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Early View

Research letter

Systemic oxidative stress in the Congenital Central Hypoventilation Syndrome

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Systemic oxidative stress in the Congenital Central Hypoventilation Syndrome

To the Editor:

The Congenital Central Hypoventilation Syndrome (CCHS), also known as Ondine's curse, is a rare disorder characterised by severe hypoventilation during sleep and autonomic dysregulation [1,2]. The incidence of CCHS is about 1/200,000 live births. In more than 90% of cases, polyalanine repeat expansion (PolyALA) mutations are present in the paired-like homeobox PHOX-2B gene, although a frameshift variant (FS) may also be found [3]. The PHOX-2B gene encodes a highly conserved homeobox domain transcription factor which plays a regulatory role in the differentiation of the motor neuron and the serotonergic neuronal fate in the development of the central nervous system [4]. The hallmark of CCHS is the "forgotten breathing", which implies the need for life-long mechanical ventilation during sleep. However, in more severe cases characterised by global hypoventilation, mechanical ventilation must also be extended during wakefulness. Ventilatory support may be provided by tracheostomy and assisted ventilation, non-invasive ventilation, or diaphragm pacemakers. Severe respiratory depression typically arises at birth, but in milder cases, CCHS may be diagnosed later on in childhood or adulthood (later-onset CCHS) [1,2]. The clue to the respiratory defect is a reduced response to hypercapnia and hypoxemia depending on the malfunctioning of brainstem areas such as the retrotrapezoid nucleus, the parafacial respiratory group and the pre-Bötzinger complex which are involved in the chemosensory drive to breathe [5,6,7]. Due to the key role played by PHOX-2B in the development of the autonomic nervous system, this genetic defect may also have several pathological consequences such as Hirschsprung's disease (HSCR), neural crest tumours like neublastomas (NB), decreased heart-rate variability, attenuated heart-rate response to exercise, constipation, oesophageal dysphagia, ophthalmologic abnormalities, altered perception of anxiety, sporadic profuse sweating, and hypothermia [1,2]. Deficiencies in neurocognitive performance in preschool and school-aged children with CCHS have also been reported [8]. Although the brain injury found in CCHS patients appears to progress in several selected areas [9], the mechanisms underlying these abnormalities remain elusive. Unless CCHS is promptly diagnosed, brain lesions may be caused by intermittent hypoxia due to primary breathing impairment in early postnatal life. However, chronic episodes of hypoxia-reoxygenation can occur during both nocturnal assisted ventilation and wakefulness when CCHS patients are engaged in activities like studying or relaxing (watching TV, playing computer games, etc.). In these conditions, the overproduction of reactive oxygen species (ROS) may provoke oxidative stress capable of inducing severe cellular damage to lipids, proteins and DNA, and activating apoptotic signalling [10]. Oxidative stress is closely associated with several chronic and acute disorders including atherosclerosis, neurodegenerative diseases (e.g. Alzheimer's and Parkinson's), cancer, diabetes mellitus and inflammatory diseases, as well as aging process [11]. Interestingly, in breathing disorders characterised by intermittent hypoxia such as the obstructive sleep apnoea syndrome (OSAS), sudden infant death syndrome (SIDS) and Rett syndrome, an increased production of ROS has already been reported [12, 13, 14]. The aim of this study was to investigate the level of oxidative stress by measuring the intracellular ROS production in clinically stable CCHS patients. We analysed the systemic redox status in 14 patients with PHOX-2B mutation-confirmed CCHS and 14 healthy controls randomly selected from a cohort of healthy sex-and age- matched subjects who had been assessed for the absence of previous diseases possibly affecting the redox status, had a healthy life-style, and were not taking drugs.

This study was approved by our institutional review board. Written informed consent/assent was obtained for all patients.

The demographic and clinical characteristics of the CCHS patients are reported in Figure 1A. All patients in the study were only ventilated during sleep and none of them were suffering from pulmonary hypertension.

Systemic oxidative stress was analysed in the CCHS patients by evaluating the intracellular ROS production in the leukocyte and erythrocyte populations. As previously reported [15], peripheral leukocytes which can be considered a "time-persistent" system, reflect the condition of the whole organism and therefore represent a valuable model for studying systemic oxidative stress-related disorders. On the other hand, erythrocytes are particularly exposed to oxidative stress during their lifetime due to the high content of polyunsaturated fatty acids in the membrane and auto-oxidation of haemoglobin inside the cell [16]. In order to rule out any possible interference by recent episodes of hypoxia, the patients recruited underwent a complete full-night polysomnography and capnography, and their apnoea-hypopnoea index (AHI), oxygen desaturation index (ODI) and carbon dioxide levels were all within the normal ranges. Fasting blood samples were collected from the antecubital vein using EDTA as an anticoagulant between 8 and 9 a.m. Samples were processed within 2 h after collection. The leukocytes were separated from the whole blood using BD FACS Lysing Solution (BD Biosciences, CA, USA). After collection, EDTA-anticoagulated blood (100 µl) was resuspended in 2 ml of BD FACS Lysing Solution (BD Biosciences, CA, USA), mixed gently and incubated at room temperature in the dark for 10 min. After being centrifuged, the supernatant was discarded and the cells washed twice with PBS.

To determine the level of intracellular ROS, the cells were incubated with H_2DCF-DA (2.5 μM) (Invitrogen, CA, USA) in RPMI medium without either serum or phenol red for 15 min. at 37 °C. After labelling, the cells were washed, re-suspended in PBS and immediately analysed using a FACSCanto flow cytometer (BD Biosciences, CA, USA). Cell viability, controlled by flow cytometry with propidium iodide staining, was found to exceed 95%. The erythrocyte ROS production was analysed as previously reported [16]. For a single analysis, the fluorescence signals of 100,000 erythrocytes were collected. Data were analysed using BD FACSDiva software (BD Biosciences, CA, USA) and the differences between sample groups were statistically evaluated using the unpaired t-test. As illustrated in Figure 1B, the leukocyte ROS production in CCHS patients increased significantly in the subpopulation analysed compared with the healthy controls (lymphocyte ROS: 1219±356 vs. 595±73, $p<0.0001$, Cohen's d 1.54; monocyte ROS: 2795±868 vs. 1145±189, $p<0.0001$, Cohen's d 1.67; granulocyte ROS: 3641±1087 vs. 1365±196, $p<0.0001$, Cohen's d 1.83). These results were confirmed by the erythrocyte ROS production analysis, where a significant increase in CCHS erythrocyte ROS was evident (590±58 vs. 270±31, $p<0.0001$, Cohen's d 3.23). Interestingly, our results are in agreement with those obtained in blood cells and sera of patients diagnosed with obstructive sleep apnoea (OSA), although different oxidative stress markers were assessed with other methods [17].

Conversely, we found no association between the ROS levels and other patient features, including age, gender, type of mutation and type of ventilator support. Chronic episodes of hypoxia-reoxygenation due to ventilatory impairment, which may occur during nocturnal assisted ventilation, as well as hypoxiemic episodes during wakefulness, may play a key role in ROS generation even though the ultimate triggers that cause the redox imbalance are unknown. ROS overproduction might cause damage to important biomolecules and organs with a plausible impact on the whole organism.

It is important to stress that the relevant advances in CCHS management in childhood have led to an increased survival rate which implies that adult patients may now have to cope with an imbalanced aging process. In conclusion, this study provides first-line evidence of the systemic oxidative status in CCHS patients, demonstrating an increase in oxidative stress in this rare disease. Many aspects must still be clarified, mostly related to the mechanisms provoking the increase of ROS; however, therapeutic strategies based on antioxidants, as well as the promotion of a healthy lifestyle (i.e. adequate physical exercise and a balanced diet), must be taken into consideration in order to mitigate the oxidative derangement in these patients.

A

ID	PHOX2B Mutation	Age (years)	Gender	Disease	Ventilation
1	Poly Ala 20/25	3	M	CCHS	NIV
2	Poly Ala 20/25	5	M	CCHS	NIV
3	Poly Ala 20/25	24	F	CCHS	NIV
4	Poly Ala 20/25	28	F	CCHS	Diaphragm pacing
5	Poly Ala 20/25	40	F	CCHS	NIV
6	Poly Ala 20/26	7	F	CCHS	Tracheostomy
7	Poly Ala 20/26	9	M	CCHS + HSCR	Tracheostomy
8	Poly Ala 20/26	23	M	CCHS	Diaphragm pacing
9	Poly Ala 20/26	25	F	CCHS	Diaphragm pacing
10	Poly Ala 20/26	29	M	CCHS	NIV
11	Poly Ala 20/27	5	F	CCHS	Tracheostomy
12	Poly Ala 20/33	18	F	CCHS	Diaphragm pacing
13	FS c.722_759	4	M	CCHS + HSCR	NIV
14	FS c.930insG	13	F	CCHS + HSCR + NB	Tracheostomy

B

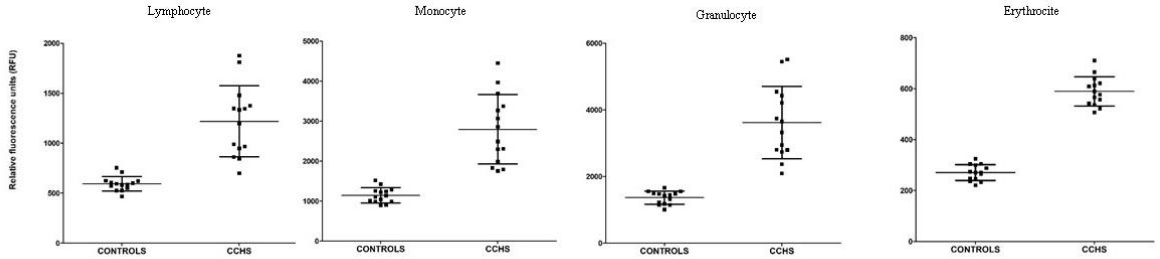

FIGURE 1 Summary of the characteristics of Congenital Central Hypoventilation Syndrome [CCHS] patients and the production of intracellular Reactive Oxygen Species [ROS] in CCHS patients and controls.

Neuroblastoma [NB], Hirschprung's disease [HSCR].

PHOX2B mutations: polyalanine (PolyAla) expansions, frame-shift [FS] variant.

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