The senotherapeutic nicotinamide riboside raises platelet nicotinamide adenine dinucleotide levels but cannot prevent storage lesion

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BACKGROUND: Supplementation of the nicotinamide adenine dinucleotide (NAD) precursor nicotinamide riboside (NR) has recently been shown to increase life-span of cells, tissues, and entire organisms. [Correction added on 13 December 2019, after first online publication: In the preceding sentence, "adenine nicotinamide" was revised to "nicotinamide adenine."] The impact of NR on platelet longevity has not been tested.

STUDY DESIGN AND METHODS: A pool-and-split design of buffy coat derived platelet concentrates (PCs) was used. One arm was treated with cumulative doses of NR-triflate, the control arm with sodium triflate. Storage lesion was monitored for 23 days. Platelet metabolic and functional parameters were tested. Clearance of human platelets was measured in a mouse model of transfusion.

RESULTS: Total intracellular NAD levels in platelets decreased two-fold from 4.8 ± 0.5 fmol (mean ± SD, n = 6) to 2.1 ± 1.8 fmol per 10^3 control cells, but increased almost 10-fold to 41.5 ± 4.1 fmol per 10^3 NR treated platelets. This high intracellular NAD level had no significant impact on platelet count, mean platelet volume, swirling, nor on lactate and glucose levels. Platelet aggregation and integrin $\alpha_{IIb}\beta_3$ activation declined steadily and comparably in both conditions. GPIbα levels were slightly lower in NR-treated platelets compared to control, but this was not caused by reduced receptor shedding because glycocalicin increased similarly. Apoptotic markers cytochrome c, Bcl-xL, cleaved caspase-3, and Bak were not different throughout storage for both conditions. Platelet survival in a mouse model of transfusion was not different between NR-treated and control platelets.

CONCLUSION: Platelets carry the cellular machinery to metabolize NR into NAD at rates comparable to other eukaryotic cells. Unlike those cells, platelet life-span cannot be prolonged using this strategy.

Platelet transfusion is essential for the acutely bleeding patient and for the patient at risk for bleeding during periods of thrombocytopenia. Platelet concentrate (PC) stock management is a tremendous challenge because of the limited shelf life. This is caused by a quick decline in platelet quality and by the risk of bacterial bloom during storage at 22°C. The latter may be prevented by pathogen inactivation techniques,[1] but this comes at a cost of platelet damage[2–4] and increased complexity during component processing.[5,6]

One classical diagnostic determinant for platelet quality is pH. The acid–base balance is used for research purposes but very often also for routine quality control checks of PC stock. The Council of Europe, AABB, and FDA guidelines therefore define a lower quality limit to pH ≥ 6.4 and pH ≥ 6.2, respectively.[7] Platelet metabolism consequently is at the center of quality assessment in blood institutions. The biochemical explanation behind this parameter is that platelets produce lactic acid relatively constant in time by anaerobic respiration. Platelet aging will therefore inevitably lead to increased acidity of the milieu allowing to monitor senescence relatively easy. Lactic acid production rate has been

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one of the few parameters that correlates reasonably well with transfusion yield.[8] Of note, oxidative phosphorylation in mitochondria still significantly contributes to the platelet's ATP pool by fueling alternative energy sources like L-glutamine and fatty acids.[9] Hence, the electron transport chain is a functional metabolic system in platelets. Its optimal function depends on many variables which is reflected in complex metabolic shifts during storage.[10] Pivotal to respiration are the nicotinamide adenine dinucleotides (NAD) NAD+ and NADH. This redox couple functions to shuttle electrons back and forth in a variety of cellular reactions and its ratio NAD+/NADH controls the activity of several enzymes.[11]

However, recent discoveries have also put NAD at the center of a search for treating senescence-related diseases because senescing cells, tissues, and organisms gradually loose NAD.[12,13] These changes in cellular NAD levels occur when pathways that regulate NAD biosynthesis and consumption are modulated. Oxidative damage for instance can lead to increased NAD consumption, by enzymes of the poly ADP-ribose polymerase (PARP), cyclic ADP-ribose synthase (CD38 and CD157), and sirtuin families.[14] Omics studies have detected members of these families in platelets.[15–17] In the absence of NAD synthesis, increased consumption will inevitably lead to NAD decline and decreased cellular function.[18] As a consequence, dietary delivery of NAD by supplementation of niacin precursors like nicotinamide mononucleotide (NMN) and nicotinamide riboside (NR) has been shown to increase viability of cells, tissues, and entire organisms.[19,20]

Because platelet quality declines fast when stored ex vivo and because this is often attributed to senescence-related metabolic dysregulation, we investigated if platelets can raise NAD content when supplemented by NR and whether this influences platelet quality and transfusion yield during long-term storage in blood banking conditions.

MATERIALS AND METHODS

Study design

Nicotinamide riboside was synthesized at NovAliX-Pharma as a trifluoromethanesulfonate salt[21] (triflate). As a vehicle control, an equimolar solution of sodium triflate (Sigma-Aldrich) was used. PCs were prepared by pooling of buffy coats following voluntary whole blood donation as described.[22] On Day 1 post phlebotomy, 2 PCs were pooled and split to prepare paired samples to be treated either by NR or vehicle control solution (Fig. S1, available as supporting information in the online version of this paper). Addition of NR or vehicle control solution was performed on Days 1, 5, 9, 12, 16, and 19 post phlebotomy by sterile connection to 500 mM of compound in distilled water. Volumes added ranged between 0.2 mL and 0.5 mL. Consequently, 154 nmol of NR or sodium triflate was added per 10^9 platelets per day. Throughout the study, both PCs were stored in standard blood banking conditions on a flatbed agitator (PF48i, Helmer Scientific) at room temperature. Samples for experiments were taken aseptically on Days 1, 2, 5, 7, 9, 12, 13, 16, 19, and 23 ranging between 1 and 8 mL depending on the experiments to be performed. On Day 1, a sample was taken from the pool, prior to splitting and representing "baseline" values (Fig. S1, available as supporting information in the online version of this paper). At the end of the study (Day 23) PCs were checked for sterility by inoculation of a 1.5 mL sample in Luria Bertani agar (Sigma-Aldrich) at 37°C for 24 hours followed by visual inspection. Even though less relevant for clinical application, follow-up beyond 7 days was chosen to study platelet longevity in the presence or absence of NR.

PC preparation

PCs were prepared by the buffy coat method from voluntary whole blood donations as described before.[22] In brief, six ABO blood group matched buffy coats were manually pooled and supplemented with 280 mL SSP+ additive solution (Macopharma). Next, the bag was centrifuged at 542 g for 450 seconds at 22°C to separate red and white cells from platelets. The buoyant platelet suspension was taken off by automated separation (Macopress Smart, Macopharma) while passing over a leukocyte reduction filter. Next, the PCs were stored at 22°C ± 2°C in a temperature controlled environment with continuous agitation.

Swirling, platelet counts, mean platelet volume, and metabolic parameters

PCs were assessed visually for swirling, yielding an integral score between 0 and 3. A low swirling score indicated an aberrant platelet morphology and deteriorating quality. PC with a swirling score ≥ 2 are considered suitable for transfusion. Platelet counts and mean platelet volume (MPV, fL) were measured using an automated hematology analyzer (pocH-100i, Sysmex). Metabolites of respiration were measured in a point-of-care blood gas analyzer (RAPIDPoint 500, Siemens).

Platelet aggregation

Platelet aggregation was examined with light transmission aggregometry at 37°C (Chrono-Log, Helena Laboratories).[23] Platelet aggregation was with 25 μg/mL fibrillar collagen (Stago) or a combination of 10 μM Thrombin Receptor Activating Peptide 6 (TRAP6, Sigma-Aldrich), 20 μM 2-(Methylthio) adenosine 5-diphosphate trisodium salt hydrate (MeSADP, Santa Cruz Biotechnology) and 5 μM epinephrine (Sigma-Aldrich). For aggregation studies, platelets were diluted to 250,000 cells/μL in autologous platelet-free supernatant. Maximal aggregation (amplitude [%]) is reported in this study.

Western blotting

For western blotting of intracellular proteins, platelets were washed twice in Tyrode's buffer (5 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) pH 7.4 with 136.5 mM NaCl, 2.7 mM KCl, 11.9 mM $NaHCO_3$, 0.4 mM NaH_2PO_4, 1 mM $MgCl_2$ and 0.1% (wt/vol) D-glucose) supplemented with 1 μg/mL tirofiban (Sigma-Aldrich) to prevent aggregation. Glycocalicin was determined in platelet-free supernatant prepared by centrifugation at 1000 g for 12 minutes in presence of 0.5 mg/mL prostaglandin E1 (PGE1) and 0.2 μm filtration. For preparation of whole cell lysate, platelets were pelleted and resuspended in ice cold lysis buffer (50 mM tris(hydroxymethyl)aminomethane (tris)–HCl pH 8.0, 150 mM NaCl, 1.0% (vol/vol) octylphenoxy poly(ethyleneoxy)ethanol (IGEPAL CA-630), 0.5% (wt/vol) sodium deoxycholate, 0.1% (wt/vol) sodium dodecyl sulphate (SDS), 2 mM ethylenediaminetetraacetic acid (EDTA) and 10 mM sodium orthovanadate) with additional 2% (vol/vol) protease and phosphatase inhibitor (Thermo Fisher Scientific).

The Mem-PER Membrane Extraction system (Thermo Fisher Scientific) was used for the preparation of cytosolic and membrane fractions. Samples were diluted in Laemmli buffer (60 mM Tris, 10% [vol/vol] glycerol, 2% [wt/vol] SDS, 41.7 mM dithiothreitol [DTT] and 0.01% [wt/vol] bromophenol blue) for SDS polyacrylamide gel electrophoresis (PAGE) and western blotting. The samples were stored at −20°C until used for analysis.

Total protein in samples was determined with a colorimetric bicinchoninic acid assay (Thermo Fisher Scientific). All samples were incubated at 100°C for 5 minutes. Total protein levels of 46 μg for cleaved caspase-3, 1.4 μg for Bcl-xL, 25 μg for Bak, and 20 μg for cytochrome c were loaded onto precast polyacrylamide gels (Bio-Rad). Proteins were transferred on polyvinylidene fluoride (PVDF) membranes using a semi-dry turbo transfer system (Bio-Rad). All antibodies were purchased from Cell Signaling Technology or United States Biological. Primary and secondary antibodies were prepared in Tris buffered saline (TBS pH 7.4, 25 mM Tris with 150 mM NaCl and 2 mM KCl) with 5% (wt/vol) skimmed milk. Loading control proteins were detected on the same membranes following stripping by incubation for 30 minutes at 60°C in western reprobe buffer (50 mM Tris pH 7.0, 2.0% [wt/vol] SDS and 100 mM β-mercaptoethanol). Bound antibody was detected with enhanced chemiluminescence and an imaging system (ChemiDoc MP, Bio-Rad) equipped with a CCD camera and analysis software (Image Lab v4.0.1, Bio-Rad) for densitometry.

Total nicotinamide adenine dinucleotide levels

Extraction and detection of total nicotinamide adenine dinucleotide (NAD) levels was performed using an NAD/NADH colorimetric assay kit (Abcam) according to the manual with minor adaptations. In brief, platelets were washed in ice cold 10 mM HEPES buffer with 0.9% (wt/vol) NaCl (HBS), pH 7.4. Platelets were then resuspended in extraction buffer and lysed by multiple freeze-thawing cycles in liquid nitrogen and a 37°C water bath. Total NAD was determined by interpolating the data obtained from colorimetric measurements (Infinite F200 Pro, Tecan Group Ltd.) in a 96-well microplate containing the NAD standard in the kit.

Thrombin generation assay

To study platelet's contribution in coagulation, a thrombin generation assay (Technoclone GmbH) was performed as described.[24] In brief, platelets were centrifuged, resuspended, and diluted to 250,000 per μL, 100,000 per μL, 50,000 per μL, or 10,000 per μL (final concentrations after addition of substrate) in a fixed volume of heterologous normal human plasma pooled (n = 38 healthy donors). The assay was performed with varying platelet concentrations to demonstrate platelet dose dependence.[25] In a fixed volume 1:10,000 (vol/vol) purified recombinant human tissue factor (TF) (Dade Innovin from Siemens Healthcare GmbH), 4.0 μM corn trypsin inhibitor (CTI, enzyme research laboratories), and 0.5 μM fluorogenic substrate (Z-G-G-RAMC, Technoclone GmbH) was mixed in a 96-well microplate and immediately analyzed in a microplate reader equipped with filters for excitation and emission wavelengths of 380 nm and 485 nm, respectively. The fluorescent signal was recorded in function of time for a total of 120 minutes at 37°C. The raw signal was converted to thrombin concentrations based on a calibration kit and a script in Excel (Microsoft) provided by the manufacturer.[26]

Murine model for platelet clearance

A previously described murine model for platelet transfusion and platelet clearance was used.[27] Platelet samples were supplemented with 0.5 mg/mL PGE1 to prevent activation and centrifuged next at 1000 g for 12 minutes. The platelet pellet was resuspended to a final concentration of 4×10^9 platelets per mL in its autologous supernatant. A bolus of 100 μL (4×10^8 platelets) was injected into immunodeficient NOD SCID IL2R common gamma chain knockout mice (NSG) via the tail vein. Murine blood was collected at 1 minute and then 1, 2, 5, and 24 hours post transfusion through retro-orbital bleeding. Blood was mixed 1:16 in anticoagulant citrate dextrose solution, solution A (ACD-A, 38.0 mM citric acid, 123.8 mM D-glucose and 74.8 mM trisodium citrate dissolved in distilled water, pH 5). Anticoagulated blood was fixed (Beckman Coulter) and samples were stored on a rotator at 4°C until read out by flow cytometry. All animal experiments were approved by the local ethical committee (107/2017, KULeuven, Leuven, Belgium).

Flow cytometry

Expression of P-selectin (phycoerythrin-anti-CD62P, Life Technologies), phosphatidylserine (peridinin-chlorophyll-Cy5.5 Annexin V, BD Biosciences), activated integrin $\alpha_{IIb}\beta_3$

(fluorescein-labeled PAC1, BD Biosciences) and GPIbα (fluorescein-labeled anti-CD42b, Life Technologies) was analyzed with an acoustic focusing flow cytometer (Attune, Life Technologies). In fixed murine blood, human platelets (allophycocyanin-labeled mouse anti-human CD41, BD Biosciences) and murine platelets (fluorescein-labeled rat anti-mouse CD42b, BD Biosciences) were measured separately. Data are expressed relative to the number of human platelets per 10,000 murine platelets in the 1 minute post transfusion sample. Samples were incubated with labeled antibodies for 10 minutes in HBS with 1 mM $MgSO_4$ and 5 mM KCl at room temperature. Samples were diluted 20-fold in the same buffer, immediately before readout. For phosphatidylserine measurements with annexin V buffers were supplemented with 2 mM $CaCl_2$. The fraction (%) of activated platelets is defined as the PAC1 positive platelet population either in resting conditions or after activation with TRAP6 at 2.5, 5.0, and 25 μM.

Threshold gates were set including 0.5% of 10,000 events incubated with corresponding isotype antibody controls. For phosphatidylserine controls, a sample containing labeled annexin V was prepared without $CaCl_2$. Percentage positive events or median fluorescent intensities (MFI) were determined for 10,000 events staining positive for CD61 (allophycocyanin-labeled anti-CD61, Life Technologies). The number of microparticles was determined using calibration beads (BioCytex) and the accompanying gating strategy provided by the manufacturer. Microparticles were determined as GPIbα positive events with sizes smaller than 0.9 μm (detection limit of flow cytometer is 0.5 μm). The result is expressed as the fraction of microparticles per 10,000 all-size events positive for GPIbα.

Statistical analysis

All statistical analyses were performed using Prism Version 7.04 (GraphPad Software Inc.). A repeated measures two-way ANOVA for test (NR) versus control (triflate) as a function of storage time was used. A Sidak correction for multiple comparison was included. Significance was assumed when $p < 0.05$.

RESULTS

NAD content in platelets

Total NAD levels increased rapidly and significantly during NR treatment compared to control. At the end of the study (day 23) an average NAD content of 41.5 ± 4.1 fmol was found per 10^3 platelets in NR treated platelets. This represented a nine fold increase from the initial NAD content of 4.8 ± 0.5 fmol per 10^3 platelets (Fig. 1). In control cells, regular senescence led to a two-fold decrease in NAD levels of 2.1 ± 1.8 fmol per 10^3 platelets. Total NAD levels were significantly higher ($p \leq 0.0001$) for NR treated platelets compared to control platelets from Day 5 on.

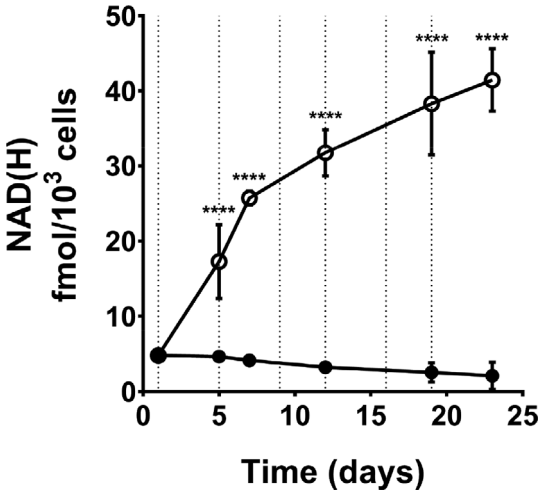

Fig. 1. Intracellular total NAD levels in platelet lysates. Total NAD was measured in platelet lysates during storage of PC. Dotted lines indicate when addition of NR or vehicle solution was performed. Samples were collected on Days 1, 5, 7, 12, 19, and 23 from PC supplemented with NR (○) or control (●). Data are shown as mean with SD (n = 6), ****p < 0.0001.

Influence of NR on PC quality

Platelet count decreased similarly in NR and control conditions (Fig. 2A). MPV slowly increased (Fig. 2B) in both conditions. Swirling remained intact until Day 12 when a decrease was detected which was not different between conditions (Fig. 2C). Platelet anaerobic metabolism was linear until Day 12 (Fig. 2D) when glucose levels (Fig. 2E) fell below detection limit (1.1 mM) and pH started to slightly rise (Fig. 2F). A small but significant difference in pH was found between NR treated and vehicle control treated PC ($p \leq 0.0001$) from Day 12 on. There were no significant differences between NR and vehicle treated platelets for the parameters shown in Fig. 2A-E.

Influence of NR on platelet activation

Platelet aggregation significantly decreased in aging platelets irrespective of NR supplementation (Fig. 3). This was the same for both activation with collagen (Fig. 3A) or with a combination of TRAP6, MeSADP, and epinephrine (Fig. 3B), even though in the latter case NR supplemented platelets retained aggregation slightly better over storage time. During aging, NR and control PC contained increasing numbers of platelets with spontaneously activated integrin $\alpha_{IIb}\beta_3$ receptors (Fig. 4A). NR treated platelets were slightly less activated than control platelets and this was significant from Day 19 on ($p \leq 0.01$). Integrin $\alpha_{IIb}\beta_3$ activation in response to TRAP6 decreased in both conditions during storage (Fig. 4B).

Regardless of platelet storage, thrombin generation was always dependent on platelet concentration (Fig. S2, available as supporting information in the online version of this

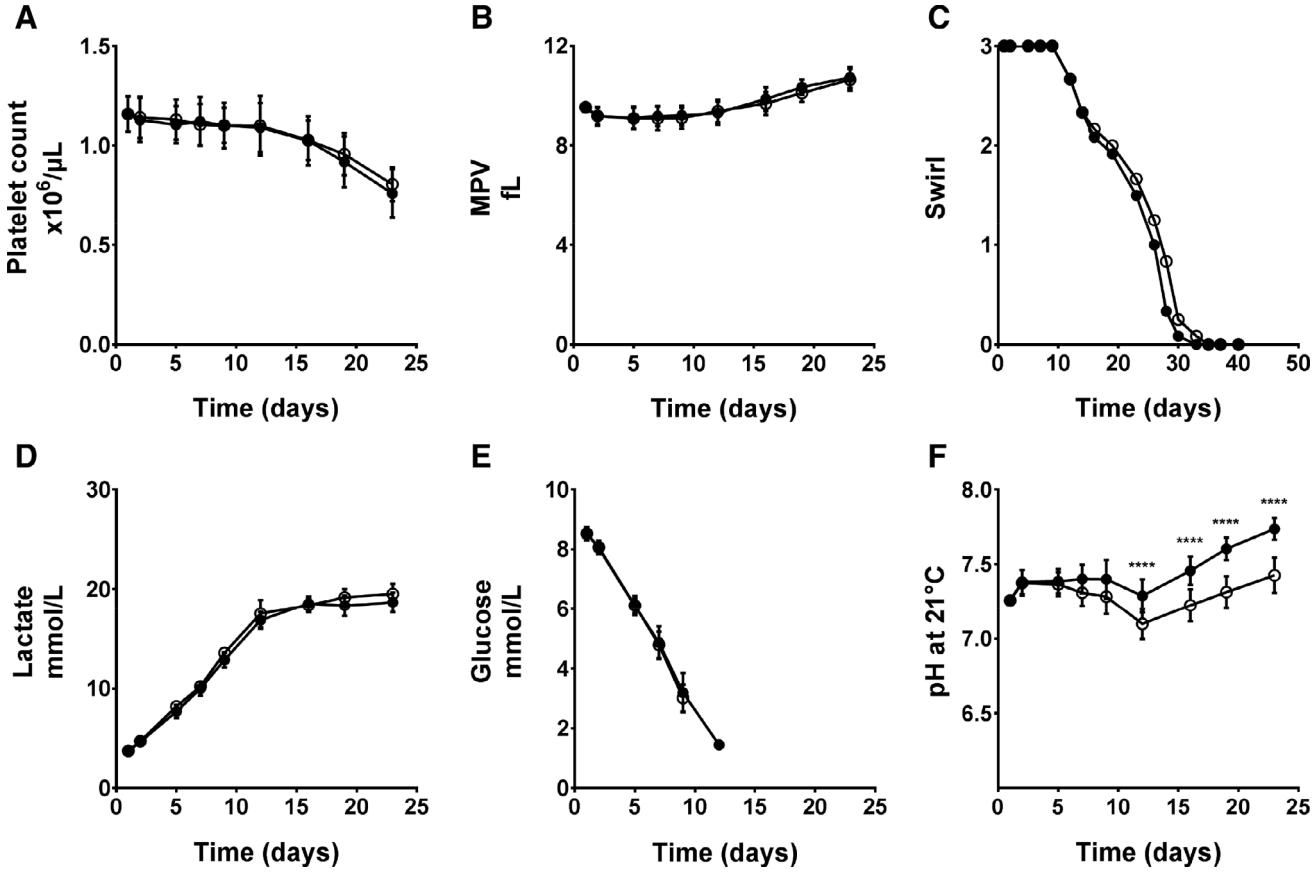

Fig. 2. Blood banking parameters. (A) Platelet count and (B) MPV were measured by a hematology analyzer. (C) Swirling was scored blinded by trained blood banking staff. The energy metabolites (D) lactate, (E) glucose, and (F) pH at 21°C were measured with a point-of-care blood gas analyzer. The detection limit for glucose is 1.1 mM. Data were collected on Days 1, 5, 7, 12, 19, and 23 from PC supplemented with NR (○) or control (●). Data are shown as mean with SD (n = 6), ****p < 0.0001.

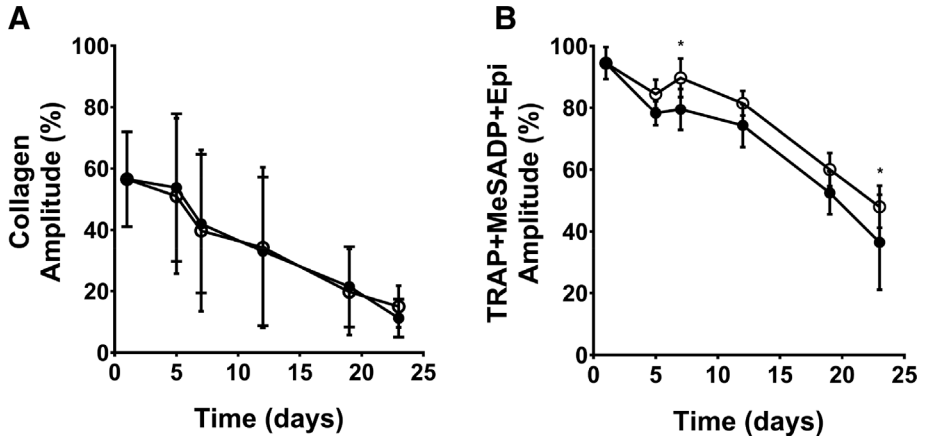

Fig. 3. Platelet aggregation. Platelet aggregation was induced with either (A) collagen or (B) a combination of TRAP, MeSADP and epinephrine and measured using light transmission aggregometry at 37°C. Data were collected on days 1, 5, 7, 12, 19, and 23 from PC supplemented with NR (○) or control (●). Data are shown as mean with SD (n = 6), *p < 0.05.

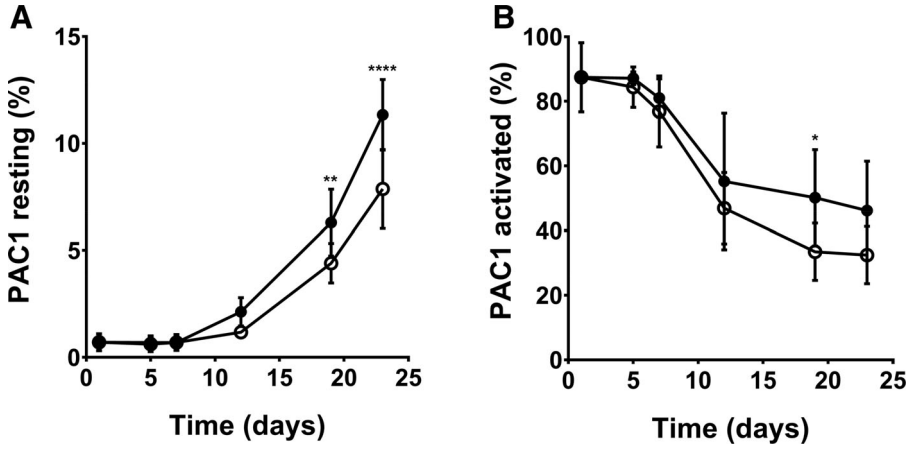

Fig. 4. Activation of platelet integrin $\alpha_{IIb}\beta_3$. Activated integrin $\alpha_{IIb}\beta_3$ was detected using PAC1 binding in flow cytometry on (A) resting platelets or on (B) platelets activated with 25 μM TRAP6. Data were collected on days 1, 5, 7, 12, 19, and 23 from PC supplemented with NR (○) or control (●). Data are shown as mean with SD (n = 6), *p < 0.05, **p < 0.01, and ****p < 0.0001.

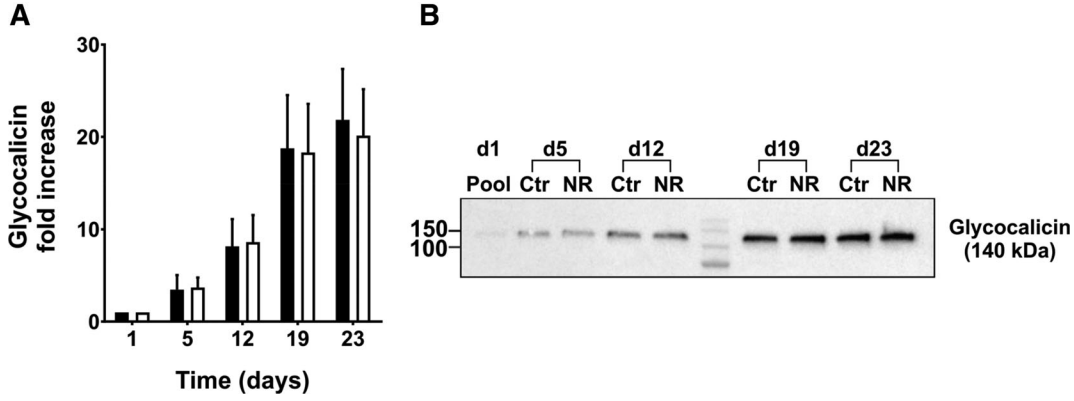

Fig. 5. Glycocalicin levels in platelet concentrate (PC) supernatant. GPIbα shedding was determined by detection of glycocalicin using SDS-PAGE and quantitative western blotting. (A) Band intensities are expressed relative to those on Day 1. (B) A representative western blot of glycocalicin in PC supernatant. Data were collected on Days 1, 5, 7, 12, 19, and 23 from PC supplemented with NR (open bars) or control (closed bars). Data are shown as mean with SD (n = 6).

paper). However, peak thrombin values increased as a function of storage. No significant differences were found between NR treated and control platelets.

Storage lesion

Phosphatidylserine expression remained stable until Day 7, but exponentially increased thereafter (Fig. S3A, available as supporting information in the online version of this paper) independent of NR supplementation. GPIbα expression decreased linearly as a function of storage in both conditions but at a slightly faster rate in NR treated platelets compared to control (Fig. S3B, available as supporting information in the online version of this paper). Correspondingly, glycocalicin levels increased 20-fold during storage but, unlike the corresponding data in flow cytometry this difference was not significant between conditions (Fig. 5). Platelet degranulation determined by P-selectin exposure increased comparably in both conditions (Fig. S3C, available as supporting information in the online version of this paper). The number of microparticles increased just slightly during storage and was not different between conditions (Fig. S3D, available as supporting information in the online version of this paper).

Apoptosis

Mitochondrial cytochrome c release into the cytoplasm displayed an increasing trend which was similar for NR

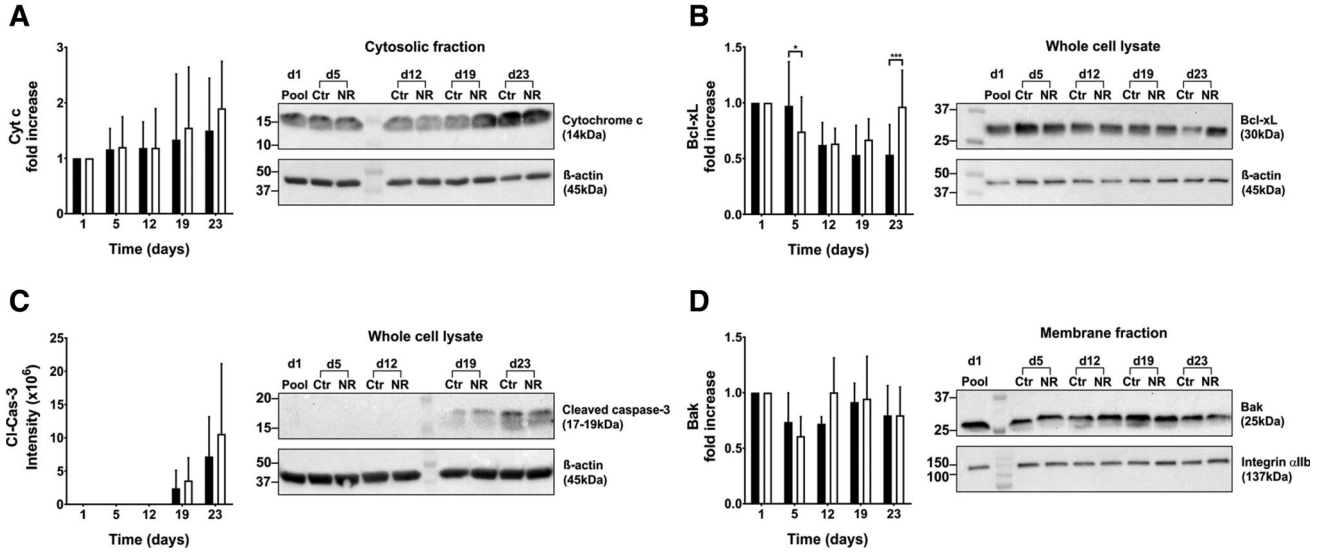

Fig. 6. Intrinsic apoptosis. Key proteins of the intrinsic apoptosis pathway were detected in SDS-PAGE western blotting. (A) Cytochrome c, (B) Bcl-xL in whole cell lysate, (C) cleaved caspase-3 (Cl-Cas-3) in whole cell lysate and (D) Cytosolic Bak. Data for cleaved caspase-3 were below the detection limit on Days 1, 5, and 12 (C). Western blotting inserts to the right of each graph are representative for the data. Data were collected on Days 1, 5, 7, 12, 19, and 23 from PC supplemented with NR (open bars) or control (closed bars) and are shown as fold increase relative to that on Day 1 except for panel C where absolute intensities are given. Data are shown as mean with SD (n = 6), *p < 0.05, ***p < 0.001.

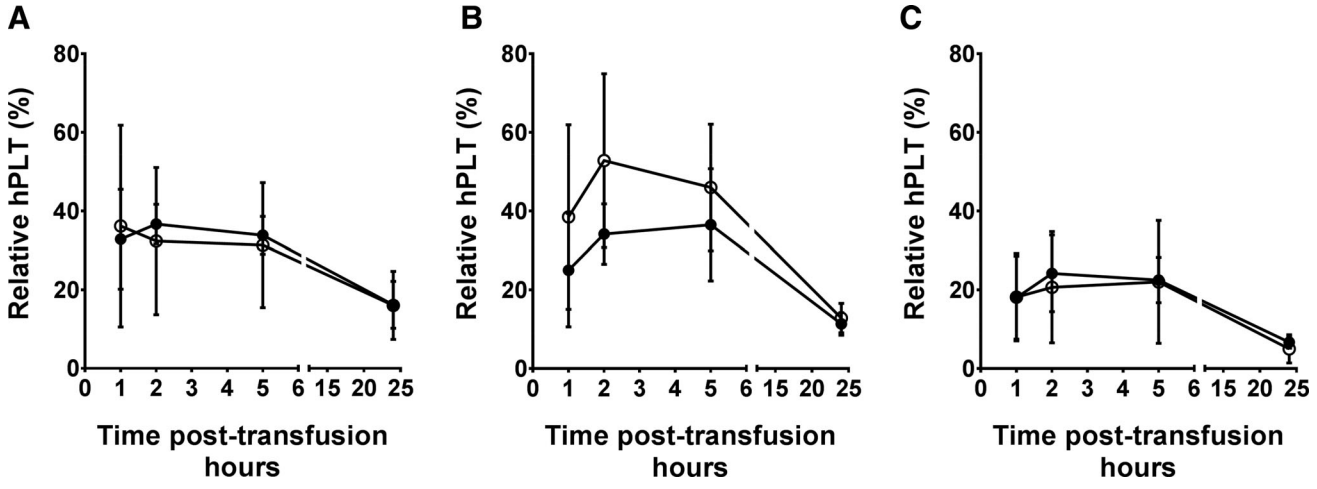

Fig. 7. Human platelet transfusion in NSG mice. Mice were transfused with aging platelets treated with NR (○) or not (●). On Day 2 (A), Day 7 (B), and Day 13 (C) of storage, 4×10^8 platelets were delivered via the tail vein (n = 3 biological repeats). Blood was taken at 1 minute, 1 hour, 2 hours, 5 hours, and 24 hours after transfusion. Data are expressed relative to the number of human platelets per 10,000 murine platelets in the 1 minute post transfusion sample.

supplemented and control platelets (Fig. 6A). This was not statistically significant. The anti-apoptotic Bcl 2 family protein Bcl-xL prevents cytochrome c release and its levels correspondingly declined in whole platelet lysates (Fig. 6B). Cleaved caspase-3 was detected from Day 19 on and increased 4 days later (Fig. 6C) indicating effector caspase activation in these later stages of storage. No changes in the pro-apoptotic membrane residing Bak protein were found (Fig. 6D). For all parameters tested, the presence of NR made no difference.

Transfusion in mice

As expected, circa half of human platelets were quickly cleared from murine circulation during the first hour after

transfusion (not shown) in both NR and control conditions and this was similar for all time points tested during storage. This rapid decrease was followed by slower platelet clearance until less than 20% of initial human platelets were detected 24 hours after transfusion (Fig. 7).

DISCUSSION

Platelets for transfusion are stored as a hyperconcentrated suspension contained in plastic bags. The suspension is continuously agitated at controlled temperatures near 22°C. In these storage conditions, platelet anaerobic metabolism eventually leads to acidosis and subsequent storage lesion.[28,29] Platelets are metabolically active cells and respond quickly to environmental disturbance by cell activation which is regulated by Ca^{2+} fluxes. This way, platelets generate ATP at six times the rate of muscle cells and 13 times the rate of red cells.[30] Long term storage of platelets ex vivo therefore is a challenge of controlling metabolic (stress) rate.

As in other cells, platelets produce reducing equivalents in the tricarboxylic acid cycle (alternative names: Kreb's or citric acid cycle) by reduction of NAD+ to NADH. In most cells, NAD availability therefore is an important determinant of mitochondrial respiration and cell survival. Yet, NAD is not only consumed during mitochondrial respiration. The NAD molecule acts as a crucial coenzyme in other biochemical pathways where it is catabolized, hence not recycled. Sirtuins and PARPs constitute the bulk of such NAD scavengers and these enzymes have been detected in platelets at the RNA level.[15–17] This way, NAD content declines during aging and has been found to be at the nexus of senescence signaling.[14] Consequently, caloric restriction and/or dietary supplementation of NAD precursors like NR can retain or restore NAD levels and attenuate some molecular effects of aging.[31]

Our study shows that when assuming a mean platelet volume of 9 fL, the intracellular NAD content in resting healthy donor platelets is 533 μM on average. We found no similar studies that have measured NAD levels in platelets, so could not compare with existing studies. An older study in human red blood cells found just 42 nmol per mL of "packed red blood cells." An estimation of the total cell volume in such conditions assuming 60% hematocrit returns an intracellular NAD concentration of 70 μM which is seven times less than platelets. However, it is suggested that in general, mammalian cells bear intracellular NAD concentrations >200 μM.[32] For instance, measurements in human brain tissue returned an intracellular NAD concentration of 370 μM, *in vivo*.[33]

The past decades have provided evidence that raising NAD levels by supplementation of niacin precursors to cells, tissues, or entire organisms can rejuvenate and even extend life-span.[34] Platelets are no different and respond to NR supplementation by increasing intracellular NAD levels. In our study, NAD content in platelets increased at an average rate of 1.5 fmol per 1000 cells per day, resulting in an almost 10-fold increase at the end of the 23-day study yielding millimolar concentrations of intracellular NAD. The rate of NAD synthesis is strikingly similar to other mammalian cells supplemented in vitro with similar concentrations of NR.[35] For instance, when HEK293 cells were supplemented with a similar concentration of NR (~600 μM), its NAD content almost doubled in the course of 2 days of tissue culture. This demonstrates that platelets carry the molecular machinery of salvage pathway NAD synthesis and readily respond to precursor supplementation by increasing intracellular NAD levels. At the same time, control senescent platelets gradually lost NAD over the course of the study indicating that also platelets suffer from decreasing NAD levels while aging (ex vivo).

This prompted further study into platelet storage lesion because the response of platelets to high intracellular NAD levels was unknown. Despite this however, NR treated platelets were no different from control untreated platelets. This also implies that supraphysiologic levels of NAD were not detrimental to platelets, at least for those parameters tested in this study. Three major platelet assay groups can be distinguished in our study: one was examining direct platelet storage lesion parameters typical to blood banking, the second dealt with activation and apoptotic markers and the third with transfusion itself in a preclinical animal model.

The typical blood banking quality control parameters were chosen to study platelet metabolism output data during long term storage in the presence or absence of NR. These data typically inform transfusion specialists on the expected success of a platelet transfusion. It often works in one way, i.e., when MPV is high, pH is low and swirling is absent a poor transfusion efficiency is to be expected. The other way around is not necessary valid, i.e., when MPV, pH and swirling is normal, then transfusion not necessarily yields a long term donor platelet survival in the patient's circulation.[36] So despite the absence of any differences between NR and control it could not be excluded that NR platelets would be better than control for other output parameters like hemostasis or aggregation.

However, this was not the case. PCs containing NR were not particularly more responsive to agonists in integrin activation or aggregation. This implies that high NAD concentrations do not boost the platelet's activation response by increasing the aggregation amplitude. It also means that high NAD levels do not cause an increase in the sustainability of the activation response over the course of storage. Using our supplementation strategy based on literature in different fields, NR caused supraphysiologic intracellular NAD levels. Whether these abnormally high NAD levels influence the platelet such that putative beneficial effects of NR supplementation are counterbalanced, needs further investigation. If so, a careful titration of NR to maintain

baseline NAD levels instead of supraphysiologic levels would be a better strategy.

Finally, because platelet clearance from circulation is hard to predict from in vitro data[8] we chose to infuse these platelets in immunodeficient mice. Clearance of human platelets in these mice is inherently variable,[27] so conclusive data were not obtained. However, obvious differences between NR and control were not found, suggesting that any effect is small or negligible. In addition, the in vivo findings correlate with our in vitro data. The small difference in GPIbα expression levels found in flow cytometry suggested that circulation time might be different, but this was not the case. Our glycocalicin measurements indeed suggest that GPIbα expression differences were not related to shedding.

In conclusion, despite promising results on NR supplementation and longevity in single cells, tissues and whole animals in other studies,[34] no measurable attenuation of storage lesion can be found in platelets treated with NR ex vivo. One explanation may be the absence of a strong gene-driven survival response. Platelets are anucleate and some of the sirtuins that regulate senescence in nucleated cells are located inside the nuclear compartment where NR supplementation may lead to gene regulated responses. If these constitute the bulk of the senotherapeutic effect, then it is sensible that platelets do not respond to NR by increasing life-span. Of note, a recent randomized, double-blind, placebo-controlled, crossover trial has provided evidence that NAD metabolism is stimulated in healthy middle-aged and older adults taking NR as a dietary supplement.[37] The authors assessed cardiovascular outcomes of which none was significantly different compared to placebo, but nonetheless some interesting trends were reported. It therefore remains to be independently proven that NR supplementation to single cell cultures, entire tissues or even humans extends life-span or ameliorates quality of life.

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CONFLICT OF INTEREST

The authors have disclosed no conflicts of interest.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Appendix S1: Supporting Information.