Evaluation of DNA damage in beta thalassemic patients undergoing therapy with iron chelators

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Abstract: Background: ß-thalassemia is a severe transfusion dependent form of thalassemias, which leads to iron overload. In addition to the organ damage, excess iron can also lead to DNA damage. For these patients, the use of chelating agents, may protect not only against iron-induced organ damage but also against the excessive iron catalyzed, oxidative DNA damage**. Aim of the work**: to evaluate the DNA damage in ß-thalassemic patients undergoing therapy with iron chelators. **Subjects and Methods:** the study included 90 subjects divided into four groups: Group I: 20 β-thalassemic patients with ferritin level<1000 µg /dl and did not start therapy with iron chelators. Group II: 30 β-thalassemic patients with high iron overload (serum ferritin level>1000 µg /dl), they were investigated in two stages: Stage IIa: before regular iron chelation with subcutaneous desferroxamine (DFO). Stage IIb: after complete six months duration of regular chelation with DFO. Group III: 20 β-thalassemic patients with high iron overload (serum ferritin level>1000 µg /dl), they were investigated in two stages: Stage IIIa: before regular chelation with oral deferiprone. Stage IIIb: after complete six months duration of regular iron chelation with deferiprone. Group IV: 20 apparently healthy children of matched age and sex, served as control group. Patients of all groups were subjected to an estimation of DNA damage by: DNA fragmentation assay, electrophoretic pattern of nucleic acid, and Pro gel analysis technique. **Results**: ß-thalassemic patients (with or without high iron overload) were suffering of double strand breaks of DNA of their peripheral leukocytes as compared to controls. Non significant decrease in the frequency of the total genomic damage (TGD) of DNA in ß-thalassemic patients with high iron overload, after six months duration of regular chelation by DFO as compared to the existing damage before regular chelation. While a significant decrease in the frequency of TGD-DNA, after the same duration of regular chelation by deferiprone, compared to the existing damage before regular chelation. There was no significant correlation between serum ferritin level and TGD-DNA in all ß-thalassemic patients and controls. **Conclusion**: A significant DNA damage present in patients of ß- thalassemia disease. Therapy with DFO as well as deferiprone is not associated with increased frequencies of DNA damage, moreover, they improved this damage, and this improvement was better with deferiprone.

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1 Introduction:

Thalassemia is a hereditary anemia results from the unbalanced production of hemoglobin chains in the red cell. Instability of the unpaired chains, followed by degradation, produces hemichromes, free heme, and molecular iron[(1).] The most common forms of the disease, α and β-thalassemia, caused by defective synthesis of the α and β-globin chains of hemoglobin respectively, they are associated with iron overload. Besides the iron overload resulting from hemolysis and blood transfusions, and contrary to all expectations, in thalassemia more iron is absorbed from the GIT [(2).] Excess iron is toxic to the heart, liver, and endocrine system. In β-thalassemia major, 70% of deaths are the result of cardiac failure or arrhythmia [(3).] In addition to the organ damage, excess iron can also lead to DNA damage by catalyzing the production of reactive oxygen species within the cell, leading to the induction of DNA damage and chromosomal aberrations [(4).] DNA integrity and damage is central to the development of cancers and most human cancers are associated with DNA instability[(5).] But unlike cancer, DNA damage can be assayed easily and relatively inexpensively in humans[(6).] Double strand breakage (DSB) and single strand breakage (SSB) are the main types of DNA damage. DSBs of DNA are of fundamental importance in many fields of biology. The incorrect repair of DSBs often results in chromosomal rearrangements, which are considered to be a major initiating factor in carcinogenesis. Progression of malignancy often correlates with increased chromosomal instabilities and plasticity, which are driven by escalating defects in DNA repair processes and cell cycle checkpoint functions [(7).]

For these patients, the use of chelating agents may protect not only against iron-induced organ damage but also against the excessive iron-catalyzed, oxidative DNA damage[(8).] Currently there are two

iron chelating agents available for the management of iron overload in thalassemia: desferoxamine (DFO), which needs to be administered parenterally and deferiprone, which has the advantage that it is taken orally. Both agents have been shown to have antioxidant and cytoprotective effects [9]. Little information is available on the potential of desferoxamine in inducing DNA damage in the clinical setting and, to the best of our knowledge, no information is available on the effect of deferiprone in inducing DNA damage in patients with β-thalassemia.

Aim of the work: was to evaluate the DNA damage in ß-thalassemic patients before and after regular therapy with iron chelators (desferoxamine, and deferiprone).

2. Subjects and Methods

Subjects:-

Seventy children of β-thalassemic disease participated in the present study (37 patients were males and 33 patients were females).

Their ages ranged from 5 to 17 years with mean age: 8.66 ± 2.7 years, patients were selected from the outpatient Pediatric hematology clinic and the inpatients of Pediatric department, Menoufiya University Hospital Another 20 healthy children of matched age and sex were enrolled as controls The study was conducted between May, 2005 and May 2006, and approved by the Ethics Committee of Menoufiya University, The parents signed an informed consent. Members of this study were categorized in the following four groups:

Group I: 20 β-thalassemic patients who did not start chelation therapy with iron chelators, and their ferritin level is less than 1000 (µg /dl). Their age ranged from 4-13 years with mean of 7.95 ±2.9 years. They were 8 males and 12 females.

Group II: 30 β-thalassemic patients with high serum iron overload (serum ferritin level > 1000 µg /dl), and were put under regular chelation therapy for 6 months with subcutaneous DFO (50 mg/kg daily for 5 days/week). Their ages ranged from 5-16 years with a mean of 8.67 ± 2.9 years. They were 16 males and 14 females. Patients were studied through two stages: ***Stage IIa***: Demonstration of total genomic damage (TGD) of DNA before giving DFO on regular basis. ***Stage IIb:*** Reevaluation of TGD of DNA of the same patients after six months of regular DFO therapy.

Group III:20 β-thalassemic patients with high serum iron overload (serum ferritin level > 1000µg /dl), and were put under regular chelation therapy of oral deferiprone (75 mg /kg/day divided into 3 doses). They were 13 males and 7 females. Their age ranged from 6-15 years with a mean: 9.37 ± 2.4 years. Patients were studied through two stages: ***Stage IIIa***: Demonstration of total genomic damage (TGD) of DNA before giving the deferiprone on regular basis. ***Stage IIIb***: Reevaluation of TGD of DNA of the same patients after six months duration from start of deferiprone.

Group IV: 20 healthy children of matched age (ranged from 4-15 years, mean of 8.74 ± 3.2 years) , sex (9 males and 11 females), and socioeconomic standard, served as a control group.

Inclusion criteria:

- Subjects had a diagnosis of β-thalassaemia major documented by hemoglobin electrophoresis.
- They were regularly transfused with washed packed RBCs.
- Subjects were receiving ongoing chelation therapy with desferoxamine (DFO) or deferiprone in an irregular manner.

Exclusion criteria:

Subjects received therapy known to have carcinogenic effects, or smoking adolescents .

Methods :- All patients and controls were subjected to the followings:

(I) Full history taking: name, age, sex, residence, family pedigree and family history of any member requiring frequent blood transfusion. presence of pallor, jaundice, blood transfusion, drug intake especially iron chelators (DFO or deferiprone) and if it is taken regularly or not.

(II) Thorough clinical examination: general examination: to detect pallor, jaundice, anthropometric measurements: weight and height with plotting them into the standard Egyptian growth charts,2002, abdominal examination: to detect hepatosplenomegaly.

(III) Laboratory investigations

A- Routine investigations:

1. Complete blood count, Reticulocytic count, Erythrocyte sedimentation rate
2. Random blood sugar: Measured by enzymatic colorimetric method.
3. Liver function tests e.g. ALT, AST, total and direct bilirubin, total protein and albumin.
4. Kidney function tests e.g. blood urea, serum creatinine, urine analysis.
5. Abdominal ultrasonography.
6. Viral markers
7. Serum ferritin (µg / dl), by ELISA.

B- Genetic study by determination of total genomic damage(TGD) of DNA:

To ensure that the clinical effects of the drugs DFO and deferiprone, rather than any in vitro artifacts, were evaluated, the time point for the collection of blood was selected such that there would be minimal amounts of the respective drugs in

the blood. As both DFO and deferiprone have relatively short elimination half lives (t1/2, almost equal to, 6 hours for DFO and 2-3 hours for deferiprone, subjects were therefore sampled at midday (13:00 h) to avoid carryover of drug into the blood sample. subjects on deferiprone forwent their early morning dose on the day of sampling. Subjects on DFO had their previous evening infusion completed by 08:00 h on the morning of sampling day. The human blood contains only nucleic acids in leucocytes, so we had to get rid of RBCs and platelets through erythrocyte-lysing buffer (ELB).

Leukocytes isolation: Leukocytes were isolated according to *Hassab El-Nabi, 2004*, from whole blood and incubated with 8 ml erythrocyte lysing buffers. Centrifugation repeated twice more till white pellet appeared. [10].

DNA extraction and apoptosis detection: Treated leukocytes(5 X 10^3 cells) in eppendorf tubes were lysed by 600 micro- liter lysing buffer (50 mM NaCI, 1 mM Naz EDTA , 0.5% SDS, pH 8.3) and gently shacked. The mixture was incubated overnight at 37C° then, 200 micro- liter of saturated NaCI was added to the samples, shacked gently and centrifuged at 12,000 rpm for 10 min. The supernatant was transferred to new eppendorf tubes and then DNA precipitated by 600 micro-liter cold isopropanol. The mix was inverted several times till fine fibers appear, and then centrifuged for 5min at 12.000 rpm . The supernatant is removed, and the pellet washed with 500 micro-liter 70% ethyl alcohol, centrifuged at 12.000 rpm for 5min. After centrifugation, the alcohol was decanted or tipped out and the tubes blotted on whatman paper or clean tissue, till the pellet appeared to be dry. The pellet was resuspended in 50 micro-liter or appropriate volume of TE buffer (10 mM tris , ImM EDTA, PH 8.3) supplemented with 5% glycerol. The resuspended DNA was incubated for 30- 60 min with Rnase and then loaded directly into the gel-wells[11]. Gel preparation :Gels were prepared with 1.8% electrophoretic grade agarose (BRL) The agarose was boiled with tris borate EDTA buffer (1x TBE buffer; 89 mM Tris, 89 mM boric acid, 2mM EDTA, pH 8.3), and then, 0.5 micro-gram /ml ethidium bromide was added to agarose mixture at 40 °C. Gel was poured and allowed to solidify at room temperature for 1h before samples were loaded[12]. After 30 min electrophoresis was performed for 1h at 50 volt using 1x TBE buffer as running buffer. Gel was photographed using a polaroid camera while the DNA was visualized using a 312 nm light under a transilluminator[13]. These photographs were analyzed in Finan lab., Cairo, Egypt to detect apoptotic activity by measuring maximal optical density of basal and apoptotic fragments of DNA at 200bp, 400bp, 600bp as these apoptotic bands were located at 200 bp and its multiples. The intensity of apoptotic bands could be measured by Gel-Pro program (figures 1,2)[14].

Statistical methods:

Data analyzed by SPSS statistical package version 11.0 (SPSS INC, Chicago, IL, USA). Quantitative data tested for normality by Klomogrov-Seminrov test of normality. Normally distributed data expressed as mean and standard deviation (X±SD) while median and range are used to express non normally distributed data. One way analysis of variance (F-test) used for comparison o f the means of more than two groups of normally distributed quantitative data followed by LSD post-hoc test for multiple pair wise comparisons of two groups. Kruskal-Wallis test used for comparison of non-normally distributed quantitative variable, student –t test used for comparison of the means of two groups of quantitative variables, while Wilcoxon rank signed test used for comparison of paired observations of non-normally distributed quantitative variables. Qualitative data expressed as number and percentage and analyzed by Chi-square (X2) test or Fisher – exact test when appropriate. Pearson correlation (r) coefficient is used to test association between two quantitative continuous variables in the same group. Level of significance was set as 95% so P value was <0.05, descriptive statistics used are:- X = mean. SD = standard deviation. No = number - % = Percentage[15].

3. Results

The results of this study were illustrated in figures (1-2) and tables (1-10).

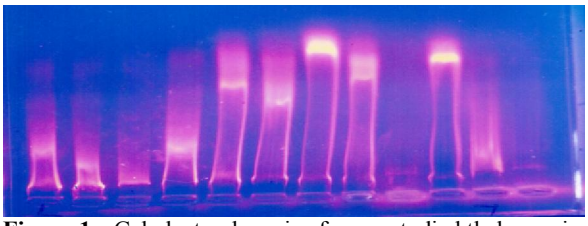

Figure 1 : Gel electrophoresis of some studied thalassemic cases before chelation therapy. It shows various grades of DNA damage in 200,400,and 600 base pairs.

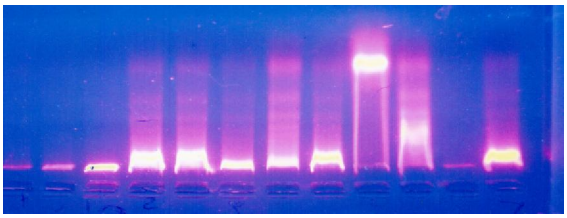

Figure 2 : Gel electrophoresis of some normal control children and some studied thalassemic patients after chelation therapy it shows intact DNA.

Table 1: Demographic data and ferritin level of thalassemic patient and normal children

		Group I	Group II	Group III	Group IV	Test of significance	P value
Age (years) Mean ±SD Range		7.95 ±2.9 4-13	8.67 ± 2.9 5-16	9.37± 2.4 6-15	8.74 ± 3.2 4-15	F test 0.78	>0.05
Gender		N % 8 40 12 60 20 100	N % 16 53.3 14 46.7 30 100	N % 13 65 7 35 20 100	N % 9 45 11 55 20 100	X^2 2.89	>0.05
Weight (kg)	Mean± SD	23.18 ± 9.8	23.52 ± 8.2	25.95 ± 10	32.81 ±8.2	F test 0.42	<0.01*
	Centile range <5th >5th	N % 4 20 16 80	N % 7 23.3 23 76.7	N % 9 45 11 45	N % 0 0 20 100	X^2 11.8	<0.01*
Height (cm)	Mean± SD	115.30±27.9	122.47±14.4	123.45±17.5	132.4 ±15.8	F test 1.06	<0.01*
	Centile range <5th >5th	N % 1 5 19 95	N % 12 40 18 60	N % 7 53.8 13 46.2	N % 0 0 20 100	X^2 16.52	<0.001**
S Ferritin (ug/dl) before chelation		Group I	Group IIa	Group IIIa	Group IV	**F test** / **P value**	**Post-hoc**
Mean ± SD		465.7±256.7	2418.3±1004	2153.3±510.51	54.5±22.1	77.73 / <0.001	P1<0.001** P2<0.001** P 3<0.05* P 4>0.05 P 5<0.001** P 6<0.001**
S Ferritin (ug/dl) after chelation		Group I	Group IIb	Group IIIb	Group IV		
Mean ± SD		465.7±256.7	1903.4±991.5	1462.05±431.8	54.5±22.1	46.5 / <0.001	P 7<0.001** P8<0.001** P 9<0.05* P 10<0.001** P11<0.001** P12<0.001**

*significant **highly significant P >0.05: non significant

Group I: non chelated patients. Group IIa: before regular DFO. Group IIIa: before regular deferiprone. Group IV: controls.

P 1: G I vs. G IIa - P 2: G I vs. G IIIa - P 3: G I vs. G IV - P 4: G IIa vs. G IIIa - P 5: G IIa vs. G IV - P 6: G IIIa vs. G IV

P 7: G I vs. G IIb - P 8: G I vs. G IIIb - P 9: G I vs. G IV - P 10: G IIb vs. G IIIb - P 11: G IIb vs. G IV - P 12: G IIIa vs. G IV

Table (2): Comparison of DNA damage between the studied groups before chelation

DNA damage	**GI** N=20		**GII a** N=30		**GIII a** N=20		**G IV** N=20		X^2 test	*P value*
	No	%	No	%	No	%	No	%		
Present									$X^2$1 (14.84)	P1<0.001**
*Apoptotic	9	45	6	20	5	25	2	10	$X^2$2 (11.19)	P2 <0.001**
*Non apoptotic	0	0	21	70	13	65 10	0	0	$X^2$3 (0.18)	P3 >0.05
									$X^2$4 (25.34)	P4<0.001**
Absent	11	55	3	10	2		18	90	$X^2$5 (31.04)	P5<0.001**
									$X^2$6 (5.16)	P6 <0.05*
Total	*20*	*100*	*30*	*100*	*20*	*100*	*20*	*100*		

*significant **highly significant P >0.05: non significant

Group I: non chelated patients. Group IIa: before regular DFO. Group IIIa: before regular deferiprone. Group IV: controls.

P 1: G I vs. G IIa - P 2: GI vs. G IIIa - P 3: G IIa vs. G IIIa - P 4 :G IIIa vs. G IV - P 5: GIIa vs. G IV- P 6: G I vs. G IV.

Table 3: Comparison of maximum optical density at 200, 400 and 600 base pair in studied groups

Max OD at		Thalassemic patients (70)			GIV N=20	Kruskall-wallis Test	P value	Post-hoc
		GI N=20	G IIa N=30	GIIIa N=20				
200 bp	Mean ±SD	9.46 ±10.37	35.66 ±23.89	31.62 ±17.74	1.49 ±2.64	46.90	<0.001	P1<0.001** P2 <0.05* P3>0.05 P4 >0.05 P5<0.001** P6<0.001**
	Range	0.31-39.17	0.01-83.12	0.21-55.05	0.03-8.93			
400 bp	Mean ±SD	10.84 ±11.30	47.85 ±23.88	39.31 ±19.14	1.89 ±2.86	57.46	<0.001	P1<0.001** P2<0.001** P3<0.001** P4 >0.05 P5<0.001** P6 >0.05
	Range	0.21-38.33	5.88-97.09	0.32-47.18	0.16-9.17			
600 bp	Mean ±SD	13.26 ±13.96	48.69 ±31.26	55.66 ±35.92	2.24 ±3.34	48.67	<0.001	P1<0.001** P2<0.001** P3>0.05 P4>0.05 P5<0.001** P6<0.001**
	Range	0.12-41.67	2.79-136.17	0.45 57.26	0.26-10.11			

*significant **highly significant P >0.05: non significant

Group I: non chelated patients. Group IIa: before regular DFO. Group IIIa: before regular deferiprone. Group IV: controls.

P1:GI vs. IIa - P 2: G I vs.G IIIa - P 3: G I vs. G IV – P4: G IIa vs. G IIIa - P5: G IIa vs. G IV - P6: G IIIa vs G IV

Table 4: Comparison of TGD of DNA between group IIa and group IIb

DNA damage	G IIa *N=30*		G IIb N=30		X^2	P
	No	%	No	%		
Present	27	90	26	86.7	0.66	>0.05
Apoptotic	6	20.0	8	26.7		
No- Apoptotic	21	70.0	18	60.0		
Absent	3	10.0	4	13.3		
Total	30	100	30	100		

P >0.05: non significant

Table 5: Comparison of maximum optical density of TGD of DNA at 200, 400 and 600 bp between group IIa and group IIb

Max OD at	Group IIa N=30	Group IIb N=30	Difference	Wilcoxon signed Rank test	P
	Mean± SD	Mean ±SD	Mean± SD		
200 bp	35.66±23.89	25.83±22.48	9.82±27.84	2.30	<0.05*
400 bp	47.85±23.88	41.08±21.89	14.76±29.68	3.19	>0.05
600 bp	45.69±31.26	39.24±14.92	19.44±31.67	3.28	>0.05

*significant P >0.05: non significant

Table 6: Comparison of DNA damage between group IIIa and group IIIb

DNA damage	G IIIa *N=20*		G IIIb N=20		X^2	P
	No	%	No	%		
Present	18.0	90.0	12	60	19.48	<0.001**
Apoptotic	5	25.0	10	50.0		
No- Apoptotic	13	65.0	2.0	10.0		
Absent	2	10.0	8	40.0		
Total	20	100	20	100		

**highly significant

Table (7): Comparison of maximum optical density of DNA at 200, 400and 600 bp between group IIIa and group IIIb

Max OD at	G IIIa N=20	G IIIb N=20	Difference	Wilcoxon signed Rank test	P value
	Mean± SD	Mean± SD	Mean ±SD		
200 bp	31.62±13.74	14.37±11.32	7.25±17.81	1.53	<0.001**
400 bp	39.31±13.14	17.27±14.69	8.04±17.30	2.24	<0.001**
600 bp	55.66±15.92	23.88±23.78	1.78±28.69	0.82	<0.001**

**highly significant

Table (8): Comparison of DNA damage between studied groups after chelation

DNA damage	GI N=20		GII b N=30		GIIIb N=20		G IV N=20		X^2 test	P value
	No	%	No	%	No	%	No	%		
Present									$X^2$1 (12.10)	P1<0.001**
*Apoptotic	9	45	8	26.7	12	60	2	10	$X^2$2 (11.10)	P2 <0.001**
*Non apoptotic	0	0	18	60	0	0	0	0	$X^2$3 (18.89)	P3 <0.001**
									$X^2$4 (8.04)	P4 <0.001**
Absent	11	55	4	13.3	8	40	18	90	$X^2$5 (27.42)	P5 <0.001**
Total	20	100	30	100	20	100	20	100	$X^2$6 (5.16)	P6 <0.001**

**highly significant

Group I: non chelated patients. Group IIa: before regular DFO. Group IIIa: before regular deferiprone. Group IV: controls.
P1:GI vs. IIb - P 2: G I vs. G IIIb - P 3: G IIb vs. G IIIb – P4: G IIIb vs. G IV - P5: G IIb vs. G IV - P6: G I vs. G IV

Table 9: Comparison of maximum optical density of DNA at 200, 400 and 600 bp in studied groups.

Max OD at		Thalassemic patients (n=70)			Group IV N=20	Kruskall-wallis test	P value	Post-hoc
		GI N=20	G IIb N=30	G IIIb N=20				
200 bp	Mean ±SD	9.46 ±10.37	25.83 ±22.48	14.37 ±11.32	1.49 ±2.64	38.33	<0.001	P1<0.001** P 2 >0.05 P 3>0.05 P 4<0.001** P 5<0.001** P 6<0.001**
	Range	0.31-39.17	0.28-92.58	0.13-32.12	0.03-8.93			
400 bp	Mean ±SD	10.84 ±11.30	41.08 ±21.89	17.27 ±14.69	1.89 ±2.86	41.58	<0.001	P1<0.001** P2 >0.05 P3 >0.05 P4<0.001** P5<0.001** P6 <0.01**
	Range	0.21 38.33	0.46-77.73	0.19-42.16	0.16 9.17			
600 bp	Mean ±SD	13.26 ±13.96	39.24 ±14.92	23.88 ±23.78	2.24 ±3.34	36.33	<0.001	P1<0.001** P2<0.05* P3<0.05* P4<0.01** P5<0.001** P6<0.001**
	Range	0.12-41.67	2.15-62.32	0.77-89.52	0.26-10.11			

*significant **highly significant P >0.05: non significant

Group I: non chelated patients. Group IIa: before regular DFO. Group IIIa: before regular deferiprone. Group IV: controls.
P1:GI vs. IIb - P 2: G I vs. G IIIb - P 3: G I vs. G IV – P4: G IIb vs. G IIIb - P5: G IIb vs. G IV - P6: G IIIb vs. G IV.

Table (10): Correlation between ferritin level and maximum optical density of DNA at 200, 400 and 600 bp in studied groups

Variable groups correlated to serum ferritin	Log MOD at 200 bp		Log MOD at 400 bp		Log MOD at 600 bp	
	r	P value	r	P value	r	P value
G **I** No 20	0.131	> 0.05	0.063	> 0.05	0.181	> 0.05
G **IIa** No 30	0.445	>0.05	0.041	> 0.05	0.054	> 0.05
G **IIb** No 30	0.102	> 0.05	0.151	> 0.05	0.108	> 0.05
G **IIIa** No 20	0.009	> 0.05	0.154	> 0.05	0.031	> 0.05
G **IIIb** No 20	0.063	> 0.05	0.016	> 0.05	0.060	> 0.05
G **IV** No 20	0.328	> 0.05	0.366	> 0.05	0.346	> 0.05

4. Discussion

The approach used in this study, was to assess the existing frequency of DNA damage in thalassemic patients before receiving regular therapy on either chelators desferrioxamine (DFO) or deferiprone and then determine if there is a significant change in the frequency of DNA damage in circulating WBCs in these same patients after 6 months of regular chelation. The basal and released DNA damage was detected by (*Gel Pro program*) as optical density.

When we studied DNA damage, we found that, among the 70 studied thalassemic patients (groups I, IIa, IIIa) 54 (77.14%) had DNA damage, while the incidence of DNA damage in control children was found in 2(10%), with a highly significant difference

(P <0.001). Comparing all studied groups, we found that group I (non chelated) thalassemic patients without high iron overload (ferritin level < 1000 ug//dl) had significant double strands breaks of DNA (TGD) in their peripheral leukocytes as compared to group IV (controls) (P <0.05), This damage was in the form of DNA laddering (regular apoptotic cell death) found in 9 patients (45%) in group I and found in 2 children (10%) in group IV (controls). (Table 2). Our findings mean that despite absence of high iron overload there was total genomic damage (TGD) of DNA in group I as compared to normal children (group IV) which raise the attention that, there may be other factors reside beyond this damage. We could exclude iron chelators as this group had never received iron chelation therapy. A possible explanation of the background TGD of the DNA in the thalassemic patients, may reside in the higher sensitivity of their leukocytes to the effect of genotoxic drugs or mutagenic food additives that may be consumed by them. It was shown that lymphocytes from different thalassemia genotypes had increased sensitivity to food mutagens compared to normal lymphocytes[16].

Our results showed that, there was highly significant difference when group IIa (before regular DFO) as well as group IIIa (before regular deferiprone) were compared to Group IV (controls) as regard DNA damage, (P <0.001), also group I had lower TGD of DNA compared to both group IIa, group IIIa (P <0.001), these results indicate that thalassemic patients with high iron overload had significant increase in the TGD of DNA (Table 2).

In Group IIa and group IIIa, the DNA damage, was detected in 2 forms on gel electrophoresis: the first was DNA laddering, indicating apoptosis which was found in 6 patients (20%) in Group IIa and in 5 patients (25%) in group IIIa. The second form took a "smear shape" indicating double strand breakage not related to apoptosis, conferring fragments of extremely variable sizes (necrosis). This was found in 21 patients (70%) of Group IIa and 13 patients (65%) of group IIIa, (table 2).

Comparison of quantitative analysis of the detected totally damaged DNA using pro-gel analysis technique with, the mean of maximum optical density (MOD) at 200, 400 and 600 base pair of all groups table 3, and we found that maximal optical density at 200 bp, was significantly different when compared between group I and both group IIa and IIIa (P1 <0.001, P2<0.05, respectively), non significant difference between group I and group IV (P > 0.05), non significant difference between group IIa and group IIIa (P >0.05), highly significant difference of both group IIa and IIIa compared to group IV (P 5, P 6 <0.001) (table 3). The values of maximal optical density at 400 bp, were similar to that of 200 bp regarding the significant difference between group I and both group IIa (P <0.001), and group IIIa (P <0.001), however the thalassemic patients with low iron load <1000 gm, (group I), are now significantly higher when compared to healthy control children (group IV) (P <0.001), Comparing values of maximal optical density at 600 bp, the comparison between groups was the same as that of MOD at 200 bp (table 3).

The results of the current study is consistent with the study of El Gendy et al.,2004, who studied the total genomic damage (TGD) of DNA by conventional gel electrophoresis where they found that there was highly significant TGD of DNA in thalassemic patients[17].

Perera et al., 2002 recorded evidence of genetically damaged DNA of sperms of thalassemic patients. They postulated some possible factors that may contribute to this DNA damage in these patients as: oxidative stress of iron overload and lower antioxidant levels[18]. **Dunkel et al., (1999)** stated that ferric iron complexes have been shown to induce DNA base damage, gene mutations and chromosomal aberrations in mammalian cells. This was suggested to account for the observation of increased frequencies of chromosomal aberrations in thalassaemia patients[19].

Hartwig and Schlepegrell, (1995) reported that excess iron can lead to DNA damage by catalyzing the production of reactive oxygen species within the cell, leading to the induction of chromosome aberration[4]. **Aruoma et al. (1989)** demonstrated that, induction of oxidative damage by different ferric iron complexes in the presence of hydrogen peroxide (H_2O_2) has been shown in isolated DNA[20].

Our main finding in this study was that, after 6 months of regular chelation, and in most cases, frequencies of total genomic damage of DNA in patients received DFO were greatly different from those received deferiprone. A decrease in the frequencies of TGD of DNA were observed following regular therapy with DFO (group IIb) but it was statistically non significant when compared to group IIa (same patients before regular desferal) (P >0.05). Regarding pattern of DNA damage, gel electrophoresis revealed slight improvement as regards frequency of severe necrotic type which decreased to 60% instead of 70%, at the same time there was increased percentage of apoptotic pattern to 26.7% instead of 20% before chelation. Also DNA damage was absent in 13.3% versus 10% before chelation (table 4). When the mean of MOD of DNA at 200, 400 and 600 bp of group IIb were compared to the that of group IIa, we found a significant difference as regards the mean of MOD at 200 bp (P

<0.05%). In the light of our findings, our results showed that the iron chelator DFO may lead to improvement of the total DNA damage rather than worsen it.

Regarding this effect of deferoxamine (DFO) on the frequency of total genomic damage of DNA in thalassemic patients, the current study is in agreement with a study by **Iamele et al, (1999)** their aim was to study the damage induced by oxidative stress on mitochondrial and nuclear DNA in liver rat. And the effect of the treatments with iron chelator DFO on this damage, it showed a significant protective effect against DNA damage. He also suggested that both peroxidation and iron are involved in cell damage, thus, iron chelating agents protect against oxidative stress mediated effects[21].

Supporting to our results, several studies have shown that DFO is an antiproliferative agent [22]. DFO can protect against DNA strand breaks and damage in renal tubular epithelial cells[23], human colon carcinoma cells [24], and liver cells[25].

However, DFO has been teratogenic at least in some animal studies, and there is evidence of fetal teratogenecity in rats [26].

As mentioned earlier, we detected in group IIa and group IIIa double strand breakage of DNA not related to apoptosis (took another severe necrotic form). Some of these cases could be explained on the basis of uncontrolled chelation which possibly is the cause of this sever form of DNA damage. This is suggested especially owing to our findings of improvements of these forms after chelation therapy especially with deferiprone however against that is the increased percentage of this form after DFO chelation.

Regarding the effect of deferiprone on the frequency of total genomic damage of DNA in thalassemic patients we found a highly significant decrease in the frequencies of double strand breaks of DNA as compared to pre chelation state, the percentage of totally damaged DNA became 60% in (group IIIb) instead of 90% in (group IIIa) which showed a highly significant difference (P<0.001). This improvement noticed also as regard pattern of DNA damage on gel electrophoresis as we found a notable decrease in the severe DNA necrosis which was reduced to 10% of patients of group IIIb instead of 65% of patients of group IIIa with 50% of the damaged DNA took the more regular apoptotic form of damage**.** Thus the thalassemic children on regular deferiprone therapy had significantly lower prevalence and severity of DNA breaks (TGD), with also significantly lower Max. OD values at 200, 400, and 600 base pairs (bp) compared to the pre chelation values and also compared to the group treated with regular DFO, these results denote that with matched iron load in the group treated with DFO for the same duration, deferiprone had significant protective effect against DNA damage compared to DFO (Tables 6, 7, 8).

These results are consistent with the findings of **Marshal et al., 2003,** who studied the effect of deferiprone and DFO on DNA damage (at chromosomal level) in a group of Indian thalassemics where he revealed that chromosomal aberrations were less frequent in patients treated with deferiprone than in patients treated with DFO, despite the difference did not reach statistical significance[27]. Also, akin to many authors, it has been demonstrated that in some iron loaded situations where the chelation of intracellular iron is important, deferiprone can prevent the formation of reactive oxygen species and, hence, cytotoxicity[23, 28, 30]**.**

For more evaluation of the underlying pathogenic factors that may be involved in TGD of DNA, we analyzed statistically the serum ferritin levels in the three studied thalassemic groups and controls, we found a non significant positive correlation between serum ferritin level and the MOD of TGD of DNA at 200, 400 and 600 bp in thalassemic patients and normal children of group IV where (P > 0.05) (table, 10). In our study, absence of correlation between ferritin level and DSBs of DNA, may be supported by the finding, of DNA damage in children without high iron overload (ferritin <1000 ug/dl) observed in group I.

Our results were in accordance with **El Gendy et al. 2004,** who found that there was no correlation between serum ferritin level and the level of double strand breaks (DSBs) of DNA in thalassemic patients[17]. Also with **Perera and colleagues, 2002,** who found no correlation between the degree of iron overload and the amount of DNA damage in sperms of thalassemic patients[20]. He also revealed that, there was a close relationship between initiation of chelation therapy and sperm DNA damage, that patients whose onset of chelation was delayed had more DNA damaged sperm than those who started chelation at a younger age, indicating that duration of exposure to oxidative stress may be an important factor in the induction of DNA damage[12].

In conclusion Thalassemic patients with or without high iron overload (reflected by serum ferritin level), had significantly high prevalence of TGM DNA of their peripheral leucocytes with significantly higher values of max OD at 200, 400, and 600 bp. Further studies are needed to evaluate DNA damage by methods such as “comet assay” which can detect other forms of DNA damage. The effect of iron chelators is not just restricted on chelating cellular iron pool, but is also involved in modulating oxidative stresses induced by several environmental

stresses including iron overload. In the light of these findings proper control of iron overload and an introduction of new therapeutic modalities to decrease iron toxicity is essential in all thalassaemic patients, raising the importance of the regular use of iron chelators with reevaluation of the beneficial effect of deferiprone on decreasing DNA damage. We recommend supplementation of antioxidants like vitamin E, and vitamin C to reduce or prevent TGD of DNA in thalassemia patients.

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