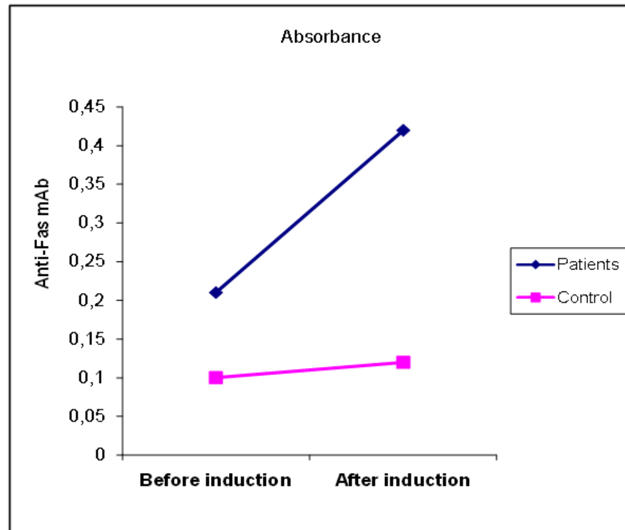

Fig. 2: (a) Levels of apoptosis (absorbance) before and after apoptosis induction by anti-Fas mAb in the supernatant of peripheral blood mononuclear cells in all rheumatoid arthritis patients (n=20) and in controls (n=20). A highly significant (P>0.01) elevation in absorbance was noted in RA pateints after induction of apoptosis.

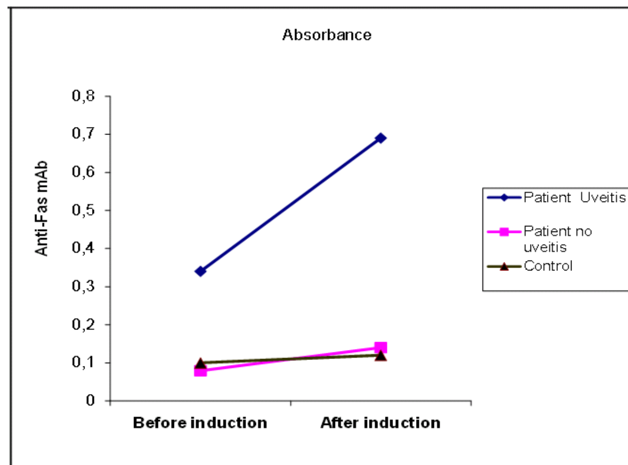

Fig. 2: (b) Levels of apoptosis (absorbance) before and after apoptosis induction by anti-Fas mAb in the supernatant of peripheral blood mononuclear cells in RA patients with and without uveitis (n=10). Significant elevation in absorbance was noted in RA pateints with uveitis (P>0.05). Insignificant different was detected between RA without uveitis and normal controls (P>0.05).

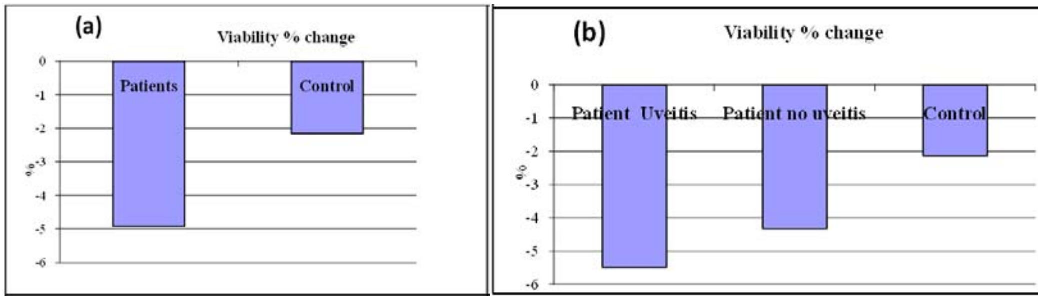

Fig. 3: The % change in cell viability of PBMNCs induced by anti-Fas mAb in all RA (a), RA with uveitis, RA without uveitis (b) and controls. Highly sigificant differences in the % change of cell viability was noticed after induction of apoptosis in all patient groups (P<0.01) which was more pronounced in RA patients with uveitis.

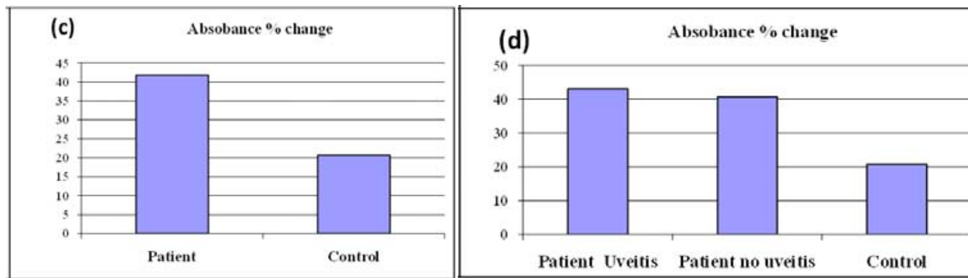

Fig. 3: The % change in apoptosis levels (absorbance) of PBMNCs induced by anti-Fas mAb in all RA (c), RA with uveitis, RA without uveitis and control (d). Sigificant chandes were revealed in all RA patients and RA with uveitis as compared with control (P<0.05), insignificant difference (P>0.05) was revealed between RA with uveitis and RA without uveitis.

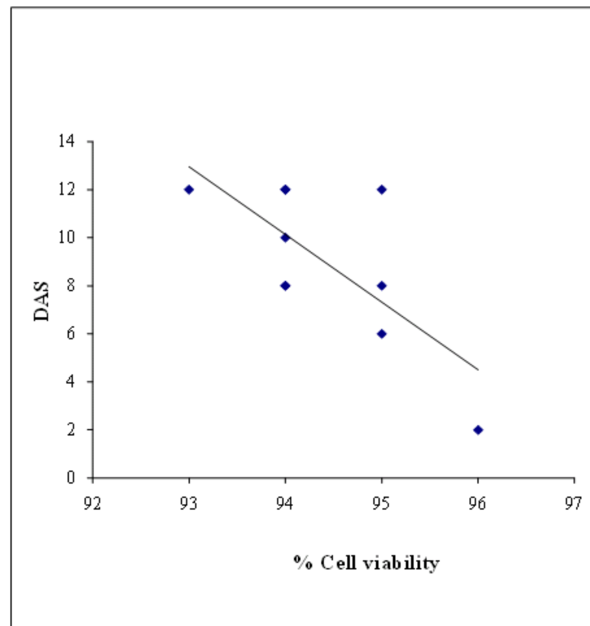

Fig. 4: (a) Correlation between % of cell viability and DAS score before induction of apoptosis among RA patients with uveitis. A negative correlation between cell viability before induction of apoptosis and DAS (r=-0.703, P>0.01).

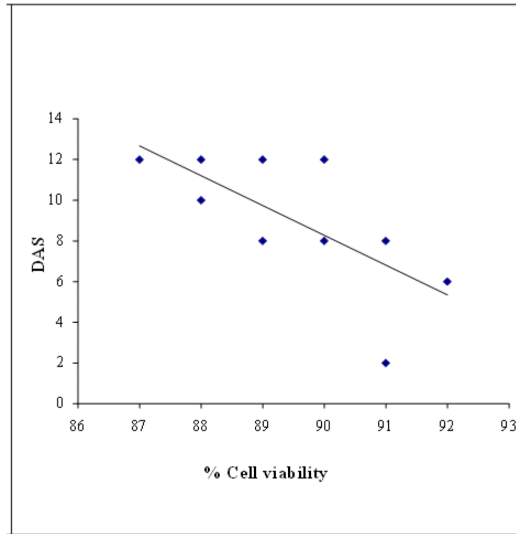

Fig. 4: (b) Correlation between % of cell viability and DAS score after induction of apoptosis among RA patients with uveitis. A negative correlation between cell viability after induction of apoptosis and DAS (r=-0.719, P<0.05).

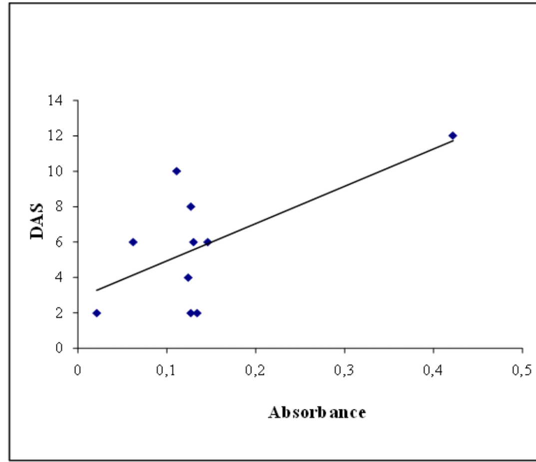

Fig. 5: (a) Correlation between absrobance levels and DAS score after induction of apoptosis in RA patients with uveitis. A positive correlation was detected (r=-0.646, P<0.05).

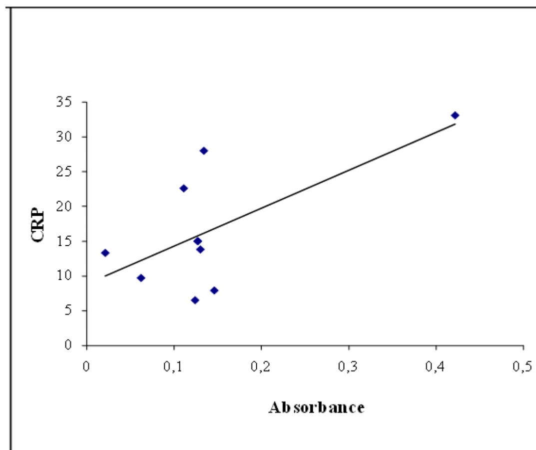

Fig. 5: (b) Correlation between absrobance and CRP levels after induction of apoptosis in RA patients with uveitis. A positive correlation was detected (r=-0.713, P<0.01).

Discussion:

It has been suggested that apoptotic pathways, particularly involving Fas-FasL, in the inflammatory cells of the RA joint represent at least one fundamental underlying process in disease pathogenesis that leads to destruction of the bone and cartilage of joints and persistent synovial cell proliferation and/or inflammation (Baier *et al* 2003) as well as in the tratment of the disease (Pope (2002). The synovial membrane of the joints is the main target of damage, but patients can also have involvement of extra articular tissues such as eyes, skin, lungs, heart and peripheral nerves.

Thus, the aim of this study was to evaluate the apoptosis process of PBMNCs in RA patients and RA patients with or without uveitis, and evauate the effect of *in vitro* induction of apoptosis with anti-Fas mAb on the viability of PBMNCs and on the apoptosis levels (absorbance) in the supernatant of cultured cells and to correlate them with different parameters of inflammation.

In the current study, the abnormally high percentage of cell viability of PBMNCs and the low levels of apoptosis (absorbance) before induction of apoptosis in RA patients, RA patients with uveitis and RA patients without uveitis may point out to the insufficient apoptosis of inflammatory cells in those patients and hence may contribute in the pathogenesis of RA disease in the affected joints and the eye. Lorenz *et al*; (1997) found that the frequency of peripheral blood apoptotic lymphocytes in RA was similar to normal controls as assessed by the expression of Fas, Fas-L, Bcl-2, Bax. In addition, Schirmer *et al*; (1998) demonstrated that RA patients have a subset of CD4 T cells with a defect in CD28 expression and dysregulation of Bcl-2 and subsequent resistance to apoptosis and growth of autoreactive T cells. Moreover, Hasunuma *et al*; (1996) and Tak and Bresnihan; (2000) reported that the inadequate apoptosis may be due to increased endogenous inhibitors such as the proinflammatory cytokines IL-1β, TNF-α and IL-6, mutations of P53 suppressor gene, deficient functional Fas L expression, over expression of anti-apoptotic molecules such as sentrin and activation of nuclear factor K-B. Lorenz *et al*; (1997) explained the low frequency of peripheral blood apoptotic lymphocytes in RA by migration of the activated T cells to the inflamed joints leaving peripheral circulation.

Furthermore, (Streilein, 1999; and Taylor; 1999) reported that the constitutive expression of FasL on ocular tissues has been suggested to play a major role in maintaining immune privilege in the eye, through the induction of apoptosis of the infiltrating activated T lymphocytes via Fas-FasL interactions. The presence of intraocular inflammation in uveitis suggests that these mechanisms are breached. Analysis of aquious humor (AqH) from patients with uveitis showed absence of detectable apoptotic lymphocytes and reduced expression of FasL in the eye during inflammation in the majority of patients. This suggested that induction of Fas-induced apoptosis is limited during the active stage of disease (Sugita *et al*, 2000). Moreover, apoptotic cells showed all of the early marker features of apoptosis such as caspase-3, and also the late features such as nuclear condensation and fragmentation (Salmon *et al*, 1997).

Our results were also in agreement with Zhang *et al* (2001) who investigated a murine autoimmune model of RA. They found impaired CD95 signaling pathway resulting in PBMNC apoptosis despite of increased levels of CD95 at the cell surface. This defect in apoptosis was ascribed to an aberrant expression of a certain inhibitory protein suppression of the caspase cascade mediated by Fas-FasL interaction and also the influence of the inflammatory milieu and presence of TNF-α in serum of RA patients that may increase CD95 expression irrespective of the presence of apoptosis.

In contrast, Ahmed *et al* (2008) investigated the expression of the extrinsic and intrinsic apoptotic markers; CD95 and Bcl-2 respectively on PBMNCs. They found that CD95 expression on PBMNCs was significantly higher in RA patients than controls and significantly correlated with DAS and ESR but no significant difference in Bcl-2 expression was detected between RA patients and controls. They concluded that CD95 seems to be related to the inflammatory process (disease activity) rather than to apoptotic process and the normal expression of Bcl-2 on PBMNCs indicated that the possibility of dysregulation of apoptosis is unlikely. They also found no significant differences between CD95 & Bcl-2 expression among patients with or without extraarticular manifestations (pericarditis and peripheral vasculitis).

However, after induction of apopotosis with anti-Fas mAb our results revealed highly significant reductions in the mean % of cell viability and highly significant elevations in the mean values of apoptosis levels in all RA patients and RA with uveitis and the effect was more pronounced in RA patients with uveitis. The same results were also noticed in the % change in cell viability and apoptosis levels. In addition a negative correlation was detected between cell viability and apoptosis levels since as apoptosis level increases, the viability of PBMNCs decreases. These results indicate that apoptosis accounted for the reduced number of viable cells, and PBMNCs were susceptible to anti-Fas mAb *in vitro.* This finding is in agreement with Hoe *et al* (1996) who attributed the decrease in cell viability to the induction of apoptosis by treatment with anti-Fas mAb.

In contrast, Hasunuma *et al* (1996) examined the *in vitro* induction of Fas-dependent apoptosis in freshly isolated synovium infiltrating mononuclear cells (SIM), synovial stromal cells (SSC) and (PBMNCs) using tissues from nine patients with RA and three with osteoarthritis (OA). Their results showed expression of Fas antigen and apoptotic cells in a number of CD3-bearing cells in RA synovial tissues and *in vitro* treatment with anti-Fas mAb produced a significant apoptosis of RA SIM and SSC. While, neither of (PBMNCs) of RA nor

SIM and SSC from OA exhibited apoptosis. They concluded that RA synovial infiltrating lymphocytes acquire high susceptibility to anti-Fas mAb and undergo apoptosis both *in vivo* and *in vitro*. Such a phenomenon of infiltrating T cells in RA synovium may play an important pathophysiological role and suggest a possible therapeutic effect for anti-Fas mAb in RA. However, the insignificant reduction in (PBMNCs) when compared with controls may be due to spontaneous apoptosis occurs normally as a result of incubation for 24 h. The same results were also demonstrated by Demian *et al* (2005) who explained the significant increase in the % reduction of CD4 T cells in synovial fluid than PBMNCs after induction of apoptosis with anti-Fas mAb by the increased infiltration of those activated T cells into the affected joints and due to the presence of minimal Fas expression on CD4 cells of (PBMNCs). The discrepancy in apoptosis results is attributed to whether the cells are examined *in vitro* or *in vivo*, preactivated *in vivo* or activated by specific reagents *in vitro*, the inflammatory milieu surrounding the cells as cytokines (Tak and Bresnihan (2000) since the Fas pathway in RA is affected by both up- and down-regulators, the relative importance of specific mechanisms probably varying at different times, and in different environmental conditions and anatomical contexts, all have to be considered on comparing the results (Wang *et al*, 2000)

Curnow *et al*, (2004) studied the proportion of apoptotic T lymphocytes in AqH, synovial fluid (SF) and isolated (PBMNCs) of chronic RA patients treated *in vitro* with anti-Fas as an apoptosis control. They found that the proportion of apoptotic T lymphocytes in AqH was similar to that observed in SF with active inhibition of apoptosis. Additionally, patients with severe disease, showed significantly greater inhibition of apoptosis than those with mild disease which suggest that apoptosis may be deficient in uveitis, or it may be actively suppressed.

However, induction of apoptosis with anti-Fas significantly increased the numbers of apoptotic lymphocytes in the SF of patients with self-limiting arthritis compared with patients with uveitis, indicating that AQH cells were highly susceptible to Fas-mediated death. Furthermore, induction of apoptosis in the isolated (PBMNCs); the majority of which are resting cells, remained relatively resistant to the induction of apoptosis by this route. They suggested the use of local anti-Fas mAb in the treatment of RA & uveitis.

Such observations strongly suggest that modulation of the Fas pathway *in vivo* may provide therapeutic benefit. Many current RA therapies are in fact known to induce apoptosis in synovial cells and ocular cells, such as methotrexate and TNF-directed therapies. These drugs appear to do so at least in part via Fas, at least in some pathogenic cell populations, such as T cells and/or synovial macrophages. Thus, approaches targeted more specifically against Fas/FasL may be of benefit (Ohshima *et al*; 2000). However, not all patients respond to these therapies, and the benefits of this form of treatment are short lived (Smith and Walker; 2004), (Catrina *et al* 2005). At present the treatment of inflammatory eye disease is through blocking therapy of TNF-α pathway which induces cell lysis after binding to TNF-α and induces apoptosis of T lymphocytes (Bucknall; 2005). Sometimes disease-modifying antirheumatic drugs (DMARDs) systemic immunosuppressive agents like Cyclosporin A (Sobrin *et al*, 2007), or a monoclonal antibody to TNF-alpha such as infliximab (Galor *et al*; 2008) may be necessary to improve tear production and to resolve severe sucu as keratoconjunctivitis. In 2008 Penolazzi *et al* suggested the application of medicinal plants and derived natural products *such as Emblica officinalis* fruits extracts as an alternative tool for therapy for the treatment of rheumatoid arthritis and osteoporosis by induction of apoptosis of human primary osteoclasts by increasing the expression levels of Fas.

It has been proven that the severity of extracellular manifestations reflects the activity of the underlying RA inflammatory process (Sahatçiu-Meka, 2010).

In the current study, correlation between apoptosis results and inflammatory parameters revealed negative correlations between cell viability, DAS and disease duration, Anti-CCp, CRP and ANA. Ahmed *et al*; (2008) revealed no correlation between apoptotic molecule (CD95) expression and the extracellular manifestations of disease activity in RA patients such as swollen joint count score (HAQ) and DAS score. The lack correlation with CD95 expression which is in the same time correlate with other disease activity measures may be attributed to the relatively smaller number of swollen joints count compared to tender joint count. Lack of correlation with disease duration may exclude the cumulative effect of the relapse of time on the triggering factors that fluctuate depending on the inflammatory process and lead to CD95 expression.

Szodoray *et al*; (2004) assessed apoptosis induction in peripheral blood B cells of RF^+ and RF^- RA patient groups using Rituximab. They found that apoptosis induction significantly increased the overall B-cell population for RF+ patients and with no evidence that RTX significantly induced apoptosis in RF^- B cells of any category.

It has been proved that activation of B cells within the inflamed RA synovia participates in the chronic progressive joint damage via production of a diversity of autoantibodies, including rheumatoid factor (RF), a well-recognized prognostic factor for aggressive disease, suggesting that autoantibody production is linked to disease progression. Furthermore, a significant proportion of patients also have high levels of antibodies to anti-nuclear antibodies (ANA), cyclic citrullinated peptides (anti-CCP) and to proteins such as the immunoglobulin (Ig) heavy chain-binding protein (Szodoray *et al* 2004). However (Sahatçiu-Meka; 2010) reported that RF may

be present in other inflammatory disorders and be present in healthy person and can't be pathognomonic sign of RA and persistently increased RF is more predisposing factor to developing RA.

The conclusions & recommendations that were drawn from this study suggest that anti-Fas–antibodies exerts an *in vitro* apoptotic effect on PBMNCs of all investigated RA patients either with or without uveitis. In addtion, the possibility of using anti-Fas monoclonal antibodies as an apoptosis induction therapy to modulate the inflammatory process locally in the eye and systemically. The study also recommends routine examination of the eye for early detection of eye complications in patients with rheumatoid arthritis and intensive treatment with disease-modifying antirheumatic drugs for RA patients with ocular manifestations.

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