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Title

Oxidative stress and Memory decline in adults with Down Syndrome: longitudinal study

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Abbreviations: ABAS, adaptive behaviour assessment scale; BPVS, British picture vocabulary scale; DLD, Dementia Questionnaire for Persons with Learning Disabilities; MOMT, modified object memory task; DS, Down syndrome; AD, Alzheimer's Disease; ID, intellectual disability; GPx, glutathione peroxidase; SOD1, Cu/Zn superoxide dismutase; SOD2, Mn superoxide dismutase; SOD, superoxide dismutases (i.e. incl. both Mn SOD and Cu/Zn SOD).

Running Title: Oxidative stress and memory in Down Syndrome

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Abstract:

By the age of 40, virtually all patients with DS have neuropathological changes characteristic of AD. The aim of our study was to investigate whether the levels of superoxide dismutase enzymes (SOD), glutathione peroxidase (GPx), or their ratio could predict cognitive decline in people with Down Syndrome (DS) over a 4-year period.

Thirty-two adults with Down syndrome participated in a longitudinal study with SOD and GPx assays at baseline. Informants rated their functional ability and memory function at baseline and at 4 years follow-up. The more able adults with DS also completed assessments of language skills and memory, at two different time points 4 years apart.

Twenty-six individuals with DS completed assessments of memory (Modified Memory Object Task, MOMT), Adaptive behaviour (ABAS) and receptive vocabulary (British Picture vocabulary, BPVS) at both time-points. SOD positively correlated with change on the MOMT score ($r = 0.578$, $p = 0.015$). There were no significant correlations between GPx level or SOD/GPx ratio and temporal changes in ABAS, BPVS or MOMT scores.

Our results suggest that SOD predicts memory decline over time and that these anti-oxidant enzymes could be a potential target for prevention of memory deterioration in adults with DS. Further research is required to test whether supplements which improve SOD function can also prevent cognitive decline. These findings may also have implications for prevention of cognitive decline in

other groups which are at high risk of developing dementia, such as adults with familial AD or mild cognitive impairment.

244 words

Key words:

Down Syndrome, aging, Alzheimer Disease, memory, superoxide dismutase, oxidative stress.

1. Introduction

Oxidative stress represents an imbalance between the production and manifestation of reactive oxygen species. In oxidative stress, the human body lacks the ability to readily detoxify the reactive intermediates and to successfully repair the resulting damage. Oxidative stress has been implicated in many diseases. Among these, the role of oxidative stress in the pathogenesis of both main forms of dementia, vascular and Alzheimer's Disease (AD), is well documented [1,2]. In people with Down Syndrome (DS), the most common genetic cause of intellectual disability (ID), the prevalence of AD increases significantly with age. By the age of 40, virtually all patients with DS have neuropathological and neuro-imaging changes characteristic of AD [3, 4].

Superoxide dismutases are an important antioxidant defense in human cells exposed to oxygen, and consist of three forms: SOD1 is located in the cytoplasm, SOD2 in the mitochondria, and SOD3 is extracellular. SOD1 and SOD3 contain copper and zinc, whereas SOD2 has manganese in its reactive centre. The genes for the enzymes are located on chromosomes 21, 6, and 4, respectively. As DS is caused by the partial or complete triplication of chromosome 21 and Cu/Zn superoxide dismutase (SOD1) is encoded by genes on the same chromosome, people with DS may present with SOD over expression. The role of SOD is to convert the superoxide radical to cytoxic hydrogen peroxide, which is further detoxified by glutathione peroxidase (GPx) or catalase. As a result of the over expression of SOD1 (a dosage-sensitive gene), there could be an imbalance of the ratio of anti-oxidant enzymes (SOD, GPx, catalase), which has been proposed to

result in oxidative damage [5]. Contrary to the prevailing hypothesis, Strydom et al. found that low SOD/GPx ratios were associated with worse cognitive ability in young adults with DS prior to the onset of dementia, which remained after controlling for confounders such as sex, age or nutritional supplements [6].

Our aim was to follow up all participants of the original study [6] after a 4-year period and to investigate whether the levels of SOD, GPx and their ratio predicted cognitive ability, particularly memory function, over time.

2. Materials and Methods

2.1. Patient population, ethics and consent procedures

The study was approved by the National NHS research ethics service. We approached all 32 individuals that were recruited at baseline in 2006 to gain consent to participate in the follow-up study. All potential participants received a detailed information pack about the study, which included a specially designed explanatory leaflet for participants with DS. Thereafter, consent was sought from participants with DS and their carers. We followed the Mental Capacity Act, UK (2005) and if participants did not have the capacity to consent for themselves, we gained consent from carers. Assessments took place in the participants' home and were undertaken by the same researcher (PZ).

2.2 SOD1/ GPx measurements

During the original study, blood samples were obtained (before 12 am) for enzyme analysis (SOD and GPx). Blood was couriered to the laboratory at

ambient temperature and was processed on the same day for SOD analysis. Aliquots were frozen on arrival for batched GPx analsis.

SOD was measured using two methods. Firstly, SOD1 activity level was measured using standard spectrophotometric methods – in summary, a cytosolic fraction from a neutrophil suspension was added to rhodamine-110-chloride reagent (Sigma 83695_Fluka) and 0.1% diolein followed by 0.1M HCl and placed in a Hitachi U-2010 spectrophotometer at wavelength 532nm. Readings were taken at 3 min intervals for at least 15 mins until linearity was established. Mean activity was calculted per 3 minutes, with a reference value of 240 to 410 units.

Secondly, as an indication of overall intracellular SOD "function", patient neutrophils were separated on a Histopaque (Sigma Chemical Co) density gradient and diluted with buffered saline to 10^6 cells per ml. Detection of SOD enzyme function was measured by means of rhodamine-110-chloride reagent (Sigma 83695_Fluka) based on how effectively the maximal production of superoxide by toxic stimulation of the neutrophils with diolein can be switched off (reduced) by an extract of a patient's packed cells (which contained SOD1 and SOD2); this has a normal range of 41–47%. Lower results are obtained in patients with poor SOD function.

GPx activity was determined in red blood cells using the method of Paglia and Valentine based on the NADPH coupled reaction [7]. The reference interval for red cell GPx was 67–90 international units of activity per gram of haemoglobin.

2.3. Psychometric assessments

The same psychometric assessments were used at baseline and follow-up four years later . Functional ability was determined with the Adaptive Behaviour Assessment Scale (ABAS), completed by carers [8]. The cognitive subscale of the Dementia Questionnaire for the Persons with Learning Disabilities (DLD) (formerly known as the DMR) [9], was also scored by carers to provide a proxy measure of memory function in all participants, including those adults with DS who were not able to complete assessments. The British Picture Vocabulary Scale II (BPVS II) [10] was used to provide a measure of the approximate developmental level of the participants, and to measure their vocabulary acquisition, an important facet of intelligence [11]. A Modified Object Memory Task (MOMT) was used as a directly assessed measure of delayed short-term memory. It was based on a version of the test which has been shown to yield delayed recall scores that are sensitive to memory decline due to dementia status, but not level of intellectual disability [12]. It was further simplified by reducing the number of objects to 6 objects presented in 2 trials before testing immediate recall followed by delayed recall of all 6 items after 5 minutes.

2.5. Data analysis

Data was coded and analysed with Statistical Package for the Social Sciences (SPSS) version 11[13]. The SOD/GPx activity ration was calculated from the logarithms of the activities because of the different orders of magnitude [14]. Correlations between the changes in psychometric and functional assessments scores (BPVS, ABAS, MOMT) were examined using Spearman's correlations. Mann Whitney U tests were used to compare scores between groups. The significance level was set at 0.05.

3. Results

3.1. Demographics

Twenty-six out of 32 (81,3%) individuals with DS were followed up, ranging from 23 to 50 years of age (mean 36.65 ± 7.05) at the time of their follow-up assessments. Two (6.3%) of the original participants refused to participate again, and four (12.5%) were lost to follow-up.

Table 1 summarizes the demographic details the 26 participants who were followed up, and the 32 patients of the original cohort. No significant baseline differences were noticed between the original cohort and the follow-up group, i.e. no bias was introduced by the loss of 6 participants.

[Table 1]

Participants with thyroid dysfunction were all receiving treatment, and none had clinical features of thyroid disorder. Those with epilepsy were diagnosed during childhood, and were stable on treatment. None of the participants had any other major neurological disorders. Hearing and vision deficits were not severe and did not prevent participation in any of the psychometric tests.

3.2 Psychometric assessments

Four of the 26 participants had severe ID and were unable to complete the BPVS II because they had very limited or no speech. Out of a possible 168, the raw score ranged between 26 and 115 with a mean score of 55.68 ± 21.19, and scores were approximately normally distributed. The equivalent developmental age

range of the participants was 3.00 to 12.58 years of age. Those rated to have a mild intellectual disability (ID) had a mean developmental age of 7.45 years and those with moderate ID, 4.63 years.

None of the four participants with severe ID were able to complete the modified memory object task (MOMT); range 2-6, mean 4.82 (± 1.05). However, this test demonstrated good correlation with carer ratings obtained from the DLD cognitive score (Spearman's Rho = -0.552, p=0.008), which allowed carer-ratings of memory functioning for all participants. All participants had a DLD cognitive score, ranging from 0-38, mean 11.88 ± 11.93, and scores were not normally distributed.

All participants had ABAS scores (excluding work skills, since none of them was employed), which ranged from 31 to 565 (mean 317.85 ± 133.48) with an approximate normal distribution.

Changes in MOMT scores (score at follow up minus score at baseline) did not show a significant relationship with age (Spearman's Rho -0.095, p=0.683), and there was no statistical difference (at the 0.05 level) between men and women, or between those taking any vitamin tablets and/or food supplements and those who were not (table 1).

3.3. Enzyme assays

SOD1 activity level measurements (obtained at baseline) was available for 18 subjects with follow-up data and ranged between 30 and 590 enzyme units (mean 324.28 ± 167.13), compared to a normal range of 240 to 410. Functional

SOD studies completed at baseline were available for 20 of the participants who completed cognitive assessments four years later. They showed a range of 32% to 54% inhibition (mean 40.70 ± 6.28). 11 participants (55%) had values lower than 41%, which indicates poor functioning of SOD.

Overall SOD function and SOD1 enzyme activity were highly correlated ($r=0.791$, $p<0.001$). Since there were more participants with SOD functional studies, this was used rather than SOD1 enzyme activity in primary analyses. SOD function did not correlate with age ($r = -0.053$; $p = 0.824$) and no significant difference was found between the 13 males and the 13 females ($p = 0.322$), or between those taking nutritional supplements ($n = 8$) or not $n = 17$; $p = 0.210$).

With normal values between 67 and 90 units/g Hb, the observed GPx levels at baseline of the 20 participants who had blood test results and completed cognitive assessments at both time points were between 39 and 87, mean 65.65 ± 15.14.

3.4 Relationship between enzymes and cognitive ability changes

Table 2 lists the correlations between changes over 4 years on tests of functional ability, memory or language ability (ABAS, MOMT, and BPVS) and levels of GPx, activity of SOD1 and SOD/GPx ratio. Overall SOD function (%) was positively correlated with change in the MOMT score (Spearman's Rho = 0.578, $p =0.015$) - better SOD function was correlated with better memory functioning over time. There was also a trend for a correlation between SOD function and change in the BPVS score (Spearman's Rho = 0.413, $p =0.088$). We proceeded with a post-hoc analysis to determine if those with SOD function below the reference range (i.e. <

41%) were more likely to have memory decline than those with SOD in the normal range (41 % and above). There was a trend towards statistical significance (Mann-Whitney U = 16.5; p = 0.053). There were no significant correlations between GPx level or SOD/GPx ratio and temporal changes in ABAS, BPVS or MOMT scores.

[Table 2]

4. Discussion

4.1. Findings

Our main finding is that a decline in memory performance over 4 years in adults with DS was positively correlated with SOD function measured at baseline. This means that the better SOD enzymes are functioning, the better the memory performance is over time.

4.2. Implications

In a recent meta-analysis it was shown that numerous chromosome 21 genes, including SOD1, have significant dosage effects (15), but the effect of dosage imbalance in people with DS at the molecular and phenotypical level remains unclear despite ongoing efforts to understand the genetics of DS.

We have previously shown that the oxidative system is associated with cognitive functioning in adults with DS [6]. Our current study showed that better SOD functioning predicts better memory performance in aging adults with DS. However, although some adults with DS in our study had increased SOD1 enzyme activity levels, a significant proportion had low levels, demonstrating

considerable variation in phenotype. Furthermore, overall SOD function was below the reference range in more than half of our participants. This is in contrast to the general expectation of increased SOD activity and function in DS. Several previous studies, particularly those in children, have shown increased SOD levels in DS [16], although other studies have shown normal or reduced levels [17]. Our results suggest that SOD expression varies considerably in adults with DS, and that those individuals with low levels of SOD are more susceptible to develop cognitive decline. The variation in SOD function in our study may be influenced by compensatory adjustments of SOD2 in response to SOD1 activity, but this is unlikely as SOD1 activity correlated well with overall SOD function suggesting that most of the variation was related to SOD1. SOD1 variation may be due to allelic variation in the gene on chromosome 21, its interaction with other genes in the rest of the genome, and the environment.

There are several experimental studies which suggests the mechanisms underlying our findings. In a key recent study, researchers crossed TG2576 mice, which expresses the human amyloid precursor protein (hAPP), with mice deficient in SOD1 to generate a mouse model expressing amyloid precursor protein while underexpressing superoxide dismutase (hAPP/SOD1--) [18]. Using control mice expressing APP but which had either partial or full superoxide dismutase expression (hAPP/SOD1+- or hAPP/SOD1++), it was shown that SOD1 deletion accelerated learning and memory deficits, which was associated with increased amyloid plaque formation and increased oxidative damage to protein or DNA in brain tissues. SOD1 deletion also accelerated soluble amyloid β formation particularly in older hAPP/SOD1-- mice, and caused neuronal

dysfunction and increased Tau phosphorylation. Further support comes from cellular studies, including a study which has shown that introduction of SOD1 to cultured neurons reduced Amyloid-Beta induced toxicity [19].

At least one study has shown that dementia in adults with DS is associated with lower levels of SOD1 [20]. Moreover, levels of SOD1 were significantly decreased in postmortem brain samples of human Alzheimer's disease patients compared to age-matched individuals without Alzheimer's disease [18]. In another human post-mortem study levels of oxidative markers including SOD1 was significantly correlated with mini mental status examination scores in the frontal cortex of individuals with mild cognitive impairment or Alzheimer's disease [2].

In summary, these studies suggest that high levels of superoxide dismutase may protect against some of the effects of APP triplication in Down's syndrome. This contrasts with the oft-quoted hypothesis that SOD1 overexpression in DS causes oxidative stress which excacerbates development of Alzheimer's pathology. The Down Syndrome phenotype varies considerably on an individual level, and our study is the first to show that there is a direct relationship between peripheral levels of superoxide dismutase in living individuals and their memory function over time.

There are several implications of our findings. Firstly, although the exact relationship of peripheral SOD levels to central nervous system activity of SOD is unknown, our study suggests that peripheral superoxide dismutase measurement is a potential biomarker for cognitive decline in DS. This needs to

be explored in larger scale studies. Secondly, it suggests that improvement in SOD function may be a potential treatment option to prevent or reduce cognitive decline in people with DS. Several substances have been found to upregulate or improve SOD function. These include resveratrol [3,5,4V-trihydroxystilbene], a naturally occurring phytoalexin present in high concentration in skin of grapes and in red wine, which upregulates SOD1 and other anti-oxidant enzymes [21].

Trans resveratrol (T-RES) has been shown to be effective in preventing cognitive deficits as well as oxidative stress in rats [22]. In addition to this, there is also evidence that RES prevents Aβ oligomerization and attenuates cognitive deterioration in a mouse model of Alzheimer's Disease [23]. Moreover, RES, as a Sirtuin 1 (SIRT1) activator, can protect from the deleterious effects triggered by oxidative stress, α-synuclein or amyloid-β peptide [24]. Therefore, resveratrol could possibly play a multiple role in DS in addition to improving the functioning of SOD. Another possible treatment option is statins which, in addition to their beneficial lipid modulation exert a variety of several so-called "pleiotropic" actions. Among these, Rosuvastatin, the most recent agent of the class to be introduced, has been shown to have an upregulation effect on the anti-oxidant enzymes [25].

Although oxidative stress has been suspected to be an important factor in DS, recent trials of anti-oxidants in infants did not result in improvements of cognitive functioning, neither did it prevent cognitive decline in older adults [26, 27]. Our findings suggest that better SOD function protects against memory decline in DS. Improving SOD enzyme functioning in individuals with DS therefore presents a new potential treatment strategy that can be tested in

randomised trials . As DS is a paradigm for Alzheimer's disease, there is also a possibility that SOD has a role in cognitive decline in other high risk groups such as people with familial AD or Mild Cognitive Impairment. This needs to be investigated.

Lastly, the MOMT appears to be a sensitive measure of memory decline in adults with DS. Memory decline has been found to be an early sign of dementia in older adults with DS along with emotional instability and behavioural change, while activities of daily living, which are included in ABAS, can remain relatively intact in early stages of dementia [28].

4.3. Strengths and limitations

We undertook a prospective cohort study, in which we managed to follow up more than 80% of the initial study group over 4 years. We have included participants with the full range of cognitive abilities associated with DS, and our sample is therefore representative of the wide variation in intellectual phenotype as well as of SOD enzyme variation in adults with DS. We established that the MOMT has good validity by demonstrating good correlation between informant rated memory scores (DLD) and memory tests scores (MOMT) completed with participants with sufficient verbal ability.

We were not able to control for all possible confounders. For example, cognitive performance may be influenced by educational exposure, though in the UK all adults with DS have equal access to education. It is also possible that dietary intake of food with anti-oxidant properties varied across participants. In patients with mosaicism, SOD1 expression cannot be predicted. In our follow-up group

we had one case with mosaicism, and therefore we were not able to study the relationship between SOD levels and mosaicism in more detail. The study was powered to detect correlations of moderate strength and it is therefore possible to have missed more subtle relationships. Lastly, age-dependent decline of SOD activity has been reported [29] as well as a possible relationship between SOD and circadian rhythm [30]. Our participants had a narrow age range, and we did not demonstrate a relationship between age and enzyme levels at baseline. As for the circadian rhythm, the blood samples were all collected in the first half of the day.

4.4 . Conclusion

We have shown for the first time that SOD functioning predicts memory functioning in adults with DS over time, which is in keeping with experimental evidence that has demonstrated the deleterious effects of SOD deficiency in the presence of APP overexpression. Our findings suggest that enhancement of superoxide dismutase functioning is a potential target for prevention of memory decline and dementia in adults with DS, and may also have implications for the treatment of other patient groups, such as those with familial AD. However, our sample was relatively small, and our results need to be confirmed with a larger cohort. Potential positive effects of nutritional supplements that improve SOD function could be tested in DS mouse models, in concert with double blinded randomized control trials in humans with DS in order to test whether these supplements help to prevent cognitive decline.

2876 words

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	Column A Follow-up (n=26)	Column B Original (n=32)	p
Male sex (%)	50.0	56.3	0.792
Age as at baseline (years, mean ± SD)	32.46 ± 7.24	32.59 ± 6.78	0.943
Ethnicity			0.884
White (%)	76.9	75.0	
Asian (%)	11.5	9.4	
Black (%)	11.5	15.6	
Hx of cardiovascular disorder (%)	19.2	21.8	0.805
Hx of visual disorder (%)	61.5	53.1	0.520
Hx of hearing disorder (%)	38.4	40.6	0.867
Hx of thyroid disorder (%)	23.1	21.8	0.913
Hx of epileptic disorder (%)	3.8	6.2	0.681
Level of intellectual disability			0.912
Mild (%)	26.9	31.2	
Moderate (%)	57.7	56.3	
Severe (%)	15.4	12.5	

Table 1. Summary of the baseline characteristics of the follow-up (column A) and original (column B) cohort. Non-continuous variables are given as percentages. Continuous variables are presented as mean ± SD. SD, standard deviation; Hx, history.

		SOD function	SOD1 enzyme units	GPx	SOD1 function/ GPx
Change in ABAS score	Spearman's Rho	0.040	-0.229	0.329	-0.248
	p	0.868	0.361	0.157	0.292
	N	20	18	20	20
Change in BPVS score	Spearman's Rho	0.413	0.325	0.227	-0.096
	p	0.088	0.219	0.366	0.704
	N	18	16	18	18
Change in MOMT score	Spearman's Rho	0.578	0.348	0.187	0.148
	p	**0.015***	0.204	0.471	0.570
	N	17	15	17	17

*Significant at 0.05 level

Table 2. Correlations between changes (score at follow up minus score at baseline) in psychometric measures, and anti-oxidant enzymes.

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