Antioxidant concentrations in children with Attention Deficit Hyperactivity Disorder (ADHD)

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ABSTRACT
ADHD is one of the most prevalent neurodevelopmental disorder. Antioxidant concentrations are shifted in ADHD patients and antioxidant centred therapies appear to alleviate symptoms significantly[1-8]. This study aims to investigate antioxidant concentrations in children with ADHD of an Antwerp based population.
For the ADHD-cohort, concentrations of lipid-soluble antioxidants (α-tocopherol, γ-tocopherol, retinyl-palmitate, β-carotene, CoQ10 and retinol) (plasma, n = 12) and glutathione (erythrocytes, n = 4) were measured and consecutively compared to a cohort of non-ADHD children (n = 25-53). Additionally, differences in mean concentrations were compared to literature[1,7,8].

Keywords
ADHD, neurodevelopment, biomarkers, oxidative stress, antioxidants, HPLC.

INTRODUCTION

ADHD is the most common neurodevelopmental disorder diagnosed in children. Most patients are diagnosed during their early school years. Because ADHD patients often experience impairments in academic or work performance and moreover face social challenges, diagnosis and treatment are of high importance. Diagnostic criteria are stated in the Diagnostic and Statistical Manual of Psychological Disorders (DSM). ADHD is described as "a persistent pattern of inattention and/or hyperactivity-impulsivity that interferes with functioning or development"[9].
Clinical tests along with questionnaires are recommended[10]. However, no biological markers have been accepted as diagnostic for ADHD yet.
To date, understanding of the aetiology is not definite and diagnostic methods in particular for children are often vague. In recent years, oxidative stress (OS) in the pathogenesis of ADHD gained interest.
OS is defined as a shift in the biological balance of oxidant production and antioxidants, towards an oxidant excess. OS is utilised in redox regulation, in either redox-signalling or redox-control. Redox regulations involve pro-oxidants (free-radicals species and non-radical species), antioxidants (enzymes and low-molecular mass compounds) and molecular targets of great diversity.

SRC 2018, November 9, 2018, The Netherlands.

In living organisms, all redox regulations are tightly controlled on an individual and a network level. This tight control creates a steady state, the redox homeostasis.[11]
The redox homeostasis is defined by the Redox Code[12] and describes, how redox regulations are balanced.
While the brain is a major oxygen metabolizer, depending on constant oxygen supply, it has only limited protective mechanisms against OS[13]. The high proportions of peroxidizable PUFAs and iron levels, present in the brain matter, are an additional risk. The brain is therefore more susceptible to damage due to OS than other organs[14].
Studies on biomarkers of OS in ADHD do help to gain better insights into ADHD's pathogenesis and hold promising diagnostic values.
Studies on the effects of antioxidant based treatments, suggest that OS is implemented in the ADHD pathogenesis. Dietary studies in children with ADHD support the key-role of OS in ADHD. Exogenous antioxidants and foods balancing out the oxidative dyshomeostasis appear to successfully alleviate symptoms[4-6]. If symptoms are altered through manipulations of a patient's oxidative homeostasis, neurochemical imbalances such as synaptic spill overs or hyper-catecholaminergic states might theoretically be altered accordingly. Recently, Hellmer and Nyström found that neurochemical imbalances are detectable prior to ADHD manifestations[15]. It is therefore of great importance to gain better understanding of the development of these neurochemical imbalances. By correcting or preventing a dysbalanced neurochemistry, children would potentially not have to experience ADHD symptoms and be effected by such throughout their life time.
Of great interest are antioxidant concentrations and status in children with ADHD, as these might provide approachable therapy targets.

Gluthatione (GSH)

GSH is an important intracellular antioxidant, known to protect proteins and cell membranes from oxidative damage. Two GSH can be oxidised to glutathione disulphide (GSSG). In normal cell oxidation metabolism, GSH outnumbers GSSG[16]. GSSG has cytotoxic potential[17] and might react with protein thiols resulting in the production of a protein mixed disulphide (PSSG) during oxidative stress[18]. In plasma of children with ADHD, Ceylan et al. found low levels of GSH-Peroxidase[19], whereas Simsek et al. did not find different concentrations in comparison to control[8]. The findings
of Ceylan et al. might present a shift in the homeostasis of GSH and GSSG away from the antioxidant protection of GSH. Similar findings were concluded from animal studies[20,21]. However, such animal studies should be

interpreted carefully because of limited comparability with human studies. The ratio of GSH to GSSG can be indicative of oxidative dyshomeostasis. Dvorakova et al. found GSH/GSSG to be decreased in paediatric ADHD patients and saw increases in this ratio after treatment with an antioxidant-modulatory pine-bark extract called Pycnogenol®[2,22].

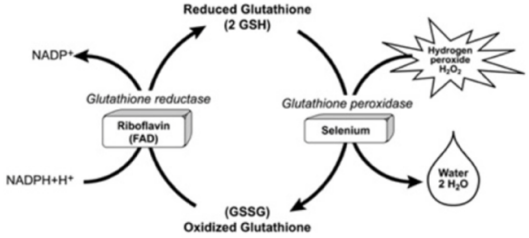

Figure 1. GSH/GSSG metabolism

Lipid-soluble antioxidants

Certain vitamins are known for neuroprotective effects due to antioxidant properties. This idea is supported by findings in a rat-study[23]. A small number of vitamins were chosen as biomarkers to investigate OS in ADHD to date. In paediatric cohorts, only vitamin A and E have been studied yet. Studies on OS in adult ADHD cohorts studied different vitamins and found increased concentrations of folate[24] and ascorbate[25], but no differences for vitamin B6[25] and B12[24] These vitamin concentrations need to be investigated in children yet. Vitamin E (α- and γ-tocopherol) is known to reduce lipid hydroxyl radicals and lipid peroxides, originating from polyunsaturated fatty acids (PUFAs). PUFAs are a dominant constituent of the brain. Vitamin E is also linked to GSH via vitamin C reduction and storage[17]. Vitamin E concentrations were found to be increased in paediatric ADHD patients[7].

Besides vitamin E, vitamin A (β-carotene, retinol and retinylpalmitate) has also shown some neuroprotective effect[23]. Vitamin A however did not present abnormal concentrations in the yet only paediatric ADHD patient-cohort studied[7]. Co-enzyme Q10 (CoQ10) is a lipophilic antioxidant with potentially OS-combating capacities[26].

METHODS

For a trial run by the NaTuRA research group at the University of Antwerp, the effect of pycogenol on ADHD in comparison to methylphenidate or placebo were investigated in a randomized, double-blinded, three-armed set-up. Blood, urine and stool samples from children with ADHD were collected at the University hospital Antwerp from all treatment-cohorts at both baseline and after ten weeks of treatment.[27]

A small number of baseline samples was selected randomly for the here reported research. Using High Pressure Liquid Chromatography (HPLC), the concentrations of lipid-soluble antioxidants in 24 plasma samples and GSH concentrations in four red blood cells (RBC) samples were determined.

Analysis of lipid-soluble antioxidant concentrations

Plasma concentrations for the following lipid-soluble antioxidant were determined: retinol, α-tocopherol, γ-tocopherol, retinylpalmitate, β-carotene and CoQ10. After extracting these lipid-soluble compounds from the plasma samples with hexane, Reversed-phase High Pressure Liquid Chromatography (RP-HPLC) combined with electrochemical detectors were utilized to create a chromatogram. The antioxidants, were separated during RP-HPLC while migrating through an ODS Hypersil (150 x 3 mm; 3 µm) column. A solution of methanol:milliQ:1M ammonium acetate (90:8:2) and a solution of methanol:1-propanol:1M ammonium acetate (78:20:2) were used as mobile phases A and B respectively. Hereby a separation based on solubility was yielded. For each of the twelve children, a mean was calculated from the respective duplo measurements. A mean for the ADHD cohort was calculated in µg/ml and µmol/L.

Analysis of GSH concentrations

GSH concentrations were determined using RBCs. Following precipitation and centrifugation, normal-phase HPLC combined with electrochemical detectors created a chromatogram. The detection cell potentials were 500, 580, 660, 740, 820, 900, 980, 1060 and mV. For the extraction of GSH during HPLC, two mobile phases were utilized. Mobile phase A was a solution of 25 mM NaH2PO4.2H2O, 1.4 mM OSA and 3% methanol. Mobile phase B was a solution of 25 mM NaH2PO4.2H2O, 1.4 mM Octanesulfonic acid (OSA) and 50% methanol. A RP-18 endcapped column (250 x 4.8 mm; 5 µm) was utilized. The mean GSH concentration in µmol/L at baseline was calculated.

Comperative analysis

No standard reference concentrations for either the lipid soluble antioxidants or GSH are established. The trial design does not include a N-ADHD control. The resulting concentrations were therefore compared to mean concentrations of a non-ADHD cohort of a previous study at NatuRA. In the previous study, concentrations of retinol, α-tocopherol, γ-tocopherol, β-carotene, CoQ10 and GSH were measured in equivalent manners in a N-ADHD cohort selected from the same population. No comparison was possible for retinylpalmitate. IBM SPPS 25 Statistics and Excel were utilized to assist the comparison of concentrations via descriptive statistics. Relative differences between mean-concentrations were assessed. As the ADHD cohort is comparatively smaller than the N-ADHD cohort, Mann-Whitney testing was selected to test for significant differences between the concentrations. As the Mann-Whitney test implies the assumption that the concentrations of the two cohorts are distributed equally, homogeneity of variance was tested. Additionally, these outcomes were compared to literature.

RESULTS

ADHD vs. N-ADHD mean concentrations

The mean concentrations of the individual antioxidants were compared by means of relative differences (%), given in table 1 and discussed below.

The mean concentration of four lipid-soluble antioxidants (α-tocopherol, γ-tocopherol, β-carotene, CoQ10) appear to be increased in the ADHD-samples in comparison to the N-ADHD control. The mean concentrations for retinol differed only slightly. The mean concentration of GSH for the ADHD cohort at baseline did not differ significantly from the N-ADHD cohort.

Outcomes of Mann-Whitney test

The differences between the medians of all but glutathione are significant in that the respective significances (p) are smaller than 0.001. For glutathione, the concentration of the ADHD cohort (median 2.350) did not differ

biomarker	N_{ADHD}	$N_{N\text{-}ADHD}$	mean ADHD ± SD (µmol/L)	mean N-ADHD ± SD (µmol/L)	Δ mean ± SD (µmol/L)	Δ %	median ADHD (µmol/L)	median N-ADHD (µmol/L)	U	z	p	r
α-tocopherol	12	29	20.996 ± 3.028	4.066 ± 1.554	16.930 ± 4.224	81	21.052	3.850	0.000	-4.986	0.000	0.779
γ-tocopherol	12	28	1.269 ± 0.376	0.181 ± 0.096	1.088 ± 0.388	86	1.223	0.154	0.000	-4.958	0.000	-0.784
retinylpalmitate	12	0	0.161 ± 0.326	-	-	-	-	-	-	-	-	-
retinol	12	29	1.079 ± 0.133	0.795 ± 0.172	0.284 ± 0.217	26	1.051	0.758	55.000	-3.410	0.000	-0.526
β-carotene	12	28	1.002 ± 0.348	0.111 ± 0.069	0.891 ± 0.355	89	1.078	0.110	1.000	-4.929	0.000	-0.780
CoQ10	12	25	0.819 ± 0.190	0.077 ± 0.040	0.742 ± 0.194	91	0.878	0.066	0.000	-4.867	0.000	-0.800
biomarker	N_{ADHD}	$N_{N\text{-}ADHD}$	**mean ADHD ± SD (mmol/L)**	**mean N-ADHD ± SD (mmol/L)**	**Δ mean ± SD (mmol/L)**	**Δ %**	**median ADHD (mmol/L)**	**median N-ADHD (mmol/L)**	**U**	**z**	**p**	**r**
glutathione	4	53	2.32 ± 0.59	2.11 ± 0.29	0.210 ± 0.660	9.1	2.350	2.070	73.000	-1.031	0.323	-0.137

Table 1. *Sample sizes (N), mean concentrations and standard deviation (SD) for both the ADHD and N-ADHD cohort as well as the relative differences (Δ%) between the two cohorts for each of the measured antioxidant. Medians of concentrations of both cohorts for each measured antioxidant. Mann-Whitney test results: test statistic (U) with corresponding z-score (z), effect size (r) and significance.*

significantly from the N-ADHD cohort (median 2.070), U = 73.000, z = -1.03, p = 0.323, r = - 0.137.

Significances of the Levene statistics indicate that the concentrations of the two cohorts are not distributed equally for retinol F(1,21.126) = 0.466, p = 0.502 and glutathione F(1,35.615) = 1.729, p = 0.197.

The concentrations of all other antioxidants are distributed equally according to the Leven test:

α-tocopherol F(1,19.753) = 12.015, p = 0.002,
γ-tocopherol F(1,17.900) = 26.502, p = 0.000,
β-carotene F(1,12.286) = 12.105, p = 0.004 and
CoQ10 F(1,12.681) = 19.152, p = 0.001

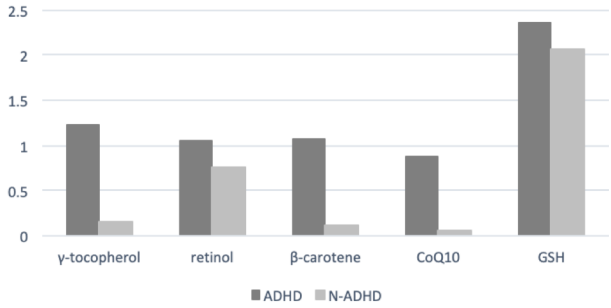

Figure 2. Medians of concentrations of *γ*-tocopherol, retinol, *β*-carotene, CoQ10 and GSH

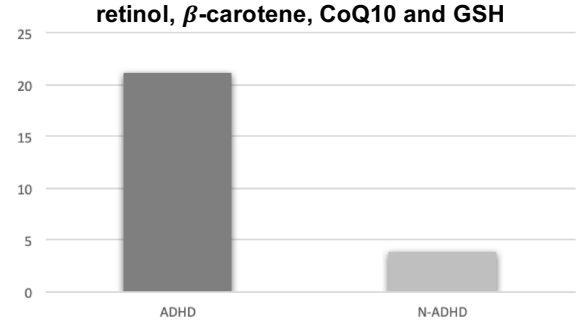

Figure 3. Medians of concentrations of *α*-tocopherol

Differences between the concentrations of the two cohorts compared to literature

Spahis et al. found concentrations of α-tocopherol and γ-tocopherol to be increased in their ADHD-cohort, as well[7]. They did not see differences in their retinol concentrations though[7]. Contrary to the above-mentioned outcomes, Simsek et al. found no significant difference in CoQ10 concentrations in their study[8] and Spahis et al. found no differences in β-carotene concentrations[7].

In their study on the effects of Pycnogenol® on OS in ADHD children, Dvořáková et al. found that GSH concentrations increase with Pycnogenol® treatment, while GSSG concentrations decrease[2]. No indications were made, whether GSH concentrations were elevated at baseline. To date, no studies on GSH concentrations in ADHD children compared to neurotypical children have been published.

DISCUSSION

Differences in concentrations of some compounds of interest were found. This might indicate a shift in oxidative homeostasis, as proposed by various studies investigating OS in ADHD, to be present in ADHD patients. However due to certain limitations, the above given results should be interpreted with care.

While a Mann-Whitney tests assumes that the data of two cohorts is distributed in an equivalent manner, this is not the case for glutathione. The respective p-value should therefore not be taken into account, when comparing the medians of glutathione of the two cohorts.

Biomarkers need to be specific. The here investigated lipid-soluble antioxidants are not brain or ADHD specific. Elevated concentrations might point to increased OS occurrence though. OS in this case needs to be generalized unless proven ADHD-specific by additional biomarkers.

While the GSH/GSSG ratio might be indicative of an oxidative dyshomeostasis, GSH by itself cannot be. Therefore, correlations between GSH and the vitamin E might be interesting to explore.

The here discussed compounds might not be diagnostic biomarkers for ADHD in children. The concentrations of these compounds in comparison to neurotypical controls, however, could be of value in understanding the ADHD pathophysiology. As proposed by Kul et al., antioxidants might only insufficiently combat OS in children with ADHD[28] Alternative treatments such as Pycogenol® or dietary regulation, increase antioxidant concentrations and alleviate ADHD symptoms. This underlines the importance of antioxidants in ADHD treatment.

Limitations

The number of samples analysed and discussed in this bachelor thesis is small. Therefore, the results lack power and might be prone to clustering illusion and/or insensitivity to sample size. As the control concentrations were obtained as part of a separate study, one cannot be sure that the ADHD cohort and control cohort are comparable. This might bias the outcomes. To date, only two studies have been looking into the here discussed biomarkers in children. Two studies have been investigating GSH concentrations in ADHD-animal models with contrary outcomes[20,21]. The comparability of animal and human studies in neurodevelopmental disorders is limited though. While retinol, α-tocopherol, γ-tocopherol, β-carotene, CoQ10 and GSH are known to have neuroprotective effects, they also play important roles in other physiological functions.

Plasma samples or RBC samples are therefore representative of the overall oxidative balance, not limited to the brain. In particular, in paediatric ADHD patients a distinction between peripheral OS and brain centred OS is of importance due to hyperkinesis[29,30].

CONCLUSION

Most of the antioxidant concentrations investigated, appear to be elevated in the ADHD cohort. Due to the limitations discussed above, this observation should be tested for reproducibility by future studies including controls. The antioxidants discussed here, are unlikely to become diagnostic biomarkers of ADHD. GSH included in the GSH/GSSG ratio could, however, play an important role as biomarker for OS. The importance of oxidative balance and antioxidants in ADHD is highlighted by the findings of the literature study and ascertained by the outcomes of the investigations.

ROLE OF THE STUDENT

Ines Kathrin Weyand worked on this research as part of her bachelor thesis, which was supervised by Dr. Harry Robberecht. The experiments were conducted under supervision of Annelies Breynaert. The HPLC measurements of the ADHD-cohort were part of the doctoral thesis of Annelies Verlaet. The HPLC measurements of the N-ADHD-cohort were conducted as part of a preliminary Master thesis of Veronique Loos. The design of the research question, data analyses and interpretation were done by Ines Kathrin Weyand.

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