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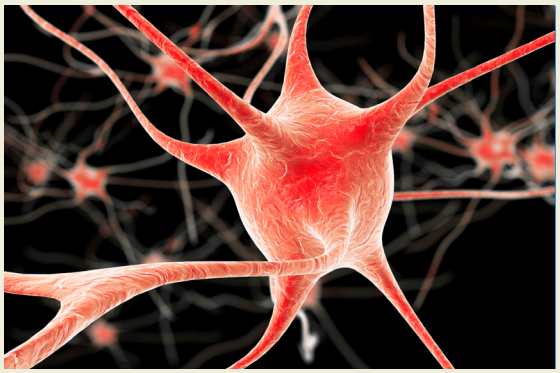

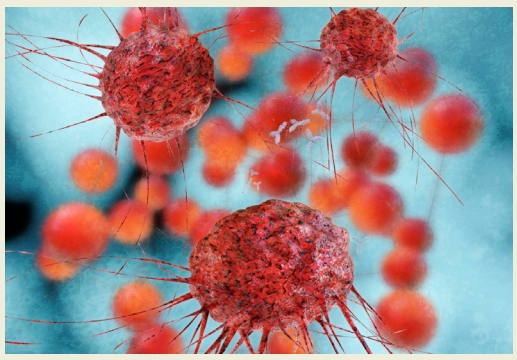

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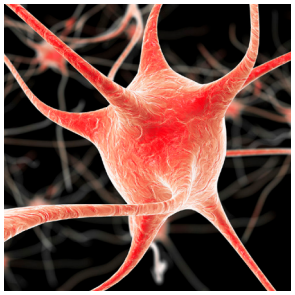

Promoting NAD+ metabolism: A new target for treating degenerative disease

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ABSTRACT:

NAD+ is found in every cell of the body and is essential for life. It serves as a cofactor for dehydrogenase, reductase and hydroxylase enzymes where it facilitates electron transfer in major metabolic pathways such as glycolysis, the triacarboxylic acid (TCA) cycle, fatty acid synthesis and steroid hormone synthesis, enabling the conversion of the food we eat into the energy and chemical products the body needs. More recently it has been found that NAD+ is also required as a substrate by enzymes that regulate the expression of genes involved in cell viability and aging and in repair of damaged DNA. Through these reactions, NAD+ influences a variety of cell processes involved in cell health, including improving mitochondrial efficiency, enhancing cell viability, down-regulating inflammation, increasing the antioxidant capacity of cells and tissues, and activating the 'longevity' enzyme SIRT1.

An increasing body of evidence indicates that enhancing NAD+ availability in the brain has the potential to moderate elements of the neurodegenerative disease processes associated with oxidative stress and aging, including Alzheimer's disease. However there are difficulties associated with raising NAD+ levels using the classical pathway and vitamin B_3 precursors nicotinic acid and nicotinamide. The recent discovery of two alternative naturally occurring B_3 vitamins; nicotinamide mononucleotide (NMN) and nicotinamide riboside (NR) may resolve these problems. NR in particular has shown good efficacy in its ability to raise NAD+ levels under a variety of conditions. Directly boosting [NAD+] may present a new and exciting approach to preventing the natural decline in cellular energy and function as we age, particularly in the brain.

WHAT IS NAD+?

Nicotinamide adenine dinucleotide (NAD+), previously known as dipyridine nucleotide (DPN) is a ubiquitous molecule found in every living cell. The structure of NAD+ was determined by Hans von Euler-Chelpin in the late 1920s. NAD+ contains two nucleotides, adenine and nicotinamide that are joined together through their respective phosphate groups, figure 1[1].

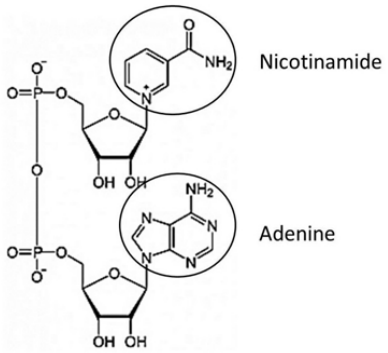

Figure 1 - Nicotinamide Adenine dinucleotide

WHAT DOES NAD+ DO IN THE CELL?

NAD+ is crucial player in a number of critical cellular processes and, along with the closely related nicotinamide adenine dinucleotide phosphate (NADP), is the most abundant cofactor in all eukaryotic cells. A primary function of NAD+,

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identified by Warburg and Christian in 1936, is its ability to act as a hydrogen acceptor facilitating the transfer of electrons in the coordinated series of oxidation-reduction (i.e. redox) reactions culminating in the mitochondrial production of ATP[2]. As ATP is the cellular 'energy currency', a decrease in available NAD+ results in a decrease in ATP production reducing cell viability and, if severe, will result in cell death[3]. As an extension to its electron transfer role in ATP production the NAD+(P)(H) family of cofactors are involved as redox couples in more than 400 enzymatic reactions throughout the body involving dehydrogenases, hydroxylases and reductases.

In addition to these well established roles in energy production and electron transfer a large body of literature has confirmed that oxidised nicotinamide adenine dinucleotide (NAD+) is also the required substrate for important processes involved in DNA repair, epigenetically modulated gene expression, calcium homeostasis and immunological function.

NAD+ AND DNA REPAIR (PARP ACTIVITY)

DNA damage by free radicals or other means (e.g. direct UV damage) occurs tens of thousands of time each day in every cell of the body[4]. A double or single stranded break results in a change to the chromatin structure activating a nuclear repair enzyme called poly(ADP-ribose) polymerase (PARP).

PARP-1 (the dominant member of a super family of 18 PARP proteins) efficiently detects the presence of DNA breaks by its N-terminal zinc-finger domain. The ADP-ribosylation of PARP triggers the recruitment of key proteins that stimulates the repair of the DNA damage in less than 15 s[5]. Importantly, in order for PARP to carry out its ADP-ribosylating function it uses the ADP ribose moiety of NAD+ for its supply. Therefore in the presence of significant DNA damage, cellular NAD+ concentrations can rapidly decline. In conditions of accelerated DNA damage the activity of PARP can be so high that NAD+ levels are critically reduced[6]. The acute consequences of this are a limiting of ATP production and reduced cellular ATP-stores which will result in cell death unless resynthesis or resupply of NAD+ occurs. Importantly, chronic over-activation of PARP at more subtle levels (e.g. chronic age associated oxidative stress) may result in suboptimal levels of NAD+ which may affect other NAD+ dependent processes including immune function and gene transcription.

NAD+ AND CD38

Another major user of NAD+ is the immune associated ecto-enzyme CD38. CD38 is a multifunctional enzyme that requires NAD+ to generate ADP ribose (ADPR) and the second messenger cyclic-ADP-ribose (cADPR) that help regulate intracellular calcium transients[7]. While the function of this ubiquitous enzyme is not completely understood it is known to play a significant role in immune function. The presence of CD38 on T-lymphocytes influences the ability of antigen presenting cells to stimulate antigen specific T-cells[8]. Upregulation of CD38 expression also signals maturation of dendritic cells during inflammatory cytokine activation and acts as a modulating adhesion and signalling molecule between dendritic cells and lymphocytes[9]. Most likely linked to an increase in circulating inflammatory cytokines, CD38 expression has been shown to increase with age[10]. Not surprisingly, decreased CD38 function has been associated with impaired immune responses[9].

CD38 activity also influences behaviour through regulation of oxytocin production[11], an important hormone influencing social engagement. Accordingly the loss or disturbance of CD38 function has been associated with social behaviour disorders in conditions such as autism[12].

Importantly in inflammatory associated conditions, CD38 activation, in addition to increased PARP activity, is also a potential cause of reduced cellular NAD+ levels in multiple tissues.

NAD+ AND SIRTUIN ACTIVITY

In addition to its role in energy production, DNA repair (PARP activity) and immune modulation (CD38), another critical function that is dependent on intracellular [NAD+] is the activity of the silent information regulators of gene transcription, also called the sirtuin family of enzymes[13]. Sirtuins are a family of class

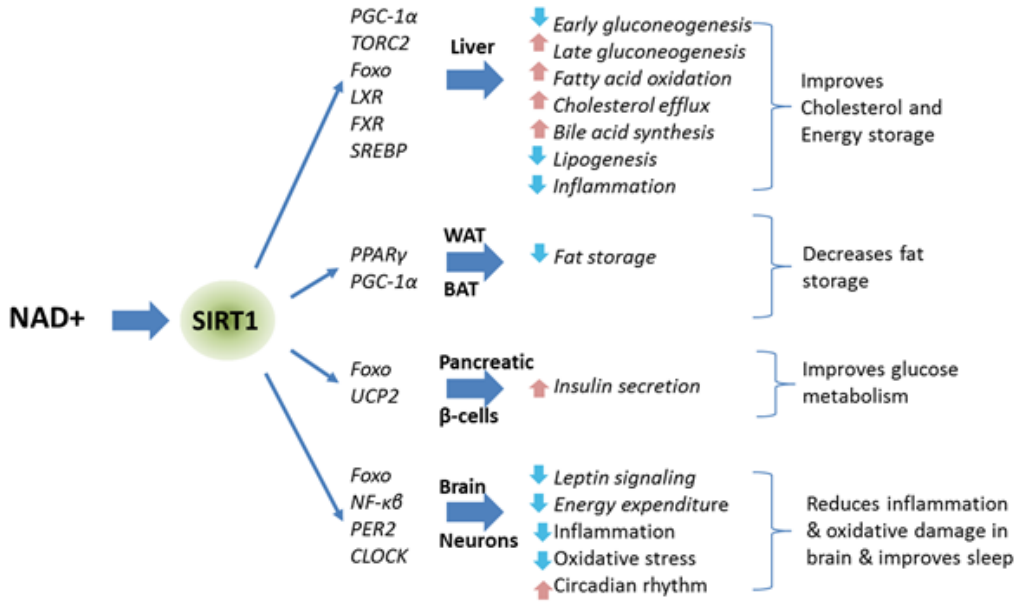

Figure 2 - Benefits of NAD+ stimulated SIRT1 activity

III NAD+ dependent histone deacetylases which catalyse the removal of acetyl groups from the lysine residue, releasing nicotinamide and acetyl-ribose as end products. At least seven classes of sirtuins (SIRT1-7) have been identified, each of which exhibit a wide range of biological functions. SIRT1, SIRT6, and SIRT7 are nuclear proteins involved in the regulation of chromatin structure and gene expression. SIRT2 is localised in the cytoplasm where it mediates gene expression by deacetylating transcription factors which shuttle from the cytoplasm to the nucleus. The remaining members of the sirtuin family (SIRT3, SIRT4 and SIRT5) are found in the mitochondrion[14,15].

As a class, the sirtuins exert a number of effects in the cell including the control of gene expression, cell cycle regulation, apoptosis, DNA repair and metabolic control. SIRT1 in particular has generated considerable interest among researchers as it appears to play a pivotal role in promoting cellular longevity and may hold the key to slowing development of the aging phenotype. Though some evidence suggests SIRT6 may also contribute to an age-resistant phenotype[16].

SIRT1 activity is dependent on NAD+ availability and is increased in response to energy stress, such as fasting[17], exercise[18] or low glucose availability which also serves to increase intracellular NAD+ levels[19].

As shown in figure 2, SIRT1 modulates the acetylation status of a number of important transcription factors, including the metabolic regulator, peroxisome proliferator-activated receptor-Ɣ (PPARƔ), tumour suppressor protein (p53), and the cell growth linked FOXO forkhead family of transcription factors among others, all of which are key metabolic regulators.

In practical terms, the deacetylation function of SIRT-1 appears to have an overall positive impact on tissue function by "turning off" the genes that help drive aging processes such as inflammation, while reducing leptin and insulin resistance reducing kilojoule intake and improving blood glucose control.

Consistent with the positive biochemical effect of SIRT1, Bordone and colleagues (2007) found that transgenic mice over-expressing SIRT1 displayed phenotypes similar to mice on a calorie-restricted diet: including reduced body weight, greater metabolic activity, reduced blood cholesterol, adipokines, insulin and fasted glucose; and were more glucose tolerant[20,21]. Importantly these beneficial, potentially life extending, properties of sirtuins activity are only achieved if NAD+ levels are adequate.

NAD+ (in particular the NAD+/NADH ratio) is recognised as a master regulator of the energy state of the cell. It has been estimated that the total intracellular content of NAD+ is in the range of 0.2-0.5 mM[22,23]. Importantly this is within the estimated Km values of SIRT1 (and other SIRTs) for NAD+. This means that NAD+ availability can rate-limit SIRT1 i.e. low [NAD+], low SIRT1 activity, whereas higher [NAD+] enables SIRT1 to reach maximal activity. Research from our group has observed that reduced levels of NAD+ are linked with significantly reduced sirtuin activity[24,25].

Strong evidence therefore indicates that maintenance of NAD+ levels, particularly under conditions of increased NAD+ catabolism, is essential to the effective realisation of the multiple benefits of healthy sirtuin activity.

WHAT CAUSES NAD+ TO BECOME DEPLETED AND WHAT EFFECT DOES THAT HAVE ON THE CELL?

Excluding problems with NAD+ synthesis, there are principally two conditions under which NAD+ depletion may occur:

1) Excessive DNA damage due to free radical or UV attack, resulting in significant PARP activation and resulting in a high turnover and subsequent depletion of NAD+. This can produce an energy crisis in the cell, due to reduced ATP production resulting in either apoptotic or necrotic cell death[26].

2) A chronic increase in immune activation and inflammatory cytokine production resulting in accelerate CD38 activity can also effectively decrease NAD+ levels[7,27].

Therefore conditions in which there is significant and sustained DNA damage and/or persistent inflammatory cytokine production results in increased NAD+ catabolism through increased PARP and/or CD38 activity resulting in a depletion of NAD+ levels. The practical biological consequence is a decrease in available energy (ATP) production and reduced sirtuin activity.

Though a variety of clinical conditions associated with deteriorating tissue disorders can fulfil these criteria one of the major drivers of NAD+ reduction is the common process of aging.

AGING AND NAD+ LEVELS: POTENTIAL BENEFITS OF RAISING NAD+ LEVELS

Aging is an unavoidable biological progression characterised by a gradual decline in biochemical and physiological function resulting in an increased predisposition to disease. The oxidative stress theory of aging was suggested by Harman in 1956 to account for the accumulation of oxidative damage products observed in human tissue[28]. It is postulated that under conditions of reduced antioxidant capacity or excess production, reactive oxygen species (ROS or free radicals) can cause indiscriminate damage to cellular constituents such as DNA, proteins and lipids that, when left unrepaired, lead to tissue dysfunction and cell death.

Research from our group was the first to show that increasing age, is associated not only with an increase in oxidative damage but also a decline in NAD+ levels. This was found in an animal model to occur in various tissues by as much as 2/3^{rds} , figure 3[24].

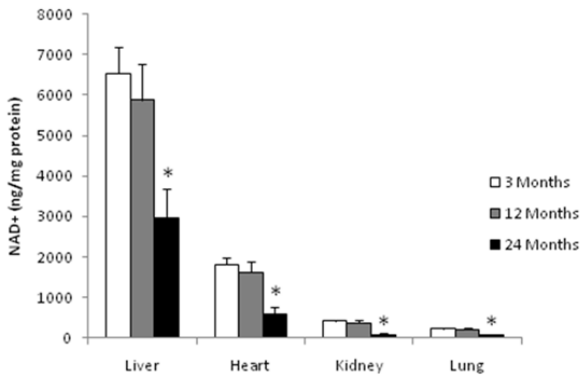

*Figure 3 - Increased oxidative damage and PARP activation decreases [NAD+] with aging. All values are means 6 S.E from tissue obtained from eight different rats for each age group. Significance *p,0.01 compared to 3 month old rats (Braidy et al., 2011).*

Subsequent research by our group confirmed this to also be the case in human tissue. Using human pelvic, non-sun exposed skin from a cohort of participants, aged between8 days to 77 years, we again observed a significant decrease in tissue [NAD(H)] with age and accumulating tissue oxidative damage, figure 4 and 5[25].

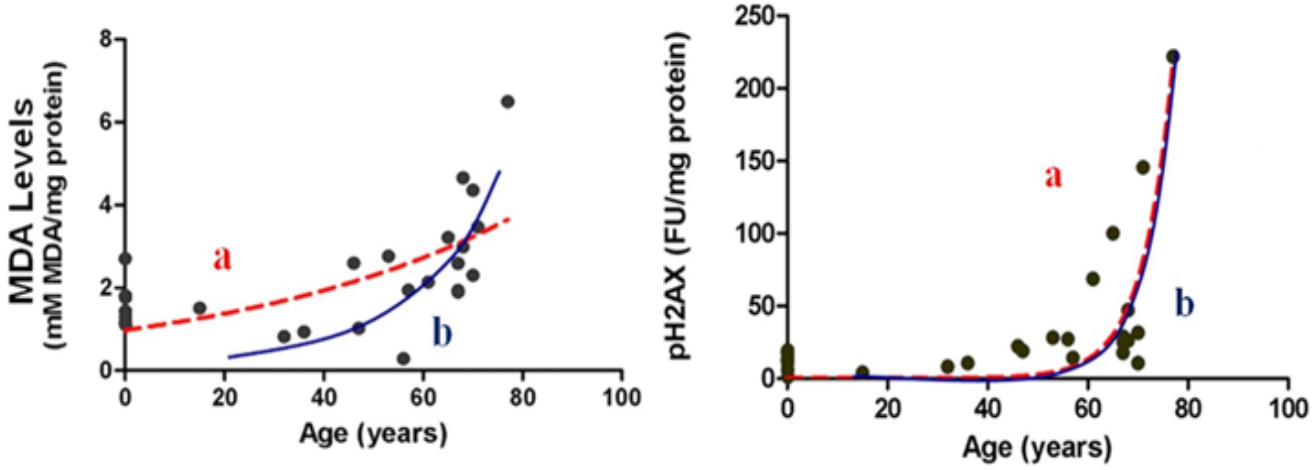

Figure 4 - Lipid peroxidation (MDA) and DNA damage (pH2AX) increases with age (data for males only shown), aged 0–77 years (Line a; represents all participants, line b shows post-pubescent males only (Massudi et al., 2012).

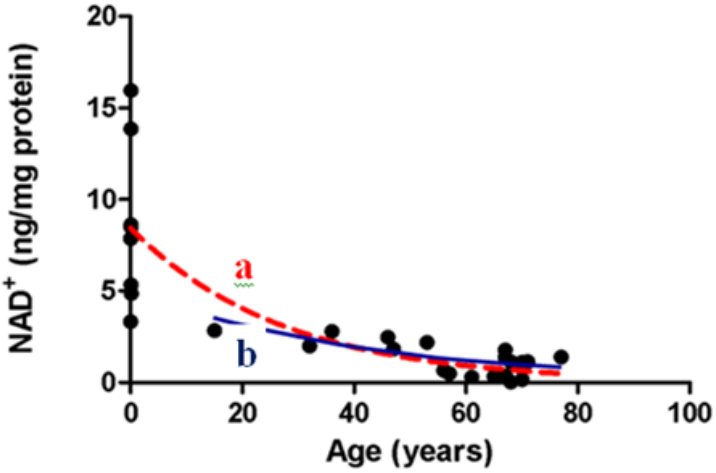

Figure 5 - NAD+ levels decreased significantly between 0–77 years (male data only shown) (line a; p = 0.0007; n = 27). Pearson's correlation coefficient for a normally distributed population, r =20.769. The post-pubescent data for male subjects also showed a decline in NAD+ levels with age (line b;r =20.706; p = 0.0001; n = 19). An exponential (first-order) least squares fit was used to generate the nonlinear trend lines (line a and b) (Massudi et al., 2012).

As outlined above decreased NAD+ levels will cause significant negative effects on cell metabolic activity due to the impaired activity of multiple dependent enzymes including the sirtuins, polymerase(ADP-ribose) polymerases (PARPs) and CD38.This may ultimately culminate in cell death through reduced energy (ATP) production, DNA repair capacity and altered immune modulation.

A number of in vitro studies have now shown that NAD+ administration significantly reduces cell death induced by oxidative damage[29,30]. NAD+ replacement therapy may therefore be a worthwhile target for ameliorating degenerative conditions, especially those associated with aging and accelerated tissue damage.

Of the many age related disorders few are as insidious and debilitating as the group of degenerative disorders affecting the brain and nervous system, in particular dementia.

The brain is particularly vulnerable to oxidative damage as a consequence of its high oxygen demand, high level of both polyunsaturated fatty acids and transition metals, and poor antioxidant defences[31]. As we age, the vulnerability of the brain to oxidative damage increases due to reduced integrity of the blood brain barrier and amplified mitochondrial dysfunction and inflammation[32,33]. It is important to note that neuronal dysfunction proceeds neuronal cell death suggesting that dementia, particularly in the early stages, can be reversed and the progression slowed or even stopped if the right tools can be found to:

1) encourage normal neurochemical function,

2) increase brain cell resilience to inflammatory and oxidative damage and

3) limit or remove the primary drivers of oxidative damage and inflammation.

Importantly enhancing NAD+ metabolism is likely to play a positive role in each of these areas.

NEURODEGENERATIVE DISEASE AND NAD+: POTENTIAL BENEFITS OF RAISING NAD+ LEVELS

The brain is the most metabolically active tissue in the body; while weighing only 2% of the body's mass the brain uses 20% of the available oxygen. This level of oxygen consumption is necessary to maintain vigorous neurochemical activity; it has been estimated that >100,000 chemical reactions occur every second. To sustain this high demand for energy a constant supply of NAD+ is needed. However, as mentioned previously the cells of the brain, particularly the aged brain, are very susceptible to oxidative damage. The resulting increase in PARP (and possibly CD38) activity causes a significant depletion of central nervous system (CNS) NAD+, figure 6 and figure 7. In 2014 our lab was the first to show that NAD+ levels were indeed reduced in the brain in association with increased oxidative damage, figure 7[34].

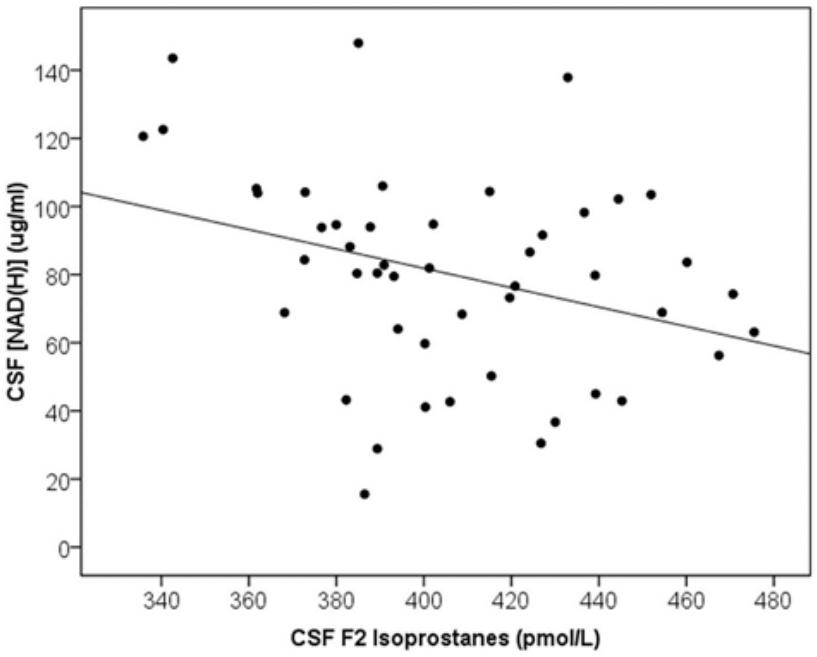

Figure 6 - Inverse association between CSF [NAD(H)] and CSF F2 Isoprostane levels. A significant inverse association was observed between CSF [NAD(H)] and F2 Isoprostane levels (p = 0.02, n = 48). Comparisons were made using the Pearson correlation coefficient and multiple linear regression controlling for age (Guest et al., 2014).

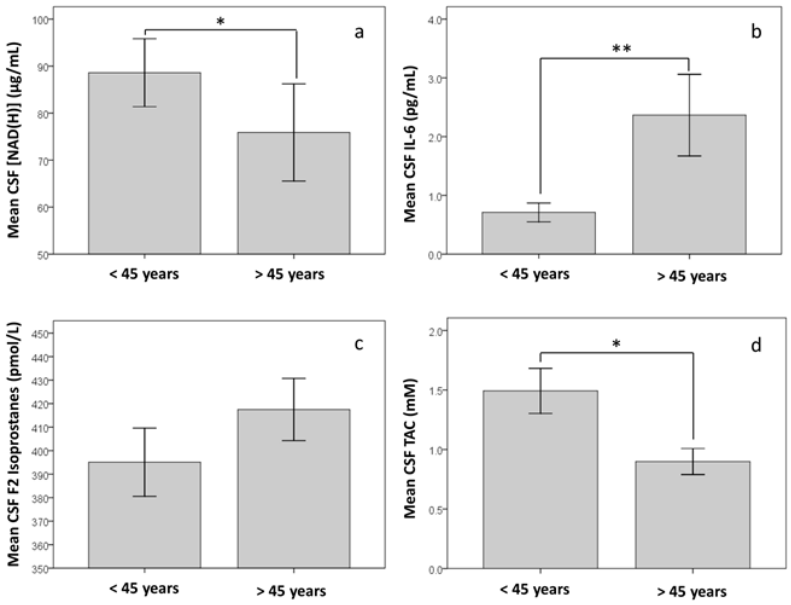

Figure 7 – (a) NAD(H) levels are significantly reduced with age in association with (b) increased inflammation and (c) oxidative damage as well as (d) reduced total antioxidant capacity (TAC). Data used with permission from Table 1 Guest et al., 2014.

As discussed above depletion of brain NAD+ may have implications beyond decreased ATP synthesis. In addition to its role in energy production, DNA repair, epigenetic control and immune modulation evidence also suggests that NAD+ may act as a direct neuro-transmitter[35]. While the functional roles mediated by NAD+ neurotransmission are not yet fully known, when coupled with NAD+'s

other important functions it is clear that NAD+ facilitated tasks are capable of improving neuronal signalling, viability and resistance to stress, in particular oxidative stress.

Previous research by others has shown that increasing NAD+ availability to brain cells can inhibit processes that lead to cell death in the degenerative dementias (e.g. Alzheimer's disease). These include: resistance to excitotoxicity[36], synaptic disconnection[37], and axonal degeneration[38]. In addition, work in animal models has shown that increasing NAD+ levels in a damaged brain can increase learning and memory[39], especially after traumatic injury.

An additional beneficial outcome to NAD+ supplementation also appears to be improved circadian rhythms (i.e. better sleep). Circadian dysfunction is a common feature of older age and is widely experienced in those with the dementia phenotype. Poor sleep behaviour has been linked to a number of disorders including, depression, bipolar disorder as well as reduced cognitive function and memory performance[40], all of which may be improved by increasing NAD+ availability.

Biological rhythms are established and maintained by a central clock consisting of around 20,000 pacemaker neurons in the supra-chiasmatic nucleus (SCN). Importantly NAD+ drives the circadian clock feedback cycle through SIRT1 and CLOCK:BMAL1[41]. Increasing NAD+ levels through targeted supplementation has been shown to improve the circadian cadence[42].

Targeted supplementation to enhance NAD+ levels has therefore the potential to: improve global cellular energy levels, DNA repair, cell viability, and resistance to oxidative damage potentially slowing down the normal aging process throughout multiple body systems.

In the brain increasing NAD+ availability is able to enhance neurochemical signalling, cell viability and resistance to degenerative disease processes including oxidative stress and excitotoxic damage, resulting in improved circadian control (better sleep), enhanced neurobiological function and improved cognition and memory.

Clearly there are multiple benefits to improving NAD+ levels, particularly under conditions of increased oxidative activity associated with advancing age or degenerative disease. However the best way to increase NAD+ levels in the body is still under investigation.

HOW CAN YOU INCREASE NAD+ LEVELS?

The synthesis of NAD+, both systemically and within the CNS, is known to occur through two major pathways; the de novo and the salvage pathways (figure 8).

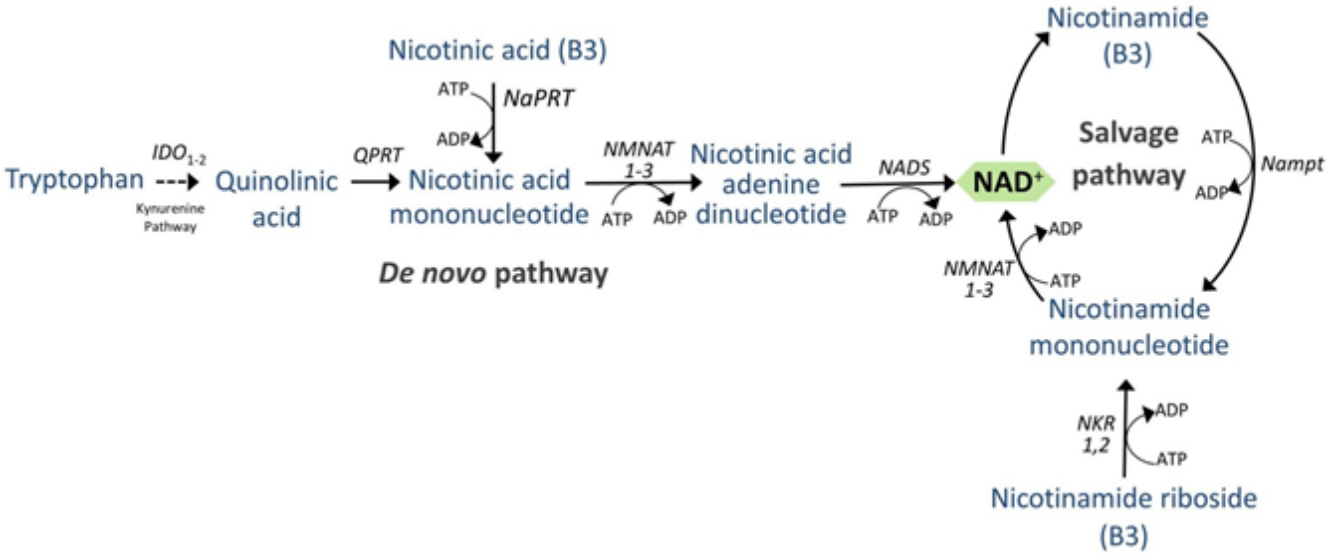

Figure 8 - NAD+ synthesis.

ADP, adenosine diphosphate; ATP, adenosine triphosphate; IDO, indolamine-2,3- dioxygenase; NAD+, nicotinamide adenine dinucleotide; NADS, nicotinamide adenine dinucleotide synthetase; Nampt, nicotinamide phosphoribosyltransferase;NaPRT, nicotinic acid phosphoribosyltransferase; NKR, nicotinamide riboside kinase; NMNAT, nicotinamide mononucleotide adenylyltransferase; QPRT, quinolinate phosphoribosyl transferase.

In order to realise the benefits inherent in NAD+ enriched tissue, supplementation with either NAD+ or its precursors should be considered as potential target therapies for the prevention and improvement of aging and neurodegenerative disease. However not all of the potential NAD+ precursors are likely to be equal to the task.
NAD+: Unfortunately oral supplementation with NAD+ its self does not appear to raise serum or tissue NAD+ levels. Early studies indicate this is due to the efficient metabolism of NAD+ in the gut and resulting poor bioavailability[43]. Therefore I.V. infusion seems to be the only effective means of increasing NAD+ using this modality.

Nicotinic acid (NA): NA is the acid form of vitamin B3, a commonly prescribed therapeutic for lowering serum lipids. Our research group were the first to show some years ago that supplemental NA efficiently increased tissue NAD+ levels, especially in brain cells[44]. However, NA therapy results in significant skin flushing. This uncomfortable side effect occurs as a result of an NA mediated stimulation of cell membrane phospholipase A2 (PLA2) which results in the conversion of the omega-6 metabolite arachidonic acid (AA) into prostaglandin E2, stimulating vasodilation of skin capillaries. This often dramatic and unwelcome side effect has therefore restricted NA applications to essentially a treatment-resistant lipid lowering therapy.

Nicotinamide (NAM): NAM is the amide form of vitamin B3 and is also a bi-product of SIRT, PARP and CD38 activity which can be converted back to NAD+ via the salvage pathway, Ffigure 8). However as a bi-product NAM acts also as a feedback inhibitor for each of these enzymes. As NAM concentrations rise, PARP, SIRT and CD38 activities are proportionately inhibited. Though NAD+ levels are still increased, the very enzyme functions we want to be enhanced (e.g. SIRT1) are actually being inhibited. Medium to longer term NAM supplementation therefore has the potential to reduce, SIRT, CD38 and PARP activity and may even contribute to genomic instability with resulting risk of cancer formation. Therefore, though exogenous NAM can be converted to NAD+ it is again not considered an ideal supplement, particularly in the medium to longer term.

Nicotinamide mononucleotide (NMN): NMN is an endogenous substrate that appears to be a safe precursor for NAD+ synthesis[45]. However evidence also suggests that as NMN is effectively contained within the cells membranes it is not subject to high diffusion gradients. This has raised the question of whether NMN is able to effectively traffic across most cells. Interestingly, extracellular NMN may be actively produced from the metabolism of exogenous NAD+ its self[46]. However, supplemental NMN has been shown to have a positive effect on insulin levels most likely through action on pancreatic β cells[47], indicating some clinical benefits to NMN supplementation. However further work is required to establish the range of conditions for which NMN may prove beneficial.

Nicotinamide riboside (NR): NR is a naturally occurring precursor of NAD+ originally isolated from fresh milk. It has been shown to efficiently increase [NAD+] without producing any of the negative flushing side effects like nicotinic acid. Recent data indicates that NR may provide greater protection against damage induced neuropathy than either NA or NAM when de novo synthesis of NAD+ from tryptophan is insufficient[48]. This suggests that NR may be an effective precursor for NAD+ enhancement in the brain and therefore a uniquely useful therapy for neurodegenerative disease.

IN CONCLUSION

NAD(+) and its reduced equivalents play crucial roles in a variety of biological processes including energy metabolism, mitochondrial function, gene expression and neuronal signalling. A mounting body of evidence indicates that raising NAD(+) levels can profoundly decrease oxidative cell death in brain cells and other organs. Promotion of NAD(+) metabolism is therefore a promising therapeutic target for age associated degenerative diseases in general and neurodegenerative disease in particular.

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