Nov. 20th, 2024

Efficacy Test of Liposomal NAD+ - Increasing The Relative Content of NAD+ in Mouse Liver

1. Test method:

A total of 27 clean-grade male C57BL/6J mice were used. The experiment began after 3 days of adaptive feeding. On the 0th day, 3 mice were killed, and the mouse livers (2 biological replicates) were taken for NAD+ detection. The remaining 24 mice were randomly divided into 4 groups, 6 mice in each group, and the drug was administered by intragastric gavage for 14 days. The control group was given water, the NAD+ original drug group was given 3000 mg/kg NAD+, the NAD+ liposome A group was given 600 mg/kg NAD+ liposome A (50% PC20), and the NAD+ liposome B group was given 600 mg/kg NAD+ liposome B (50% PC60), which were prepared and used every day. On the 7th day, 3 mice in each group were killed, and the mouse livers (2 biological replicates) were taken for NAD+ detection. On the 14th day, the remaining 3 mice in each group were killed, and the mouse livers (2 biological replicates) were taken for NAD+ detection.

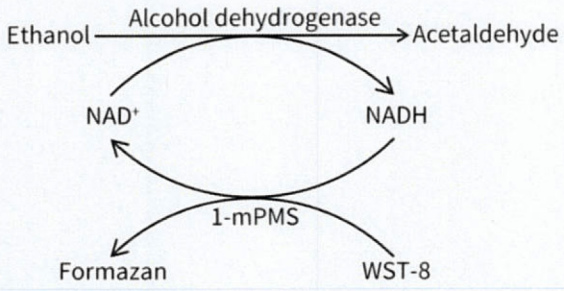
Group	Number of mice	Processing
Control	6	Water, 5 times the weight of the mouse
NAD+	6	Prepare 600mg/mL NAD+, 5 times the weight of the mouse
NAD+ Liposome A (50% PC20)	6	Prepare 120mg/mL NAD+ liposome A, 5 times the weight of the mouse
NAD+ Liposome B (50% PC60)	6	Prepare 120mg/mL NAD+ liposome B, 5 times the weight of the mouse

2. NAD+ Detection Principle

NAD+/NADH Assay Kit (WST-8 Method) is a colorimetric reaction based on WST-8, which detects the amount, ratio and total amount of NAD+ (oxidized coenzyme I) and NADH (reduced coenzyme I) in cells, tissues or other samples by colorimetry.

NAD (Nicotinamide adenine dinucleotide) is a coenzyme present in all cells, including NAD+ (oxidized form) and NADH (reduced form). NAD+ is not only a coenzyme that transfers electrons during redox reactions, but also can be used as a substrate for many enzymes to participate in intracellular reactions. For example, deacetylases such as Sirt1 of the Sirtuins family need NAD+ as a substrate for deacetylation reactions to regulate the acetylation level of proteins and thus participate in the life activities of cells. NAD+ plays an important role in cells and the body. Its synthesis and degradation and its products are involved in cell apoptosis, metabolic regulation, and gene expression regulation, and the reduction of NAD+ is one of the main factors of cell death. Although NMNAT (nicotinamide mononucleotide adenylyl transferase) is a synthase of NAD+, including Nmnat1, Nmnat2, and Nmnat3, NAMPT (Nicotinamide phosphoribosyl transferase) is generally considered to be the rate-limiting enzyme for NAD+ synthesis. The importance of NAD+ in regulating the redox state of cells and its function in regulating signaling pathways and transcription make NAD+ and its synthesizing and consuming enzymes potential drug targets for a variety of diseases.

Determination of the total amount of NAD+ and NADH: Ethanol is oxidized to acetaldehyde by alcohol dehydrogenase (ADH). In this reaction, NAD+ is reduced to NADH, and the generated NADH reduces WST-8 to orange-yellow formazan by the action of the electron coupling reagent 1-mPMS (1-Methoxy-5-methylphenazinium Methyl Sulfate), with a maximum absorption peak at around 450 nm. The formazan generated in the reaction system is proportional to the total amount of NAD+ and NADH in the sample.



Schematic diagram of NAD+/NADH Assay Kit (WST-8 Method) (S0175)

Determination of NADH: After heating in a 60°C water bath for 30 min, NAD+ in the sample decomposes and NADH remains. NADH reduces WST-8 to formazan. The amount of formazan generated by the reaction is determined by colorimetry, and ultimately the amount of NADH in the sample can be determined.

Determination of NAD+ and NAD+/NADH ratio: Based on the total amount of NAD+ and NADH and the amount of NADH obtained in the first two steps, the amount of NAD+ in the sample and the ratio of NAD+/NADH can be calculated.

3. NAD+ Detection Steps

NAD+ detection was performed after the liver was taken on days 0, 7, and 14, with 2 liver samples per mouse and 20 mg per sample.

After washing the tissue with pre-cooled PBS on ice, mince it with scissors, place it in a homogenizer, and add 400 μL of extract solution for homogenization. Then centrifuge at 12,000g, 4°C for 5 min, and take the supernatant as the sample to be tested. Take 20 μL of supernatant and dilute it with 180 μL of extract solution for detection.

Setting up the NADH standard curve: Dilute the 10 mM NADH standard with NAD+/NADH extract to an appropriate concentration gradient (0, 1, 2, 4, 6, 8, 10 μM). When testing, add 20 μL of the standard to each well of the 96-well plate, which is equivalent to NADH concentrations of 0, 20, 40, 80, 120, 160, 200 pmol per well.

Determination of the total amount of NAD+ and NADH in the sample: Pipette 20 μL

of the sample to be tested diluted with NAD+/NADH extract into a 96-well plate.

	Blank control	Standards	sample
Samples to be tested	——	20 μL	20 μL
Extract	20 μL	——	——
Alcohol dehydrogenase working solution	90 μL	90 μL	90 μL

Incubate at 37 °C in the dark for 10 min. The purpose of this incubation step is to convert NAD+ in the sample into NADH. Mix the colorimetric solution appropriately, then add 10 μL of the colorimetric solution to each well and mix well. Incubate at 37 °C in the dark for 20 min. At this time, orange-yellow formazan will be formed, and the absorbance at 450 nm will be measured.

Determination of NAD+, NADH content or NAD+/NADH ratio in samples: Pipette 100 μL of the sample to be tested into a centrifuge tube and heat it on a PCR instrument at 60°C for 30 min to decompose NAD+. Pipette 20 μL of the supernatant diluted with NAD+/NADH extract as the sample to be tested into a 96-well plate. Incubate at 37 °C in the dark for 10 min. The purpose of this incubation step is to convert NAD+ in the sample into NADH. Mix the colorimetric solution appropriately, then add 10 μL of the colorimetric solution to each well and mix well. Incubate at 37 °C in the dark for 20 min. At this time, orange-yellow formazan will be formed, and the absorbance at 450 nm will be measured.

The relative content (RC) of NAD+ in the liver is calculated by the following formula:

$$RC = \frac{C_1}{C_2} \times \frac{Dose_2}{Dose_1}$$

Where: C_1 and C_2 are the contents of NAD+ in the liver of the NAD+ original drug group and the NAD+ liposome group, respectively. $Dose_1$ and $Dose_2$ are the dosages of the NAD+ original drug group and the NAD+ liposome group, respectively.

4. Test results

The NAD+ calibration curve is as follows, with an R^2 of 0.9995, which is greater than 0.99. The curve can fit the observed values well.

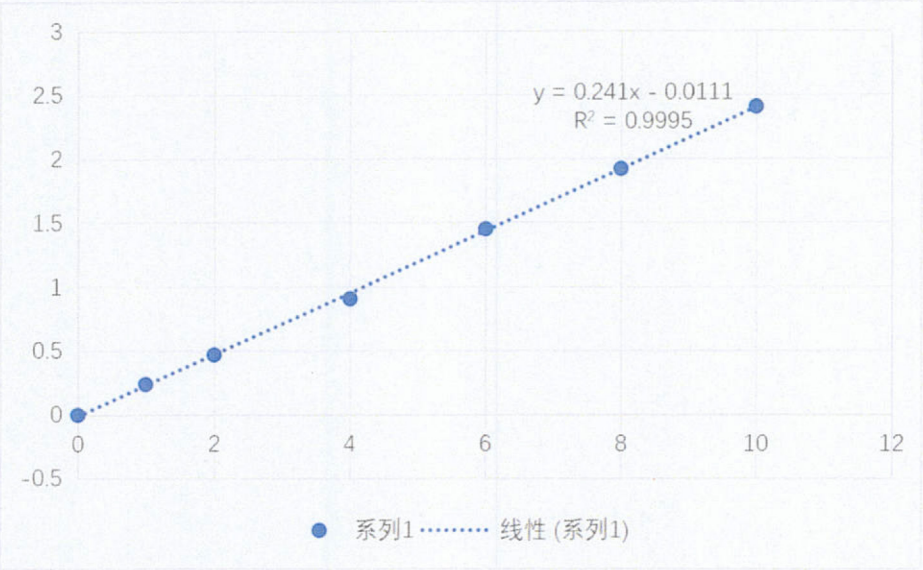
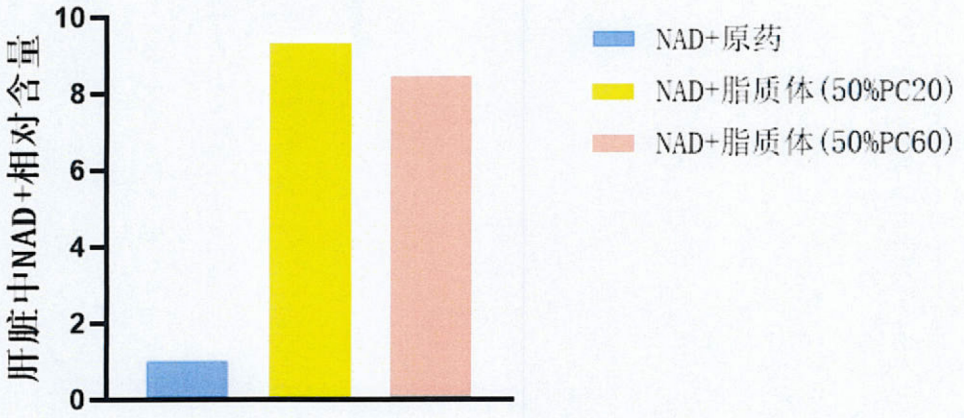


Figure 1. NAD+ calibration curve

Table 3. Relative content of NAD+ in mouse liver on day 14

Parameter	Control	NAD+	NAD+ Liposome A (50% PC20)	NAD+ Liposome B (50% PC60)
NAD+ dosage (mg/kg)	0	3000	300	300
NAD+ content in liver (pmol/mg)	274.22	1653.06	1539.50	1395.24
Relative content of NAD+ in liver	/	1	9.31	8.44



The results show that after 14 days of continuous administration, the relative content of NAD+ in the liver of mice in the NAD+ liposome A (50% PC20) group and the NAD+ liposome B (50% PC60) group was higher than that in the NAD+ original drug group. Among them, the relative content of NAD+ in the liver of mice in the NAD+ liposome A (50% PC20) group was the highest, reaching 9.31 times, which shows that after NAD+ is prepared into liposomes, it can significantly increase the NAD+ content in the liver.