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Application of Buccal Micronucleus Cytome Assay in Rheumatoid Arthritis patients on Etanercept: A Case–Control Study

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Abstract
Rheumatoid arthritis (RA) is an inflammatory disease characterized by its chronicity and symmetrical pattern of involvement. The purpose of the present study was to assess the genotoxic effect of the antitumor necrosis factor-α agent (Etanercept) in the adult rheumatoid arthritis patients by the mean of a buccal micronucleus cytome assay**.** Buccal smears from a normal mucosa of 30 rheumatoid arthritis patients on Etanercept and 30 healthy subjects were taken to apply the buccal micronucleus cytome assay on them. Significantly higher frequencies of micronuclei, pyknotic cells and nuclear buds were seen in those patients when compared to controls. In respect to these findings, the rheumatoid arthritis patients on Etanercept were showing a higher frequency of the genomic damage and cell death biomarkers than the healthy controls which indicates a higher risk of developing pathosis.
Keywords: Rheumatoid arthritis, genotoxic effect, buccal micronucleus cytome assay.

1. Introduction

Rheumatoid arthritis (RA) is an inflammatory and autoimmune disease characterized by its chronicity and symmetricity, it can manifest at any age predominantly in the females after age of 16, both genetic susceptibility and environmental elements (hormones, smoking, infections... etc.) are contributing in the pathogenesis (Hayward and Wallace, 2009).

Several pharmacological agents are used to reduce the symptoms and to slow down the progression of RA, among these drugs, the Disease modifying anti-rheumatic agents (DMARDs) specially the biological part has proven their effectiveness and their better tolerability and less side effects when compared to the conventional DMARDs (Gramling and O'Dell, 2012).

Etanercept (Etan) is a soluble recombinant protein of the human tumor necrosis factor (TNF) receptor type 2, bound to the Fc portion of an IgG 1, acts by inhibiting and antagonizing TNF function (Akiyama and Mimura, 2006).

Buccal micronucleus cytome (BMNCyt) assay has assumed to be an easy, quick, cheap cytological assay to be used with no invassiveness in the studies that evaluate the ability of different drugs, chemical compounds and lifestyle factors to permanently damage the genomic material and cellular morphology in the human (Fenech *et al*., 2011).

2. Methodology

2.1 *Subjects selection*:
A total of 30 RA patients on Etanercept 50 mg/week were included in this study and compared to 30 healthy controls matched for age and sex. Patients who were using other drugs or having other autoimmune connective tissue disease, those who were smokers or alcoholic and patients who were exposed to the head and neck radiation were excluded from the study. Informed consent was gained from all those participants

2.2 Clinical evaluation:
The RA patients were diagnosed by a rheumatologist according to the American College of Rheumatology/European League Against Rheumatism collaborative initiative classification criteria in 2010 (Aletaha *et al*., 2010), the clinical disease activity index(CDAI) was calculated for each patient by calculating the number of the tender and swollen joints, this number was added to the result of the patient's/physician's assessment on a visual analogue scale (1-10cm). The score of this index from 2.8-10 is considered as low CDAI, 10-22 as moderate CDAI and >22 as being high CDAI (Aletaha *et al.,* 2005).

2.3 Sample collection:
After recording all the baseline information for the participants, they rinsed their mouth with water before samples were taken. A disposable brushes (BAG Lich/Germany) were used to take smears from a normal buccal mucosa where the brushes rotated ten times against the inner side of the cheek. The samples were smeared directly to clean, dry and labeled microscope slides, slides were fixed immediately with 96% ethanol for 30 min and stained with Papanicolaou stain. Two slides were set for every subject.

2.4 Slides examination:
The slides were examined by using the light microscope in a zigzag method after calculating 1,000 cells/slide, the scoring based on the criteria described by (Tolbert *et al.,* 1992) to determine the cytological biomarkers frequencies (Micronucleated cells, Pyknotic cells, Karyolytic cells, Karyorrhetic cells, Basal cells, Binucleated cells and cells with condensed chromatin). The reliability of readings was judged by an inter-examiner calibration with an experienced pathologist and an intra-examiner calibration where a total of 10 slides (5 for cases and 5 for controls) was re-viewed a month later by the same examiner, the median change observed in all of the examined parameters was very small and not statistically significant.

2.5 Statistical analysis:
Statistical analysis was performed using SPSS 20 and Minitab version 17 software. The Anderson Darling test was used to assess the normal distribution of continuous variables. The statistical significance of comparisons between the 2 groups was determined by Student's t-test and one way ANOVA for the normally distributed variables and by Mann Whitney U tests for non normally distributed variables. The chi-square test was also used to evaluate the relationship between 2 categorical variables, the statistical significance of intra examiner differences was assessed by Wilcoxon signed rank test. A Spearman correlation was also used to correlate between variables.

3. Results

3.1 *The demographic data*:
The ages of the participants were ranging from 30-50 years, the mean age of the study group was (42.5) and that of the controls was (40.4). The number and percentage of female RA patients was 26(86.7%), while the number and percentage of RA male patients was 4(13.3%). This sex distribution matched that of the controls with 23(76.7%) females and 7(23.3%) males, the P- value for the age and sex distribution was>0.05, therefore; the sociodemographic variables were unlikely to confound any detected variances between the study group and controls.

3.2 *The disease characteristics*:
The disease characteristics in the study group were shown in table 1, the median of disease duration was (6.5) years and the median of the treatment's duration was(2) years. The CDAI mean±SD was (20.4 ± 10.1), The results of the CDAI distribution between RA patients showed that (43.3%) of them were presented clinically with high disease activity, (40%) of them were with a moderate disease activity and only(16.7%) of them were presented with low disease activity.

Table 1: Disease characteristics of rheumatoid arthritis patients

Variables			**Values**
Disease duration (Median)			6.5 years
Treatment duration(Median)			2 years
CADI(Mean±SD)			20.4 ± 10.1
CADI	Low	No	5
		%	16.7%
	Moderate	No	12
		%	40.0%
	High	No	13
		%	43.3%

3.3 *The cytological findings*:
After calculation of median(inter quartile range) of each parameter, the RA patients on Etan showed a higher median of Micronucleated cells (Mn), Pyknotic cells (PK) and nuclear buds(Nbud) when compared to the normal control as shown in (table 2, figure 1), the P-values for the statistical differences of those parameters compared to the controls were(<0.001 for both Mn and PK) and (0.003) for the Nbud.

Table 2: The cytological finding in the controls and RA patients (median and IQR)			
Variables	Control (n=30)	Etanercept (n=30)	P value
Micronucleated cells	7 (5 – 10)	15 (10 – 30)	**<0.001**
Pyknotic cells	54 (34 – 76)	89 (64 – 127)	**<0.001**
Karyorrhectic cells	1 (0 – 2)	1 (0 – 3)	0.357
Karyolytic cells	0 (0 – 2)	0 (0 – 1)	0.181
Basal cells	2 (0 – 3)	1 (0 - 2)	0.176
Nuclear Bud	0 (0 - 0)	0 (0 – 1)	**0.003**
Binucleated cells	0 (0 - 0)	0 (0 – 1)	0.185
Condensed chromatin	0 (0 - 0)	0 (0 - 0)	-
Mann Whitney U test			

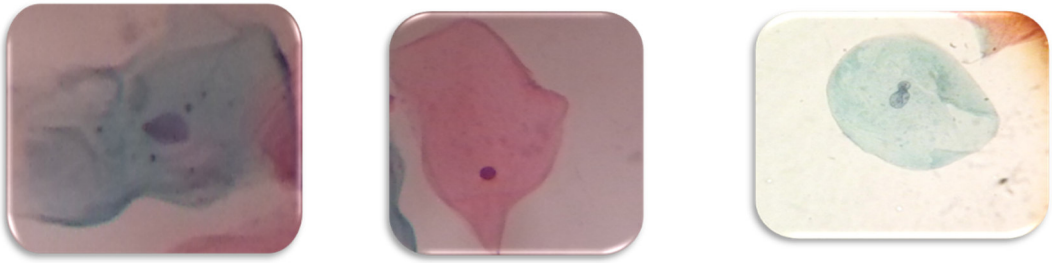

Figure1: The cytological findings, a:Micronucleus, b:Pyknotic nucleus, c:Nuclear bud.

3.4 *The correlation between the cytological findings and the CDAI:*
Univariant linear regression was used to assess the correlation between the cytological findings and the CDAI, the results revealed no association between them (P>0.05) as in the table 3.

Table 3: Univariate linear regression of various variables according to CADI			
Variable	Correlation coefficient	DF	P- value
Micronucleated cells	0.129	0.473	0.497
Pyknotic cells	0.048	0.066	0.8
Karyorrhectic cells	-0.336	3.568	0.069
Karyolytic cells	0.051	0.073	0.788
Basal cells	-0.189	1.035	0.318
Nuclear Bud	0.255	1.942	0.174
Binucleated cells	-0.032	0.028	0.868
Condensed chromatin	-0.149	0.633	0.433
DF: degree of freedom = (82,1)			

3.5 *The correlation between the cytological findings and the treatment duration:*
The median of the duration of the treatment with 50mg/week of Etan was 2 years and no statistical correlation was found between the duration of the treatment and the cytological findings as in table 4.

Table 4: Correlation between treatment duration and cytological markers		
Variables	Spearman correlation	P- value
Micronucleated cells	0.076	0.69
Pyknotic cells	-0.096	0.614
Karyorrhectic cells	0.019	0.921
Karyolytic cells	-0.148	0.436
Basal cells	-0.307	0.099
Nuclear Bud	-0.215	0.255
Binucleated cells	0.124	0.515
Condensed chromatin	0.332	0.073
Non parametric Spearman correlation		

4. Discussion

4.1 *Demographic data:*

Rheumatoid arthritis is an age and gender related disease where the middle aged and female patients are the commonly affected subjects (Klippel *et al.,* 2008) that matches the results in the present study where the mean age of the RA patients(42.5 years) and the females represented (86.7%) of the patients in the study group.

4.2 *Disease characteristics:*

The meadian of the disease duration was(6.5) years, the studies found that the increased duration of RA could reduce the responsiveness to the treatments used, increased the joint destruction along with unstable disease activity scores and reduced functional abilities, as well as increasing the risk of developing the complications such as coronary-artery calcification, increased risk of infections because of the perminant changes in thier immune system and prolonged use of immune supressive drugs like the steroids and biologicals(Listing *et al.,* 2012; Welsing *et al.,* 2001).

The median of Etan treatment was 2 years, which was similar to the duration of Etan treatment in the study of (Moreland *et al.,* 2001) who cleared that Etan medical advantage was safe and well accepted by the patients for the period of treatment(a median of 25 months).

Rheumatoid arthritis involved the inflammation of the joints with the later destruction and impaired function, therefore; it is important to assess the extent of the activity of this disease clinically, to measure the improvement, response to the present therapies and the necessity of moving to other drugs. The mean of the CDAI in RA patients on Etan was (20.4), which was agreed with Furst *et al*., (2011) who stated that the mean of the CDAI was(21.0) for the RA patients presented with mean of disease duration equal to 7.7 years.

A percentage of (43.3%, 40% and 16.7%) of RA patients were having high, moderate and low CDAI values respectively, Furst *et al*., (2011) showed different CDAI values in the high and moderate subgroups with (53% and 28.8% respectively) but the percentage of those with low CDAI was close to our results(18.2%), this study stated that the increasing in the disease duration is the main factor that cause more active disease and less rate of remission.

4.3 *The cytological findings*:

4.3.1 *Micronucleated cells:*

Micronuclei are small, chromatin containing bodies outside the main nucleus that may be shaped as a result of defects in mitosis or chromosomal breakage (Sabharwal *et al.,* 2015), the results revealed that the median of Mn was higher in the RA patients than in the controls ($P<0.001$), these results were in line with Demirkaya *et al.,* in (2009) who studied the genotoxic effect of Etan in Juvenile Idiopathic Arthritis, who showed an increasing in the DNA damage when compared to the controls.

Pyknotic cells frequency was higher in RA patients than the controls($P<0.001$), PK cells represented a marker of the cell death(apoptosis), apoptosis is a regulated process of cell death used by the body to eliminate the cells with a damaged morphology or damaged genetic components, this process may be dysregulated to cause either atrophy or uncontrolled growth and cancers, apoptosis mechanism leads to the cell death in different stages, where the initial changes of it include beating the cell junctions and membrane structures, later on, the cytoplasm shrinks and the nucleus merges into small masses, which later breaks up into fragments and permanently lost (Smith and Walker, 2004).

Nuclear buds(Nbud) just like the micronuclei can reflect a genomic material injury where the increased copies of particular DNA fragments (DNA amplification) said to be the main mechanism of their formation. The amplified parts of the DNA are usually located at the nucleus boundaries and tend to be rejected by the mean of budding during the DNA replication phase of the cell cycle (Fenech *et al*., 2011). The median of Nbud in the RA patients was higher than that in the controls ($P=0.003$).

This higher rate of Mn and Nbuds represented the increased rate of the DNA damage produced by the drug, this damage can be produced by the RA it self as well, RA as an autoimmune and inflammatory disease can contribute to the genotoxicity and cancer formation by the mean of autoantibodies production (which may attack different cytoplasmic and nuclear parts), apoptosis regulation problems and the disruption in the equilibrium of the reactive oxygen species, all the previous causes are sharing the damaging effect produced by RA (karaman *et al*., 2011), therefore; the Mn and Nbuds should be evaluated in an RA patients who are newly diagnosed with no treatments.

Rheumatoid arthritis patients presented with higher cell death rate signified by the higher PK median, the increased rate of apoptosis in RA patients can either be related to the chronic inflammation produced by the disease itself as the inflammatory cytokines are associated with a damaging effect to the epithelial cells and their junction leading to an increased rate of apoptosis to eradicate these damaged cells, therefore; the more active inflammation is related to an increased rate of apoptosis and cellular injury (Bruewer *et al.,* 2003) or related to the protective action done by the body in an attempt to reduce the irritating and injurious outcome caused by the drugs.

The present study demonstrated that the increased frequency of the studied parameters is clearly indicating the genotoxic capacity of the drug under study to different extents, the permanent damage produced by this drug may or may not cause a mutation, this mutation can either be eliminated by the mean of multiple check points and defense mechanisms or passed into the new cells,it is important to know that multiple mutations are needed to cause a cancer and the actual formation of the cancerous mass requires a damage at both genetic and cellular levels (AshaRani *et al.,* 2009).

4.3.2 *The correlation between the cytological findings and the CDAI:*

It was hypothesised that the increased inflammation which appears clinically in the term of higher CDAI will result in a positive correlation with the frequencies of the cytological parameters, but the results of the univariant linear regression showed no significant correlation (P>0.05), this may not eliminate the importance of the disease to magnitute the damage produced by the drug as the genetic damage produced by the drug under study collectively with the effect of chronic inflammation will result in a more apparent outcome, Where the irreversible DNA structure changes produced by the genotoxic agent provide an initiation stimulus to develop a cancer and the repeated changes in the cellular micro environment produced by the long standing chronic inflammation will further promote the cancerous development(Meira *et al.,* 2008).

4.3.3 *The correlation between the cytological findings and treatment duration:*

The goal behind any treatment used in RA is to achieve the total remission or to reach the state of low and controlled disease activity where the better quality of life can be gained, the duration of Etan treatment was statistically not related to the increasing of the cytological parameters frequencies (P>0.05) ,which may disagree with Demirkaya *et al.,* in (2009), who studied the genotoxic effect of Etan in Juvenile Idiopathic Arthritis, who showed an increasing in the DNA damage when compared to controls and the damage was more prominent after the 90th days, 180th days till the termination of the study, this disagreement may be because the different study design or because the small sample size.

5- Conclusion

The present study showed an increased frequency of the cytological parameters of nuclear damage and cell death which may indicate an injurious effect of Etanercept at a cytological level. The collaborated damage produced by both the drug as well as the chronic inflammation of RA may increase the chance of developing different pathosis including the cancer.

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