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Serum nicotinamide adenine dinucleotide levels through disease course in multiple sclerosis

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ABSTRACT

The levels of the essential pyridine nucleotide, NAD^+ and its reduced form NADH have not been documented in MS patients. We aimed to investigate NAD^+ and NADH levels in serum in patients with different disease stages and forms of MS. NAD^+ and NADH levels were measured in the serum from 209 patients with relapsing remitting MS (RRMS), 136 with secondary progressive MS (SPMS), 51 with primary progressive MS (PPMS), and 99 healthy controls. All patients were in a clinically stable phase. Serum NAD^+ levels declined by at least 50% in patients with MS compared to controls (17.9 ± 3.2 μg/ml; $p=0.0012$). Within the MS sub-groups NAD^+ levels were higher in RRMS (9.9 ± 2.9 μg/ml; $p=0.001$) compared to PPMS (6.3 ± 2.1 μg/ml; $p=0.003$) and SPMS (7.8 ± 2.0 μg/ml; $p=0.005$). A two-fold increase in NADH levels ($p=0.002$) and at least three-fold reduction in the NAD^+/NADH ratio ($p=0.009$) were observed in MS patients compared to controls. Serum NAD^+ and NADH levels are may be associated with disease progression in MS. Given the importance of NAD^+ in the maintenance of normal cellular function, it is likely that this molecule is of therapeutic relevance in MS.

1. Introduction

Nicotinamide adenine dinucleotide (NAD^+) and reduced nicotinamide adenine dinucleotide (NADH) are ubiquitous pyridine nucleotides which are well known to participate in oxidation–reduction reactions during ATP production (Berger et al., 2005). NAD^+ also serves as a cofactor for NAD-dependent glycohydrolases (CD38) involved in intracellular calcium regulation (Berger et al., 2004). NAD^+ serves as an important secondary messenger signaling molecule inducing the continuous release of intracellular calcium to mediate lymphocyte chemotaxis (Partida-Sanchez et al., 2007) or microglia activation (Lior et al., 2008). Recently, NAD^+ has also been shown to act as the sole substrate for the DNA nick

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sensor poly(ADP-ribose) polymerase-1 (PARP-1), and the class III protein lysine deacetylases, the sirtuins (silent information regulators of gene transcription).

On the contrary, NADH is a coenzyme which can stimulate energy production by replenishing depleted cellular stores of ATP. However, when the re-oxidation of NADH is impaired due to reduced complex I activity, the NADH/NAD^+ ratio increases, thus reducing the activity of several NAD^+ dependent dehydrogenase enzymes. Moreover, NADH is also able to promote the formation of H_2O_2 in the presence of iron via Fenton chemistry leading to further oxidative stress formation and NAD^+ depletion (Tretter and Adam-Vizi, 2004). The NAD^+/NADH ratio plays an omnipresent role in regulating the intracellular redox status, and therefore represents a function of the metabolic state (Massudi et al., 2012a; Massudi et al., 2012b). Given the major function of these two nucleotides in maintaining normal cellular homeostasis during inflammation, further studies into the biological roles of NAD^+ and NADH may increase our understanding for the potential role of NAD^+ related therapies in MS (Massudi et al., 2012b).

During chronic CNS inflammation, oxidative stress may potentially play a critical role in the demyelination and neurodegeneration observed in MS (Koch et al., 2006). NAD^+/NADH levels are altered by Th-1 derived cytokines such as IFN-γ, which enhance free radical generation during inflammation, and increase PARP activity and therefore NAD^+ catabolism (Grant and Kapoor, 2003; Grant and Kapoor, 1998; Grant et al., 2000). Persistent activation of PARP, has been shown to stimulate NAD^+ depletion in human neuronal cells (Braidy et al., 2009; Wang et al., 2003). Reduced NAD^+ levels appear to result in a loss of cellular function and metabolism culminating in cell death [16]. Retention of intracellular NAD^+/NADH pools may therefore facilitate PARP dependent nuclear repair and ATP production following an oxidative insult in vulnerable neurons in MS (Penberthy and Tsunoda, 2009). There is also controversial evidence that lowered NAD^+ levels may be associated with systemic effects such as fatigue, a common complaint as MS progresses (Lassmann, 2008).

To our knowledge, there is no information regarding the role of NAD^+ and NADH in MS. As these pyridine nucleotides play critical roles in maintaining cell survival via several mechanisms, it is important to determine changes in the levels of NAD^+ and NADH in the pathogenesis of MS. We report here on a cross-sectional study of changes in serum NAD^+/NADH levels as measured by a well-established spectrophotometric technique in various sub-groups of MS patients.

2. Results

The data presented in Fig. 1 and Table 1 show that the serum NAD^+ levels were significantly lower in patients with MS compared to controls ($p=0.0012$) when corrected for age and gender. Higher levels of NADH (Fig.2, $p=0.002$) and a lower NAD^+/NADH ratio (Fig. 3, $p=0.009$) were observed in MS patients compared to controls. Although higher NAD^+ (including NAD^+ and NADH) levels were observed in males compared with females in the control group and in patients with MS, this did not reach statistical significance (Table 2).

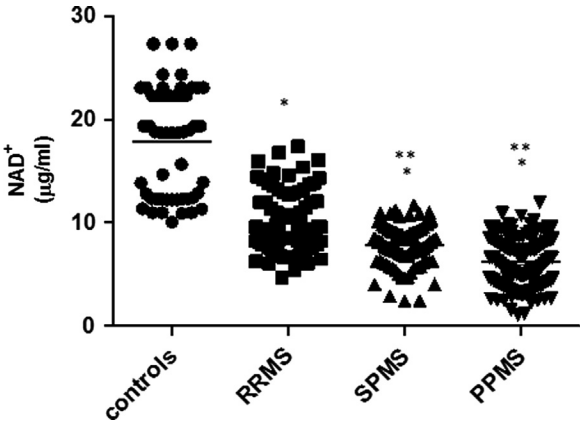

Fig. 1 – Serum NAD^+ levels in different MS disease course and in controls. *Note:-* **PPMS indicates primary progressive multiple sclerosis; SPMS, secondary progressive multiple sclerosis; RRMS, relapse-remitting multiple sclerosis.** $^*p<0.05$ **Compared to serum from healthy controls.** $^{**}p<0.05$ **Compared to serum from RRMS patients.**

NAD^+ levels were significantly different between the MS subgroups ($P=0.0012$), with higher levels in RRMS ($p=0.001$) compared to PPMS ($p=0.003$) and SPMS ($p=0.005$) when corrected for age and gender. Serum NAD^+ levels were not statistically significant between PPMS and SPMS ($p>0.05$). NADH levels and NAD^+/NADH ratio were not significantly different between the MS subgroups ($P>0.05$).

3. Discussion

The present study demonstrates that NAD^+ and NADH levels are significantly altered in MS. While NADH levels and NAD^+/NADH ratio were not significantly different between MS subgroups, NAD^+ levels were markedly lower in PPMS and SPMS compared to RRMS. Altered NAD^+ metabolism has been observed in progressive neuronal cell death both *in vivo* and *in vitro* (Arraki et al., 2004). Below we will discuss how our findings subjectively fit into the current understanding of MS pathogenesis.

One of the major contributing factors towards NAD^+ depletion is chronic oxidative stress leading to reduce cellular viability. Oxidative stress can be mediated by increased reactive oxygen species (ROS) and subsequently worsen when the cellular capability of detoxifying ROS is compromised. ROS produced in MS and experimental autoimmune encephalomyelitis (EAE) – the most common animal model of MS – are thought to be released from activated inflammatory cells and are directly involved in demyelination and axonal damage (Gilgun-Sherki et al., 2004). Increased oxidative stress damages DNA and activates PARP-1, which utilizes NAD^+ as the substrate (Szabo, 2006).

Under mild to moderate ROS mediated DNA damage, activation of PARP-1 can lead to DNA repair and restoration of normal cellular function. Similarly, up regulation of PARP-1 activity has been observed in activated glial cells surrounding demyelinated EAE plaques (Kauppinen et al., 2005). Additionally, Diestel et al. (2003) showed that the levels of 7-ketocholesterol, an important

Table 1 – Demographic characteristics of subjects used in the cross sectional analysis. *Note*: **PPMS indicates primary progressive multiple sclerosis; SPMS, secondary progressive multiple sclerosis; RRMS, relapse-remitting multiple sclerosis; SD, standard deviation.(*ANOVA,**Scheffe's post hoc test for RRMS *vs* SPMS or PPMS).**

	RRMS	SPMS	PPMS	Control	*p* Value*
n	209	136	51	99	
Sex (female/male)	163/36	92/44	34/17	77/22	0.003
Mean age ± SD (Sedlmayr)	43 ± 10.5	52 ± 9.4	52 ± 9.9	46 ± 10.7	0.31**
EDSS at baseline (Mean ± SD)	2.71 ± 1.7	5.18 ± 2.01	4.74 ± 2.14		0.001**
Serum NAD^+ levels (μg/ml) (Mean ± SD)	9.9 ± 2.9	7.8 ± 2.0	6.3 ± 2.1	17.9 ± 3.2	0.0012
Serum NADH levels (μg/ml) (Mean ± SD)	22.7 ± 3.1	24.7 ± 3.5	24.7 ± 2.9	12.2 ± 2.4	0.002
Serum NAD^+/NADH ratio (Mean ± SD)	0.44 ± 0.3	0.32 ± 0.1	0.26 ± 0.9	1.47 ± 0.9	0.009

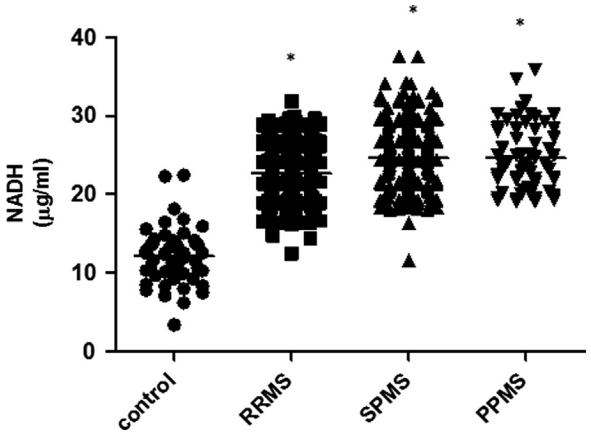

Fig. 2 – Serum NADH levels in different MS disease course and in controls. *Note*: **PPMS indicates primary progressive multiple sclerosis; SPMS, secondary progressive multiple sclerosis; RRMS, relapse-remitting multiple sclerosis.*$p < 0.05$ Compared to serum from healthy controls.**

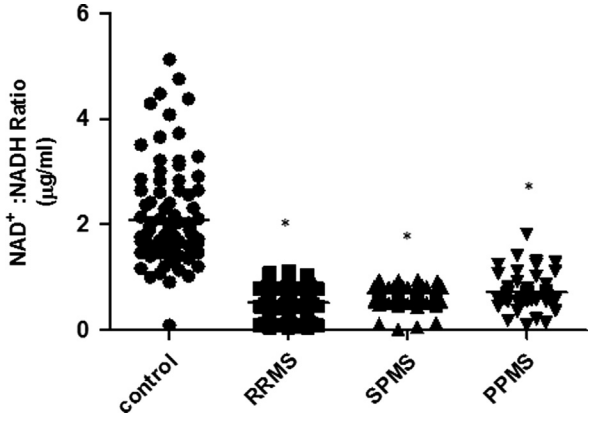

Fig. 3 – Serum NAD^+/NADH ratio in different MS disease course and in controls. *Note*: **PPMS indicates primary progressive multiple sclerosis; SPMS, secondary progressive multiple sclerosis; RRMS, relapse-remitting multiple sclerosis.*$p < 0.05$ Compared to serum from healthy controls.**

lipid breakdown product, is increased in the brain and cerebrospinal fluid (CSF) of MS patients and in EAE mice. 7-Ketocholesterol can rapidly diffuse into neuronal cell nuclei causing DNA damage and activation of PARP-1 (Diestel et al., 2003). ROS-induced hyper activation of PARP-1 can result in NAD^+ and ATP depletion leading to cell death via apoptosis (Bouchard et al., 2003; Burkle, 2005). Neuronal cells are particularly vulnerable to elevated PARP-1 activity, since NAD^+ is an essential cofactor for oxidative phosphorylation and ATP generation (Arraki et al., 2004). Accordingly PARP inhibitors have been used to prevent cytotoxicity in EAE mice (Farez et al. (2009)). If ROS is the key player that indirectly depletes NAD^+, how is ROS generated in MS?

MS is an archetypal inflammatory brain disease associated with the recruitment of activated T-cells and macrophages from the periphery into the neural parenchyma (Lassmann, 2008). These activated immune cells can initiate a number of cytokine-mediated inflammatory responses including the macrophage activating cytokine interferon gamma (IFN-γ). Poor treatment responses with interferon-β have been shown to induce a 4-fold increase in IFN-γ serum mRNA expression (Cucci et al. (2010)). IFN-γ can enhance ROS generation, which can rapidly deplete NAD^+ levels in many cell types, including neuronal and glial cells, as a result of DNA damage and increased PARP activation (Grant et al., 2000). A significant loss in intracellular NAD^+ can subsequently lead to cell death, thus supporting the notion that rapid NAD^+ depletion is detrimental to cell viability.

Mitochondrial dysfunction, which is believed to play a critical role in the pathogenesis of neurodegenerative disorders, also represents a major source of ROS (Heales et al., 1999). In progressive MS, axonal damage may be attributed to impaired axonal energy metabolism (Koch et al., 2006). Dysfunction in the axonal mitochondrial electron transport system can enhance ROS formation (Koch et al., 2006). Reduced electron flow through the mitochondrial respiratory chain, particularly through the inhibition of complex I has been reported by Kumleh et al. (2006) showing similar reduction in complex I activity in a Persian cohort with progressive MS (Kumleh et al., 2006). Given the results of the present study, impaired pyridine nucleotide metabolism may be a consequence of impaired mitochondrial respiratory function, and may, at least in part, be attributed to increased NADH: NAD^+ ratio inducing free radical production. An important observation when examining results of the circulating pyridine nucleotides NAD^+ and NADH is that MS patients are exposed to energy deficits which may be due to impaired mitochondria function (Dutta et al., 2006; Qi et al., 2006; Witte et al., 2009). Under conditions of acute/chronic ROS and NAD depletion, neurons are exceptionally vulnerable to axonal degeneration which is a typical hallmark of MS (Suh et al., 2007). Moreover, it is well established that MS patients are

Table 2 – NAD (NAD$^+$ and NADH) levels in relation to patient characteristics. *Note*: **Analyses are corrected for age and gender. PPMS indicates primary progressive multiple sclerosis; SPMS, secondary progressive multiple sclerosis; RRMS, relapse-remitting multiple sclerosis.**

	Serum NAD$^+$ levels (μg/ml) (Mean ± SD)			Serum NADH levels (μg/ml) (Mean ± SD)			Serum NAD$^+$/NADH ratio (Mean ± SD)		
	Females	Males	*p* Value	Females	Males	*p* Value	Females	Males	*p* Value
RRMS	11.9 ± 3.1	12.5 ± 3.3	0.12	21.9 ± 0.8	22.1 ± 1.2	0.10	0.54 ± 0.1	0.56 ± 0.1	0.51
SPMS	7.7 ± 1.9	7.2 ± 2.2	0.09	23.2 ± 1.5	24.1 ± 0.7	0.21	0.33 ± 0.1	0.30 ± 0.1	0.49
PPMS	6.4 ± 2.1	6.7 ± 2.9	0.25	23.0 ± 1.1	23.6 ± 0.9	0.30	0.28 ± 0.02	0.28 ± 0.01	0.50
Controls	20.1 ± 1.5	21.1 ± 1.3	0.20	11.9 ± 0.7	12.1 ± 0.8	0.42	1.7 ± 0.2	2.1 ± 0.2	0.35

highly vulnerable to inter-current systemic inflammatory or non-inflammatory conditions, particularly during clinical exacerbation of RRMS (Petzold et al., 2004; Rejdak et al., 2007). Therefore, the effect of reduced serum NAD$^+$/NADH ratio on significant CNS and extra-cerebral pathology cannot be overruled.

The loss in NAD$^+$ biosynthesis can be counterbalanced by administering pharmacological doses of NAD$^+$ precursors (Penberthy and Tsunoda, 2009). Addition of NAD$^+$ (5–20 mM) or nicotinamide (5–25 mM) can strongly prevent axotomy mediated axonal degeneration by delaying stress induced depletion of axonal NAD$^+$ and ATP (Arraki et al., 2004). Nicotinamide significantly improved behavioral deficits and axonal loss in EAE mice, most likely through inhibition of PARP-1 activity (Chen et al., 2008). Kaneko et al. (2006) showed that nicotinamide can prevent degeneration of demyelinated axons and attenuate behavioral defects in EAE mice, thus demonstrating a therapeutic potential for nicotinamide as a protective agent for MS (Kaneko et al., 2006). Nicotinic acid appears to be the preferred substrate for NAD$^+$ synthesis when applied in high concentrations, and generates greater levels of NAD$^+$ per mole than nicotinamide or tryptophan by 200 and 500 fold respectively (Grant and Kapoor, 1998). However, unlike glial cells, neurons are unable to efficiently convert dietary NAD$^+$ precursors to NAD$^+$. Moreover, a major adverse effect associated with nicotinic acid is flushing. This may be attributed to its ability to activate GPR109a G-protein coupled receptor responsible for the production of large amounts of prostaglandin PGD2 in antigen presenting cells (APC) such as dendritic cells, macrophages and microglia (Gille et al., 2008). Given that EAE neuropathogenesis can be attenuated by inhibition of PARP-1, or complementation of NAD$^+$ deficiency, combinatorial approaches may prove therapeutically beneficial for the treatment of MS. Collectively, maintaining intracellular NAD$^+$ levels is crucial to prevent ROS production and cytotoxic effect of PARP-1 mediated NAD$^+$ depletion (Fig. 4). By reducing free-radical production, NAD$^+$ precursors may be able to ameliorate MS pathogenesis.

Another well-known NAD$^+$ precursor deriving from a physiological metabolic pathway and also an essential amino acid is tryptophan. Catabolism of tryptophan via kynurenine pathway leads to *de novo* NAD$^+$ synthesis. It is reported that CSF and serum tryptophan levels are reduced in MS patients (Ott et al., 1993) implying possible role for NAD+ generation via tryptophan catabolism. Indeed, the kynurenine pathway may be altered in MS associated with neurodegeneration (Hartai et al., 2005) and ultimately compromises NAD$^+$ production. However this pathway remains to be fully delineated and pharmacological intervention to manipulate this pathway to promote NAD$^+$ anabolism may be a feasible therapeutic approach for MS.

In summary, our results clearly indicate an association between serum NAD$^+$ and NADH levels, and disease progression in MS. Considering the vital role of NAD$^+$ not only for maintaining mitochondria energy production but also genomic integrity, it is highly likely that this pyridine nucleotide may be pathogenically important in MS. The main question as to whether altered NAD$^+$ levels in these disorders is actively and directly associated with the pathogenesis of MS or represents an epiphenomenon of neuronal dysfunction

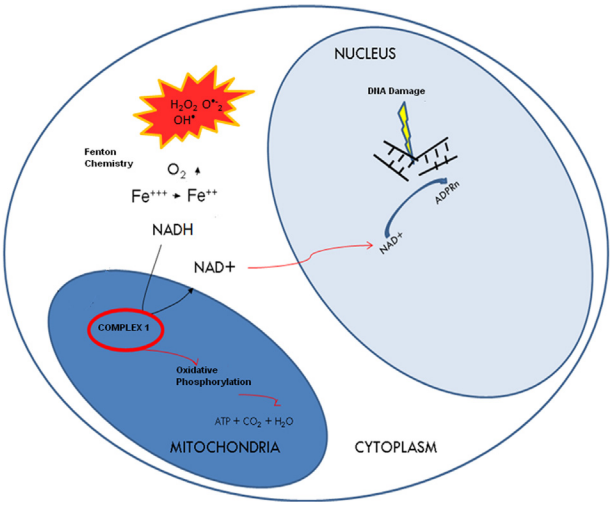

Fig. 4 – Schematic representation for the association between oxidative stress, PARP over activation, mitochondrial dysfunction, and altered NAD^+ content in MS. Our observations MS lead us to suggest the following mechanism for the association between oxidative stress, mitochondrial dysfunction and decline in NAD^+ content in MS. Given the observations in the present study, increased ROS production in MS may, at least in part, be a consequence of impaired mitochondrial respiratory function, resulting in an elevated NADH:NAD^+ ratio thereby inducing more free radical production via Fenton chemistry. Consequently oxidation of DNA will contribute to the potential PARP-mediated decline in NAD^+ levels.

and neurodegeneration remains unclear. The results from this study clearly indicate significant *systemic* energy depletion.

4. Reagents and chemicals

Nicotinamide, bicine, β-nicotinamide adenine dinucleotide reduced form (β-NADH), 3-[-4,5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide (MTT), alcohol dehydrogenase (ADH), and TRIS were obtained from Sigma-Aldrich (Castle-Hill, Australia). Phenazine methosulfate (PMS) was obtained from ICN Biochemicals (Ohio, USA).

5. NAD^+ and NADH measurement

Total serum NAD (NADH+NAD^+) concentration was measured spectrophotometrically using the thiazolyl blue microcycling assay established by Bernofsky and Swan (1973) and adapted for the 96 well plate format by Grant and Kapoor (2003). Briefly, each assay contained 100 mM bicine, pH 7.8; 500 mM ethanol; 0.42 mM MTT, 1.66 mM PMS and 14 units ADH. For NAD^+ measurement, 20 μl of ADH in 0.15% ethanol was added to the reaction mixture. The amounts of NAD and NADH were measured as the change in absorbance at 590 nm at 37 °C for 10 min with a Model 680XR microplate reader (BioRad, Hercules). NAD^+ levels were determined by subtracting the NADH concentration from the total NAD concentration. The ratio of NAD^+/NADH was calculated based on results of NAD^+ and NADH concentrations.

6. Data analysis

Results are presented as the mean ± the standard error of the mean unless otherwise stated. Kruskal–Wallis analysis of variance followed by Dunn's multiple comparison tests were used to determine statistical significance between patient groups. To control for possible confounding factors including age and gender, ANOVA was performed on the rank scores of NAD^+, and NADH levels and clinical measures with Scheffe's post hoc test. Differences between stratified groups were considered significant if p was less than 0.05

7. Experimental procedure

7.1. *Participants*

This study was conducted in accordance with the Helsinki declaration. Approval was obtained from the local ethics committee. The serum samples were obtained from a repository of 733 patients with MS from the Accelerated Cure Project for MS (ACPMS) with 99 control serum from healthy patients. Samples were collected after overnight fasting. All patients gave informed consent and patient anonymity was preserved throughout the study.

The diagnosis of MS had been assessed by an MS research clinic and evaluated based on Kurtzke Expanded Disability Status Score (EDSS) and MRI scans according to the International Panel criteria. All MS patients were classified as per the criteria of Lublin and Reingold, into RRMS, SPMS or PPMS according to the McDonald criteria for definite MS. MS patients with other neurological pathologies, not formally diagnosed as MS, or who had received steroid therapy over the past six months from the date of sample collection were excluded from this study.

Table 1 shows the characteristics of the subject cohort. Relatively more females than males were present in the control, RRMS, SPMS and PPMS groups, although this difference was not statistically significant. As expected, EDSS scores were worse in patients with PPMS and SPMS compared to RRMS. No significant difference was observed between the ages of the patients in the MS group compared with age in the controls. Overall, 495 samples were used in this study based on the above criteria.

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