Nicotinamide Mononucleotide Supplementation: A Potential Treatment of Vascular Cognitive Impairment and Dementia PhD Thesis Summary

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Introduction

In recent years, it has been recognized that the health of the cerebral microcirculation is critical for the brain health during aging. It is now known that aging is associated with structural and functional impairments of cerebral microvasculature which substantially contribute to the pathogenesis of age-related cognitive decline. Vascular cognitive impairment and dementia is the second most common cause of dementia and up to 75% of all patients with dementia have evidence of vascular pathology at autopsy. Due to the rapidly aging population of Europe, it is increasingly important to explore potential drugs which could prevent or delay cognitive impairment and dementia in the elderly.

Maintenance of cerebral homeostasis requires a tightly controlled supply of oxygen and nutrients as well as washout of harmful metabolites. The brain has limited energy reserves and cerebral oxygen content, so the neuronal function can be sustained only for a short period of time if cerebral blood flow (CBF) decreases. Both structural and functional impairment of the cerebromicrovasculature can damage the fine structure of the neuronal network causing deterioration of cognitive and motor performance in patients. The dynamic balance between angiogenesis and microvascular regression is critical for the maintenance of a healthy cerebral microcirculatory network. Previous studies showed that advanced aging is associated with a significant impairment of endothelial angiogenic processes resulting in cerebromicrovascular rarefaction in the aged brain. Microvascular rarefaction contributes to a decline in cerebral blood flow compromising oxygen and nutrient delivery to the active

neurons and leading to the formation of ischemic foci, neuronal dysfunction and demyelination. In addition to being essential for the structural integrity in the brain, complex spatial regulation of CBF is needed for a healthy brain. Intense neuronal activity requires rapid adjustment of regional oxygen and glucose delivery. This is provided by an evolutionarily conserved physiological mechanism known as neurovascular coupling (NVC). NVC is achieved through orchestrated, tightly controlled intercellular communication between activated neurons, astrocytes, vascular endothelial cells, pericytes and smooth muscle cells. Impairment of functional hyperemia ('neurovascular uncoupling') has been described in a wide spectrum of pathophysiologic conditions associated with aging, including hypertension, obesity, cognitive impairment and Alzheimer's disease.

NAD^+ acts as an important, rate-limiting coenzyme in multiple electron transfer reactions. NAD^+ is a donor of ADP-ribose moieties in ADP-ribosylation reactions, precursor of the second messenger molecule cyclic ADP-ribose, and substrate for the longevity assurance factor sirtuin enzymes. Maintenance of NAD+ level is critical for normal cellular proliferation and function, regulation of mitochondrial metabolism and cellular bioenergetics, adaptive stress responses, and normal activation of pro-survival, anti-aging pathways. With advanced age, there is decreased availability of cellular NAD^+, which has been proposed to be a critical driving force of aging processes. Aging-induced NAD^+ depletion has been suggested to contribute to a wide range of chronic diseases and pathological conditions associated with old age, including endothelial dysfunction. There is strong preclinical evidence that restoration of cellular NAD^+ levels in aged rodents by administration of NAD^+ precursors exerts potent anti-aging effects,

reversing age-related organ dysfunction and increasing mouse lifespan. Nicotinamide mononucleotide (NMN) is an example of an intracellular NAD-boosting molecule, and is a precursor of NAD^+ biosynthesis. Cells take up NMN with great efficiency, however the underlying mechanism is unknown. Intracellularly, NMN is converted to NAD^+ through the NAD^+ salvage pathway by nicotinamide mononucleotide adenylyl transferase enzymes. Furthermore, NMN has great pharmacokinetics in humans giving it great therapeutic potential.

MicroRNAs (miRNA) are short, endogenous, non-coding transcripts that repress gene expression at post-transcriptional level in both physiological and pathological conditions. Strong evidence suggests that miRNAs have a role in regulation of lifespan in mammalian. Importantly, miRNAs were also reported to regulate several important aspects of endothelial biology and vascular function. Furthermore, several studies have demonstrated that age-related miRNA dysregulation contributes to the development of vascular aging phenotypes. Despite these advances, fundamental cellular and molecular processes of aging that are responsible for dysregulation of vascular miRNA expression have not been elucidated.

The present study was designed to test the hypothesis that restoration of cellular NAD^+ level with the supplementation of NMN can improve the health of cerebromicrovasculature in aging. Our hypothesis was tested in an array of in vitro and in vivo studies.

Materials and Methods

Primary Cerebromicrovascular Endothelial Cell Cultures:
We used Fischer 344 x Brown Norway (F344xBN) rats as a model of aging. In F344xBN rats the primary effects of aging can be studied without complications caused by age-related pathology. 3 and 24-month-old, male F344xBN rats were obtained from the National Institute on Aging. The rats were housed in an environmentally controlled vivarium under pathogen-free conditions. All experimental animals were maintained according to National Institutes of Health guidelines, and all animal use protocols were approved by the Institutional Animal Care and Use Committees of the participating institutions.
After the anesthesia the brains were rapidly dissected to establish primary cerebromicrovascular endothelial cell (CMVEC) cultures. After the harvested tissue was mechanically and enzymatically dissociated the endothelial cell–enriched fraction was collected using an OptiPrep gradient solution. Next, endothelial cells were collected using the MACS LD magnetic separation columns. The endothelial fraction was cultured on fibronectin coated plates in Endothelial Growth Medium with reduced nicotinamide concentration for 10 days.
CMVECs derived from young and aged rats were treated with NMN in vitro in 5×10^{-4} mol/L concentration for 1–5 days.

Assessment of Angiogenic Processes in CMVECs
Cell Proliferation Assay: Cell proliferation capacity was assessed in CMVECs using the flow cytometry–based Guava CellGrowth assay. Cells were stained with carboxyfluorescein diacetate

succinimidyl ester (CFSE) and incubated for 24 hours with VEGF. Finally, cells were collected, washed, stained with propidium iodide (to gate out dead cells), and analyzed with a flow cytometer.

Wound-healing and Cell Migration Assay: Electric Cell-substrate Impedance Sensing technology (ECIS) was used to monitor the migration of CMVECs in a wound-healing assay. CMVECs were seeded in 96-well array culture dishes and placed in an incubator (37°C), and changes in resistance and impedance were continuously monitored. When impedance reached a plateau, cells in each well were subjected to an elevated field pulse ("wounding"), which killed the cells present on the small active electrode due to severe electroporation. The detachment of the dead cells was immediately evident as a sudden drop in resistance. VEGF was immediately added to each well. CMVECs surrounding the active electrode that had not been subjected to the wounding then migrated inward to replace the detached dead cells resulting in resistance recovery. The time to reach 50% resistance recovery was determined for cells in each experimental group, and this parameter and the known physical dimensions of the electrode were used to calculate the migration rate.

Tube Formation Assay: To asses tube formation, CMVECs were plated on Geltrex Reduced Growth Factor Basement Membrane Matrix. Half of the aged control cells and NMN-treated aged cells were pre-treated with EX-527, a potent and selective sirtuin 1 (SIRT1) inhibitor. The extent of tube formation was quantified by measuring total tube length in five random fields per well.

Assessment of Cellular H_2O_2 Production in CMVECs:

To assess cellular peroxide production, we used the cell-permeant oxidative fluorescent indicator dye CM-H2DCFDA (5 (and 6)-

chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate-acetyl ester, Invitrogen, Carlsbad, CA, USA). Cells were incubated with CM-H2DCFDA, and the fluorescence was assessed by flow cytometry.

SIRT1 and SIRT2 shRNA Transfection of CMVECs:

To determine the role of sirtuin signaling in the endothelial effects of NMN treatment, the downregulation of SIRT1 and SIRT2, in CMVECs was achieved by RNA interference using SIRT1 and SIRT2 short hairpin RNA sequences. CMVECs were transfected using the electroporation-based Amaxa Nucleofector technology. Experiments were performed on day 2 after the transfection.

Assessment of Mitochondrial Function in CMVECs:

Mitochondrial ROS Production: Mitochondrial production of ROS (mtROS) in CMVECs was measured using MitoSOX Red, a mitochondrion-specific hydroethidine-derivative fluorescent dye. Cells were incubated with MitoSOX (5×10^{-6} mol/L) for 30 minutes, at 37°C, in the dark. The cells were then washed with PBS and MitoSOX fluorescence was measured by flow cytometry.

Mitochondrial Membrane Potential: To elucidate the effects of NMN on mitochondrial membrane potential in CMVECs we used the mitochondrial membrane potential indicator fluorescent dye JC-1. JC-1 is a cationic carbocyanine dye that accumulates in energized mitochondria. When it is present in its monomer form in the mitochondria at low concentrations (low mitochondrial potential), the dye exhibits green fluorescence. When it accumulates in the energized mitochondria and forms J-aggregates at higher concentrations (high mitochondrial potential), it exhibits red fluorescence. A decrease in the aggregate red fluorescence and an

increase in monomer green fluorescence is indicative of depolarization whereas an increase in the aggregate red fluorescence and a decrease in monomer green fluorescence is indicative of hyperpolarization. Cells were labeled with JC-1 for 30 minutes at 37 °C and fluorescence was analyzed with flow cytometry. The red/green fluorescence ratio was calculated as an indicator of mitochondrial membrane potential.

Mitochondrial Bioenergetics Assay: We performed real-time measurements of the oxygen consumption rate (OCR) as a marker of oxidative phosphorylation in CMVECs using a Seahorse XF96 extracellular flux analyzer. CMVECs were seeded into XF96 cell culture microplates in Seahorse XF-Assay media supplemented with 25 mM glucose and 1 mM sodium pyruvate (pH 7.4) the day before the assay. Plates were maintained for 45 minutes at 37 °C in 0% CO2 prior to the measurement. Basal respiration, coupling efficiency, and spare respiratory capacity were compared using the Mito Stress Test Kit. OCR was monitored before and after the addition of the electron transport inhibitors oligomycin and FCCP, an ionophore that is a mobile ion carrier, and a mixture of antimycin-A (which is a complex III inhibitor) and rotenone, a mitochondrial inhibitor that prevents the transfer of electrons from the Fe–S center in complex I to ubiquinone. Basal respiration (baseline respiration minus antimycin-A post injection respiration), ATP synthesis coupled respiration (baseline respiration minus oligomycin post injection respiration), maximal respiratory capacity (FCCP stimulated respiration minus antimycin-A post injection respiration) and reserve respiratory capacity (FCCP stimulated respiration minus baseline respiration) were calculated. Sample protein content was used for normalization purposes.

Measurement of Cellular NO Release in CMVECs:
The production of NO in CMVECs was measured using the fluorescent indicator DAF-FM (4-amino-5-methylamino- 2′,7′-difluorescein). After incubation the cells were washed and the DAF-FM fluorescence was measured by flow cytometry.

Measurement of Cellular ATP Levels in CMVECs:
ATP levels in endothelial cells were assessed using the ENLITEN ATP bioluminescent assay. For ATP determination the cells were homogenized in Passive Lysis Buffer. The samples were diluted 1:10 and mixed with an equal volume of the luciferase reagent. The plates were incubated at room temperature for 10 min and the luminescence signal was detected with a Tecan Infinite M200 plate reader. ATP quantification was carried out from a standard curve using ATP disodium salt hydrate. BCA protein determination was performed for normalization purposes. Cell viability in each population was determined by flow cytometry to ensure similar viability of CMVECs in each group in a parallel experiment using the ViaCount Assay.

In vivo Animal Model:
Young (3 months) and aged (24 months) male C57BL/6 mice were obtained from the aging colony maintained by the National Institute on Aging at Charles River Laboratories. Animals were housed under specific pathogen-free barrier conditions in the Rodent Barrier Facility at University of Oklahoma Health Sciences Center Mice in the aged cohort were assigned to two groups. One group of the aged mice was injected daily with NMN (i.p. injections of 500 mg NMN/kg body weight per day) or the equivalent volume of PBS for 14 consecutive days at 6 PM and 8 AM. On day 14 animals

were sacrificed 4 hours after last injection. All procedures were approved by the Institutional Animal Use and Care Committees of the University of Oklahoma Health Sciences Center.
To confirm efficiency of NMN treatment, NAD^+ levels were measured in snap frozen aortas from young and aged mice using the NAD/NADH-Glo bioluminescent assay.

Animal Behavior Testing:
Radial arms water maze test: Spatial learning and memory in each group of mice were tested by the radial arms water maze test. The maze consisted of eight arms that radiated out from an open central area. Paint was added into the water to make it opaque. At the end of one arm there was a submerged escape platform. The maze was surrounded by privacy blinds and intra- and extra-maze clues were provided to help the animals during the experiment. The mice were monitored by a video tracking system from above the maze and parameters were measured using Ethovision software. During the learning period mice were given the opportunity to learn the location of the submerged platform during two sessions each consisting of four consecutive acquisition trials. On each trial, the mouse was started in one arm not containing the platform and allowed to wade for up to one minute to find the escape platform. All mice spent 30 seconds on the platform following each trial before beginning the next trial. The platform was located in the same arm on each trial. Over the three days of training, mice in the young control group gradually improved performance as they learned the procedural aspects of the task. Upon entering an incorrect arm (all four paws within the distal half of the arm) or failing to select an arm after 15 seconds the mouse was charged an error. Learning capability was

assessed by comparing performance on days 2 and 3 of the learning period.

Elevated plus maze, learning protocol: Mice were also assessed for learning capacity using an elevated plus maze-based learning protocol. Two open arms and two closed arms were attached at right angles to a central platform. The apparatus was 40 cm above the floor. Mice were placed individually at the end of an open arm. The time for mice to cross a line halfway along one of the closed arms was measured on day 1 and day 2. Learning was defined as reduced transfer latency on day 2 compared to day 1.

Novel object recognition test: The test consists of a habituation, a familiarization and a trial phase. During the habituation phase the animals explored the empty open-field arena for 5 minutes. Then, in the familiarization phase the mice explore two identical objects for 2 minutes. After a 4 hours delay, a trial phase occurred. During this period animals explored the familiar object and a novel object for 2 minutes. Exploration of the objects was defined as directing the nose at a distance less than 2 cm to the object.

Rotarod performance: Motor coordination was assessed in each group of mice by using a four-lane rotarod device. Mice were pre-trained by placing them on the moving rotarod at 10 rounds per minute (rpm) until they performed at this speed for 120 seconds. The test phase consisted of 3 trials. The testing apparatus was set to accelerate from 4 to 40 rpm in 300 seconds. One mouse was then placed on each lane. The rotational velocity was set to increase every 10 seconds and the latency to fall was recorded. Latency to fall was recorded in by an infrared beam across the fall path along with the max rpm sustained by each mouse.

Grip strength test: Maximal muscle strength of forelimbs of the mice were measured by a grip strength meter. The strength measurements

of each group of mice were repeated three times by the same investigator.

Analysis of gait function: To determine the gait coordination, we tested the animals using an automated computer assisted method (CatWalk). Using the CatWalk system, the detection of paw print size, pressure and pattern during volunteer running on an illuminated glass walkway by a camera placed under the glass surface provides an automated analysis of gait function and the spatial and temporal aspects of interlimb coordination.

Assessment of Neurovascular Coupling:

Animals were anesthetized with isoflurane (4% induction and 1% maintenance), endotracheally intubated and ventilated. A thermostatic heating pad was used to maintain body temperature. Arterial blood pressure was measured via femoral artery catheter. Mice were placed on a stereotaxic frame and were equipped with an open cranial window. Changes in CBF were assessed above the left barrel cortex using a laser Doppler probe. The cranial window was filled with artificial cerebrospinal fluid. The right whisker pad was stimulated by a bipolar stimulating electrode. To assess the role of NO mediation, CBF responses to whisker stimulation were repeated in the presence of the nitric oxide synthase inhibitor Nω-Nitro-l-arginine methyl ester (l-NAME).

Assessment of Oxidative Stress in the Cortex:

To characterize the effect of NMN treatment on cellular redox homeostasis in aging, 3-nitrotyrosine, a marker for peroxynitrite activity, was assessed in homogenates of cortical samples using OxiSelect Protein Nitrotyrosine ELISA Kits, according to the manufacturer's guidelines. In the microcirculation of aged rodents'

endothelium-derived NO was shown to react with O^{2-} forming $ONOO^-$ thus decreasing the bioavailability of NO.

Endothelial Function in the Aorta:

To assess the specific effect of NMN treatment on endothelial function we used isolated aorta rings. Aortas were cut into ring segments 1.5 mm in length and mounted in myographs chambers for measurement of isometric tension. The vessels were superfused with Krebs buffer solution. After an equilibration period of 1 hour during which an optimal passive tension was applied to the rings (as determined from the vascular length-tension relationship), they were pre-contracted with 10^{-6} M phenylephrine and relaxation in response to acetylcholine was measured.

Quantitative Real-time RT-PCR and miRNA Expression Profiling:

A quantitative real time RT-PCR technique was used to analyze miRNA expression profiles in the aortas of mice from each experimental group. The expression profile of mouse miRNAs in aortas was analyzed using the TaqMan Array Rodent MicroRNA A+B Cards Set v3.0. The qPCR data were quantified using the ΔΔCt method. Predicted and experimentally validated microRNA targets were obtained from the TargetScan database, and Gene Ontology enrichment analysis was performed on differentially expressed microRNA targets. To identify relationships between miRNA targets and terms in the biomedical literature, we utilized the Implicit Relationship IDEntification by in-Silico Construction of an Entity-based Network from Text (IRIDESCENT) system.

mRNA-sequencing of Isolated CMVECs:

Animals from a separate cohort were killed and transcardially perfused with PBS. From the mechanically and enzymatically dissociated tissue the endothelial cell–enriched fraction was collected using an OptiPrep gradient solution. The endothelial/neurovascular enriched fraction was collected by magnetic separation using the MACS LD magnetic separation columns.

RNA isolation and next generation sequencing: RNA was isolated from the samples using AllPrep DNA/RNA Mini Kit. RNA quantity and quality were measured using the RNA 6000 Nano Assay with an Agilent 2100 Bioanalyzer. Using 1µg RNA, cDNA was synthesized from purified RNA using ABI High-capacity cDNA Reverse Transcription Kit. Library construction was performed in a stranded manner. The mature mRNA was enriched via pull down by beads coated with oligo-dT homopolymers. The mRNA molecules were then chemically fragmented, and the first strand of cDNA was generated using random primers. Following RNase digestion, the second strand of cDNA was generated, replacing dTTP in the reaction mix with dUTP. Double stranded cDNA then underwent adenylation of 3′ends following ligation of Illumina adapter sequences. Subsequent PCR enrichment of ligated products was further selected for those strands not incorporating dUTP, leading to strand-specific sequencing libraries. Final libraries for each sample were assayed on the Agilent Tapestation for size and quantity. Libraries were then pooled in equimolar amounts as ascertained via fluorometric analyses. Final pools were absolutely quantified using qPCR on a Roche LightCycler 480 instrument with Kapa Biosystems Illumina Library Quantification reagents. Sequencing was performed on an Illumina NovaSeq 6000 instrument with paired-end 50 base pairs reads.

Data Analysis: Raw sequencing reads were trimmed and aligned to the mouse genome version GRCm38. Samples were checked for outliers and separation by principle components. Raw expression counts were summarized at the gene level to transcript-length adjusted, library-size scaled counts per million. Differential expression analysis was performed using the empirical Bayes approach. Genes were considered differentially expressed if the absolute value of the fold-change ≥ 1.5 and the False Discovery Rate p-value adjusted ≤ 0.05.

We used the Upstream Regulator Analysis algorithm in the Ingenuity Pathway Analysis software to find upstream regulators that potentially explain the observed gene expression changes in our samples.

Results

1. NMN supplementation rescued angiogenic capacity and attenuates oxidative stress in aged CMVECs.

2. NMN supplementation improved mitochondrial energetics and attenuated mitochondrial ROS production in CMVECs.

3. NMN supplementation improved cognitive and motor performance in aged animals.

4. NMN supplementation rescued neurovascular coupling in the barrel cortex.

5. NMN supplementation alters miRNA expression profiles in the aorta.

6. NMN supplementation alters gene expression profile of neurovascular unit.

Discussion

It is well accepted that aging is associated with structural and functional impairments of cerebral microvasculature which substantially contribute to the pathogenesis of vascular cognitive impairment and dementia. Despite of its epidemiological importance, currently there isn't any available pharmacological intervention to prevent or treat this devastating disease.

Maintenance of intracellular NAD^+ level is critical for normal cellular proliferation, mitochondrial metabolism, cellular bioenergetics, adaptive stress responses, and normal activation of pro-survival pathways. With advanced age, there is decreased availability of intracellular NAD^+. However, with small molecular drugs like nicotinamide mononucleotide (NNM) the level of intracellular NAD^+ can be boosted. The cellular uptake of NMN is very efficient and it is converted to NAD^+ through the NAD^+ salvage pathway. Furthermore, NMN has great pharmacokinetics in humans giving it great therapeutic potential. Increased oxidative stress, cerebromicrovascular rarefaction and neurovascular uncoupling in animal models of aging as well as in clinical studies of older individuals has been linked to deterioration of high-level brain function, causing vascular cognitive impairment and dementia. In our studies we performed a series of in vitro and in vivo experiments to systematically evaluate the effect of NMN treatment on these hallmarks of cerebromicrovascular aging and to assess the underlying gene expression changes of NMN treatment. To our knowledge, this is the most comprehensive study available in the literature investigating the effects of NMN on the aged cerebromicrovascular.

Previous studies showed that aging is characterized by mitochondrial dysfunction and increased free radical production. The importance of the excessive free radicals in the cerebromicrovasculature were highlighted in some of our recent studies in which different inhibitors or scavengers of mitochondrial reactive oxygen species (SS-31, resveratrol or mitoTEMPO) could improve cognitive and gait function by rescuing NO mediated neurovascular coupling responses in aging. In our current in vitro study based on cultured, aged cerebromicrovascular endothelial cells, we measured decreased mitochondrial membrane potential, decreased oxygen consumption and increased free radical production compared to cells isolated from young animals. In contrast, NMN treatment of aged cells significantly improved mitochondrial function characterized by increased mitochondrial membrane potential, increased oxygen consumption rate and decreased free radical production. Our in vitro findings were also confirmed by our in vivo results. The 14 days NMN treatment of the aged mice significantly reduced the level of nitrogen free radical species in the cortex, which was indicated by the decreased level of modified proteins assessed by 3-nitrotyrosin staining.

Aging is also associated with cerebromicrovascular rarefaction due to declining VEGF-induced angiogenic processes. Previous studies showed that decreased capillary density significantly contributes to the pathogenesis of the aging-associated cognitive impairment and dementia. Our in vitro results show that age-related decline in cellular NAD^+ levels are associated with impaired VEGF-induced angiogenic response in aged rat cerebromicrovascular endothelial cells. However, restoration of cellular NAD^+ levels with 14 days of NMN treatment significantly

improved cell proliferation and rescued the increased migration and tube forming capacity of the aged cells.

According to our current understanding of aging associated vascular cognitive impairment, the dysfunction of the neurovascular unit ("neurovascular uncoupling") connects the subcellular processes to the organ level dysfucntion. Here we provide data to confirm that aging associated cognitive impairment strongly correlates with neurovascular uncoupling, which was assessed in the barrel cortex by the Whisker-Barrel Test. In aged animals, 14 days of NMN treatment was sufficient to successfully restore neurovascular coupling. Our finding strongly correlates with our in vitro results showing that NMN treatment successfully restored NO release in cultured aged cerebromicrovascular endothelial cells. In another set of ex vivo experiments, NMN supplementation also rescued endothelial NO-mediated vasodilation in the aortas of aged mice. In addition to vasoregulation endothelium-derived NO plays versatile biological roles. NO is a paracrine regulator of cellular metabolism and mitochondrial function, which modulates the function of dozens of proteins by promoting nitrosylation on their cysteine residues. This protein nitrosylation plays a role in platelet aggregation, smooth muscle cell proliferation and leukocyte adhesion, promotes stability of atherosclerotic plaques and exerts potent anti-inflammatory, anti-apoptotic and pro-angiogenic effects. Thus, rescue of cerebromicrovascular NO bioavailability by treatment with NAD^+ precursors likely has clinical significance beyond restoration of neurovascular coupling.

Finally, to show the beneficial effect of NMN on the higher-level brain functions, including hippocampal learning, memory and cortical gait function were tested extensively in our animals. According to our results, aged mice had impaired learning and

memory function, which were successfully rescued by 14 days NMN treatment. NMN treated mice performed significantly better in multiple behavioral assays including radial arm water maze, elevated plus maze compared to age-matched counterparts. Neurovascular dysfunction in older adults as well as in preclinical models of aging has been linked to gait alterations as well. Recent experimental studies in mouse models of pharmacologically-induced neurovascular uncoupling established a mechanistic link between impaired neurovascular coupling and gait abnormalities. In our experiments, aging associated gait abnormalities were also reversed by the 14 days NMN treatment.

To understand the underlying gene expression changes of NMN treatment, high throughput gene expression assays were performed. Previous studies demonstrated that alterations in miRNA expression profiles are linked to the development of multiple cardiovascular diseases and the aging phenotypes. Frist, miRNA profiling of aorta samples was performed by a TaqMan PCR Array. NMN treatment reversed aging associated changes in the miRNA expression profile. These findings raise the possibility that changes in post-transcriptional control of gene expression that encode critical targets for vascular health contribute to the beneficial effects of treatment with NAD^+ boosters. Furthermore, functional annotation of mRNA sequencing data, acquired from isolated neurovascular unit showed that NMN treatment in aged mice reversed, age-related, pro-inflammatory, pro-oxidative, pro-apoptotic, and endothelial-dysfunction-promoting transcriptional alterations. Analysis of the differential gene expression profile indicated that SIRT1 plays a critical role as upstream regulator in the development of the beneficial effects of NMN treatment. This finding strongly correlates both with the data available in the

literature and with our in vitro findings. In our cultured cerebromicrovascular endothelial cells originating from aged rats, shRNA knockdown of SIRT1 prevents the beneficial effects of NMN treatment.

In conclusion, we successfully demonstrated that NMN has a wide range of beneficial effects not only on the aged cerebromicrovascular endothelial cells but on the high-level of brain functions in the aged mice. Our study provides strong evidence that restoration of cellular NAD^+ level in the cerebromicrovascular endothelial cells by a small molecular booster NMN can provide a long-anticipated, efficient and safe intervention to prevent and/or treat aging-associated vascular cognitive impairment and dementia in elderly.

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Publications

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