

Antioxidant Effects of Potassium Ascorbate with Ribose in Costello Syndrome

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Abstract. *Background:* Costello syndrome is a rare genetic condition characterized by coarse facies, short stature, loose folds of skin especially on hands and feet, severe feeding difficulties and failure to thrive. Other features include cardiac anomalies, developmental disability and increased risk of neoplasms. Given the link between oxidative stress (OS) and carcinogenesis, we tested the hypothesis that OS occurs in this syndrome, supposing its role both in cancer development and in other clinical features. *Patients and Methods:* We describe four cases with Costello syndrome in which we verified the presence of OS by measuring a redox biomarker profile including total hydroperoxides, non-protein-bound iron, advanced oxidation protein products, thiols, carbonyl groups and isoprostanes. Thus, we introduced an antioxidant agent, namely potassium ascorbate with ribose (PAR) into the therapy and monitored the redox profile every three months to verify its efficacy. *Results:* A progressive decrease in OS biomarkers occurred, together with an improvement in the clinical features of the patients. *Conclusion:* OS was proven in all four cases of Costello syndrome. The antioxidant therapy with PAR demonstrated positive effects. These promising results need further research to confirm the relevance of OS and the efficacy of PAR therapy in Costello syndrome.

Costello syndrome is a rare genetic disease. Its actual prevalence is unknown, but approximately 200 cases have been reported in the literature. It is characterized by failure to thrive in infancy, short stature, delayed development, intellectual disability, coarse facial features (full lips, large

mouth, sparse or curly hair), and skin and cardiac anomalies. Relative or absolute macrocephaly is typical. Dermatological findings include loose folds of skin, especially on the hands and feet, acanthosis nigricans, dark skin and papillomata. Hyperextensibility of the fingers and unusually flexible joints are also frequent. Affected infants may be larger than average at birth, but as a result of severe postnatal feeding difficulties, they grow more slowly than normal children. Cardiac dysfunction is common, especially in the form of arrhythmia, structural heart defects and hypertrophic cardiomyopathy (1, 2). Mild to moderate intellectual deficit is usual; most patients exhibit a characteristic sociable and friendly personality (3).

Beginning in early childhood, patients with Costello syndrome are predisposed to the development of tumors, with an approximately 15% lifetime risk. The most common tumor associated with the syndrome is rhabdomyosarcoma. Neuroblastoma and transitional cell carcinoma of the bladder have been also reported (4). Diagnosis of Costello syndrome is based on clinical features; regarding molecular genetic testing, sequence analysis of Harvey rat sarcoma viral oncogene homolog (*HRAS*), the only gene currently known to be associated with the syndrome, detects mutations in 89-90% of individuals with this clinical diagnosis (5, 6). Cardiac examinations should be performed to identify for heart defects, and physical and occupational therapies are recommended. The prognosis depends on the severity of the cardiomyopathy and on the occurrence of malignant tumors.

According to Knudson's two-hit hypothesis, cancer risk in Costello syndrome depends on a first germinal mutation in *HRAS* inherited by a parent and on a second somatic mutation. We hypothesized that this second DNA mutation could be linked to environmental factors. Given the link between oxidative stress (OS) and neoplastic risk underlined by recent literature (7, 8), we assumed that OS may also play a role in cancer development in patients with Costello syndrome. We also hypothesized that free radicals (FRs) are involved in determining non-neoplastic clinical features of the syndrome, such as elastin anomalies, alterations of the

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skin and appendages, developmental retardation, and cardiac defects. The pathogenesis of these clinical features, in fact, could be linked to OS damage to the cell cycle, apoptosis mechanism and DNA integrity.

The aim of this study was to assess the occurrence and the nature of OS in patients with Costello syndrome, as well as to evaluate the possible positive effects of therapy with an antioxidant agent. A redox biomarker profile was assessed in four patients with clinical diagnosis of Costello syndrome, confirmed by the presence of *HRAS* mutation. Once OS was ascertained, an antioxidant agent, namely potassium ascorbate with ribose (PAR) was introduced into therapy. PAR combines the antioxidant action of vitamin C with the stabilizing intracellular effects of potassium. Ribose acts as a catalyst, strengthening the action of potassium ascorbate (9).

Patients and Methods

Patient 1. Male, this patient was born in the 38th week of pregnancy with sonographic detection of polyhydramnios. He demonstrated the occipitofrontal circumference (OCF) up to the 97th centile, while the other growth parameters were at the 10th-25th centile. The patient presented coarse facial features, depressed nasal bridge, anteverted nostrils, low-set ears, large mouth, full lips, macroglossia, short neck, loose skin on hands and feet, and palmoplantar hyperkeratosis, deep palmar and plantar creases, curly hair and bilateral cryptorchidism. Mild neurodevelopment delay was also present. The patient exhibited epileptic manifestations and in the first two years of life, had severe feeding difficulties. Storage disease and chromosomopathies were excluded. The immunohistochemical study of cutaneous fibroblasts showed an increase in collagen I and fibronectin in the extracellular matrix. Fibroblasts produced tropoelastin in normal quantities but with abnormal secretion. We evaluated the OS biomarker profile for the first time when the patient was four years old.

Patient 2. This female patient was born in the 40th week of pregnancy with sonographic detection of polyhydramnios. From birth to three years of age, length and weight were at the 75th-95th centile, while OCF was always up to the 97th centile. She had coarse facial features, epicanthus with sinophris, depressed nasal bridge, anteverted nostrils, low-set ears, long philtrum, large mouth, full lips, macroglossia, high arched palate, short neck, bell chest, batracian abdomen, voluminous umbilical hernia, loose skin on hands and feet, and palmoplantar hyperkeratosis, deep palmar and plantar creases, diffuse hypertrichosis and curly hair. She presented mild neurodevelopment delay at eight months of age and hypertrophic cardiomyopathy at two years of age. Urinary metabolite and lysosomal enzyme analyses and a cytogenetic study excluded storage disease and numerical or structural chromosomopathy. The immunohistochemical study of cutaneous fibroblasts showed an increase in collagen I and fibronectin in the extracellular matrix. Fibroblasts produced tropoelastin in normal quantities but with abnormal secretion. Molecular analysis of oncogene *HRAS* showed the presence of mutation. OS biomarker profile was performed for the first time when the patient was 14 months of age.

Patient 3. This patient was female, born in the 33rd week of a twin pregnancy. We observed the patient at the age of six months for the first time. She presented growth failure and neurodevelopment delay. Growth parameters were under the 3rd centile except for OCF (97th centile). Hydrocephalus was surgically corrected. The patient had coarse facial features, depressed nasal bridge, anteverted nostrils, short philtrum, micrognathia, large mouth, full lips, low-set ears, loose skin on hands and feet, and palmoplantar hyperkeratosis, deep palmar and plantar creases, curly hair and bilateral flatfoot. At the age of eight years, the patient developed a mammary papilloma. We excluded storage disease, metabolic accumulation diseases and chromosomopathies. Ultrastructural examination with optical and electron microscopy of cutaneous and subcutaneous tissue showed a reduction of fibrillar pattern, with an altered organization of dermo-epidermal junction. We evaluated the OS biomarker profile for the first time when the patient was 8 years old.

Patient 4. This patient was male, born in the 38th week of pregnancy with ultrasonographic detection of polyhydramnios. Length and weight were under the 25th centile, while OCF was at the 97th centile. A gargoylic aspect was present, with coarse facial features, diffuse hypertrichosis, and loose skin on hands and feet; feeding difficulties were present, with a severe growth defect. Arrhythmia with hypertrophic cardiomyopathy at the age of a few months caused premature death. *HRAS* mutation confirmed Costello syndrome; chromosomes were normal, as well as metabolic analyses. Only a redox evaluation was possible, showing abnormal OS biomarker levels.

Methods. Heparinized blood samples were drawn and immediately centrifuged (1,000 ×g for 10 min). The plasma was stored in plastic metal-free containers at -80°C until analyses, which were performed at the Laboratory of Oxidative Stress of the University Hospital of Siena. Twenty healthy children of the same age as our patients (aged from 1 to 8 years) were used as controls.

OS was detected by measuring a redox panel represented by non-protein-bound iron (NPBI), advanced oxidation protein products (AOPP), total hydroperoxides (TH), isoprostanes (IP₂), thiols (SH) and carbonyl groups (CO). NPBI is a pro-oxidant substance that can act as a catalyst of the Fenton reaction in which FRs are produced. Under physiological conditions, it is not detectable. It was measured according to our previously published high-performance liquid chromatography (HPLC) method (10). AOPP are terminal products of protein exposure to FRs; they were measured as described by Witko-Sarsat *et al.* (normal values <29±0.49 μmol/l) (11). TH represent a measurement of overall OS: they are the intermediate oxidative products of lipids, peptides and amino acids. TH production was measured with a D-Roms Kit from Diacron SRL, Grosseto, Italy (normal values between 250 and 300 CARR U; 1 CARR U is equivalent to 0.08 mg/l of H₂O₂). IP₂ is the most specific marker of OS; IP₂ is generated by FR attack on polyunsaturated fatty acids. Their accumulation in human tissues is a cause of cellular dysfunction. Recent evidence has implicated IP₂ in cancer development (12, 13). IP₂ was measured according to the method of Milne *et al.* (normal values <60 pg/ml) (14). Thiols (SH groups) represent protective factors maintaining the antioxidant system and proteins in a reduced state; they were measured according to the method of Hu *et al.* (normal values between 450 and 650 μmol/l) (15). We used the Protein Carbonyl Assay Kit by Cayman Chemical, Michigan, USA, for CO (normal values <0.1 nmol/mg).

Table I. Values of markers of oxidative stress before and after antioxidant administration in our patients.

Marker/patient	At diagnosis	At 3 months	At 6 months
IP ₂ (pg/ml)			
1	1091	287	90
2	2000	369	150
3	1773	120	60
4	1502	---	---
TH (CARR U)			
1	1040	620	358
2	394	350	287
3	462	380	300
4	502	---	---
AOPP (μmol/l)			
1	47.5	35	29
2	70	67	32
3	61	61	35
4	68	---	---
Thiols (μmol/l)			
1	107	320	483
2	181	214	400
3	109	400	520
4	150	---	---
CO (nmol/l)			
1	0.1	0.14	0.1
2	0.1	0.1	0.1
3	0.1	0.1	0.1
4	0.1	---	---
NPBI (μmol/l)			
1	0.7	0	0
2	1.2	0	0
3	1.55	0	0
4	1.60	---	---

AOPP, Advanced oxidation protein products; CO, carbonyl groups; IP₂, isoprostanes; NPBI, non-protein-bound iron; TH, total hydroperoxides.

Once OS was ascertained in our patients, PAR therapy was started, administering a daily dose of 150 mg L-ascorbic acid, 3 mg D-ribose and 300 mg potassium bicarbonate, on the basis of a protocol used for other genetic syndromes and approved by the local Ethics Committee of the University Hospital of Siena. After the introduction of PAR therapy, the patients were monitored quarterly. The clinical outcome, psychomotor development, evolution of heart disease and the occurrence of abdominal diseases were carefully monitored, as well as the appearance of neoplasms and changes in OS biomarkers.

The study was performed on three out of the four patients, because patient 4 died prematurely.

Results

OS values were higher in patients than in the controls (Tables I and II), suggesting the need for protection from OS. PAR therapy started immediately after OS was ascertained and was given once a day as a continuous therapy. After three

Table II. Values of oxidative stress biomarkers in controls.

Biomarker	Value
IP ₂ (pg/ml)	<60
NPBI (μmol/l)	<2.3
AOPP (μmol/l)	<29±0.49
TH (CARR U)	250-300
CO (nmol/mg)	<0.1
THIOLS (μmol/l)	450-650

AOPP, Advanced oxidation protein products; CO, carbonyl groups; IP₂, isoprostanes; NPBI, non protein-bound iron; TH, total hydroperoxides.

months of administration, we observed a reduction of OS biomarkers in parallel with an improvement of clinical aspects in all treated patients (Table I). The efficacy of the treatment was confirmed by the follow-up of the patients. In particular, we observed no progression of cardiac aspects and even noted regression of heart hypertrophy in patient 2 and an improvement of skin and appendage aspects. No side-effects, nor ingestion problems were detected during the treatment. Administration of PAR was not possible for patient 4 because of the severe cardiac condition which caused his premature death.

Discussion

The foundation for treating Costello syndrome with PAR in these children arose from the clinical evidence of an improvement in patient 1 after PAR administration to the child by his parents as a vitamin supplement. A follow-up examination, planned according to a Costello syndrome management program, showed a clear improvement in the clinical findings of the patient (improvement of skin and appendage aspects, better evolution of psychomotor development, no progression of heart hypertrophy, nor tumor development). PAR being an antioxidant, we hypothesized a possible involvement of OS in the pathogenesis of the syndrome and carried out a redox biomarker profile of the patient. Then we compared the OS biomarker values found after PAR administration with those found for a previous blood sample of the patient, taken as a routine procedure performed in our Clinic for all children having genetic syndromes with cancer risk, such as Costello syndrome and Beckwith-Wiedemann syndrome. A reduction in OS biomarkers was observed. These findings prompted us to set up a protocol to treat these children with PAR once OS was confirmed.

OS is involved in a large number of human pathologies, such as carcinogenesis, and cardiovascular and neurodegenerative diseases (16-24). It also has a role in the pathogenesis of several genetic syndromes. We previously studied its occurrence in Down syndrome and in Prader-Willi

syndrome, as well as in Beckwith-Wiedemann syndrome (25-30). Given the link between OS, genetic diseases and carcinogenesis, we assumed that OS could be involved in the pathogenesis of Costello syndrome, another genetic disease characterized by a high neoplastic risk. Moreover, none of the patients treated with PAR has developed tumors so far, with the exception of a mammary papilloma in patient 2. This confirms what recent literature has reported on the effects of antioxidant therapy against tumor development (31-34). We also hypothesized that OS may have a role in the development of non-neoplastic clinical features of the syndrome, such as elastin anomalies, alterations of skin and appendages, developmental retardation and cardiac defects. One of the main characteristics of the syndrome is the abnormal assembly of elastic fibers, which contributes to the connective tissue abnormalities. This may result from an improper interaction between microfibrillar components and elastin (35, 36). It is interesting to note that in the literature, an inverse correlation between the content of intracellular iron and the percentage of tropoelastin synthesis has been reported (37). Since in our patients we found an increased value of NPBI – which under physiological conditions is not detectable – we believed that an antioxidant therapy, while reducing NPBI levels, could have positive effects on elastin deposits, leading to clinical improvement. Our hypothesis is strongly supported by the positive effects found in our patients after a period of therapy with PAR. Our evaluation of PAR therapy efficacy was based both on the clinical monitoring of the patients, as well as on the reduction in plasma OS biomarkers. Lower levels of OS biomarkers were observed in all the patients. In addition, we verified a marked improvement in the aspect of skin and appendages in patients 1, 2 and 3, as well as a better evolution of psychomotor development and no progression of cardiac problems. None of the patients presented tumors within a follow-up period of 10 years.

We recorded great benefits especially in patient 2: the girl, who has been under therapy with PAR since the age of 14 months, presented an attenuation of gargoylic aspects, improvements in hair anomalies (detected by microscopy) and of skin anomalies. The most important aspect was the improvement of cardiac hypertrophy, detected by electro- and echocardiogram.

The choice of an antioxidant strategy must include the following characteristics: lack of toxicity, easy administration also in childhood, cheap, few and not relevant side-effects (38, 39). PAR seems to be a particularly promising therapy since, in addition to the positive and encouraging results, it is of low cost, has no side-effects and is orally administered. It is noteworthy that PAR also led to important and interesting results in other genetic syndromes, such as Beckwith-Wiedemann syndrome and Prader-Willi syndrome (29, 30).

Our results are preliminary and further research with a large number of cases is needed.

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