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In vivo prooxidant state in Werner syndrome (WS): Results from three WS patients and two WS heterozygotes

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Abstract

The hypothesis was tested that Werner syndrome (WS) phenotype might be associated with an *in vivo* prooxidant state. A set of redox-related endpoints were measured in three WS patients, two of their parents, and 99 controls within a study of some cancer-prone and/or ageing-related genetic disorders. The following analytes were measured: (a) leukocyte 8-hydroxy-2′-deoxyguanosine; (b) glutathione from whole blood, and (c) plasma levels of glyoxal, methylglyoxal, 8-isoprostane, and some plasma antioxidants (uric acid, ascorbic acid, α- and γ-tocopherol). Leukocyte 8-hydroxy-2′-deoxyguanosine levels showed a significant increase in the 3 WS patients vs. 85 controls ($p < 10^{-7}$). The disulfide glutathione:glutahione ratio was significantly altered in WS patients ($p = 0.005$). Glyoxal and methylglyoxal levels were significantly increased ($p = 0.018$ and $p = 0.007$, respectively). The plasma levels of uric acid ($p = 0.002$) and ascorbic acid ($p = 0.003$) were also increased significantly in WS patients and in their parents. No significant alterations were found in the plasma levels of α- and γ-tocopherol, nor of 8-isoprostane. This is the first report of *in vivo* alterations of oxidative stress parameters in WS patients. Further investigations on more extensive study populations are warranted to verify the relevance of an *in vivo* prooxidant state in WS patients.

Keywords: *Werner syndrome, prooxidant state, oxidative DNA damage, glutathione, glyoxal, methylglyoxal*

Abbreviations: *8-OHdG, 8-hydroxy-2′-deoxyguanosine; GSH, glutathione; GSSG, glutathione disulfide; Glx, Glyoxal; MGlx, Methylglyoxal*

Introduction

Werner Syndrome is very rare autosomal recessive disorder, associated with premature ageing, excess cancer risk, high incidence of type II diabetes mellitus, and a number of complications affecting various organs and systems (heart and vessels, skin, eyes, and joints) [1]. Werner syndrome (WS) is caused by mutations in the *WRN* gene, encoding a 160 kDa protein (WRNp), a homolog of the *E. coli* RecQ DNA helicase, associated with 3′-5′-exonuclease and ATPase activities [1–2]. In the current literature, the

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WS-associated genetic defect is mostly related to the DNA helicase activity and hence to the maintenance of DNA integrity [3]. The diagnosis of WS relies on the molecular analysis of defective WRNp, and on clinical phenotype features [4]. The heterozygous state can be proven only when gene mutations are known in families. The illness always presents a progressive, invalidating course, which is only relieved by palliative treatments [1].

Some of WS-associated phenotypic features suggested possible links with oxidative stress, related to the excess sensitivity of WS cells to 4-nitroquinoline- *N*-oxide (4NQ) and mitomycin C (MMC) [5,6]. These agents are characterized by toxicity mechanisms that relate to oxidative stress [7,8]. A few recent reports have shown redox abnormalities in WS cells, related to their response to an antioxidant or to hydrogen peroxide [9–11]. A possible relationship of WS phenotype with oxidative stress had been suggested by an early study by Nordenson [12], who reported on the counteracting effects of some antioxidant enzymes on chromosomal instability in WS cells. Subsequently, Oliver et al. reported that the levels of oxidatively modified proteins in fibroblasts from individuals with WS were significantly higher than in age-matched controls [13].

However, also due to the rarity of this disorder, no attempt has yet been made, to the best of our knowledge, to ascertain any *in vivo* prooxidant state in body fluids or blood cells from WS patients and their family members. In the present study, a set of oxidative stress-related endpoints were evaluated in whole blood, plasma, and WBC from three WS patients and two of their parents. The results support the hypothesis that an *in vivo* prooxidant state may be associated with WS clinical phenotype.

Materials and methods

Study population

Three patients with WS, aged 25–47 years, two of their parents, and two control groups were enrolled in this study. The enrolled persons were subjected to a 15-ml drawing of heparinized peripheral blood following informed consent as approved by the Ethical Committee of ASL Napoli 1, Naples, Italy, in accordance with the Helsinki Declaration of 1975, as revised in 1983. Diagnosis in WS patients was defined by the occurrence of a set of clinical features, fulfilling the minimal diagnostic criteria (Table I). Control donors consisted of two groups, namely: (i) 45 healthy donors (HD) aged 19–49 years (33.8 ± 11.9 years), and (ii) 54 parents of patients affected by other diseases, aged 37–88 years (50.6 ± 14.0 years) termed as "pathologic controls" (PC). The PC donors were included in the control group after verifying the identity of analytical results between PC and HD groups. Thus, their analytical data were pooled together as one control group. Analyses were conducted, within a study of some oxidative stress-related diseases, in blind mode of sample tagging and of analytical determinations.

Table I. Main clinical features of the WS patients recruited in the present study.

Patient No.	1	2	3
Parents' consanguineity	+	+	+
Sex	F	M	F
Age (years)	25	47	35
General weakness	+	+	+
Short stature	+	+	+
Muscle hypoplasia	+	+	+
Osteoporosis/Arthrosis	−	+	+
Sclerodermia	+	+	+
Alopecia	+	+	+
Peculiar face	+	+	+
Dysphonia	−	+	+
Cataract	−	+	+
Diabetes	−	+	−
Renal failure	−	+	−
Hypogonadism	+	−	+

Simultaneous analyses were run on samples from WS patients, patients with other genetic diseases, their parents, and healthy donors. Thus, the procedures applied in this study rule out any bias as, e.g. uncontrolled autooxidation of samples from WS patients.

Determination of 8-hydroxy-2′-deoxyguanosine in DNA from leukocytes

Buffy coat was obtained by Ficoll gradients, suspended in PBS and frozen at −70°C. Thawed WBC were suspended in nine volumes of separation buffer, and DNA purification was then accomplished as reported previously [14]. The levels of 8-hydroxy-2′-deoxyguanosine (8-OHdG) were expressed as mol of 8-OHdG adducts × 10^6 per mol dG. Analyses were run in triplicate, and coefficients of variation were lower than 15%.

Determination of glyoxal and methylglyoxal

Plasma from freshly drawn heparinized blood was deep frozen at −70°. Glyoxal (Glx) and methylglyoxal (MGlx) plasma levels were determined according to the analytical procedure reported by Espinosa-Mansilla et al. [15]. Calibration curves for 6-methylpterin (0.8 to 81.9 μM) and standard addition method allowed calculations of Glx or MGlx levels by integration of peak areas.

Determination of glutathione disulfide and total glutathione

Blood samples (0.5 ml) were treated, immediately after venipuncture, with 12% (v/v) perchloric acid

(PCA) containing 2 mM bathophenanthroline-disulfonic acid (BPDS) (1:5) to determine total glutathione. In order to determine glutathione disulfide (GSSG) levels, blood (0.5 ml) was acidified immediately after being drawn with 0.5 ml of 12% PCA, 2 mM BPDS and 40 mM N-ethylmaleimide (NEM). Analyses were run according to the method described previously [16].

Determination of ascorbic acid and uric acid

The high performance liquid chromatography (HPLC) of uric acid and ascorbic acid was based on the method of Iriyama et al. [17] with modifications. Final concentrations for ascorbic acid and uric acid were calculated with external standards which were run simultaneously.

Determination of α- and γ-tocopherol

Vitamin E (α- and γ-tocopherol) was measured in plasma shipped in dry ice, based on the method of Kelly et al. [18], using HPLC with ultra-violet detection.

Determination of 8-isoprostane

Quantitative analysis of 8-isoprostane (8-epi-PGF2a) was performed on plasma samples by a competitive enzyme immunoassay (EIA) (8-isoprostane EIA kit, Cayman Chemical Company, MI, USA) based on the competition between 8-isoprostane and an 8-isoprostane-acetylcholinesterase conjugate for a limited number of 8-isoprostane-specific rabbit antiserum binding sites.

Statistical methods

Differences between groups were tested using one-way analysis of variance (ANOVA) followed by the *post-hoc* Tukey honest significant difference (HSD) test. When data sets with unequal N were considered, the Spjotvoll-Stoline test (Tukey HSD for unequal N) was used [19]. All analyses were performed using statistical routines in STATISTICA® 5.1 software.

Results

The levels of leukocyte 8-OHdG in three WS patients and in two of their parents were measured compared to the 8-OHdG levels in 85 controls. As shown in Table II, the individual levels of 8-OHdG in WS patients were 11.5, 5.3, and 11.6 mol 8-OHdG $\times 10^6$/mol dG, with a mean value of 9.5 ± 3.6, with a highly significant increase vs. the analytical results from 85 controls (3.2 ± 1.5, $p < 10^{-7}$). The 8-OHdG levels in two mothers of WS patients were 4.5 ± 0.4 mol 8-OHdG $\times 10^5$/mol dG, which failed to reach statistical significance ($p = 0.16$).

A significant increase was observed in Glx levels measured in two WS patients (4.6 ± 2.6 μM, $p = 0.018$) vs. the Glx levels in 23 controls (2.4 ± 0.3 μM) (Table II). Also the plasma levels of MGlx were significantly increased in WS patients with a mean concentration of 732 ± 78 nM, vs. a control value of 455 ± 115 nM, as shown in Table II ($p = 0.007$).

Glutathione levels in WS patients showed significant differences vs. 76 controls in the same age range (Table II), including a significant decrease in total glutathione ($p = 0.03$) and in GSH ($p = 0.02$), along with a significant increase in GSSG and a highly significant increase in the GSSG:GSH ratio in WS patients (11.3 ± 10.3) vs. controls (3.0 ± 3.5) ($p = 0.005$).

The plasma levels of uric acid and ascorbic acid (Table III) resulted to be significantly increased in three WS patients and in two mothers (with superimposable levels) vs. 41 controls. A significant increase was observed in ascorbic acid levels in WS patients and their mothers that were 57 ± 19 μM vs. 22 ± 13 μM in controls ($p = 0.003$). Uric acid levels were 291 ± 93 μM in the WS group (patients + parents) vs. 99 ± 32 μM in 41 controls ($p = 0.002$) (Table III).

Table II. Levels of 8-OHdG, glyoxal, methylglyoxal, and glutathione in WS patients, their parents and in controls.

Analytes	WS patients	WS parents	Controls	References
	(Number of samples analysed)			
Leukocyte				
8-hydroxy-2′-deoxyguanosine (mol × 10^6/mol dG)	9.5 ± 3.6⋆ (3)	4.5 ± 0.4 (2)	3.2 ± 1.5 (85)	[14]
Plasma α-oxoaldehydes				
Glyoxal (μM)	4.6 ± 2.6† (2)	3.0 (1)	2.4 ± 0.3 (23)	[15]
Methylglyoxal (nM)	732 ± 78‡	570	455 ± 115	
Whole blood glutathione				
GSSG (μM)	177 ± 155¶ (3)	51 (1)	86 ± 82 (76)	[16]
GSH (μM)	1649 ± 251†	2626	3436 ± 1506	
GSSG:GSH × 100	11.3 ± 10.3‡	1.9	3.0 ± 3.5	

⋆$p < 10^{-7}$; †$p < 0.02$; ‡$p < 0.01$; ¶$p < 0.05$.

Table III. Levels of some selected plasma antioxidants in three WS patients, two mothers and controls.

Analytes	WS patients	WS parents	WS patients and parents	Controls	References
	Number of samples analysed				
Ascorbic acid (μM)	57 ± 22 (3)	56 ± 21 (2)	57 ± 19* (5)	22 ± 13 (41)	[17]
Uric acid (μM)	300 ± 130	278 ± 10	291 ± 93*	99 ± 32	[17]
α-Tocopherol (μM)	33 ± 13	30 ± 8	32 ± 8	28 ± 10	[18]
γ-Tocopherol (μM)	1.5 ± 1.5	0.9 ± 0.0	1.3 ± 1.1	1.2 ± 0.8	[18]

*$p < 0.005$.

No significant changes were detected in α- and γ-tocopherol (Table III), nor in 8-epi-$PGF_{2\alpha}$ (data not shown).

Discussion

The present study provides the first report of an *in vivo* prooxidant state in three WS patients assessed by a multiple set of endpoints in whole blood, leukocytes and plasma, including significant excess levels of oxidative DNA damage (leukocyte 8-OHdG), oxidative carbohydrate and amino acid degradation (Glx and MGlx), GSSG:GSH ratio, uric acid, and ascorbic acid (Tables II and III). The abnormalities in oxidative stress parameters in the present group of WS patients may suggest some mechanistic link towards some clinical features observed in WS including early ageing, atherosclerosis, cancer proneness, cataract formation, and type II diabetes mellitus, whose relationships with oxidative stress have been reported in a vast body of literature [20–22]. Interestingly, lipophilic markers of oxidative stress, as Vitamin E and 8-isoprostane, were detected in WS patients in the control range.

An involvement of oxidative stress in WS had been suggested by previous reports of abnormal response to either antioxidants [9,12], or to hydrogen peroxide [10,11]. An early report had shown that fibroblasts from WS patients underwent enhanced protein oxidation vs. control cells [13].

Evidence for excess sensitivity of WS cells to some xenobiotics, such as 4NQ and MMC [5,6], may suggest an impairment of WS cells in redox pathways [7,8], due to the involvement of redox-dependent toxicity mechanisms. Some of these agents (e.g. MMC) have been reported previously to exert excess toxicity in cells from patients with Fanconi anaemia (FA), another cancer-prone, oxidative stress-related disease [8].

Indirect information for a WS-associated prooxidant state might be related to the multiple functions of the *WRN* gene product, WRNp. This protein associates with a number of interactors (Ku 70/80, MYC, p53, p38, p21, and the ATR/ATM pathway), whose functions have been related to oxidative stress [23–27]. Orren et al. [25] found that oxidative DNA damage, as 8-oxoadenine and 8-oxoguanine, blocks the exonuclease activity of WRNp. Ku heterodimer, active in specifically removing oxidative DNA damage, was involved in rescuing WRNp exonuclease to remove 8-oxoadenine and 8-oxoguanine modifications [25]. Another implication of WRNp in redox pathways was suggested by Blander et al. [27] who demonstrated that WRNp overexpression increases p53 expression which, in turn, is upregulated by oxidative stress [27].

Together, several lines of evidence had suggested implications of redox pathways in WRNp functions, as well as excess sensitivity of WS cells to redox-active xenobiotics. Analogous to WS, other cancer-prone genetic disorders have been reported to display phenotypic features related to a prooxidant state, as FA [8,14,28]. However, the present results on WS patients largely exceed our recent data from the FA patients [28].

In conclusion, this study supports the occurrence of *in vivo* alterations in a set of oxidative stress parameters, related to oxidative degradation of biomolecules (8-OHdG, Glx and MGlx), impairment of the glutathione system, and increased uric acid and ascorbic acid plasma levels.

The data obtained in two mothers of WS patients, namely excess uric acid and ascorbic acid levels, suggested possible intermediate values of a prooxidant state in WS heterozygotes. Beyond the endpoints investigated in the present study, other aspects of the WS phenotype deserve *ad hoc* investigations. The prospects of verifying the present data in more extended groups of WS families, and extending knowledge of the WS clinical phenotype as related to an *in vivo* prooxidant state might lead to the development of novel diagnostic tools, and to chemoprevention trials designed to alleviate disease progression.

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