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Research Article

The Role of NAD+ in Anti-Aging Therapies

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Abstract

The aging process involves accumulation of DNA damage, mitochondrial defects, progressive tissue degeneration and atrophy, and the development of metabolic dysfunction and weakness. Aging is also accompanied by decreased levels of nicotinamide adenine dinucleotide (NAD^+), which can result in cell damage and even shorter life spans. NAD^+ acts as an enzyme cofactor in many essential biological pathways and is a substrate for several regulatory proteins. Many studies have suggested that the upregulation of NAD^+ precursors can increase levels of NAD^+ in tissues or cells to delay aging. Clinical trials have been conducted on the safety and efficacy of NAD^+. Here we provide a review of NAD^+ metabolism and its role in aging-related therapy.

Keywords: NAD^+; Aging; Clinical trials

Introduction

The aging process is influenced by a variety of factors and considered to be an irreversible process. Aging of an organism is accompanied by metabolic disorders and the impairment of physiological function, as well as the development of age-related diseases [1-4]. There is abundant evidence that NAD^+ plays an important role in aging, as it is involved in various biological functions and is a key regulator of stress resistance [5,6]. Levels of NAD^+ steadily decline with age, resulting in altered metabolism and increased disease susceptibility [7-9]. NAD^+ plays a key role in various energy metabolism pathways [6,10]. Additionally, NAD^+ is a cofactor for many enzymes, such as poly (ADP-ribose) polymerases (PARPs), CD38, and sirtuins [11]. Sirtuins are NAD^+-dependent histone deacetylase for a wide range of transcriptional regulators [10,12]. Overexpression of SIRT1 in the brains of mice has been shown to delay aging [13]. PARP is a major NAD^+-degrading enzyme, which plays diverse roles in many molecular and cellular processes [14]. Inhibition of PARP-1 increases mitochondrial metabolism via modulation of SIRT1 activity [15]. Another NAD^+-degrading enzyme, CD38, had been associated with the decline in NAD^+ levels during aging [16].

Mammalian cells cannot import NAD^+ *in vivo*, so they must synthesize it either from tryptophan or the various forms of niacin taken up in the diet including nicotinamide mononucleotide (NMN) and nicotinamide riboside (NR) [17-20]. Recently, it was found in mice that supplementing with NAD^+ precursors (including NMN, NR, and nicotinamide) or inhibiting the activity of NAD^+-consuming enzymes can increase the level of NAD^+ in tissues and improve energy metabolism, thereby delaying aging and extending healthy life [15,21-24].

Currently, the anti-aging activity of NAD^+ precursors is primarily evaluated through measurement of aging markers in mouse behavior, accumulation of DNA damage, and mitochondrial activity. RNA sequencing has also been used to identify genes and pathways involved in the anti-aging mechanisms of NAD^+ [20,22,25,29]. Furthermore, recent research has shown that biological age can be measured by analyzing the 353 DNA methylation sites of the Horvath clock [30,31].

NAD^+ Biosynthesis-Salvage Pathway

In vivo, NAD^+ is an essential cofactor of dehydrogenase [32,33]. Nicotinamide coenzyme is an electron carrier which plays an important role in various oxidation-reduction reactions. Therefore NAD^+ is a cofactor of many key enzymes in glycolysis, the tricarboxylic acid cycle, and oxidative phosphorylation [34]. Age-associated

decline in NAD+ availability has an important effect on the aging process of many species [8,35,36]. There are three pathways for the synthesis of NAD+ in cells, involving many different precursors [37-39]. Here we focus on the salvage pathway, which is important from a translational research perspective because it is the main source of NAD+ [40,41].

There are three pathways for the synthesis of NAD+ in cells:

a. de novo from tryptophan;

b. from nicotinic acid via the Preiss-Handler pathway; and

c. from nicotinamide (NAM) via the salvage pathway [37].

NAM itself is a by-product of NAD+-degrading enzymes such as sirtuins and PARP. As shown in Figure 1, the first step of the salvage pathway is catalyzed by nicotinamide phosphoribosyl-transferase (NAMPT), which converts nicotinamide and 5-phosphoribosyl-1-pyrophosphate into NMN [42]. Subsequently, nicotinamide mononucleotide adenylyltransferase (NMNAT) produces NAD+ from NMN and ATP [43,44]. NR can be converted by nicotinamide-riboside kinase (NRK) into NMN which participating in the Salvage pathway [45]. NAMPT is the rate-limiting enzyme of the salvage pathway [42]. It has been hypothesized that reduced NAD+ synthesis is one of the causes of lower NAD+ levels with aging, and this may be due to decreased activity of NAMPT [42,46]. Indeed, NAMPT levels are known to decline with age in many types of tissues [47-49], whereas exercise increases skeletal muscle NAMPT expression [50].

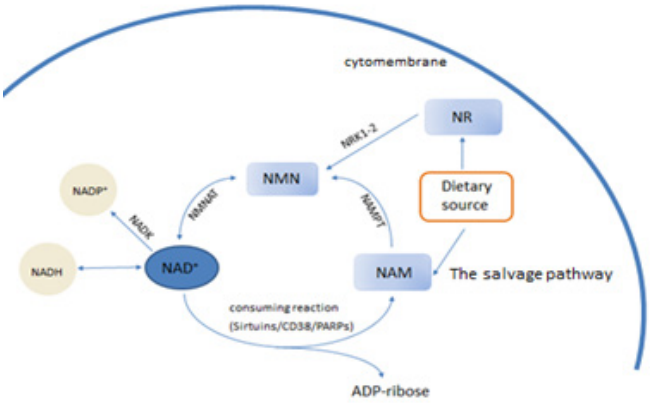

Figure 1: The Salvage pathway NAM and NR are the main precursor for the salvage pathway.

In mammals, NAMPT has two different forms: intra- and extracellular [51]. The intracellular form is the one that participates in the salvage pathway of NAD+ synthesis [42], while the extracellular form likely functions as a circulating cytokine [52]. Studies have shown that secretion of NAMPT is regulated by SIRT1 *in vivo* [53,54], and SIRT1 activity in turn depends on NAMPT which regulates level of NAD+ [55]. Increasing level of NAMPT may delay aging of individuals via SIRT1-dependent pathways [56]. NAMPT has been shown to regulate osteoblast differentiation in primary culture of mouse bone marrow-derived mesenchymal stem cells via NAD+-SIRT1 pathway. NAMPT deficiency may increase the risk of bone aging or fractures [48,57-59].

Levels of NAMPT significantly decrease with age in mice and humans [60,61]. Adipose tissue-specific overexpression of NAMPT in aged mice resulted in increased levels of circulating eNAMPT, increased levels of NAD+ in multiple tissues, and extended lifespan [60].

In mammals NMNAT is the central enzyme of the NAD biosynthetic pathway [43,62]. There are three isoforms, NMNAT1, NMNAT2, and NMNAT3, encoded by different genes and localized to nucleus, Golgi apparatus, and mitochondria, respectively [43]. NMNAT1 directly control SIRT1 deacetylase activity at a set of target gene promoters [63]. Homozygous knockout of *Nmnat1* in mice results in embryonic death [64]. Low levels of NMNAT2, highly expressed in the brain and nervous system, could impair axon regeneration as well as axon survival in aging and disease [65,66]. NMNAT3 has been identified as the rate-limiting enzyme for mitochondrial NAD+ biosynthesis [67]. In addition, down-regulation of NMNAT3 gene expression in cells significantly impairs the capacity for mitochondrial respiration, suggesting that NMNAT3 plays a key role in mitochondrial NAD+ homeostasis [68].

Anti-aging effects of NAD+

Through its role as a substrate for sirtuins, CD38, and PARP, NAD+ regulates a variety of cellular process including energy metabolism, DNA damage repair, gene expression, and oxidative stress response [11,34,69,70].

a. The Sirtuins Pathway

Sirtuins are evolutionarily conserved NAD+-dependent deacetylases. Increasing sirtuin expression has been shown to affect lifes-

pan across various species [13,36,71,72]. Sirtuins have received significant attention since the discovery that the increased sirtuin silent information regulator 2 (Sir2) can extend yeast lifespan [72]. The closest mammalian homologue of this regulator is SIRT1[69], mainly localized in the nucleus but also present in cytosol [73]. Its nuclear export signal allows shuttling to the cytosol under specific circumstance [12]. It has been shown *in vivo* that NAD^+-SIRT1 signaling promotes mitochondrial activity [25]. Previous research has suggested that increasing SIRT1 in the brain, especially in the dorsomedial and lateral hypothalamic nuclei, can delay aging and extend lifespan in mice [13]. More recently, heart-specific overexpression of *Sirt1* was found to delay aging and protect against oxidative stress in the heart [74]. NR-SIRT1 signaling can inhibit cardiac stem cell senescence by improving mitochondrial function and muscle stem cell function, thereby enhancing life span in mice [25]. More research is needed to determine whether increased level of NAD^+ *in vivo* can improve SIRT1 activity, thereby delaying aging [75].

b. The PARPs Pathway

PARPs are expressed by most eukaryotic cells and are involved in DNA damage detection and repair, cell death pathways and so on [14]. Aging is associated with an accumulation of DNA damage [76]. Depletion of NAD^+ is involved in cell death through PARP-1 [70]. Although this enzyme plays an important role in cells, over-activation of PARP-1 can lead to depletion of NAD^+, reduction of ATP, reducing the activity of SIRT1, loss of mitochondrial function, and even cell death [70,77,78]. Increased level of NAD^+, when SIRT1 is intact, can reduce the cell death caused by activation of PARP-1 in cardiac myocyte [79].

c. CD38 and NAD^+

CD38 is a multi-functional protein. Studies have shown that CD38 is the NADase in mammalian tissues [80,81]. It is thought to contribute to the age-related decline in NAD^+ levels [23,80,82]. CD38 also acts as an antigen for B-lymphocyte activation and as an ecto-enzyme in endothelial and inflammatory cells [82,83]. Senescent cells are known to express small molecules including secreted cytokines, growth factors, and extracellular matrix modifiers to promote chronic sterile inflammation and fibrosis. This reaction process, known as the senescence-associated secretory phenotype [84-86], involves secretion of factors by senescent cells which induce the expression of CD38 in non-senescent cells [82,87]. This increased CD38 activity can disrupt the fine balance between NAD^+ and its reduced form, NADH, within a cell [82]. Recently, the small molecule CD38 inhibitor 78c was shown to reverse the age-related loss of NAD^+ [28,83]. By increasing tissue levels of NAD^+, 78c may be able to ameliorate metabolic disorder and other disruptions involved in the aging process. In addition, animals treated with 78c show activation of longevity genes, which inhibit DNA damage [28].

NAD^+ and NADH are in dynamic equilibrium within the cell [75]. Intracellular NAD^+ can be increased *in vivo* through oral administration of NAD^+ precursor or by inhibiting the degradation of NAD^+ [15,23,88]. Regulation of the NAD^+/ NADH ratio in this way can improve mitochondrial function and has been shown to treat senile deafness in elderly mice [89].

NAD^+ Repletion and Aging

One of the major causes of aging is progressive tissue degeneration and atrophy due to reduced somatic or stem cell function [22,90,91]. Adult stem cells are not only essential in continuously proliferating tissues (such as hematopoietic, intestinal, and skin systems) but also in normally quiescent tissues (such as skeletal muscle and the brain) that require regeneration after damage or exposure to disease [92]. NR supplementation improved metabolic function in muscle and neural stem cells, in both young and old mice, thereby increasing lifespan [25]. NR treatment has also been shown to rejuvenate stem cells from aged mice and restore the impaired ability to repair gut damage [22]. Previous studies have shown that DNA damage of nerve cells, nerve stem cells, and muscle stem cells in mice can be reduced by NR supplementation [25,93]. NR has also been shown to ameliorate mitochondrial dysfunction and enhance oxidative metabolism in obese mice [94,95] and prolong the lifespan of mice through neuronal DNA repair and mitochondrial quality improvement [96,97].

Supplementation with NMN can restore age-related capillary rarefaction and increase blood flow in elderly mice, and it maybe a novel therapy to restore SIRT1 activity and reverse age-related arterial dysfunction by reducing oxidative stress [98,99]. Mitochondrial disorders due to impaired oxidative phosphorylation (OXPHOS) are a cause of aging [100]. Long-term treatment with NMN in elderly C57BL/6J mice can improve metabolic dysfunction and ameliorate age-associated physiological decline [20]. NMN can also restore mitochondrial function, prevent neural death, and delay cognitive decline in a mouse model of Alzheimer's disease [101,102]. Supplementation with NAM was shown to improve blood sugar levels and metabolic capacity in HFD-fed mice. However, it had no effect on lifespan [103].

There are data showing that supplementation with NAD^+ precursors enhanced the mitochondrial function of cells or stem cells in a SIRT1-dependent manner [25,94]. Furthermore, supplementing NAD^+ precursors in elderly mice improved mitochondrial function in hematopoietic stem cells and muscle stem cells, as well as extended lifespan [25,104]. NMN specifically was found to enhance the biological activity of mesenchymal stromal cells through the upregulation of SIRT1, thereby stimulating osteogenesis of the cells and protecting bone from aging to delay the aging of mice [105]. In elderly mice, NMN treatment improved capillary density through the NAD^+-H_2S signaling network to increase blood flow, endurance, and physiological status [27,29,106].

Inhibition of some NAD^+-degrading enzymes could also lead to increased levels of NAD^+ [15,23]. CD38, PARPs, and SARM1 all

degrade NAD^+ inside the cell [23,77,80,107]. The activity of CD38 increases with aging, contributing to the age-related decline in NAD^+ [16]. Several small-molecule inhibitors of CD38 have been described [83,108-110]. Thiazoloquin(az)olin(on)e is one such inhibitor which could potentially be used as a therapeutic agent to increase intracellular NAD^+ level [28]. Inhibition of PARP1 has recently been reported to correct mitochondrial impairment [111,112] and has strong metabolic implications through its modulation of SIRT1 activity [15].

Measure Biological Age

Thus far, the anti-aging activity of NAD^+ is mainly examined using RNA sequencing and gene set enrichment analysis to identify pathways of candidate biomarkers [20,22,25]. However, there is not yet a gold standard for aging biomarkers. The DNA clock may offer a better objective biomarker for the study of aging [30,31]. DNA methylation plays a critical role in the regulation of gene transcription [113-118]. Senescence can be predicted and evaluated by detecting cytosine-5 methylation within CpG dinucleotides [30,31]. These age-related CpG characteristics are independent of gender or tissue type. Recent research has shown that biological age can be approximated by measuring levels of DNA methylation, a process known as the Horvath (or DNA) clock [30,31,119,120]. Age-related DNA methylation was first described for humans after cross comparison of thousands of CpG sites in Illumina Bead Chip microarray data [121,122]. Many of these age-associated CpG sites were then used as epigenetic age-predictors [30,31,120,123]. Three hundred and fifty-three unique CpG sites were found to be predictive of biological age, independent of chromatin status or tissue source [30,31].

Petkovich et al. developed a robust predictor of mouse biological age based on 90 CpG sites derived from partial blood DNA methylation profiles [124]. Stubbs et al. further developed a multi-tissue predictor to estimate age based on DNA methylation at 329 unique CpG sites from various different mouse tissues [125]. One group claims to have found three methylation sites, *Prima1, Hsf4,* and *Kcns1*, which are enough to predict biological age in mice [126]. However, this study has yet to be replicated. The most accurate clock results from applying elastic net regression to all CpGs for multi-tissue in mice [127].

Together these studies suggest that the DNA clock provides an objective biomarker for the study of aging [30,31]. Recently, metformin has shown that reversed subject's biological age, based on assessment of Horvath clock [128,129]. Its use will allow us to examine the anti-aging effectiveness of NAD^+ and its precursors more objectively and accurately.

Clinical Research

NAD^+ precursors can be delivered orally to humans or animals to alter the dynamic balance of NAD^+/NADH *in vivo* [24,130]. Preliminary clinical studies in humans showed that NR supplementation could improve muscle NAD^+ metabolism in the elderly [131,132]. Healthy volunteers, who underwent an 8-day course of NR, with doses increasing from 250 mg to 1000 mg, showed increased levels of circulating NAD^+ and experienced no adverse side effects [133]. Similarly, NR supplementation increased NADH and NADPH levels and improved exercise performance in elderly subjects [134]. Therefore, NMN is considered safe in clinical trials [135]. However, high dose supplementation with NAD^+ precursors may increase rates of glycolysis and mitochondrial respiratory metabolism, thereby promoting the secretion of proinflammatory cytokines in cells [136]. Thus, use of supplements should be carefully observed to ensure that they strike a proper balance between anti-aging effects and potential detrimental effects.

Conclusions

NAD^+ is a cofactor for many important enzymes. Reduced levels of NAD^+ have been associated with aging. Evidence suggests that supplementation with NAD^+ precursors, or inhibition of NAD^+ degradation, could improve metabolic function. While supplementation with NAD^+ precursors has been found to delay aging in mice, anti-aging effects of NAD^+ have yet to be demonstrated in human subjects. Use of a more accurate biomarker for aging, such as the DNA methylation clock, will significantly advance the field. Recently, several human clinical trials have been initiated.

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