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The Association between Anti-Oxidant/Redox Status and Sister Chromatid Exchanges in Down Syndrome Individuals

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Abstract

The main objective of this project is to study the possible association between anti-oxidant/redox status and DNA instability in Down syndrome. The activities of 5 antioxidant enzymes were studied in 19 Down syndrome (DS) cases and in age- and sex-matched normal controls. Sister Chromatid Exchanges (SCE) were measured in lymphocyte cultures derived from all DS and control subjects. All DS and control individuals had normal hematological parameters, but the proliferation and mitotic indices were significantly lower in the DS- than in the controls-derived lymphocyte cultures, while the average generation time was higher than that in the controls. The specific activity of superoxide dismutase in the DS individuals was 40% higher than that in the controls, while the specific activity of glutathione S-transferase in the DS group was significantly lower than that in the controls ($P \leq 0.05$). Catalase and glutathione peroxides' activities were not different between the two groups ($P > 0.05$). SCE rate in the DS derived cultures was significantly higher ($P < 0.001$) than that of the controls. DS individuals have a higher oxidative stress, higher superoxide dismutase activities and higher rates of SCE in their derived lymphocyte cultures compared to those of the controls. We claim that such differences may have resulted from the over expression of superoxide dismutase gene, leading to imbalanced cellular antioxidant mechanisms and, consequently, resulted in a high concentration of free radicals that destabilized the DNA as expressed by the high rate of SCE.

Key Words: Down syndrome, Antioxidant enzymes, Catalase, Glutathione peroxidase, Glutathione S- transferase, Oxidative stress, Sister chromatid exchanges (SCE).

1. Introduction

Trisomy 21, known as Down Syndrome (DS), is one of the most common human chromosomal disorders in live born children (1 in 800–1000) (Capone, 2004), leading to the characteristic phenotypes of DS individuals, which include a constant common feature of early mental decline and premature aging. DS affected individuals may also suffer from developmental abnormalities, specific cardiac and gastrointestinal congenital malformations, thyroid dysfunction and increased risk for various types of leukemia (Antonarakis and Epstein, 2006; Roizen and Patterson, 2003; Shaw *et. al*, 2006). The characteristic phenotypes of DS were suggested to be the ultimate results of the triplication of the Down Syndrome Critical Region (DSCR) 21q22.1 - 21q22.3 (Vesa *et. al*, 2005), which includes the gene coding for the enzyme, copper zinc superoxide dismutase (SOD1), implicated in the conversion of the harmful superoxide anion radical ($O_2^{\cdot-}$) to molecular oxygen (O_2) and hydrogen peroxide (H_2O_2) (Gardiner and Davisson, 2000; Sherman *et. al*, 1983). H_2O_2 can be converted by catalase (CAT) and by (selenium-containing) glutathione peroxidase (GPx) to water. The triplication of this DSCR leads to imbalance in the ratio of SOD1 to CAT and GPx, located on other chromosomes which results in excess H_2O_2 in the cells. Consequently, DS individuals are expected to have oxidative stress (OS), due to the imbalance between the generation and the removal of the reactive oxygen species (ROS) and result in harmful effects on different cellular macromolecules including DNA, where they cause DNA instability and induce single and double strand DNA breaks (Devasagayam *et. al*, 2004); they were suggested to be the cause of the high rate of cancer appearance in DS individuals (Wiseman and Halliwell, 1996).

In the present study, the association of the catalytic activities of the red blood cell antioxidant enzymes superoxide dismutase (EC 1.15.1.1) (SOD1), catalase (EC 1.11.1.6) (CAT), glutathione peroxidases (EC 1.11.1.9) (GPx), glutathione S-transferases (EC 2.5.1.18) (GST) were investigated along with the rate of sister chromatid

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exchanges (SCE) in lymphocyte cultures derived from DS individuals and sex and age matched controls.

2. Materials and Methods

2.1. Subjects and Blood Samples

Nineteen DS affected children (5 to 16 years old) were recruited from a special school for children with special needs in Jordan, and another group of 19 age- and sex-matched normal healthy children were used as controls. Blood sample of 5mL were collected from each participating child in heparinized tubes. An informed consent was obtained in all cases from the children's parents in accordance with the Helsinki declaration (Helsinki, 2000). Direct Complete Blood Count (CBC) was performed using an automated blood counter (COPAS Micros, France).

2.2. Determination of Enzymes Activities

All enzymes' activities were assayed in a hemolysate of the red blood cells by the addition of (5: 3) mixture of absolute ethyl alcohol and trichloromethane, followed by centrifugation at 18000 Xg for 60 minutes to precipitate Hb (Alkaraki *et. al*, 2010). All enzyme assays were measured spectrophtometrically through using UNICAM UV/Visible Spectrophotometer. Negative control reactions were prepared by substituting the hemolysate fraction by distilled water.

SOD1 activity was measured based upon pyrogallol auto-oxidation as reported earlier (Marklund and Marklund, 1974; Sun *et. al*, 1988), while CAT activity was assayed as reported previously (Aebi, 1984), GPx and GST activities were measured according to earlier reports (Habig *et. al*, 1974; Paglia and Valentine, 1967).

2.3. Preparation of Lymphocyte Cultures and Stained Metaphase Chromosomes

All cultures were prepared in duplicates using 25 cm^2 culture flasks. Each culture was initiated as reported earlier (Verma and Babu, 1989) with minor modifications. For each culture, fresh heparinized whole blood (0.8 mL) was added to 9.2 mL of RPMI 1640 culture media (Euroclone, Europe) supplemented with 10% fetal bovine serum (Euroclone, Europe), 1% penicillin-streptomycin (1000 U/µl, 10000 µg/µL) (Euroclone, Europe), 1% of 200 mM L-Glutamine (Sigma), 7.5 µg/mL phyto-haemagglutinin (Sigma) and 25 µg/mL 5´-Bromodeoxy-Uridine (BrdU) from Sigma Company. The culture flasks were incubated in the dark at 37°C in an upright position for 72 hours in a humidified 5% CO2 atmosphere incubator. Two hours prior to cell harvesting, cultures were treated with 100 µL of 10 µg/mL colcemid (Gibco, USA). Lymphocytes, from each culture, were collected by centrifugation at 275 Xg for 8 minutes using MPW–350R centrifuge. The lymphocytes were then used to prepare metaphase chromosomes that were differentially stained according to the fluorescence plus Giemsa method (Ishii and Bender, 1980). Permanent chromosomal preparations were made by mounting in D.P.X. mounting medium (WWR International Ltd., England). All slides were coded independently and scored by a blind study using a 100x oil-immersion objective mounted on a Labrolux 11 bright-field microscope (Zeiss, Germany).

2.4. Analysis of Sister Chromatid Exchanges and Cell Kinetics

The Mitotic Index (MI) and the Proliferative Index (PI) of each culture were measured to reflect the cytotoxicity of the conditions in the culture. The MI was calculated as the percentages of metaphases, providing that at least 1000 well spread cells were examined from each culture (Preston *et. al*, 1987). PI was determined by classifying 100 cells that passed through the replication cycle once (M1), twice (M2), three (M3) or more (>M3) using the following formula: PI = [M1%+ (2× M2%) + (3 × M3%)] / 100, while the Average Generation Mean time (AGT) was estimated as the number of hours cells incubated in BrdU divided by PI (Lamberti *et. al*, 1983; Preston *et. al*, 1987). SCE were scored for each culture using at least 50 clearly spread second division metaphase cells (M2) containing 46 ±1 chromosomes in presence of BrdU (Carrano *et al*., 1979).

2.5. Statistical Analysis

The average mean values of the hematological and cytological parameters and enzyme activities were expressed as mean values ± standard error (S.E.) of the mean. Based on normality assumption validation, comparisons between groups were accomplished using either the independent t test, when the normality assumption was valid, or, otherwise, the Mann-Whitney (MW) test. The differences were considered significant when the P value was ≤ 0.05. All statistical analyses were accomplished using the SPSS software version 11.5.

3. Results

Complete Blood Count (CBC), including Red Blood Cell count (RBC), hemoglobin (Hb), hematocrit (HCT), Mean Cell Volume (MCV), Mean Cell Hemoglobin (MCH), Mean Cell Hemoglobin Concentration (MCHC) and Red cell Distribution Width (RDW) were measured for all individuals in the DS and control groups. The average mean values of all the CBC values for all individuals, in both the DS and control groups, were within the standard normal ranges, as shown in Table 1. However, DS cases had significantly higher ($P \leq 0.05$) Mean of Corpuscular Volume (MCV) (84.1 μm^3) than those of the control (78.1 μm^3), while the DS group had significantly ($P \leq 0.05$) lower mean of MCHC (32.5 g/dl) than that of the control (31.7 g/dl).

Table 1. Average mean values (Mean ±S E) of the hematological parameters in the Down syndrome and the control groups

Hematological parameters	Controls	Down syndrome	*P – value* (Statistical Test)
RBC (10^6/mm^3)	5.0 ± 0.1	4.7 ± 0.1	0.061 (*t*)
Hb (g/dl)	12.7 ± 0.2	12.5 ± 0.3	0.686 (*t*)
HCT (%)	39.1 ± 0.6	39.7 ± 0.9	0.608 (*t*)
MCV (μm^3)	78.1 ± 1.0	84.1 ± 1.8	0.006 ^{S} (*t*)
MCH (pg)	26.9 ± 1.4	26.7 ± 0.7	0.064 (MW)
MCHC (g/dl)	32.5± 0.1	31.7± 0.2	0.002^S (*t*)
RDW (%)	14.9 ± 1.0	14.9 ± 0.3	0.077 (MW)

^{S}: Significant difference between DS and control groups at 0.05; MW: Mann-Whitney test; t: t-test; RBC: red blood cell count; Hb: hemoglobin; HCT: hematocrit; MCV: mean cell volume; MCH: mean cell hemoglobin; MCHC: mean cell hemoglobin concentration; RDW: red cell distribution width.

The average mean values of the specific activities of the antioxidant enzymes in the DS and the control groups are summarized in Table 2. The mean value of the activity of SOD1 in DS group was significantly ($P \leq 0.05$) higher (140%), than that of the control group, while the specific activity of GST was statistically lower in the DS group (59.8%) than that of the control group ($P \leq 0.05$). Contrary to the differences in SOD1 and GST activities between the DS and control groups, the average mean values of CAT and GPx specific activities were not significantly different between the DS cases and the controls.

Table 2. Average values of the enzyme activities in DS and control groups

Enzyme	Enzyme activities Means ± S E in the studied groups		*P – value*
	DS individuals	Controls	(t test)
SOD1	2.247 ± 0.077	1.605 ± 0.083	0.042^S
CAT^a	195.3 ± 11.09	170.8 ± 7.03	0.070
GPX^b	51.3 ± 5.57	58.2 ± 9.87	0.546
GST^b	5.2 ± 0.65	8.8 ± 1.05	0.007 ^{S}

^{a}Unit /g Hb; ^{b} U/ mg Hb; ^{S} Significant difference at 0.05; SOD1: superoxide dismutase; CAT: catalase; GPX: glutathione peroxidases; GST: glutathione S-transferases.

Chromosomal DNA stability was investigated by examining the average rate of SCE in all individuals within the DS and the control groups. SCE test is known as a highly sensitive indicator for DNA instability (Wolff *et. al*, 1977). The average mean values of SCE and the rates of cell cycle parameters for the lymphocytes cultures derived from DS and control groups are summarized in Table 3. The average mean values of SCE rates per cell in the DS and the control groups were 7.9 and 5.1 exchanges, respectively. Using the t test, this difference was statistically significant ($P < 0.01$) and showed that SCE rates in the lymphocyte cultures derived from DS group are higher than SCE rates in the cultures derived from the control group. Also, as shown in Table 3, the lymphocytes derived from the DS individuals exhibited significantly longer average mean value of AGT and lower values of MI and PI compared to the lymphocytes derived from the control group.

Table 3. Average mean values (Mean ±S E) of the genotoxicity and cytotoxicity parameters in lymphocyte cultures derived from Down syndrome and control subjects.

Cytological parameters	Mean ± S E		Ratio of Parameters In DS /control	*P – value*s (test)
	Down syndrome	Controls		
SCE	7.993 ± 0.200	5.132± 0.0579	155.9	0.000 ^{S} (*t*)
MI	1.458 ± 0.04597	2.549± 0.05006	57.2	0.000 ^{S} (*t*)
PI	1.493 ± 0.05952	2.489 ± 0.04817	59.9	0.000 ^{S} (*t*)
AGT	49.539± 1.866	29.100 ± 0.570	170.2	0.000 ^{S} MW

^{S} Significant difference between DS and control groups at 0.05; SCE; sister chromatid exchanges; MI: mitotic index; PI: proliferative index; AGT: average mean time; MW: Mann-Whitney test.

4. Discussion

The normal ranges for the average mean values of all hematological parameters in both studied DS and control groups indicated that all DS and control individuals participated in this study were hematologically healthy, free of any of the common hemoglobinopathies such as thalassemia, sickle cell anemia and microspherocytosis, which, if present, would interfere with the results.

In a previous study of the same DS and control groups for aminothiols including total cysteine, cysteinyl-glycine, homocystiene and glutathione we found that these low molecular weight thiol-containing amino acids were not significantly different between the DS and the control group, suggesting that these molecules were not associated with DS syndrome phenotype (Alkaraki *et. al*, 2010). These same groups of DS and controls were further investigated in this study for the association between Down syndrome and the catalytic activities of the oxidative enzymes SOD1, CAT, GPx and GST in the red blood cells. In addition, the DNA stability in both groups was investigated by measuring the frequencies of the spontaneous rate of SCE among the Down Syndrome group and the healthy controls, since the changes of SCE rates were reported to be highly sensitive indicator for DNA instability (Wolff *et. al*, 1977). Our findings were in agreement with the results reported earlier (Sinet, 1982), which showed that SOD1 activity in DS could reach about 150% of that in the controls. It is well known that SOD1 works in conjunction with the two enzymes CAT and GPx, which neutralize the hydrogen peroxide produced in normal human cells (Gardiner and Davisson, 2000; Sherman *et. al*, 1983).

In this study, the lower GST activity in DS group compared to those in the controls indicated that there is an oxidative stress in DS subjects associated by the increase in SOD1 activity. Such association can be explained by the accumulation of genotoxic hydroxyl radicals as a result of the high SOD1 activity, which may contribute to the modulation of the second defense line against the generated free radicals provided by GST that functions as a detoxicant by catalyzing the conjunction of reduced glutathione with a variety of exogenous and endogenous harmful electrophiles. It is clear that the oxidative stress in DS individuals is due to the increase of SOD1 activity that resulted in excess production of H_2O_2 but not due to any adaptive increase in the catalytic activities of the CAT or GPx as suggested earlier (Mattei *et. al*, 1982; Sinet *et. al*, 1975). H_2O_2 is a potent oxidative agent that can generate highly reactive hydroxyl radicals in the presence of metal cations via the Fenton reaction (Emerit *et. al*, 2001; Urbañski and Berêsewicz, 2000). Such imbalance in the production of large amounts of reactive oxygen free radicals has harmful effects on the different cellular macromolecules including DNA, proteins, lipids and carbohydrates (Sies, 1993).

However, contrary to SOD1 activity in the DS group, the results of this study showed that CAT and GPx activities in the DS group are not significantly different from those in the control group, supporting the fact that the enhanced generation of ROS in DS individuals is a result of the increase in the SOD1 catalytic activity, which is not accompanied by an equivalent increase in the CAT and GPx activities that fail to neutralize the excess hydrogen peroxide produced in the trisomic cells. The elevated ratio of SOD1/CAT plus SOD1/GPX in DS group with respect to controls is a sign of oxidative imbalance in DS. This notion is in agreement with the recently reported results of Garlet *et al.* (2013) and Paristto *et al*. (2014); however the results of this study were different from previous reports, which showed an increase in the CAT and GPx activities in DS individuals compared to the normal controls (Mattei *et. al*, 1982; Sinet *et. al*, 1975).

The lower values of MI and PI observed in the lymphocyte cultures derived from DS individuals in parallel with the extension of AGT compared to those derived from the normal control group, indicated that DS lymphocytes cultures have a slower growth rate than those of the control cultures, which can be related to their higher oxidative stress. Similarly, the higher induced rate of SCE in the DS lymphocyte cultures compared to those derived from the controls confirmed the instability of DNA in DS cells compared to those of the controls. It is well reported that the elevation of SCE rate is a highly sensitive indicator for DNA instability (Wolff *et al*., 1977). Therefore, our results indicated that such DNA instability is most likely due to the oxidative stress status found in DS-derived lymphocytes compared to the controls due to their triplication of chromosome 21 and consequently their triplication of *SOD1* gene. However, the unexpected reduced activity of GST in DS cultures may be contributed to the down regulation of the *GST* gene by the over expression of the SOD1 enzyme. Further studies are required to clarify this notion.

In conclusion, this study showed that DS condition is associated with DNA instability that may have resulted from oxidative imbalance in the patients. Further studies related to the correlation between oxidative stress and DNA instability in DS should be stimulated by our results since recent studies in this area are very scarce.

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